VOLUME: 14 NO: 3 YEAR: 2017

ISSN: 1304-530X

TURKISH JOURNAL OF PHARMACEUTICAL SCIENCES

An Official Journal of the Turkish Pharmacists' Association, Academy of Pharmacy

TURKISH
JOURNAL OF PHARMACEUTICAL SCIENCES

TURKISH
JOURNAL OF PHARMACEUTICAL SCIENCES

AIMS AND SCOPE

The Turkish Journal of Pharmaceutical Sciences is the only scientific periodical publication of the Turkish Pharmacists' Association and has been published since April 2004.
Turkish Journal of Pharmaceutical Sciences is an independent international open access periodical journal based on double-blind peer-review principles. The journal is regularly published 3 times a year and the publication language is English. The issuing body of the journal is Galenos Yayinevi/Publishing House.
The aim of Turkish Journal of Pharmaceutical Sciences is to publish original research papers of the highest scientific and clinical value at an international level.
The target audience includes specialists and physicians in all fields of pharmaceutical sciences.
The editorial policies are based on the "Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals (ICMJE Recommendations)" by the International Committee of Medical Journal Editors (2016, archived at http://www.icmje.org/) rules.

Editorial Independence

Turkish Journal of Pharmaceutical Sciences is an independent journal with independent editors and principles and has no commerical relationship with the commercial product, drug or pharmaceutical company regarding decisions and review processes upon articles.

ABSTRACTED/INDEXED IN

Web of Science-Emerging Sources Citation Index (ESCI)
SCOPUS SJR
Chemical Abstracts Service (CAS)
EBSCO
EMBASE
Analytical Abstracts
International Pharmaceutical Abstracts (IPA)
Medicinal & Aromatic Plants Abstracts (MAPA)
TÜBİTAK/ULAKBİM TR Dizin
Türkiye Atıf Dizini

OPEN ACCESS POLICY

This journal provides immediate open access to its content on the principle that making research freely available to the public supports a greater global exchange of knowledge.
Open Access Policy is based on the rules of the Budapest Open Access Initiative (BOAI) http://www.budapestopenaccessinitiative.org/. By "open access" to peer-reviewed research literature, we mean its free availability on the public internet, permitting any users to read, download, copy, distribute, print, search, or link to the full texts of these articles, crawl them for indexing, pass them as data to software, or use them for any other lawful purpose, without financial, legal, or technical barriers other than those inseparable from gaining access to the internet itself. The only constraint on reproduction and distribution, and the only role for copyright in this domain, should be to give authors control over the integrity of their work and the right to be properly acknowledged and cited.

CORRESPONDENCE ADDRESS

Editor-in-Chief, Feyyaz ONUR, Prof.Dr.
Address: Ankara University, Faculty of Pharmacy, Department of Analytical Chemistry, 06100 Tandoğan-Ankara, TURKEY
E-mail: onur@pharmacy.ankara.edu.tr

PERMISSION

Requests for permission to reproduce published material should be sent to the editorial office. Editor-in-Chief, Prof. Dr. Feyyaz ONUR

ISSUING BODY CORRESPONDING ADDRESS

Issuing Body : Galenos Yayınevi
Address: Molla Gürani Mah. Kaçamak Sk. No: 21/1, 34093 İstanbul, TURKEY
Phone: +90 212 621 99 25 Fax: +90 212 621 99 27
E-mail: info@galenos.com.tr

INSTRUCTIONS FOR AUTHORS

Instructions for authors are published in the journal and on the website http://turkjps.org

MATERIAL DISCLAIMER

The author(s) is (are) responsible for the articles published in the JOURNAL.
The editor, editorial board and publisher do not accept any responsibility for the articles.

Publisher Contact
Address: Molla Gürani Mah. Kaçamak Sk. No: 21/1 34093 İstanbul, Turkey
Phone: +90 (212) 621 99 25
Fax: +90 (212) 621 99 27
E-mail: info@galenos.com.tr/yayin@galenos.com.tr
Web: www.galenos.com.tr

Printing at: Bizim Basım Limited Şirketi
Litros yolu 2. Matbaacılar Sitesi ZD1 Topkapı -İstanbul
Phone: +90 212 709 75 25 Fax: +90 212 709 75 30
Printing date: November 2017
ISSN: 1304-530X
E-ISSN: 2148-6247

TURKISH
JOURNAL OF PHARMACEUTICAL SCIENCES

INSTRUCTIONS TO AUTHORS

Turkish Journal of Pharmaceutical Sciences is the official double peer-reviewed publication of The Turkish Pharmacists' Association. This journal is published every 4 months (3 issues per year; April, August, December) and publishes the following articles:

• Research articles

• Reviews (only upon the request or consent of the Editorial Board)

• Preliminary results/Short communications/Technical notes/Letters to the Editor in every field or pharmaceutical sciences.

The publication language of the journal is English.

The Turkish Journal of Pharmaceutical Sciences does not charge any article submission or processing charges.

A manuscript will be considered only with the understanding that it is an original contribution that has not been published elsewhere.

The Journal should be abbreviated as "Turk J Pharm Sci" when referenced.

The scientific and ethical liability of the manuscripts belongs to the authors and the copyright of the manuscripts belongs to the Journal. Authors are responsible for the contents of the manuscript and accuracy of the references. All manuscripts submitted for publication must be accompanied by the Copyright Transfer Form [copyright transfer]. Once this form, signed by all the authors, has been submitted, it is understood that neither the manuscript nor the data it contains have been submitted elsewhere or previously published and authors declare the statement of scientific contributions and responsibilities of all authors.

Experimental, clinical and drug studies requiring approval by an ethics committee must be submitted to the JOURNAL with an ethics committee approval report including approval number confirming that the study was conducted in accordance with international agreements and the Declaration of Helsinki (revised 2013) (http://www.wma.net/en/30publications/10policies/b3/). The approval of the ethics committee and the fact that informed consent was given by the patients should be indicated in the Materials and Methods section. In experimental animal studies, the authors should indicate that the procedures followed were in accordance with animal rights as per the Guide for the Care and Use of Laboratory Animals (http://oacu.od.nih.gov/regs/guide/guide.pdf) and they should obtain animal ethics committee approval.

Authors must provide disclosure/acknowledgment of financial or material support, if any was received, for the current study.

If the article includes any direct or indirect commercial links or if any institution provided material support to the study, authors must state in the cover letter that they have no relationship with the commercial product, drug, pharmaceutical company, etc. concerned; or specify the type of relationship (consultant, other agreements), if any.

Authors must provide a statement on the absence of conflicts of interest among the authors and provide authorship contributions.

All manuscripts submitted to the journal are screened for plagiarism using the 'iThenticate' software. Results indicating plagiarism may result in manuscripts being returned or rejected.

The Review Process

This is an independent international journal based on double-blind peer-review principles. The manuscript is assigned to the Editor-in-Chief, who reviews the manuscript and makes an initial decision based on manuscript quality and editorial priorities. Manuscripts that pass initial evaluation are sent for external peer review, and the Editor-in-Chief assigns an Associate Editor. The Associate Editor sends the manuscript to at least two reviewers (internal and/or external reviewers). The reviewers must review the manuscript within 21 days. The Associate Editor recommends a decision based on the reviewers' recommendations and returns the manuscript to the Editor-in-Chief. The Editor-in-Chief makes a final decision based on editorial priorities, manuscript quality, and reviewer recommendations. If there are any conflicting recommendations from reviewers, the Editor-in-Chief can assign a new reviewer.

The scientific board guiding the selection of the papers to be published in the Journal consists of elected experts of the Journal and if necessary, selected from national and international authorities. The Editor-in-Chief, Associate Editors may make minor corrections to accepted manuscripts that do not change the main text of the paper.

In case of any suspicion or claim regarding scientific shortcomings or ethical infringement, the Journal reserves the right to submit the manuscript to the supporting institutions or other authorities for investigation. The Journal accepts the responsibility of initiating action but does not undertake any responsibility for an actual investigation or any power of decision.

The Editorial Policies and General Guidelines for manuscript preparation specified below are based on "Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals (ICMJE Recommendations)" by the International Committee of Medical Journal Editors (2016, archived at http://www.icmje.org/).

Preparation of research articles, systematic reviews and meta-analyses must comply with study design guidelines:

CONSORT statement for randomized controlled trials (Moher D, Schultz KF, Altman D, for the CONSORT Group. The CONSORT statement revised recommendations for improving the quality of reports of parallel group randomized trials. JAMA 2001; 285: 1987-91) (http://www.consort-statement.org/);

PRISMA statement of preferred reporting items for systematic reviews and meta-analyses (Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group. Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 2009; 6(7): e1000097.) (http://www.prisma-statement.org/);

STARD checklist for the reporting of studies of diagnostic accuracy (Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig LM, et al., for the STARD Group. Towards complete and accurate reporting of studies of diagnostic accuracy: the STARD initiative. Ann Intern Med 2003;138:40-4.) (http://www.stard-statement.org/);

STROBE statement, a checklist of items that should be included in reports of observational studies (http://www.strobe-statement.org/);

MOOSE guidelines for meta-analysis and systemic reviews of observational studies (Stroup DF, Berlin JA, Morton SC, et al. Meta-analysis of observational studies in epidemiology: a proposal for reporting Meta-analysis of observational Studies in Epidemiology (MOOSE) group. JAMA 2000; 283: 2008-12).

Authorship

Each author should have participated sufficiently in the work to assume public responsibility for the content. Any portion of a manuscript that is critical to its main conclusions must be the responsibility of at least 1 author.

TURKISH
JOURNAL OF PHARMACEUTICAL SCIENCES

INSTRUCTIONS TO AUTHORS

GENERAL GUIDELINES

Manuscripts can only be submitted electronically through the Journal Agent website (http://journalagent.com/tjps/) after creating an account. This system allows online submission and review.

The manuscripts are archived according to ICMJE, Web of Science-Emerging Sources Citation Index (ESCI), SCOPUS, Chemical Abstracts, EBSCO, EMBASE, Analytical Abstracts, International Pharmaceutical Abstracts, MAPA(Medicinal & Aromatic Plants Abstracts), Tübitak/Ulakbim Turkish Medical Database, Türkiye Citation Index Rules.

Format: Manuscripts should be prepared using Microsoft Word, size A4 with 2.5 cm margins on all sides, 12 pt Arial font and 1.5 line spacing.

Abbreviations: Abbreviations should be defined at first mention and used consistently thereafter. Internationally accepted abbreviations should be used; refer to scientific writing guides as necessary.

Cover letter: The cover letter should include statements about manuscript type, single-Journal submission affirmation, conflict of interest statement, sources of outside funding, equipment (if applicable), for original research articles.

The ORCID (Open Researcher and Contributor ID) number of the all authors should be provided while sending the manuscript. A free registration can be done at http://orcid.org.

REFERENCES

Authors are solely responsible for the accuracy of all references.

In-text citations: References should be indicated as a superscript immediately after the period/full stop of the relevant sentence. If the author(s) of a reference is/are indicated at the beginning of the sentence, this reference should be written as a superscript immediately after the author's name. If relevant research has been conducted in Turkey or by Turkish investigators, these studies should be given priority while citing the literature.

Presentations presented in congresses, unpublished manuscripts, theses, Internet addresses, and personal interviews or experiences should not be indicated as references. If such references are used, they should be indicated in parentheses at the end of the relevant sentence in the text, without reference number and written in full, in order to clarify their nature.

References section: References should be numbered consecutively in the order in which they are first mentioned in the text. All authors should be listed regardless of number. The titles of Journals should be abbreviated according to the style used in the Index Medicus.

Reference Format

Journal: Last name(s) of the author(s) and initials, article title, publication title and its original abbreviation, publication date, volume, the inclusive page numbers. Example: Collin JR, Rathbun JE. Involutional entropion: a review with evaluation of a procedure. Arch Ophthalmol. 1978;96:1058-1064.

Book: Last name(s) of the author(s) and initials, chapter title, book editors, book title, edition, place of publication, date of publication and inclusive page numbers of the extract cited.

Example: Herbert L. The Infectious Diseases (1st ed). Philadelphia; Mosby Harcourt; 1999:11;1-8.

Book Chapter: Last name(s) of the author(s) and initials, chapter title, book editors, book title, edition, place of publication, date of publication and inclusive page numbers of the cited piece.

Example: O'Brien TP, Green WR. Periocular Infections. In: Feigin RD, Cherry JD, eds. Textbook of Pediatric Infectious Diseases (4th ed). Philadelphia; W.B. Saunders Company;1998:1273-1278.

Books in which the editor and author are the same person: Last name(s) of the author(s) and initials, chapter title, book editors, book title, edition, place of publication, date of publication and inclusive page numbers of the cited piece. Example: Solcia E, Capella C, Kloppel G. Tumors of the exocrine pancreas. In: Solcia E, Capella C, Kloppel G, eds. Tumors of the Pancreas. 2nd ed. Washington: Armed Forces Institute of Pathology; 1997:145-210.

TABLES, GRAPHICS, FIGURES, AND IMAGES

All visual materials together with their legends should be located on separate pages that follow the main text.

Images: Images (pictures) should be numbered and include a brief title. Permission to reproduce pictures that were published elsewhere must be included. All pictures should be of the highest quality possible, in

JPEG format, and at a minimum resolution of 300 dpi.

Tables, Graphics, Figures: All tables, graphics or figures should be enumerated according to their sequence within the text and a brief descriptive caption should be written. Any abbreviations used should be defined in the accompanying legend. Tables in particular should be explanatory and facilitate readers' understanding of the manuscript, and should not repeat data presented in the main text.

MANUSCRIPT TYPES

Original Articles

Clinical research should comprise clinical observation, new techniques or laboratories studies. Original research articles should include title, structured abstract, key words relevant to the content of the article, introduction, materials and methods, results, discussion, study limitations, conclusion references, tables/figures/images and acknowledgement sections. Title, abstract and key words should be written in both Turkish and English. The manuscript should be formatted in accordance with the above-mentioned guidelines and should not exceed 16 A4 pages.

Title Page: This page should include the title of the manuscript, short title, name(s) of the authors and author information. The following descriptions should be stated in the given order:

1. Title of the manuscript (Turkish and English), as concise and explanatory as possible, including no abbreviations, up to 135 characters

2. Short title (Turkish and English), up to 60 characters

3. Name(s) and surname(s) of the author(s) (without abbreviations and academic titles) and affiliations

4. Name, address, e-mail, phone and fax number of the corresponding author

5. The place and date of scientific meeting in which the manuscript was presented and its abstract published in the abstract book, if applicable

Abstract: A summary of the manuscript should be written in both Turkish and English. References should not be cited in the abstract. Use of abbreviations should be avoided as much as possible; if any abbreviations are used, they must be taken into consideration

INSTRUCTIONS TO AUTHORS

independently of the abbreviations used in the text. For original articles, the structured abstract should include the following sub-headings:

Objectives: The aim of the study should be clearly stated.

Materials and Methods: The study and standard criteria used should be defined; it should also be indicated whether the study is randomized or not, whether it is retrospective or prospective, and the statistical methods applied should be indicated, if applicable.

Results: The detailed results of the study should be given and the statistical significance level should be indicated.

Conclusion: Should summarize the results of the study, the clinical applicability of the results should be defined, and the favorable and unfavorable aspects should be declared.

Keywords: A list of minimum 3, but no more than 5 key words must follow the abstract. Key words in English should be consistent with "Medical Subject Headings (MESH)" (www.nlm.nih.gov/mesh/MBrowser.html). Turkish key words should be direct translations of the terms in MESH.

Original research articles should have the following sections:

Introduction: Should consist of a brief explanation of the topic and indicate the objective of the study, supported by information from the literature.

Materials and Methods: The study plan should be clearly described, indicating whether the study is randomized or not, whether it is retrospective or prospective, the number of trials, the characteristics, and the statistical methods used.

Results: The results of the study should be stated, with tables/figures given in numerical order; the results should be evaluated according to the statistical analysis methods applied. See General Guidelines for details about the preparation of visual material.

Discussion: The study results should be discussed in terms of their favorable and unfavorable aspects and they should be compared with the literature. The conclusion of the study should be highlighted.

Study Limitations: Limitations of the study should be discussed. In addition, an evaluation of the implications of the obtained findings/results for future research should be outlined.

Conclusion: The conclusion of the study should be highlighted.

Acknowledgements: Any technical or financial support or editorial contributions (statistical analysis, English/Turkish evaluation) towards the study should appear at the end of the article.

References: Authors are responsible for the accuracy of the references. See General Guidelines for details about the usage and formatting required.

Review Articles

Review articles can address any aspect of clinical or laboratory pharmaceuticals. Review articles must provide critical analyses of contemporary evidence and provide directions of or future research. Most review articles are commissioned, but other review submissions are also welcome. Before sending a review, discussion with the editor is recommended.

Reviews articles analyze topics in depth, independently and objectively. The first chapter should include the title in Turkish and English, an unstructured summary and key words. Source of all citations should be indicated. The entire text should not exceed 25 pages (A4, formatted as specified above).

CORRESPONDENCE

All correspondence should be directed to the Turkish Journal of Pharmaceutical Sciences editorial board;

Post: Turkish Pharmacists' Association

Address: Willy Brandt Sok. No: 9 06690 Ankara, TURKEY

Phone: +90 312 409 8136

Fax: +90 312 409 8132

Web Page: http://turkjps.org/home/

E-mail: onur@pharmacy.ankara.edu.tr

TURKISH
JOURNAL OF PHARMACEUTICAL SCIENCES

CONTENTS

Original articles

TURKISH
JOURNAL OF PHARMACEUTICAL SCIENCES

CONTENTS

Review articles

TURKISH
JOURNAL OF PHARMACEUTICAL
SCIENCES

Volume: 14, No: 3, Year: 2017

CONTENTS

Original articles

Review articles

Turk J Pharm Sci 2017;14(3):213-221
DOI: 10.4274/tjps.97720

ORIGINAL ARTICLE

Quetiapine Fumarate Extended-release Tablet Formulation Design Using Artificial Neural Networks

Ketiapin Fumarat Etkin Maddesini İçeren Uzatılmış Salımlı Tablet Formülasyonlarının Yapay Sinir Ağları ile Tasarımı

Esher ÖZÇELİK[1], Burcu MESUT[1], Buket AKSU[2], Yıldız ÖZSOY[1]*

[1]İstanbul University, Faculty of Pharmacy, Department of Pharmaceutical Technology, İstanbul, Turkey
[2]İstanbul Kemerburgaz University, Faculty of Pharmacy, Department of Pharmaceutical Technology, İstanbul, Turkey

ABSTRACT

Objectives: This design study was implemented within the scope of the quality by design approach, which included the "International Conference on Harmonization" guidelines. We evaluated the quality of a modified-release tablet formulation of quetiapine fumarate, which was designed using artificial neural networks (ANN), and determined a new formulation that was similar to the reference product.

Materials and Methods: Twelve different formulations were produced and tested. The reference product's results and our experimental results were used as outputs for the training of the ANN programs of Intelligensys Ltd.

Results: Dissolution tests were performed with the new formulation (F13) suggested by the INForm V.4 ANN program in three different pHs of the gastrointestinal system. The compliance of this formulation was confirmed by comparing the results with an f2 similarity test.

Conclusion: Use of these programs supports research and development processes with multiple evaluation methods and alternative formulations may be determined faster and at lower cost.

Key words: Quetiapine fumarate, quality by design, artificial neural networks, neuro-fuzzy logic, extended-release tablets, wet granulation

ÖZ

Amaç: Bu tasarım çalışması "International Conference on Harmonization" rehberleri kapsamındaki tasarımda kalite yaklaşımı çerçevesinde uygulanmıştır. Yapay sinir ağları (ANN) kullanılarak ketiapin fumaratın değiştirilmiş salımlı tablet formülasyonunun kalitesi değerlendirilmiş ve referans ürüne benzer olan yeni bir formülasyon tespit edilmiştir.

Gereç ve Yöntemler: On iki farklı formül üretilmiş ve test edilmiştir. Referans ürünün analiz sonuçları ve deneysel sonuçlarımız çıktı olarak Intelligensys Ltd. şirketine ait programlarında ANN'nin eğitimi için kullanılmıştır.

Bulgular: INForm V.4 ANN programı tarafından önerilen yeni bir formülasyon (F13) ile gastrointestinal sistemdeki üç farklı pH da çözünme testleri yapılmıştır. Bu formülasyonun uygunluğu da f2 benzerlik testi sonuçları karşılaştırılarak doğrulanmıştır.

Sonuç: Bu programların kullanılması, çoklu değerlendirme yöntemleri ve daha düşük maliyetle Ar-Ge süreçlerini desteklemekte ve alternatif formülasyonlar daha hızlı tespit edilebilmektedir.

Anahtar kelimeler: Ketiapin fumarat, tasarımda kalite, yapay sinir ağları, bulanık mantık, uzatılmış salım tabletler, yaş granülasyon

*Correspondence: E-mail: yozsoy@istanbul.edu.tr, Phone: +90 212 440 02 72 ORCID-ID: orcid.org/0000-0002-9110-3704
Received: 14.10.2016, Accepted: 09.03.2017

INTRODUCTION

Quetiapine fumarate, a Biopharmaceutics Classification System class 2 drug, has been used in the treatment of schizophrenia. Its conventional and extended-release (ER) preparations are available on the market. Its ER formulations ensure safe, clinically effective treatment within accepted therapeutic intervals due to the decreased adverse effects and increased patient treatment adherence.[1]

ER formulations of quetiapine fumarate are prepared as membrane or matrix delivery systems. Mostly hydrophilic polymers are used in the formulation of ER systems. In particular, semi-synthetic cellulose derivatives, acrylic acid derivatives (carbomers), and natural polysaccharides are preferred.[1-4]

Carbopol 974P, an acrylic acid derivative, is used as a binding agent, which increases viscosity, film coating, and acts as a suspension agent material due to its capability of forming a gel at high viscosity.[5] In addition, it controls the release rate of the drug through hydration and swelling mechanisms. Xanthan gum, a microbiologic polysaccharide, is produced by *Xsanthomonas campestris* with pure culture fermentation. It is also widely used as a stabilizer, suspension agent, viscosity increaser[6], and as a matrix agent in ER formulations[7] due to its capability to form a strong matrix structure.

The wet granulation method for the preparation of ER tablets is a common process in the pharmaceutical industry.[8] Decreasing the time and cost of industrial scale production are the most important parameters in the development of ER tablets.[9] In recent years, neural networks, fuzzy-logic programs, and multiple evaluation techniques have been used for the optimization of pharmaceutical formulations due to their advantages over classic formulation development strategies, such as being time saving and having lower cost. In addition, efficiency and quality are other important parameters in pharmaceutical development and production. There are some tools for these objectives such as faster analysis of data, efficient use of incomplete data banks, training of newly developed networks, exploring independent design areas away from complicated designs, finding the most useful choice, establishing restrictions in optimization, and producing useful norms.[10]

An artificial neural network (ANN) is an artificial intelligence tool that identifies arbitrary non-linear multi-parametric distinguishing functions directly from dependent and independent complex variables. Thus, it can separate signals and noise from experimental data.[11,12] These programs evaluate dissolution profiles[13] and take into account the physicochemical properties of products, such as friability and disintegration, and other consequential parameters that affect the release rate of drugs (particle size, type and amount of polymer, granulation technique, compression force, amount and type of lubricant).[14]

FormRules V3.32, a neural network-fuzzy logic program, INForm V.4 ANN, and V.4 gene expression programing (GEP) programs were used in the research and development studies.[15] FormRules is a hybrid technology that is a very effective tool to obtain understandable rules from complex and non-linear data.[16]

The INForm programs use neural network-fuzzy logic and can perform optimization. Optimization is a mathematic method that searches for an "optimum" and most advantageous solutions to solve problems. As for INForm V.4 GEP, it is based on genetic algorithms, searching for the best holistic solutions according to the principle of 'survival of the fittest' in the multidimensional search space.[17]

Quality by Design (QbD) is included in the International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Q8 guideline and, by enabling the use of data observed throughout the life cycle, it provides more scientific data regarding the product quality and to critical process knowledge.[18] In the pharmaceutical industry, QbD is a very important contributor and emphasizes comprehension of process control, and quality products may be achieved more quickly with a systematic approach.[19-23]

In this study, ER formulations of quetiapine fumarate were prepared with xanthan gum or Carbopol 974P as matrix agents at their different ratios and an optimum formulation was developed that would be similar to the reference drug (Seroquel 200 mg XR tablet, AstraZeneca) in the market. In this context, the effect of granule size of bulk was investigated on the dissolution profiles of drug from formulated ER tablets. Critical quality characteristics and process parameter inputs were determined as polymer type-concentrations and sieving mesh sizes of dried granules, respectively. Then, a design study was conducted to follow the changes of the obtained outputs of the formulations and evaluated using FormRules V3.32, INForm programs.

EXPERIMENTAL

Equipment

The equipment used included the following: tablet press machine (Rimek-Minipress-II, Karnavati-India), dissolution test apparatus (Distek Syringe Pump-Evolution 4300 Dissolution Sampler - USA), friability machine (Caleva FT-15, UK), hardness test apparatus (Caleva THT-15, UK), magnetic stirrer (Hei-standard, Heidolph MR), pH meter (Seven Easy, Mettler Toledo-Switzerland), HPLC (Waters e2695 PDA detector, Waters-USA), ultrasonic bath (RK 1028 CH, Bandelin Sonorex Super), wet stoker (Mettler LP16, Mettler), oven (Nuve) KD 200, balance (Mettler PM100, Mettler-Switzerland), mechanical stirrer (Stirrer LH, Velp Scientifica), FormRules V3.32, INForm V.4 ANN, and INForm V.4 GEP softwares (INtelligent Ltd-UK).

Ingredients

Quetiapine (fumarate salt) (Aurobindo Pharma Limited, Unit-XI- India), MCC 101 (FMC Biopolymer, Newark, USA), lactose monohydrate (DMV, Netherlands), sodium citrate (Merck, Germany), Carbopol 974P (Lubrizol, USA), xanthan gum (JUNGBUNZLAUER, Switzerland), Mg stearate (FACI, Genoa, Italy) were pharma grade and all other chemicals were analytical grade.

Data set

In this study, polymer type, polymer concentrations, and sieving mesh size parameters were described as independent variables

(inputs); tablet weight, tablet hardness, friability, assay and dissolution percentage (%) at all of the time points used in the dissolution tests (30 min and 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 hours) were described as dependent variables (outputs). Therefore, two different polymer types (xanthan gum and Carbopol 974P), three different concentrations for each polymer [according to the preliminary study findings for xanthan gum; 16%, 24.5%, and 31% (w/w), for Carbopol 974P; 10%, 23%, and 50% (w/w)], and two different sieving mesh sizes (0.8 and 1.4 mm) for dry granules were suggested.

A systematic scientific approach was applied to control tablet production variables in the process design. For this purpose, a study design was formed by determining the critical parameters for the formulation within the framework of the QbD approach. These variables were also formed as the critical quality attributes. The main goal of our study was to be able to understand the possible effects of these parameters on the release profiles of ER quetiapine fumarate tablets. Then, training of the FormRules V.3.32 and INForm programs was conducted with the observed test results. The results showed that formulation F1 was the most similar formulation to the reference product chosen by the programs. In addition, formulation F13 was suggested as a new formula after optimization from INForm programs. Afterwards, F1 and F13 dissolution profiles were tested at three different pH values, as proposed by the ICH Q6 guidelines (pH 1.2, 4.5, and 6.8).

The dissolution profiles of F1 and F13 were compared with the reference product to determine whether it adhered to the compliance criteria.[24] The critical quality and process parameters (inputs) and quality target product profiles (outputs) in the process design are shown in Table 1, and the visualizations of the interactions between these properties are shown with their positions in a fishbone diagram in Figure 1.

Tablet formulation and manufacturing

All tablet formulations containing 230 mg of quetiapine fumarate were prepared with the wet granulation method (Table 2). A flow diagram of the production method is shown in Figure 2. In brief, quetiapine fumarate, lactose monohydrate, sodium citrate, microcrystalline cellulose 101, and the polymer (xanthan gum or Carbopol 974P) were weighed and sieved as the internal phase and mixed in a mechanical mixer for 10 min. Granulation was

Table 1. Dependent and independent variables of formulations in process design

Critical quality and proses parameters (Inputs)	Quality target product profile properties (Outputs)
Polymer type (Xanthan gum or Carbopol 974P) Polymer concentration Xanthan gum: (16-24.5-31%; w/w) Carbopol 974P: (10-23-50%; w/w) Sieve size (0.8 or 1.4 mm)	Tablet weight (mg) Hardness (n) Friability (%) Assay (%) Dissolution Amount of dissolved (%) in all time points (30 min, 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 h)

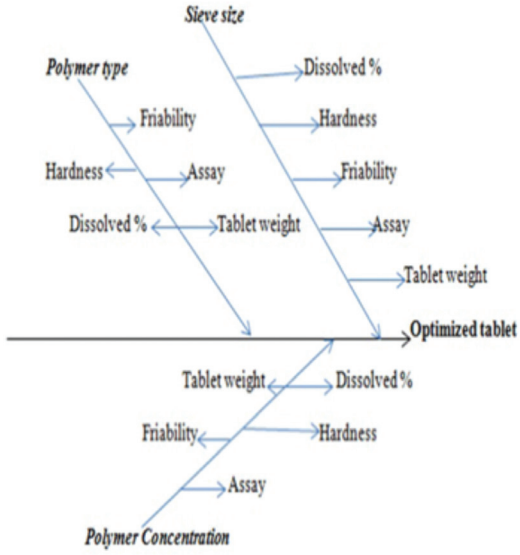


Figure 1. Critical characteristics of extended-release tablet formulation in fishbone model

Table 2. Unit formula ingredients of tablets

Ingredients (mg)	F1* F2**	F3* F4**	F5* F6**	F7* F8**	F9* F10**	F11* F12**
Quetiapine fumarate (200.0 mg Quetiapine equivalent)	230.0	230.0	230.0	230.0	230.0	230.0
Microcrystalline cellulose 101	100.0	100.0	100.0	100.0	100.0	100.0
Lactose monohydrate	52.63	52.63	52.63	52.63	52.63	52.63
Sodium citrate	88.86	88.86	88.86	88.86	88.86	88.86
Carbopol 974P	-	-	-	53.05	143.2	477.48
Xanthan gum	93.0	155.0	217.0	-	-	-
Mg stearate	6.0	6.0	6.0	6.0	6.0	6.0
Tablet weight	570.49	632.49	694.49	530.54	620.69	954.96

*F1, F3, F5, F7, F9, and F11 were sieved through 0.8 mm mesh screen; **F2, F4, F6, F8, F10, and F12 were sieved through 1.4 mm mesh screen

achieved by slowly adding distilled water to the dry mixture; when the granules reached the desired consistency, they were sieved with a 4-mm mesh screen. The wet granules were dried in an oven at 45˚C and then divided into two parts; one part was sieved through a 0.8 mm mesh screen and the other was sieved through a 1.4 mm mesh screen. Magnesium stearate was sieved and added to the granules as the outer phase. The product was mixed in a mechanical mixer for 5 min and tablets were compressed using a tablet compressor (Karnavati Tablet Compression Machine, India).

Tests for prepared tablets

Weight variation

For all formulations, 10 tablets were weighed one-by-one on a precision scale (Mettler-Switzerland), and average weight, standard deviation, and relative standard deviation values were calculated.

Determination of hardness

Ten tablets were taken from all formulations. Tablet hardness was measured using a hardness testing instrument (Caleva, UK). Average hardness, standard deviation, and relative standard deviation values were calculated.

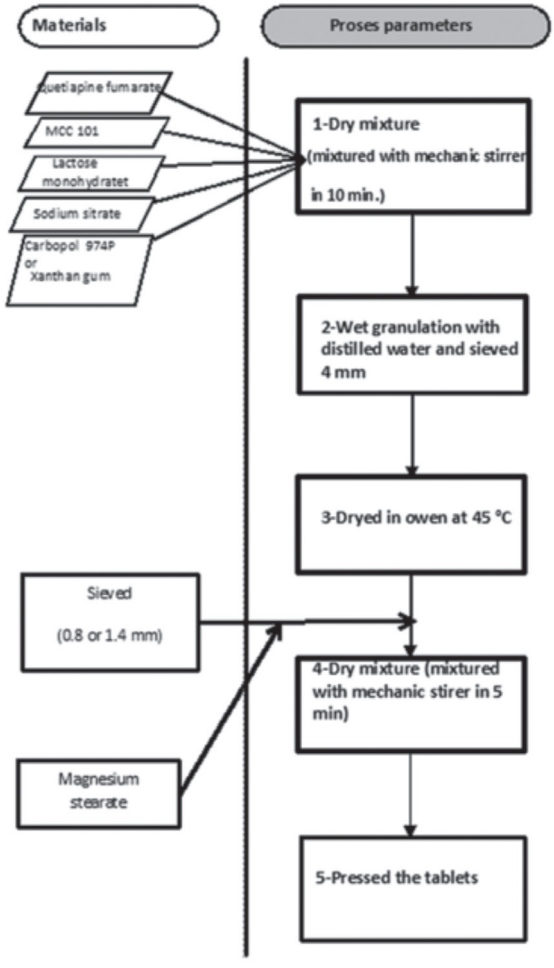


Figure 2. Process flow chart of extended-release tablets prepared

Friability test

From all formulations, 10 tablets were weighed and placed in a friability tester (Caleva, UK); the instrument was run, a test was conducted at 25 rpm for 4 min, and the tablets were weighed after the procedure. Percentage weight loss was calculated according to Equation 1[25]:

$$F(\%) = \frac{(Wi-Wf)}{Wi} x100 \qquad \textbf{(Equation 1)}$$

Where,

Wi = initial weight of tablets

Wf = weight after friabilityv

Assay

HPLC (Waters) was used for quetiapine fumarate analysis.[26] Method parameters are summarized as follows;

Detector: PDA

Mobile phase: pH 6.5 phosphate buffer-acetonitrile (60:40; v/v)

Column: Cromasil 100, C_{18} (250x4.6 mm), 5 µm

Wavelength: 225 nm

Flow rate: 1.5 mL/min

Retention time: 4 min

The determination of active substance amount in ER tablets containing 230 mg of quetiapine fumarate and dissolution rate test method validation (accuracy, precision, specificity, linearity, range, detection limit, quantitation limit, robustness, system suitability testing) were performed according to the ICH Q2 guidelines.[27]

Dissolution tests

For the similarity between the developed ER formulations of quetiapine fumarate and reference product, a dissolution test was performed in the medium (0.1 N HCl, 900 mL) registered in the literature. USP apparatus II with the paddle method was used for this test at 37˚C±0.5 at 50 rpm.[28] The experiments were performed six times. The analysis of samples, which were taken from the dissolution medium at the determined time intervals (at the end of 30 min and 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 hours) was performed and fresh dissolution medium was added. Dissolution tests were also conducted under the same conditions using different dissolution media including buffered solutions at pH 1.2, pH 4.5, and pH 6.8.

Evaluation of dissolution data

The similarity of dissolution profiles of ER quetiapine fumarate tablet formulations and that of the reference ER product were determined using the f2 test (similarity factor)[29] given in Equation 2:

$$f_2 = 50 \times \log\left\{\left[\frac{1}{\sqrt{1+{}^{1}\!/_{n}\sum_{j=1}^{n}(R_j - T_j)^2}}\right] \times 100\right\} \qquad \textbf{(Equation 2)}$$

Where *Rj* and *Tj* are the cumulative percentages dissolved at each of the selected "*n*" time points of the reference and test product, respectively. For similarity of the formulations, this factor should be between 50 and 100.

Software tools

Commercial artificial intelligence software tools were used to interrogate the production data generated in the studies. FormRules V3.32[30] and INForm programs are software packages developed by Intelligensys Ltd., UK.

RESULTS

Analytical validation results

Linearity concentrations range between 10-120%. The limit of detection value was 5.0 mg/mL and the limit of quantification value was 16.667 mg/mL. The other validation procedure results are shown in Table 3.

Physical tests results

Physical tests of the formulations were performed. The average diameter of the tablets was between 17.88-17.99 mm for formulations composed of both polymers, and the thickness values were between 5.36-6.01 mm for tablets prepared with xanthan gum, and between 5.21-8.20 mm for the Carbopol 974P series. The assay results of the tablets were between 98.07-102.00%. Other physical tests results are shown in Table 4. All parameters were within the Pharmacopoeia limits.[31,32]

Dissolution tests results

The dissolution profiles of all formulations were compared with that of the reference product. All dissolution studies were performed in the best-dissolved medium of quetiapine fumarate for 24 hours (pH 1.2) (Figure 3). The similarity factor (f2) values for all formulations were calculated and are shown as a histogram in Figure 4. The f2 similarity values of formulations coded with F1, F2, F3, F4, F6, and F12 were calculated as 69, 52, 63, 68, 68, and 55, respectively. The formulations coded as F1, F4, and F6 were highly similar to the reference product.

FormRules program results

Training of programs was conducted using the physical and chemical test results of all formulations, and the results were evaluated. The r^2 value was 0.99 for the average weight data and the polymer concentration was the dependent variable. Polymer concentrations and hardness values can influence each other moderately, as such, the dependent variables and models also have influence.

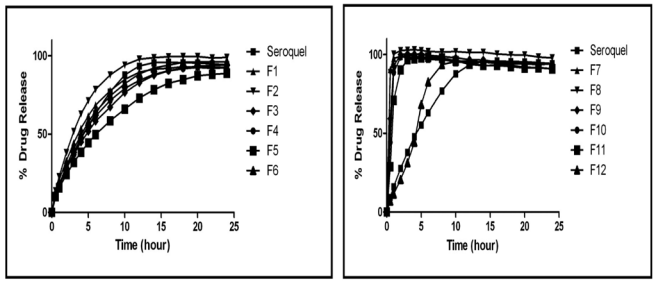


Figure 3. Dissolution profiles of all formulations developed and reference product at pH 1.2 (n=6)

Table 3. Results of analytical validation procedure

Validation characteristics		Results	
Specificity		There was no peak observed at the time of active substance retention time from the solvent or placebo.	
System repeatability		RSD %: 1.989	
Linearity		R^2: 1	
Range		RSD %: 1.72 (10%) RSD %: 0.65 (120%) No deviation observed in the specified range	
Accuracy		Xanthan gum	Carbopol 974P
		Yield %: 98.11-100.27-98.93	Yield %: 99.71-100-100.75
Precision		RSD %: 1.918-91.50%	RSD %: 1.921-94.47%
Intermediate precision		RSD %: 0.748 Contrast %: 3.83	RSD %: 1.838 Contrast %: 3.46
Robustness	Mobil phase flow rate	1.35 mL/min: Contrast %: 1.88 1.65 mL/min: Contrast %: 2.03	1.35 mL/min: Contrast %: 0.19 1.65 mL/min: Contrast %: 4.59
	Column temperature	20°C: Contrast %: 0.25 30°C: Contrast %: 0.82	20°C: Contrast %: 0.81 30°C: Contrast %: 0.52
Solution stability		Standard and sample solutions were stable at 25°C and +5°C for 48 hours	

RSD: Relative standard deviation

Table 4. Tablet weight, hardness, friability (%) results of all formulations

Formula	Tablet weight		Hardness		Friability (%)
	(mg)	SD (+)	(n)	SD (+)	
F1	560.20	2.05	187.00	21.02	0.030
F2	571.58	2.22	133.10	16.44	0.140
F3	623.40	0.81	207.00	31.41	0.030
F4	634.70	1.08	239.00	19.12	0.42
F5	693.20	0.76	227.00	14.68	0.050
F6	693.35	4.81	169.00	28.07	0.068
F7	531.41	5.13	131.30	37.86	0.190
F8	534.79	3.32	157.90	44.07	0.260
F9	623.95	3.32	242.50	19.63	0.063
F10	622.78	3.33	254.70	4.24	0.061
F11	960.64	3.12	255.40	0.70	0.120
F12	962.32	1.56	256.30	2.16	0.120

SD: Standard deviation

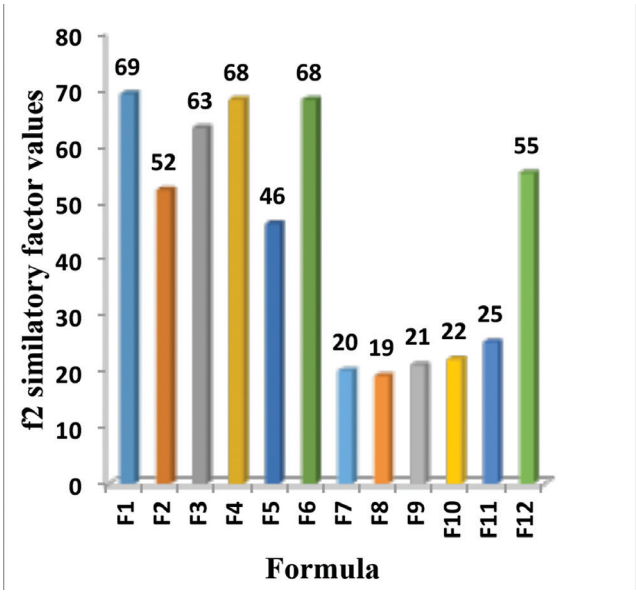


Figure 4. Similarity factor (f2) values of all tablet formulations

The dependent variables of polymers are highly important for the dissolution rate of drugs. If the polymer type and concentration change, the drug dissolution profiles would also change. When Carbopol 974P was used, the dissolution percentage of drug was high, and the dissolution behavior of drug was not affected due to the particle size.[33] When xanthan gum was used as the polymer, the dissolution percentage of drug was decreased.[34,35] The change in drug release by polymer type observed in this study complies with the literature. The release of active substance from hydrophilic matrix tablets is related to a product's own behavior, and the typical characteristics of the drug together with the properties of the polymer, i.e., molecular weight, hydrophilicity, and degree of cross-linking.[36] An inverse proportionality was detected between polymer concentration and dissolution percent of drug. Therefore, it was suggested that *in vitro* release behavior of drug was related to polymer concentration. Increased polymer concentration led to decreased diffusion of drug in the dissolution medium. Thus, the types and concentrations of polymers are crucial factors that affect dissolution of drugs.[37]

The similarity values were obtained using the FormRules program. Formulation F1 was found as the most similar to the reference product (100%). This formulation also showed the highest similarity to the reference product (69%) in terms of f2 similarity factor when the dissolution study was performed at pH 1.2.

INForm program results

All results were also evaluated using the INForm programs. It was observed that the r^2 values ranged from 74.31 to 99.97 for weight, hardness, friability (%), assay, and dissolution rates (%) of tablets.

When the r^2 values were high, the model was well-built, and the program was conditioned. F1 was the most similar formulation to the reference product according to the INForm data.

DISCUSSION

ER oral tablets are required to meet similar behaviors in the gastrointestinal tract in which physiologic fluids are at different pH values. Thus, *in vitro* dissolution studies are needed to be performed at three different pH values that mimic *in vivo* conditions. Following the evaluation of all dissolution studies and assessment of program data, formulation F1 was determined as the most similar to the reference product. The dissolution tests were performed in dissolution media at pH 1.2, 4.5, and 6.8. Although formulation F1 satisfied the ICH guideline[24] limits at pH 1.2 and pH 6.8 (f2 values were 69 and 95, respectively), the f2 value for this formulation was calculated as 23 in pH 4.5 dissolution medium. Therefore, the formulation was not assessed as similar to the reference product.

Furthermore, the INForm programs recommended a new optimized formulation, which had the highest similarity to the reference product. However, the two formulations were very similar in terms of drug dissolution rates. Therefore, we chose the formula recommended by the ANN program, and the tablets were prepared according to this proposed formula (F13) in which xanthan gum was used as the polymer matrix agent [20.35% (w/w)] and the granule size was 1.0 mm.

To prove the similarity of the F13 formulation to the reference product, *in vitro* dissolution studies were performed using the dissolution method as explained above. The dissolution profiles of drug performed at three different pH values are shown in Figures 5-7. F2 similarity values of formulation F13 at pH 1.2, 4.5, and 6.8 were calculated as 76, 50, and 62, respectively.

The formulation selected by the FormRules (F1) and the INForm programs (F1) that showed the most similarity to the reference product did not show the expected performance in *in vitro*

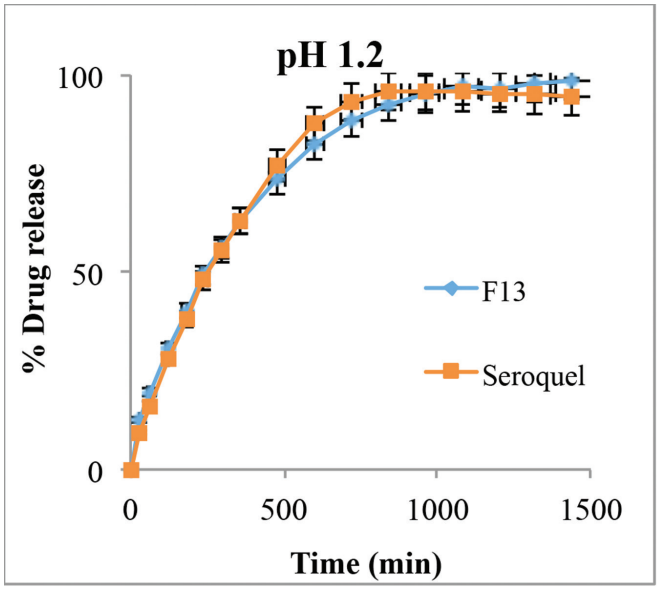


Figure 5. Dissolution profiles of F13 and reference product at pH 1.2 (n=6)

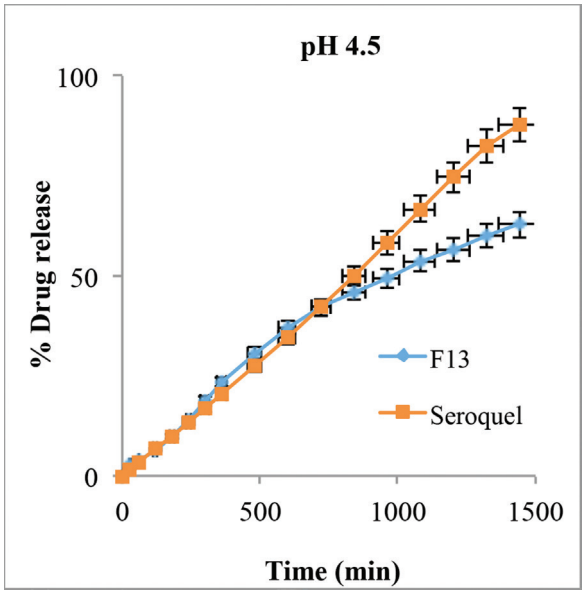


Figure 6. Dissolution profiles of F13 and reference product at pH 4.5 (n=6)

release studies. However, tablets with required performances were obtained in a shorter time with fewer trials due to the optimized formula suggestion of the INForm programs.

Similar programs have been used for the controlled-release formulation of clopidogrel using a matrix agent (Methocel K100M).[38] In another study, the formulation components (diluent, binder, and their concentrations) and operation variables (type of the granulator and addition of the binder) of caffeine were evaluated and optimized formulas were obtained.[39] In addition, the controlled-release tablets of theophylline were developed using a study of simultaneous optimization technique.[40] All these studies indicated that these programs performed better estimation than those of current statistical methods.

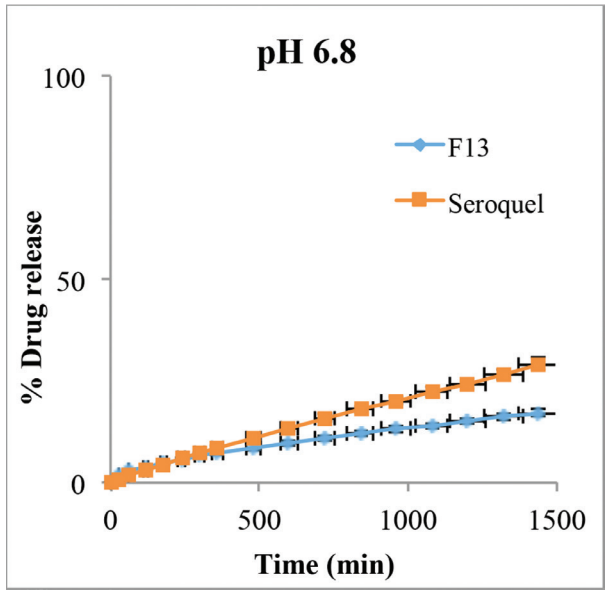


Figure 7. Dissolution profiles of F13 and reference product at pH 6.8 (n=6)

It also should be kept in mind that these programs have some disadvantages. The most important is that models can be over-trained. In that case, the value of r^2 might reach 0.95 or higher, and the background color turns to red. In such a case, model parameters are needed to be reviewed, checked, and retrained.

In our study, a release tablet formulation of quetiapine fumarate using a different polymer than that of its marketed product was optimized with lower cost and fewer trials, and in a shorter time by using ANN programs.

CONCLUSION

The greatest benefit of the FormRules program was understandable models, impressive experimental results and clearly described mathematical concept data. The program generated a rule base and contained all observations, experiences, and mathematical connections related to the subject, i.e., all information. The better and broader the rule base was prepared, the more precise and correct results were obtained. It presented data visually as three-dimensional graphs, which allowed us to extract the information quickly and easily, therefore enabling us to see which input was really effective on our formulation and also facilitating our decisions on how to obtain the most efficient results.

Using these programs, we attained information that tablets composed of xanthan gum were more successful than those that consisted of Carbopol 974P in extending the release of quetiapine fumarate from an ER tablet formulation. We evaluated the parameters including polymer concentration and mesh size of granules. A genetic algorithm-based optimization was performed and an optimized formula similar to the reference product was recommended. Without genetic algorithm-based optimization, more formulation trials would have been necessary to obtain the optimum formula. Consequently, it

has been shown that an ER tablet formulation of quetiapine fumarate could be optimized quickly and at a lower cost.

ACKNOWLEDGEMENTS

This study was supported by Research Fund of the İstanbul University project number: 26586. An application for a patent has been made to the Turkish Patent Institute using the results of this study (Application number: TR 2013-G-316804).

Conflict of Interest: No conflict of interest was declared by the authors.

REFERENCES

1. Gawali P, Gupta A, Kachare S, Kshirsagar S. Formulation and evaluation of matrix-based sustained release tablets of Quetiapine fumarate and the influence of excipients on drug release. J Chem Pharm Res. 2012;4:3073-3081.
2. Völgyi G, Baka E, Box KJ, Comer JE, Takács-Novák K. Study of pH-dependent solubility of organic bases. Revisit of Henderson-Hasselbalch relationship. Anal Chim Acta. 2010;673:40-46.
3. Pasha MK, Velrajan G, Balasubramaniam V, Dayakar B, Shivakumar B. Formulation and evaluation of extended release matrix tablets of quetiapine fumarate tablets. Int J Pharm. 2013;3:14-19.
4. Hiremath PS, Saha RN. Controlled release hydrophilic matrix tablet formulations of isoniazid: design and *in vitro* studies. AAPS PharmSciTech. 2008;9:1171-1178.
5. Wen H, Park K. Oral controlled release formulation design and drug delivery: Theory and Practice. In: Wen H, Park K. New Jersey, USA; Wiley; 2010. pp. 8.
6. García-Ochoa F, Santos VE, Casas JA, Gómez E. Xanthan gum: production, recovery, and properties. Biotechnol Adv. 2000;18:549-579.
7. Peh KK, Wong CF. Application of similarity factor in development of controlled-release diltiazem tablet. Drug Dev Ind Pharm. 2000;26:723-730.
8. Kristensen HG, Schaefer T. Granulation: A review on pharmaceutical wet granulation. Drug Dev Ind Pharm. 1987;13:803-872.
9. Mandal U, Gowda V, Ghosh A, Bose A, Bhaumik U, Chatterjee B, Pal TK. Optimization of metformin HCl 500 mg sustained release matrix tablets using Artificial Neural Network (ANN) based on Multilayer Perceptrons (MLP) model. Chem Pharm Bull. 2008;56:150-155.
10. Aksu B. Innovative pharmaceutical manufacturing practices in Ramipril tablet manufacturing process, Doctorate Thesis, pp. 34, Department of Pharmaceutical Technology, Ege University of Izmir, 2010.
11. Golla S, Neely BJ, Whitebay E, Madihally S, Robinson RL Jr, Gasem KA. Virtual design of chemical penetration enhancers for transdermal drug delivery. Chem Biol Drug Des. 2012;79:478-487.
12. Almeida JS. Predictive non-linear modeling of complex data by artificial neural networks. Curr Opin Biotechnol. 2002;13:72-76.
13. Peh KK, Lim CP, Quek SS, Khoh KH. Use of artificial neural networks to predict drug dissolution profiles and evaluation of network performance using similarity factor. Pharm Res. 2000;17:1384-1388.
14. Mesut B, Aksu B, Ozsoy Y. Design of Sustained Release Tablet Formulations of Alfuzosin HCl by means of Neuro-Fuzzy Logic. Lat Am J Pharm. 2013;32:1288-1297.
15. Intelligensys Ltd. UK, 2009. Available from http://www.intelligensys.co.uk/
16. Dematas M, Shao Q, Shukla R. Artificial intelligence the key to process understanding. Pharm Tech Eur. 2007;19:44-49.
17. Colbourn E, Rowe CR. Neural Computing and Formulation Optimization. Encyclopedia of Pharmaceutical Technology. In: Swarbrick J. New York; Informa Healthcare; 2007. pp. 2399-2412.
18. Aksu B, De Matas M, Cevher E, Özsoy Y, Güneri T, York P. Quality by design approach for tablet formulations containing spray coated ramipril by using artificial intelligence techniques. Int J Drug Delivery. 2012;4:59-69.
19. Wu H, Khan MA, Hussain AS. On-line process control and process analytical technology: integration of chemical engineering practice into semiconductor and pharmaceutical industries. Chem Eng Commun. 2007;194:760-779.
20. Yu LX. Pharmaceutical quality by design: product and process development, understanding, and control. Pharm Res. 2008;25:781-791.
21. Verma S, Lan Y, Gokhale R, Burgess DJ. Quality by design approach to understand the process of nanosuspension preparation. Int J Pharm. 2009;377:185-198.
22. Aksu B, Paradkar A, de Matas M, Özer Ö, Güneri T, York P. A quality by design approach using artificial intelligence techniques to control the critical quality attributes of ramipril tablets manufactured by wet granulation. Pharm Dev Technol. 2013;18:236-245.
23. Aksu B, Paradkar A, de Matas M, Ozer O, Güneri T, York P. Quality by design approach: application of artificial intelligence techniques of tablets manufactured by direct compression. AAPS PharmSciTech. 2012;13:1138-1146.
24. ICH Q6A Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances. Available from www.ich.org. Accessed January 24, 2008.
25. Uddin MS, Al Mamun A, Tasnu T, Asaduzzaman M. In-process and finished products quality control tests for pharmaceutical tablets according to pharmacopoeias. J Chem Pharm Res. 2015;7:180-185.
26. Bharathi Ch, Prabahar KJ, Prasad ChS, Srinivasa Rao M, Trinadhachary GN, Handa VK, Dandala R, Naidu A. Identification, isolation, synthesis and characterization of impurities of quetiapine fumarate. Pharmazie 2008;63:14-19.
27. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceutical for Human Use, Validation of Analytical Procedures: Methodology, Q2 (1995).
28. Draft guidance on quetiapine fumarate, Recommended July 2008, FDA. Available from http://www.fda.gov/downloads/Drugs/.../Guidances/UCM089520.pdf, Accessed: July 2009.
29. Guidance for Industry, Dissolution Testing of Immediate Release Solid Oral Dosage Forms, U.S. Department of Health and Human Services, Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER), August 1997, pp.8. Available from http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm070237.pdf f2.
30. Shao Q, Rowe RC, York P. Investigation of an artificial intelligence technology Model trees. Novel applications for an immediate release tablet formulation database. Eur J Pharm Sci. 2007;31:137-144.
31. Tablet friability, Final text for addition to The International Pharmacopoeia, World Health Organization, Document QAS/11.414 FINAL March

2012. Available from: http://www.who.int/medicines/publications/pharmacopoeia/TabletFriability_QAS11-414_FINAL_MODIFIED_March2012.pdf.

32. United States Pharmacopoeia Convention. United States Pharmacopoeia 38-National Formulary 33. USA; Stationery Office; 2010.
33. Soppela I, Airaksinen S, Hatara J, Räikkönen H, Antikainen O, Yliruusi J, Sandler N. Rapid particle size measurement using 3D surface imaging. AAPS PharmSciTech. 2011;12:476-484.
34. Kanwar N, Kumar R, Sarwal A, Sinha VR. Preparation and evaluation of floating tablets of pregabalin. Drug Dev Ind Pharm. 2016;42:654-660.
35. Pandey S, Shah RR, Gupta A, Arul B. Design and Evaluation of buccoadhezive controlled release formulations of prochlorperazine maleate. Int J Pharm Pharm Sci. 2016;8:375-379.
36. Körner A, Piculell L, Iselau F, Wittgren B, Larsson A. Influence of different polymer types on the overall release mechanism in hydrophilic matrix tablets. Molecules. 2009;14:2699-2716.
37. Fu XC, Wang GP, Liang WQ, Chow MS. Prediction of drug release from HPMC matrices: effect of physicochemical properties of drug and polymer concentration. J Control Release. 2004;95:209-216.
38. Tan C, Degim IT. Development of sustained release formulation of an antithrombotic drug and application of Fuzzy logic. Pharm Dev Technol. 2012;17:242-250.
39. Kesevan JG, Peck GE. Pharmaceutical granulation and tablet formulation using neural networks. Pharm Dev Technol. 1996;1:391-404.
40. Takayama K, Morva A, Fujikawa M, Hattori Y, Obata Y, Nagai T, Formula optimization of theophylline controlled release tablet based on artificial neural networks, J Control Release. 2000;6:175-186.

Turk J Pharm Sci 2017;14(3):222-230
DOI: 10.4274/tjps.80299

ORIGINAL ARTICLE

Cytotoxic Activities of Certain Medicinal Plants on Different Cancer Cell Lines

Bazı Tıbbi Bitkilerin Farklı Kanser Hücre Hatlarında Sitotoksik Aktiviteleri

Deniz UĞUR[1], Hatice GÜNEŞ[1]*, Fatma GÜNEŞ[2], Ramazan MAMMADOV[3]

[1]Muğla Sıtkı Koçman University, Faculty of Science, Department of Biology, Muğla, Turkey
[2]Trakya University, Faculty of Pharmacy, Department of Pharmaceutical Botany, Edirne, Turkey
[3]Pamukkale University, Faculty of Science, Department of Biology, Denizli, Turkey

ABSTRACT

Objectives: In recent years, the use of plants for the prevention and treatment of cancer is gaining more attention due to their diverse range of phytochemical constituents and fewer adverse effects. In this study, four medicinal plant species from the Kars province of Turkey were investigated for their cytotoxic potential against six different cancer cell lines and one normal cell line.

Materials and Methods: MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-dipenyltetrazolium bromide] assay was performed to assess cytotoxic activity and apoptotic effect was determined using flow cytometry and caspase-3 analyses.

Results: Significant cytotoxicity (≥70%) was observed with the leaf extract of *Artemisia absinthium* on A-549, CCC-221, K-562, MCF-7, PC-3 cells, whereas seed extracts caused significant cytotoxicity (≥70%) on CCC-221, K-562, MCF-7, PC-3 cells. Selective cytotoxicity was obtained with leaf extract on A-549 and K-562 cells; and with seed extract on K-562, MCF-7 and PC-3 cells compared with normal Beas-2B cells. The levels of cytotoxicity for both extracts were time- and dose-dependent at lower concentrations. Moreover, selective cytotoxicity (78%) was detected on A-549 cells with the seed extract of *Plantago major*. Cytotoxicity of extracts from *Hyoscyamus niger* and *Amaranthus retrosa* ranged between 10% and 30%.

Conclusion: *A. absinthium* extracts and *P. major* seed extract have potential for development as therapeutic agents for cytotoxicity on certain cancer cells following further investigation.

Key words: Cytotoxicity, cancer cells, medicinal plants, apoptosis, *Artemisia absinthium*

ÖZ

Amaç: Son yıllarda kanserin önlenmesi ve tedavisi için, farklı sınıfta fitokimyasal içerikleri ve daha az yan etkileri nedeniyle bitkilerin kullanımı oldukça önem kazanmaktadır. Bu çalışmada, Türkiye'nin Kars ilinden toplanan dört farklı tıbbi bitki türünün altı farklı kanser hücre hattı ve bir normal hücre hattı üzerindeki sitotoksik etkileri incelenmiştir.

Gereç ve Yöntemler: Sitotoksik aktivite MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-dipenyltetrazolium bromide] yöntemiyle belirlenirken, apoptotik hücre ölümü akış sitometri ve kaspaz-3 analizleriyle tespit edilmiştir.

Bulgular: *Artemisia absinthium* yaprak özütü A-549, CCC-221, K-562, MCF-7, PC-3 hücre hatları üzerinde anlamlı sitotoksik etki (≥%70) gösterirken, tohum özütü ise CCC-221, K-562, MCF-7, PC-3 hücreleri üzerinde anlamlı sitotoksik etki (≥%70) göstermiştir. Normal hücre hattı Beas-2B hücresine kıyasla, seçici sitotoksik aktivite yaprak özütüyle A-549 ve K-562 hücreleri üzerinde gözlenirken, tohum özütüyle K-562, MCF-7 ve PC-3 hücreleri üzerinde gözlenmiştir. Her iki özütün sitotoksisite seviyesi düşük konsantrasyonlarda zaman ve doza bağlı olarak değişmektedir. İlave olarak, *Plantago major* bitki özütüyle A-549 hücreleri üzerinde seçici sitotoksisite (%78) elde edilmiştir. *Hyoscyamus niger* ve *Amaranthus retrosa* özütlerinin sitotoksisite aktivitesinin %10 ve %30 olduğu gözlenmiştir.

Sonuç: *A. absinthium* özütleri ve P. major tohum özütü gelecek çalışmalarda bazı kanser hücrelerinin sitotoksisitesi için tedavi edici ilaç geliştirilmesi potansiyeline sahiptirler.

Anahtar kelimeler: Sitotoksisite, kanser hücreleri, tıbbi bitkiler, apoptozis, *Artemisia absinthium*

*Correspondence: **E-mail:** haticegunes@mu.edu.tr, **Phone:** +90 534 282 11 33 **ORCID-ID:** orcid.org/0000-0001-5191-365X
Received: 26.10.2016, **Accepted:** 02.02.2017

INTRODUCTION

Cancer is the most common and lethal disease in the modern era.[1] New strategies or compounds need to be discovered because most known cancer treatments have adverse effects and all tumors do not react in the same way to treatment. Plant-based medicines have good potential as a primary source for chemotherapeutic drugs. Some of the currently-used chemotherapeutic agents such as paclitaxel for breast cancer, vinca alkaloids for leukemia, and flavopiridol for colorectal cancer were initially derived from plants.[2-5] In addition, herbal products are still used as primary health care products in most third world countries.[6] Accordingly, plants as herbal medicines are good sources to search for antitumor compounds.

Traditional studies of plants use two parts, subterranean and surterranean. While almost all subterranean parts of the plant consist of root, surterranean parts of the plant have the bark, leaves, flowers, and seeds. All of these are derived from shoot apical meristem, but they are highly differentiated and consist of different secondary metabolites in different compositions.[7] In addition, medical uses of such plants are also specific to different parts of the plant, or in some cases, highly specific to certain regions of some parts. Therefore, it is important to clarify the part of the plant to be used for the source of therapeutic agents.

Studies have demonstrated that most phytochemicals act by interfering with several cell-signaling pathways and lead to cell cycle arrest and/or differentiation induction, apart from their apoptosis-inducing potential.[8] Apoptosis is a central event essential in the maintenance of tissue homeostasis for all organ systems in the human body.[9] Suppression of apoptosis in carcinogenesis plays a central role in the development and progression of cancer. Tumor cells use a variety of molecular mechanisms to suppress apoptosis.[10] Hence, induction of apoptosis in tumor cells is a specific therapeutic approach towards cancer chemotherapy.

Artemisia species belong to the *Asteraceae* family and are widely distributed in Asia, Europe, and North America. Medicinal values of *Artemisia* species have been approved throughout the world. Pharmacologic studies of *Artemisia annula* indicated the presence of novel biologically active compounds such as monoterpenes, sequterpenes, lactones, flavonoids, and coumarins.[11] The extracts of some *Artemisia* species have been reported to exhibit cytotoxic effects on breast cancer cells.[12] Moreover, *Plantago* species have been used against cancer and Gálvez et al.[13] showed cytotoxic activities of luteolin-7-0-beta-glucoside, a major flavonoid against human renal adenocarcinoma and human melanoma. In contrast to *Artemisia* and *Plantago* species, not much information is available for *Hyoscyamus niger* and *Amaranthus retrosa* on cytotoxic activities against cancer cells.

Turkey is at the cross section of three phytogeographic regions, and is therefore very rich in terms of biodiversity.[14] More than 12.000 species and sub-species are considered to be in the region and they are used medically, as well as in the form of food and feed.[15-17] The Kars province in the Eastern region of Turkey has a tradition of using herbal medicine for a variety of diseases, from headaches to cancer.[15] *Artemisia* species from other countries are established to be cytotoxic in cancer[12,18]; however, there are no reports on the cytotoxicity of other plants on cancer cells. Although the chemical composition and biologic activities of many plant species are known in the literature, the level and quality of compounds, as well as their biologic activities can change significantly according to geographic origin and growing conditions. Therefore, this is the first study to evaluate the anticancer activities of four medicinal plants from Kars, which has 1800 m altitude. Ethanolic extracts of these plant parts were examined for their cytotoxicity on six cancer cell lines and one normal cell line according to time and concentration as variables. Furthermore, the mechanism of cytotoxicity was analyzed for *Artemisia absinthium* on A-549 cells using flow cytometry and a caspase-3 assay.

MATERIALS AND METHODS

Cell lines and culture conditions

A-549 (human lung carcinoma), CCC-222 (human colorectal carcinoma), DU-145 (human prostatic carcinoma), MCF-7 (human breast carcinoma), K-562 (human leukemic carcinoma), and PC-3 (human prostatic carcinoma) cell lines were procured originally from ATCC. All cell lines were maintained in RPMI 1640 culture medium (Biochrom, Germany), supplemented with 10% fetal bovine serum (FBS) (FBS; Biochrom, Germany), penicillin (100 U/mL) and streptomycin sulphate (100 mg/mL) (Biochrom, Germany). Cells were incubated at 37°C in 5% CO_2, 95% air in a humidified incubator.

Plant material

Four different plant species, *A. absinthium* L. (*Asteraceae*), *H. niger* L. (*Solanaceae*), *A. retrosa* L. (*Amaranthaceae*) and *Plantago major* L. (*Plantaginaceae*) were collected from Kafkas University Campus in July and authenticated by Dr. Fatma Güneş in the Botanic Laboratory. The herbarium numbers of plants are *A. absinthium* L. (FG 2562); *A. retrosa* L. (FG 2563); *H. niger* L. (FG 2564); *P. major* L. (FG 2565). These plants are deposited in Trakya University, Herbarium of Faculty of Pharmacy.

Plant extraction

The plant samples were air-dried under shade and milled to a powder using a porcelain muller. Powdered plant materials (10 g) were extracted with 100 mL of ethanol several times by shaking at 53.1-54.6°C until the color faded. The extracts were filtered and solvent was removed in a vacuum using a rotary evaporator at 42-43°C in an Erlenmeyer bulb. After that, extracts was lyophilized to give the crude dry extract in freeze-dryer. The powdered crude extracts were stored at -20°C until used. All extracts were dissolved in 10% DMSO as stock solution and further dilutions were freshly prepared to achieve working solutions.

MTT assay

The cytotoxic activity of plant extracts was determined using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-dipenyltetrazolium

bromide] assay (Applichem, USA). This assay detects the reduction of MTT by mitochondrial dehydrogenase to a blue formazan product, which reflects the function of mitochondria and cell viability.[19] Exponentially growing cells at $2x10^4$ cells/mL were plated in triplicate into 96-well plates (Greiner, Germany) in 200 µL of growth medium and incubated for 24 hr before the addition of extracts. Plant extracts were dissolved in 10% DMSO and added to the cell culture at a final concentration of 4 mg/mL to be tested against six cell lines. Cells were incubated for 72 hr at 37°C in a 5% CO_2 incubator. For the dose and time responsiveness of the cells, plant extracts were added at 0.1 mg/mL, 0.2 mg/mL, 0.5 mg/mL, 1 mg/mL, and 2 mg/mL final concentrations, and incubated for 24, 48, and 72 hr. After that, 10 µL of PBS containing 5 mg/mL MTT was added into each well. After 4 hr incubation, the medium was discarded and the formazan blue crystals that formed in the cells were dissolved in 100 µL DMSO. Reduced MTT was quantified by reading the absorbance at 540 nm on a microplate reader (Thermo Scientific Multiscan Spectrum). The cytotoxic effects of the tested extracts were determined by comparing the optical density of the treated cells against the optical density of the untreated cells. Cytotoxicity relative to controls was calculated using the following formula:

$$\% \text{ Cytotoxicity} = [(A_c - A_t)/A_c)] \times 100 \quad (17)$$

Where A_c and A_t are mean absorbance of the control wells and mean absorbance of test wells, respectively.

Caspase-3 assay

Caspase-3 activity was determined using caspase-3 colorimetric assay kit (Abcam, USA). This assay measures the amount of free p-nitroanilide (pNA) in the cell produced by the cleavage of DEVD-pNA bond by activated caspase-3 during apoptosis. The experiment was conducted in accordance with the manufacturer's protocol. In brief, A549 cells were treated with 4 mg/mL of *A. absinthium* extract for 36 hr. After that, cells were trypsinized and centrifuged at 1100 g for 10 min. Cells were resuspended in 1 mL of cold PBS and centrifuged at 4000 g for 5 min at 4°C. In order to extract total protein, cell pellets were suspended and lysed with 100 µL of lysis buffer and incubated on ice for 10 min. The cell lysates were then centrifuged at 10.000 g for 1 min at 4°C. The protein concentration was measured using a Bradford assay. Then, 200 µg protein from each sample was mixed with 50 µL of 2x reaction buffer containing 10 mM DTT and 5 µL of 4 mM caspase-3 substrate (DEVD-pNA) and incubated at 37°C for 4 h. The p-NA light emission was quantified using an ELISA plate reader at 405 nm. The fold increase in caspase-3 activity was determined by comparing the absorbance of p-NA from an apoptotic sample with an untreated control cells.

Flow cytometry

A-549 cells were cultured in 6-well plates ($5x10^5$ cells/well) and treated with 0.25 mg/mL, 1 mg/mL, 2 mg/mL, and 4 mg/mL concentrations for both *A. absinthium* leaf and seed for 24 hr. After treatment, cells were washed with PBS, trypsinized, washed, and resuspended in binding buffer. Five microliters of propodium iodine (PI) and 5 µL Annexin-V-FITC were added. After incubation for 15 min in the dark, 500 µL of binding buffer was added and ten thousand cells per group were analyzed using flow cytometry (BD FACSCanto A, BD Biosciences, USA). To detect PI and AnnexinV-FITC, green solid state 488 laser was used for excitation. Filter configurations for PI and FITC were 556/LP and 585/40, and 502/LP and 530/30, respectively.

Statistical analysis

Data were analyzed using the Microsoft Office Excel (2007) SPSS software package. Multiple comparisons of treatments were performed using one-way ANOVA and Student's t-test. A difference was considered to have significance at $*p<0.05$, $*p<0.01$, and $*p<0.005$. Data are presented as mean ± standard deviation of three replicates.

RESULTS

Screening of cytotoxic effects of plant extracts on different cancer cell lines

Six different cancer cell lines were treated with 4 mg/mL leaf or seed extracts for 72 hr to distinguish the effectiveness of each part of the plant on cytotoxicity (Figure 1). The results indicated that *A. absinthium* extracts showed toxicity between 40% and 87% on all cell lines, except DU-145 cells (Figure 1a, b). Unlike *A. absinthium* extracts, other extracts exhibited either cytotoxic or mitogenic effects depending on the cell lines. The least toxic extract was the *A. retrosa* seed, which showed 25% cytotoxicity on DU-145 cells. In addition, leaf extract of *A. retrosa* was cytotoxic on DU-145 and K-562 cell lines by 22% and 28%, respectively (Figure 1e, f). With the exception of the *H. niger* seed extract, all extracts resulted in cytotoxicity between 20% and 30% on DU-145 cells.

The most effective extracts on cytotoxicity of K-562 cell lines were *A. absinthium* leaf (86%) and *A. retrosa* leaf (28%). In addition, the highest cytotoxicity (78%) for A-549 cells was obtained from *P. major* seed extract (Figure 1h). Among the other plant extracts, *P. major* seed was the best extract for selective cytotoxicity on A-549 cancer cell lines because it did not cause cytotoxicity on the normal Beas-2B cell line.

Two statistical analyses were performed to see if the effects of extracts on cell lines were significant compared with the control containing no extract (Table 1) and the normal cell line, Beas-2B (Table 2). The most significant cytotoxic effects were exhibited by *A. absinthium* leaf and seed extracts, and *P. major* seed extract (Table 1). To date, most chemotherapeutic agents do not distinguish between normal and cancer cell lines. In the interest of having less damage to normal cells after cancer therapy, it is important to discover plant extracts with selective cytotoxicity for cancer cells. Therefore, the cytotoxic effect of plant extracts on cancer cells were compared with that of normal Beas-2B cells. According to the statistical analysis, *A. absinthium* seed extracts caused significant cytotoxicity on K-562, MCF-7, and PC-3 cell lines (Table 2). In addition, significant cytotoxic effects compared with Beas-2B cells were observed with *A. retrosa* leaf extract on DU-145 and K-562 cell lines, and *P. major* seed extracts on A-549 and DU-145 cell lines.

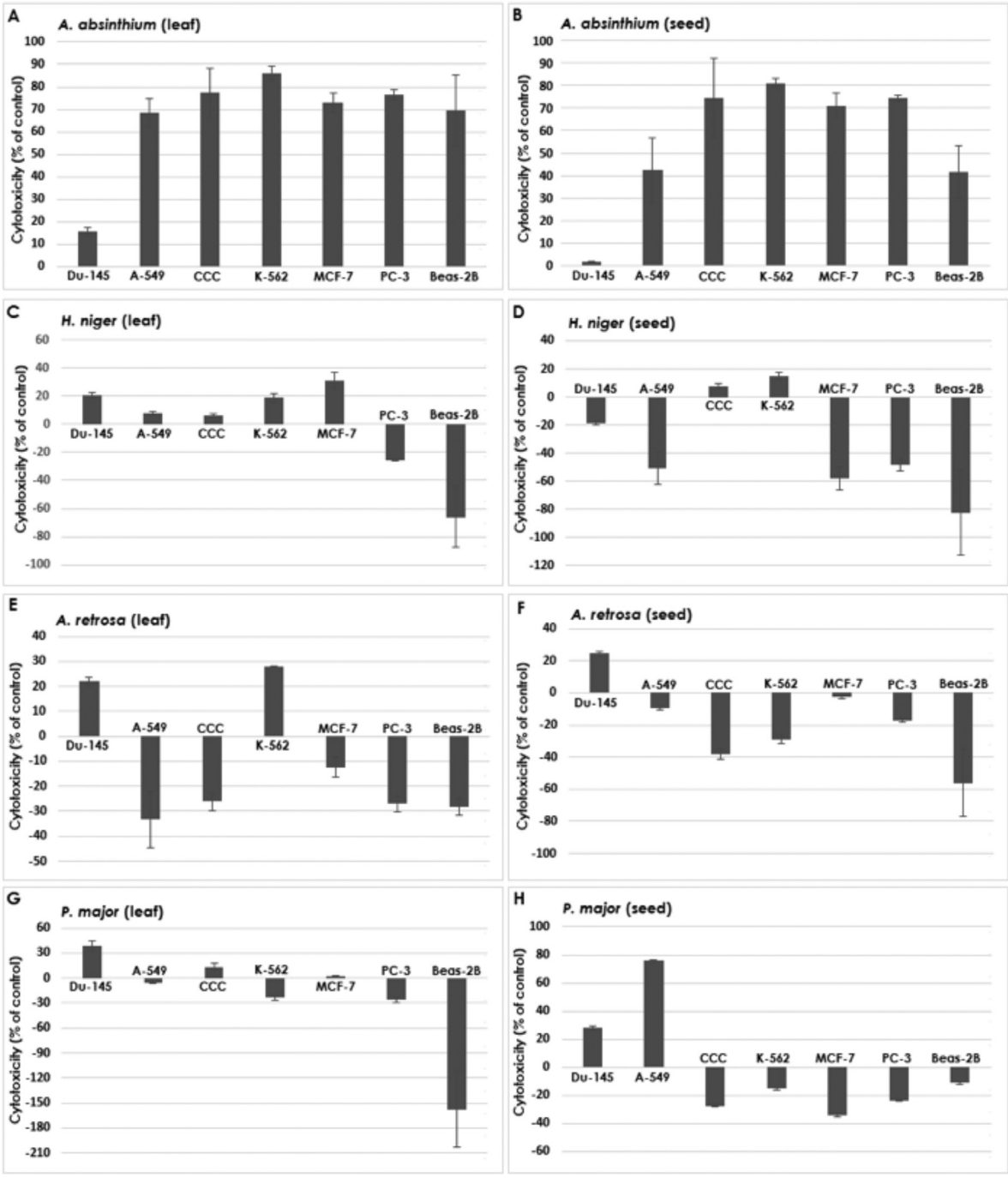


Figure 1. Cytotoxicity of plant extracts on different cancer cell lines. Cells were treated with leaf or seed extracts of plants at 4 mg/mL concentration for 72 hr. Cytotoxicity was determined using MTT assays and results expressed as percentages of cytotoxicity compared with untreated control. Data expressed as mean ± standard deviation, n=3

Time- and dose-dependence of cytotoxicity

After detecting significant cytotoxicity on A-549 and K562 cell lines treated with *A. absinthium* leaf and seed extracts, we investigated how time of treatment with varying concentrations of extracts affected the cytotoxicity. Cells were treated with 0.1, 0.2, 0.5, 1, 2, and 4 mg/mL extracts for 24, 48, and 72 hr. Extracts caused cytotoxicity in a different manner (Figure 2). For example, leaf extract above 0.2 mg/mL concentration resulted in cytotoxicity between 60% and 70% on A-549 cells on day 1, remained the same level on day 2, and was between 60% and 83% on day 3 (Figure 2a). A similar manner of cytotoxicity was observed with seed extracts (Figure 2b). In addition, the cytotoxic effects of seed and leaf extracts on K-562 cells were proportional with the duration of exposure and the concentration of the extract (Figure 2c, d).

Table 1. Difference of cytotoxicity on treated cells compared with untreated controls

	Beas-2B	A-549	CCC-221	DU-145	K-562	MCF-7	PC-3
A. absinthium (leaf)	***	***	***	(-)	***	***	***
A. absinthium (seed)	*	(-)	***	(-)	***	***	***
H. niger (leaf)	(-)	(-)	(-)	*	(-)	(-)	+++
H. niger (seed)	(-)	+	(-)	+	(-)	+	++
A. retrosa (leaf)	+	(-)	(-)	*	***	(-)	+
A. retrosa (seed)	(-)	+	+	**	+	(-)	+
P. major (leaf)	+	(-)	(-)	*	(-)	(-)	(-)
P. major (seed)	(-)	***	(-)	***	(-)	++	+++

*Means cytotoxicity with *p<0.05, **p<0.01, ***p<0.005
+Means mitogenecity with +p<0.05, ++p<0.01, +++p<0.005
-No significant difference

Table 2. Difference of cytotoxicity on cells compared with normal cell line Beas-2B

	A-549	CCC-221	DU-145	K-562	MCF-7	PC-3
A. absinthium (leaf)	+	(-)	(-)	***	(-)	(-)
A. absinthium (seed)	(-)	(-)	*	***	***	***
H. niger (leaf)	*	(-)	***	*	*	(-)
H. niger (seed)	(-)	(-)	***	*	(-)	(-)
A. retrosa (leaf)	(-)	+	***	***	(-)	+
A. retrosa (seed)	(-)	+	***	+	(-)	(-)
P. major (leaf)	(-)	*	***	***	***	**
P. major (seed)	***	+	***	++	+	+++

*Means cytotoxicity with *p<0.05, **p<0.01, ***p<0.005
+Means mitogenecity with +p<0.05, ++p<0.01, +++p<0.005
-No significant difference

In addition, the dose-dependence experiment of *P. major* seed extract was conducted on the cytotoxicity of the A-549 cell line. Cells were treated with 4 mg/mL seed extract for 72 hr. It was observed that the level of cytotoxicity was proportional with the increasing concentration of the extract and reached around 40% at 2 mg/mL, and 73% at 4 mg/mL (Data not shown).

Flow cytometric analysis of cell death

Cellular cytotoxicity can be accounted for by various cellular processes, the most common of which is cell death. Cell death is classified as necrosis, apoptosis, and autophagy. Apoptosis is planned cell death, whereas autophagy and necrosis are considered as sudden deaths and occur when cells are under extensive stress. In this present study, flow cytometric analysis was performed in order to confirm cell death after treatment of A-549 cells with different concentrations of *A. absinthium* leaf and seed extracts. The results indicated a difference between the cytotoxicities created by seeds and leaf extracts (Figure 3). At a lower concentration of 0.2 mg/mL, seed extracts had already killed around 25% of cells, whereas the leaf extract exhibited the same cytotoxicity as the control (Figure 3b, Table 3). It took up to 2 mg/mL concentration to show a different profile than the controls for the leaf extract (Figure 3d). For both extracts, the percentage of dead cells gradually peaked as the concentration of extract increased. At 4 mg/mL, leaf and seed extracts caused 20% and 6% survival rates, respectively (Figure 3e, Table 3). As a result, seed extract seems to kill A-549 cells through apoptosis at a lower concentration than leaf extract (Figure 3c).

Investigation of apoptosis by caspase 3 assay

Apoptosis occurs through a series of cleavages of serine proteases called caspases. That serial cleavage, which is called a caspase cascade, is present in both the external and internal apoptotic pathway. In both pathways, the last protease that executes cell death is caspase-3. Cleavage of the apoptotic substrate by activated caspase-3 is a good indicator of the presence of apoptosis. Therefore, after treatment of A-549 cells with *A. absinthium* leaf extract at 4 mg/mL, the amount of active caspase-3 was measured in cell lysate. After 36 hr of treatment, caspase-3 was demonstrated to be activated in the cells and was found 4.5-folds higher than in untreated cells (Figure 4).

DISCUSSION

Use of medicinal plants as an approach in the prevention and treatment of cancer has been followed for many years and many therapeutic plants with anticancer activity are reported in

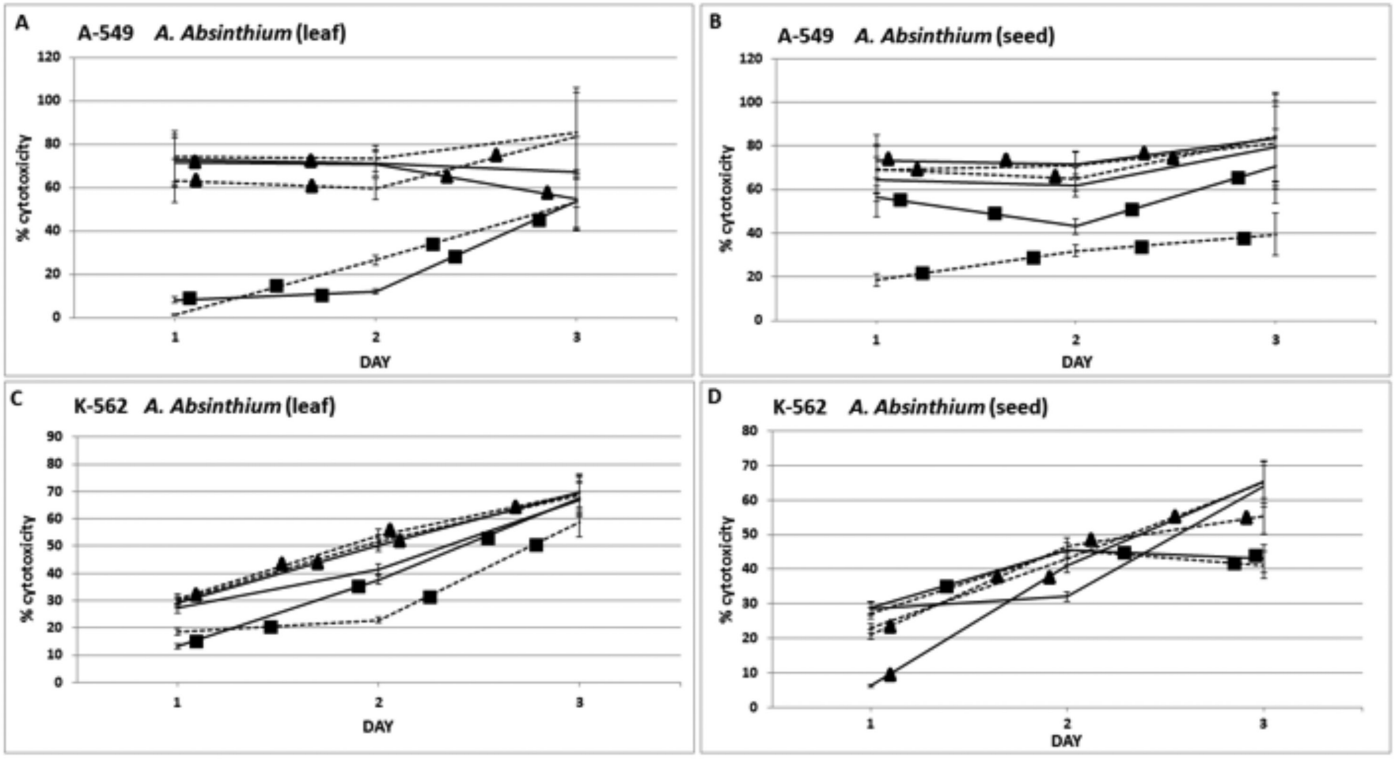


Figure 2. Time-based dose response curves of cancer cells. A-549 cells (a, b) or K-562 cells (c, d) were treated with leaf or seed extracts of *A. absinthium* at various concentrations

Data are expressed as mean ± standard deviation, n=3

----■----: 0.1 mg/mL, —■—: 0.2 mg/mL, ----▲----: 0.5 mg/mL, —▲— : 1 mg/mL, ----------: 2 mg/mL, ———: 5 mg/mL

Table 3. Detection of apoptosis with flow cytometric analysis

Treatment (concentration)	Necrosis (%)	Late apoptosis (%)	Live cells (%)	Early apoptosis (%)
Untreated	1.8	9.1	87.5	1.50
A. absinthium leaf (0.2 mg/mL)	1.0	7.0	89.3	2.6
A. absinthium leaf (0.5 mg/mL)	1.1	5.9	87.8	5.2
A. absinthium leaf (2 mg/mL)	52.90	6.2	37.5	3.4
A. absinthium leaf (4 mg/mL)	47.70	23.2	21.0	8.1
Untreated	1.60	12.4	85.4	0.7
A. absinthium seed (0.2 mg/mL)	5.7	12.0	74.5	7.8
A. absinthium seed (1 mg/mL)	31.8	20.6	31.3	16.3
A. absinthium seed (2 mg/mL)	56.1	10.6	29.6	3.8
A. absinthium seed (4 mg/mL)	60.8	30.7	6.2	2.4

A-549 cells were treated with *A. absinthium* leaf or seed extract for 24 hr

the literature.[20,21] As the interest in organic and simple lifestyles grows, the interest in plant-based medicine also increases.[13] In addition, adverse effects and drug interactions are major restrictions in synthetic anticancer drugs; therefore, plants have been investigated across the world to exploit novel and potential sources of anticancer agents.

Previous studies on *Artemisia* species have shown medicinal properties such as antibacterial and anticancer effects. Many phytochemicals exert their cytotoxic effects by acting as cell cycle and apoptosis regulators, as well as anti-inflammatory agents.[22] In this present study, ethanol extracts of different plant species were evaluated for their cytotoxic potentials on different cancer cell lines. Emami et al.[23] studied the anticancer activity of five species of *Artemisia* (*A. kulbadica, A. sieberi, A. turanica, A. santolina,* and *A. diffusa*) against Hep2 and HepG2 cell lines. The authors used extracts from aerial parts of each

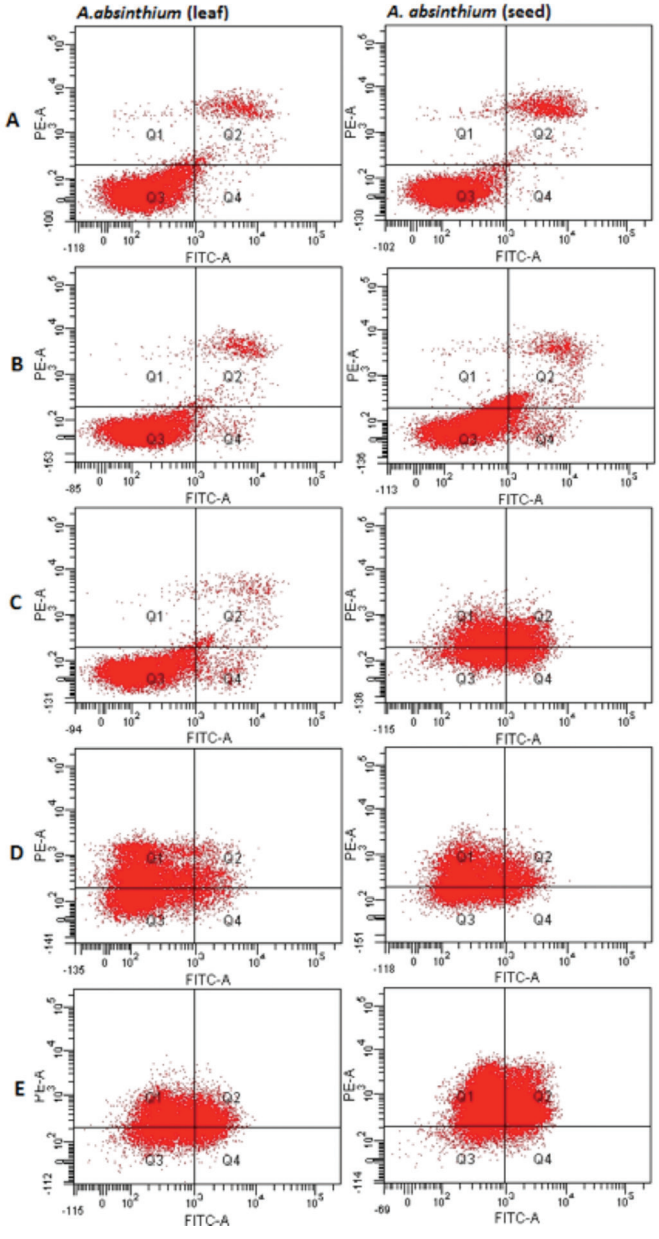


Figure 3. Flow cytometric analysis of apoptosis. A-549 cells were treated with leaf or seed extracts of *A. absinthium* at various concentrations. Results showing cells in necrosis (Q1), late apoptosis (Q2), live cells (Q3) and early apoptosis (Q4), for control (a), 0.25 mg/mL (b), 0.5 (c), 2 mg/mL (d), 4 mg/mL (e)

species at concentrations between 0.2 mg and 3.2 mg/mL. The minimum toxic dose of extracts from five species ranged from 0.4 to 1.6 mg/mL. Therefore, we used *A. absinthium* extracts at 4 mg/mL to screen the cytotoxic effect of the extracts. In order to determine the dose- and time-dependence of cytotoxicity, the extracts were used at five different concentrations starting from 4 mg/mL to 0.1 mg/mL. *In vitro* cytotoxicity of leaf or seed extracts of *A. abshintium* was observed on almost all cell lines examined, except DU-145 cells. The level of cytotoxicity on A-549 cells was time- and dose-dependent, especially for leaf or seed extracts at less than 0.5 mg/mL concentrations (Figure 2a, b). Extracts at more than 0.2 mg/mL concentrations

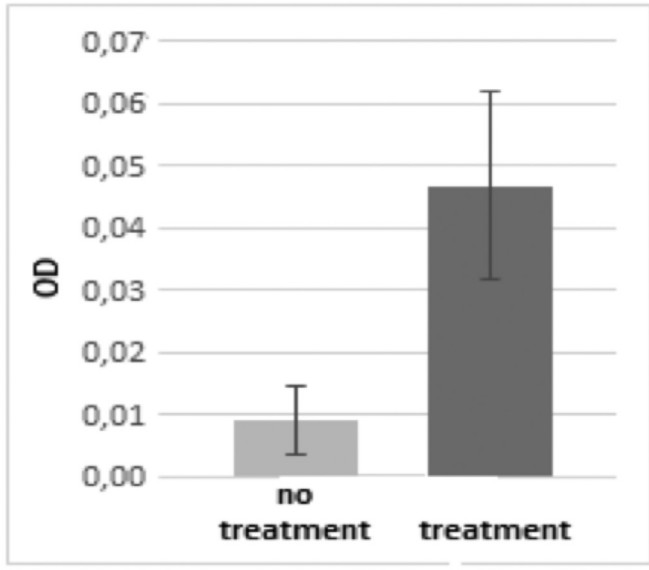


Figure 4. The effects of *A. absinthium* leaf extract on caspase-3 activity in A-549 cells as detected using a colorimetric assay. Treatment of cells with *A. absinthium* leaf extract exhibited 4.5-fold higher OD values than untreated cells. Data expressed as mean ± standard deviation, n

caused cytotoxicity above 60% even on day one. It stayed at the same level on day two and reached to 82% by day three. These findings indicated that leaf or seed extracts of *A. abshintium* may be effective on cytotoxicity of A-549 cells in a dose- and time-dependent manner at less than 0.2 mg/mL concentrations.

In addition, the cytotoxic activity of methanol extracts from different parts of five *Arthemisia* species was evaluated by Gordanian et al.[12]. *A. absinthium* was found to have a greater cytotoxic effect on MCF-7 cells with a 50% inhibitory concentration (IC_{50}) value of 221.5 µg/mL. They showed that a flower extract of *A. absinthium* was more cytotoxic than those of leaf, stem, and root extracts. Similarly, we found that seed extracts at less than 0.5 mg/mL concentrations were more effective with regards cytotoxicity on A-549 cells compared with that of leaf extracts, especially on day 1 and day 2 (Figure 2a, b). In fact, flower and leaf extracts of some *Artemisia* species contain rich sources of artemisinin, a sesquiterpene lactone Mannan et al.[24], and artemisinin caused *in vitro* and *in vivo* anticancer activities in a previous study.[25]

Gordanian et al.[26] reported that the cytotoxic activity of methanol extract from different organs of *A. absinthium* at higher altitude was 20-30% higher as compared with those of lower altitudes. The type and level of secondary metabolites may vary in the same plant species based on different agroclimatic and geographic regions.[27] Gordonian et al.[12] showed that leaf extract of *A. absinthium* from Iran at 0.1 mg/mL resulted in 30% cytotoxicity on MCF-7 cell lines, whereas we found 20% cytotoxicity on MCF-7 cells at the same concentration (data not shown). However, this could be due to different geographic regions for plant origins and consequently an accumulation of different secondary metabolites, or the use of different solvents for extraction because they used different fractions of

methanol extracts in cytotoxicity assays. In addition, the greater cytotoxicity (>60%) of the same plant extracts on other cancer cell lines exhibits cell type specificity of the extracts.

Ethanol extracts from *A. absinthium* leaf and seed were evaluated for apoptotic potential using flow cytometric analysis and caspase-3 activity on A-549 cells. According to our findings, extracts induced apoptosis, and total cell death increased in a concentration-dependent manner. In other words, treatment of cells with more toxic concentration of extracts caused apoptosis and killed cells undergoing apoptosis faster through necrosis. Investigation of Bad and Bcl-2 protein expression levels in a future study will elucidate the mechanism of apoptosis in all cells lines examined here because the balance between pro-apoptotic and anti-apoptotic proteins is vital for cell survival. In a previous study, a methanolic extract of *A. absinthium* inhibited cell proliferation through apoptosis by modulating Bcl-2 family proteins and the MEK/ERK pathway in MCF-7 and MDA-MB-231 cell lines.[18]

P. major has been used as a remedy against stomachache, toothache, and inflammation in the Kars region.[15] In addition, traditional use of *P. major* in the treatment of tumors was reviewed by Samuelsen[28] and Haddadian et al.[29]; however, not much information is available for scientific validation and anticancer activities. In this present study, we investigated the cytotoxic effect of extracts obtained from leaf and seed of *P. major*. Seed extracts exhibited 78% and 28% cytotoxicity on A-549 and DU-145 cells, respectively. Even though the leaf extract did not cause cytotoxicity on A-549 cells, it gave rise to 38% cytotoxicity against DU-145 cells.

Gálvez et al.[13] examined methanolic extracts of *P. major* against three different cancer cell lines and reported that the extract was more effective on MCF-7 cells with an LC_{50} value of 207 µg compared with renal adenocarcinoma (TK-10) and human melanoma (UACC-62) cell lines. Unlike the finding of Gálvez et al.[13], ethanolic extract of leaf and seed did not cause cytotoxicity on the MCF-7 cell line in the present study. However, the seed extract induced proliferation of MCF-7 cells by 38%. The differential cytotoxic effect of seed or leaf extracts on a variety of cancer cell lines may depend on differences in the fatty acid, amino acid, phenolic, and flavonoid composition of leaf or seed extracts. Kobeasy et al.[30] reported the differences in levels of these compounds in leaf and seed extracts of *P. major.*

Leaf and seed extract of *A. retrosa* exerted cytotoxic activity on DU-145 and K-562 cell lines. However, both extracts resulted in cell proliferation in other cell lines. Rajasekaran et al.[31] reported antitumor effects of methanolic extracts from *Amaranthus spirous* on Hep2, HepG2, HT-29, and Hu7 cell lines. In addition, anticancer activity of *H. niger* extract has not yet been reported in the literature. However, some studies examined the antiinflammatory and analgesic activity of the plant. It has been shown that methanol extracts of *H. niger* inhibit the activity of monoamine oxidase and lower the cellular levels of OH produced by mitochondria[32], which suggests that the extract prevents oxygen damage by controlling reactive oxygen species activity and therefore prevents apoptosis. In our study, the cytotoxic effects of leaf and seed extracts on cancer cell lines were variable. They caused a cytotoxic reaction in some cell lines but induced cell proliferation in others. The range of cytotoxicity of *H. niger* extracts changed between 7% and 30% on various cancer cell lines, suggesting that this plant does not contain anticancer compounds.

CONCLUSION

Ethanolic leaf and seed extracts of *A. absinthium* showed significant anticancer activity against five different cancer cell lines. Antitumor activities of leaf extract on A-549 and K-562 cells, and seed extracts on K562, MCF-7, and PC-3 was found to be selective compared with the normal Beas-2B cell line. In addition, seed extracts of *P. major* caused significant and selective anticancer activity against A-549 and DU-145 cell lines but not on the Beas-2B cells. Other plant extracts induced anticancer activity with various levels. Moreover, leaf or seed extract of the plant exhibited different effects on cells, suggesting that different parts of the plant may have different types and concentrations of secondary metabolites depending on the function of the plant part. In conclusion, use of crude ethanolic extracts enabled us to detect the anticancer potential of phytochemicals of medicinal plants from the Kars region of Turkey. Future studies will identify the level of chemical components of *A. absinthium* and *P. major* extracts responsible for anticancer activity and elucidate more detailed molecular mechanisms of cell death.

ACKNOWLEDGEMENT

This work was supported by Scientific Research Projects (BAP) of Muğla Sıtkı Koçman University under project number 13/82.

Conflict of Interest: No conflict of interest was declared by the authors.

REFERENCES

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin. 2012;62:10-29.
2. Huang T, Gong WH, Li XC, Zou CP, Jiang GJ, Li XH, Feng DP. Induction of apoptosis by a combination of paclitaxel and carboplatin in the presence of hyperthermia. Asian Pac J Cancer Prev. 2012;13:81-85.
3. Kintzios E. Terrestrial plant-derived anticancer agents and plant species used in anticancer research. Crit Rev Plant Sci. 2006;25:79-113.
4. Pandi M, Kumaran RS, Choi YK, Kim HJ, Muthumary J. Isolation and detection of taxol, an anticancer drug produced from *Lasiodiplodia theobromae*, an endophytic fungus of the medicinal plant *Morinda citrifolia*. Afr J Biotechnol. 2011;10:1428-1435.
5. Park HJ, Kim MJ, Ha E, Chung JH. Apoptotic effect of hesperidin through caspase3 activation in human colon cancer cells, SNU-C4. Phytomedicine. 2008;15:147-151.
6. Mans DR, da Rocha AB, Schwartsmann G. Anti-cancer drug discovery and development in Brazil: targeted plant collection as a rational strategy to acquire candidate anti-cancer compounds. Oncologist. 2000;5:185-198.
7. Fletcher JC. Coordination of cell proliferation and cell fate decisions in the angiosperm shoot apical meristem. Bioessays. 2002;24:27-37.

8. Lucas DM, Still PC, Pérez LB, Grever MR, Kinghorn AD. Potential of plant-derived natural products in the treatment of leukemia and lymphoma. Curr Drug Targets. 2010;11:812-822.
9. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer. 1972;26:239-257.
10. Frank SA. Dynamics of cancer, p.90, Princeton University Press, New Jersey, 2007.
11. Bora KS, Sharma A. The genus *Artemisia*: a comprehensive review. Pharm Biol. 2011;49:101-109.
12. Gordanian B, Behbahani M, Carapetian J, Fazilati M. *In vitro* evaluation of cytotoxic activity of flower, leaf, stem and root extracts of five *Artemisia* species. Res Pharm Sci. 2014;9:91-96.
13. Gálvez M, Martín-Cordero C, López-Lázaro M, Cortés F, Ayuso MJ. Cytotoxic effect of *Plantago* spp. on cancer cell lines. J Ethnopharmacol. 2003;88:125-130.
14. Avcı M. Çeşitlilik ve Endemizm açısından Türkiye'nin bitki örtüsü. Istanbul Üniversitesi Geography Department, Geography Journal. 2005;13:27-55.
15. Güneş F, Özhatay N. An ethnobotanical study from Kars (Eastern) Turkey. BioDiCon. 2011;4:30-41.
16. Kendir G, Güvenç A. Etnobotanik ve Türkiye'de yapılmış etnobotanik çalışmalara genel bir bakış. Hacettepe Üniversitesi Eczacılık Fakültesi Dergisi. 2010;30:49-80.
17. Tuzlacı E, İşbilen DFA, Bulut G. Turkish folk medicinal plants, VIII: Lalapaşa (Edirne). Marmara Pharm J. 2010;14:47-52.
18. Shafi G, Hasan TN, Syed NA, Al-Hazzani AA, Alshatwi AA, Jyothi A, Munshi A. *Artemisia absinthium* (AA): a novel potential complementary and alternative medicine for breast cancer. Mol Biol Rep. 2012;39:7373-7379.
19. Holst Hansen C, Brünner N. MTT Cell Proliferation assay, Cell Biology: A Laboratory Handbook. pp. 16-18, San Diego Academic Press, 1998.
20. Kumar S, Jawaid T, Dubey SD. Therapeutic Plants of Ayurveda. A Review on Anticancer. Pharmacognosy J. 2011;3:1-11.
21. Khazir J, Mir BA, Pilcher L, Riley DL. Role of plants in anticancer drug discovery. Phytochemistry Letters. 2014;7:173-181.
22. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100:57-70.
23. Emami SA, Vahdati-Mashhadian N, Vosough R, Oghazian MB. The Anticancer Activity of Five Species of *Artemisia* on Hep2 and HepG2 Cell Lines. Pharmacologyonline. 2009;3:327-339.
24. Mannan A, Ahmed I, Arshad W, Asim MF, Qureshi RA, Hussain I, Mirza B. Survey of artemisinin production by diverse *Artemisia* species in northern Pakistan. Malaria J. 2010;9:310.
25. Ferreira JF, Luthria DL, Sasaki T, Heyerick A. Flavonoids from *Artemisia annua* L. as antioxidants and their potential synergism with artemisinin against malaria and cancer. Molecules. 2010;15:3135-3170.
26. Gordanian BB, Behbahani M, Carapetian J, Fazilati M. Cytotoxic effect of *Artemisia absinthium* L. grown at two different altitudes on human breast cancer cell line MCF7. Res Med. 2012;36:124-131.
27. Mammadov R. Tohumlu bitkilerde sekonder metabolitler. pp.412, Nobel Lt., Ankara, 2014.
28. Samuelsen AB. The traditional uses, chemical constituents and biological activities of *Plantago major* L. A review. J Ethnopharmacol. 2000;71:1-21.
29. Haddadian K, Haddadian K, Zahmatkash M. A review of *Plantago* plant. Indian J Traditional Knowledge. 2014;13:681-685.
30. Kobeasy MI, Abdel-Fatah OM, Abd El-Salam SM, Mohamed Z.E.O.M. Biochemical studies on *Plantago major* L. and *Cyamopsis tetragonoloba* L. International J Biodiversity and Conservation. 2011;3:83-91.
31. Rajasekaran S, Dinesh MG, Kansrajh C, Baig FHA. *Amaranthus spinosus* leaf extracts and its anti-inflamatory effects on cancer. Indian J Res in Pharm and Biotechnol. 2014;2:1058-1064.
32. Begum S, Saxena B, Goyal M, Ranjan R, Joshi VB, Rao ChV, Krishnamurthy S, Sahai M. Study of anti-inflammatory, analgesic and antipyretic activities of seeds of *Hyoscyamus niger* and isolation of a new coumarinolignan. Fitoterapia. 2010;81:178-184.

Turk J Pharm Sci 2017;14(3):231-236
DOI: 10.4274/tjps.42714

ORIGINAL ARTICLE

In Vitro Cytotoxic and Anti-inflammatory Activities of *Tanacetum argenteum* (Lam.) Willd. subsp. *argenteum* Extract

Tanacetum argenteum subsp. *argenteum* Ekstrelerinin *In Vitro* Sitotoksik ve Anti-inflamatuvar Etkileri

Gökay ALBAYRAK[1], Ayşe NALBANTSOY[2], Şüra BAYKAN[1]*

[1]Ege University, Faculty of Pharmacy, Department of Pharmaceutical Botany, İzmir, Turkey
[2]Ege University, Faculty of Engineering, Department of Bioengineering, İzmir, Turkey

ABSTRACT

Objectives: The objective of this study was to examine the anti-inflammatory and cytotoxic potential of n-hexane, ethyl acetate, and methanolic extracts of *Tanacetum argenteum* subsp. *argenteum*.

Materials and Methods: *Tanacetum* L. is the third largest genus of *Asteraceae* family and is represented by 60 taxa in Turkish flora. Sesquiterpene lactones and pyrethrins are the main chemical groups of the genus. *T. argenteum* subsp. *argenteum* is an endemic taxa that is distributed in the Central and South Anatolia.

Results: *In vitro* anti-inflammatory activity was assayed using iNOS and NF-κB inhibition tests on RAW264.7 and HeLa cells. The cytotoxic activities were tested against ten cell lines using MTT assays.

Conclusion: As a result, the n-hexane extract was found more active than the positive control parthenolide in iNOS test (IC_{50}: 0.627±0.16 µg/mL) and cytotoxic experiments against PC3 and MPANC-96 cell lines (IC_{50}: 2.85±0.51 µg/mL and 5.35±1.24 µg/mL, respectively).

Key words: *Tanacetum argenteum* subsp. *argenteum*, anti-inflammatory, NF-κB, iNOS, cytotoxicity, MTT

ÖZ

Amaç: *Tanacetum argenteum* subsp. *argenteum* bitkisinin hekzan, etil asetat ve metanol ekstrelerinin anti-inflamatuvar ve sitotoksik potansiyellerini incelemektir.

Gereç ve Yöntemler: *Tanacetum* L. *Asteraceae* familyasının en büyük 3. cinsidir ve Türkiye florasında 60 taksonla temsil edilir. Seskiterpen laktonlar ve piretrinler cinsin ana kimyasal gruplarıdır. *T. argenteum* subsp. argenteum İç ve Güney Anadolu'da yayılış gösteren endemik bir taksondur.

Bulgular: *In vitro* anti-inflamatuvar aktivite iNOS ve NF-κB inhibisyon metodlarıyla RAW264.7 ve HeLa hücrelerine karşı test edilmiştir. Sitotoksik aktivite MTT metoduyla 10 hücre hattına karşı denenmiştir.

Sonuç: Sonuç olarak, n hekzan ekstresi iNOS testinde (IC_{50}: 0.627±0.16 µg/mL) ve PC3 ve MPANC-96 hücreleri (IC_{50}: 2.85±0.51 µg/mL ve 5.35±1.24 µg/mL, sırasıyla) üzerinde yapılan sitotoksik ölçümlerde pozitif kontrol partenolitten daha etkili bulunmuştur.

Anahtar kelimeler: *Tanacetum argenteum* subsp. *argenteum*, anti-inflamatuvar, NF-κB, iNOS, sitotoksisite, MTT

INTRODUCTION

The genus *Tanacetum* L. consists of around 160 species in the world[1] and 60 taxa of the genus exist in Turkey, 26 of which are endemic.[2] The genus is distributed in Europe and West Asia and all over Turkey, excluding the Aegean side.[3,4] *Tanacetum* sp. are used as insecticides, tonic, appetizers, anthelmintics, diuretics, carminatives, stimulants, emmenagogues, antipyretics, and antimigraine agents in Turkey.[5] *Tanacetum parthenium* (L.) Schultz Bip. (feverfew) is the most prominent species. It is known as having antimigraine properties and is used to relieve menstrual pain in traditional medicine, and standardized capsules of leaf extract are available on the market.[3,6] Eudesmane sesquiterpenes are the main components of the genus. *Tanacetum argenteum.* (Lam.) Willd., subsp. *argenteum* is an endemic perennial plant

*Correspondence: E-mail: sura.baykan@ege.edu.tr, Phone: +90 505 640 48 68 ORCID-ID: orcid.org/0000-0002-3624-4811
Received: 28.11.2016, Accepted: 23.02.2017

with 20-30 cm stem length and deeply divided or 2-pinnatisect leaves. The involucre is 3-4 (-5) mm broad and campanulate. Its flowers are yellow, female flowers are absent, and achenes are brown, 2-2.25 mm. It grows on rocks and limestone cliffs, ranging from 990 to 2500 m. The plant is mainly distributed in Inner and South Anatolia.[4] The main chemical constituents of *T. argenteum* subsp. *argenteum* are sesquiterpenoids and flavonoids. Phytochemical studies on this species also resulted with the isolation of β-sitosterol and β-amyrin, desacetyllaurenobiolide, spiciformin, tatridin-A, tatridin-B, desacetyl–β-cylclopyrethrosin, desacetyltulipinolide-1β, 10α-epoxide and 8α-angeloyloxycostunolide.[7] Sesquiterpenoids are thought to be the bioactive constituents of this taxon.[3] The major compounds of the essential oil of the plant are reported as monoterpenes; α-pinene, β-pinene and santolinatriene by previous studies.[7-9] Caryophyllene oxide and α-thujone were found as the main constituents of the oil of *T. argenteum* subsp. *canum* var. *canum* in Gören and Tahtasakal[10] study.

Recently, Orhan et al.[11] measured parthenolide levels using liquid chromatograph-mass spectrometry and total flavonoid contents of three subspecies of *T. argentum*. Among all *T. argenteum* subsp. *argenteum* has the highest parthenolide and total flavonoid contents. The authors also investigated the cholinesterase inhibitory potential of these plants. Among them, the leaf extract of *T. argenteum* subsp. *flabellifolium* has the strongest cholinesterase inhibitor activity.[11] Gören and Tahtasakal[10] isolated guaian-type sesquiterpene lactones from *T. argenteum* subsp. *canum* var. *canum* and *T. argenteum* subsp. *Flabellifolium*.[12,13]

The aim of this study was to investigate the anti-inflammatory and the cytotoxic activity of *n*-hexane, ethyl acetate, and methanolic extracts of *T. argenteum* subsp. *argenteum*. For this purpose, nuclear factor kappa B (NF-κB) and induced nitric oxide synthesis (iNOS) methods were used as anti-inflammatory assays and MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used for cytotoxic activity. Thin-layer chromatography (TLC) analysis of extracts were also performed to obtain an overview on the chemical compounds of the extract.

EXPERIMENTAL

Plant material

Plants were collected from Nemrut Mountain, 1600 m, Kahta, Adıyaman on May 30th, 2012, and identified by Şüra Baykan. Voucher specimens have been deposited in the Ege University Herbarium, Faculty of Pharmacy, İzmir, Turkey (IZEF 6029) (www.izef.ege.edu.tr).

Extraction

Dried and powdered plant parts (250 g) were extracted sequentially with *n*-hexane, ethyl acetate and methanol (2x300 mL for each), sonicated at room temperature for 6 h, and then filtered. The combined extracts were evaporated using a rotary evaporator to dryness at 40˚C.

Thin-layer chromatography analysis

TLC analysis of n-hexane, EtOAc, and methanolic extracts of the plant was made. A silica gel-coated aluminum plate was used as the stationary phase and *n*-hexane:ethyl acetate (5:5) solvent system was used as the mobile phase. Comparisons was performed using parthenolide as the reference compound.

Cells

All cells were obtained from American Type Culture Collection (ATCC, Rockville, MD).

Chemicals and other materials

MTT, *n*-hexane, parthenolide, Griess reagent, 100 µ/mL penicillin G sodium, 100 µ/mL streptomycin were from Sigma (St. Louis, MO); ethyl acetate and methanol were from Merck, Germany; RPMI 1640, fetal bovine serum (FBS), 1% L-glutamine and 1% gentamicin were obtained from PAA Laboratories GmbH, Cölbe, Germany; DMEM/F12 and RPMI-1640 were from Invitrogen (Carlsbad, CA); 1 mM calcium and magnesium were from Packard Instrument Company (Meriden, CT); bovine calf serum and FBS were from Atlanta Biologicals (Lawrenceville, GA); and the Luciferase Assay Kit was obtained from Promega (E1500).

Assay for in vitro cytotoxicity

n-hexane, EtOAc, and MeOH extracts of *T. argenteum* subsp. *argenteum* were tested against ten cell lines using MTT assays. These cell lines included A549 (human alveolar epithelial cells), CaCo-2 (human epithelial colorectal adenocarcinoma), MCF-7 (human breast adenocarcinoma), MPANC-96 (human pancreatic cancer), MDA-MB-231 (metastatic human breast cancer), 253J-BV (bladder cancer cells), U87-MG (human glioblastoma-astrocytoma, epithelial-like), prostate cancer (PC_3), human cervical carcinoma (HeLa) as cancerous cells and human embryonic kidney (HEK)-293 as noncancerous cells. Parthenolide was used as a positive control. Cells were cultivated in DMEM-F12 medium, passaged twice a week. Cells (10^4 cells/well) were seeded to the wells of 96-well plates and cultivated for 24 h in an incubator. Extracts with different dilutions (0.5, 5, 50 µg/mL) and the positive control were added and cells were incubated for 48 h. After 48 h, the number of viable cells was determined using an MTT assay. For this purpose; in order to count the number of living cells, 25 µL MTT (stock solution of 2.5 mg/mL) physiologic saline (9% NaCl) was added to the wells and incubated for 4 h. After a while, the medium was removed to dissolve the formazan crystals; 150 µL DMSO was added and the absorbance was read at 520 and 620 nm. All measurements were performed in triplets and the half-maximum inhibitory concentration (IC_{50}) was determined using GraphPad Prism 5.

Assay for the inhibition of NF-κB activity

The NF-κB Luciferase Reporter HeLa Stable Cell Line (Signosis, CA) was used for this assay. Cells were cultured in DMEM-F12 supplemented with 10% FBS, 100 U/mL penicillin G sodium and 100 µg/mL streptomycin at 37˚C in an atmosphere of 5% CO_2 and 95% humidity.

Cells ($5x10^5$ cells/well/90 µL) were added to 96-well plates and incubated for 24 h. Then, test samples at different concentrations (0.5, 5, 50 µg/mL) were added to the medium and incubated for 30 min. After that, cells were induced with PMA (70 ng/

mL) for 24 h, and the medium then was removed and the cells were washed with 200 µL PBS. Cells were incubated with 20 µL lysis buffer on a shaker at room temperature for 1 h. Absorbance was measured using a luminometer subsequent to adding 20 µL Luciferase substrate. Parthenolide was used as a positive control.

Assay for inhibition of iNOS activity

The mouse macrophage (RAW264.7) cells cultured in phenol red-free RPMI medium with 10% bovine calf serum and 100 U/mL penicillin G sodium and 100 µg/mL streptomycin. Cells (10^5 cells/well) were seeded in 96-well plates and incubated for 24 h. Test samples at different concentrations (0.5, 5, 50 µg/mL) were prepared from 10 mg/mL stock solution and 20 µL were added for each.

After 60 µL serum-free medium were added and 30 min of incubation, LPS (5 µg/mL) was added to the cells to induce iNOS and cells were incubated for 24 h. The concentration of nitric oxide produced as a result of iNOS activity was determined by measuring the level of nitrite in the cell culture supernatant using Griess reagent. The absorbance was read at 540-630 nm. IC_{50} values were obtained from dose curves. Parthenolide was used as a positive control and DMSO was used in the tests as a vehicle control.[14] Extracts were also evaluated using MTT analyses to detect cytotoxic activity against RAW264.7 cells, in addition to the iNOS inhibition test.

RESULTS AND DISCUSSION

Parthenolide was detected in *n*-hexane and EtOAc extracts of the plant. R_F values of *n*-hexane and EtOAc extracts and parthenolide were 0.69 and their distances from origin of all three spots were equal and all three spots were blue. Based on this results, it was concluded that the major compound of *n*-hexane and EtOAc extracts of *T. argenteum* subsp. *argenteum* was parthenolide. The TLC result is also given as Figure 1.

The results of the cytotoxic activity tests are shown in Table 1. Among the extracts, significant cytotoxic activity of the *n*-hexane extract was observed against CaCo-2, MPANC-96, HEK-293, MDA-MB-231, and PC_3 (IC_{50} values of 3.959±0.62, 5.353±1.24, 1.651±0.43, 4.154±0.18, 2.847±0.51 µg/mL, respectively). Our results showed that the *n*-hexane extract is more effective than parthenolide against MPANC-96 and PC_3. It also had cytotoxic activity as strong as parthenolide against CaCo-2, HEK-293, MDA-MB-231. The viability percentage of *n*-hexane extract is shown in Figure 2. Microscopy images of the cells are shown in Figure 3.

In many studies, cytotoxic activities of various *Tanacetum* species have been reported. Rateb et al.[15] found that hydroalcoholic and aqueous extracts of different parts of *T. parthenium* L. had cytotoxic effects. The alcoholic extracts of the flowers of the plant showed significant cytotoxic effects (15% cell viability) with 50 µg/mL concentration on Ehrlich ascites carcinoma. This activity is considered as a result of synergy of flavonoid and sesquiterpene lactone contents. Rosselli et al.[16] investigated the cytotoxic activity of eudesmanolides isolated from flowers of *Tanacetum vulgare* subsp. *siculum* against an A549 cell line.

This is the first report to demonstrate the cytotoxic activity of *T. argenteum* subsp. *argenteum* against CaCo-2, MPANC-96, MDA-MB-231, 253J-BV, U87-MG, and PC_3 cell lines. In a previous report, the plant was analyzed for its cytotoxic property on MCF-7, the cell viability inhibition value of methanolic extract was found as 52.58% at a concentration of 100 µg/mL, whereas we found no serious activity with MeOH extracts. The difference is likely due to the extraction methods.[17]

Recently, Şen et al.[18] investigated the cytotoxic activity of aerial parts of *T. argenteum* subsp. *argentum* against four cell lines [A549, HeLa, HT-29 (human colorectal adenocarcinoma), MCF-7] using MTT assays. The authors found that an *n*-hexane extract of the plant inhibited growth of A-549 and HT-29 cells, and a chloroform extract of the plant inhibited growth of A-549 and HeLa cells at the concentrations of 30 µg/mL. Both extracts of the plant inhibited more than 50% proliferation of cells. When compared their results with ours on the same cell lines; the stronger results observed in our study with the same extracts are likely associated with the quantitative superiority of secondary metabolites responsible for the cytotoxic activity. Thus, the disparity between the two studies could be attributed to the different localities of the plant materials.

Apolar sesquiterpene lactones with γ lactone moiety pass into the *n*-hexane fraction could be responsible of the effect because sesquiterpene lactones have a broad spectrum of biologic activities including anti-inflammatory and cytotoxic properties. The lactone ring and exocyclic methylene group of sesquiterpene lactones are considered to be the liable units for biologic activities of sesquiterpenoids.[3]

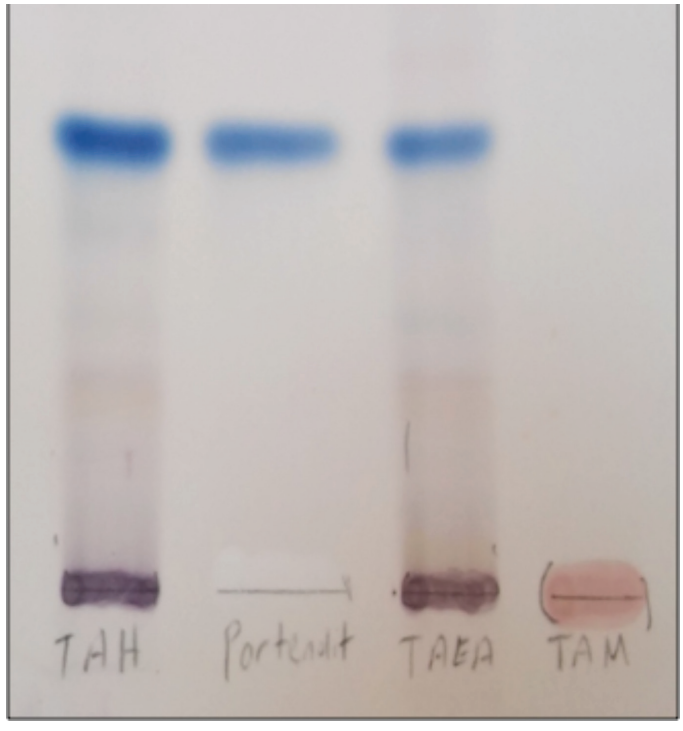


Figure 1. TLC results of parthenolide and *n*-hexane (TAH), EtOAc (TAEA), Methanolic (TAM) extracts of *T. argenteum* subsp. *argenteum*. Mobile phase system: hexane:ethyl acetate (5:5)

It is known that parthenolide, a germacranolide-type sesquiterpene lactone, plays a significant role for anti-inflammatory and cytotoxic activities.[19] Notably *T. parthenium, T. vulgare, Tanacetum densum* subsp. *amani, T. argenteum* subsp. *Flabellifolium*, and *T. argenteum* subsp. *canum* var. *canum* contain parthenolide.[3] The activities of the *n*-hexane extract and the ethyl acetate extract may be associated with the plant's major component parthenolide.

The anti-inflammatory activity of *T. argenteum* subsp. *argenteum* was evaluated using NF-κB and iNOS methods. As the results can be seen in Table 2, n-hexane extract had significant anti-inflammatory activity with an IC_{50} of 6.159±0.45 µg/mL for NF-κB, and 0.627±0.16 µg/mL for iNOS methods, respectively. Anti-inflammatory activities of various Tana*cetum* species using *in vitro* and *in vivo* methods have been reported.[20,21] The anti-inflammatory activities of *Tanacetum* spp. are related to their flavonoid and sesquiterpene contents. In a previous report, Nasri et al.[22] investigated the hydroalcoholic extract of *Tanacetum balsamita* and found anti-inflammatory activity related to quercetin, a flavonoid, and we found the anti-inflammatory activity of the plant through apolar fractions related to sesquiterpene lactones, most probably parthenolide. Bukhari et al.[23] noted that the *n*-hexane extract of *Tanacetum artemisioides* showed stronger *in vivo* anti-inflammatory activity than that of polar aqueous fractions.

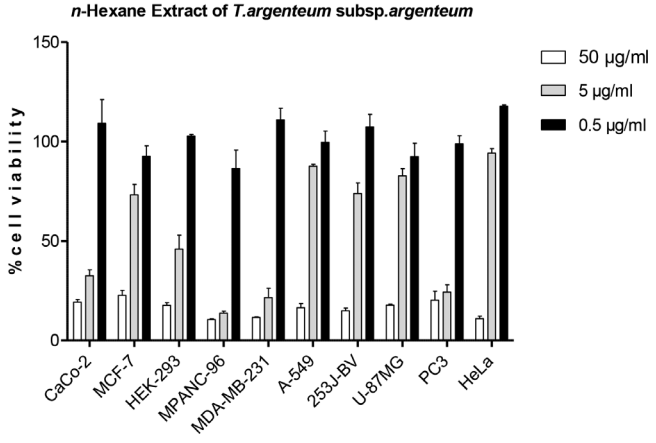


Figure 2. Cell viability of n-hexane extract with different concentrations of *T. argenteum* subsp. *argenteum* on ten cells

As postulated, sesquiterpene lactones are generally known to impair the activity of the NF-κB.[24] Hehner et al.[25] noted that parthenolide, the main compound of *T. parthenium*, is a well-known anti-inflammatory agent that inhibits the NF-κB pathway. López-Franco et al.[26] showed parthenolide's inhibiting role using the NF-κB method against murine cells. The *n*-hexane extract is more effective than parthenolide in iNOS methods, whereas parthenolide is stronger than *n*-hexane extracts with NF-κB. The *n*-hexane extract's potency could be related to a synergistic effect of different secondary metabolites of the extract. The results can also be seen in Figure 4 and 5.

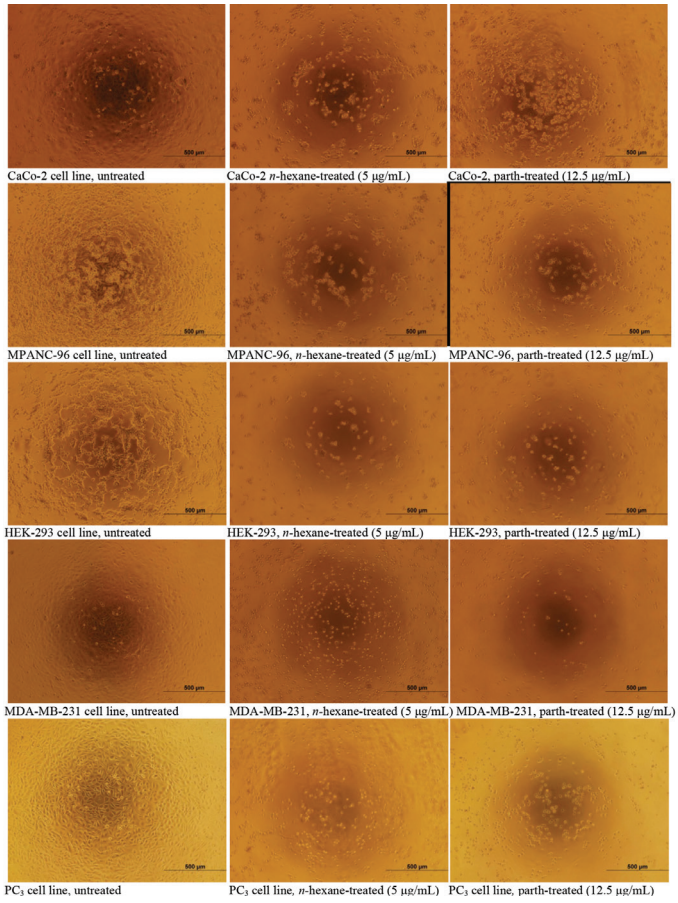


Figure 3. Views of CaCo-2, MPANC-96, HEK-293, MDA-MB-231 and PC_3 cells under a microscope (500 µm). Untreated cells, hexane and parthenolide treated cells

Table 1. IC_{50} values for cytotoxic activities of various extracts of *Tanacetum argenteum* subsp. *argenteum* extracts on different cells, µg/mL

Extract	A549	CaCo-2	MCF-7	MPANC-96	HEK-293	MDA-MB 231	253J-BV	U-87 MG	PC_3	HeLa
n-hexane	17.65±1.52	3.96±0.62	13.99±2.61	5.35±1.24	1.65±0.43	4.15±0.18	12.02±1.19	15.85±1.54	2.85±0.51	18.94±1.00
EtOAc	29.24±5.88	37.54±10.69	38.16±5.10	25.66±3.08	7.39±0.77	16.21±2.58	36.19±10.39	-	18.99±1.53	21.48±4.91
MeOH	-	-	-	-	-	-	-	-	-	-
Partheno-lide	3.26±2.49	3.16±2.93	7.37±0.16	5.51±5.71	1.16±0.09	3.61±3.34	6.35±0.20	3.38±1.97	3.44±0.36	5.79±0.00

Table 2. IC_{50} values for anti-inflammatory activities of various extracts of *Tanacetum argenteum* subsp. argenteum

Extract	iNOS (Raw 264.7) IC_{50} (µg/mL) with SD	NF-κB (HeLa) IC_{50} (µg/mL) with SD
n-hexane	0.627±0.16	6.159±0.45
EtOAc	1.602±0.48	37.505±1.51
MeOH	17.15±1.65	-
Parthenolide	0.674±0.01	1.779±0.14

-: No activity, SD: Standard deviation

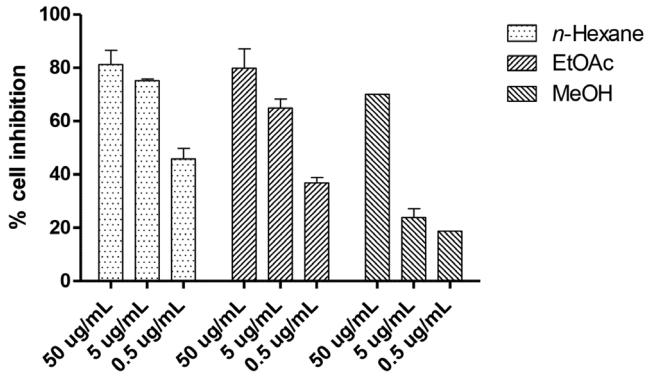


Figure 4. Various extracts of *T. argenteum* subsp. *argenteum* with different concentrations HeLa cell line, NF-κB method

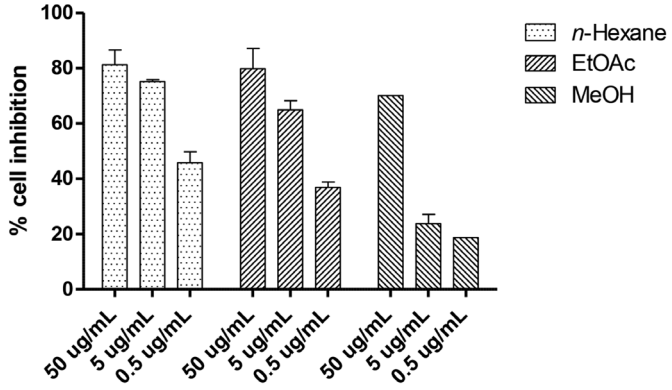


Figure 5. Various extracts of *T. argenteum* subsp. *argenteum* with different concentrations RAW264.7 cell line, iNOS method

CONCLUSION

Our results indicated that the *n*-hexane extract of *T. argenteum* subsp. *argenteum* has promising cytotoxic activity on CaCo-2, MPANC-96, HEK-293, MDA-MB-231, and PC_3 cells, and also anti-inflammatory activity. Parthenolide, a well-known anti-inflammatory and cytotoxic agent was detected in *n*-hexane and EtOAc extracts of the plant using TLC analysis. *n*-hexane extract's activity may be associated with its major component parthenolide and other substances in the plant. Phytochemical experiments are ongoing with *n*-hexane extracts of *T. argenteum* subsp. *argenteum.*

Conflict of Interest: No conflict of interest was declared by the authors.

REFERENCES

1. Orhan IE, Tosun F, Gulpinar AR, Kartal M, Duran A, Mihoglugil F, Akalgan D. LC-MS quantification of parthenolide and cholinesterase inhibitory potential of selected *Tanacetum* L. (Emend. Briq.) taxa. Phytochemistry Letters. 2015;11:347-352.
2. Güner A, Aslan S, Ekim T, Vural M, Babaç M. Türkiye bitkileri listesi (damarlı bitkiler). Nezahat Gökyiğit Botanik Bahçesi ve Flora Araştırmaları Derneği Yayını İstanbul. 2012:47-83.
3. Gören N, Arda N, Çaliskan Z. Chemical characterization and biological activities of the genus *Tanacetum* (*Compositae*). Studies in Natural Products Chemistry. 2002;27:547-658.
4. Davis PH. Flora of Turkey and the East Aegean Islands. Vol. 3. Flora of Turkey and the East Aegean Islands Vol 3, 1970.
5. Baytop T. Türkiyede bitkiler ile tedavi (geçmişte ve bugün). 1984;40.
6. Bruneton J, Pharmacognosy P. Medicinal plants. Pharmacognosy phytochemistry, 2003.
7. Gören N, Tahtasakal Ef, Pezzuto JM, Cordell GA, Shwarzt B, Prokscht P. Sesquiterpene lactones from *Tanacetum argenteum*. Phytochemistry. 1994;36:389-392.
8. Bagci E, Kocak A. Essential oil composition of two endemic *Tanacetum* (*T. nitens* (Boiss. & Noe) Grierson and *T. argenteum* (Lam.) Willd. subsp. *argenteum*) (*Asteraceae*) taxa, growing wild in Turkey. Industrial crops and products. 2010;31:542-545.
9. Polatoğlu K, Demirci F, Demirci B, Gören N, Başer KH. Essential oil composition and antibacterial activity of *Tanacetum argenteum* (Lam.) *Willd*. ssp. *argenteum* and *T. densum* (Lab.) *Schultz Bip*. ssp. amani heywood from Turkey. J Oleo Sci. 2010;59:361-367.
10. Gören N, Tahtasakal E. Sesquiterpenoids from *Tanacetum argenteum* subsp. *canum* var. *canum*. Phytochemistry. 1997;45:107-109.
11. Orhan IE, Tosun F, Gülpınar AR, Kartal M, Duran A, Mihoglugil F, Akalgan D. LC-MS quantification of parthenolide and cholinesterase inhibitory potential of selected *Tanacetum* L. (Emend. Briq.) taxa. Phytochemistry Letters. 2015;11:347-352.
12. Goren N, Tahtasakal E, Krawiec M, Watson WH. Guaianolides from *Tanacetum argenteum* subsp. *canum* var. *canum*. J Nat Prod. 1998;61:560-563.
13. Gören N, Tahtasakal E, Krawiec M, Watson WH. A guaianolide from *Tanacetum argenteum* subsp. *flabellifolium*. Phytochemistry. 1996;42:757-760.
14. Quang DN, Harinantenaina L, Nishizawa T, Hashimoto T, Kohchi C, Soma G, Asakawa Y. Inhibition of nitric oxide production in RAW 264.7 cells by azaphilones from xylariaceous fungi. Biol Pharm Bull. 2006;29:34-37.

15. Rateb ME, El-Gendy A-NA, El-Hawary SS, El-Shamy AM. Phytochemical and biological investigation of *Tanacetum parthenium* (L.) cultivated in Egypt. Journal of Medicinal Plants Research. 2007;1:018-026.
16. Rosselli S, Bruno M, Raimondo FM, Spadaro V, Varol M, Koparal AT, Maggio A. Cytotoxic effect of eudesmanolides isolated from flowers of *Tanacetum vulgare* ssp. *siculum*. Molecules. 2012;17:8186-8195.
17. Yumrutas O, Oztuzcu S, Pehlivan M, Ozturk N, Poyraz IE, Iğci YZ, Cevik MO, Bozgeyik I, Aksoy AF, Bagıs H, Arslan A. Cell viability, anti-proliferation and antioxidant activities of Sideritis syriaca, *Tanacetum argenteum* subsp. *argenteum* and *Achillea aleppica* subsp. *zederbaueri* on human breast cancer cell line (MCF-7). 2015;5:1-5.
18. Şen A, Bingöl Özakpınar Ö, Birteksöz Tan S, Kültür Ş, Uras F, Bitiş L. Biological activities of aerial parts extracts of endemic *Tanacetum argenteum* subsp. *argenteum*. Marmara Pharmaceutical Journal 2017;21:286-290.
19. Dey SSM, Giri B. Anti-inflammatory and Anti-tumor Activities of Parthenolide: An Update. J Chem Biol Ther. 2016;2:107.
20. Hegazy FME, Hamed AR, Mohamed TA, Debbab A, Nakamura S, Matsuda H, Paré PW. Anti-inflammatory sesquiterpenes from the medicinal herb *Tanacetum sinaicum*. RSC Advances. 2015;5:44895-44901.
21. Petrovic SD, Dobric S, Bokonjic D, Niketic M, García-Piñeres A, Merfort I. Evaluation of *Tanacetum larvatum* for an anti-inflammatory activity and for the protection against indomethacin-induced ulcerogenesis in rats. J Ethnopharmacol. 2003;87:109-113.
22. Nasri S, Amin G, Azimi A. Antiinflammatory and Antinociceptive of Hydro Alcoholic *Tanacetum balsamita* L. Extract. World Academy of Science, Engineering and Technology, International Journal of Medical, Health, Biomedical, Bioengineering and Pharmaceutical Engineering. 2014;8:186-189.
23. Bukhari IA, Khan RA, Gilani AU, Shah AJ, Hussain J, Ahmad VU. The analgesic, anti-inflammatory and calcium antagonist potential of *Tanacetum artemisioides*. Arch Pharm Res. 2007;30:303-312.
24. Hehner SP, Hofmann TG, Dröge W, Schmitz ML. The antiinflammatory sesquiterpene lactone parthenolide inhibits NF-kappa B by targeting the I kappa B kinase complex. J Immunol. 1999;163:5617-5623.
25. Hehner SP, Heinrich M, Bork PM, Vogt M, Ratter F, Lehmann V, Schulze-Osthoff K, Dröge W, Schmitz ML. Sesquiterpene lactones specifically inhibit activation of NF-kappa B by preventing the degradation of I kappa B-alpha and I kappa B-beta. J Biol Chem. 1998;273:1288-1297.
26. López-Franco O, Hernández-Vargas P, Ortiz-Muñoz G, Sanjuán G, Suzuki Y, Ortega L, Blanco J, Egido J, Gómez-Guerrero C. Parthenolide modulates the NF-kappaB-mediated inflammatory responses in experimental atherosclerosis. Arterioscler Thromb Vasc Biol. 2006;26:1864-1870.

Turk J Pharm Sci 2017;14(3):237-242
DOI: 10.4274/tjps.74745

ORIGINAL ARTICLE

Polymorphisms and Protein Expressions of Glutathione *S*-Transferase M1 and T1 in Non-Small Cell Lung Cancer

Küçük Hücreli Dışı Akciğer Kanserinde Glutatyon *S*-Transferaz M1 ve T1 Polimorfizmleri ve Protein İfadeleri

Murat KILIÇ[1]*, Ahmet Oğuz ADA[2], Serpil OĞUZTÜZÜN[3], Funda DEMİRAĞ[4], Sezgin ÇELİK[5], Pınar BIÇAKÇIOĞLU[6], Mümtaz İŞCAN[2]

[1]Ankara University, Vocational School of Health Services, Department of Pharmacy Services, Ankara, Turkey
[2]Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology, Ankara, Turkey
[3]Kırıkkale University, Faculty of Arts and Sciences, Department of Biology, Kırıkkale, Turkey
[4]Atatürk Chest Diseases and Thoracic Surgery Training and Research Hospital, Clinic of Pathology, Ankara, Turkey
[5]Yıldız Technical University, Faculty of Arts and Sciences, Department of Molecular Biology and Genetics, İstanbul, Turkey
[6]Atatürk Chest Diseases and Thoracic Surgery Training and Research Hospital, Clinic of Thoracic Surgery, Ankara, Turkey

ABSTRACT

Objectives: The deletion polymorphisms of glutathione S-transferase (GST) *GSTM1* and *GSTT1* genes result in the absence of the corresponding protein, which decreases the detoxification of carcinogens. Studies evaluating polymorphisms and protein expressions in the same patients are limited. Therefore, in this study, we aimed to investigate the association between polymorphisms and protein expressions of *GSTM1* and *GSTT1* in lung tissues of patients with non-small cell lung cancer (NSCLC).

Materials and Methods: For protein expression and gene deletion studies, tumor and surrounding tumor free (normal) tissue of 33 patients with NSCLC were used. In paraffin-embedded tissues, immunohistochemistry was used to detect protein expressions, and multiplex polymerase chain reaction amplification was used to identify gene deletions.

Results: *GSTM1* and *GSTT1* protein expressions were not detected in patients with *GSTM1* and *GSTT1* gene deletions, whereas protein expressions were detected in lung tissues of all patients carrying *GSTM1* and *GSTT1* genes. The protein expression level of *GSTT1* was 2.0-fold higher in tumors of patients lacking *GSTM1* genes than those with *GSTM1* genes (p=0.018). Protein expression of *GSTM1* was statistically higher in tumor tissues than in normal tissues of patients with *GSTM1* genes (p=0.001).

Conclusion: These results show that a) there is an association between gene deletions and protein expressions of *GSTM1* and *GSTT1* in patients with NSCLC, b) in the absence of *GSTM1* genes, enhancement of expression of GSTT1 in tumors is likely to show that *GSTT1* increases its capacity to detoxify the toxic electrophiles in tumors, and c) *GSTM1* protein expression is higher in tumors compared with normal lung tissues of patients with NSCLC.

Key words: *GSTM1*, *GSTT1*, polymorphism, protein expression, non-small cell lung carcinoma

ÖZ

Amaç: Glutatyon S-transferaz (GST) *GSTM1* ve *GSTT1* genlerinde delesyon polimorfizmleri, karsinojenlerin detoksifikasyonunu azaltan ilgili enzimlerin yokluğuna neden olur. Aynı hastalarda polimorfizm ve protein ifadelerinin değerlendirildiği çalışmalar sınırlıdır. Bu nedenle, bu çalışmada, küçük hücreli dışı akciğer kanser (KHDAK) hastalarının akciğer dokularında, *GSTM1* ve *GSTT1* polimorfizmleri ile protein ifadeleri arasındaki ilişkinin incelenmesini amaçladık.

Gereç ve Yöntemler: Protein ekspresyon ve gen delesyon çalışmaları için, 33 KHDAK'li hastanın arşiv dokularından elde edilen tümörlü ve çevresindeki normal doku çiftleri kullanıldı. Parafine gömülü dokularda, protein ekspresyonlarını belirlemek için immünohistokimyasal metod, gen delesyonlarını belirlemek için multipleks polimeraz zincir reaksiyonları yöntemi kullanıldı.

Bulgular: *GSTM1* ve *GSTT1* gen delesyonlarına sahip hastalarda *GSTM1* ve *GSTT1* protein ifadeleri bulunmazken, *GSTM1* ve *GSTT1* genlerini taşıyan tüm hastaların akciğer dokularında protein ifadeleri saptanmıştır. *GSTT1*'in protein ifadesi, *GSTM1* geninden yoksun olan hastaların tümünde, *GSTM1* genine sahip olanlardan 2.0 kat daha yüksek olduğu gözlenmiştir (p=0.018). *GSTM1*'in protein ifadesi, *GSTM1* genine sahip hastaların tümör dokularında, normal dokularından istatistiksel olarak daha yüksek belirlenmiştir (p=0.001).

*Correspondence: E-mail: muratkilic_genetic@yahoo.com, Phone: +90 544 566 95 77 ORCID-ID: orcid.org/0000-0002-1377-2021
Received: 09.02.2017, Accepted: 23.03.2017

Sonuç: Bu sonuçlar, a) KHDAK'li hastaların *GSTM1* ve *GSTT1* protein ifadeleri ile gen delesyonları arasında bir ilişkinin olduğunu, b) *GSTM1* geninin yokluğunda, tümör dokularında *GSTT1* protein ifadesinin artışı bu dokularda *GSTT1*'in toksik elektrofilikleri detoksifiye etme kapasitesini arttırma eğiliminde olduğunu ve c) KHDAK'li hastaların normal dokularına kıyasla tümörlü dokularında *GSTM1* protein ifadesinin daha yüksek olduğunu göstermektedir.

Anahtar kelimeler: *GSTM1*, *GSTT1*, polimorfizm, protein ifadesi, küçük hücreli dışı akciğer kanseri

INTRODUCTION

Lung cancer is one of the leading causes of cancer deaths in the world and it is an increasing public health problem, particularly in men.[1] Histologically, lung tumors constitute two major groups. In general, approximately 20% of cases are small-cell lung cancer (SCLC) and nearly 80% are non-small cell lung cancer (NSCLC), - the most frequently seen form of lung cancer. NSCLC has three types: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma.[2]

It was reported that cigarette smoke in particular, which contains carcinogenic xenobiotics such as polycyclic aromatic hydrocarbons, nitrosamines and aldehydes, increases carcinogenic DNA adducts in patients with NSCLC, suppresses tumor suppressor genes (such as p53), and causes aggressive tumor generation by mutations and decreases survival in these patients.[3] However, the increasing incidence of lung cancer among smokers and non-smokers suggests the possible cause of different etiologic factors other than tobacco smoking.[4] Among the risk factors, expression and genetic polymorphisms of xenobiotic metabolizing enzymes involved in the metabolism of carcinogens, namely phase I and phase II enzymes, have been reported to be associated with inter-individual variability in response to carcinogens.[5-7]

Glutathione S-transferases (GSTs), one of the phase II complex supergene family enzymes, play a major role in the detoxification of xenobiotics including carcinogens and chemotherapeutics.[8] Hence, they also have important roles in chemoresistance.[9] Cytosolic GSTs exist in seven classes GSTA, GSTM, GSTP, GSTS, GSTT, GSTO, and GSTZ; many isoenzymes have been described within these classes.[10] These GSTs are expressed polymorphically, which may lead to wide inter-individual variation in the metabolic activation of carcinogens, and toxicity and efficacy of drugs, particularly the chemotherapeutics.[8,9,11,12]

GSTM1 and *GSTT1* have deletion polymorphisms that result in the absence of their corresponding enzymes. The associations between their polymorphisms and risk of lung cancer has been studied in various populations. However, some studies revealed an association between *GSTM1*[13,14] and *GSTT1*[15] polymorphism and lung cancer risk, whereas others found no relation with respect to GSTM1[8,16] and GSTT1.[8,17]

Studies with respect to the expression of GSTM1 and GSTT1 in lung tissues of NSCLC are scarce and their results are contradictory.[18,19] Likewise, studies evaluating the combination of polymorphisms and protein expressions of GSTs in the same patients are limited, and the results are inconclusive or even conflicting.[20,21] Nakajima et al.[21] (1995) found an association between GSTM1 expression and polymorphism i.e. patients with *GSTM1* null genotype had no detectable GSTM1 protein in their lungs, whereas all patients who possessed this gene expressed GSTM1 protein in lung. However, this correlation was not noted by Cantlay et al.[20]

Moreover, the absence of one GST gene might influence the level of the other GST, which is important for a better understanding of inter-individual variation in response to carcinogens and chemotherapeutics. In line with this, the absence of GSTM1 had also shown to affect the expression of other GST (GSTM3-3) level significantly in the lung.[21]

On the other hand, the potential of GSTs as a useful tumor marker has been well established. For example, GSTP1 is the major GST isoform in the lung coupled with its increased expression in lung tumors than in normal lung tissues, which renders GSTP1 protein useful as a tumor marker for lung cancer.[20,22] Although, not as high and frequent as GSTP1, GSTM1 and GSTT1 are also expressed in normal human lung tissues.[18,20,23,24] Nevertheless, this does not exclude the possibility of their being promising useful markers for lung cancer. Thus, studies in this regard are needed.

In this study, we aimed to demonstrate whether a) polymorphisms and protein expressions of GSTM1 and GSTT1 correlate in lung tissues of patients with NSCLC, b) the absence of GSTM1 or GSTT1 gene might influence the protein expression level of the other, and c) there exists GSTM1 and GSTT1 protein expression differences between tumor and normal tissues of patients that may render them potentially useful tumor markers for NSCLC.

MATERIALS AND METHODS

Patients

For immunohistochemical and genetic polymorphism studies, tumor and surrounding tumor-free normal paraffin-embedded pairs of tissues obtained from 33 patients with NSCLC who had not received chemotherapy or radiotherapy were obtained from the archives of the pathology department of Atatürk Chest Diseases and Thoracic Surgery Training and Research Hospital (Ankara, Turkey), and the study was approved by the local ethics committee.

DNA isolation from paraffin-embedded tissues

DNA used for polymorphic analysis was isolated from paraffin-embedded tissues of patients using a DNA purification kit purchased from Zymo Research (Irvine, CA, USA) in accordance with the manufacturer's instructions. The isolated DNA samples were stored at -20˚C until use.

Genetic polymorphism analysis of GSTM1 and GST1

GSTM1 and the *GSTT1* genetic polymorphism analyses were determined using multiplex polymerase chain reaction (PCR) method.[25] In brief, isolated DNA was amplified in a 50 µL reaction mixture containing 200 µM deoxynucleotide mix, 10x

Standard Taq Reaction Buffer, 0.5 mM $MgC1_2$, 2.5 units Taq DNA polymerase and 50 pmol of each *GSTM1* primer: 5' GAA CTC CCT GAA AAG CTA AAG C and 5' GTT GGG CTC AAA TAT ACG GTG G; and *GSTT1* primers, 5'-TIC CTT ACT GGT CCT CAC ATC TC and 5'-TCA CCG GAT CAT GGC CAG CA. The *CYP1A1* gene was co-amplified using the primers 5'-GAA CTG CCA CTT CAG CTG TCT and 5'-CAG CTG CAT TTG GAA GTG CTC as internal controls to prove successful PCR. The PCR conditions consisted of an initial melting temperature of 94˚C (5 min) followed by 35 cycles of melting (94˚C, 2 min), annealing (59˚C 1 min) and extension (72˚C 1 min) with a final extension step (72˚C) of 10 min. The PCR products of *GSTT1, GSTM1*, and *CYP1A1* genes were analyzed using 2% agarose gel electrophoresis. *GSTM1* and *GSTT1* genes were detected through the presence or absence of a band at 215 bp (*GSTM1*) and a band at 480 bp (*GSTT1*). A band at 312 bp corresponding to *CYP1Al* gene was always present and used as an internal control for PCR amplification.

Immunohistochemistry

A semi-quantitative evaluation of immunohistochemical staining for GSTM1 and GSTT1 was assessed as previously described in the method of Oguztüzun et al.[19] (2010). Polyclonal rabbit antibodies against human GSTM1 and GSTT1 with no cross-reactivity with other GSTs were obtained from Abcam (Abcam-Cambridge, MA, USA). These antibodies are also suitable for immunohistochemical analysis of formalin-fixed paraffin- embedded tissues according to the manufacturer (Abcam-Cambridge, MA, USA). For immunohistochemical staining, 4-µm-thick sections from each formalin-fixed paraffin-embedded lung cancer tissue were used. Tissue sections were deparaffinized in xylene, rehydrated in graded series of alcohol, and immersed in distilled water. Endogenous peroxidase activity was blocked by incubating the sections in 1% hydrogen peroxide (v/v) in methanol for 10 minutes at room temperature (RT). The sections were subsequently washed in distilled water for 5 min, and antigen retrieval was performed for 3 min using 0.01M citrate buffer (pH 6.0) in a domestic pressure cooker. The sections were transferred in 0.05 M Tris-HCl (pH 7.6) containing 0.15 M sodium chloride (TBS). After washing in water, the sections were incubated at RT for 10 min with super block (SHP125) (ScyTek Laboratories, USA) to block nonspecific background staining. The sections were then covered with the primary antibodies diluted 1:100 for anti-GSTM1 and 1:200 for anti-GSTT1 (Abcam, USA). After washing in TBS for 15 min, the sections were incubated at RT for biotinylated link antibody (SHP125) (ScyTek Laboratories, USA). Then, treatment was followed with Streptavidin/HRP complex (SHP125) (ScyTek Laboratories, USA). Diaminobenzidine was used to visualize peroxidase activity in the tissues. Nuclei were lightly counterstained with hematoxylin, and then the sections were dehydrated and mounted. Both positive and negative controls were included in each run. TBS was used in place of the primary antibody for negative controls. Immunohistochemically stained sections were examined using light microscopy without knowing the clinical information of the patients, and the distribution, localization, and characteristics of immunostaining were recorded. Brown color in the cytoplasm of the epithelial cells was evaluated as positive staining. Scoring was performed by two authors independently without knowledge of patient data. Scoring differences between the observers were resolved by consensus. Staining intensity was graded as; 0 for none, 1 for weak, 2 for moderate, and 3 for strong staining.

Statistical analysis

MINITAB 14 statistical software (MINITAB release 14.12.0, MINITAB INC. State College, Pennsylvania, United States) was used for statistical evaluations. In tumor and normal tissues, the differences between protein expressions were investigated using the Mann-Whitney U test. The results were considered as significant for $p<0.05$.

RESULTS

The characteristics of the 33 patients with NSCLC are shown in Table 1. The mean age of the patients was 57±7 years. The majority of patients (28/33, 85%) was male. Regarding histologic subtypes, 58% (19/33) of the patients with NSCLC had squamous cell carcinoma (SCC), and 42% (14/33) had adenocarcinoma (AC). Some 76% (25/33) of the patients with NSCLC were smokers.

The *GSTM1* and *GSTT1* gene deletion frequencies in patients were 69.70% (23/33) and 33% (11/33), respectively; 30% (10/33) and 66.67% (22/33) of the patients were carriers of the *GSTM1* and *GSTT1* genes, respectively.

Figures 1 and 2 show the immunohistochemistry of GSTs in lung tumors and normal tissues using polyclonal antibodies. When

Table 1. Characteristics of 33 patients with NSCLC

Characteristics		Number of patients (%)
Total	33	
Age		
	<57	15 (45%)
	>57	18 (55%)
Sex		
	Male	28 (85%)
	Female	5 (15%)
Histology		
	Squamous cell carcinoma	19 (58%)
	Adenocarcinoma	14 (42%)
Stage at diagnosis		
	Stage I	14 (42%)
	Stage II	11 (33%)
	Stage III	8 (24%)
Smoking status		
	Never	8 (24%)
	Current	25 (76%)

tumor and normal lung tissues were matched and the level of protein expressions of GSTM1 and GSTT1 were assessed, GSTM1 and GSTT1 expression were not observed in patients with *GSTM1* and *GSTT1* gene deletions. However, staining of cells with GSTM1 and GSTT1 antibodies revealed the presence of GSTM1 and GSTT1 proteins in tumors and normal tissues of patients carrying the *GSTM1* and *GSTT1* genes, as shown in Figures 1a, 1c, 2a, and 2c. Cells stained with GSTM1 and GST1 antibodies showed positivity with varying intensities for GSTM1 and GSTT1 in 100% (10/10) and 100% (22/22) of tumors, and in 30% (3/10) and 68% (15/22) of normal lung tissues, respectively. Specific immunostaining of tissue sections was absent when the antibody was replaced in the immunohistochemical procedure by TBS, as shown in Figure 1b, 1d, 2b, and 2d.

The protein expression differences of GSTM1 and GSTT1 in tumor and normal tissues of patients with *GSTM1+, GSTT1+, GSTM1+/GSTT1-, GSTT1+/GSTM1-* and *GSTM1+/GSTT1+* are given in Table 2. When staining intensity was assessed, the majority of tumor samples (80%, 8/10), had higher GSTM1 expression than their respective normal lung tissues. However, in 41% (9/22) of sample pairs, GSTT1 expression was higher in tumor than normal lung tissue.

In addition, the protein expression level of GSTM1 was found significantly higher (6.33-fold) in tumor tissue than in normal tissues of patients with *GSTM1+* gene (p=0.001). The difference in GSTT1 expression between tumor tissues and normal tissues of patients with *GSTT1+* was not statistically significant (p=0.589).

It should also be noted that in normal tissues, the level of expression of GSTT1 exceeded that of GSTM1 by 5.3-fold (1.59±0.27 and 0.30±0.15, respectively). No significant difference, however, was noted between tumor protein levels of GSTT1 and GSTM1 (1.82±0.19 vs. 1.90±0.28).

It was observed that GSTM1 expression was remarkably (2.67-fold) higher in tumors than normal tissue of patients with *GSTM1+/GSTT1-* genotypes. However, the difference was not significant (p=0.117). Also, no significant difference was noted between the mean scores of GSTT1 of tumor and normal lung tissue of patients carrying *GSTM1-/GSTT1+* genotypes (p=0.491) (Table 2). In patients carrying both *GSTM1* and *GSTT1* genes, the level of expression of GSTM1 was statistically higher in tumor tissues than in normal tissues (p=0.012), whereas no significant difference was noted for GSTT1 expression between these tissues (p=0.999).

GSTT1 protein level was about 2.06-fold higher in tumors of patients carrying *GSTM1-/GSTT1+* genotypes than in those carrying *GSTM1+/GSTT1+* genotypes (p=0.019). However, tumor GSTM1 protein level was not significantly different between *GSTM1+/GSTT1-* and *GSTM1+/GSTT1+* genotypes (p=0.347) as shown in Table 2.

DISCUSSION

In our study, the expressions of GSTM1 and GSTT1 proteins in lung correlated perfectly with the genotypes and were not detectable in patients without *GSTM1* and *GSTT1* genes, whereas all patients who possessed these genes expressed GSTM1 and GSTT1 proteins in lung. The results of our study in regard to GSTM1 are in line with those of Nakajima et al.[21] (1995), but in contrast to those of Cantlay et al.[20] (1994). In addition, the present study also showed that protein and polymorphism association also exists for GSTT1 in human lung.

The absence of one GST gene might influence the expression level of the other GST, which is important for a better

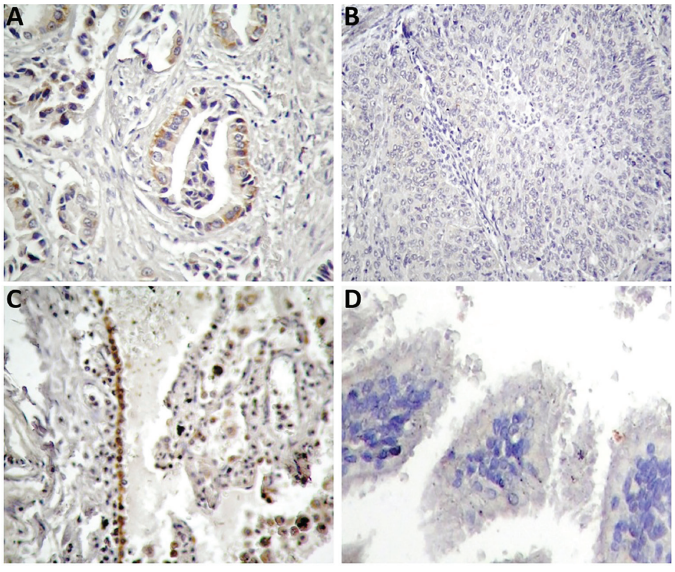


Figure 1. Immunohistochemical staining of GSTM1 protein in tumors [Panels: (a) with antibody x 400, (b) without antibody, with TBS x 400) and normal lung tissues [Panels: (c) with antibody x 400, (d) without antibody, with TBS x 400] (hematoxylin counterstain)

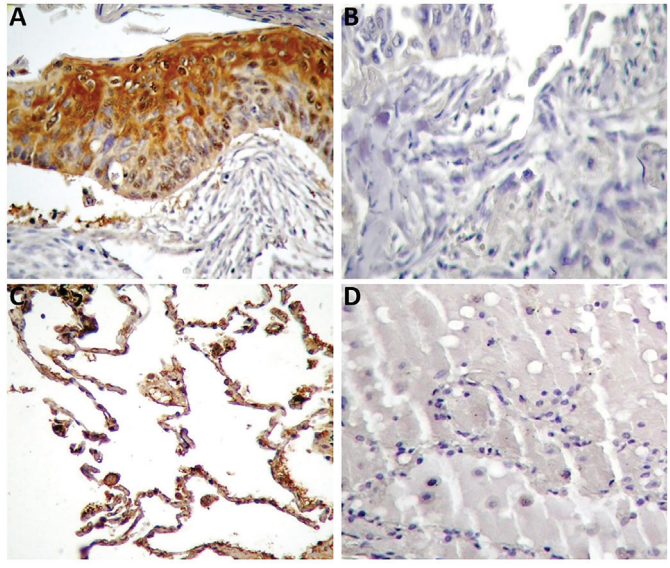


Figure 2. IImmunohistochemical staining of GSTT1 protein in tumors [Panels: (a) with antibody x 400, (b) without antibody, with TBS x 400] and normal lung tissues [Panels: (c) with antibody x 400, (d) without antibody, with TBS x 400] (hematoxylin counterstain)

Table 2. Protein expression differences of GSTM1 and GSTT1 in tumor and normal tissues of patients with *GSTM1+*, *GSTT1+*, *GSTM1+/GSTT1-*, *GSTT1+/GSTM1-* and *GSTM1+/GSTT1+*

Genotype	n	GSTM[1]		R[1] p value	GSTT[1]		R[1] p value
		Tumor	Normal		Tumor	Normal	
GSTM1+	10	1.90±0.28[a] (1-3)[b]	0.30±0.15 (0-1)	6.33 **0.001**			
GSTT1+	22				1.82±0.19 (1-3)	1.59±0.27 (0-3)	1.14 0.589
GSTM1+/GSTT1-	5	1.60±0.40 (1-3)	0.60±0.25 (0-1)	2.67 0.117			
GSTM1-/GSTT1+	17				2.06±0.22 (1-3)	1.71±0.31 (0-3)	1.20 **0.491**
GSTM1+/GSTT1+	5	2.20±0.37 (1-3)	0.00±0.00 (0-0)	- **0.012**	1.0±0.0 (1-1)	1.20±0.58 (0-3)	0.83 0.999
R^2 p value		0.73 0.347	- 0.144		2.06 **0.019**	1.43 0.583	

The staining scores were calculated based on the sum of the staining intensity of positively stained tumor and normal tissues. Staining intensity was graded as; 0 for none, 1 for weak, 2 for moderate, and 3 for strong staining. Differences of GST expressions between tumor and normal tissues were examined using the Mann-Whitney U test with 95% confidence level; + shows the presence of the gene; - shows the absence of the gene

a: mean ± standard error mean; b: minimum and maximum staining intensity; R^1: the ratio of staining intensity between tumors and control tissues; R^2: ratio of *GSTM1* and *GSTT1* staining intensities in tumor and control tissues in patients with *GSTM1+/GSTT1+*when compared to patients with *GSTM1+/GSTT1-* and *GSTM1-/GSTT1+* (staining intensity ratios of tumor/tumor and control/control are given by the columns); p value less than 0.05 was considered statistically significant.

understanding of the inter-individual variation in response to carcinogens and chemotherapeutics. Previously, the absence of GSTM1 gene/protein was shown to significantly decrease the expression of GSTM3-3 protein levels but not of GSTP1-1 or GSTA1/2 in lungs.[21] In the same study, it was also observed that the expression of GSTM2 existed only in the presence of *GSTM1* genes.[21] In the current study, the observation of the elevation of GSTT1 protein expression levels in tumors of patients lacking *GSTM1* is also noteworthy. In the absence of *GSTM1* gene, GSTT1 seems to enhance its xenobiotic detoxification capacity in lung tumors. Although we do not know the reason/s behind it, it is plausible that, as noted previously[26], many GST inducers also serve as substrates of GSTs and it might therefore be expected that the absence of *GSTM1* gene results in inducing agents, both endogenous and exogenous, possessing longer half-lives, thereby showing a greater induction capacity on GSTT1. However, the reasons behind it still remain to be thoroughly investigated.

In the current study, the protein expression of GSTM1 in normal lung tissues was also too low to be detected, confirming the findings of previous studies.[18,20,21,23] whereas expression of *GSTT1* easily detectable in more than half of normal lung tissues, as reported previously.[18] Hence, GSTT1 protein expression was observed as higher (5.3-fold) than GSTM1 protein expression in normal lung tissue. In contrast, the protein expressions of these GSTs in tumors were detectable in all tumors almost at the same level. However, their patterns of expression between tumors and normal tissues were different. The observation of the significantly higher level of expression of GSTM1 protein compared with normal lung tissue in the current study is likely to show that GSTM1 protein could be a useful marker for NSCLC. However, Nakajima et al.[21] found no significant difference between GSTM1 protein expression in tumors and normal lung tissue using immunoblot analysis. Spivack et al.[18] reach no conclusion as to whether the expression of GSTM1 protein using immunoblot analysis or mRNA levels were higher in tumor than normal lung or vice versa due to its uncommon expression in human lung. On the other hand, the lack of significant difference observed herein between the expression of GSTT1 in tumor and normal lung tissue is in contrast to the findings of a previous study[19] in which GSTT1 expression was found higher in tumors than in normal tissues. These inconsistent findings could be related to the low levels of protein expressions of GSTs, especially GSTM1, in normal lung tissues, and the use of different techniques and/or antibodies in the analysis of protein expressions of these GSTs in lung tissues in these studies. Further studies are likely to be needed to clarify these inconsistencies.

CONCLUSION

In conclusion, this study shows that, a) there is an association between gene deletions and protein expression levels of GSTM1 and GSTT1 in lungs of patients with NSCLC, b) in the absence of *GSTM1* gene, elevation of expression of GSTT1 in tumors is likely to show that GSTT1 increases its capacity to detoxify the toxic electrophiles in tumors, and c) GSTM1 protein expression is higher in tumors compared with normal lung tissues of patients with NSCLC.

ACKNOWLEDGEMENT

This study was carried out under the project supported by Kırıkkale University, Coordination Unit of Scientific Research Projects (Grant number: 2011/16).

Conflict of Interest: No conflict of interest was declared by the authors.

REFERENCES

1. Oyama T, Sugio K, Uramoto H, Kawamoto T, Kagawa N, Nadaf S, Carbone D, Yasumoto K. Cytochrome P450 expression (CYP) in non-small cell lung cancer. Front Bio Sci. 2007;12:2299-2308.
2. Oyama T, Sugio K, Isse T, Matsumoto A, Nose N, Uramoto H, Nozoe T, Morita M, Kagawa N, Osaki T, Muto M, Yasumoto K, Kawamoto T. Expression of cytochrome P450 in non-small cell lung cancer. Front Bio Sci. 2008;13:5787-5793.
3. Ada AO, C Kunak S, Hancer F, Bilgen S, Suzen SH, Alpar S, Gulhan M, Kurt B, Iscan M. CYP and GST polymorphisms and survival in advanced non-small cell lung cancer patients. Neoplasma. 2010;57:512-521.
4. Choi JH, Chung HC, Yoo NC, Lee HR, Lee KH, Choi W, Lim HY, Koha EH, Kim JH, Roha JK, Kim SK, Lee WY, Kim BS. Changing trends in histologic types of lung cancer during the last decade (1981-1990) in Korea: a hospital-based study. Lung Cancer. 1994;10:287-296.
5. Harty LC, Caporaso NE, Hayes RB, Winn DM, Bravo-Otero E, Blot WJ, Kleinman DV, Brown LM, Armenian HK, Fraumeni JF Jr, Shields PG. Alcohol dehydrogenase 3 genotype and risk of oral cavity and pharyngeal cancers. J Natl Cancer Inst. 1997;89:1698-1705.
6. Katoh T, Kaneko S, Takasawa S, Nagata N, Inatomi H, Ikemura K, Itoh H, Matsumoto T, Kawamoto T, Bell DA. Human glutathione S-transferase P1 polymorphism and susceptibility to smoking related epithelial cancer; oral, lung, gastric, colorectal and urothelial cancer. Pharmacogenetics. 1999;9:165-169.
7. Castell JV, Donato MT, Gómez-Lechón MJ. Metabolism and bioactivation of toxicants in the lung. The *in vitro* cellular approach. Exp Toxicol Pathol. 2005;57(Suppl 1):189-204.
8. Ada AO, Kunak SC, Hancer F, Soydas E, Alpar S, Gulhan M, Iscan M. Association between GSTM1, GSTT1, and GSTP1 polymorphisms and lung cancer risk in a Turkish population. Mol Biol Rep. 2012;39:5985-5993.
9. Lo HW, Ali-Osman F. Genetic polymorphism and function of glutathione S-transferases in tumor drug resistance. Curr Opin Pharmacol. 2007;7:367-374.
10. Hayes JD, Flanagan JU, Jowsey IR. Glutathione transferases. Annu Rev Pharmacol Toxicol. 2005;45:51-88.
11. Gonzalez FJ. Hereditary polymorphisms of human drug metabolizing enzymes and cancer susceptibility. Birth Defects Orig Artic Ser. 1990;26:17-42.
12. Di Pietro G, Magno LA, Rios-Santos F. Glutathione S-transferases: an overview in cancer research. Expert Opin Drug Metab Toxicol. 2010;6:153-170.
13. Lewis SJ, Cherry NM, Niven RM, Barber PV, Povey AC. GSTM1, GSTT1 and GSTP1 polymorphisms and lung cancer risk. Cancer Lett. 2002;180:165-171.
14. Pinarbasi H, Silig Y, Cetinkaya O, Seyfikli Z, Pinarbasi E. Strong association between the *GSTM1*-null genotype and lung cancer in a Turkish population. Cancer Genet Cytogenet. 2003;146:125-129.
15. Sørensen M, Autrup H, Tjønneland A, Overvad K, Raaschou-Nielsen O. Glutathione S-transferase T1 null-genotype is associated with an increased risk of lung cancer. Int J Cancer. 2004;110:219-224.
16. Carlsten C, Sagoo GS, Frodsham AJ, Burke W, Higgins JP. Glutathione S-transferase M1 (GSTM1) polymorphisms and lung cancer: a literature-based systematic HuGE review and meta-analysis. Am J Epidemiol. 2008;167:759-774.
17. Altinisik J, Balta ZB, Aydin G, Ulutin T, Buyru N. Investigation of glutathione S-transferase M1 and T1 deletions in lung cancer. Mol Biol Rep. 2010;37:263-267.
18. Spivack SD, Hurteau GJ, Fasco MJ, Kaminsky LS. Phase I and II carcinogen metabolism gene expression in human lung tissue and tumors. Clin Cancer Res. 2003;9:6002-6011.
19. Oguztüzun S, Aydin M, Demirag F, Yazici U, Ozhavzali M, Kiliç M, Işcan M. The expression of GST isoenzymes and p53 in non-small cell lung cancer. Folia Histochem Cytobiol. 2010;48:122-127.
20. Cantlay AM, Smith CA, Wallace WA, Yap PL, Lamb D, Harrison DJ. Heterogeneous expression and polymorphic genotype of glutathione S-transferases in human lung. Thorax. 1994;49:1010-1014.
21. Nakajima T, Elovaara E, Anttila S, Hirvonen A, Camus AM, Hayes JD, Ketterer B, Vainio H. Expression and polymorphism of glutathione S-transferase in human lungs: risk factors in smoking-related lung cancer. Carcinogenesis. 1995;16:707-711.
22. Di Ilio C, Del Boccio G, Aceto A, Casaccia R, Mucilli F, Federici G. Elevation of glutathione transferase activity in human lung tumor. Carcinogenesis. 1988;9:335-340.
23. Anttila S, Hirvonen A, Vainio H, Husgafvel-Pursiainen K, Hayes JD, Ketterer B. Immunohistochemical localization of glutathione S-transferases in human lung. Cancer Res. 1993;53:5643-5648.
24. Mainwaring GW, Williams SM, Foster JR, Tugwood J, Green T. The distribution of theta-class glutathione S-transferases in the liver and lung of mouse, rat and human. Biochem J. 1996;318:297-303.
25. Abdel-Rahman SZ, el-Zein RA, Anwar WA, Au WW. A multiplex PCR procedure for polymorphic analysis of *GSTM1* and *GSTT1* genes in population studies. Cancer Lett. 1996;107:229-233.
26. Talalay P, De Long MJ, Prochaska HJ. Identification of a common chemical signal regulating the induction of enzymes that protect against chemical carcinogenesis. Proc Natl Acad Sci U S A. 1988;85:8261-8265.

Turk J Pharm Sci 2017;14(3):243-250
DOI: 10.4274/tjps.93585

ORIGINAL ARTICLE

Histopathology Study of Alginate Microspheres Containing Ovalbumin on Liver and Kidney Following Oral Administration and Evaluation of Uptake by Peyer's Plaque

Oral Uygulamayı Takiben Ovalbümin İçeren Alginat Mikroküre lerinin Karaciğer ve Böbrekte Histopatoloji Çalışması ve Peyer Plakları Tarafından Alımının Değerlendirilmesi

Dewi Melani HARIYADI[1]*, Esti HENDRADI[1], Idha KUSUMAWATI[2], Fauzia AZZAHRA[1]

[1]Airlangga University, Faculty of Pharmacy, Department of Pharmaceutics, Surabaya, Indonesia
[2]Airlangga University, Faculty of Pharmacy, Department of Pharmacognosy and Phytochemistry, Surabaya, Indonesia

ABSTRACT

Objectives: The development of oral vaccine formulations has been widely investigated to overcome oral route problems. This research investigated the *in vivo* immune response of ovalbumin-alginate microspheres by uptake compared with a commercial oral vaccine product.

Materials and Methods: Ovalbumin-loaded alginate microspheres were prepared using aerosolization. Ovalbumin antigen in vivo uptake was investigated in order to understand the distribution and uptake by Peyer's plaque (PP) after oral administration using fluorescence microscopy. The histopathology of ovalbumin-alginate microspheres in the liver and kidney was also investigated.

Results: The use of alginate microspheres to deliver vaccines could be a promising delivery system for the development of oral vaccines because uptake by PP is an essential step in oral vaccination.

Conclusion: Fluorescence visualization revealed the uptake of ovalbumin-loaded alginate microspheres with and without lyoprotectant maltodextrin by PP was equal to the oral vaccine product and no liver or kidney damage was found.

Key words: Vaccine delivery, microspheres, histopathology

ÖZ

Amaç: Oral aşı formülasyonunun geliştirilmesi, oral kullanım problemlerinin üstesinden gelebilmek için geniş çapta araştırılmıştır. Bu araştırmada, ticari oral aşı ürününe kıyasla ovalbümin aljinat mikroküreleri alımındaki *in vivo* bağışıklık tepkisi araştırıldı.

Gereç ve Yöntemler: Ovalbümin yüklü aljinat mikroküreleri aerosolizasyon tekniği kullanılarak hazırlandı. Floresans mikroskobu kullanılarak oral uygulama sonrasında Peyer plakları (PP) ile alımın ve dağılımın anlaşılması için ovalbümin antijeninin *in vivo* alınımı araştırıldı. Ovalbümin aljinat mikrokürelerinin karaciğer ve böbrekteki histopatolojisi de araştırıldı.

Bulgular: Oral aşının geliştirilmesi için aşının salımında aljinat mikrokürelerin kullanılması umut vaad eden bir salım sistemi olabilir çünkü oral aşılamada PP tarafından alınım önemli bir adımdır.

Sonuç: Floresans görüntülemesi, lyoprotectant maltodextrin içeren veya içermeyen ovalbümin yüklü aljinat mikrokürelerin PP tarafından alımının oral aşıya eşit olduğunu ve karaciğer ve böbrekte hasar oluşturmadığını ortaya koymuştur.

Anahtar kelimeler: Aşı salınımı, mikroküreler, histopatoloji

***Correspondence: E-mail:** dewi-m-h@ff.unair.ac.id, **Phone:** 62 87855989394 **ORCID-ID:** orcid.org/0000-0001-9357-3913
Received: 06.02.2017 , **Accepted:** 06.04.2017

INTRODUCTION

Oral delivery systems are one of the alternative routes of drug or vaccine administration, which are non-invasive and can avoid pain and discomfort and repeated administration is easy if required.[1] Peyer's plaques (PP) in the small intestine are the main target of oral delivery systems as a place for the transport of pathogens to lymphoid tissue.[2,3] This function is carried out by M-cells, which are located between epithelial cells, bringing antigens and microparticles measuring less than 10 μm.[4]

Microspheres contain biodegradable polymers and ideally have particle sizes of less than 200 μm.[5,6] Natrium alginate is a natural polymer that is non-toxic, biocompatible, and relatively inexpensive.[7] Alginates form a three-dimensional structure when reacted with a multivalent ion. Divalent cations such as calcium, barium and strontium bind between a collection G of alginate chains, and form bridges between the chains, which causes the gelling alginate solution. Ca^{2+} is one of the best options as agents continually cross with alginate.[7] Ca^{2+} is a two-dimensional planar binding poly guluronate acid group (G) of alginate that yields a so-called egg-box. In previous research, the production of ovalbumin-alginate microspheres using ionotropic gelation by aerosolization provided advantages of spherical-shaped, smooth, and small-sized particles (<30 m) that met the requirements of particles for oral delivery systems.[7,8] Maltodextrin was added to improve the stability of the microspheres during storage during freeze drying. The addition of maltodextrin lyoprotectant was found to form smooth surfaces and smaller microspheres (<6 μm) when compared with microspheres without a lyoprotectant.[8]

An alternative for oral antigen delivery systems is microspheres. The objective of this research was to determine the immune response after administration of ovalbumin-alginate microspheres as well as oral vaccine products. Furthermore, to determine the uptake and distribution of microspheres in the gastrointestinal tract as well as the target organ. Histology using fluorescence microscopy is a qualitative approach that may provide direct evidence of the existence and location of particles in the network.[9,10] This research evaluated ovalbumin-alginate microspheres with and without maltodextrin lyoprotectant and a commercial oral vaccine product. Unencapsulated ovalbumin was used as a negative control.

MATERIALS AND METHODS

Ovalbumin, sodium alginate, protein quantification kit and BSA (Sigma Aldrich), $CaCl_2/2H_2O$ pharmaceutical grade (Solvay Chemicals Internationals), sodium citrate p.g, CMC Na p.g, and maltodextrin (Bratachem Chemicals), Rhodamin B (E Merck), vaccine product (i.m) from Sanovi Pasteur, Optimal Cutting Temperature (O.C.T) Compound (Sakura), phosphate-buffered saline pH 7.2, Na EDTA, aquadest, red gout blood cell, and mice mus musculus strain Balb C from Pusat Veterenaria Farma (PUSVETMA) Surabaya. Six mice of each group's formula was used in the in vivo study based on Federer calculation with the following animal criteria: healthy, no inflammation or irritation, 2-3 months old, and weight 20-30 grams. This research was approved by Animal Care Ethics Committee of Airlangga University in 2015.

Methods

Preparation of ovalbumin-loaded alginate microspheres

Sodium alginate (2.5%) was dissolved in distilled water and ovalbumin (2.5%) was dissolved in it. This solution was then sprayed into a solution of 1.5 M $CaCl_2$ at a pressure of 40 psi. The mixture was stirred at 1000 rpm for 2 hours. Formed microspheres were collected and then separated using centrifugation at 2.500 rpm for 6 min and washed twice. The microspheres were resuspended in lyoprotectant solution (1 g/10 mL) with concentration according to the formula. The suspension was dried in a freeze dryer at a temperature of -80°C for 29 hours. For group preparation, formula was dispersed in CMC Na solution prior to administration.

Formulas in this study as follows:

F1.1: Formula of blank alginate microspheres 1st replicate,

F1.2: Formula of blank alginate microspheres 2nd replicate,

F3.1: Formula of ovalbumin-loaded alginate microspheres 1st replicate,

F3.2: Formula of ovalbumin-loaded alginate microspheres 2nd replicate,

K1: Control of ovalbumin 1st replicate,

K2: Control of ovalbumin 2nd replicate.

Preparation of animal in vivo study

The mice were adapted for a week in a room at 25°C±2°C in a separate cages. The mice were then orally given the formulas with administration volume adjusted to the body weight of mice. For histopathologic study, after administration, the mice were sacrificed through anesthesia with ketamine prior to cervical dislocation, and the liver and kidneys were then taken. The liver and kidneys were cut and sliced. The liver and kidney samples prepared for hematoxylin and eosin staining and visualized using a fluorescence microscope (FSX 100, Olympus).

Histopathology study of ovalbumin-alginate microspheres in liver and kidney

Histopathologic examination of the liver and kidneys aimed to show the degree of damage to the liver and kidneys from the ovalbumin control, blank microspheres, and ovalbumin-alginate microspheres. This evaluation used an optical microscope Nikon H600L complete with a DS Fi2 300 megapixel digital camera and Nikon Image System Software to analyze the data.

The scoring method for the degree of liver damage in this examination was performed according to the methods of Knodell et al.[9] and Klopfleisch[11], whereby the degree of damage of each sample was determined by adding the entire score of the four types of histopathologic lesions, as shown in Table 1.

The scoring method for the degree of kidney damage was performed according to the Klopfleisch[11] method, whereby the degree of damage was determined by adding the entire score of the four types of histopathologic lesions, as shown in Table 2.

Table 1. Score based on histopathological lesions of liver

Histopathology of lesion	Score	Note
Degenerative	0	No degenerative occured
	1	Degenarative changes occured at less than 25% of all view areas
	2	Degenarative changes occured at 26-50% of all view areas
	3	Degenarative changes occured at 51-75% of all view areas
	4	Degenarative changes occured at above 76% of all view areas
Necrosis	0	No necrosis occured
	4	Necrosis occured at less than 25% of all view areas
	6	Necrosis occured at 26-50% of all view areas
	8	Necrosis occured at above 50% of all view areas
	10	Necrosis occured at 26-50% of all view areas along with bridging necrosis
	12	Necrosis occured at above 50% of all view areas along with bridging necrosis
	14	Diffuse necrosis >76% and distribute widely at almost all lobulus area (Multilobular necrosis)
Inflammation	0	No inflammation occured
	1	Inflammation area occured at less than 1/3 of total area Kiernan's triangle (portal area)
	2	Inflammation area occured at 1/3-2/3 of total area Kiernan's triangle
	3	Inflammation area occured at above 2/3 of total area Kiernan's triangle
Fibrosis	0	No fibrosis occured
	2	Intra sinusoidal fibrosis or periportal fibrosis occured at less than 25% of all areas
	4	Intra sinusoidal fibrosis or periportal fibrosis occured at 25-50% of all areas
	6	Intra sinusoidal fibrosis or periportal fibrosis occured at above 50% of all areas
	8	Intra sinusoidal fibrosis or periportal fibrosis occured at 50-75% of all areas

Total degree of damage is the total amount of all the above lesion degree of damage is where the interval is betwen **0 - 28**

Uptake of microspheres

Formulas of ovalbumin-alginate microspheres with and without lyoprotectant were compared with ovalbumin and an oral vaccine product. Rhodamine B is a fluorochrome, which was used to label all groups. The mice were adapted for a week in a room at 25˚C ± 2˚C in separate cages. Mice were then fasted for 16 hours followed by oral administration. Volume oral administration was 500 µL/25-gram body weight. To determine the intestinal uptake in the mice 7 and 8 hours after oral administration, the mice were anesthetized using ketamine and sacrificed by cervical dislocation. After the mice were dead, the intestine samples were split and cut. The intestine samples were embedded in OCT. The intestine was cut transversely with a thickness of 5 µm using a cryotome (Tissue-Tek Cryo3, Sakura) at a temperature of -59˚C. Intestinal tissue histology was then observed using a fluorescence microscope with a red filter.

Data analysis

Data from the evaluation of ovalbumin-alginate microsphere characteristics are expressed as mean ± standard deviation from triplicate experiments (data not shown). The histology study was analyzed semi-quantitatively based on scores and presented in duplicate data. For the uptake study, triplicate experiments were conducted and selected micrograph figures were presented.[11]

RESULTS AND DISCUSSION

The histopathologic examination of the livers of the mice showed the degree of damage caused by the ovalbumin control, blank microspheres, and ovalbumin-alginate microspheres.

The scores for the degree of damage to the liver can be seen in Table 3.

For the histopathology of kidney, the degree of damage to the kidneys caused by the ovalbumin control, blank microspheres, and ovalbumin-alginate microspheres is shown in Figure 1-7.

The scoring for the degree of damage to the kidneys can be seen in Table 4.

The histopathologic results in the liver and kidney showed damage/necrosis of the liver and the kidney was minimal or even absent.

Table 2. Score based on histopathological lesions of kidney

Histopathology of lesion	Score	Note
Degenerative of tubular epithelial cell	0	No degenerative occured
	1	Degenarative changes occured at less than 25% of all view areas
	2	Degenarative changes occured at 26-50% of all view areas
	3	Degenarative changes occured at 51-75% of all view areas
	4	Degenarative changes occured at above 76% of all view areas
Necrosis of tubular epithelial cell	0	No necrosis occured
	2	Number of necrosis cell of less than 25% of all view areas
	4	Number of necrosis cell of 26-50% of all view areas
	6	Number of necrosis cell of above 50% of all view areas
	8	Number of necrosis cell of above 76% of all areas
Necrosis of glomerular	0	No necrosis glomerular occured
	3	Necrosis glomerular occured of less than 25% of all glomerulus
	5	Necrosis glomerular occured of 26-50% of all glomerulus
	7	Necrosis glomerular occured of above 50% of all glomerulus
	9	Necrosis glomerular occured of above 76 % of all glomerulus
Glomerular infiltration	0	No infiltration glomerular occured
	1	Infiltration glomerular occured of less than 25% of all glomerulus
	2	Infiltration glomerular occured of 26-50% of all glomerulus
	3	Infiltration glomerular occured of above 50% of all glomerulus
	4	Infiltration glomerular occured of above 76% of all glomerulus
Interstitial infiltration	0	No infiltration occured in interstitial
	1	Infiltration occured of less than 25% of all interstitial
	2	Infiltration occured of 26-50% of all interstitia
	3	Infiltration occured of above 50% of all interstitial
	4	Infiltration occured of above 76% of all interstitial
Mesangial proliferation and or hyalization (glomerular sclerosis)	0	No proliferation and or hyalization glomerular sclerosis occured
	1	Proliferation and or hyalization glomerular sclerosis occured at less than 25% of all glomerulus
	2	Proliferation and or hyalization glomerular sclerosis occured at 25-50% of all glomerulus
	3	Proliferation and or hyalization glomerular sclerosis occured at above 50% of all glomerulus
	4	Proliferation and or hyalization glomerular sclerosis occured at 50-75% of all glomerulus
Interstitial fibrosis	0	No fibrosis occured
	3	Fibrosis occured at less than 10% of all areas
	5	Fibrosis occured at 11-30% of all areas
	10	Fibrosis occured at above 30% of all areas

Total degree of damage is the total amount of all the above lesion degree of damage is where the interval is betwen **0 - 28**

Table 3. Scores of the degree of damage to the liver

Preparat code	Score				Total score
	Degeneration	Necrosis	Inflammation	Fibrosis	
F1.1	1	4	1	0	6
F1.2	0	4	2	0	6
average					**6**
F3.1	2	4	3	0	9
F3.2	0	4	3	0	7
average					8
K1	2	4	1	0	7
K2	2	4	3	0	9
average					8

Table 4. Scores the degree of damage to the kidneys

Preparat code	Forms of lession							Total score
	a	b	c	d	e	f	g	
F1.1	0	2	0	2	0	4	0	8
F1.2	0	2	0	0	1	1	0	4
average								6
F3.1	0	4	5	2	2	2	0	15
F3.2	0	4	2	2	2	2	0	12
average								13.5
K1	0	4	3	3	3	5	0	19
K2	0	4	1	1	3	3	0	12
average								15.5

The results of uptake of ovalbumin-alginate microspheres and oral vaccine product in the fluorescence microscopy examination 7 and 8 hours after application can be seen in Figure 8 and 9.

Observations of uptake, as one of the immune response indicators, were made using a fluorescent indicator, which produces a fluorescent color at a specific wavelength. Emission wavelength fluorescence results are captured and selected by the filter, which then presents them in an appropriate dye setting. The microscopy observations of the immune response of the ovalbumin control, ovalbumin-alginate microspheres, ovalbumin-alginate microspheres with 5% maltodextrin, and the oral vaccine product were expected to show an oral vaccine antigen ovalbumin protein in the target site, the PPs. A microscopy morphology overview demonstrated golden yellow fluorescence, which suggested the presence of ovalbumin in the intestine, especially in the PPs.

Observations of uptake in the ileum of the mice performed 7 and 8 hours after administration can be seen in Figure 8 and 9. The uptake of unencapsulated ovalbumin was not seen; this may suggest that unencapsulated ovalbumin was not taken in the ileum and did not induce an immune response in lymphoid tissue.[1]

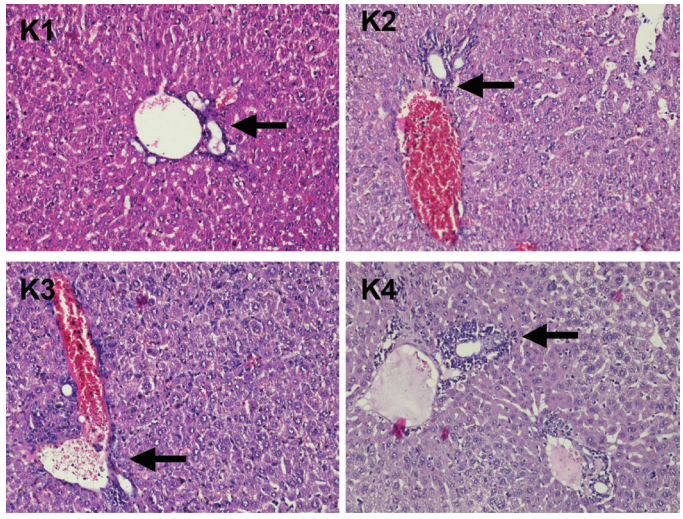


Figure 1. Differences of infiltration of inflammatory cells in the portal area (arrow) during treatment (H&E staining, Magnification 200x; H600L Nikon microscope; Fi2 300 megapixel camera DS).

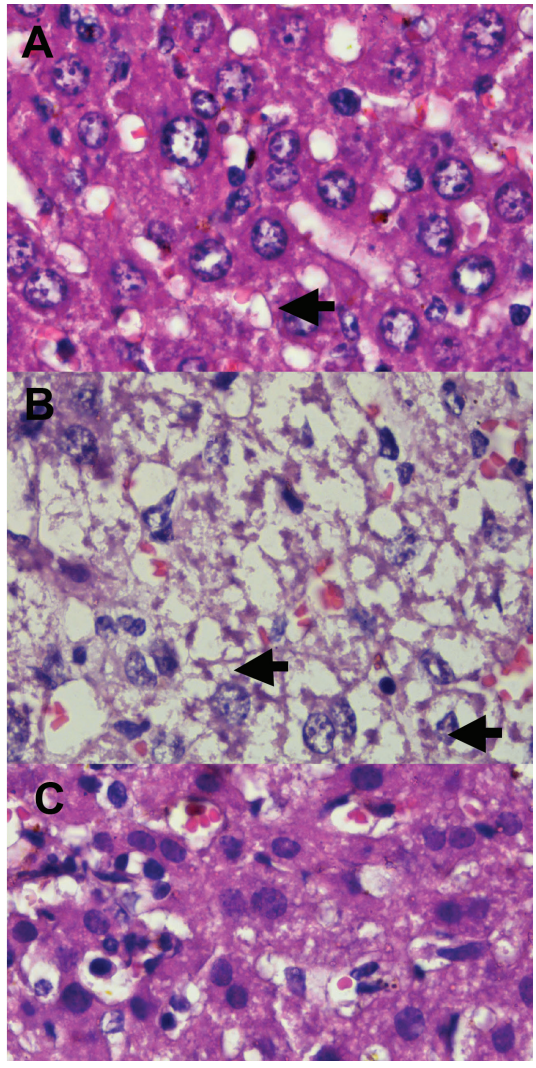


Figure 2. Normal hepatocyte cells (a), degenerative (b) and necrotic (c) (H&E staining, magnification 1000x; H600L Nikon microscope; Fi2 300 megapixel camera DS).

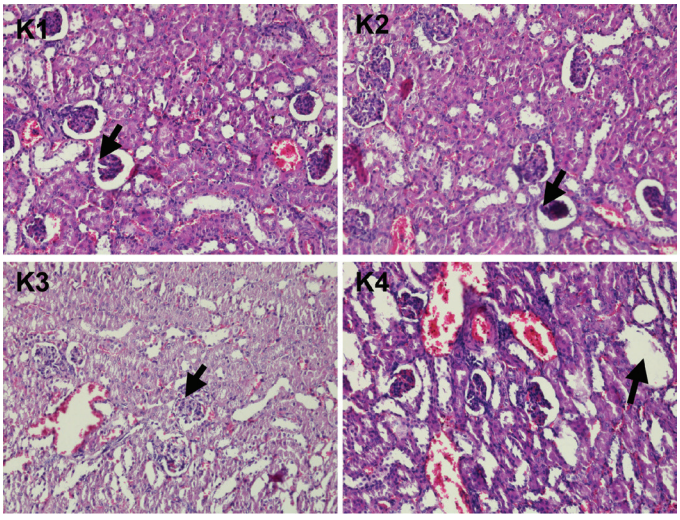


Figure 3. Different degrees of damage to the renal corpuscle in the renal cortex among treatments. Damage to renal corpuscle characterized by necrosis of glomerular cells. At a small magnification (100x-200x), visible wrinkle damaged glomeruli (black arrow) to Bowman's space is stretched, as compared to the normal renal corpuscle and even seen as an empty space (black arrow in K4) when composite of whole cell lysis by glomerular have cell activity phagosit. In this study, the glomerular injury in the group K3 relatively mild compared to other groups (H&E staining, Magnification 200x; H600L Nikon microscope; Fi2 300 megapixel camera DS).

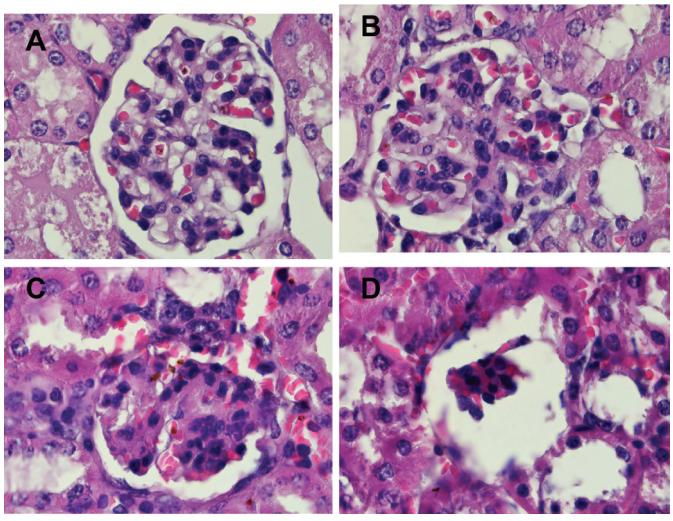


Figure 4. An overview of each glomerulus normal (a), hyperplasia (b), sclerosis (c) and necrosis (d) (H&E staining, magnification 1000x; H600L Nikon microscope; Fi2 300 megapixel camera DS).

For ovalbumin-loaded alginate microspheres, ovalbumin-alginate microspheres started entering through the villi at 7 hours, and entered deeper from seven to eight hours. Interestingly, uptake of ovalbumin-alginate microspheres with maltodextrin lyoprotectant showed deeper entry inside the villi at the 8th hour, the same as the oral vaccine product.

The uptake of ovalbumin-alginate microspheres in the villi toward the deeper part compared with unencapsulated microspheres indicated that the uptake of ovalbumin-loaded into the delivery system was more evident in the villi and PPs.

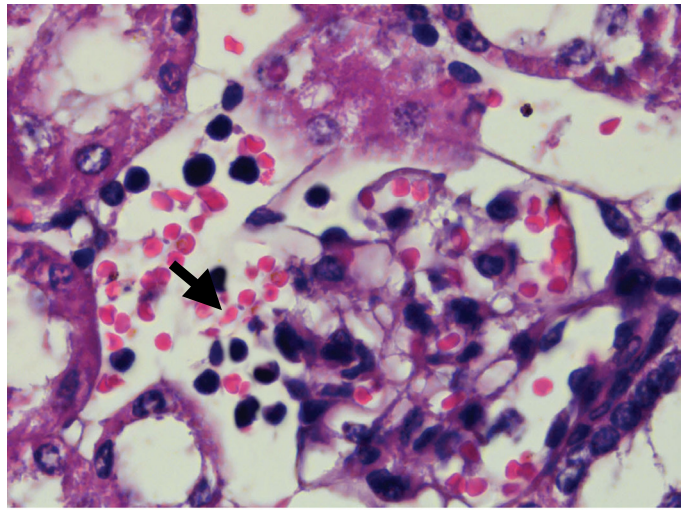


Figure 5. Infiltration of inflammatory cells (arrows) in the glomerulus (H&E staining. Magnification 1000x; Nikon microscope H600L; Fi2 300 megapixel camera DS).

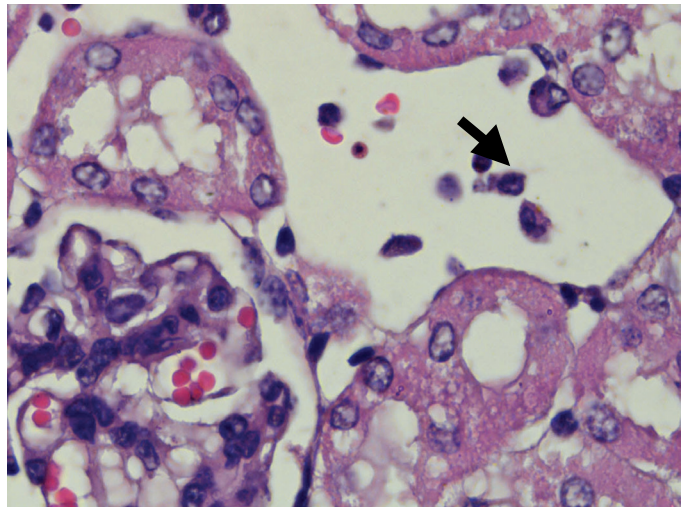


Figure 6. Infiltration of inflammatory cells (arrows) in the interstitial space (H&E staining, magnification 1000x; H600L Nikon microscope; Fi2 300 megapixel camera DS).

Uptake of microparticles in the intestine was influenced by particle size and hydrophobicity.[4] Microspheres smaller than 5 µm were transported to the lymph, where the antigen contained would be released and produce an immune response, whereas particles sized larger than 5 µm would stay in PPs and release antigen.

From the observations, a fluorescent golden yellow glow indicated the presence of ovalbumin in the network of PPs. However, the ovalbumin control group showed a lower intensity compared with formula ovalbumin-alginate microspheres both with and without lyoprotectant maltodextrin or oral vaccine product. Ovalbumin uptake in PPs was clearly shown for the ovalbumin-alginate microspheres with lyoprotectant and the oral vaccine product. This illustrated that ovalbumin had reached the target site and been taken up by M cells in PPs. In terms of particle size in ovalbumin-loaded alginate microspheres, small-

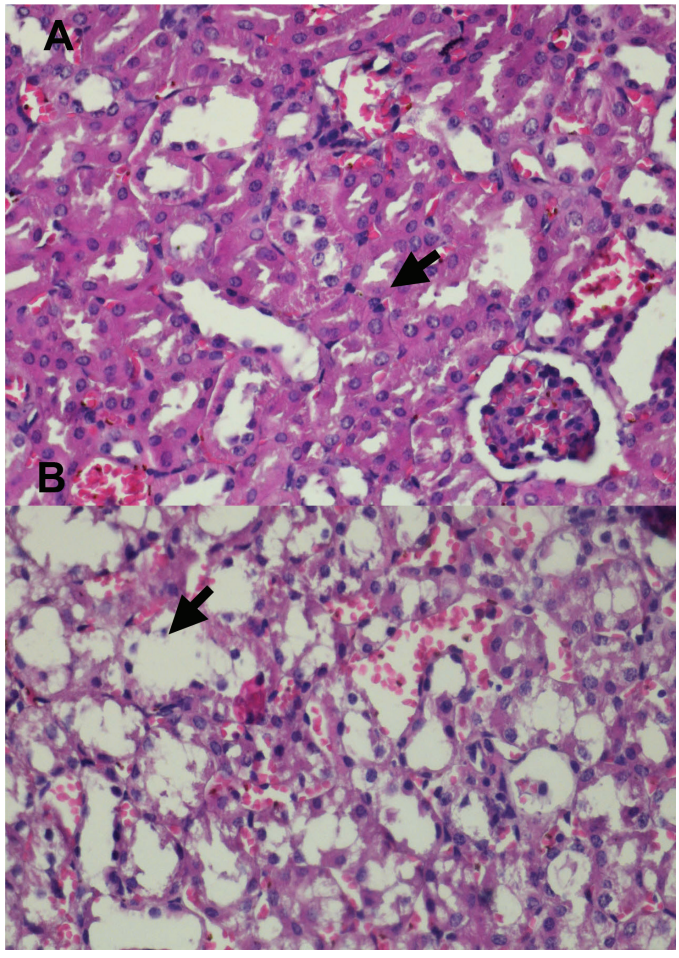


Figure 7. Cells of (a) tubular epithelial normal (black arrow) and (b) epithelial cells of tubular necrotic (black arrows) (staining H&E. Magnification 400x; microscope Nikon H600L; camera DS Fi2 300 megapixels).

sized particles passed directly into glands in addition to PPs, and were suitable to induce response.[12,13] Antigen to the target site and microspheres can bypass all barriers in the gastrointestinal tract and enter the epithelial tissue in PPs.

From the description above, it is summarized that the in vivo immune response test conducted on mice showed that microspheres as delivery systems of oral vaccines can provide an immune response equal to that of oral vaccine products.

CONCLUSION

It can be concluded that formula ovalbumin-alginate microspheres with lyoprotectant maltodextrin showed delivery of antigen to the target site, PP, at the same intensity as an oral vaccine. Furthermore, histopathology tests showed no necrotic damage of the liver and kidneys.

ACKNOWLEDGEMENT

This work was supported by a grant DIKTI (Directorate of Higher Education). We would like to thank the Faculty of Pharmacy at Airlangga University (UNAIR) for their support with research facilities.

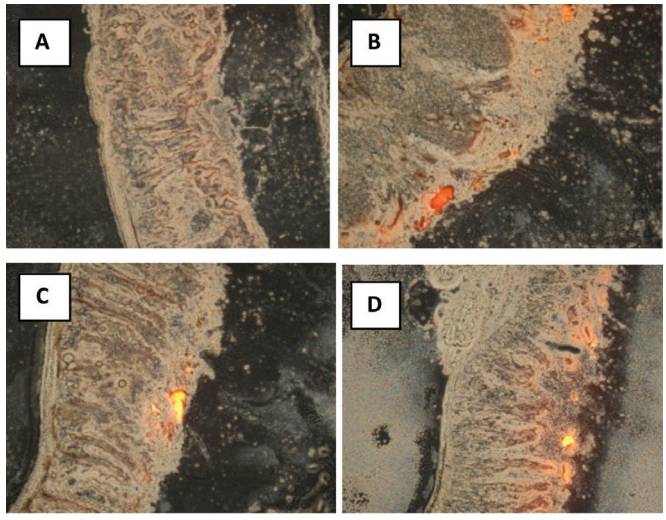


Figure 8. Uptake after 7 hours application (a) Ovalbumin, (b) ovalbumin-alginate microspheres, (c) ovalbumin-alginate microspheres with 5% maltodextrin, and (d) an oral vaccine product.

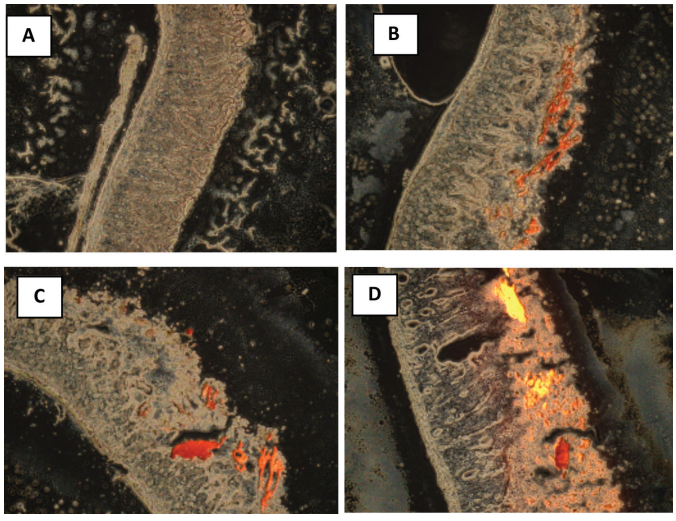


Figure 9. Uptake after 8 hours application (a) ovalbumin, (b) ovalbumin-alginate microspheres, (c) ovalbumin-alginate microspheres with 5% maltodextrin, and (d) an oral vaccine product.

Conflict of Interest: No conflict of interest was declared by the authors.

REFERENCES

1. Abdelwahed W, Degobert G, Stainmesse S, Fessi H. Freeze-drying of nanoparticles: formulation, process and storage considerations. Adv Drug Deliv Rev. 2006;58;1688-1713.
2. Van der Lubben IM, Konings FA, Borchard G, Verhoef JC, Junginger HE *in vivo* uptake of chitosan microparticles by murine Peyer's patches: visualization studies using confocal laser scanning microscopy and immunohistochemistry. J Drug Target. 2001;9:39-41.
3. Van der Lubben IM, Verhoef JC, van Aelst AC, Borchard G, Junginger HE. Chitosan microparticles for oral vaccination: preparation, characterization and preliminary *in vivo* uptake studies in murine Peyer's patches. Biomaterials. 2001;22:687-694.

4. Borges O, Cordeiro-da-Silva A, Romeijn SG, Amidi M, de Sousa A, Borchard G, Junginger HE. Uptake studies in rat Peyer's patches, cytotoxicity and release studies of alginate coated chitosan nanoparticles for mucosal vaccination. J Control Release. 2006;114:348-358.

5. Tewes F, Boury F, Benoit JP. 2005. Biodegradable Microspheres: Advances in Production Technology. Dalam: S. Benita, penyunt. Microencapsulation Methods and Industrial Application. France: Taylor and Francis Group, pp. 1-41.

6. O'Hagan DT, Singh M, Ulmer JB. Microparticle-based technologies for vaccines. Methods 2006;40:10-19.

7. Hariyadi DM, Hendradi E, Purwanti T, Fadıl FDGP, Ramadani CN. Effect of Cross Linking Agent and Polymer on the Characteristic of Ovalbumin Loaded Alginate Microsphere. International Journal of Pharmacy and Pharmaceutical Sciences, 2014;6:469-474.

8. Hariyadi DM, Purwanti T, Kusumawati I, Nirmala RN, Maindra HMC. Physical Characterization and *in vivo* study of ovalbumin encapsulated in alginate microspheres, International Journal of Drug Delivery Technology. 2015;5;48-53.

9. Knodell RG, Ishak KG, Black WC, Chen TS, Craig R, Kaplowitz N, Kiernan TW, Wollman J. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. Hepatology. 1981;1:431-435.

10. Mutwiri G, Bowersock TL, Babiuk LA. Microparticles for oral delivery of vaccines. Expert Opin Drug Deliv. 2005;2:791-806.

11. Klopfleisch R. Multiparametric and semiquantitative scoring systems for the evaluation of mouse model histopathology a systematic review. BMC Vet Res. 2013;9:123.

12. Brayden DJ, Jepson MA, Baird AW. Keynote review: intestinal Peyer's patch M cells and oral vaccine targeting. Drug Discov Today. 2005;10:1145-1157.

13. Robbins PF, Lu YC, El-Gamil M, Li YF, Gross C, Gartner J, Lin JC, Teer JK, Cliften P, Tycksen E, Samuels Y, Rosenberg SA. Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. Nat Med. 2013;19:747-752.

Turk J Pharm Sci 2017;14(3):251-256
DOI: 10.4274/tjps.84856

ORIGINAL ARTICLE

A Synchronous Fluorescence Spectrofluorometric Method for the Simultaneous Determination of Clonazepam and Paroxetine Hydrochloride in Combined Pharmaceutical Dose Form

Kombine Farmasötik İlaç Şekillerinde Klonozepam ve Paroksetin Hidroklorür'ün Aynı Anda Belirlenmeleri İçin Senkronize Spektroflorimetrik Yöntem

Jalpa U. PATEL*, Usmangani K. CHHALOTIYA

Indukaka Ipcowala College of Pharmacy, Gujarat, India

ABSTRACT

Objectives: First derivative synchronous spectrofluorimetry has been found to be superior because of its highly specific spectral discrimination and readily available solvent, it is economical, eco-friendly, and lacks an extraction procedure.

Materials and Methods: In the present study, a new simple, sensitive, and time-saving first derivative synchronous spectrofluorimetry method has been developed for simultaneous estimation of clonazepam (CLO) and paroxetine hydrochloride (PH) in pharmaceutical dose forms.

Results: CLO was determined at the emission wavelength of 512.79 nm (zero-crossing wavelength point of PH). Similarly, PH was measured at 336.00 nm (zero-crossing wavelength point of CLO). The first derivative amplitude-concentration plots were rectilinear over the range of 1-5 µg/mL for CLO and 5-25 µg/mL for PH. The method was validated statistically as per the ICH guidelines. The limits of detection were 0.055 and 0.033 µg/mL and quantification limits were 0.169 and 0.102 µg/mL for CLO and PH, respectively. The percentage recovery in commercial formulation was found to be in the range 100.45% and 99.38% for CLO and PH, respectively, by the proposed method, and percent relative standard deviation values for precision and accuracy studies were found to be less than 2.

Conclusion: This spectrofluorimetry method has been found to have several advantages such as simple spectra, high selectivity, and low interference. By virtue of its high sensitivity, this method could be applied to the analysis of both CLO and PH in their co-formulated dose forms.

Key words: First-derivative synchronous spectrofluorimetry, clonazepam, paroxetine hydrochloride

ÖZ

Amaç: Yüksek spesifiklikte spektral ayırıcılığı, kolay bulunan çözücülerin kullanımı, ekonomik ve çevre dostu oluşu ve ekstraksiyona gerek duymaması sebebiyle birinci türev senkron spektroflorimetri yönteminin üstün olduğu bulundu.

Gereç ve Yöntemler: Bu çalışmada farmasötik preparatlardan klonazepam (CLO) ve paroksetin hidroklorürün (PH) eş zamanlı tayini için basit, hassas ve zamandan tasarruf sağlayan birinci türev senkronize spektroflorimetri yöntemi geliştirilmiştir.

Bulgular: CLO, 512.79 nm'deki (PH'nin sıfır olduğu dalga boyu noktasında) emisyon dalga boyunda tayin edilmiştir. Benzer şekilde PH ise 336.00 nm'deki (CLO'nun sıfır olduğu dalga boyu noktasında) emisyon dalga boyunda ölçüm yapılarak tayin edilmiştir. Birinci türev için pik yüksekliğine karşı çizilen konsantrasyon grafiği CLO için 1-5 µg/mL, PH için 5-25 µg/mL aralığında doğrusal olarak bulunmuştur. Yöntemin istatistiksel validasyonu ICH kılavuzlarına uygun olarak istatistiksel gerçekleştirilmiştir. Teşhis sınırı CLO ve PH için sırasıyla 0.055 ve 0.033 µg/mL, tayin sınırı ise 0.169 ve 0.102 µg/mL'dir. Önerilen yöntem kullanılarak piyasa preparatlarındaki yüzde geri kazanım sonuçları CLO ve PH için sırasıyla %100.45 ile %99.38 arasında ve yüzde bağıl standart sapma değerleri de kesinlik ve doğruluk çalışmalarında 2'den daha düşük bulunmuştur.

Sonuç: Bu spektroflorimetri yönteminin, basit spektrumlar, yüksek seçicilik ve düşük girişim gibi birçok avantajı olduğu bulunmuştur. Yüksek duyarlılığından dolayı, bu yöntem CLO ve PH'nin birlikte formüle edilmiş dozaj formlarından analizi için uygundur.

Anahtar kelimeler: Birinci türev senkronize spektroflorimetri, klonazepam, paroksetin hidroklorür

*Correspondence: **E-mail:** jalpa.patel70@ymail.com, **Phone:** +91 9033994032 **ORCID-ID:** orcid.org/0000-0002-8134-6294
Received: 11.08.2016, **Accepted:** 08.12.2016

INTRODUCTION

The frequent association between panic disorders and depression is extensively documented in both clinical and epidemiologic studies, and it is considered to be more of a common phenomenon than an exception. Current treatment recommendations for comorbid depression and anxiety are based on clinical experience with the treatment of anxiety and depressive disorders when they occur independently. There are studies that have tried combinations of selective serotonin reuptake inhibitors (SSRI) (paroxetine) and benzodiazepine (clonazepam) in patients of depression and anxiety/panic disorders. Hence, the availability of a fixed dose combination of an anti-depressant such as paroxetine hydrochloride (PH) and an anti-anxiety drug such as clonazepam (CLO) would be a useful treatment option for the management of co-morbid depression and anxiety.[1-4]

CLO [5-(2-chlorophenyl)-7-nitro-2, 3-dihydro-1H-1,4-benzodiazepin-2-one], Figure 1 is a benzodiazepine drug that has anxiolytic, anticonvulsant, muscle relaxant, sedative, and hypnotic properties. CLO exerts its action by binding to the benzodiazepine site of GABA receptors, which causes an enhancement of the electrical effect of GABA binding on neurons, resulting in an increased influx of chloride ions into the neurons. These result in an inhibition of synaptic transmission across the central nervous system.[3-5]

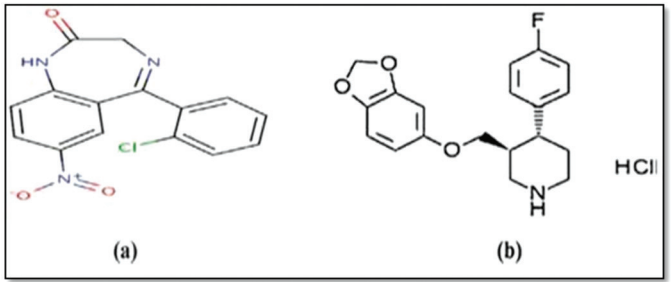


Figure 1. Chemical structure of (a) CLO and (b) PH

PH [(-)-Trans-4R-(4'-fluorophenyl)-3S-[(3', 4'-methylenedi-oxyphenoxy) methyl] piperidine hydrochloride], Figure 1b is an SSRI and potentiates 5-HT in the CNS. PH is indicated for the treatment of major depressive disorder, social anxiety disorder, obsessive-compulsive disorder, panic disorder, generalized anxiety disorder, and post-traumatic stress disorder. It exerts its antidepressant effect through selective inhibition for the reuptake of the neurotransmitter serotonin by presynaptic receptors.[3-5]

CLO and PH both are official drugs in the Indian Pharmacopoeia, United States Pharmacopeia, and British Pharmacopoeia when used individually, but the combination of CLO and PH is not official in any pharmacopoeia.[5-7] CLO and PH have been formulated in a fixed-dose combination and used in the treatment of depression, anxiety, and comorbidities.

Few analytical methods including spectrophotometry[8], colorimetry[9], stability-indicating high-performance liquid chromatography[10,11] high-performance thin layer chromatography[12] and ultra-performance liquid chromatography[13] have been reported for the simultaneous estimation of CLO and PH in combined pharmaceutical formulations. Chromatographic methods offer a high degree of specificity, yet they require large amounts of high purity organic solvents and generate a large amount of waste. Therefore, there is a need for an alternative to these techniques for the routine quality control analysis of the concerned drug.

To the best of our knowledge, no spectrofluorimetric method has yet been reported for the quantification of CLO and PH in combined formulations. Spectrofluorimetric methods have been found more selective than normal UV-spectroscopy due to the quantification of substance at excitation and emission wavelengths. Derivative spectrofluorimetry provides greater selectivity and spectral discrimination than common spectrofluorimetry. It is a powerful approach for the resolution of one analyte whose peak is hidden by a large overlapping peak of another analyte in multi-component analysis. Synchronous fluorescence spectroscopy has been found to have several advantages such as simple spectra, high selectivity, and low interference. The combination of synchronous scanning spectrofluorimetry with derivative techniques is advantageous in terms of sensitivity, spectral discrimination, and more reliable identification of chemical species in multi component analysis.[14,15]

The goal of the present work was to develop a simple, cost effective, sensitive, and rapid method for the simultaneous determination of CLO and PH in tablet form through first-derivative synchronous fluorimetry (FDSF) based on their native fluorescence. The emission spectra of CLO and PH overlap, which makes it difficult to analyze and determine their contents by conventional fluorimetry. These problems were minimized by using FDSF.

EXPERIMENTAL

Materials

All chemicals and reagents were of analytical grade. The CLO and PH were obtained as gift samples from Vital Formulation, Anand and Torrent Pharmaceutical, and Ahmedabad, respectively. Pari CR Plus formulation (CLO 0.5 mg and PH 12.5 mg) were purchased from local pharmacies. Analytical grade methanol purchased from Merck, Mumbai, was used throughout these experiments.

Apparatus

Fluorescence spectra and measurements were recorded using a spectroflorophotometer (RF-5301PC series, Shimadzu Corporation, Japan), which allowed high sensitivity analysis based on a unique optical system that involves highly efficient Blazed Holographic Grating as well as low-noise circuitry that includes a digital filter. Spectroflorophotometer equipped with a 150 W xenon arc lamp and slit widths for both monochromators were set at 10 nm. A 1 cm^2 quartz cell was used for all measurements. Spectra and intensities were automatically

obtained using Shimadzu fluorescence spectroscopy software, RFPC version 2.04.

Preparation of standard solutions

Standard stock solutions each containing 1000 µg/mL of CLO and PH were prepared separately in the methanol. Working standard solutions (100 µg/mL) of the mentioned drugs were obtained by dilution of the respective stock solution in methanol. Working solutions were prepared separately by making serial dilutions from the standard solution to obtain concentrations between 1-5 and 5-25 µg/mL for CLO and PH, respectively, and fluorescence intensity was quantified using a spectroflorophotometer.

Spectrofluorimetric methods

For spectrofluorimetry, the solutions were scanned between 220 nm to 800 nm and emission wavelength was selected based on the maximum fluorescence intensity. The excitation and emission wavelength of both the drugs were found. The synchronous spectra of CLO and PH were obtained by keeping a constant interval between emission and excitation wavelength (i.e $\Delta\lambda$= 20). The obtained synchronous spectra of both drugs were converted to the 1st derivative spectra by optimizing $\Delta\lambda$= 20. The first derivative synchronized spectrum of CLO has zero intensity at 336.00 nm, whereas PH gives significant derivative response. The derivative spectrum of PH has zero intensity at 512.79 nm, and CLO gives significant derivative response by maintaining a constant interval $\Delta\lambda$= 20. Therefore, 512.79 nm and 336.00 nm were chosen for the estimation of CLO and PH, respectively. Different aliquots were transferred for both drugs to 10 nm volumetric flasks and the volumes were adjusted to the mark with methanol to obtain concentrations of 1-5 µg/mL of CLO and 5-25 µg/mL PH, respectively. The calibration curves between concentration and fluorescence intensity were plotted and corresponding regression equations were derived.

Method validation

The proposed methods were validated in accordance with International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines Q2 (R1) for the evaluation of various parameters: linearity, precision, accuracy, limit of detection, limit of quantification, specificity, and robustness.[16]

Linearity and range

The linear relationship between concentration and amplitude of both drugs were evaluated over the concentration range expressed in the concentrations range of 1-5 µg/mL for CLO, 5-25 µg/mL was selected for PH. The linearity ranges for the determination of CLO and PH by the proposed methods were repeated five times. Calibration curves were constructed by plotting the analyte intensity against the respective concentration.

LOD and LOQ

As per the ICH guidelines, the limit of detection and quantification of the developed method were calculated from the standard deviation of the response and slope of the calibration curve of each drug using the following formulas:

Limit of detection= $3.3x\sigma/S$

Limit of quantification= $10x\sigma/S$

Where, "σ" is standard deviation of response, "S" is Slope of calibration curve.

Precision

The precision of the developed methods were evaluated by performing intraday precision on the same day and interday precision studies on different days in three replicates. Repeatability and intermediate precision was performed for CLO at 1, 3, 5 µg/mL, and 5, 15, 25 µg/mL for PH. The percent relative standard deviation (% RSD) was calculated.

Accuracy

Accuracy of method was ascertained by performing a recovery study using the standard addition method; a known amount of standard drug was added to preanalyzed samples of CLO and PH at three concentration levels (50%, 100%, and 150%) in triplicate. The percentage recovery and RSD were calculated for each concentration.

Robustness

Robustness was performed by making deliberate changes in wavelength and different model of UV visible spectrophotometer. % RSD was calculated.

Applicability of the proposed method for analysis of formulation

For the analysis of the marketed formulation, 20 tablets were weighed and finely powdered. From the tablet sample, an amount equivalent to 0.5 mg of CLO and 12.5 mg PH were accurately weighed and taken into a 100 mL volumetric flask. About 30 mL of methanol was added and the mixture was sonicated for 15 min. The mixture was diluted to volume with methanol, mixed well and filtered to obtain the sample stock solution, 5 µg/mL of CLO and 125 µg/mL of PH. The resultant solution was used for the analysis of CLO and for analysis of PH; 1 mL from the above solution was withdrawn and the volume was made up to 10 mL to make 12.5 µg/mL for PH. The resultant solutions were then used to estimate both drugs at their particular λ_{max} for both methods. The analysis was repeated in triplicate.

RESULTS AND DISCUSSION

Synchronous fluorescence spectrofluorimetric method

Different solvent systems were tested in order to find the best conditions such as solubility, fluorescence activity, stability, and spectral discrimination (clear separation) of both drugs. From the results, it was found that CLO and PH gave comparatively high fluorescence intensity in methanol. CLO exhibited native fluorescence at emission wavelength of 460 nm after excitation at 258 nm and similarly PH exhibited fluorescence at emission wavelength of 545 nm after excitation at 288 nm in methanol. It was revealed that the fluorescence spectra of these drugs overlapped considerably. As a result, the conventional spectrofluorimetric method did not permit the simultaneous determination of both drugs.

The overlapped spectra were resolved by using a first-order derivative synchronous spectrofluorimetric method, which was used to choose the suitable wavelengths that make the estimation proportional to CLO and PH concentrations with the zero cross over point. It was necessary to record the synchronous spectra of CLO and PH shown in Figure 2 and 3, corrected for the blank signal. There was large overlap of the spectra; the quantification of CLO and PH through synchronous spectrofluorimetry was not possible. This overlap was highly discriminated by using a first-derivative synchronous spectrofluorimetric method (Figure 4).

The first-derivative synchronized spectrum of CLO has zero intensity at 336.00 nm, whereas PH gives significant derivative response. The derivative spectrum of PH has zero intensity at 512.79 nm, whereas CLO gives a significant derivative response by maintaining a constant interval $\Delta\lambda$= 20. Therefore, 512.79 nm and 336.00 nm were chosen for the estimation of CLO and PH, respectively, in tablet form (Figure 5, 6).

Method validation

The validation of the methods was performed according to ICH recommendations.

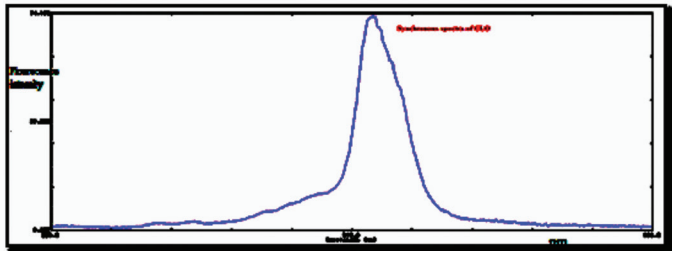


Figure 2. Synchronous spectrum of CLO

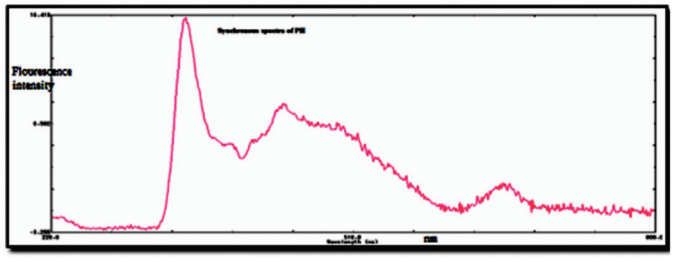


Figure 3. Synchronous spectrum of PH

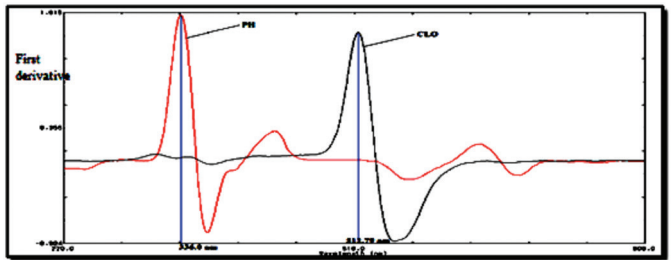


Figure 4. Overlay of 1st order derivative spectrum of CLO and PH

Linearity

The calibration ranges for CLO and PH were established through considerations of the practical range necessary according to Beer-Lambert's law. Linearity was evaluated using the least square regression method. The responses for CLO were found to be linear in the concentration range of 1-5 µg/mL with a correlation co-efficient (r^2) value of 0.9986 as depicted in Figure 7. Similarly, the responses for PH were linear in the concentration range of 5-25 µg/mL with a correlation coefficient (r^2) value of 0.9982, as shown in Figure 8. The values of correlation coefficients of CLO and PH were close to unity, indicating good linearity. The characteristic parameters for the constructed equations are summarized in Table 1.

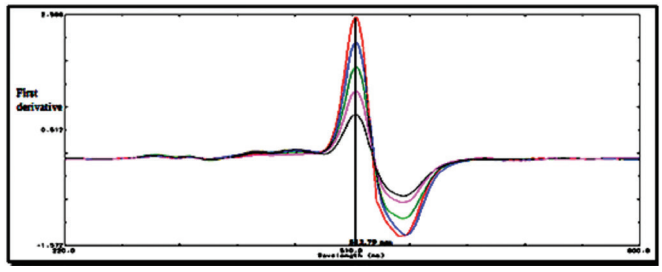


Figure 5. 1st order derivative spectrum of CLO at 512.79 nm (1-5 µg/mL)

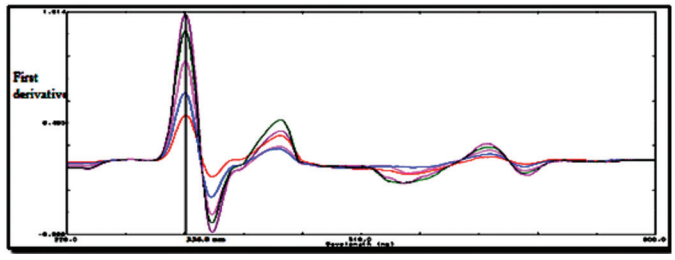


Figure 6. 1st order derivative spectrum of PH at 336.00 nm (1-5 µg/mL)

Table 1. Linear regression parameters for CLO and PH

Parameters	CLO	PH
Linearity range (µg/mL)	1-5	5-25
Correlation coefficient (r^2)	0.9986	0.9982
Slope ± SD[b] (S_b)	0.470±0.005	0.063±0.009
Confidence limit of slope	0.435 to 0.506	0.058 to 0.068
Intercept ± SD[b] (S_a)	0.274±0.008	0.087±0.012
Confidence limit of intercept	0.156 to 0.392	0.006 to 0.169
Limit of detection (µg/mL)	0.055	0.033
Limit of quantification (µg/mL)	0.169	0.102
Bartlett's test[b] (χ^2)	0.0110	0.0054

[b]: Mean of five determinations, SD: Standard deviation, RSD %: Relative standard deviation, b χ^2 critical value: 9.488 at α=0.05, [a]: Confidence interval at 95% confidence level and 4 degree of freedom (t=2.78)

Limit of detection (LOD) and Limit of quantification (LOQ)

The limit of detection and limit of quantification were determined based on the standard deviation of response (y-intercept) and slope of the calibration curve according to ICH guidelines. LOD and LOQ for CLO were found as 0.055 µg/mL and 0.169 µg/mL, and 0.033 µg/mL and 0.102 µg/mL for PH, respectively, as tabulated in Table 1.

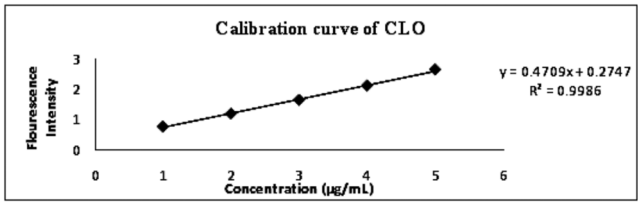


Figure 7. Calibration curve of CLO at 512.79 nm

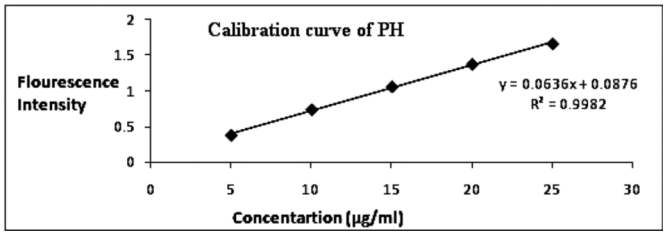


Figure 8. Calibration curve of PH at 336.00 nm

Table 2. Precision study

Amount of drug (µg/mL)	Intraday precision		Interday precision	
	Amount of drug found ± SD (µg/mL)	RSD %	Amount of drug found ± SD (µg/mL)	RSD %
CLO				
1	1.056±0.003	0.454	1.069±0.007	0.946
3	2.899±0.007	0.473	2.956±0.026	1.563
5	5.003±0.019	0.723	5.072±0.036	1.360
PH				
5	4.951±0.002	0.663	4.969±0.005	1.309
15	15.010±0.008	0.826	15.187±0.019	1.801
25	24.787±0.019	1.170	24.817±0.022	1.375

n=3 concentration/3 replicates, SD: Standard deviation, RSD %: Relative standard deviation

Precision

The intraday and interday precision were determined by the analysis of three different concentrations of CLO and PH, within the linearity range, through three replicate analyses of three pure samples of both drugs on a single day and on three consecutive days, respectively. As indicated in Table 2, data showed RSD % less than 2%. The precision of the method was considered acceptable based on its intended use.

Accuracy

Recovery study by spiking the standard at 3 concentration levels, 50, 100 and 150% showed RSD % of less than 2% with acceptable percent recovery, indicating that the proposed method was accurate and could be applicable for routine analysis of formulation (Table 3).

Robustness

The method remained unaffected by deliberate small changes in parameters such as wavelength (±1 nm) and model of UV spectrophotometer. The tabulated values indicate that the method was robust in terms of changed wavelength and model of UV spectrophotometer. The data are presented in Table 4.

Analysis of marketed dosage form

The proposed method was applied to the assay of commercially available tablets containing CLO (0.5 mg) and PH (12.5 mg). The percentage potency in the commercial formulations was found as 100.45% for CLO and 99.38% for PH using the proposed method. The RSD % for the formulations was less than 2 for both drugs, as shown in Table 5. The percentage recoveries of the amount of CLO and PH in tablet dose form, expressed as a percentage assay, were in good agreement with the label claims, thereby suggesting that there was no interference from any of the excipients that normally present in tablets.

Table 3. Recovery study for CLO and PH by proposed method

Drugs	Taken (µg/mL)	Level %	Amount of standard added (µg/mL)	Total amount of drug found (µg/mL)	Recovery ± SD %	RSD %
CLO	2	50%	1	3.024	101.21±0.015	0.015
		100%	2	4.007	100.90±0.026	0.025
		150%	3	5.029	100.84±0.034	0.034
PH	10	50%	5	15.118	101.20±0.071	0.070
		100%	10	20.267	100.15±0.211	0.211
		150%	15	25.244	100.78±0.232	0.232

n=3 concentration/3 replicates, SD: Standard deviation, RSD %: Relative standard deviation

Table 4. Robustness of proposed method

Sr. No	Variable parameters	Amount of drug found ± SD	RSD %
CLO			
1	Equipment 1 UV Spectrophotometer model-UV-1700	5.051±0.0358	1.354
	Equipment 2 UV Spectrophotometer model-UV-1800	5.029±0.0356	1.348
2	Wavelength 513.79 nm	4.906±0.0289	1.315
	Wavelength 511.79 nm	4.762±0.0280	1.276
PH			
1	Equipment 1 UV Spectrophotometer model-UV-1700	4.621±0.001	0.618
	Equipment 2 UV Spectrophotometer model-UV-1800	4.951±0.002	0.663
2	Wavelength 335.00 nm	4.321±0.004	1.138
	Wavelength 337.00 nm	4.969±0.005	1.309

SD: Standard deviation

Table 5. Analysis of marketed dose form

Formulation	Drug	Label claim	Assay ± SD %	RSD %
Pari CR plus	CLO	0.5 mg	100.451±0.926	1.295
	PH	12.5 mg	99.381±0.300	0.331

n=3 replicates, % RSD: Relative standard deviation; SD: Standard deviation

CONCLUSION

In the present study, a new simple, sensitive, and time-saving first-derivative synchronous spectrofluorimetry method was developed for simultaneous estimation of CLO and PH in pharmaceutical dose forms. First-derivative synchronous spectrofluorimetry has been found to be superior because of its highly specific spectral discrimination, readily available solvent, cost effectiveness, eco-friendly nature, and lack of extraction procedure. This spectrofluorimetry method has been found to have several advantages such as simple spectra, high selectivity, and low interference. By virtue of its high sensitivity, this method can be applied to the analysis of both CLO and PH in their co-formulated dose forms.

ACKNOWLEDGEMENT

The authors are thankful to Vital Formulation, Anand and Torrent Pharmaceutical, and Ahmedabad for providing gift samples of CLO and PH, respectively. The authors are thankful to the management of Anand Pharmacy College for providing facilities for this research work.

Conflict of Interest: No conflict of interest was declared by the authors.

REFERENCES

1. Rang HP, Dale M, Ritter JM, Flower RJ. Rang and Dale's Pharmacology. 6th ed. Churchill Livingstone Elsevier; 2007. pp. 536-542.
2. Brown JH, Taylor P, Robert LJ, Marrow JD. Goodman and Gilman's The Pharmacological Basis of Therapeutics. New York; McGraw-Hill; 2001. pp. 280.
3. Jagawat T. A Comparative Study to Assess the Efficacy and Safety of Combination Capsules of Paroxetine and Clonazepam in comparison to Paroxetine in patients suffering from Co-morbid Depression and Anxiety. Delhi Psychiatry Journal. 2011;14:106-109.
4. Romosan F, Dehelean L, Ienciu M. Comorbidity of Panic Disorder with Depression: Clinical Implications. TMJ. 2003;53:246-249.
5. Indian Pharmacopoeia. Ministry of Health and Family Welfare. Indian Pharmacopoeial Commission. Ghaziabad; 2014. pp. 1434, 2439.
6. United States Pharmacopeia 38, National Formulary 33, The United States Pharmacopoeial Convention, 2888, 4765, Rockville, vol. 2, 2015.
7. British Pharmacopoeia, Medicines and Health Care Products Regulatory Agency, pp. 506-508, 587-588, London, vol. 1-2, 2014.
8. Boda JM, Bhalodiya HA, Patel PB. UV spectroscopic method for simultaneous estimation of clonazepam and Paroxetine hydrochloride hemihydrate in combined pharmaceutical formulation. Inventi Rapid: Pharm Ana and QA, 2012.
9. Sheeja VK, Swapna AS, Eapen SC, Kumar P. Method development and validation for the simultaneous estimation of clonazepam and paroxetine in combined dosage form using colorimetry. Asian J of Research in Chem. 2014;7:48.
10. Yanamadala G, Praveen Srikumar PP. Determination of paroxetine hydrochloride and clonazepam in pharmaceutical dosage forms. Inter J of Pharm. 2014;4:448-457.
11. Reddy GS, Prasad Reddy SLN, Shiva Kumar Reddy L. Development and validation of a stability indicating liquid chromatographic method for the simultaneous estimation of paroxetine and clonazepam in bulk and its pharmaceutical formulations. Inter J of Pharm and Pharm Sci. 2014;6:397-402.
12. Shah P, Patel J, Patel K, Gandhi T. Development and validation of an HPTLC method for the simultaneous estimation of Clonazepam and Paroxetine hydrochloride using a DOE approach. J of Taibah Uni Sci. 2017;11:121-132.
13. Umadurai M, Nagarajan V. Development and validation of a rapid UPLC Assay method for the simultaneous estimation of paroxetine and clonazepam in tablet dosage form. Inter J of Chem and Pharm Sci. 2014;5:42-47.
14. Anumolu PD, Sirisha N, Haripriya A, Sathesh Babu PR, Subrahmanyam VS. First derivative synchronous spectrofluorimetric quantification of Telmisartan/AmLodipine Besylate combination in tablets. J of Pharm Sci. 2013;12:35-40.
15. Karim MM, Jeon CW, Lee HS, Alam SM, Lee SH, Choi JH, Jin SO, Das AK. Simultaneous determination of acetylsalicylic acid and caffeine in pharmaceutical formulation by first derivative synchronous fluorimetric method. J Fluoresc. 2006;16:713-721.
16. International Conference on Harmonization, ICH Q2 (R1): Validation of Analytical Procedures: Text and Methodology. Geneva; ICH Secretariat; 2005.

Turk J Pharm Sci 2017;14(3):257-263
DOI: 10.4274/tjps.49369

ORIGINAL ARTICLE

The Ameliorative Effects of Pycnogenol® on Liver Ischemia-Reperfusion Injury in Rats

Sıçanlarda Karaciğer İskemi Reperfüzyon Hasarında Piknogenol®'ün İyileştirici Etkileri

Mehmet TOKAÇ[1], Merve BACANLI[2], Ersin Gürkan DUMLU[3], Sevtap AYDIN[2], Merve ENGİN[4], Birkan BOZKURT[5], Abdüssamed YALÇIN[3], Özcan EREL[6], Mehmet KILIÇ[3], Nurşen BAŞARAN[2]*

[1]Yeni Yüzyıl University, Faculty of Medicine, Gaziosmanpaşa Hospital, Clinic of General Surgery, İstanbul, Turkey
[2]Hacettepe University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology, Ankara, Turkey
[3]Yıldırım Beyazıt University, Faculty of Medicine, Department of General Surgery, Ankara, Turkey
[4]Ankara Atatürk Training and Research Hospital, Clinic of Biochemistry, Ankara, Turkey
[5]Cumhuriyet University, Faculty of Medicine, Department of General Surgery, Sivas, Turkey
[6]Yıldırım Beyazıt University, Faculty of Medicine, Department of Biochemistry, Ankara, Turkey

ABSTRACT

Objectives: Pycnogenol® (PYC®), a standardized extract from the bark of *Pinus maritima*, consists of different phenolic compounds. PYC® has shown to have protective effects on chronic diseases such as diabetes, asthma, cancer, and immune disorders. The aim of this study was to determine the effects of PYC® against the DNA damage and biochemical changes in blood, liver, and lung tissues of ischemia-reperfusion (IR)-induced Wistar albino rats.

Materials and Methods: A sham group, IR injury-induced group, and IR+PYC® group were formed. Ischemia was induced and sustained for 45 min, then the ischemic liver was reperfused, which was sustained for a further 120 min at the end of this period. After anesthesia and before the IR inducement, 100 mg/kg PYC® was given to the IR+PYC® group through intraperitoneal injections. The total oxidant (TOS) and total antioxidant status (TAS), total thiol levels (TTL), advanced oxidation protein products (AOPP), and biochemical parameters [myeloperoxidase (MPO), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH)] in the rats were analyzed using spectrophotometric methods and DNA damage was assessed using single-cell gel electrophoresis.

Results: The levels of TOS, TTL, MPO, AOPP, ALT, AST, and LDH were significantly decreased in the IR+PYC® group compared with the IR group ($p<0.05$). The levels of TAS were significantly increased in the IR+PYC® group compared with the IR group ($p<0.05$). PYC® reduced the DNA damage when compared with the IR group ($p<0.05$).

Conclusion: The present results suggest that PYC® treatment might have a role in the prevention of IR-induced oxidative damage by decreasing DNA damage and increasing antioxidant status.

Key words: Pycnogenol, ischemia reperfusion injury, DNA damage

ÖZ

Amaç: Piknogenol® (PYC®), *Pinus maritima* bitkisinin kabuğundan elde edilen ve birkaç tip fenolik içerikten oluşan, suda çözünür standardize bir özüttür. Piknogenolün diyabet, astım, kanser ve immün hastalıklar gibi farklı hastalıklar üzerinde koruyucu etkilerinin olduğu gösterilmiştir. Bu çalışmanın amacı PYC®'nin karaciğer iskemi-reperfüzyonu yapılan sıçanların kan, karaciğer ve akciğer dokularında DNA hasarı ve biyokimyasal değişiklikler üzerindeki etkisinin değerlendirmektir.

Gereç ve Yöntemler: Sham grubu, iskemi-reperfüzyon (IR) ve (IR+PYC®) grubu olmak üzere 3 grup oluşturuldu. Karaciğerde 45 dakika iskemi ardından 120 dk reperfüzyon yapıldı. IR+PYC® grubunda PYC® anestezi sonrası iskemi yapmadan önce 100 mg/kg dozunda intraperitoneal verildi. Sıçanlarda total oksidan durum (TOS), total antioksidan durum (TAS), total tiyol düzeyleri (TTL), ilerlemiş oksidasyon protein ürünleri (AOPP) ve biyokimyasal parametreler [miyeloperoksidaz düzeyi (MPO), aspartat aminotransferaz (AST), alanin aminotransferaz (ALT), laktat dehidrogenaz (LDH)] spektrofotometrik yöntemle, ayrıca tek hücre jel elektroforezi tekniğiyle de DNA hasarı analiz edildi.

*Correspondence: E-mail: nbasaran@hacettepe.edu.tr, Phone: +90 533 347 32 63 ORCID-ID: orcid.org/0000-0001-8581-8933
Received: 21.11.2016, Accepted: 09.02.2017

Bulgular: TOS, TTL, MPO, AOPP, ALT, AST and LDH düzeyleri IR+PYC® grubunda IR grubuna göre anlamlı ölçüde düşük bulundu ($p<0.05$). TAS düzeyi IR+PYC® grubunda IR grubuna göre anlamlı ölçüde yüksek bulundu ($p<0.05$). DNA hasarı değerlendirildiğinde PYC® verilen grupta DNA iskemi grubuna göre anlamlı ölçüde azaldığı saptandı ($p<0.05$).

Sonuç: Mevcut bulgular, piknogenol tedavisinin IR ile indüklenmiş oksidatif hasarı DNA hasarını azaltarak ve antioksidan durumu arttırarak da önlediğini göstermektedir.

Anahtar kelimeler: Piknogenol, iskemi reperfüzyon hasarı, DNA hasarı

INTRODUCTION

Liver surgical options applied for intrahepatic lesions or liver transplantation require a period of ischemia.[1,2] When blood flow is restored, injury to the liver may occur. This phenomenon is called ischemia-reperfusion (IR) injury.[3] IR, one of the main causes of hepatic failure, occurs in different situations including heart failure, liver transplantation, liver trauma, and blood occlusion to the liver.[4-6] Free oxygen radicals and various cytokines, which are produced after reperfusion, play a pivotal role in IR injury.[7,8] The infiltration of polymorphonuclear leukocytes in a tissue is characteristic of acute inflammation and indicates the collective action of chemotactic mediators.[9] The generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is possible in IR. These compounds may act on proteins, enzymes, nucleic acids, cytoskeleton, and lipid peroxides, leading to mitochondrial dysfunction and lipid peroxidation.[10] Additionally, ROS and RNS may also damage endothelial cells and destroy the integrity of the microvasculature.[11]

Although there is evidence about the administration of drugs to protect the liver in animals, even today there is little evidence about the use of these substances in human IR.[12,13] Prevention of oxygen radical release via administration of radical scavengers has been found to be beneficial against IR injury.[7,8] Antioxidants are known as potential scavengers of ROS, so that they protect biologic membranes against oxidative damage. Natural products are widely used as dietary supplements because of their potential antioxidant properties. Plant polyphenols may act as antioxidants by different mechanisms such as free radical scavenging, metal chelation and protein binding.[14]

Pycnogenol® (PYC) is a standardized extract from the bark of the French maritime pine (*Pinus maritima)*.[15] Studies indicate that PYC® components are highly bioavailable and it is assumed to display greater biologic effects as a mixture than its individual purified components, which indicates that there are synergistic interactions between its components. Previous studies demonstrated that PYC® was a very strong antioxidant at scavenging reactive oxygen and nitrogen species.[16] It is used in dietary supplements and health protective products because of its direct and strong antioxidant activity.[15] It has beneficial effects on various diseases such as diabetes, asthma, hypertension, cancer, and immune diseases.[17]

The aim of this study was to evaluate the effects of PYC® against the DNA damage and biochemical changes in blood, liver, and lung tissues of IR-induced rats.

MATERIALS AND METHODS

Ethics

The study was approved by the Local Ethical Committee of Laboratory Animal Research of Ankara Training and Research Hospital (2014/291).

Chemicals

The chemicals used in the experiments were purchased from the following suppliers: PYC®, a registered trade mark of Horphag Research Ltd., (Geneva, Switzerland), was provided by Henkel Corporation (La Grange, IL, U.S.A.); normal melting agarose (NMA) and low-melting-point agarose (LMA) from Boehringer Manheim (Mannheim, Germany); 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), chloramine-T, acetic acid, potassium iodide (KI), hydrogen peroxide (H_2O_2), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), sodium chloride (NaCl), sodium hydroxide (NaOH), and potassium chloride from Merck Chemicals (Darmstadt, Germany); dimethyl sulfoxide (DMSO), ethidium bromide (EtBr), Triton-X-100, phosphate-buffered saline (PBS) tablets, from Sigma-Aldrich Chemicals (St Louis, Missouri, USA); ethylendiaminetetraacetic acid disodium salt dihydrate (EDTA-Na_2), natrium lauroyl sarcosinate, and Tris from ICN Biomedicals Inc. (Aurora, Ohio, USA), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and albumin kits from Roche Diagnostics (Mannheim, Germany).

Animals

A total of 24 Wistar albino rats, male, weighing 200-300 g, were used in the study. The rats were kept in rooms with automatically adjusted temperature (23±2˚C) and humidity (50%) and a rotation of light and dark illumination for 12 hr each. They were housed in plastic cages with stainless steel grid tops. Animals were fed with standard laboratory chow and allowed to access feed and drinking water ad libitum.

Experimental protocol

The rats were randomized into three groups of 8 rats each.

- Group 1 (Sham group) (n=8): Only hepatoduodenal ligament dissection was performed and no drug was given.

- Group 2 (IR group) (n=8): Forty-five min after the Pringle maneuver, reperfusion was generated for 120 min and no drug was given.

- Group 3 (IR+PYC® group) (n=8): PYC® was given at a dose of 100 mg/kg intraperitoneal (i.p.) after anesthesia

was performed. Forty-five min after the Pringle maneuver, reperfusion was generated for 120 min.

Rats were anesthetized with 80 mg/kg ketamine hydrochloride (Ketalar, Eczacıbaşı-Warner Lambert, İstanbul, Turkey) plus 10 mg/kg xylazine (Rompun, Bayer, İstanbul, Turkey) via intramuscular (i.m.) injection. A midline incision was performed and rats underwent either sham surgery or IR. Ischemia was induced with the Pringle maneuver and sustained for 45 min after this time, the ischemic liver was reperfused, and this was sustained for a further 120 min at the end of this period, the animals were sacrificed by taking blood from the heart. After anesthesia and before the IR inducement, 2 mL 0.9% NaCl was given intraperitoneally (i.p.) to group 1 and group 2, and 100 mg/kg PYC® in 2 mL 0.9% NaCl i.p. was given to group 3. The dose of PYC® was selected according to our previous study.[18]

After the end of this procedure, blood samples obtained through cardiac puncture were collected into preservative-free heparin tubes for the biochemical and the DNA damage evaluations. The heparinized blood samples were kept in the dark at 4°C and processed within 6 hr. The liver and kidney tissues were carefully dissected from their attachments and totally excised. Excised tissues were divided into three parts for biochemical analysis, DNA damage analysis, and assessment of antioxidant and oxidant parameters.

Biochemical analysis

For biochemical analysis, the heparinized blood samples were centrifuged at 800 g for 15 min. The plasma was collected and examined for total antioxidant status (TAS), total oxidant status (TOS), total thiol levels (TTL), myeloperoxidase (MPO), advanced oxidation protein products (AOPP), albumin, ALT, AST and LDH.

The liver and kidney tissues were extracted following the homogenization and sonication procedure as previously described by Sier et al.[19] (1996). TOS, AOPP, ceruloplasmin and MPO levels were examined.

Determination of oxidative stress parameters

Measurement of TAS

Plasma and tissue homogenate TAS levels were measured using a novel automated colorimetric method developed by Erel[20] (2004). In this method, antioxidants in the sample reduce the dark blue-green-colored ABTS radical to a colorless reduced ABTS form. The change of absorbance at 660 nm is related with the total antioxidant level of the sample. This method determines the antioxidative effect of the sample against the potent free radical reactions initiated by the produced hydroxyl radical. The results are expressed as mmol Trolox equivalent per liter (mmol Trolox Eq/L).

Measurement of TOS

Plasma and tissue homogenate TOS levels were measured using a novel automated colorimetric method described by Erel[21] (2005). In this method, oxidants present in the sample oxidize the ferrous ioneo-dianisidine complex to the ferric ion. The oxidation reactions enhanced by glycerol molecules are abundantly present in the reaction medium. The ferric ion forms a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with H_2O_2, and the results are expressed in terms of micromolar H_2O_2 equivalent per liter (μmol H_2O_2 Eq/L).

Measurement of TTL

TTL or sulfhydryl (SH) groups in plasma and tissue homogenates were measured using the methods originally described by Ellman[22] (1959) and modified by Hu[23] (1994). Here, thiols interact with DTNB, forming a highly colored anion with maximum peak at 412 nm (e412= 13.600 M/cm). The result is expressed in μmol/L.

Measurement of MPO levels

Serum MPO activity was determined using a modification of the o-dianisidine method[24] based on kinetic measurement at 460 nm with the rate of the yellowish-orange product formation from the oxidation of o-dianisidine with MPO in the presence of H_2O_2. One unit of MPO was defined as that degrading 1 μmol of H_2O_2 min^{-1} at 25°C. A molar extinction coefficient of 1.13x104 of oxidized o-dianisidine was used for the calculation. MPO activity is expressed in units per liter serum.

Measurement of AOPP levels

The quantification of AOPP in plasma used the method described by Witko-Sarsat et al.[25] (1996). Two hundred microliters of plasma diluted 1:5 in PBS, or chloramine-T standard solutions (0 to 100 μmol/liter), followed by 20 μL of acetic acid. Ten microliters of 1.16 M potassium iodide were then added, followed by 20 μL of acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm against a blank containing 200 μL of PBS, 10 μL of KI, and 20 μL of acetic acid. The chloramine-T absorbance at 340 nm was linear within the range of 0 to 100 μmol/liter. AOPP concentrations are expressed in μmol/liter of chloramine-T equivalents.

Measurement of albumin, ALT, AST and LDH levels

The levels of ALT, AST, LDH, and albumin were determined using commercially available assay kits (Roche) with an autoanalyzer (Roche/Hitachi Cobas C501).

Determination of DNA damage

Whole blood samples were used for evaluation of DNA damage. The liver and lung tissues were carefully dissected from their attachments and totally excised. Preparation of single-cell suspension from the organs was performed according to standard procedures.[26-28] In brief, approximately 0.2 g of each organ was placed in 1 mL chilled mincing solution [Hank Balanced Salt Solution (HBSS) with 20 mM EDTA and 10% DMSO] in a petri dish and chopped into pieces with a pair of scissors. The pieces were allowed to settle and the supernatant containing the single-cell suspension was taken. The concentrations of renal and hepatic tissue cells in the supernatant were adjusted to approximately $2x10^6$ cells/mL in HBSS containing 20 mM EDTA/10% DMSO.

The Alkaline Comet assay technique of Singh et al.[29], as further described by Collins[30] and Bacanlı et al.[31] was followed. The cells were suspended in 75 µL of 0.5% LMA. The suspensions were then embedded on slides precoated with a layer of 1% NMA. Slides were allowed to solidify on ice for 5 min. Coverslips were then removed. The slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM EDTA, 100 mM Tris, 1% sodium sarcosinate, pH 10.0 with Triton-X-100 and 10% DMSO) for a minimum of 1 h at 4˚C. The slides were then removed from the lysing solution, drained, and left in the electrophoresis solution (1 mM sodium EDTA and 300 mM NaOH, pH 13.0) for 20 min at 4˚C to allow unwinding of the DNA and expression of alkali-labile damage. They were then left in the electrophoresis solution (1 mM sodium EDTA and 300 mM NaOH, pH: 13) for 20 min at 4˚C to allow unwinding of the DNA and expression of alkali-labile damage.

Electrophoresis was also conducted at a low temperature (4˚C) for 20 min using 24 V and adjusting the current to 300 mA by raising or lowering the buffer level. The slides were neutralized by washing three times in 0.4 M Tris-HCl (pH: 7.5) for 5 min at room temperature. After neutralization, the slides were incubated in 50%, 75%, and 98% of alcohol for 5 min each.

The dried microscopic slides were stained with EtBr (20 µg/mL in distilled water, 60 µL/slide), covered with a cover-glass prior to analysis with a Leica® fluorescence microscope under green light. The microscope was connected to a charge-coupled device camera and a personal computer-based analysis system (Comet Analysis Software, version 3.0, Kinetic Imaging Ltd, Liverpool, UK) to determine the extent of DNA damage after electrophoretic migration of the DNA fragments in the agarose gel. In order to visualize DNA damage, 100 nuclei per slide were examined at x400 magnification. Results are expressed as the length of the comet (tail length), the product of the tail length, and the fraction of total DNA in the tail (tail moment) and percent of DNA in the tail (tail intensity).

Statistical analysis

Statistical analysis was performed using the SPSS for Windows 20.0 computer program. Differences between the means of data were compared using the ANOVA test, and post hoc analysis of group differences was performed using the least significant difference test. The Kruskal-Wallis H test was used to compare parameters that displayed abnormal distribution between groups. The results are given as the mean ± standard deviation. P values of less than 0.05 were considered as statistically significant.

RESULTS

Biochemical parameters in plasma

The plasma biochemical parameters are shown in Table 1. TAS levels in the IR group were found to be significantly lower than the sham group ($p<0.05$). The levels of TAS were found to significantly increase in the IR+PYC® group compared with the IR group ($p<0.05$). There was no significant difference in terms of TAS levels between the IR+PYC® group and the sham group.TOS, TTL, MPO, AOPP, ALT, AST, and LDH levels in the IR group and IR+PYC® group were found significantly higher than the sham group ($p<0.05$). The levels of TOS, TTL, MPO, AOPP, ALT, AST, and LDH were found to significantly decrease in the IR+PYC® group compared with the IR group ($p<0.05$). There was no significant difference with regards TTL and MPO levels between the IR+PYC® and sham groups.

Biochemical parameters in liver and lung

The TAS, TOS, and TTL levels in the lung and liver tissues are shown in Table 2 and 3, respectively. TOS and TTL levels in the lung and liver tissues were found to be significantly higher in the IR group compared with the sham group ($p<0.05$) (Table 2, 3).

In the lung samples, TOS and TTL levels were found to significantly decrease in the IR+PYC® group compared with the IR group ($p<0.05$). There were no significant differences in terms of TOS and TTL levels between the IR+PYC® group and the sham group (Table 2). TAS levels in the lung tissues were found significantly lower in the IR group compared with the sham group ($p<0.05$). There was no significant difference regarding TAS levels between the IR+PYC® group and the sham group (Table 2).

In the liver samples, TOS levels were found to significantly

Table 1. Biochemical findings of plasma samples of experimental groups

	Sham group	IR group	IR+PYC group
TAS (mmol Trolox Eq/L)	4.22±1.08	1.83±0.26[a]	3.43±0.88[b]
TOS (µmol H_2O_2 Eq/L)	4.50±1.16	30.08±7.46[a]	14.34±2.81[a,b]
TTL (µmol/L)	128.98±18.81	241.13±26.66[a]	130.61±22.56[b]
MPO (U/L)	37.92±16.57	159.37±94.15[a]	55.06±21.34[b]
AOPP (µmol Chloramine T Eq/L)	141.18±37.84	299.68±62.63[a]	234.54±63.96[a,b]
Albumin (g/dL)	3.84±0.35	3.62±0.56	2.84±0.36[a,b]
ALT (mg/dL)	32±18	2997±650[a]	1380±443[a,b]
AST (mg/dL)	172±52	2747±827[a]	1525±317[a,b]
LDH (mg/dL)	325±95	8261±1947[a]	5227±1146[a,b]

IR: Ischemia-reperfusion, PYC: Pycnogenol, TAS: Total antioxidant status, TOS: Total antioxidant status, TTL: Total thiol levels, MPO: Myeloperoxidase activity, AOPP: Advance oxidation protein product, ALT: Alanine transaminase, AST: Aspartate transaminase, LDH: Lactate dehydrogenase, [a]Statistically different from sham group ($p<0.05$), [b]Statistically different from IR group ($p<0.05$)

decrease in the IR+PYC® group compared with the IR group ($p<0.05$). There were no significant differences in terms of TAS, TOS, and TTL levels between the IR+PYC® group and the sham group (Table 3).

Assessment of DNA damage

The DNA damage expressed as tail length, tail intensity, and tail moment in the blood, liver, and lung cells of rats are given in Figure 1, 2, and 3. In the blood and tissues studied, DNA damage was found to be significantly higher in the IR group compared with the sham group ($p<0.05$). PYC® was found to reduce the DNA damage significantly because the damage in the IR+PYC® group was lower than in the IR group ($p<0.05$). There was no significant difference in DNA damage between the sham group and the IR+PYC® group.

Table 2. Oxidative stress parameters in lung samples of experimental groups

	Sham group	IR group	IR+PYC group
TAS (mmol Trolox Eq/L)	3.12±1.24	2.02±0.56[a]	2.97±0.70[b]
TOS (μmol H_2O_2 Eq/L)	4.78±1.15	6.20±1.54[a]	4.29±1.19[b]
TTL (μmol/L)	56.43±10.06	71.62±7.56[a]	60.87±8.30[b]

IR: Ischemia-reperfusion, PYC: Pycnogenol, TAS: Total antioxidant status, TOS: Total antioxidant status TTL: Total thiol levels, [a]Statistically different from sham group ($p<0.05$), [b]Statistically different from IR group ($p<0.05$)

Table 3. Biochemical findings of liver samples of experimental groups

	Sham group	IR group	IR+PYC group
TAS (mmol Trolox Eq/L)	5.44±1.09	5.74±0.28	5.88±0.19
TOS (μmol H_2O_2 Eq/L)	7.05±1.61	9.68±2.41[a]	7.84±1.53[b]
TTL (μmol/L)	42.42±24.64	62.39±19.07[a]	58.59±18.95

IR: Ischemia-reperfusion, PYC: Pycnogenol, TAS: Total antioxidant status, TOS: Total antioxidant status, TTL: Total thiol levels, [a]Statistically different from sham group ($p<0.05$), [b]Statistically different from IR group ($p<0.05$)

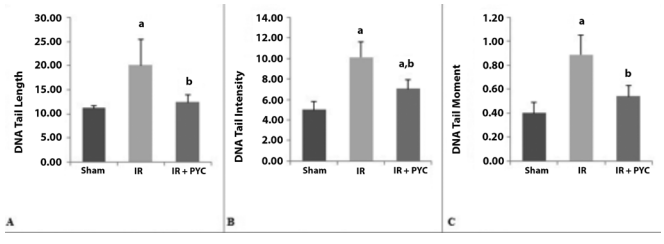


Figure 1. DNA damage in the blood cells of the experimental groups expressed as (a) tail length, (b) tail intensity, (c) tail moment. The values are expressed as mean ± standard deviation

[a]$p<0.05$, compared with sham group; [b]$p<0.05$, compared with IR group; IR: Ischemia-reperfusion, PYC: Pycnogenol

DISCUSSION

Previous studies have shown that ROS plays an important role in liver IR injury, which can result in different undesirable effects.[32,33] When tissues are exposed to ischemia followed by reperfusion, ROS are extensively generated in the early stage of reperfusion, which cause serious damage to tissues in various organs, including the liver, brain, heart, and kidney.[34] Oxidative stress contributes to the pathogenesis of liver injury. When liver is deprived of oxygen, antioxidant enzyme activities decrease, and ROS attack cellular molecules.[35] Thus, free radical scavengers are thought to be beneficial in the treatment of IR damage. A number of compounds, especially antioxidants, have been used to reduce hepatic IR injury in animal studies, but few are currently used in humans because of limited and controversial data about their efficacy.[36]

Phenolic compounds such as flavonoids found in daily diets have various beneficial effects against various diseases such as cancer, diabetes, and cardiovascular and neurodegenerative diseases, and experimental data are accumulating regarding them as natural important phytochemical antioxidants for human health.[37] Research on antioxidant substances has focused on the potential benefits of both purified phytochemicals and plant extracts such as the pine bark extract known as PYC®.[18] The protective effects of PYC® on the biochemical changes in TAS, TOS, TTL, MPO, AOPP, ALT, AST, and LDH levels in hepatic IR were investigated in our study. We used PYC® to reduce hepatic IR because it has been shown previously to have antioxidant properties.

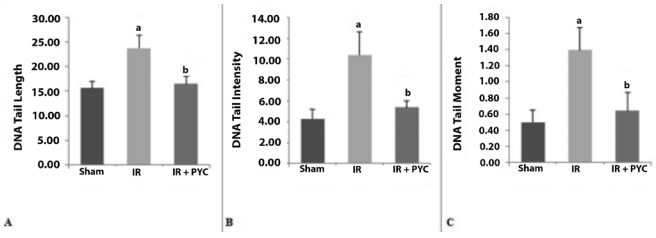


Figure 2. DNA damage in the liver cells of the experimental groups expressed as (a) tail length, (b) tail intensity, (c) tail moment. The values are expressed as mean ± standard deviation

[a]$p<0.05$, compared with sham group; [b]$p<0.05$, compared with IR group; IR: Ischemia-reperfusion, PYC: Pycnogenol

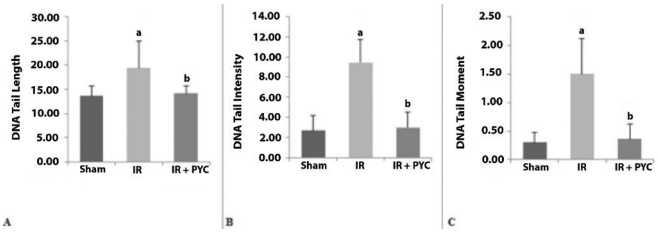


Figure 3. DNA damage in the lung cells of the experimental groups expressed as (a) tail length, (b) tail intensity, (c) tail moment. The values are expressed as mean ± standard deviation

[a]$p<0.05$, compared with sham group; [b]$p<0.05$, compared with IR group. IR: Ischemia-reperfusion, PYC: Pycnogenol

Studies indicated that PYC® components were highly bioavailable. It is a procyanidin-enriched extract of *Pinus pinaster* bark consisting of a variety of flavonoids, which are known as potent antioxidants.[38] PYC® shows various beneficial health effects against different types of diseases.[17] PYC® has been extensively used in Europe as a dietary food supplement. It has been suggested to have free radical scavenging and antioxidant properties[39,40], to protect protein oxidation[41], and to ameliorate oxidative organ injury and DNA damage. PYC® was reported to reduce IR-induced renal injury and preserve renal function.[42]

During the reperfusion phase of the liver, emerging reactive oxygen radicals activate some mediators and can cause an inflammatory response and tissue damage. For this reason, AST, ALT, and LDH levels may increase.[37,43] Atila et al.[44] (2002) showed that pretreatment with an antioxidant such as carnitine protected hepatic enzyme levels (ALT and AST) in rats with increased ischemia reperfusion. Similarly, Yang et al.[15] (2008) reported that PYC® decreased serum AST and ALT levels on CCl_4- induced hepatotoxicity in rats. In our study, plasma AST, ALT, and LDH levels increased in the IR group but PYC® administration decreased the levels of AST, ALT, and LDH in comparison with the IR group. These findings support the protective effect of PYC® against liver IR injury.

The decrease in TAS levels and the increase in TOS levels were reported in hepatic IR damage.[45] Tüfek et al.[46] (2013) demonstrated that the serum, liver, lung, and kidney tissues of the IR group had higher TOS values and lower TAC values when compared with the sham group. When dexmedetomidine, an antioxidant compound, was administered to the IR group, it was observed that TOS values decreased and TAC values increased. Similar with this study, our data showed that TOS values were increased and TAS values were decreased in IR group and PYC® treatment ameliorated these changes.

SH groups are known to be sensitive to oxidative damage and depleted following ischemic insult.[47] In our study, TTLs were increased following IR in plasma, liver, and lung tissues of rats. Hosseinzadeh et al.[48] (2005) reported that rats pretreated with crocin, a phenolic compound in saffron extract, exhibited higher thiol contents than their respective controls in a dose-related pattern, indicating that crocin helped to replenish the total thiol pool. However saffron-mediated SH replenishment was not as impressive as expected. Saffron pretreatment slightly increased total thiol concentration following ischemic insult, but this elevation was not significant as compared with the control group.

As far as we know, this is the first study to determine MPO and AOPP levels in IR injury- induced rats. We found that MPO and AOPP levels in the IR group were found to be significantly higher than the sham group. The levels of MPO and AOPP were significantly decreased in the IR+PYC® group compared with the IR group.

We found that the DNA damage was significantly higher in the blood, liver, and lung cells of the IR group compared with the sham group. On the other hand, the parameters were significantly decreased in the PYC® treated IR group when compared with the IR group. PYC® treatment seemed to prevent IR- induced DNA damage in the blood, liver, and lung cells of the rats. There are limited data about the protective effects of PYC® against DNA damage. Consistent with our data, Taner et al.[18] (2014) reported the protective effects of PYC® on sepsis-induced oxidative DNA damage. The protective effects of water extracts from pine needle against DNA damage and apoptosis induced by hydroxyl radical were also demonstrated in non-cellular and cellular systems through inhibiting oxidative DNA damage induced by hydroxyl radical and preventing the cells from oxidative damage.[49]

CONCLUSION

The results of this study have shown that oxidative stress parameters are significantly altered in experimental hepatic IR injury in rats. PYC® was found to be protective against IR injury-induced oxidative damage and genotoxic effects in blood, liver, and lungs of rats. In conclusion, PYC® may protect against oxidative liver injury in rats. We also conclude that a possible protective role and clinical availability of PYC® for liver injury should be investigated in further studies.

Conflict of Interest: No conflict of interest was declared by the authors.

REFERENCES

1. Huguet C, Addario-Chieco P, Gavelli A, Arrigo E, Harb J, Clement RR. Technique of hepatic vascular exclusion for extensive liver resection. Am J Surg. 1992;163:602-605.
2. Delva E, Camus Y, Nordlinger B, Hannoun L, Parc R, Deriaz H, Lienhart A, Huguet C. Vascular occlusions for liver resections. Operative management and tolerance to hepatic ischemia: 142 cases. Ann Surg. 1989;209:211-218.
3. Lee SH, Culberson C, Korneszczuk K, Clemens MG. Differential mechanisms of hepatic vascular dysregulation with mild vs. moderate ischemia-reperfusion. Am J Physiol Gastrointest Liver Physiol. 2008;294:G1219-G1226.
4. Jaeschke H, Bautista AP, Spolarics Z, Spitzer JJ. Superoxide generation by neutrophils and Kupffer cells during *in vivo* reperfusion after hepatic ischemia in rats. J Leukoc Biol. 1992;52:377-382.
5. Tejima K, Arai M, Ikeda H, Tomiya T, Yanase M, Inoue Y, Nagashima K, Nishikawa T, Watanabe N, Omata M, Fujiwara K. Ischemic preconditioning protects hepatocytes via reactive oxygen species derived from Kupffer cells in rats. Gastroenterology. 2004;127:1488-1496.
6. Sahin T, Begeç Z, Toprak Hİ, Polat A, Vardi N, Yücel A, Durmuş M, Ersoy MÖ. The effects of dexmedetomidine on liver ischemia-reperfusion injury in rats. J Surg Res. 2013;183:385-390.
7. Bahde R, Spiegel HU. Hepatic ischaemia-reperfusion injury from bench to bedside. Br J Surg. 2010;97:1461-1475.
8. Çekın AH, Gür G, Türkoğlu S, Aldemır D, Yilmaz U, Gürsoy M, Taşkoparan M, Boyacioğlu S. The protective effect of L-carnitine on hepatic ischemia-reperfusion injury in rats. Turk J Gastroenterol. 2013;24:51-56.
9. Ishikawa F, Miyazaki S. New biodefense strategies by neutrophils. Arch Immunol Ther Exp (Warsz). 2005;53:226-233.

10. Jaeschke H. Reactive oxygen and ischemia/reperfusion injury of the liver. Chem Biol Interact. 1991;79:115-136.
11. Jaeschke H. Reactive oxygen and mechanisms of inflammatory liver injury: Present concepts. J Gastroenterol Hepatol. 2011;26(Suppl 1):173-179.
12. Selzner N, Rudiger H, Graf R, Clavien PA. Protective strategies against ischemic injury of the liver. Gastroenterology. 2003;125:917-936.
13. Jegatheeswaran S, Jamdar S, Satyadas T, Sheen AJ, Adam R, Siriwardena AK. Use of Pharmacologic Agents for Modulation of Ischaemia-Reperfusion Injury after Hepatectomy: A Questionnaire Study of the LiverMetSurvey International Registry of Hepatic Surgery Units. HPB Surg. 2014;2014:437159.
14. Maurya DK, Devasagayam TP. Antioxidant and prooxidant nature of hydroxycinnamic acid derivatives ferulic and caffeic acids. Food Chem Toxicol. 2010;48:3369-3373.
15. Yang YS, Ahn TH, Lee JC, Moon CJ, Kim SH, Jun W, Park SC, Kim HC, Kim JC. Protective effects of Pycnogenol on carbon tetrachloride-induced hepatotoxicity in Sprague-Dawley rats. Food Chem Toxicol. 2008;46:380-387.
16. Packer L, Rimbach G, Virgili F. Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (*Pinus maritima*) bark, pycnogenol. Free Radic Biol Med. 1999;27:704-724.
17. Rohdewald P. A review of the French maritime pine bark extract (Pycnogenol), a herbal medication with a diverse clinical pharmacology. Int J Clin Pharmacol Ther. 2002;40:158-168.
18. Taner G, Aydın S, Bacanlı M, Sarıgöl Z, Sahin T, Başaran AA, Başaran N. Modulating effects of pycnogenol® on oxidative stress and DNA damage induced by sepsis in rats. Phytother Res. 2014;28:1692-1700.
19. Sier CF, Kubben FJ, Ganesh S, Heerding MM, Griffioen G, Hanemaaijer R, van Krieken JH, Lamers CB, Verspaget HW. Tissue levels of matrix metalloproteinases MMP-2 and MMP-9 are related to the overall survival of patients with gastric carcinoma. Br J Cancer. 1996;74:413-417.
20. Erel O. A novel automated method to measure total antioxidant response against potent free radical reactions. Clin Biochem. 2004;37:112-119.
21. Erel O. A new automated colorimetric method for measuring total oxidant status. Clin Biochem. 2005;38:1103-1111.
22. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys. 1959;82:70-77.
23. Hu ML. Measurement of protein thiol groups and glutathione in plasma. Method Enzymol. 1994;233:380-385.
24. Bradley PP, Priebat DA, Christensen RD, Rothstein G. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. J Invest Dermatol. 1982;78:206-209.
25. Witko-Sarsat V, Friedlander M, Capeillère-Blandin C, Nguyen-Khoa T, Nguyen AT, Zingraff J, Jungers P, Descamps-Latscha B. Advanced oxidation protein products as a novel marker of oxidative stress in uremia. Kidney Int. 1996;49:1304-1313.
26. Patel S, Pandey AK, Bajpayee M, Parmar D, Dhawan A. Cypermethrin-induced DNA damage in organs and tissues of the mouse: evidence from the comet assay. Mutat Res. 2006;607:176-183.
27. Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF. Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. Environ Mol Mutagen. 2000;35:206-221.
28. Bakare AA, Patel S, Pandey AK, Bajpayee M, Dhawan A. DNA and oxidative damage induced in somatic organs and tissues of mouse by municipal sludge leachate. Toxicol Ind Health. 2012;28:614-623.
29. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res. 1988;175:184-191.
30. Collins AR. Investigating oxidative DNA damage and its repair using the comet assay. Mutat Res. 2009;681:24-32.
31. Bacanlı M, Aydın S, Taner G, Göktaş HG, Şahin T, Başaran AA, Başaran N. The protective role of ferulic acid on sepsis-induced oxidative damage in Wistar albino rats. Environ Toxicol Pharmacol. 2014;38:774-782.
32. Abu-Amara M, Yang SY, Tapuria N, Fuller B, Davidson B, Seifalian A. Liver ischemia/reperfusion injury: processes in inflammatory networks a review. Liver Transpl. 2010;16:1016-1032.
33. Kaplowitz N. Mechanisms of liver cell injury. J Hepatol. 2000;32(1 Suppl):39-47.
34. Fukuda K, Asoh S, Ishikawa M, Yamamoto Y, Ohsawa I, Ohta S. Inhalation of hydrogen gas suppresses hepatic injury caused by ischemia/reperfusion through reducing oxidative stress. Biochem Biophys Res Comm. 2007;361:670-674.
35. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-254.
36. Kocoglu H, Ozturk H, Ozturk H, Yilmaz F, Gulcu N. Effect of dexmedetomidine on ischemia-reperfusion injury in rat kidney: a histopathologic study. Ren Fail. 2009;31:70-74.
37. Yildiz F, Coban S, Terzi A, Ates M, Aksoy N, Cakir H, Ocak AR, Bitiren M. Nigella sativa relieves the deleterious effects of ischemia reperfusion injury on liver. World J Gastroenterol. 2008;14:5204-5209.
38. Lee HH, Kim KJ, Lee OH, Lee BY. Effect of pycnogenol on glucose transport in mature 3T3-L1 adipocytes. Phytother Res. 2010;24:1242-1249.
39. D'Andrea G. Pycnogenol: a blend of procyanidins with multifaceted therapeutic applications? Fitoterapia. 2010;81:724-736.
40. Parveen K, Khan MR, Siddiqui WA. Pycnogenol prevents potassium dichromate K2Cr2O7-induced oxidative damage and nephrotoxicity in rats. Chem Biol Interact 2009;181:343-350.
41. Voss P, Horakova L, Jakstadt M, Kiekebusch D, Grune T. Ferritin oxidation and proteasomal degradation: protection by antioxidants. Free Radical Res. 2006;40:673-683.
42. Ozer Sehirli A, Sener G, Ercan F. Protective effects of pycnogenol against ischemia reperfusion-induced oxidative renal injury in rats. Ren Fail. 2009;31:690-697.
43. Choukér A, Martignoni A, Schauer RJ, Dugas M, Schachtner T, Kaufmann I, Setzer F, Rau HG, Löhe F, Jauch KW, Peter K, Thiel M. Alpha-gluthathione S-transferase as an early marker of hepatic ischemia/reperfusion injury after liver resection. World J Surg. 2005;29:528-534.
44. Atila K, Coker A, Sagol O, Coker I, Topalak O, Astarcioglu H, Karademir S, Astarcioglu I. Protective effects of carnitine in an experimental ischemia-reperfusion injury. Clin Nutr. 2002;21:309-313.
45. Kandis H, Karapolat S, Yildirim U, Saritas A, Gezer S, Memisogullari R. Effects of Urtica dioica on hepatic ischemia-reperfusion injury in rats. Clinics (Sao Paulo). 2010;65:1357-1361.
46. Tüfek A, Tokgöz O, Aliosmanoglu I, Alabalik U, Evliyaoglu O, Çiftçi T, Güzel A, Yıldırım ZB. The protective effects of dexmedetomidine on the liver and remote organs against hepatic ischemia reperfusion injury in rats. Int J Surg. 2013;11:96-100.
47. Soszyński M, Bartosz G. Decrease in accessible thiols as an index of oxidative damage to membrane proteins. Free Radical Biol Med. 1997;23:463-469.
48. Hosseinzadeh H, Sadeghnia HR, Ziaee T, Danaee A. Protective effect of aqueous saffron extract (*Crocus sativus L.*) and crocin, its active constituent, on renal ischemia-reperfusion-induced oxidative damage in rats. J Pharm Pharm Sci. 2005;8:387-393.
49. Jeong JB, Seo EW, Jeong HJ. Effect of extracts from pine needle against oxidative DNA damage and apoptosis induced by hydroxyl radical via antioxidant activity. Food Chem Toxicol. 2009;47:2135-2141.

Turk J Pharm Sci 2017;14(3):264-273
DOI: 10.4274/tjps.88598

ORIGINAL ARTICLE

Ethical Overview of Pharmaceutical Industry Policies in Turkey from Various Perspectives

Türkiye'de İlaç Sanayi Politikalarına Çeşitli Perspektiflerden Etik Bakış

Murat ORAL*, Gülbin ÖZÇELİKAY

Ankara University, Faculty of Pharmacy, Department of Pharmacy Management, Ankara, Turkey

ABSTRACT

Objectives: Countries' national drug policies include all key stakeholders (pharmacists, physicians, pharmaceutical firms, and patients) in the public, the private sector, and the field of pharmacy. The aim of this study was to outline pharmaceutical patents and data protection, orphan drugs, drug pricing, and surplus goods regarding pharmacoeconomics, promotions, inspections in the pharmaceutical industry, and policies in pharmacies with respect to buying and selling drugs, and to discuss ethics in particular.

Materials and Methods: Written laws in force relating to drugs in Turkey constitute the materials of this study.

Results: Essential medicines must always be accessible. Both governments and pharmaceutical companies must fulfill the obligations imposed on them in an ethical way. Research and development activities must also be carried out for orphan drugs. While pricing drugs, authorities must take pharmacoeconomic evaluations into account.

Conclusion: Drugs must be accessible and in the first grade at all times under all circumstances because a product cannot replace it. The concept of surplus goods should be revised to ensure the common needs of the pharmaceutical industry, warehouses, and pharmacies. Promotions in the field by the pharmaceutical industry should be made based on scientific evidence in an ethical way. Inspectors should perform meticulous pharmaceutical industry inspections.

Key words: Drug pricing, ethics, health policies, patient rights, pharmaceutical industry, pharmacists

ÖZ

Amaç: Ülkelerin ulusal ilaç politikaları kamu sektöründeki, özel sektördeki, eczacılık alanındaki tüm önemli paydaşları (eczacılar, hekimler, ilaç firmaları ve hastalar) içerir. Bu çalışmanın amacı Türkiye'de ilaçta patent ve veri korumasını, yetim ilaçları, farmakoekonomik açıdan ilaç fiyatlandırması ve mal fazlası kavramlarını, tanıtımları, ilaç sanayi denetimlerini ve eczanelerde ilaç alış ve satış politikalarını eczacı, ilaç firması ve hasta açısından ele almak ve özellikle etik açıdan tartışmaktır.

Gereç ve Yöntemler: Bu çalışmanın gereçlerini Türkiye'de ilaçla ilgili yürürlükte bulunan yazılı hukuk kuralları oluşturmaktadır.

Bulgular: Temel ilaçlar her zaman ulaşılabilir olmalıdır. İlaç firmaları da devletler de kendilerine düşen yükümlülükleri etik olarak yerine getirmelidirler. Yetim ilaçlar için de Ar-Ge faaliyetleri yürütülmelidir. İlaçların fiyatları belirlenirken yetkililer farmakoekonomik değerlendirmeleri dikkate almalıdır.

Sonuç: İlaç ikamesi olmayan bir ürün olduğu için her zaman her koşulda ulaşılabilmeli ve birinci kalitede olmalıdır. Mal fazlası kavramı ilaç sanayisinin, ecza depolarının ve eczanelerin ihtiyaçlarını ortak paydada sağlayacak şekilde yeniden düzenlenmelidir. İlaç sanayisinde tanıtımlar bilimsel kanıtlara dayalı ve etik bir şekilde yapılmalıdır. Denetçiler ilaç sanayi denetimlerini titizlikle gerçekleştirmelidir.

Anahtar kelimeler: İlaç fiyatlandırılması, etik, sağlık politikaları, hasta hakları, ilaç endüstrisi, eczacılar

INTRODUCTION

There are different opinions about drug the patent protection regime brought to drugs with the Trade-Related Aspects of Intellectual Property Rights (TRIPS) Agreement, which arranges intellectual property rights. At the center of the discussions there are two counter-arguments:

1- Medicines are unique products because research and development (R&D) costs and regulatory approval costs are significant parts of the total production costs. In fact, after having a drug developed and approved by the regulator, the marginal cost of medicine production remains negligible. R&D costs and regulatory approval costs are mostly funded by the companies

*Correspondence: E-mail: ecz.murat.oral@gmail.com, Phone: +90 505 702 32 82 ORCID-ID: orcid.org/0000-0001-5001-8477
Received: 15.10.2016, Accepted: 09.02.2017

that first develop the drugs. The approval costs for generic medicines are not insignificant; however, generic medicine manufacturers face lower costs than the original medicine manufacturers for the same medicines. If there is not any profit for a limited period, new drug developers cannot meet R&D and regulatory agencies approval costs and incentives to develop new medicines will substantially reduce. Therefore, drug patent protection is particularly important regarding technical development.[1]

2- Some non-governmental organizations (NGOs) are pursuing these concerns against the TRIPS Agreement and patent protection for drugs; their beleif is that the increased patent protection would increase the price of medicines. Additionally, the number of new essential medicines under patent protection would increase, but it would be difficult to reach these essential drugs in developing countries due to the high prices. Thus, at the point of access to essential medicines, the gap between developed and developing countries would increase. The NGOs think the enforcement of related World Trade Organization rules would have an adverse impact on local production capacities and generic, innovative, high-quality drug resources, on which developing countries are dependent, would disappear.[2]

Patent types in the pharmaceutical industry

Fundamentally, for drugs, there are product patents and process patents. Product patents are related to any new medical or veterinary-oriented molecule or with active substances derived from nature. Process patents are related to the methods used to produce the crystal form or derivatives of any product, or in the production and preparation of drugs.[2] Beyond these patents, there are form patents, derivative patents, drug substance mixture patents, formulation patents, and indication patents.[3]

Human rights and intellectual property rights

Human rights are specific rights of people, whatever their nationality, place of living, sex, national or ethnic origin, color, religion, language or any other characteristic.[4] Intellectual property rights allow the creators or owners of patents, trademarks, and copyrighted works to benefit from their work or investments for creation. These rights are set out in Article 27 of the Universal Declaration of Human Rights, which provides protection of secular and spiritual interests of the author/owner of scientific, literary or artistic production.[5,6]

Right to health and medical care as a human right

Article 25 of the Universal Declaration of Human Rights and Article 35 of the Charter of Fundamental Rights of the European Union (UN) guarantee the right to health and medical care. Section 12 of the International Economic, Social, and Cultural Rights Agreement, Article 24 of the Convention on the Rights of the Child, Article 12 of the Agreement on the Elimination of All Forms of Discrimination Against Women also guarantee the right to health and medical care.[5,7-10]

UN Economic, Social and Cultural Rights Committee consider the right to health as a basic right category, which can be divided into sub-headings of other specific categories. These classes are the control, prevention, and treatment of disease, and include access to essential drugs.[11] According to The World Health Organization (WHO), 'essential medicines' are drugs that meet the needs of the majority of the population. These drugs must always be in appropriate amounts, and adequate doses with a price people and communities can afford.[12]

Situation in poor countries, global-scale problem

Public institutions, especially of developing countries, are faced with a lack of financial resources, which is why they encounter difficulties regarding delivering high-price drugs as preventative or curative medicines to citizens due to patent protection, particularly to disadvantaged individuals and groups. Both at the national and international level, this scenario leads to great social injustice. As a result, these problems are increasing the ethical obligations of the pharmaceutical companies.[13]

Drug-research and development orientation

Data of the report prepared by the United Nations High Commissioner of the WHO revealed that the pharmaceutical industry channels their studies first and foremost to 'profitable diseases', those that probably have the highest return in the market. Diseases such as tuberculosis or malaria, which affect people living in developing countries, are classified as bad investments and ignored. There is drug R&D orientation according to disease type, and three types of diseases are mentioned[14]:

1. First-type diseases, such as hepatitis B, affect many people in both rich and developing countries.

2. Second-type diseases are neglected diseases such as HIV/AIDS and tuberculosis, both poor and rich countries are affected, but they are more common in developing countries.[15]

3. Third-type diseases are also known as over neglected diseases such as river blindness and sleeping sickness, which affect more than one billion people and are seen widely in citizens of developing countries.

Rare diseases-orphan drugs

The United States of America (USA) defines rare diseases as diseases and conditions that affect fewer than 100.000 people. The European Union (EU) defines rare diseases as diseases that affect no more than 5 in 10.000 people, that threaten life, and chronically debilitate the body.[16] To date, there are 5000-8000 known rare diseases. Thirty million Americans and 30-40 million Europeans are affected by these diseases. Although 250 new rare diseases are discovered each year, there are acceptable treatments only for 200-300 of rare diseases.

Drug pricing and surplus drugs

Nowadays, health needs are steadily increasing, and at the same time, there is a continuous improvement in technology. Those in decision-making positions in health economics are facing problems related to providing more health services, because of scarce resources, and financing those services due to the increasing trend in health needs and improvements in technology. Hence, decision-makers benefit from health economics and pharmacoeconomics, which is a sub-discipline of health economics regarding the effective and efficient use of available scarce resources.

Pharmacoeconomics has emerged as a sub-branch of health economics. Pharmacoeconomics compares different pharmaceutical products from the same therapeutic class through the consideration of cost-benefit, cost-utility, cost-minimization, and cost-effectiveness analyses or compares a treatment method with surgical alternatives. It is even used to demonstrate the benefits of the establishment of some pharmaceutical services or to compare these services with themselves. Table 1 shows cost units and result units. At decision-making stages during pharmacoeconomic analyses, decision-makers evaluate expenses and results. Costs in pharmacoeconomics are direct costs, indirect costs, and intangible costs. Direct costs express drug expenditures, medical examination fees, laboratory tests, diagnostic transaction costs, and the money directly paid by patients and relatives of patients for treatment. Indirect costs are factors such as reduction of a patient's ability to work or early death. Intangible costs are factors such as stress and pain, which are difficult to calculate in monetary terms. Regarding pharmacoeconomics, evaluators take direct costs into account and make assessments.

Due to the day-by-day increase in the need for healthcare and scarce resources available around the world, the presence of various regulations on drug pricing emerges as a necessity. In Turkey, there is a Reference Pricing System (RPS), which is a common worldwide practice used for drug pricing.

Surplus goods are 'pharmacists chance to earn money,' 'pharmacist savior campaigns made by pharmaceutical warehouses,' 'the same kinds of goods supplied free-of-charge next to purchased goods;' but what truth is there in these statements? Surplus drugs are goods given toll-free by drug companies to pharmacies via distribution channels, and at the same time, they are quantity discount practices.[17] In the process of the supplier's sales, surplus goods are motivational tools to increase sales volumes; however, regarding the receivers, surplus goods mean stock loading. All around the world, the pharmaceutical sector uses surplus goods. For instance, consider a campaign: if the pharmacist buys ten pieces of drug A into his pharmacy, the surplus goods will also be ten [drug A 10 (purchased) + 10 (free of charge)].

Promotions and inspections in the pharmaceutical sector

Drug promotions cover the activities of license/permit holder companies to provide information for health professionals. In Turkey, as in the entire world, drug promotions must be made in compliance with the various procedures and principles within the framework of ethical principles.

The inspection function is the last role of the management functions and located in the corresponding position. The inspection of pharmaceutical manufacturing plants is highly critical for everyone.

Ethics

Ethics is a synonym of moral philosophy, which is a branch of philosophy. In this discipline, researchers try to determine which practices are good or bad, right or wrong, virtuous or immoral, fair or criminal. While making these assessments, ethics benefit from ethical principles such as respect for autonomy, justice, beneficence, non-maleficence, accuracy, and privacy. Thus, ethical principles are relevant for the evaluation of pharmaceutical industry policies.

EXPERIMENTAL

Written laws in force relating to drugs in Turkey constitute the material of this study. Legislations as accessible through the e-legislation website of The Republic of Turkey Prime Ministry General Directorate of Legislative Development and Publication Regulatory Information System including current changes, were used as references for this study. Legislations related with our study are: Industrial Property Law (The Official Gazette of Republic of Turkey 10.01.2017; 29944), Licensing Regulation of Human Medicinal Products (The Official Gazette of Republic of Turkey 19.01.2005; 25705), Communique on the Pricing of Human Medicinal Products (The Official Gazette of Republic of Turkey 11.12.2015; 29559), Regulation on Promotional Activities of the Human Medicinal Products (The Official Gazette of Republic of Turkey 03.07.2015; 29405), Regulation on Manufacturing of the Human Medicinal Products (The Official Gazette of Republic of Turkey 27.04.2013; 28630), Turkish Medicines and Medical Devices Agency Regulation of Health Auditors (The Official Gazette of Republic of Turkey 30.05.2013; 28662). Especially legal rules related to patents, orphan drug practices, access to drugs, pricing, promotion, and inspections will be discussed and assessed regarding pharmaceutical companies, pharmacists, and patients.

RESULTS

Drug patents

In Turkey, there is Industrial Property Law.[18] The protection provided to medical and veterinary pharmaceutical manufacturing processes and product patent certificates began on January 1, 1999. The use of magistral drugs is beyond the scope of patent rights. Information and test results, whose

Table 1. Basic pharmacoeconomic analysis methods, cost, and result units used in these methods

Pharmacoeconomic analysis method	Cost unit	Result unit
Cost-benefit analysis	Money	Money
Cost-utility analysis	Money	QALY and other utilities
Cost-minimization analysis	Money	The comparison of equivalent group results
Cost-effectiveness analysis	Money	Natural units (e.g., change in blood pressure, blood sugar, weight loss)

QALY: Quality-adjusted Life Years

creation and accumulation requires a significant effort, expense and whose owners did not disclose to the public, are requested by the related authorities for certification of manufacturing and sales licenses of a patent application. Information on veterinary and agricultural drugs shall be kept confidential by the requesting authority. The authority requesting information and test results shall take the necessary measures to prevent their unlawful use. The duration of the patent given by examination is twenty years starting from the application date and cannot be extended. The duration of patents given without examination is seven years. If the review request is performed within seven years and at the end of this period if it is decided to award a patent, the patent duration will be integrated to twenty years starting from the application date. The patent owner or the authorized person must use the invention protected by patent. The obligation for use is within three years from the announcement of the patent award in critical bulletins. In the evaluation of use, market conditions are taken into consideration. Patents can be converted into compulsory licenses if patent-related inventions are not used and are subject to public interest. A compulsory license is a legal right granted by the Government without the permission of the patent holder to benefit from the patent.[19]

In Turkish law, the original drug's patent protection period should have ended for a generic drug to be licensed. Generic pharmaceutical companies that make drug applications do not have to present results of toxicologic and pharmacologic tests or clinical trials in the event that the generic drug is similar to the original drug and consent has been obtained from the original pharmaceutical company having a licence in Turkey.[20]

However, for topics related to public health, license applications for generic medicines can be accepted concerning the data of the original drug by making an exception to the original drug data exclusivity without making additional studies. The Council of Ministers of the Republic of Turkey may decide upon patent applications or the inventions subject to patents as compulsory license issues when they are useful to the public. Regarding the interest of public health or national defense of putting an invention into practice; if there would be an increase in the use of the invention or dissemination in general or reforming for a beneficial use is of great importance, it is accepted that there is the public interest.[21,22]

Drug pricing and surplus drugs

In Turkey, RPS is used for drug pricing. Moreover, drug pricing activities are carried out according to the Communique on the Pricing of Pharmaceuticals for Human Use. There are five reference countries for drug pricing in Turkey: Spain, France, Portugal, Greece, and Italy. Every year, a constant Euro / Turkish Lira (TL) exchange rate is determined; for instance, 1 Euro=2.3421 TL). By using RPS, the lowest prices of the drug found in reference countries are taken into account while calculating the price of the drug. When the mentioned condition is evaluated in a pharmacoeconomic analysis, it is understood that cost-minimization analyses are directly or indirectly carried out. While applying the RPS, the Euro value is determined by multiplying the average annual value of the Euro (annual average daily selling Euro exchange rates of the Central Bank of Turkey and as declared in the Official Gazette of the Republic of Turkey) with the adaptation factor, which is determined as 70%. When pricing medicinal products for the first time, if the ex-factory price is below 3.63 TL, reference price monitoring is not made.[23]

Pricing of Original Products: If the original product enters the market for the first time (regarding active substance), its price must not exceed 100% of the lowest price of the reference product's price in reference countries.

Pricing of twenty-year products: The prices of twenty-year original products (regarding active substances), whose sale price to wholesalers is above 6.79 TL, may be a maximum 80% of the reference prices. At the first pricing of twenty-year products, if the selling price to wholesalers is less than 6.79 TL, the selling price to wholesalers may be 100% of the reference price.

Pricing of generic products: The price cannot exceed 60% of the original product's reference price in Turkey. If the price of the original product is less than the reference price, for the pricing of the generic drug, 60% of the reference price is taken into account. Thus, if the price of the original product is less than the reference price due to commercial concerns (entering the reimbursement list, the emergence of small price differences at pharmacies), 60% of the reference product is considered for the pricing of the generic product. At the first pricing of the generic products, if the selling price to wholesalers is less than 3.56 TL, the selling price to wholesalers may be 100% of the reference price.

When the first generic of the original product enters the market, the reference price of the original product is reduced from 100% to 60%. The price of the original product may be exactly 60% of the reference price or less due to commercial concerns of the owner company.

Pricing of combined drugs: for example, the related drug consists of 20 tablets, each of which contains 100 mg paracetamol and 200 mg atorvastatin as active drug substances. The basic principle for combined drugs: if two or more drug substances unite and form quite another indication, different from their indications alone, in that case, this combined drug is evaluated as an original product. Namely, if the combined drug consisting of analgesic and antihyperlipidemic drug substances is antineoplastic or antiviral, and if this indication is proved scientifically, this combined drug is then assessed as an original drug. However, these kinds of drugs are very few, which is why almost all combined drugs are evaluated as generic medicines.

Primarily, it is checked as to whether there is an identical combined drug (namely, a combined drug consisting of 20 tablets, each of which containing 100 mg paracetamol and 200 mg atorvastatin) on the Turkish market. If there is such a combined drug available in Turkey, then the price of the 'new' combined drug cannot exceed the price of that already on the market. If there is no equivalent combined drug on the Turkish market, then the five reference countries are checked

for the probability of finding a like-for-like equivalent original combined drug. If there is no equivalent combined drug in the reference countries, the Turkish market is verified for combined drugs that can be scaled from small-packaged in nature, or small doses (e.g., combined drug consisting of 10 tablets, each containing 50 mg paracetamol and 100 mg atorvastatin). If there is no combined drug that can be scaled in Turkey, the market is then checked for combined drugs that can be scaled from a large-packaged nature or large doses (e.g., 40 tablets, each containing 200 mg paracetamol and 400 mg atorvastatin). The process for pricing is always the same. In essence, a like-for-like equivalent is searched for, if there is no equivalent, then the nearest small equivalent is investigated. If there is no nearest small equivalent, then the nearest large equivalent drug is searched for, and finally, price rationing is made. Related to the pricing of combined drugs, if there is no reference combined drug in Turkey (if there are neither small- nor large-form like-for-like equivalent drugs), then original products sold separately in the same doses are sought. That is, the market is searched for original drugs containing 100 mg paracetamol and another drug containing 200 mg atorvastatin. If there are original medicinal products on the market, first, a like-for-like equivalent is searched for; if there are no equivalent drugs in the market, then the nearest low- and high-dose equivalents medicines and the nearest single equivalent drugs are sought, respectively. Finally, rationing is made for drug pricing by calculating 60% of the reference drug's price because the combined drug is accepted as a generic drug. If there are no original drugs containing drug substances (paracetamol and atorvastatin) of the combined drug, then the highest-priced generic products in Turkey are taken into account.

Surplus goods from the perspectives of the pharmaceutical industry

The pharmaceutical industry uses surplus goods as tools to increase sales volume in a highly competitive environment, promoting the products on the market, thus transferring the stock cost and risk to the post-production pharmaceutical supply chain (pharmaceutical warehouses, distribution channels, and pharmacies).

Surplus goods from the perspectives of the drug distribution channels

Distribution channels are interfaces between the pharmaceutical industry and pharmacies. Distribution channels think that surplus goods are marketing policy tools of the pharmaceutical industry, tools for increasing sales volumes, and a way sharing stock costs and risks with pharmacies, which are last in pharmaceutical supply chains. The direct and indirect benefits of drug distribution channels from surplus goods are dependent on parallel actions compatible with pharmacies and a "win-win" policy because if the selling of surplus goods to pharmacies from distribution channels is not healthy and measurable, problems linked with post mature products, stock cost, and the inability of drug prescriptions returns as a load on distribution channels.

Surplus goods from the perspectives of pharmacists

Although surplus goods from the perspectives of pharmacists are quantity discount practices that reduce unit cost purchasing and increase the gains on sales, they are factors in pharmacies that increase stock holding costs and stock carrying risks, such as decreased prices, post-mature problems, wastage, and chames in public institution's discount (the discount given by pharmaceutical companies to the Government for products within the scope of reimbursement, billed to the Social Security Institution); additionally, they affect cash flow. The frequent replacement of prescribed drugs by doctors as a result of unethical practices by the pharmaceutical industry reveals risks and costs related with surplus goods for pharmacists. In addition, unfortunately, the selling strategies and planning of industry and distribution channels are not available in pharmacies. For instance, while the reimbursement agency is paying the invoice amounts of drugs to pharmacists at an average of 60 days, if the pharmacists do not evaluate the conditions of purchasing goods with surplus goods correctly, then there is no profit for those goods; on the contrary, pharmacists will have to bear the stock costs of the goods as surplus goods.

The conditions of pharmaceutical warehouses regarding surplus property and regarding commercial discounts (discounts given by drug companies to pharmacies for making their products more attractive) are significant for pharmacists. This discount is reflected in pharmacies by bypassing warehouses. Nowadays, the number of drug companies providing trade discounts has decreased greatly. Additionally, warehouse deal discounts (additional discounts reflected by warehouses to pharmacies as a result of commercial treaties) tend to decrease.

Regarding tax legislations, because surplus goods increase profit margins by reducing costs, they attract extra attention from tax authorities. There are many problems between tax authorities and pharmacies because there are inadequate facilities for accounting; if surplus goods are not handled in accounting books, then surplus goods are considered as cash sales, and pharmacies are subject to criminal procedures regarding income taxes and value-added taxes.[24]

Surplus goods from the perspectives of patients

Pharmacists purchase goods according to the socio-economic, socio-cultural, and educational levels of the people living in their neighborhoods. For example, in a neighborhood where the social and economic level of people is high, people want the same drugs (mostly expensive original medicines) prescribed by the doctor. The reimbursement agency pays the price of the cheapest equivalent drug, and the patient pays the rest. In affluent areas, patients can pay the price difference easily because they have money. In addition, patients in these regions go to private doctors' offices and purchase the prescribed drugs by paying the full fee of the prescribed drugs. Pharmacists whose pharmacies are in these kinds of regions do not purchase many generic drugs because patients generally request the expensive original medicines. Surplus goods are given mostly with cheap generic drugs, accordingly, such pharmacists mostly do not purchase drugs with surplus goods because the

patients can buy the drugs with cash and the selling rate of expensive cosmetic products is high. Also, cash flow at these pharmacies is very high. Cash flow is a crucial factor for trade, as such these pharmacists earn enough to compensate for the positives of purchasing drugs with surplus goods. In contrast, in places where people's socio-economic levels are low, people do not have enough money to buy original drugs. Thus, residents in these neighborhoods usually do not want to pay money at pharmacies, and they accept generic equivalent drugs, which are paid in full by the reimbursement agency. Also, people in these regions do not purchase expensive cosmetic products, and cash flow, which is critical for trade, is very low. Therefore, pharmacists whose pharmacies are in those regions usually purchase generic drugs with large amounts of surplus goods because they try to make up for the cash loss and try to gain through purchasing drugs.

Rare diseases and orphan drugs

There is a paucity of accessible data about the prevalence of rare diseases in Turkey, but researchers estimate that at least 5 million people have at least one rare disease. More importantly, millions of patients and their many relatives try to obtain a rapid diagnosis and effective treatment.[21] Orphan drugs are medicinal products developed to treat rare diseases in humans. In Turkey, there is no regulation directly related to orphan drugs.[22] On the Amendment of the Communique on the Pricing of Pharmaceuticals for Human Use[23], orphan drugs are defined as drugs used for diseases with an incidence of less than 1/100.000 people, and which are not exactly defined. The same communique expresses that orphan products can be priced up to the reference price put forth with official documents obtained from the country of import and manufacture, and the price of these commodities will be revised every year making calculations on sales amounts. In Turkey, according to the Regulation on the Licensing of Human Medicinal Products, the Ministry of Health finalizes full drug registration applications, whose preliminary investigation is completed within 210 days following the acceptance of this application by examining the fulfillment of regulatory requirements. In Turkey's drug regulations, the parts that can be associated with the orphan drugs are: registration of filed drugs being the first in the treatment or diagnosis of diseases; innovation or reduction of public spending on health; and requirement in terms of public health. Examination are completed after no more than 180 days to ensure rapid access to the whole community. Also in the same Regulation, it is stated that the authority can issue licenses depending on certain conditions such as rarely seen indications of the referring drug and accordingly, when the applicant cannot provide absolute evidence for efficacy and safety.[20]

The first diagram shows the percentage distribution of EU orphan drugs in Turkey. Twenty-eight percent of licensed EU orphan drugs are not available for use in Turkey. The second diagram is about the percentage distribution of licensed EU orphan medicinal products used with different access procedures in Turkey. Thirty percent of EU-licensed orphan drugs have also been licensed in Turkey; 70% of EU-licensed orphan drugs are used in Turkey through off-label use approval (Diagram 1, 2).

Diagram 1. Percentage of Licensed European Union Orphan Drugs in Turkey[22]

EU: European Union

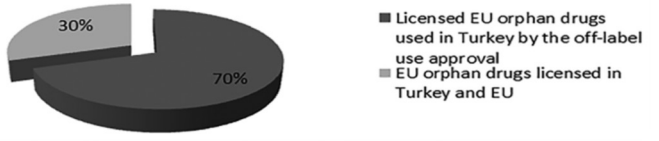
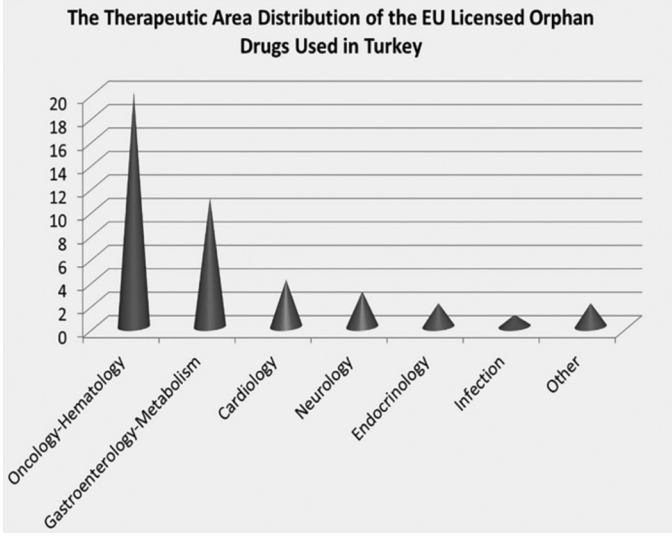


Diagram 2. Percentage of Licensed European Union Orphan Drugs used by different access procedures in Turkey[22]

EU: European Union

The above graph shows the therapeutic area distribution of the EU-licensed orphan medicinal products used in Turkey. The majority of orphan drugs used in Turkey are oncology-hematology drugs. There are twenty oncology-hematology orphan drugs, eleven for gastroenterology-metabolism, four for cardiology, two for endocrinology, one for infections, and two orphan medicinal products used for other therapeutic purposes (Graphic 1).



Graphic 1. The therapeutic area distribution of orphan drugs in Turkey in 2011[22]

EU: European Union

Patients' access to orphan drugs in Turkey and ethical approach

In Turkey, people can access orphan drugs through licensed sales, even if not authorized in Turkey, by the procurement of approved orphan drugs from the USA and EU. Alternatively, orphan drugs can be accessed though case-by-case prescription approval of medicines whose efficacy and safety are verified with an ongoing clinical trial protocol, or by the clinical use of yet unapproved drugs on patients with severe diseases. In Turkey, regardless of their authorization status, all orphan medicinal products are covered with 100% reimbursement.[16]

Drug promotion in the pharmaceutical industry

Drug promotion in the Turkish pharmaceutical industry must be performed according to the Regulation on Promotional Activities of Human Medicinal Products as published by the Ministry of Health of Turkey, Turkish Medicines and Medical Devices Agency (TITCK). Promotion covers all the informing activities, all the promotional staff activities about the medical-scientific features of the medicinal products for human use within the scopes of the Regulation held by the owners of the licenses/permits for healthcare professionals, advertisements that are given to medical and professional books and journals, announcements made by direct mailings or through the press or other means of communication, scientific/educational activities, and meetings and other events held by the industry. Promotional materials include printed materials such as books, booklets, and brochures, containing sufficient and necessary information about the product; film and slides; audio /visual materials provided with storage devices such as flash memory drives and CD/DVDs; every kind of publication that may be used as information/data/reference; free samples; programs and materials for patient education; reminder visiting materials such as pens, pencils, notepads, and calendars whose monetary value should not exceed 2.5% of the current monthly minimum gross wage. Product promotional staff pertains to people who have received a certificate of qualification and who promote products to physicians, dentists, and pharmacists via direct visits. Qualification certificate refers to that given directly to university graduates of the medical promotion department and the marketing program by the Turkish Ministry of Health or the certificate provided by examination at the end of the in-service training approved by the Ministry.[25]

Healthcare professionals includes physicians, dentists, pharmacists, nurses, and midwives. Health professionals must declare all kinds of support given by the owners of the licenses/permits when they write an article at the end of each article, when they talk or present something at the beginning of each speech/presentation. All scientific and educational activities relating to promotions of medicinal products for human use cannot be used, except to transfer existing medical information, and to present new information. The owners of the licenses/permits cannot directly or indirectly cover transportation and accommodation costs of respondents participating in these activities. Meetings at which healthcare professionals attend must be related with their expertise/task fields. A healthcare provider can benefit from this support a total of three times in the same year; two of these three supports can be held by the same license/permit owner and only one can be used abroad. Meetings at which healthcare professionals attend as speakers or report research with the support of license/permit owners are not considered within this scope.[25]

Pharmaceutical industry inspections

Inspections of the pharmaceutical industry regarding Good Manufacturing Practices (GMP) are performed according to the Regulation on Manufacturing Sites of Medicinal Products for Human Use.[26] TITCK inspects manufacturing plants that have manufacturing permission within a program by giving prior notice. In addition, the Agency can inspect manufacturing plants when necessary without further notification. Health inspectors are authorized to examine all documents related to manufacturing, quality control, and quality assurance, and also for sampling when needed, and examining other recordings. The matters required for notification are indicated in the inspection report, and the Agency announces these issues. The Agency may inspect the manufacturing plants of imported drugs. According to TITCK Regulation on Health Inspectors[27], health inspectors have to graduate from faculties such as law, political information, economics, business, economics and administrative sciences, health sciences, medicine, dentistry, pharmacy, engineering, biology, and chemistry.

DISCUSSION

Drug patents

Drugs are defined as essential social products used for or foreseen to be used for changing physiologic systems or pathologic conditions to the benefit of users. At first glance, although drugs are not different from other inventions subject to patent rules, their use to protect people against diseases or to diagnose/improve a patient's condition determines its exchange value. Drugs cannot be replaced with any other product. Thus there is no direct relationship between the drug price and the patients' purchasing power. The demand for drugs is independent of its exchange value and the purchase request depending on this value. Direct interest in the medicine in the right to human health and life as a commodity or a product places needy drug consumers demanding the drug in a particular position and differentiates them from consumers in the classic sense. This situation makes a person socially powerless in the period up to access to drugs in the face them meeting a patient if they require protection against the patient. In the process for available drugs, patent owners, manufacturers, physicians, and pharmacists have several functions.

In the case of first-type diseases in promoting R&D in the pharmaceutical industry, market mechanisms in wealthy countries, public funding for basic studies, and patent protections provide benefits. However, the level of technological and economic development of developing countries does not allow for the development of drugs and vaccines, thus patent protections and high prices represent an obstacle for the supply of current drugs. R&D activities made for second-type diseases are shaped not by global needs, but mostly by the

demands of rich countries' markets. When it comes to third category diseases, to which developing countries are exposed, the market mechanism that functions in affluent countries does not work. The TRIPS regime accelerated this deprivation.

What can governments do?

- When a new drug is invented, governments could give awards to the company rather than give a product patent, thus, because there will be no a related patent, generics of the new invented original drug may be launched to the market more easily.

- Compulsory License Agreements made by governments: with the results of government enforcement, the transfer of patent right without the inventor company's request may be performed rather than transferring with provision for material benefits to another company; therefore, generics of the new invented original drug may be put into the market easily.

- Parallel imports: the importation of a product from a country where the product is sold cheaper to another where the product is sold more expensively without the permission of the manufacturer or distributor may be conducted.

"Respect" Liabilities of Pharmaceutical Companies Regarding Ethics[14]:

1. When creating missions, companies should enfranchise human rights in general, in particular the clear right to the highest attainable standard of health, and should avoid any behaviors that could lead to a violation of these fundamental rights.

2. Companies should respect the national laws of governments of countries in which they reside or operate.

3. Companies should avoid any behaviors that would encourage acting contrary to human rights norms, including the right to health born of the national or international laws of governments.

4. Companies should give an undertaking about not performing lobbying activities against the implementation of flexibility provisions including parallel imports and compulsory licensing relating to the TRIPS Agreement, and allowing access to drugs by governments in related countries with a request for the protection of intellectual property interests.

"Realization" Liabilities of Pharmaceutical Companies Regarding Ethics[14]:

1. When companies formulate and apply their strategies, policies, programs, projects and activities, they should show a special effort for people and groups who are poverty-stricken and classified as disadvantaged, and also for women and children who face discrimination such that they benefit from the highest standards of health.

2. Companies should participate in the UN Global Compact.

3. Companies should constitute a board-level system of governance that will take responsibility for their access to the drug strategy.

4. Companies must give a public undertaking about contributing to R&D against neglected diseases and should consult with relevant civil society organizations. Companies should provide investigation and development studies against neglected diseases within themselves or should support outside companies and disclose studies related to these diseases.

5. Companies should make technology transfer agreements with local firms of low and middle-income countries.

6. When creating their price policies, companies should take into account the development level of the countries. They should have different drug pricing in low, medium, and high-income countries.

Rare diseases - orphan drugs

As mentioned earlier, pharmaceutical companies have mostly been conducting R&D for profitable diseases. Therefore, orphan drugs always remain in the background. They exhibit approaches that do not comply with the right to health in the perspectives of ethics. Some questions must be asked: Is money everything? What about human health? What about the right to health?

In the clinical research process of orphan drugs yet to receive regulatory approval, the utmost importance should be given to ethical principles in the implementation of supply to people with severe illnesses. At this point, informed consent, respect for autonomy, beneficence, and harm to ethical principles are becoming crucial.

In Turkey, when pricing orphan drugs, 5% more of the reference price may be given to these drugs by leaving pharmacoeconomic concerns aside, and thus we can easily conclude that rare diseases and orphan drugs are taken into account in Turkey. The right of access to orphan medicinal products is an essential right for all people of the world, regardless of differences regarding ethics. The Government must also do their utmost in this regard.

Drug pricing and surplus drugs

Pricing of drugs

At the time of this study, the Euro/TL exchange rate is (1 Euro= 4.030 TL). However, when pricing drugs, the Euro/TL exchange rate is fixed as (1 Euro= 2.3421 TL). The currency differences with the countries concerned should be arranged by taking available national resources into account, and there must not be any problem regarding the supply of essential drugs of foreign origin. Another point that can be debated is the quality of pharmaceutical substances. Drugs are essential products, and therefore should always be accessible in all circumstances and at first grade.

The ethical impact of surplus goods among pharmacists

Surplus goods given at a irrational levels create significant economic differences among pharmacies, and the differences are gradually increasing. The pharmacists' respect for manufacturer providing irregular surplus goods is gradually diminishing. Tax offices evaluate pharmacies as earning considerable amounts of money, and this sentiment creates moral corruption and unnecessary competition among pharmacists. Also, when we consider patients, for pharmacists to be ethically justified, they should pay attention to giving

generic drugs, with which patients will be provided optimal benefit. Thus, pharmacists should distance themselves from financial matters when considering the health of the patient.

The optimal situation regarding surplus goods

Relating to the sustainability of commercial activities in pharmacies, the thing to be understood from products with surplus at a logical level is the supply of commercial discounts at rates of 4-7%, in line with the payments of reimbursement institutions and with a maturity of at least 60 days.

Promotion in the pharmaceutical industry

In the Turkish pharmaceutical industry, promotions should not only be for profit purposes and should not be conducted by non-qualified people. Drug promotions should be made based on scientific evidence and in an ethical manner by qualified product promotion members. Healthcare professionals should demonstrate sensitivity to related issues, and they should not provide themselves unethical benefits and earnings as a result of unethical behaviors. Also, while making drug promotions, transferring pharmacoeconomic evaluations related to drugs to healthcare professionals is important to allow for assessment of the drug from a wider perspective.

The pharmaceutical industry inspections

Considerations for pharmaceutical industry inspections and the ethical approach

Pharmaceutical industry inspections should go beyond the scope of relevant legislation. Inspections should be carried out transparently by qualified inspectors on a scientific basis, and it should not be forgotten that humans will directly use the medicine manufactured in the inspected drug manufacturing facility. Inspections are crucial because the drugs must always be first grade. Inspectors should inspect all sites justly, and unethical practices should absolutely be avoided. Inspectors who perform GMP inspections must educate themselves at all levels about the process from the manufacturing of drugs until delivery to patients, regarding knowledge and skills, and they should comply with ethical principles. Inspectors should sign an ethical contract on their first entry to work. Additionally, inspectors' conflict of interest declarations must be taken by the Agency and renewed periodically.

CONCLUSION

The drugs patent protection regime brought to drugs with the TRIPS Agreement is encouraging the pharmaceutical industry at the point of original drug discovery. However, obstacles related to the protection of the right to health and access to essential medicines should not be overlooked. The problems of developing countries regarding access to essential medicines must be remedied. Governments must find the necessary solutions related to these issues, and essential medicines must always be accessible by the public. Governments and the pharmaceutical companies must fulfill the ethical obligations imposed on them. Pharmaceutical companies should perform R&D for profitable diseases and orphan drugs, and they have to conduct studies for health problems in developing countries. In the clinical research processes of orphan drugs yet to receive regulatory approval, utmost importance should be given to ethical principles in the implementation supply to people with severe illnesses. At this point, moral principles such as informed consent, respect for autonomy, beneficence, and non-maleficence are becoming crucial.

Regarding our national interests and pharmacoeconomic terms, choosing the cheapest between drugs with the same efficacy as a reference for pricing drugs is a logical and consistent action for our economic interests. If the RPS is applied, currency differences with the countries concerned should be arranged taking available national resources and pharmacoeconomic considerations into account. There must be no problem regarding the supply of essential drugs of foreign origin. Essential medicines should always be accessible in our country.

The Turkish drug industry should be supported, and more resources should be transferred to R&D activities. R&D activities must be conducted regarding biologic medicines, biosimilar products, and biotechnology products, which are of increasing importance in the world. The supply of surplus drugs should be rearranged to provide a common ground for the pharmaceutical industry, pharmaceutical warehouses, and pharmacies. As a result of pharmacoeconomic evaluations, if there is a generic equivalent drug having a lower price, it must be chosen and provided to the patient.

In the Turkish pharmaceutical industry, promotions should not only be for profit purposes and should not be conducted by non-qualified people. Drug promotions should be made based on scientific evidence and in an ethical manner by qualified product promotion members who have received a qualification certificate.

Inspections of the pharmaceutical industry must be performed by inspectors who are extremely well informed regarding all aspects of drug manufacturing through to delivering products to patients. Thus, various problems may be fixed during the manufacturing processes. Also, inspectors must comply with ethical principles during inspections. It is crucial to carefully follow whether deficiencies have been fixed owing to the fundamental requirement that drugs must always be top-grade quality.

Conflict of Interest: No conflict of interest was declared by the author.

REFERENCES

1. Sykes AO. TRIPs, Pharmaceuticals, Developing Countries, and the Doha "Solution, John M. Olin Program in Law and Economics Working Paper No 140, 2012.
2. T'Hoen EFH, TRIPS, Pharmaceutical Patents and Access to Essential Medicines: Seattle, Doha and Beyond. In: Economics Of Aids And Access To Hiv/Aids Care In Developing Countries Issues And Challenges, Ed(s): J.P. Moatti, B. Coriat, Y. Souteyrand, T. Barnett, J. Dumoulin, Y.A. Flori, pp. 39-69, Anrs Collection Sciences Sociales Et Sida, Paris, 2013.

3. Yalçıner UG. İlaç ve Patent Türkiye'de ve Dünyada Son Gelişmeler. Ankara Barosu Fikri Mülkiyet ve Rekabet Hukuku Dergisi. 2002:2.
4. United Nations Human Rights Office of the High Commissioner. Your Human Rights. http://www.ohchr.org/EN/Issues/Pages/WhatareHumanRights.aspx (last visited: 05.02.2017).
5. United Nations. Universal Declaration of Human Rights. http://www.un.org/en/universal-declaration-human-rights/ (last visited: 05.02.2017).
6. World Intellectual Property Organization (WIPO). What is Intellectual Property? WIPO Publication No: 450 (E). http://www.wipo.int/edocs/pubdocs/en/intproperty/450/wipo_pub_450.pdf (last visited: 05.02.2017).
7. Charter of Fundamental Rights of the European Union. Official Journal of the European Communities 2000/C 364/01. http://www.europarl.europa.eu/charter/pdf/text_en.pdf (last visited: 05.02.2017).
8. International Covenant on Economic, Social and Cultural Rights. United Nations Office of the High Commissioner for Human Rights 1976; in accordance with article 27.
9. Convention on the Rights of the Child. United Nations Office of the High Commissioner for Human Rights 1990; in accordance with article 49.
10. Convention on the Elimination of All Forms of Discrimination Against Women. United Nations Office of the High Commissioner for Human Rights 1981; in accordance with article 27.
11. General Comment No. 14: The Right to the Highest Attainable Standard of Health (Art. 12). United Nations Office of the High Commissioner for Human Rights 2000; E/C. 12/2000/4.
12. Laing R, Waning B, Gray A, Ford N, 't Hoen E. 25 years of the WHO essential medicines lists: progress and challenges. Lancet. 2003;361:1723-1729.
13. Chapman RA. Approaching Intellectual Property as a Human Right: Obligations related to Article 15(I) (c). Copyright Bulletin Unesco Publishing XXXV (3), 4-36, 2001.
14. Büyüktanır BGÖ, Birinci G, Ömürgönülşen U. Trips Agreement and Ethical Responsibilities of Pharmaceutical Companies Concerning the Right to Health. Hacettepe University Journal of Faculty of Economics and Administrative Sciences. 2012;30:1-26.
15. Yücekal Y. Protection of Intellectual Property Rights at the International Level World Intellectual Property Organization (WIPO). Republic of Turkey Ministry of Foreign Affairs Journal of International Economic Issues VIII, 2003.
16. Koçkaya G, Wertheımer A, Kılıç P, Tanyeri P, Vural İM, Akbulat A, Artiran G, Kerman S. An Overview of the Orphan Medicines Market in Turkey. Value In Health Reg Issues. 2004;4C:47-52.
17. Gülpınar G, Uzun MB, Yalım NY. The effects of Social Security Institution implementations on community pharmacists' job satisfaction: a qualitative study. Turkish Journal of Bioethics. 2015;2:36-46.
18. 6769 sayılı Sınai Mülkiyet Kanunu. Türkiye Cumhuriyeti mevzuatı. Türkiye Cumhuriyeti Resmi Gazete 10.01.2017; 29944. (No. 6769 Industrial Property Law. Legislation of the Republic of Turkey. The Official Gazette of Republic of Turkey 10.01.2017; 29944).
19. Bird RC. Developing nations and the compulsory license: maximizing access to essential medicines while minimizing investment side effects. J Law Med Ethics. 2009;37:209-221.
20. Beşeri Tıbbi Ürünler Ruhsatlandırma Yönetmeliği. Türkiye Cumhuriyeti mevzuatı. Türkiye Cumhuriyeti Resmi Gazete 19.01.2005; 25705. (Licensing Regulation of Human Medicinal Products. Legislation of the Republic of Turkey. The Official Gazette of Republic of Turkey 19.01.2005; 25705).
21. İlbars H, Irmak DK, Akan H. Orphan Drugs: R&D Challenges with Updates from Turkey and Middle East Countries. Journal for Clinical Studies. 2014;6:58-63.
22. Kılıç P, Koçkaya G, Yemşen Ö, Tan C, Öztunca FH, Aksungur P. Kerman S. Orphan drug regulations in Turkey. J Pharm Health Serv Res. 2013;4:151-153.
23. Beşeri Tıbbi Ürünlerin Fiyatlandırılması Hakkında Tebliğ. Türkiye Cumhuriyeti mevzuatı. Türkiye Cumhuriyeti Resmi Gazete 11.12.2015; 29559. (Communique on the Pricing of Human Medicinal Products. Legislation of the Republic of Turkey. The Official Gazette of Republic of Turkey 11.12.2015; 29559).
24. Çakıl AU. Eczane İşletmelerinde Muhasebe ve Vergi Uygulamaları. Türk Eczacılar Birliği Meslek İçi Sürekli Eğitim Dergisi 21-22, 85-102, 2009.
25. Beşeri Tıbbi Ürünlerin Tanıtım Faaliyetleri Hakkında Yönetmelik. Türkiye Cumhuriyeti mevzuatı. Türkiye Cumhuriyeti Resmi Gazete 03.07.2015; 29405. (Regulation on Promotional Activities of the Human Medicinal Products. Legislation of the Republic of Turkey. The Official Gazette of Republic of Turkey 03.07.2015; 29405).
26. Beşeri Tıbbi Ürünler İmalathaneleri Yönetmeliği. Türkiye Cumhuriyeti mevzuatı. Türkiye Cumhuriyeti Resmi Gazete 27.04.2013; 28630. (Regulation on Manufacturing of the Human Medicinal Products. Legislation of the Republic of Turkey. The Official Gazette of Republic of Turkey 27.04.2013; 28630).
27. Türkiye İlaç ve Tıbbi Cihaz Kurumu Sağlık Denetçileri Yönetmeliği. Türkiye Cumhuriyeti mevzuatı. Türkiye Cumhuriyeti Resmi Gazete 30.05.2013; 28662. (Turkish Medicines and Medical Devices Agency Regulation of Health Inspectors. Legislation of the Republic of Turkey. The Official Gazette of Republic of Turkey 30.05.2013; 28662).

Turk J Pharm Sci 2017;14(3):274-279
DOI: 10.4274/tjps.41636

ORIGINAL ARTICLE

Use of Non-steroidal Anti-inflammatory Drugs for Chemoprevention of Inflammation-induced Prostate Cancer

İnflamasyonla Tetiklenen Prostat Kanserleşmesinin Önleyici Tedavisinde Non-streoidal Anti-inflamatuvar İlaç Kullanımı

Bilge DEBELEÇ BÜTÜNER[1]*, Mert Burak ÖZTÜRK[2]

[1]Ege University, Faculty of Pharmacy, Department of Pharmaceutical Biotechnology, İzmir, Turkey
[2]Ege University, Faculty of Engineering, Department of Bioengineering, Cancer Biology Laboratory, İzmir, Turkey

ABSTRACT

Objectives: Chronic inflammation has been known as one of the major causes of cancer progression and 25% of cancer cases initiate due to chronic inflammation according to epidemiologic data. It has been determined that chronic inflammation induces carcinogenesis through the abrogation of cell proliferation, apoptosis, and angiogenesis mechanisms. Therefore, it is believed that inhibition of inflammation-induced carcinogenic mechanisms is an efficient therapeutic strategy in drug development studies of cancer chemoprevention. It has also been observed that use of anti-inflammatory drugs reduces the incidence of cancer, and the risk of developing prostate cancer decreases 15-20% with regular use of aspirin and non-steroidal anti-inflammatory drugs (NSAID).

Materials and Methods: In this study, we investigated the effects of some clinically used NSAIDs on cellular mechanisms that play a role in inflammation-induced prostate carcinogenesis. Inhibition activities on the nuclear factor kappa-B signaling pathway, which activates tumorigenic mechanisms, as well as alterations on androgen receptor signaling, which regulates the proliferation of prostate cells, were investigated. In addition, protein kinase B (Akt) activation, which is stimulated a the inflammatory microenvironment, was examined.

Results: The results showed that anti-inflammatory agents alter the protein levels of androgen receptors as well as tumor suppressor NKX3.1, and might trigger an unexpected increase in $Akt^{(S473)}$ level, which induces tumorigenesis.

Conclusion: It is suggested that inflammatory pathways and prostate carcinogenesis-specific mechanisms should be taken into account for the use of anti-inflammatory drugs for chemoprevention of inflammation-induced prostate cancer.

Key words: NSAID, prostate cancer, inflammation, androgen receptor, NKX3.1

ÖZ

Amaç: Kronik enflamasyonun kanser gelişiminin önemli nedenlerinden biri olduğu ve epidemiyolojik verilere göre kanser olgularının %25'inin kronik inflamasyona bağlı olarak geliştiği bilinmektedir. Kronik inflamasyon, hücre proliferasyonu, apoptoz, anjiyogenez gibi mekanizmalarda bozukluklara yol açarak tümörleşmeyi tetiklemektedir. Bu nedenle kanseri önleyici ilaç geliştirme çalışmalarında, inflamasyon ile tetiklenen mekanizmaların inhibisyonununda etkin bir terapötik strateji olduğu düşünülmektedir. Klinik çalışmalarda da, birçok kanser tipinde anti-inflamatuvar ilaçların kanser insidansını azaltıcı etkisi belirlenmiş ve prostat kanserinde de düzenli aspirin veya non-streoidal anti-inflamatuvar ilaç (NSAID) kullananların prostat kanserine yakalanma riskinin yaklaşık %15-20 azaldığı saptanmıştır.

Gereç ve Yöntemler: Bu çalışma kapsamında, klinik kullanımdaki NSAID'lerden indometazin, sulindak, ibuprofen, naproksen, selekoksib ve nimesulidin inflamasyona bağlı prostat kanserleşmesinde rol oynayan moleküler mekanizmalardan bazıları üzerindeki etkileri araştırılmıştır. Tümörijenik mekanizmaları aktifleştiren nükleer faktör kappa B sinyal yolağı üzerindeki inhibisyon etkileri ve prostat hücrelerinin proliferasyonunu düzenleyen androjen reseptörü sinyal yolağı üzerindeki etkileri araştırılmıştır. Ayrıca inflamatuvar mikroçevrede etkinleşerek tümörleşmeye neden olan protein kinaz B (Akt) aktivitesindeki değişim de incelenmiştir.

Bulgular: Elde edilen sonuçlar, anti-inflamatuvar ajanların, androjen reseptörü ve tümör baskılayıcı NKX3.1'in protein seviyelerinde konsantrasyona bağlı bir değişime neden olduğu, ayrıca tümörleşmeyi tetikleyen $Akt^{(S473)}$ düzeyinde de beklenmedik bir aktivasyonu tetikleyebildiğini göstermiştir.

Sonuç: Anti-inflamatuvar ajanların, prostat inflamasyonu ile tetiklenen kanserleşme sürecini önleme amacıyla kullanımlarında yalnızca inflamatuvar yolakların değil prostat kanserleşmesine özgün mekanizmaların da dikkate alınması gereği ortaya konmuştur.

Anahtar kelimeler: NSAID, prostat kanseri, inflamasyon, androjen reseptörü, NKX3.1

*Correspondence: **E-mail:** bilge.debelec@ege.edu.tr, **Phone:** +90 505 393 87 72 **ORCID-ID:** orcid.org/0000-0001-8112-9241
Received: 29.11.2016, **Accepted:** 26.01.2017

INTRODUCTION

Clinical studies have shown that use of anti-inflammatory drugs results in a decrease of the incidence of many types of cancer such as colon, prostate, and stomach cancer in particular.[1] Some anti-inflammatory drugs show antitumor activities through affecting pathways such as nuclear factor (NF) kappa-B (NF-κB), cyclooxygenase-2 (COX2), Wnt/B-catenin, Protein Kinase B (known as Akt or PkB), reactive oxygen and nitrogen species (RONS) production, and angiogenic vascular endothelial growth factor (VEGF), which function in cellular mechanisms such as proliferation, apoptosis, angiogenesis, and migration/invasion.[2-7] In addition, inflammation-related alterations of the androgen receptor (AR), NKX3.1, and Akt, which regulate cell proliferation in co-operation, have a significant role in prostate tumorigenesis.[8-10]

The negative correlation between the use of anti-inflammatory drugs and cancer incidence proves that active inflammation supports carcinogenesis, and anti-inflammatory drugs can prevent inflammation-related tumorigenesis.[4,5] Inflammation leads to neoplastic transformation by altering gene expression levels of oncogenes and tumor suppressors. In addition, these genetic changes influence the expression patterns of many inflammation-related genes and result in the recruitment of inflammatory cells in the tumor microenvironment. These alterations cause tumor cells to produce free radicals, and inflammatory cytokines create a feedback loop that supports carcinogenesis.[11-13]

Androgen receptors have a critical role in the survival of prostate cells. However, AR signaling is also abrogated during inflammation. Activation of AR signaling supports the development of prostate adenocarcinomas because prostate cells depend on AR for proliferation. On the other hand, androgen ablation therapy leads to a tumor reduction at the beginning; however, paradoxically, it induces and accelerates the transition to castration-resistant-stage prostate cancer. In this stage, prostate cells do not depend on AR presence to survive and proliferate.[14,15]

NKX3.1 is a tumor suppressor protein whose expression is lost in most primary prostate cancers.[16,17] Loss of NKX3.1 function is observed through the related chromosome deletion and enhanced proteasome degradation induced by pro-inflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α.[18] Further, it has been shown that NKX3.1 expression was alleviated in proliferative inflammatory atrophy and prostatic intraepithelial neoplasia.[10,19]

In the present study, we aimed to investigate the potential chemo-preventative effects of commonly used NSAIDs on NF-κB signaling and inflammation-induced degradations of AR and NKX3.1, which have a significant role on inflammation-related prostate tumorigenesis.

EXPERIMENTALS

Cell culture and treatments

LNCaP cells were obtained from American Type Culture Collection (ATCC Manassas, VA) and propagated using RPMI 1640 supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 µg/mL). Synthetic androgen R1881 was used at 10 nm concentration. A specific NF-κB inhibitor (BAY11-7082) and celecoxib were purchased from Biovision. Indomethacin, sulindac, ibuprofen, naproxen, and nimesulide were purchased from Sigma and used at the indicated concentrations determined according to the assay approach.

Conditioned media (CM) collection and measurement of cytokines in CM

The U937 monocyte cell line was cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. To achieve macrophage differentiation and cytokine production, cells ($8x10^6$) were grown in 75 cm^2 culture flasks for 2 h prior to treatment. Next, phorbol 12-myristate 13-acetate was added at a final concentration of 16 nM for 16 h, and adherent clusters were followed. Cells were washed twice, and 20 mL of fresh medium was then added. After allowing the cells to rest for 2-3 h, lipopolysaccharide was added (10 ng/mL), and the cells were incubated for 24 h. Finally, the supernatant [conditioned medium (CM)] was collected and filtered (0.2 µm) for further use.

Before feeding the LNCaP cells with CM, TNF-α (Invitrogen), IL-6, and IL-1β (Boster Biological Technology Co., US) levels were assessed using an enzyme-linked immunosorbent assay in accordance with the manufacturer's recommendations. Finally, CM with known concentrations was used to induce an inflammatory microenvironment in cell culture.

Antibodies

The antibodies were purchased from the manufacturers as follows: p-Akt$^{(S473)}$ from SCBT; AR antibody from Millipore; B-actin antibody from Sigma-Aldrich; HRP-anti-mouse and HRP-anti-rabbit secondary antibodies were purchased from Amersham BioSciences UK, and used as recommended. NXK3.1 antibody was kindly supplied by Prof. Saatcioglu from the Oslo Biotechnology Center, Norway.

NF-κB luciferase reporter assay

LNCaP cells (10^4) were seeded in 96-well culture plates and incubated for 48 h. Cell culture media was replaced with serum/antibiotic-free culture medium 2 h before the transfection. Negative and positive control vectors (100 ng/mL), and NF-κB (100 ng/mL) reporter vector (SA biosciences Cignal NF-κB reporter luc kit-CCS-013L) were transfected using fugene HD transfection reagent (Roche). Normal culture medium was added onto cells 5 h after transfection and incubated for 24 h. Treatments were performed for 4 h and cells were collected with passive lysis buffer according to the recommendations of dual-luciferase reporter assay kit (Promega, UK). Renilla/firefly luciferase activity measurements were obtained using a luminometer (Thermo) according to the manufacturer's protocol.

Cell lysis, protein extraction and blotting

For protein extraction, cells were grown in 6 cm plates (Sarstedt, Germany) and washed once with PBS prior to cell lysis. Next,

250 µL ice-cold modified RIPA buffer [10 mM Tris Cl (pH 8.0), 1% Triton X-100, 0.1% SDS, 0.1% Na deoxycholate, 1 mM EDTA, 1 mM EGTA and 140 mM NaCl] containing both protease and phosphatase inhibitors was added to the plates, and the cells were then collected into Eppendorf tubes using a cell scraper. The lysates were sonicated for 20 seconds (25% power, 0.5 cycles), centrifuged at 12.000 g for 10 min at 4˚C, and the cleared supernatants were transferred into new tubes. The protein concentration was determined using a BCA assay (Sigma, UK). Western blots were performed under standard conditions using 50 µg of protein lysate per lane. First, the proteins were separated on a 10-12% SDS-PAGE gel and transferred to a PVDF membrane (Amersham BioSciences, UK) using a wet transfer blotter. The PVDF membrane was blocked with 5% dry milk in TBS-T (Tris-Borate-Saline solution containing 0.1% Tween 20), and then primary and secondary antibody incubations were performed using TBS-T containing 0.5% dry milk or 5% BSA at RT for 1 h or at 4˚C overnight. The membranes were developed using ECL plus reagent (Amersham BioSciences, UK) for 5 min and photographed using Kodak X-ray films in a dark room.

Statistical analysis

Student's t test was applied to assess the statistical significance between pairs when necessary using Microsoft Excel program.

RESULTS

Relative NF-κB inhibition activities of the selected NSAIDs

As inhibition of the NF-κB pathway is a key strategy to protect cells against inflammation-induced tumorigenic alterations, commonly used NSAIDs were investigated for their inhibition activity of the NF-κB signaling pathway using the luciferase reporter method. LNCaP cells were seeded 10^4/well on 96-well plates and incubated for 48 h. The cells were then transfected with NF-κB luciferase reporter and control vectors for 24 h and treatment were performed as 25 ng/mL TNF-α and indicated concentrations of anti-inflammatory drugs (sulindac 5 µM, naproxen 12.5 µM, ibuprofen 50 µM, nimesulide 50 µM, indomethacin 100 µM, celecoxib 50 µM) for 4 h. NF-κB activity was measured for each well and relative luciferase activity was determined as fold change of TNF-α induced cells to non-induced cells after firefly/renilla normalization. NF-κB activity was induced to 179-fold using TNF-α treatment and suppressed to 63 (sulindac), 76 (naproxen), 128 (ibuprofen), 77 (nimesulide), 71 (indomethacin), and 41 fold (celecoxib) at indicated concentrations. An NF-κB specific inhibitor (BAY11-7082) (5 µM) was used as a positive control, which led to a 98-fold decrease in NF-κB signaling activity (Figure 1).

Chemo-preventative activities of NSAIDs on inflammation-related prostate tumorigenesis

Loss of NKX3.1 in proliferative inflammatory atrophy regions was shown in previous studies. Preventing the loss of NKX3.1 in inflammatory conditions could be a useful strategy for the inhibition of tumorigenic alterations in prostate cells because it is a tumor suppressor that functions in response to DNA damage and oxidative stress regulation. Therefore, in order to understand whether NSAID treatments could prevent inflammation-induced NKX3.1 proteasomal degradation, the effects of NSAID treatments on NKX3.1 expression were investigated in LNCaPs. Cells were treated with CM (125 pg/mL TNF-α including conditioned media) for 24 h w/wo NSAIDs, which were added 2 h before CM. A decrease in NKX3.1 protein level due to cytokine-induced degradation was observed after CM as detected in our previous studies.[9,10] It was also determined that indomethacin, celecoxib, and nimesulide at their NF-κB inhibitory concentrations enhanced this degradation. NKX3.1 levels remained the same with sulindac, ibuprofen, and naproxen. None of the agents led NKX3.1 to be protected from degradation in inflammatory conditions, although NF-κB related pro-inflammatory activation was suppressed as shown by relative luciferase activity. In addition, activation of PI3K/Akt signaling, which induces proliferation, was also checked because NSAIDs could affect the activation of Akt phosphorylation. It has been determined that CM treatment increased p-Akt$^{(S473)}$ levels, consistent with our previous studies.[8] Sulindac and naproxen were found to suppress phosphorylation to its basal levels in inflammatory conditions (Figure 2).

AR signaling has a regulatory role for prostate cell proliferation. Moreover, AR signaling and tumor suppressor AR and NKX3.1 are key factors of inflammation-related prostate tumorigenesis. Therefore, in order to enlighten the dose-dependent effects of the gents on their expressions, LNCaPs were treated for 24 h. Indomethacin led to a concentration-dependent decrease on AR and NKX3.1 levels. Sulindac and ibuprofen treatments resulted in an unchanged expression profile of NKX3.1. Naproxen treatment enhanced NKX3.1 protein levels up to 3 and 6 µM concentrations, and did not change AR levels. On the other hand, celecoxib and nimesulide alleviated NKX3.1 expression in

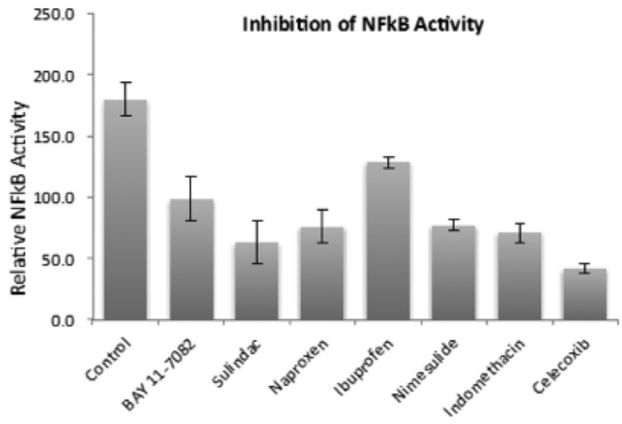


Figure 1. Relative NF-κB inhibition activities of NSAIDs. 10^4 LNCaP cells were transfected with NF-κB lusiferase reporter vector and incubated for 48 h. Cells were treated with 40 ng/mL TNF-α and anti-inflammatory agents for 4 h with the concentrations that Sulindac 5 µM, Naproxen 12.5 µM, Ibuprofen 50 µM, Nimesulide 50 µM, Indomethacin 100 µM, Celecoxib 50 µM. Specific NF-κB inhibitor BAY11-7082 was used as a positive control with a concentration of 5 µM. Dual luciferase activity was measured and relative luciferase activity was calculated as fold change of luciferase activity of TNF-α treated cells versus untreated cells after firefly/renilla normalization. Error bars represent the standard deviation of 3 replicates, $p<0.001$

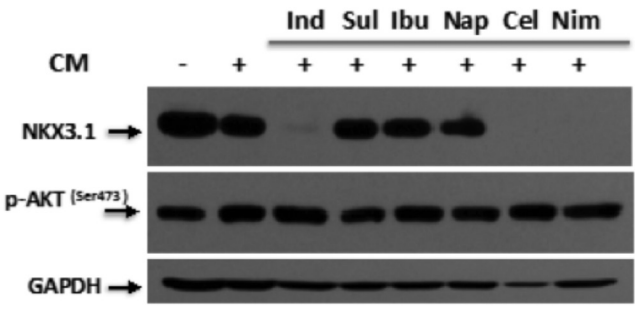


Figure 2. Change of tumor suppressor NKX3.1 expression due to NSAIDs in inflammatory microenvironment. $6x10^5$ LNCaP cells were seeded and incubated for 48 h. NSAIDs were applied onto cells 2 h before CM treatment and cell were incubated further 24 h after CM. NKX3.1 and p-Akt$^{(S473)}$ expressions were detected by immunoblotting. GAPDH was used as a loading control. CM: 125 pg/mL TNF-α including conditioned media, Indomethacin: 100 μM, Sulindac: 10 μM, Ibuprofen: 50 μM, Naproxen: 12.5 μM, Celecoxib: 50 μM, Nimesulide: 50 μM.

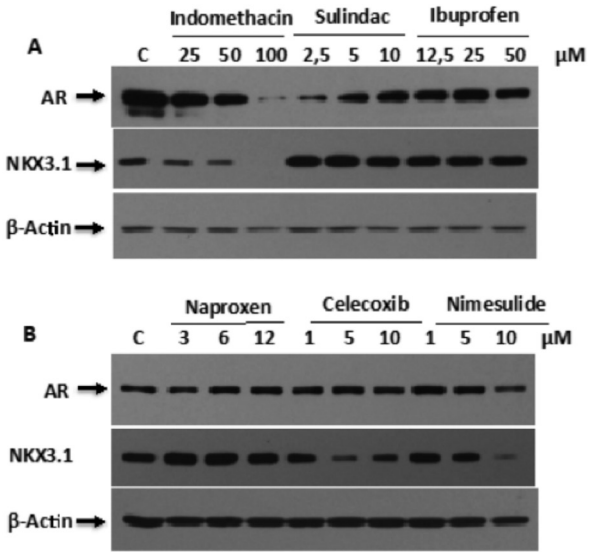


Figure 3. Alterations on AR and NKX3.1 expressions after NSAID treatments. $6x10^5$ LNCaP cells were seeded and incubated for 48 h. NSAIDs at indicated concentrations were applied onto cells for 24 h and expressions of AR and NKX3.1 were detected by immunoblotting. B-actin was used as a loading control

a concentration-dependent manner. AR protein levels remained largely stable with these agents (Figure 3a, b).

Finally, activities of anti-inflammatory drugs on the recovery of AR and NKX3.1 in the inflammatory microenvironment were investigated. LNCaP cells were treated with indicated concentrations of agents (26 h) in the absence and presence of CM (24 h) and synthetic androgen R1881 (26 h). Indomethacin severely enhanced NKX3.1 degradation during inflammation; however, stimulation of AR signaling enhanced the expressions of both AR and NKX3.1 in the presence of indomethacin. Sulindac, ibuprofen, and naproxen treatments were determined to partially protect NKX3.1 from degradation when compared with other agents. Further, degradation of NKX3.1 was not prevented by celecoxib and nimesulide in inflammatory conditions. In

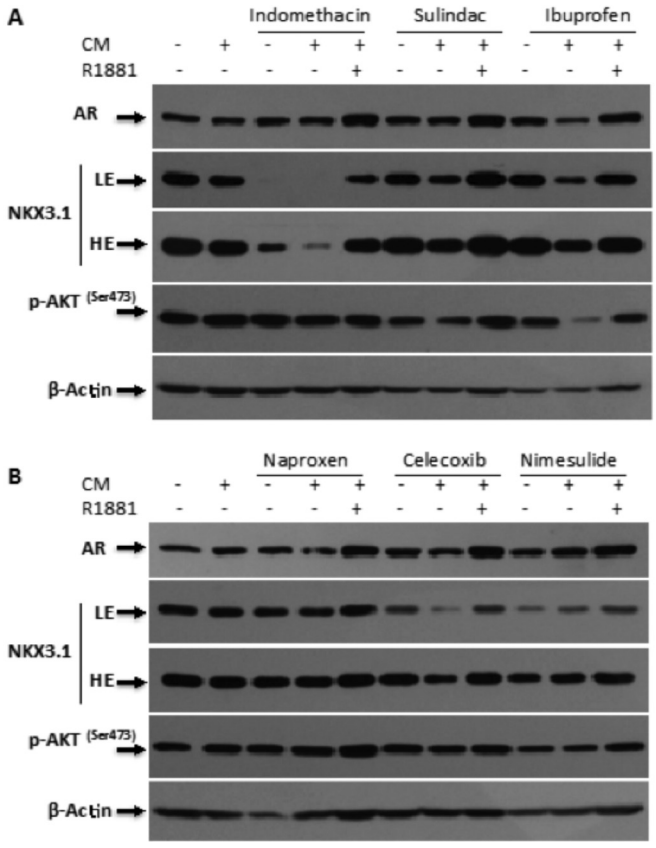


Figure 4. Effects of NSAIDs on AR signaling in inflammatory microenvironment. $6x10^5$ LNCaP cells were seeded and incubated for 48 h. NSAIDs and R1881 (10 nM) were applied onto cells 2 h before CM treatment and cell were incubated further 24 h after CM. NKX3.1 expression was detected by immunoblotting. B-actin was used as a loading control. CM: 125 pg/mL including conditioned media, Indomethacin: 50 μM, Sulindac: 5 μM, Ibuprofen: 12.5 μM, Naproxen: 6 μM, Celecoxib: 1 μM, Nimesulide: 1 μM

the presence of anti-inflammatory drugs, activation of AR signaling by R1881 treatment led to increased AR and NKX3.1 protein levels, even in the inflammatory microenvironment, as expected. Sulindac, ibuprofen, and nimesulide were detected to inhibit p-Akt$^{(S473)}$ phosphorylation even after CM treatment (Figure 4a, b).

DISCUSSION

Inflammation and the subsequent unbalanced anti-inflammatory response is known to activate tumorigenic mechanisms.[20-22] Previous studies showed that use of anti-inflammatory drugs reduced the incidence of many types of cancer such as prostate and colon cancer[23-25], as such, the development of therapeutic strategies for the inhibition inflammation-related tumorigenic mechanisms is a useful approach in the prevention of inflammatory diseases and cancer chemoprevention.[26-28] In this study, activities of NSAIDs on NF-κB inhibition and the AR signaling pathway were investigated in order to establish the protective concentrations of anti-inflammatory drugs that inhibit tumorigenic alterations in prostate cells.

Inhibition of AR and NKX3.1 degradation during inflammation has been suggested as a key strategy to protect prostate cells

from deregulated oxidative stress and enhanced proliferation. Although inhibition of AR signaling is the main strategy of primary prostate tumors through chemical castration with anti-androgens, it has been also reported that loss of AR signaling results in insufficient oxidative stress regulation with subsequent oxidative DNA damage and genomic heterogeneity, leading to castration-resistant prostate cancer.[29,30] Therefore, protective concentrations of NSAIDs on AR and NKX3.1 protein stabilities were investigated and sulindac, ibuprofen, and naproxen were found as the most efficient molecules for maintaining regulatory AR signaling and NKX3.1 function. However, it was also determined that increasing concentrations of the agents were not always correlated to AR and NKX3.1 recovery, possibly because of the cytotoxic effects of relatively higher concentrations. Therefore, it was suggested that as AR and NKX3.1 were survival factors for prostate cells that degrade under cytotoxic cellular conditions; concentrations that suppress cell proliferation via NF-κB pathway and effect cellular viability were not optimal to protect cells from AR and NKX3.1 degradation. In addition, enhanced Akt phosphorylation due to inflammation was mostly suppressed by sulindac (5 μM), ibuprofen (12.5 μM), and nimesulide (1 μM). Because sulindac, ibuprofen, and naproxen showed consistent protection for AR and NKX3.1 protein levels at their anti-inflammatory concentrations, it is concluded that use of this agents for the cure of prostatitis favors protecting cells from loss of AR signaling induced by inflammatory microenvironment.

Surprisingly, it is known that NSAIDs such as indomethacin[31] can activate Akt signaling, which enhances S473 phosphorylation, consistent with our results. Increased p-Akt$^{(S473)}$ levels result in NKX3.1 suppression[9]; the negative correlation between NKX3.1 recovery and Akt phosphorylation in our results suggests that NSAIDs that lead to unexpected activation of Akt signaling such as indomethacin should be taken into account for their effects on tumor suppressor NKX3.1. On the other hand, sulindac was shown to suppress this activation in our results, as well as in a previous study[32], which supports its inhibition potential on inflammation-induced tumorigenic events. Celecoxib was also reported to induce apoptosis via inhibition of Akt activation.[33] However, because celecoxib showed a severe suppression on NKX3.1 protein levels at its NF-κB inhibitory concentration, and also mild protection on AR and NKX3.1 without suppression of Akt phosphorylation at its lower concentrations in our results, we concluded that celecoxib was not an effective candidate among the commonly used selective COX2 inhibitors for cancer chemoprevention. Ibuprofen also has a suppression activity on the proliferation of prostate cancer cells[34] according to the literature. It is suggested that significant suppression of p-Akt$^{(S473)}$ levels could account for this effect on prostate cells. In addition, it is suggested that this effect results from the stabilization of AR and NKX3.1 by androgens in the presence of NSAIDs because it has been also reported that the presence of dihidrotestosterone enhances the apoptotic effects of anti-inflammatory agents.[35] However, the concentration of the agents was observed to be critical in balancing anti-inflammatory activity and further carcinogenic alterations such as protein stability of AR, as well as NKX3.1 and Akt activation, as found in our study.[36]

We suggest that these results are significant for the selection of the best therapy among many anti-inflammatory therapy alternatives for prostatic inflammation because our findings show the secondary effects of commonly used NSAIDs on carcinogenesis-related AR signaling abrogation and Akt activation in prostate cells.

CONCLUSION

Inflammation-related changes in cellular mechanisms could lead to carcinogenesis. Therefore, these alterations should be reversed as a strategy for the chemoprevention of carcinogenesis. In the case of inflammation-induced prostate carcinogenesis, functional protection of AR signaling and tumor suppressor NKX3.1 is a valuable strategy that should be taken into consideration to achieve the right therapy combination for prostatic inflammation.

ACKNOWLEDGEMENT

This study was supported by TUBITAK with the grant number 113Z078 to BDB and Ege University with the grant number 2014/BIL/002 to BDB.

Conflict of Interest: No conflict of interest was declared by the authors.

REFERENCES

1. Kismet K, Akay MT, Abbasoglu O, Ercan A. Celecoxib: a potent cyclooxygenase-2 inhibitor in cancer prevention. Cancer Detect Prev. 2004;28:127-142.
2. Narayanan BA, Narayanan NK, Pittman B, Reddy BS. Regression of mouse prostatic intraepithelial neoplasia by nonsteroidal anti-inflammatory drugs in the transgenic adenocarcinoma mouse prostate model. Clin Cancer Res. 2004;10:7727-7737.
3. Sooriakumaran P, Kaba R. The risks and benefits of cyclo-oxygenase-2 inhibitors in prostate cancer: a review. Int J Surg. 2005;3:278-285.
4. Schetter AJ, Heegaard NH, Harris CC. Inflammation and cancer: interweaving microRNA, free radical, cytokine and p53 pathways. Carcinogenesis. 2010;31:37-49.
5. Hussain M, Javeed A, Ashraf M, Al-Zaubai N, Stewart A, Mukhtar MM. Non-steroidal anti-inflammatory drugs, tumour immunity and immunotherapy. Pharmacol Res. 2012;66:7-18.
6. Lin Y, Bai L, Chen W, Xu S. The NF-kappaB activation pathways, emerging molecular targets for cancer prevention and therapy. Expert Opin Ther Targets. 2010;14:45-55.
7. Vendramini-Costa DB, Carvalho JE. Molecular link mechanisms between inflammation and cancer. Curr Pharm Des. 2012;18:3831-3852.
8. Debelec-Butuner B, Alapinar C, Ertunc N, Gonen-Korkmaz C, Yörükoğlu K, Korkmaz KS. TNFα-mediated loss of β-catenin/E-cadherin association and subsequent increase in cell migration is partially restored by NKX3.1 expression in prostate cells. PloS one. 2014;9:e109868.

9. Debelec-Butuner B, Alapinar C, Varisli L, Erbaykent-Tepedelen B, Hamid SM, Gonen-Korkmaz C, Korkmaz KS. Inflammation-mediated abrogation of androgen signaling: an *in vitro* model of prostate cell inflammation. Mol Carcinog. 2014;53:85-97.
10. Khalili M, Mutton LN, Gurel B, Hicks JL, De Marzo AM, Bieberich CJ. Loss of Nkx3.1 expression in bacterial prostatitis: a potential link between inflammation and neoplasia. Am J Pathol. 2010;176:2259-2268.
11. Coussens LM, Jacks T. Genetic and cellular mechanisms of oncogenesis. Curr Opin Genet Dev. 2008;18:1-2.
12. Hussain SP, Harris CC. Inflammation and cancer: an ancient link with novel potentials. Int J Cancer. 2007;121:2373-2380.
13. Schetter AJ, You WC, Lennette ET, Gail MT, Rabkin CS. Association of Epstein-Barr virus antibody levels with precancerous gastric lesions in a high-risk cohort. Cancer Sci. 2008;99:350-354.
14. Abate-Shen C, Shen MM. Molecular genetics of prostate cancer. Genes Dev. 2000;14:2410-2434.
15. De Marzo AM, DeWeese TL, Platz EA, Meeker AK, Nakayama M, Epstein JI, Isaacs WB, Nelson WG. Pathological and molecular mechanisms of prostate carcinogenesis: implications for diagnosis, detection, prevention, and treatment. J Cell Biochem. 2004;91:459-477.
16. Abate-Shen C, Banach-Petrosky WA, Sun X, Economides KD, Desai N, Gregg JP, Borowsky AD, Cardiff RD, Shen MM. Nkx3.1; Pten mutant mice develop invasive prostate adenocarcinoma and lymph node metastases. Cancer Res. 2003;63:3886-3890.
17. Abdulkadir SA, Magee JA, Peters TJ, Kaleem Z, Naughton CK, Humphrey PA, Milbrandt J. Conditional loss of Nkx3.1 in adult mice induces prostatic intraepithelial neoplasia. Mol Cell Biol. 2002;22:1495-1503.
18. Markowski MC, Bowen C, Gelmann EP. Inflammatory cytokines induce phosphorylation and ubiquitination of prostate suppressor protein NKX3.1. Cancer Res. 2008;68:6896-6901.
19. Bowen C, Bubendorf L, Voeller HJ, Slack R, Willi N, Sauter G, Gasser TC, Koivisto P, Lack EE, Kononen J, Kallioniemi OP, Gelmann EP. Loss of NKX3.1 expression in human prostate cancers correlates with tumor progression. Cancer Res. 2000;60:6111-6115.
20. De Marzo AM, Platz EA, Sutcliffe S, Xu J, Grönberg H, Drake CG, Nakai Y, Isaacs WB, Nelson WG. Inflammation in prostate carcinogenesis. Nat Rev Cancer. 2007;7:256-269.
21. Klein EA, Silverman R. Inflammation, infection, and prostate cancer. Curr Opin Urol. 2008;18:315-319.
22. Fang LY, Izumi K, Lai KP, Liang L, Li L, Miyamoto H, Lin WJ, Chang C. Infiltrating macrophages promote prostate tumorigenesis via modulating androgen receptor-mediated CCL4-STAT3 signaling. Cancer Res. 2013;73:5633-5646.
23. Nambiar D, Singh RP. Advances in prostate cancer chemoprevention: a translational perspective. Nutr Cancer. 2013;65(Suppl 1):12-25.
24. Huls G, Koornstra JJ, Kleibeuker JH. Non-steroidal anti-inflammatory drugs and molecular carcinogenesis of colorectal carcinomas. Lancet. 2003;362:230-232.
25. Wright JL, Chéry L, Holt S, Lin DW, Luedeke M, Rinckleb AE, Maier C, Stanford JL. Aspirin and NSAID use in association with molecular subtypes of prostate cancer defined by TMPRSS2:ERG fusion status. Prostate Cancer Prostatic Dis. 2016;19:53-56.
26. Chan AT, Detering E. An emerging role for anti-inflammatory agents for chemoprevention. Recent Results Cancer Res. 2013;191:1-5.
27. Tinsley HN, Grizzle WE, Abadi A, Keeton A, Zhu B, Xi Y, Piazza GA. New NSAID targets and derivatives for colorectal cancer chemoprevention. Recent Results Cancer Res. 2013;191:105-120.
28. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell. 2010;140:883-899.
29. Khandrika L, Kumar B, Koul S, Maroni P, Koul HK. Oxidative stress in prostate cancer. Cancer Lett. 2009;282:125-136.
30. Murata M, Thanan R, Ma N, Kawanishi S. Role of nitrative and oxidative DNA damage in inflammation-related carcinogenesis. J Biomed Biotechnol. 2012;2012:623019.
31. Lincová E, Hampl A, Pernicová Z, Starsíchová A, Krcmár P, Machala M, Kozubík A, Soucek K. Multiple defects in negative regulation of the PKB/Akt pathway sensitise human cancer cells to the antiproliferative effect of non-steroidal anti-inflammatory drugs. Biochem Pharmacol. 2009;78:561-572.
32. Zhou H, Liu W, Su Y, Wei Z, Liu J, Kolluri SK, Wu H, Cao Y, Chen J, Wu Y, Yan T, Cao X, Gao W, Molotkov A, Jiang F, Li WG, Lin B, Zhang HP, Yu J, Luo SP, Zeng JZ, Duester G, Huang PQ, Zhang XK. NSAID sulindac and its analog bind RXRalpha and inhibit RXRalpha-dependent AKT signaling. Cancer Cell. 2010;17:560-573.
33. Hsu AL, Ching TT, Wang DS, Song X, Rangnekar VM, Chen CS. The cyclooxygenase-2 inhibitor celecoxib induces apoptosis by blocking Akt activation in human prostate cancer cells independently of Bcl-2. J Biol Chem. 2000;275:11397-11403.
34. Andrews J, Djakiew D, Krygier S, Andrews P. Superior effectiveness of ibuprofen compared with other NSAIDs for reducing the survival of human prostate cancer cells. Cancer Chemother Pharmacol. 2002;50:277-284.
35. Andrews P, Krygier S, Djakiew D. Dihydrotestosterone (DHT) modulates the ability of NSAIDs to induce apoptosis of prostate cancer cells. Cancer Chemother Pharmacol. 2002;49:179-186.
36. Kashiwagi E, Shiota M, Yokomizo A, Inokuchi J, Uchiumi T, Naito S. EP2 signaling mediates suppressive effects of celecoxib on androgen receptor expression and cell proliferation in prostate cancer. Prostate Cancer Prostatic Dis. 2014;17:10-17.

Turk J Pharm Sci 2017;14(3):280-284
DOI: 10.4274/tjps.16362

ORIGINAL ARTICLE

Physicochemical Characterization and *In Vitro* Dissolution Test of Quercetin-Succinic Acid Co-crystals Prepared Using Solvent Evaporation

Çözücü Buharlaştırma ile Hazırlanan Kersetin-Süksinik Asit Ko-kristalinin *In Vitro* Çözünme Testi ve Fizikokimyasal Karakterizasyonu

Dwi SETYAWAN*, Indah Pudji OKTAVIA, Rizka FARIZKA, Retno SARI

Universitas Airlangga, Faculty of Pharmacy, Department of Pharmaceutics, Surabaya, Indonesia

ABSTRACT

Objectives: Quercetin is one of the flavonoids with a polyhydroxyaromatic structure. Quercetin has been proposed to exhibit a bioWactivity against oxidative stress. However, quercetin has poor solubility in aqueous media. The purpose of this study was to investigate the physicochemical properties and dissolution rates of quercetin-succinic acid co-crystals.

Materials and Methods: The quercetin-succinic acid co-crystals were prepared in 1:1 molar ratio using solvent evaporation. X-ray diffraction, differential thermal analysis, infrared spectroscopy, and scanning electron microscopy were performed to determine the physicochemical properties of quercetin-succinic acid co-crystals. Dissolution was studied in medium citrate buffer with 2% SLS for 60 min using USP II (paddle) apparatus at 100 rpm and 37˚C.

Results: Based on diffractogram, thermogram, infrared spectrum, and microscopic capture, the physicochemical properties of quercetin-succinic acid co-crystals showed difference to those of quercetin. In addition, the *in vitro* dissolution test showed that the dissolution profile of co-crystals was significantly higher than pure quercetin.

Conclusion: This study suggests that the formation of quercetin-succinic acid co-crystals using solvent evaporation enhanced the physicochemical properties and dissolution rate of quercetin.

Key words: Characterization, dissolution, co-crystal, quercetin, succinic acid, solvent evaporation

ÖZ

Amaç: Kersetin, polihidroksiaromatik yapılı flavonoidlerden biridir. Kersetinin oksidatif strese karşı bir biyoaktivite sergilediği ileri sürülmüştür. Ancak, kersetin sulu ortamda zayıf çözünürlüğe sahiptir. Bu çalışmanın amacı kersetin-süksinik asit ko-kristalin fiziko-kimyasal özelliklerini ve çözünme oranlarını araştırmaktır.

Gereç ve Yöntemler: Kersetin-süksinik asit ko-kristali 1:1 molar oranında çözücü buharlaştırma ile hazırlanmıştır. Kersetin-süksinik asit ko-kristalinin fiziko-kimyasal özelliklerini belirlemek için X-ışını kırınımı, diferansiyel termal analiz, kızıl ötesi spektroskopi ve taramalı elektron mikroskopu kullanılmıştır. Çözünme, %2 SLS içeren sitrat tamponu içinde 100 rpm ve 37˚C'de USP II (palet) aparatında 60 dakika çalışılmıştır.

Bulgular: Difraktogram, termogram, IR vemikroskobik tespite dayanarak, kersetin-süksinik asit ko-kristalin fiziko-kimyasal özelliklerinin kersetinden farklı olduğu görülmüştür. Ek olarak, *in vitro* çözünme testi, ko-kristalin çözünme profilinin saf kersetinden çok daha yüksek olduğunu göstermiştir.

Sonuç: Bu nedenle bu çalışma, çözücü buharlaştırma kullanılarak kersetin-süksinik asit ko-kristalinin oluşumunun kersetinin fiziko-kimyasal özelliklerini ve çözünme oranını artırdığını düşündürmektedir.

Anahtar kelimeler: Karakterizasyon, çözünme, ko-kristal, kersetin, süksinik asit, çözücü buharlaştırma

*Correspondence: **E-mail:** dwisetyawan-90@ff.unair.ac.id, **Phone:** +6281220516116 **ORCID-ID:** orcid.org/0000-0001-8009-6054
Received: 04.01.2017, **Accepted:** 16.02.2017

INTRODUCTION

Poor solubility of active pharmaceutical ingredients (APIs) is one of the problems in the development of oral dose form because it may affect the bioavailability profile. Solubility and dissolution are major factors that determine the release of APIs in dissolution media.[1] Several strategies have been developed in order to improve the dissolution rate of oral dose forms, such as enhancing the solubility of APIs.

Quercetin (Q) (3',4',3,5,7-pentahydroxyflavon) is a polyphenolic flavonoid with potential antioxidant activity against oxidative stress. However, Q exhibits a poor solubility in aqueous media.[2,3] Previous studies showed that several strategies were used to improve the solubility of Q, such as solid dispersion[3] and complexation with β-cyclodextrin.[4]

Co-crystallization is one of methods used to improve the solubility profile of APIs.[5] Co-crystals are produced by combining two or more stoichiometrically neutral compounds through hydrogen bonding and π–π interaction.[6] Co-crystal formers are commonly inert or exhibit no pharmacology activity.[7] Succinic acid (S) is a co-crystal former that is freely soluble in aqueous medium. It has been reported the co-crystals of S and sildenafil citrate with 1:1 molar ratio increase the solubility of sildenafil citrate by 5-fold.[8,9] It has been demonstrated that the formation of co-crystals can improve several properties of APIs, such as bioavailability, stability, hygroscopic properties, compressibility, and flowability.[5] Smith et al.[10] successfully produced the 1:1 molar ratio co-crystal of Q-caffeine. Moreover, the co-crystals enhanced solubility up to 14-fold and up to 10-fold compared with those of Q and Q dihydrate, respectively.

The two most common methods in the formation of co-crystals are solvent evaporation and grinding.[11] Solvent evaporation, commonly called slow evaporation, involves two or more molecules in certain stoichiometric amounts. The slow evaporation process facilitates the formation of hydrogen bonds between the components. This method produces larger amount of crystals and relatively more homogenous in particle size distribution compared with those produced by other methods. Moreover, slow evaporation can be conducted at room temperature.[12,13] It has been reported that solvent evaporation was successfully conducted to produce Q-isonicotinamide co-crystals and Q-theobromine dehydrate co-crystals.[14]

The present study investigated the physicochemical properties and the dissolution profile of QS co-crystals (Co-QS). Co-QS was prepared in 1:1 molar ratio using solvent evaporation. Powder X-ray diffraction (PXRD), differential thermal analysis (DTA), infrared spectroscopy (FTIR) and scanning electron microscopy (SEM) were performed to characterize Co-QS. Dissolution studies were also undertaken to evaluate the dissolution rate of Co-QS.

MATERIALS AND METHODS

The materials used in this study were Quercetin (Tokyo Chemical Industry Co., Ltd., Japan), sodium lauryl sulphate (E Merck, Germany), citric acid (E Merck, Germany), S (E Merck, Germany), pro-analytical methanol (E Merck, Germany).

Preparation of quercetin-succinic acid co-crystal with solvent evaporation method

Co-QS was prepared using solvent evaporation. For the formation of Co-QS, 365.5 mg (1 mmol) of Q and 134.5 mg (1 mmol) of S were dissolved in methanol. The dissolved Q was then poured into the S solution. The mixture was stirred and evaporated at room temperature. When evaporation was completed, the resulting Co-QS was then dried at 40˚C for 12 hours and transferred into a desiccator.

Preparation of physical mixture quercetin-succinic acid

The physical mixture (Pm) of Q and S was prepared by mixing the pure components with the same proportion used in the formation of Co-QS.

Thermal analysis

Thermal analysis of the sample was performed on DTA (Mettler Toledo FP-90, USA). The temperature calibration was conducted using indium prior to the test. Samples (5 to 7 mg) were placed in sealed aluminum pans and scanned at heating rate of 10˚C/min in the temperature range of 50-350˚C.

Powder X-ray diffraction analysis

Diffractogram patterns were collected on PXRD (Philips X`Pert, Holland) with electricity conditions of 40 kV, 40 mA. Samples were packed into a sample holder and pressed on clean glass slides to ensure uniformity of powder thickness. Measurements were performed over 5˚ to 40˚ of 2θ range. The diffractogram patterns of the Pm of Q, S, and Co-QS were compared.

Fourier transform infrared spectroscopy analysis

FTIR spectra were obtained using an FT-IR Jasco 5300 (USA). Ten milligram samples were mixed homogeneously with potassium bromide powder. The mixture was pressed to produce a pellet. Spectra were collected over the range of 4000 to 400 cm^{-1}.

Scanning electron microscopy analysis

Approximately 10 mg samples were dispersed on glass slides and coated with gold aluminum (10 nm). Co-crystals were observed under SEM (JEOL, Japan) with electricity conditions of 20 kV and 12 mA. Photomicrographs were taken at magnification of 600x for S, and 2500x for Q and Co-QS.

Dissolution profile studies

The dissolution studies were conducted using USP II (paddle) apparatus containing 900 mL of citrate buffer (pH 5.0±0.05) with SLS 2% at temperature 37±0.5˚C with paddle speed 100 rpm for 60 min. The samples were withdrawn at 5, 10, 15, 30, 45, and 60 min. The samples were filtered immediately through a 0.45 µm membrane filter and the Q concentrations were measured using UV-Vis spectrophotometry at the maximum wavelength of Q of 336.95 nm. The data were calculated as percentage (%) dissolved and dissolution efficiency at 60 min (DE_{60}). Statistical analysis was conducted using one-way ANOVA with α= 0.05 (95% CI).

RESULT AND DISCUSSION

Diffractogram analysis

The PXRD pattern of Co-QS was compared with the patterns of pure components, Q and S, and Pm-QS (Figure 1). The results showed that the diffractogram of Q gave strong interference at 2θ= 10.78˚, 12.43˚, 14.15˚, 15.84˚, 24.43˚, 10.78˚, 24.43˚, 26.44˚, 27.41˚, and 28.31˚. The diffractogram result of S showed a specific interference at 2θ= 16.02˚, 19.99˚, 26.10˚, 31.41˚, 32.40˚, and 38.52˚. These diffractogram results are comparable with those of previous studies.[4,8]

The PXRD patterns of Pm-QS in a 1:1 molar ratio showed all intense unique peaks of the components. The diffractogram of the Pm reportedly appeared as a superposition between peaks of active ingredient and those of the co-crystal former.[4,8] However, the diffractogram of Co-QS showed several unique peaks at 2θ= 10.01˚, 13.23˚, 21.98˚, and 44.52˚. The different PXRD pattern of Co-QS from those of the constituent Q and S suggests the formation of a co-crystal phase.

DTA thermogram analysis

The thermogram for Q, S, Pm-QS, and Co-QS were determined using DTA. DTA is known as the fastest measurement method to detect co-crystal formation.[8] The result showed that the thermogram of Q had an endothermic peak at 325.4˚C (ΔH= 111 J/g), representing the melting point of Q. The thermogram of S showed an endothermic peak at 189.8˚C (ΔH= 22.8 J/g), indicating the melting point of S (Figure 2). Previous studies showed that Q demonstrated specific endothermic peaks at 326˚C and 147˚C. The endothermic peak at 147˚C is produced due to the release of entrapped water molecules in the crystal. This may shift the melting temperature of Q to the lower level compared with those of anhydrous Q.[15,16]

Nevertheless, the thermogram of Pm-QS and Co-QS showed a new endothermic peak at 251.3˚C (ΔH= 1.06 J/g), which might represent the melting point of Co-QS (Figure 3). The result showed that the melting point of Co-QS lay between the melting points of its components. This result is in agreement with a previous study that showed the melting point of a co-crystal product, which presented between the melting points of its raw materials.[4] Moreover, the melting point of Co-QS shifted to the lower level, suggesting the fusion of S and Q in co-crystal form. Taken together, the shifting of the melting temperature of Co-QS suggests the possible interaction between Q and S in the formation of a co-crystal.

FTIR spectrum analysis

An infrared spectrophotometer was used to evaluate the interaction between Q and S molecules in the formation of the co-crystal. The IR spectrum of Q demonstrated specific peaks corresponding to - OH at 3411 cm^{-1}, C=O at 1667 cm^{-1} and 1612 cm^{-1}, and C-O-C at 1522 cm^{-1}, 1319 cm^{-1} and 1168 cm^{-1}. This result is in agreement with previous studies.[4] The IR spectrum of S showed the specific absorbance for -OH at 2932 cm^{-1} and 2647 cm^{-1}, and for C=O at 1202 cm^{-1} , 1693 cm^{-1}, and 1309 cm^{-1}, confirming previous reports.[5,8] Accordingly, FTIR analysis of Pm-QS showed a combination of specific absorbance from Q and S. This result suggests that the Pm did not facilitate any interaction between Q and S molecules in the mixture. This finding is in agreement with previous reports showing that there is no interaction between the active compound with its coformer in the Pm as demonstrated by the superimposition spectrum in the IR spectrum.[13,17]

The IR spectrum of Co-QS demonstrated several unique new IR peaks at 3424 cm^{-1} representing functional group -OH and at 1830 cm^{-1}, representing the C=O functional group. The absorbance representing the OH functional group was notably shifted and the peak intensities were markedly decreased compared with those of Q and S. These results suggest that there are a number of functional groups involved in chemical bonding between Q and S in co-crystal form. Furthermore, the absorbance peak representing the -OH functional group of Q and the absorbance peak representing the carboxylic functional

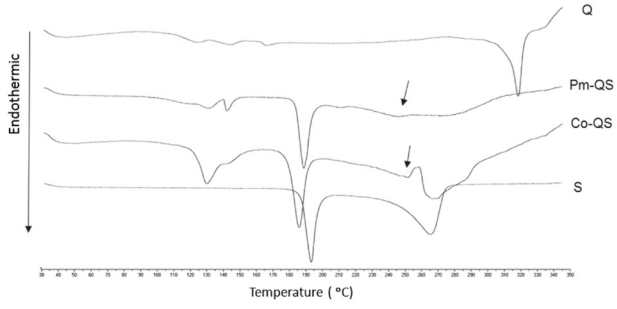


Figure 2. Thermogram of quercetin, succinic acid, physical mixture and Co-crystal QS

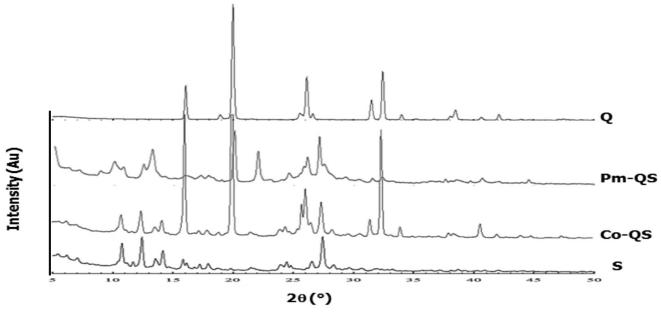


Figure 1. Diffractogram of quercetin, succinic acid, physical mixture, and Co-crystal

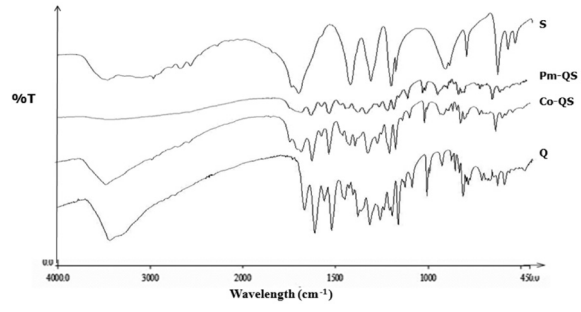


Figure 3. IR Spectrum of quercetin, succinic acid, physical mixture and Co-crystal

group of S were shifted, which suggests possible bonding between these functional groups in co-crystal form.

Photomicrograph SEM analysis

Photomicrographs of Q, S, and Co-QS are shown in Figure 4. The photomicrographs were captured at 600 times magnification for AS and 2500 times for Q and Co-QS. The results showed that the crystal size of S was approximately 300 µm diameter. This is in agreement with research that stated that the particle size of S lay between 50 to several hundred µm.[18] Moreover, the photograph of S showed a different crystal habit compared with those of Q. On the other hand, the photomicrograph of Co-QS demonstrated smaller and more homogeneous particles as compared with those of its raw materials.

Dissolution study (In vitro)

Dissolution profiles of Q, Pm-QS, and Co-QS are shown in Figure 5. The results show that Pm-QS increased the solubility of Q. Moreover, Co-QS achieved the highest solubility as compared with those of Pm-QS and Q. The DE_{60} value of Co-QS, Pm-QS, and Q were 87.25±0.07%, 73.90±3.27%, and 64.43±0.94%, respectively. One-way ANOVA ($p<0.05$) showed that Co-QS significantly increased the dissolution rate of Q as compared with those of Q and Pm-QS. The enhancement of Q solubility by the Pm was likely due to the wetting effect of coformer S.[4] The higher dissolution rate of Co-QS compared with those of pure Q might be due to the formation of a new crystal lattice and the decrease in enthalpy energy as indicated previously by PXRD and DTA studies. In addition, it is suggested that the interaction between hydroxyl functional group from Q and the carboxylic functional group from S, conformer, facilitates the improvement of solubility profile of Q in aqueous medium.

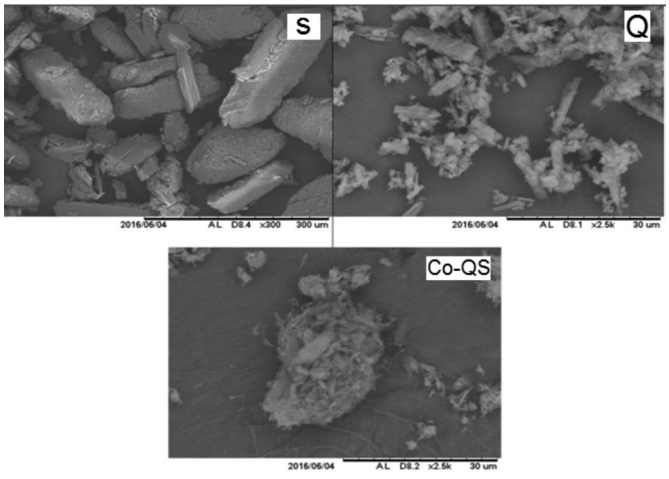


Figure 4. Photomicrograph of quercetin, succinic acid and Co-crystal

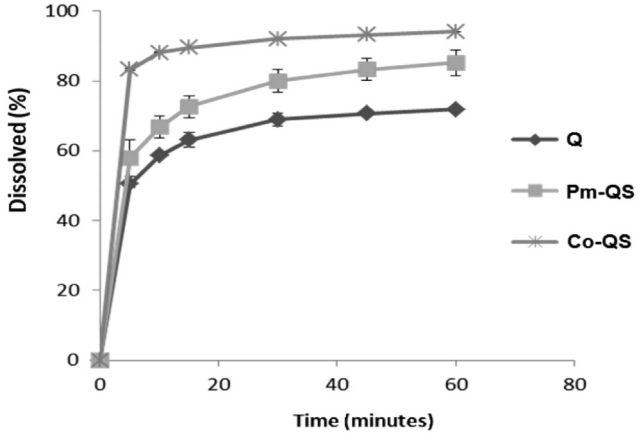


Figure 5. Dissolution rate of quercetin, physical mixture of quercetin-succinic acid, and quercetin-succinic acid co-crystal in buffer citrate medium (pH 5.0±0.05) with SLS 2% at temperature 37±0.5°C

CONCLUSION

The formation of Co-QS was well-characterized by the shifting in melting temperature, the formation of a new crystal lattice as shown by the PXRD pattern, and the shifting in absorbance peaks, which represented the functional groups of Q and S. The dissolution study confirmed that the formation of Co-QS using solvent evaporation significantly improved the solubility profile of the compound. The possible potential of this research includes the development of pharmaceutical dose forms, especially solid dose forms.

ACKNOWLEDGEMENT

The authors thank the Higher Degree Education Directorate for Funding the Research Grant, Faculty of Pharmacy Airlangga University for facilities support and our students Indah P, Rizka F for their support.

Conflict of Interest: No conflict of interest was declared by the authors.

REFERENCES

1. Thakuria R, Delori A, Jones W, Lipert MP, Roy L, Rodríguez-Hornedo N. Pharmaceutical cocrystals and poorly soluble drugs. Int J Pharm. 2013;453:101-125.
2. Materska, M. Quercetin and its derivatives: chemical structure and bioactivity a review. Polish Journal of Food and Nutrition Sciences, 2008;58.
3. Li B, Konecke S, Harich K, Wegiel L, Taylor LS, Edgar KJ. Solid dispersion of quercetin in cellulose derivative matrices influences both solubility and stability. Carbohydr Polym. 2013;92:2033-2040.
4. Kakran M, Sahoo NG, Li L. Dissolution enhancement of quercetin through nanofabrication, complexation, and solid dispersion. Colloids Surf B Biointerfaces. 2011;88:121-130.
5. Qiao, Ning. 2014. PhD Thesis: Investigation of Carbamazepin-Nicotinamid Co-crystal Solubility and Dissolution By a UV Imaging System. United Kingdom: Faculty of Health and Life Sciences, DeMonfort University, Leicester.
6. Sinha AS, Maguire AR, Lawrence SE. Cocrystallization of nutraceuticals. Crystal Growth & Design. 2015;15:984-1009.
7. Vinesh V, Sevukarajan M, Rajalakshmi R, Chowdary GT, Haritha K. Enhancement of solubility of tadalafil by cocrystal approach. Int Res J Pharm. 2013;4:218-223.

8. Fulias A, Vlase G, Vlase T, Şuta LM, Şoica C, Ledeţi I. Screening and characterization of cocrystal formation between carbamazepine and succinic acid. Journal of Thermal Analysis and Calorimetry. 2015;121:1081-1086.
9. Khristi AP, Soni T, Suhagia BN. Development, Characterisation and Evaluation of Sildenafil Aspirin Co-Crystals. Indo American Journal of Pharmaceutical Research. 2015:5:2700-2708.
10. Smith AJ, Kavuru P, Wojtas L, Zaworotko MJ, Shytle RD Cocrystals of quercetin with improved solubility and oral bioavailability. Mol Pharm. 2011;8:1867-1876.
11. Kothur RR, Swetha AS, Bondili NP. An Outline of Crystal Engineering of Pharmaceutical Co-crystal and Applications: A Review. IJPRD. 2012;4:84-92.
12. Fucke K, Myz SA, Shakhtshneider TP, Boldyreva EV, Griesser UJ. How good are the crystallisation methods for co-crystals? A comparative study of piroxicam. New Journal of Chemistry. 2012;36:1969-1977.
13. Setyawan D, Sari R, Yusuf H, Primaharinastiti R. Preparation And Characterization Of Artesunate-Nicotinamide Cocrystal By Solvent Evaporation And Slurry Method. Asian Journal of Pharmaceutical and Clinical Research. 2014;7(Suppl 1):62-65.
14. Sekhon BS. Nutraceutical cocrystals: an overview. RGUHS J. Pharm Sci. 2012;2:16-25.
15. Scalia S, Haghi M, Losi V, Trotta V, Young PM, Traini D. Quercetin solid lipid microparticles: a flavonoid for inhalation lung delivery. Eur J Pharm Sci. 2013;49:278-285.
16. Nugrahani, I, Bahari MU. The dynamic study of co-crystal formation between anhydrous and monohydrate theophylline with sodium saccharine dihydrate by FTIR. J Chem Biochem. 2014;2:117-137.
17. Lin HL, Wu TK, Lin SY. Screening and characterization of cocrystal formation of metaxalone with short-chain dicarboxylic acids induced by solvent-assisted grinding approach. Thermochimica Acta. 2014;575:313-321.
18. Ober CA, Gupta RB. Formation of itraconazole-succinic acid cocrystals by gas antisolvent cocrystallization. AAPS PharmSciTech. 2012;13:1396-1406.

Turk J Pharm Sci 2017;14(3):285-289
DOI: 10.4274/tjps.24008

ORIGINAL ARTICLE

Synthesis and Pharmacologic Evaluation of Some Benzimidazole Acetohydrazide Derivatives as EGFR Inhibitors

EGFR İnhibitörü Olarak Bazı Asetohidrazit Türevi Benzimidazollerin Sentezi ve Farmakolojik Değerlendirilmesi

Serkan DEMİREL[1], Gülgün AYHAN KILCIGİL[1]*, Zümra KARA[2], Berna GÜVEN[2], Arzu ONAY BEŞİKCİ[2]

[1]Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, Ankara, Turkey
[2]Ankara University, Faculty of Pharmacy, Department of Pharmacology, Ankara, Turkey

ABSTRACT

Objectives: In this study, some novel 2-(2-phenyl)-1*H*-benzo[d]imidazol-1-yl)-N'-(arylmethylene) acetohydrazide derivatives (1-12) were designed and synthesized.

Materials and Methods: Compounds 1-12 were obtained by condensing 2-(2-phenyl)-1*H*-benzo[d]imidazol-1-yl)acetohydrazide (III) with the corresponding aromatic aldehyde derivatives in the presence of catalytic amounts of hydrochloric acid in ethanol.

Results: Following the structure elucidation, epidermal growth factor receptor kinase inhibitor activity was measured. The ADP-Glo™ kinase assay determines kinase activity based on the quantification of the amount of ADP produced during a kinase reaction.

Conclusion: Almost all of the compounds' kinase inhibitor activities were rather limited.

Key words: Benzimidazole, acetohydrazide, epidermal growth factor receptor kinase, inhibitory

ÖZ

Amaç: Bu çalışmada bazı yeni 2-(2-fenil)-1*H*-benzo[d]imidazol-1-il)-N'-(arilmetilen) asetohidrazit türevleri tasarlanmış ve sentezlenmiştir.

Gereç ve Yöntemler: 1-12 numaralı bileşikler 2-(2-fenil)-1*H*-benzo[d]imidazol-1-il) asetohidrazitin (III) uygun aromatik aldehitlerle etanol içinde katalitik miktarda hidroklorik asit eşliğinde kondensasyonuyla elde edilmiştir.

Bulgular: Yapı aydınlatılmasını takiben, epidermal büyüme faktör reseptörü kinaz inhibitörü aktivitesi, kinaz reaksiyonu sırasında üretilen ADP miktarının nicelenmesine dayanan kinaz aktivitesini belirleyen ADP-Glo™ kinaz yöntemi ile tespit edilmiştir.

Sonuç: Bileşiklerin kinaz inhibitörü aktiviteleri oldukça sınırlıdır.

Anahtar kelimeler: Benzimidazol, asetohidrazit, epidermal büyüme faktörü reseptörü kinaz, inhibitör

INTRODUCTION

Cancer is a lethal disease characterized by an uncontrolled proliferation of cells, invasive nature, and metastasis. In developed countries, following cardiovascular diseases, it is the second most common cause of death. According to research conducted worldwide in 2015, 8.8 million people died of cancer, which is nearly 1 in 6 of all deaths.[1] With standard therapies, cancer is often not completely removed and new treatments are sought.

Tyrosine kinase belongs to the protein kinase family and it provides for protein phosphorylation.[2] Phosphorylation of proteins through kinases plays an important role in signal transduction mechanisms. Changes that occur in signal transduction play an important role in cancer development and metastases because it controls functions such as cell proliferation and apoptosis.

Epidermal growth factor receptor (EGFR), the first cell surface receptor associated with cancer, is a tyrosine kinase receptor. EGFR is involved in cancer development and metastasis.[3,4]

*Correspondence: E-mail: kilcigil@pharmacy.ankara.edu.tr, Phone: +90 532 551 39 20 ORCID-ID: orcid.org/0000-0001-5626-6922
Received: 10.05.2017, Accepted: 01.06.2017

Studies have shown that inhibition of EGFR can arrest or slow the development of cancer. EGFRs have been observed to be overproduced in lung, breast, prostate, bladder, ovarian, colon, and rectum cancers, and they are found to be structural in most normal epithelial cells (e.g., skin, hair follicles). Thus, inhibition of EGFR activity inhibits cell proliferation and leads to apoptosis, programmed cell death.[5] EGFR, such as other tyrosine kinase receptors, may also be inhibited by both monoclonal antibodies and tyrosine kinase inhibitors. Other inhibitors of tyrosine kinase such as gefitinib, erlotinib, lapatinib are small molecules that target EGFR and are used in cancer therapy.[4] It is known that compounds used as inhibitors of tyrosine kinase such as sunitinib carry benzimidazole isostere indole rings, and pazopanib and axitinib have indazole rings. Furthermore, by determining that benzimidazoles exhibit protein kinase inhibitor activity[6-9], in addition to their different biologic activities, the compounds planned to be synthesized in this study are thought to be inhibitory on the EGFR receptor, which is now known to be directly related to cancer development.

By developing novel EGFR tyrosine kinases inhibitors, we designed and synthesized new benzimidazole derivatives bearing a phenyl ring at the 2nd position and N-arylmethylideneacetohydrazide (1-12) (Table 1) at the 1st position, and evaluated their EGFR kinase inhibitory activities by comparing them with erlotinib. Following the structure elucidation, EGFR kinase inhibitor activities of the synthesized compounds were measured (Table 1).

EXPERIMENTAL

General synthetic

All starting materials and chemical reagents used in the synthesis were high-grade commercial products purchased from Aldrich or Merck (Germany). Analytical thin-layer chromatography was performed using Merck precoated TLC plates, and spots were visualized with ultraviolet light. Melting points were determined using a Thermo Scientific Electrothermal IA9100 digital melting point apparatus (Bibby Scientific Limited, Staffordshire, UK) and were uncorrected. The structures of all synthesized compounds were assigned on the basis of nuclear magnetic resonance (NMR) and mass spectral analyses. ^{1}H-NMR spectra were recorded on a Varian Mercury 400 MHz instrument (Varian Inc., Palo Alto, CA, USA) using a tetramethylsilane internal standard and *DMSO-d_6*; coupling constants (*J*) are reported in Hertz. All chemical shifts are reported as δ (ppm) values. ES-MS were obtained using a Waters ZQ Micromass LC-MS spectrometer (Waters Corporation, Milford, MA, USA) with the positive electrospray ionization method. All instrumental analyses were performed at the Central Instrumentation Laboratory of the Pharmacy Faculty of Ankara University, Ankara, Turkey.

Table 1. General structure and inhibitory effects of the synthesized compounds

Compounds	Ar	Inhibition %
1	4-chlorophenyl	8.63
2	4-fluorophenyl	1.93
3	4-bromophenyl	8.63
4	3-nitrophenyl	13.71
5	2-naphtyl	0
6	3-methylthiophene	0
7	4-benzyloxyphenyl	12.42
8	2-chloro-5-nitrophenyl	0
9	3,4-dibenzyloxyphenyl	9.92
10	3-bromo-4-fluorophenyl	0
11	2,4-dichlorophenyl	7.69
12	4-chloro-3-nitrophenyl	3.01
Erlotinib 1 μM 100 nM		 90.06 74.56

General procedure for the preparation of 2-(2-phenyl)-1H-benzimidazol-1-yl)-N'-(arylmethylidene) acetohydrazide derivatives (1-12)

Aromatic aldehyde derivative (0.02 mol) was added to a solution of the acyl hydrazide III (0.02 mol) in absolute EtOH (5 mL) containing a catalytic amount of 37% hydrochloric acid. The mixture was refluxed for 60 min, poured into cold water, and neutralized with 10% aqueous sodium bicarbonate solution. Crude product was filtered off and crystallized from dimethylformamide (DMF) water.

N'-[(4-Chlorophenyl)methylidene]-2-(2-phenyl-1H-benzimidazol-1-yl)acetohydrazide (1)

M.P: 267°C; ^{1}H NMR δ 5.06 and 5.52 (2s, 2H, CH_2), 7.25-7.28 (m, 2H, Ar-H), 7.48-7.57 (m, 6H, Ar-H), 7.70-7.75 (m, 5H, Ar-H), 8.03 and 8.24 (2s, 1H, CH=N), 11.88 (brs, 1H, NH); ESI-MS (m/z): 389.4 (100%) (M+H), 391.5 (40%) (M+2+H).

N'-[(4-Fluorophenyl)methylidene]-2-(2-phenyl-1H-benzimidazol-1-yl)acetohydrazide (2)

M.P: 214-217°C; ^{1}H NMR δ 5.05 and 5.51 (2s, 2H, CH_2), 7.24-7.29 (m, 4H, Ar-H), 7.52-7.57 (m, 4H, Ar-H), 7.70-7.80 (m, 5H, Ar-H), 8.04 and 8.24 (2s, 1H, -CH=N), 11.79, 11.93 (2s, 1H, NH); ESI-MS (m/z): 373.36 (M+H).

N'-[(4-Bromophenyl)methylidene]-2-(2-phenyl-1H-benzimidazol-1-yl)acetohydrazide (3)

M.P: 287°C; ^{1}H NMR δ 5.06 and 5.52 (2s, 2H, CH_2), 7.25-7.30 (m, 2H, Ar-H), 7.52-7.80 (m, 11H, Ar-H), 8.02 and 8.22 (2s, 1H, CH=N), 11.86 (brs, 1H, NH); ESI-MS (m/z): 433.38 (100%) (M+H), 435.34 (95%) (M+2+H).

N'-[(3-Nitrophenyl)methylidene]-2-(2-phenyl-1H-benzimidazol-1-yl)acetohydrazide (4)

M.P: 280°C; ^{1}H NMR δ 5.09 and 5.57 (2s, 2H, CH_2), 7.25-7.29 (m, 2H, Ar-H), 7.51-7.59 (m, 4H, Ar-H), 7.69-7.80 (m, 4H, Ar-H), 8.17, 8.52 (m, 3H, Ar-H), 8.53 and 8.55 (2s, 1H, -CH=N), 12.04, 12.20 (2s, 1H, NH); ESI-MS (m/z): 400.5 (M+H).

N'-[(2-Napthalene-2-yl)methylidene]-2-(2-phenyl-1H-benzimidazol-1-yl)acetohydrazide (5)

M.P: 285°C; ^{1}H NMR δ 5.00 and 5.56 (2s, 2H, CH_2), 7.24-7.28 (m, 2H, Ar-H), 7.49-7.58 (m, 6H, Ar-H), 7.69-7.92 (m, 3H, Ar-H), 7.94-7.98 (m, 4H, Ar-H), 8.11-8.38 (m, 2H, Ar-H and CH=N), 11.86, 11.99 (2s, 1H, NH); ESI-MS (m/z): 405.44 (M+H).

N'-[(3-Methylthiophene-2-yl)methylidene]-2-(2-phenyl-1H-benzimidazol-1-yl)acetohydrazide (6)

M.P: 267-269°C; ^{1}H NMR δ 2.31 (2s, 3H, CH_3), 5.01 and 5.38 (2s, 2H, CH_2), 6.96 (dd, 1H, J=4.8 Hz, J=1.6 Hz, Ar-H), 7.25-7.29 (m, 2H, Ar-H), 7.52-7.58 (m, 5H, Ar-H), 7.69-7.72 (m, 2H, Ar-H), 7.78-7.81 (m, 1H, Ar-H), 8.27 and 8.48 (2s, 1H, CH=N), 11.63, 11.82 (2s, 1H, NH); ESI-MS (m/z): 375.48 (M+H).

N'-[(4-Benzyloxyphenyl)methylidene]-2-(2-phenyl-1H-benzimidazol-1-yl)acetohydrazide (7)

M.P: 240-241°C; ^{1}H NMR δ 5.03 and 5.49 (2s, 2H, CH_2), 5.15 (s, 2H, OCH_2), 7.05-7.10 (m, 2H, Ar-H), 7.25-7.57 (m, 11H, Ar-H), 7.64-7.81 (m, 5H, Ar-H), 7.98 and 8.18 (2s, 1H, CH=N), 11.67, 11.81 (2s, 1H, NH); ESI-MS (m/z): 461.51 (M+H).

N'-[(2-Chloro-5-nitrophenyl)methylidene]-2-(2-phenyl-1H-benzimidazol-1-yl)acetohydrazide (8)

M.P: 299-300°C; ^{1}H NMR δ 5.08 and 5.57 (2s, 2H, CH_2), 7.22-7.30 (m, 2H, Ar-H), 7.47-7.59 (m, 4H, Ar-H), 7.67-7.92 (m, 4H, Ar-H), 8.19-8.23 (m, 1H, Ar-H), 8.42 (s, 1H, Ar-H), 8.61 and 8.67 (d, d, J=2.8 Hz, CH=N), 12.18 (br s, 1H, NH); ESI-MS (m/z): 434.37 (100%) (M+H), 436.39 (39%) (M+2+H).

N'-[(3,4-Dibenzyloxyphenyl)methylidene]-2-(2-phenyl-1H-benzimidazol-1-yl)acetohydrazide (9)

M.P: 178-180°C; ^{1}H NMR δ 5.03 and 5.19 (2s, 2H, CH_2), 5.14 (s, 2H, OCH_2), 5.17 (s, 2H, OCH_2), 7.07-7.45 (m, 15H, Ar-H), 7.53-7.58 (m, 4H, Ar-H), 7.71-8.18 (m, 4H, Ar-H and CH=N), 11.71 (br s, 1H, NH), ESI-MS (m/z): 567.48 (M+H).

N'-[(3-Bromo-4-fluorophenyl)methylidene]-2-(2-phenyl-1H-benzimidazol-1-yl)acetohydrazide (10)

M.P: 258-260°C; ^{1}H NMR δ 5.06 and 5.54 (2s, 2H, CH_2), 7.25-7.28 (m, 2H, Ar-H), 7.41-7.57 (m, 5H, Ar-H), 7.70-7.79 (m, 4H, Ar-H), 8.01-8.21 (m, 2H, Ar-H and CH=N), 11.90 (br s, 1H, NH); ESI-MS (m/z): 451.29 (100%) (M+H), 453.26 (98%) (M+2+H).

N'-[(2,4-Dichlorophenyl)methylidene]-2-(2-phenyl-1H-benzimidazol-1-yl)acetohydrazide (11)

M.P: 147-148°C; ^{1}H NMR δ 5.10 and 5.58 (2s, 2H, CH_2), 7.31-7.34 (m, 2H, Ar-H), 7.47-7.50 (m, 1H, Ar-H), 7.56-7.66 (m, 4H, Ar-H), 7.73-7.81 (m, 4H Ar-H), 7.93-8.03 (m, 1H, Ar-H), 8.38 and 8.58 (2s, 1H, CH=N), 12.04, 12.29 (2s, 1H, NH); ESI-MS (m/z): 423.3 (100%) (M+H), 425.30 (65%) (M+2+H).

N'-[(4-Chloro-3-nitrophenyl)methylidene]-2-(2-phenyl-1H-benzimidazol-1-yl)acetohydrazide (12)

M.P: 269-270°C; ^{1}H NMR δ 5.09 and 5.57 (2s, 2H, CH_2), 7.25-7.29 (m, 2H, Ar-H), 7.52-7.57 (m, 4H, Ar-H), 7.70-7.85 (m, 4H, Ar-H), 8.03 (dd, Jo=8 Hz, Jm=2 Hz, Ar-H), 8.09 (s, 1H, Ar-H), 8.30, 8.38 and 8.43 (s, d, d, J=1.6 Hz, -CH=N), 12.05, 12.18 (2 br s, 1H, NH); ESI-MS (m/z): 434.4 (100%) (M+H), 436.5 (33.5%) (M+2+H).

EGFR kinase assay

It is important to evaluate the possible interaction of solubilized EGFR kinase activity with the amount of enzyme and with the reaction of the solvents (5% DMSO) of the substances for which inhibitor activity is to be assessed. For this purpose, kinase titration was first performed with EGFR kinase at a range of 0.2-200 ng. As the amount of enzyme was increased, kinase reaction was increased (more ATP was formed, more ADP was formed; results not shown).

After these preliminary experiments and system validation, the inhibitory activities of the synthesized substances on EGFR kinase were evaluated. Erlotinib, a substance known to inhibit EGFR kinase in these experiments was also used as a positive control in all experiments.

Screening of inhibitor activity

Kinase reactions were performed using 50 μM ATP, 5 ng EGFR kinase ± 1 μM inhibitor in each reaction medium. Erlotinib was tested in two different concentrations: 100 nM and 1 μM to confirm dose-dependency of the inhibitory activity. Five percent DMSO (solvent) was used as a control in all experiments. These reactions constituted the maximum enzyme activity (100% activity).

Standard curve for ATP-ADP transformation

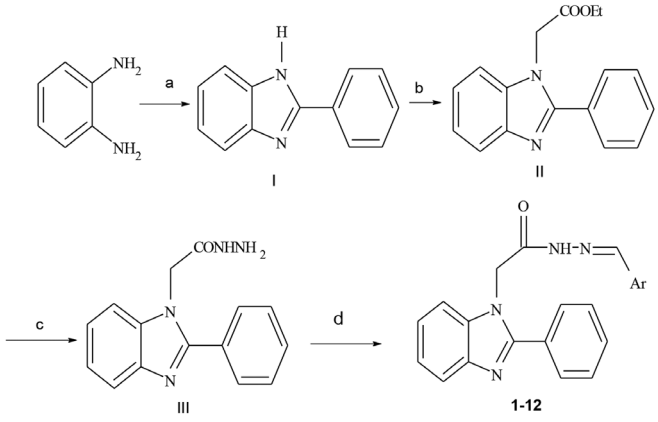
In the first step, the standard curves for the ATP-ADP transformation to be used in determining the amount of ADP formed in the kinase reaction were established. While these curves were being generated, the ATP and ADP stock solutions were mixed at specified ratios and the resulting luminescence was read. The purpose of these curves was to determine the luminescence signal interval obtained by the amount of the designated EGFR kinase. ATP/ADP conversion curves were generated using 1 μM, 5 μM, 50 Mm, and 500 μM ATP-ADP concentrations. These experiments are important for determining luciferase activity and signal range obtained in the kinase assay.

RESULTS AND DISCUSSION

Chemistry

The benzimidazole acetohydrazide derivatives were synthesized according to the following synthetic scheme: 2-Phenyl-1*H*-benzo[d]imidazole (**I**) was prepared via the reaction of *o*-phenylenediamine, benzaldehyde and sodium metabisulfite in DMF.[10] The reaction of **I** with ethyl chloroacetate yielded ethyl 2-(2-phenyl)-1*H*-benzo[d]imidazol-1-yl) acetate (**II**).[11] Treatment of the ester (**II**) with hydrazine hydrate gave the desired hydrazide compound, 2-(2-phenyl)-1*H*-benzo[d]imidazol-1-yl) acetohydrazide (**III**).[12] Compounds **1-12** were found by condensing acyl hydrazide **III** with the corresponding aromatic aldehydes in the presence of sulfuric acid[13] (Scheme 1). The structure of the newly synthesized compounds was confirmed and supported by spectroscopic data. The mass spectrum of each of compound

appeared as M+H peak, consistent with the molecular formula of the assigned structure. *N*-acylhydrazones may be present as four isomers depending on the geometric isomerism relative to the imino group (*E, Z* isomers) and conformers related to the amide linkage (*syn/anti* amide conformers).[14] In ^{1}H-NMR spectra measured in polar solvent dimethyl sulfoxide-d_6, two signals representing the imino hydrogen (CH=N) were observed at 7.98-8.61 and 8.18-8.67 ppm, the methylene protons (CH_2CO) were also typically observed as two singlet signals at 5.01-5.10 and 5.19-5.58 ppm, respectively.



Scheme 1. Synthetic route to compounds **1-12**

(a) $Na_2S_2O_3$ adduct of benzaldehyde/DMF; (b) Ethyl chloroacetate/KOH; (c) Hydrazine/ EtOH; (d) Corresponding aromatic aldehyde/EtOH/HCl_{cat}

Biological activities

The ADP-Glo™ kinase assay[15] determines kinase activity based on the quantification of the amount of ADP produced during a kinase reaction. EGFR is a tyrosine kinase receptor and was the first receptor to be proposed for cancer therapy. Currently, there are two approaches that target EGFRs in cancer treatment: monoclonal antibodies and small-molecule inhibitors of EGFR tyrosine kinase enzymatic activity. Erlotinib selectively inhibits tyrosine kinase activity of EGFRs.

In the present study, we measured the activity of EGFR kinase and evaluated the inhibitory efficiencies of newly synthesized compounds by comparing them with erlotinib.

The assays were performed in three steps (Figure 1). A kinase reaction with 5 ng EGFR kinase and 50 μM ATP was the first step. The amounts of both EGFR and ATP were determined in the preliminary optimization assays (results not shown). The total volume of the kinase reaction was 25 μL. The amount of the EGFR and $PolyE_4Y_1$ substrate was 1 μg, based on the recommendations of the literature. In the second step, an equal volume of ADP-Glo™ reagent was added to terminate the kinase reaction and deplete the remaining ATP. On the third and final step, a kinase detection reagent was added to convert the ADP produced during the kinase reaction to ATP and to determine the amount of newly synthesized ATP through a luciferase/ luciferin reaction.

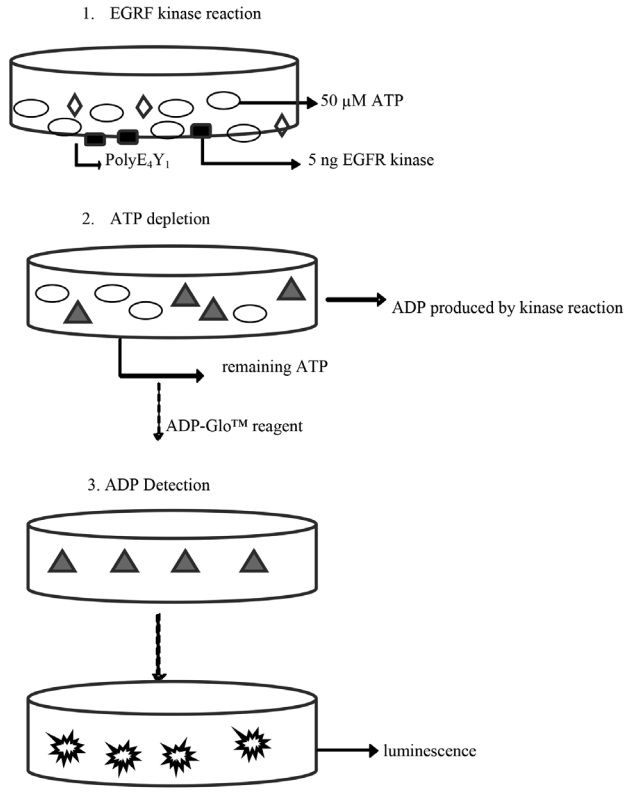


Figure 1. Schematic representation of the protocol

Inhibitory efficiency was determined by comparing the enzyme activities in the presence of the inhibitors with the maximum enzyme activity. Maximum kinase activity (100% kinase) was determined as the luminescence acquired in the kinase reaction where EGFR kinase, $PolyE_4Y_1$, ATP and diluting agent, and 5% DMSO were present in the reaction medium.

CONCLUSION

In the reactions where inhibitory efficiencies were determined, the reaction medium contained either erlotinib or one of the newly synthesized compounds in a single concentration of 1 μM. At this concentration, erlotinib inhibited EGFR kinase activity by nearly 90%. Inhibitory efficiencies of the new compounds are shown in Table 1. The results indicated that almost all of the compounds' kinase inhibitor activities were somewhat limited. Compounds that have 3-nitrophenyl (**4**) and 4-benzyloxyphenyl (**7**) substituents as aryl groups were found to have slight inhibitory potencies in the kinase assay at 13.71% and 12.42%, respectively.

Conflict of Interest: No conflict of interest was declared by the authors.

REFERENCES

1. http://www.who.int/cancer
2. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. Science. 2002;298:1912-1934.

3. Normanno N, De Luca A, Bianco C, Strizzi L, Mancino M, Maiello MR, Carotenuto A, De Feo G, Caponigro F, Salomon DS. Epidermal growth factor receptor (EGFR) signaling in cancer. Gene. 2006;366:2-16.

4. Bianco R, Gelardi T, Damiano V, Ciardiello F, Tortora G. Rational bases for the development of EGFR inhibitors for cancer treatment. Int J Biochem Cell Biol. 2007;39:1416-1431.

5. Lin Y, Wang X, Jin H. EGFR-TKI resistance in NSCLC patients: mechanisms and strategies. Am J Cancer Res. 2014;4:411-435.

6. Hasegawa M, Nishigaki N, Washio Y, Kano K, Harris PA, Sato H, Mori I, West RI, Shibahara M, Toyoda H, Wang L, Nolte RT, Veal JM, Cheung M. Discovery of novel benzimidazoles as potent inhibitors of TIE-2 and VEGFR-2 tyrosine kinase receptors. J Med Chem. 2007;50:4453-4470.

7. Li Y, Tan C, Gao C, Zhang C, Luan X, Chen X, Liu H, Chen Y, Jiang Y. Discovery of benzimidazole derivatives as novel multi-target EGFR, VEGFR-2 and PDGFR kinase inhibitors. Bio Med Chem. 2011;19:4529-4535.

8. Yadav S, Sinha D, Singh SK, Singh VK. Novel benzimidazole analogs as inhibitors of EGFR tyrosine kinase. Chem Biol Drug Des. 2012;80;625-630.

9. Abdel-Ghaffar NF. Synthesis, reactions, structure-activity relationship of 2-benzimidazole analogs as anticancer agents and study their molecular docking. Der Pharma Chem. 2013;:243-257.

10. Ridley HF, Spickett RGW, Timmis GM. A new synthesis of benzimidazoles and aza analogs. J Heterocyclic Chem. 1965;2:453-456.

11. Heaney H, Ley SV. N-Alkylation of indole and pyroles in dimethyl sulphoxide. J Chem Soc Perkin I. 1973;499-500.

12. Smith PAS. In: Adams R, Bachmann WE, Fieser LF, Johnson JR, Snyder HR. (Eds). Organic Reactions. John Wiley & Sons, Inc. London; Chapman & Hall, Limited; 1949;3:337-389.

13. Carvalho SA, Feitosa LO, Soares M, Costa TE, Henriques MG, Salomão K, de Castro SL, Kaiser M, Brun R, Wardell JL, Wardell SM, Trossini GH, Andricopulo AD, da Silva EF, Fraga CA. Design and synthesis of new (E)-cinnamic N-acylhydrazones as potent antitrypanosomal agents. Eur J Med Chem. 2012;54:512-521.

14. Patorski P, Wyrzykiewicz E, Bartkowiak G. Synthesis and Conformational Assignment of N-(E)-Stilbenyloxymethylenecarbonyl-Substituted Hydrazones of Acetone and o-(m- and p-) Chloro- (nitro-) benzaldehydes by Means of 1H and 13C NMR Spectroscopy. J Spectrosc. 2013;2013:1-12.

15. ADP-Glo™ Kinase Assay. Technical Manual. Promega.

Turk J Pharm Sci 2017;14(3):290-293
DOI: 10.4274/tjps.07279

ORIGINAL ARTICLE

Cytotoxic, Phytotoxic and Insecticidal Activities of *Chrysophthalmum montanum* (DC.) Boiss.

Chrysophthalmum montanum (DC.) Boiss.'un Sitotoksik, Fitotoksik ve İnsektisidal Aktiviteleri

Fatma AYAZ[1], Nurgün KÜÇÜKBOYACI[1]*, Hayri DUMAN[2], Bilge ŞENER[1], Muhammad Iqbal CHOUDHARY[3]

[1]Gazi University, Faculty of Pharmacy, Department of Pharmacognosy, Ankara, Turkey
[2]Gazi University, Faculty of Science, Department of Biology, Ankara, Turkey
[3]University of Karachi, International Center for Chemical and Biological Sciences, Karachi, Pakistan

ABSTRACT

Objectives: To investigate the *in vitro* cytotoxic, phytotoxic, and insecticidal activity of *Chrysophthalmum montanum* (DC.) Boiss.

Materials and Methods: The crude methanol (80%) extract of the aerial parts of *C. montanum* was fractionated to obtain *n*-hexane, chloroform, *n*-butanol, and remaining water fractions. The crude extract and subsequent solvent fractions of the plant were evaluated for their biological activities using screening bioassays such as cytotoxicity on brine shrimp lethality, phytotoxicity against *Lemna minor* L., and insecticidal activity against *Rhyzopertha dominica* and *Tribolium castaneum*.

Results: The cytotoxicity assay revealed that the crude extract, *n*-hexane, and chloroform fractions of the plant had positive lethality with LD_{50} values of 71.51, 126.62, and 75.95 µg/mL, respectively. The extract and its fractions, except for the remaining water fraction, showed phytotoxic activity, which was expressed as percentage growth regulation in a concentration-dependent manner. *n*-hexane and chloroform fractions in particular had 100% growth inhibition (GI) at 1000 µg/mL, followed by the *n*-butanol fraction (62.6% GI) and crude extract (40.0% GI) of the plant at the same concentration. Otherwise, all samples had no insecticidal activity against *R. dominica* and *T. castaneum*.

Conclusion: This study demonstrates that *C. montanum* contains bioactive compounds related to potential biological activities such as cytotoxic and phytotoxic.

Key words: *Chrysophthalmum montanum*, Asteraceae, cytotoxic activity, phytotoxic activity, insecticidal activity

ÖZ

Amaç: Bu çalışmada, *Chrysophthalmum montanum* (DC.) Boiss.'un *in vitro* sitotoksik, fitotoksik ve insektisidal aktivitelerinin incelenmesi amaçlanmıştır.

Gereç ve Yöntemler: *C. montanum*'un toprak üstü kısmının %80 metanollü ham ekstresi *n*-hekzan, kloroform, *n*-butanol ve kalan sulu fraksiyonları elde etmek üzere ardarda fraksiyonlanmıştır. Bitkinin ham ekstre ve fraksiyonları tuzlu su karidesi letalite testinde sitotoksisitesi, *Lemna minor* L.'e karşı fitotoksisitesi ve *Rhyzopertha dominica* ile *Tribolium castaneum*'a karşı insektisidal aktivitesi gibi biyolojik tarama çalışmalarında biyolojik aktiviteleri bakımından incelenmiştir.

Bulgular: Sitotoksisite testinde ham ekstre, *n*-hekzan ve kloroform fraksiyonları sırasıyla 71.51, 126.62 ve 75.95 µg/mL LD_{50} değerleri ile belirgin letaliteye sahip bulunmuştur. Kalan su fraksiyonu hariç, ekstre ve fraksiyonların % büyüme inhibisyonu ile ölçülen fitotoksik aktivitesi konsantrasyona bağlı olarak gözlenmiştir. Özellikle *n*-hekzan ve kloroform fraksiyonları, 1000 µg/mL'de %100 büyüme inhibisyonuna sahip bulunmuş ve takiben n-butanol ve ham ekstre aynı dozda sırasıyla %62.6 ve %40.0 büyümeyi inhibe edici etkiye sahip bulunmuştur. Buna karşın, tüm örnekler *R. dominica* ve *T. castaneum*'a karşı insektisidal aktivite göstermemiştir.

Sonuç: Bu çalışma *C. montanum*'un sitotoksik ve fitotoksik biyolojik aktivite potansiyeline sahip biyoaktif bileşikler içerdiğini göstermiştir.

Anahtar Kelimeler: *Chrysophthalmum montanum*, Asteraceae, sitotoksik aktivite, fitotoksik aktivite, insektisidal aktivite

*Correspondence: **E-mail:** nurgun@gazi.edu.tr, **Phone:** +90 312 202 31 77 **ORCID-ID:** orcid.org/0000-0001-5489-3367
Received: 13.10.2016, **Accepted:** 13.02.2017

INTRODUCTION

Medicinal plants contain various chemical constituents that have potential to use for their biological activities. Natural resources that yield valuable phytochemical products are often used in the treatment serious diseases. Moreover, folk medicines can attribute to the discovery of a large number of clinically effective compounds.

Chrysophthalmum Schultz Bip., a member of the family Asteraceae, the tribus Inulaeae[1], is represented by three species, namely *Chrysophthalmum montanum* (DC.) Boiss., *C. dichotomum* Boiss. & Heldr., and *C. gueneri* Aytac and Anderb[2] in Turkey. *C. montanum*, known as "nezle otu and tutça", is a herbaceous perennial plant mainly distributed in eastern parts of Turkey. Its aerial parts are traditionally used for the treatment of the common cold and sinusitis, as well as healing wounds on the body of human and animal in Turkey.[3-5]

To date, a few studies on the morphologic characteristics and preliminary evaluation of biologic antioxidant and antimicrobial activities have been reported on *C. montanum*.[2,3,6-10] Only one recent phytochemical study has been conducted on the isolation of sesquiterpene lactones from *C. montanum*.[11] However, there have been no other experimental studies for the scientific evaluation of phytotoxic, cytotoxic, and insecticidal effects of *C. montanum*.

The Asteraceae family has been intensively investigated in the treatment of various diseases in recent years. The family is well-known as a good source of sesquiterpene lactones, which are associated with antitumor, cytotoxic, antimicrobial, anti-inflammatory, and phytotoxic activities.[12,13] In our ongoing research on *C. montanum*, we revealed that *C. montanum* had cytotoxicity against some cancer cell lines using sulforhodamine B assays.[14] The aim of the present study was to investigate the therapeutic importance of *C. montanum,* which is relatively safe from toxic effects, for its phytotoxic, cytotoxic, and insecticidal activities by using screening bioassays.

EXPERIMENTAL

Chemicals

In the extraction and fractionation procedure, methanol, *n*-hexane, chloroform, and *n*-butanol were of analytical grade and purchased from Merck Co. (Darmstadt, Germany). Analytical thin-layer chromatography (TLC) was performed on precoated Kieselgel 60 F_{254} plates (Art. 5554, Merck). The plates were sprayed with anisaldehyde reagent [76% methanol (Merck) and 19% ortho-phosphoric acid (Riedel-De Haën, Buchs, SG Switzerland), 5% *p*-anisaldehyde (Merck)] and 20% H_2SO_4 (Merck) solution in MeOH (Merck).

Plant material

The aerial parts of *C. montanum* (DC.) Boiss. were collected from the valley of Tohma River, Akçadağ, Malatya, Turkey at the flowering stage in July 2014. The plant material was identified by one of the authors (Professor, PhD Hayri Duman). An authenticated voucher specimen (Hayri Duman 10324) was deposited in the Herbarium of GAZI, Ankara, Turkey.

Preparation of extracts

The air-dried aerial parts of *C. montanum* (500 g) were extracted four times (4x3000 mL) with 80% methanol at 25°C by stirring for 2 days. Following filtration, the combined methanol extracts were evaporated *in vacuo* at 40°C to dryness. The concentrated MeOH extract (90.8 g, CM) were further fractionated through successive solvent extractions with *n*-hexane (11x250 mL), chloroform (8x250 mL), and *n*-butanol saturated with H_2O (8x250 mL) in a seperatory funnel. Each extract, as well as its remaining aqueous phase (R-H_2O) after solvent extractions were evaporated to dryness under reduced pressure to yield an "*n*-hexane fraction" (1.7 g, CMH), "$CHCl_3$ fraction" (15.8 g, CMC), "*n*-BuOH fraction" (21.4 g, CMB), and "R-H_2O fraction" (36.4 g, CMR), respectively.

Phytochemical analysis

The extracts of *C. montanum* (1 mg/mL) were applied to silica gel plates. The *n*-hexane and $CHCl_3$ extracts were developed with the mixture of *n*-hexane:ethylacetate (65:35) and chloroform:acetone (80:20), respectively, as mobile phases. TLC plates were evaluated under UV light at 254 and 366 nm for the determination of fluorescent compounds. Anisaldehyde reagent and 20% H_2SO_4 were sprayed on the plates to visualize the separated compounds, and then the plates were heated for 5 min at 100°C. Sesquiterpenes appeared with pink and purple coloration.

Brine shrimp lethality assay

Brine shrimp (*Artemia saline* Leach) eggs (50 mg) were sprinkled in a hatching tank (a rectangular dish 22x32 cm) half-filled with filtered brine solution. The crude extract and subsequent solvent fractions of *C. montanum* (20 mg) were dissolved in 2 mL of methanol (stock solution). The stock solutions of the extracts were diluted to 10, 100, and 1000 µg/mL concentrations in three vials. The solvent was evaporated under a fume hood by keeping overnight. After hatching (2 days), 30 shrimps were added in each vial with the volume adjusted to 5 mL using sea water. The vials were incubated at 25-27°C for 24 hours under illumination. Other vials were supplemented with solvent and reference cytotoxic drug (Etoposide: 7.46 µg/mL), which served as negative and positive controls, respectively. The number of brine shrimps that survived was counted in each vial and LD_{50} values with 95% confidence intervals were determined using Finney computer software.[15,16]

Pytotoxicity assay

The phytotoxicity assay was performed for the crude extract and subsequent solvent fractions of *C. montanum* against *Lemna minor* L.[17] The medium was prepared by mixing various constituents in 1000 mL distilled water. KOH pellets were added for the adjustment of pH at 6.0-7.0. The extracts (30.0 mg) were dissolved in 1.5 mL of methanol, which served as a stock solution. The stock solutions of the extracts were diluted to get final concentrations as 10, 100, and 1000 µg/mL (nine flasks, three for each dilution). After evaporating the solvent overnight under sterile conditions, 20 mL medium and 10 plants were added to each flask, each one containing a rosette of two fronds

of *L. minor*. Other flasks were supplemented with medium and reference plant growth inhibitor (Paraquate) as negative and positive controls, respectively. All flasks were incubated in a growth cabinet for seven days at 30˚C. The number of fronds per flasks was counted and recorded at the end of the incubation period. Growth regulation (GR) as a percentage (%) was determined using the formula given below:

The criteria indicate that the GR (%) of 0-39 for low activity,

$$\text{GR (\%)} = \frac{\text{100-}\textit{Number of the fronds in the test samples}}{\textit{Number of the fronds in the negative control}} x\ 100$$

40-59 for moderate activity, 60-69 for good activity, and >70 for significant activity were detected.

Insecticidal activity

The crude extract and subsequent solvent fractions of *C. montanum* were tested against *Rhyzopertha dominica* and *Tribolium castaneum* using impregnated filter paper.[18] The samples (200 mg) were dissolved in 3 mL of methanol and served as stock solution. The samples (1019.10 µg/cm^2) were applied to filter paper of appropriate size (9 cm or 90 mm) on petri plates using a micropipette. The plates were left for 24 hours to evaporate the solvent. The next day, 10 insects of each species were placed in each plate (test and control) using a clean brush. Permethrin (239.5 µg/cm^2) was used as positive control; methanol was used as negative control. The plates were incubated at 27˚C for 24 hours with 50% relative humidity in the growth chamber. For the calculation, the number of survivals of each species was counted and mortality (M) (%) was determined using the following formula:

$$\text{M (\%)} = \frac{\text{100} - \textit{Number of insects alive in the test samples}}{\textit{Number of insects alive in the control}} x\ 100$$

RESULTS AND DISCUSSION

In this study, we investigated the crude (80% methanol) extract and its fractions of *C. montanum* for their primary screening bioassays including cytotoxic, phytotoxic, and insecticidal activities. The cytotoxic properties of *C. montanum* were investigated at concentrations of 10, 100, and 1000 µg/mL, using etoposide as a standard. The methanol extract, *n*-hexane, and chloroform fractions of the plant had positive lethality with LD_{50} values of 71.52, 126.62, and 75.95 µg/mL against the brine shrimps, respectively (Table 1).

The phytotoxicity of the investigated samples on *L. minor* was observed to have dose-dependent activity because low activity was detected in the *n*-hexane fraction with 12.5 and 18.7% inhibition at 10 and 100 µg/mL, respectively. Moderate phytotoxic activity was found in the methanol extract (40.0% inhibition) at 1000 µg/mL. Good phytotoxic activity was found in the chloroform fraction (68.7% inhibition) at 100 µg/mL and *n*-butanol fraction (62.6% inhibition) at 1000 µg/mL. Significant phytotoxic activity was shown in the *n*-hexane and chloroform fractions of the plant; 100.0% inhibition for each fraction at 1000 µg/mL (Table 2).

Table 1. Cytotoxic activities of the extract and fractions from *C. montanum*

Samples	No of survivors from 30 shrimps			LD_{50} (µg/mL)
	10 µg/mL	100 µg/mL	1000 µg/mL	
CM	25	10	06	71.52
CMH	18	23	06	126.62
CMC	15	17	00	75.95
CMB	22	22	17	-
CMR	27	26	21	-

Standard drug: Etoposide (LD_{50}=7.46 µg/mL)

Table 2. Phytotoxic activities of the extract and fractions from *C. montanum*

Samples	Growth regulation (%)		
	10 µg/mL	100 µg/mL	1000 µg/mL
CM	0	0	40.0
CMH	12.5	18.7	100.0
CMC	0	68.7	100.0
CMB	0	0	62.6
CMR	0	0	0

Standard drug: Paraquate (0.015 µg/mL)

Table 3. Insecticidal activities of the extract and fractions from *C. montanum*

Samples (1019.10 µg/cm^2)	*Tribolium castaneum*		*Rhyzopertha dominica*	
	Mortality (%)	Insecticidal activity	Mortality (%)	Insecticidal activity
CM	0	NO	0	NO
CMH	0	NO	0	NO
CMC	0	NO	0	NO
CMB	0	NO	0	NO
CMR	0	NO	0	NO

Reference insecticide: Permethrin (239.5 µg/cm^2)

The methanol extract and fractions of *C. montanum* were also screened for their insecticidal effects against *R. dominica* and *T. castaneum* using permethrin as a standard drug. There were no insecticidal effects on all samples against *T. castaneum* and *R. dominica* (Table 3).

The brine shrimp lethality assay is not specific for any particular physiologic effects. However, the cytotoxic effect of the natural constituents on the shrimp larvae was especially correlated with their anticancer potentials. This preliminary method,

which has been developed for screening, fractionation, and monitoring of physiologically active natural products, is clearly a more rapid, inexpensive, and general bioassay.[16] Moreover, phytotoxic and insecticidal constituents are mostly important to develop natural herbicides and insecticides that are safe, cost effective, and user-friendly for the environment.[19]

According to our results, the *n*-hexane and chloroform fractions of *C. montanum* were found as promising samples due to having cytotoxicity on brine shrimp. In our recent study, *n*-hexane and chloroform fractions of the plant also exhibited cytotoxicity on selected cancer cell lines.[14] In addition, our findings demonstrate that *n*-hexane and chloroform fractions of *C. montanum* possess significant phytotoxicity against *L. minor*. Our preliminary phytochemical detection using TLC showed that sesquiterpenes were as prominent components in the bioactive chloroform fraction of the plant.

CONCLUSION

In summary, the present study firstly depicts the potential of the extracts of *C. montanum* on biologic activities such as cytotoxicity against brine shrimp and phytotoxic effects, which indicate that the plant might be considered as a new potential source in the research of new drugs. Accordingly, further investigations to identify the responsible bioactive compound(s), principally sesquiterpenes, are ongoing on *C. montanum*.

ACKNOWLEDGEMENTS

This study was supported by TUBİTAK-2214/A, TUBİTAK-2211/A and ICCBS-HEJ.

Conflict of Interest: No conflict of interest was declared by the authors.

REFERENCES

1. Grierson AJC. *Chrysophthalmum* Schultz Bip. In: Davis PH. Ed. Flora of Turkey and the East Aegean Islands. Edinburgh; Edinburgh University Press; 1975:5:52-53.
2. Aytac Z, Anderberg AA. A new species of *Chrysophthalmum* Schultz Bip. (*Asteraceae-Inuleae*) from Turkey. Bot J Linn Soc. 2001;137:211-214.
3. Kirbag S, Zengin F, Kursat M. Antimicrobial activities of extracts of some plants. Pakistan J Bot. 2009;41:2067-2070.
4. Yeşil Y, Akalın E. Folk Medicinal Plants In Kürecik Area (Akçadağ/Malatya). Turk J Pharm Sci. 2009;3:207-220.
5. Arasan S, Kaya I. Some important plants belonging to *Asteraceae* family used in folkloric medicine in Savur (Mardin/Turkey) Area and their application areas. J Food Nutr Res. 2015;3:337-340.
6. Englund M, Pornpongrungrueng P, Gustafsson MHG, Anderberg AA. Phylogenetic relationships and generic delimitation in *Inuleae* subtribe *Inulinae* (*Asteraceae*) based on ITS and cpDNA sequence data. Cladistics. 2009;25:319-352.
7. Karlıoğlu N, Paksoy MY. *Chrysophthalmum* (*Asteraceae*) Cinsi Polen Morfolojisi, 21st. İzmir, Turkey; National Congress of Biology; 2012. pp. 202.
8. Selvi S, Paksoy MY. Türkiye'de Yetişen *Chrysophthalmum* (*Asteraceae*) Cinsinin Karşılaştırmalı Gövde ve Yaprak Anatomisi, 21st. İzmir, Turkey; National Congress of Biology; 2012. pp. 038.
9. Ozdemir A, Turkoglu V, Demir H. *In vitro* effect of some plant extracts on acetylcholinesterase enzyme in human erythrocytes and serum. Fresen Environ Bull. 2013;22:2510-2515.
10. Gürbüz P, Doğan ŞD, Pasayeva L, Paksoy MY. Guaiane-type Sesquiterpene Lactones from *Chrysophthalmum montanum*. Rec Nat Prod. 2016;10:714-720.
11. Selvi S, Paksoy MY, Polat R, Cakilcioglu U. Micromorphological and anatomical characteristics of the genus *Chrysophthalmum* Schultz Bip. (*Asteraceae*) growing in Turkey. Proc Natl Acad Sci India Sect B Biol Sci. 2014;84:431-438.
12. Rodriguez E, Towers GHN, Mitchell JC. Biological activities of sesquiterpene lactones. Phytochemistry.1976;15:1573-1580.
13. Picman AK. Biological activities of sesquiterpene lactones. Biochem Syst Ecol. 1986;14:255-281.
14. Ayaz F, Kucukboyaci N, Sarimahmut M, Bani B, Duman H, Ulukaya E, Calis I. Cytotoxic Activity of the Genus *Chrysophthalmum* Schultz Bip. from Turkey Against Various Human Cancer Cell Lines. 3rd. İzmir, Turkey; EACR-Sponsored Anticancer Agent Development Congress; 2015. pp. 42.
15. Finny DJ. Probit Analysis (3rd ed). Cambridge; Cambridge University Press; 1971:333.
16. Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL. Brine shrimp: a convenient general bioassay for active plant constituents. Planta Med. 1982;45:31-34.
17. Atta-ur-Rahman. Studies in Natural Product Chemistry. The Netherlands; Elsevier Science Publishers BV; 1991;9:383-409.
18. Atta-ur-Rahman, Choudhary MI, William JT. Bioassay techniques for drug development. The Netherlands; Harward Academic Publisher; 2001:67-68.
19. Saeed M, Muhammad N, Khan H, Khan SA. Analysis of Toxic Heavy Metals in branded Pakistani Herbal Products. J Chem Soc Pak. 2010;32:471-475.

Turk J Pharm Sci 2017;14(3):294-303
DOI: 10.4274/tjps.50470

ORIGINAL ARTICLE

Substance Abuse Profiles of Patients Admitted to the Alcohol and Drug Addiction Research, Treatment, and Education Center in Turkey

Alkol ve Madde Bağımlılığı Araştırma, Tedavi ve Eğitim Merkezi Birimi'nde Yatan Hastaların Madde Kullanım Profili-Türkiye

Fadime CANBOLAT[1]*, Aykut KUL[1], Murat ÖZDEMİR[1], Uğur ATİK[1], Ahmet AYDIN[2], S. Tuncel ÖZDEN[1], K. Nevzat TARHAN[1]

[1]Üsküdar University, Advanced Toxicology Analysis Laboratory, İstanbul, Turkey
[2]Yeditepe University, Faculty of Pharmacy, Department of Toxicology, İstanbul, Turkey

ABSTRACT

Objectives: To determine the substance abuse profiles of patients treated a Drug Addiction Research, Treatment, and Education Center (AMATEM) in association with the percentage of substance use distribution and multiple substance use in their urine samples. For this, we retrospectively evaluated the urine sample analysis reports of 600 male and female patients aged 13 to 65 years who were treated at the AMATEM unit of İstanbul Neuropsychiatry Hospital between January 1st, 2015, and December 12th, 2015.

Materials and Methods: The urine samples were sent to Üsküdar University Advanced Toxicology Analysis Laboratory and were analyzed using a UPLC tandem mass spectrometer (UPLC-MS/MS). To determine the substance use profiles of the patients applying to AMATEM, statistical assessment was performed on the analysis reports of the patients.

Results: When the analysis reports of the 600 urine samples were examined, 293 patients were identified to have used addictive substances. The substances most frequently detected in the urine samples were respectively: cannabis, alcohol, morphine, cocaine, synthetic cannabinoids, 3,4-Methylenedioxymethamphetamine, and amphetamine.

Conclusion: The findings in our study resemble the rates of cannabis use by the young population throughout the world. Our results show differences to the literature regarding the consumption of synthetic cannabinoids because the variety of synthetic cannabinoids change rapidly around the world each year.

Key words: Mass spectrometer, substance abuse, urine, statistical assessment

ÖZ

Amaç: Çalışmamızın amacı, AMATEM biriminde tedavi gören hastaların madde kullanım profilini onların idrar örneklerindeki madde kullanım yüzde dağılımlarına ve çoklu madde kullanımlarına göre belirlemektir. Bunun için, İstanbul Nöropsikiyatri Hastanesi AMATEM biriminde 1 Ocak 2015 - 12 Aralık 2015 tarihleri arasında tedavi gören 13 ve 65 yaş aralığında erkek ve kadın 600 hastanın idrar örneklerinin analiz raporları retrospektif olarak değerlendirildi.

Gereç ve Yöntemler: İdrar örnekleri Üsküdar Üniversitesi İleri Toksikoloji Analiz Laboratuvarı'na gönderildi ve orada UPLC kütle spektrometresi ile (UPLC-MS/MS) analiz edildi. AMATEM birimine başvuruda bulunan hastaların madde kullanım profillerini tanımlamak için hastaların analiz raporları üzerinden istatistiksel değerlendirme yapılmıştır.

Bulgular: Altı yüz idrar örneğinin analiz raporu incelendiğinde, hastaların 293'ünde bağımlılık yapıcı madde kullanımı belirlenmiştir. İdrar örneklerinde çoğunlukla tespit edilen maddeler sırasıyla; esrar, alkol, morfin, kokain, sentetik kannabinoitler, 3,4-metilendioksimetamfetamin ve amfetamindir.

Sonuç: Çalışmamızın sonuçları bütün dünyada genç nüfus tarafından esrar kullanım oranı ile benzerlik göstermektedir. Ayrıca, sentetik kannabinoitlerin çeşitliliği her yıl dünyada hızla değiştiği için çalışmamızın sonuçları ve literatüre sonuçları sentetik kannabinoit tüketimi konusunda farklılık göstermektedir.

Anahtar kelimeler: Kütle spektrometresi, madde kullanımı, idrar, istatistik değerlendirme

*Correspondence: **E-mail:** fadime.canbolat@uskudar.edu.tr, **Phone:** +90 532 574 92 63 **ORCID-ID:** orcid.org/0000-0002-7720-4904
Received: 21.07.2016, **Accepted:** 02.03.2017

INTRODUCTION

Substance abuse and dependence is a chronic disease that lasts a lifetime. Substance dependence is a global concept. The addictive substances are mainly alcohol, cannabis, opiates, amphetamine groups, cocaine, and synthetic cannabinoids.[1] In the United Nations Office on Drugs and Crime (UNODC) 2015 world drug report, it was announced that 246 million people around the world within the age group of 15-64 had taken a drug at least once in the last year. This number indicates that approximately one in every twenty people aged between 15-64 years has taken a drug in the last year.[2] Cannabis is the most commonly used drug across the world. Amphetamine-type Stimulants are the second most common drugs. Amongst the synthetic drugs, ecstasy has become the most widely used synthetic drug in the industrialized world. Opiate and cocaine groups follow in the ranking.[3] Along with these, new synthetic addictive substances began to emerge near the end of 2006. Use of synthetic cannabinoids has grown exponentially worldwide.[4]

Substance abuse and dependence is a health issue with biologic, mental, and social aspects that can affect anyone, but primarily young people, in Turkey, and globally.[5] In Turkey, the most commonly produced and consumed illegal drug is cannabis.[6] The identification of the psychoactive substance relies greatly on information provided by the patient, examination of blood, urine, and saliva samples or other traces (drug samples possessed by the patient, clinical symptoms and findings, information obtained from third parties). When the detection periods of substances and metabolites are taken into consideration, urinalysis is widely preferred due to its longer periods of detection for substances and metabolites in the urine.[7] However, data reflecting the dimensions of substance dependence in Turkey heavily rely on arrests by the Narcotics Police and some survey data. Accordingly, in order to obtain more reliable results, we differently evaluated the prevalence of addictive substances by using direct analysis and reports of patient's urine.

In this study, we determined substance abuse profiles of patients treated at the AMATEM unit in association with the percentage of substance use distribution and multiple substance use in urine samples. For this, we retrospectively evaluated the urine sample analysis reports of 600 male and female patients aged 13 to 65 years who were treated at the AMATEM unit of İstanbul Neuropsychiatry Hospital between January 1st, 2015, and December 12th, 2015. The urine samples were sent to Üsküdar University Advanced Toxicology Analysis Laboratory and analyzed. Statistical assessment was performed on the analysis reports to determine the substance use profiles of the patients.

MATERIALS AND METHODS

Ethics statement and sample selection

We applied to the Ethics Committee of Üsküdar University and the study was approved. The urine samples of the 600 patients, which were obtained during the initial admission periods at the AMATEM Unit, were analyzed between January 1st, 2015, and December 12th, 2015, in the Advanced Toxicology Analysis Laboratory of Üsküdar University.

Urine samples preparation

For the detection of a total 25 addictive molecules such as ethyl alcohol, cocaine, amphetamine, 3,4-Methylene-dioxymethamphetamine (MDMA), cannabis, morphine, 19 synthetic cannabinoid molecules in the urine samples were analyzed using the validated method in a Waters UPLC-MS/MS. The detection of ethyl alcohol in urine was performed by identifying its metabolite ethyl glucuronide; the detection of cannabis in urine was achieved through the identification of its metabolite 11-nor-THC-9-carboxylic acid (THC-COOH), and the detection of cocaine in urine was achieved by determining its metabolite benzoylecgonine. The sample preparation methods are given in Table 1.

UPLC-MS/MS condition

The urine samples were analyzed using a Waters UPLC/ICLASS Xevo TQD tandem mass spectrometer (UPLC-MS/MS). The (multiple reaction monitoring) MRM parameters of the molecules and the internal standards are shown in Table 2.

Table 1. Sample preparation

The detection of ethyl alcohol	Detection of amphetamine, MDMA, and benzoylecgonine	Detection of THC-COOH, morphine, and synthetic cannabinoids
1000 µL formic acid (1%) + 100 µL urine sample + 100 µL ethyl beta D-Glucuronide-D5 (internal standard)	1000 µL urine sample + Benzoylecgonine-D3 (internal standard) + 500 µL cold acetonitrile	1000 µL urine sample + 100 µL UR 144-D3 (as an internal standard) + 100 µl β-Glucuronidase (wait at 60°C for 3 hours) . . . (then wait for room temperature) + 500 µL cold acetonitrile

Table 2. MRM transitions, retention times, and conditions of each molecule

Molecule	Precursor ion (m/z)	Production (m/z)	RT	Cone (V)	CE (V)	Polarity (ESI)	LOQ ng/mL	LOD ng/mL
Ethyl glucuronide	221.00	**75**	1.85	30	15	-	410	200
Ethyl beta D-Glucuronide-D5 (IS)	225.98	**84.96**	1.85	30	17	-		
THC-COOH	343.10	245.10 **299.10**	2.62	45	28 20	-	10.80	2.50
Benzoylecgonine	290.0404	104.88 **167.95**	0.77	38	30 18	+	8.32	1.00
Benzoylecgonine-D3 (IS)	293.2042	105.11 171.26	0.77	38	22 18			
AM-2201 6-Hydroxyindole	376.20	77.00 **127.05**	3.57	50	75 50	+	1.92	0.60
AM2201-4 Hydroxypentyl	375.98	**127.00** 143.94 154.93	3.20	6	44 36 22	+	1.60	0.50
Amphetamine	136.00	91.00 **119.00**	1.81	30	16 7	+	61.60	20
BB22 3-Carboxyindole	258.10	**118.06** 176.01	4.33	42	20 14	+	3.02	0.50
JWH-018 5-Hydroxyindole	358.15	127.00 **155.00** 160.00	9.08	55	48 25 38	+	2.24	0.50
JWH-018 5-Hydroxypentyl	358.2	127.00 **155.00** 230.00	3.24	45	50 22 25	+	1.92	0.50
JWH019 N-6-Hydroxyhexyl	372.07	127.00 **154.00**	4.10	38	52 20	+	2.36	0.50
JWH-073 3-Hydroxybutyl	344.04	127.00 **155.00**	3.16	50	45 25	+	1.73	0.50
JWH-073 N-Butanoic Acid	358.20	127.05 144.00 **155.00**	2.53	45	50 30 26	+	2.02	0.50
JWH081 N-4-Hydroxypentyl	388.07	157.01 **184.97**	3.84	48	42 22	+	4.00	0.50
JWH122 N-5-Hydroxypentyl	372.06	115.0 **141.00**	4.19	45	70 45	+	2.75	0.50
JWH-18 N-Pentanoic acid	372.20	127.05 144.00 **155.00**	2.90	46	50 35 24	+	2.18	0.50
JWH210 N-4-Hydroxypentyl	386.09	**144.00** 248.00	3.70	45	36 24	+	2.26	0.50
JWH-250 4 Hydroxypentyl	352.09	**121.05** 130.15 186.08 204.10	2.41	68	18 34 14 16	+	2.07	0.70
JWH-250 5 Carboxypentyl	366.15	**121.00** 200.00	2.12	45	22 15	+	2.11	0.70

Table 2. Continue

JWH-250 5 Hydroxyindole	352.15	121.00	4.73	45	20	+	1.92	0.50
MAM2201 N-Pentanoic acid	386.02	**115.06** 154.81 182.90	3.70	50	44 40 24	+	2.38	0.50
MDMA	194.1	104.88 135.53 **162.97**	1.84	10	22 18 12	+	65.00	25.00
Morphine	286.20	153.10 **165.20**	1.76	55	40 40	+	5.34	0.80
UR-144 _Pentanoic acid – D5(IS)	347.21	55.08 **125.05**	4.45	8	42 22	+		
UR144 N-5-Hydroxypentyl	328.10	97.00 **125.00**	5.06	45	30 18	+	2.79	0.90
UR144 N-Pentanoic acid	342.05	125.00 244.70	4.49	45	20 24	+	2.88	0.90
XLR11 N-4- Hydroxypentyl	346.1	144.00 **248.00**	3.69	45	36 24	+	4.00	1.00

Underlined transitions were used for quantification; RT: retention time; CE: collision energy; ESI: electrospray ionization; IS: internal standard; LOQ: limit of quantitation; LOD: limit of detection

Table 3. Substance abuse profile of 293 patients

Substance	Alcohol		Cocaine		Morphine		Amphetamine		MDMA		Cannabis		Synthetic cannabinoids	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Non-users	196	66.89	225	76.79	221	75.43	277	94.54	270	92.15	192	65.53	261	89.08
Users	97	33.11	68	23.21	72	24.57	16	5.46	23	7.85	101	34.47	32	10.92
Total	293	100.0	293	100.0	293	100.0	293	100.0	293	100.0	293	100.0	293	100.0

n: analyzed urine sample; %: percentage of substance abuse

Statistical analysis

Statistical assessment was performed on the analysis reports to determine the substance use profiles of the patients admitted to the AMATEM unit. The results were statistically evaluated using the chi-square test. The level of significance was $p \leq 0.05$ at a 95% significance level.

RESULTS

When the analysis reports of the urine samples obtained during the initial admission periods of 600 patients were observed, 293 of the patients were identified to have used addictive substances. Statistical analysis was conducted by taking the data of the 293 patients with addictive substances into consideration in order to determine their substance abuse profiles.

In Table 3, it can be seen that of the 293 patients, 97 patients (33.1%) consumed alcohol, 68 (23.2%) used cocaine, 72 used (24.6%) morphine, 16 (5.5%) used amphetamine, 23 (7.8%) used MDMA, 101 (34.5%) smoked cannabis, and 32 (10.9%) used synthetic cannabinoids. In compliance with the data, the top three substances consumed by the 293 patients were cannabis, alcohol, and morphine respectively (Figure 1).

As seen in Figure 2, 260 (88.74%) of 293 patients were male and 33 (11.26%) were female. As shown in Table 4, there was no statistical correlation between individual substance consumption and sex at a 95% significance level ($p > 0.05$) (Figure 3).

When the age groups of the 293 patients were considered, there were 57 (19.45%) people in 13-22 years age group, 159 (54.27%) people in 23-32 years age group, 51 (17.41%) people in 33-42 age group, 17 (5.80%) people in 43-52 years age group, and 9 people aged 53 years and above (3.07%) (Figure 4).

As shown in Table 5, there were correlations between age and consumption of alcohol, cannabis, and morphine at the 95% significance level ($p \leq 0.05$). There was no correlation between age and consumption of cocaine, amphetamine, MDMA, and synthetic cannabinoids at the 95% significance level ($p > 0.05$) (Figure 5).

Nineteen types of synthetic cannabinoid molecules were analyzed in the urine samples in order to detect the use of

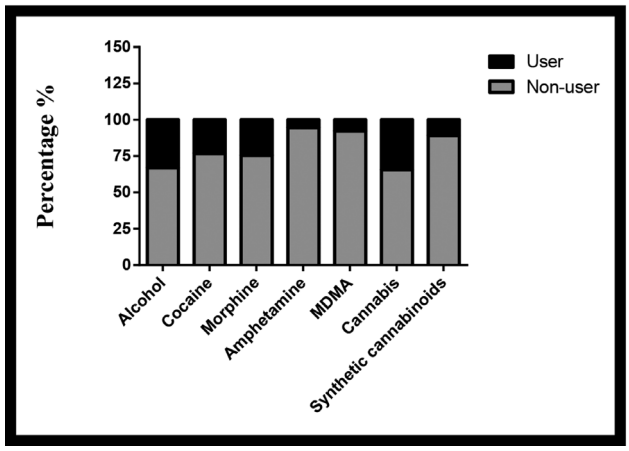


Figure 1. Percentage distribution of substance abuse by 293 patients

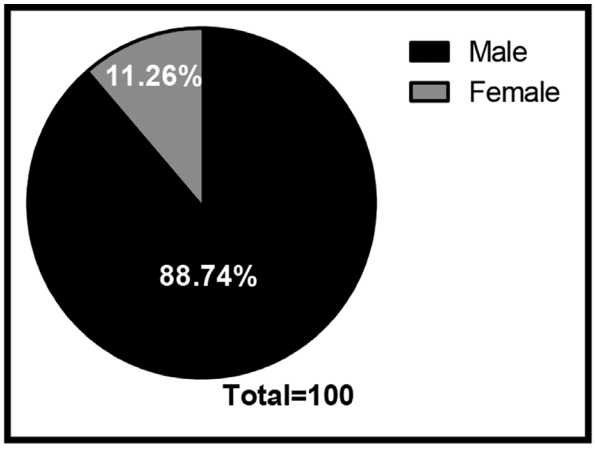


Figure 2. Two hundred nineteen three patients' percentage distribution according to gender

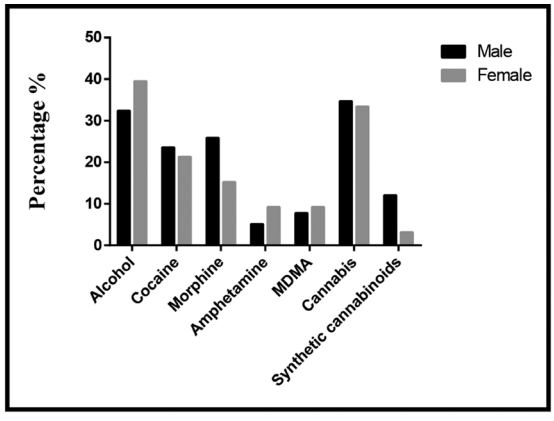


Figure 3. Substance abuse profile of 293 patients according to gender

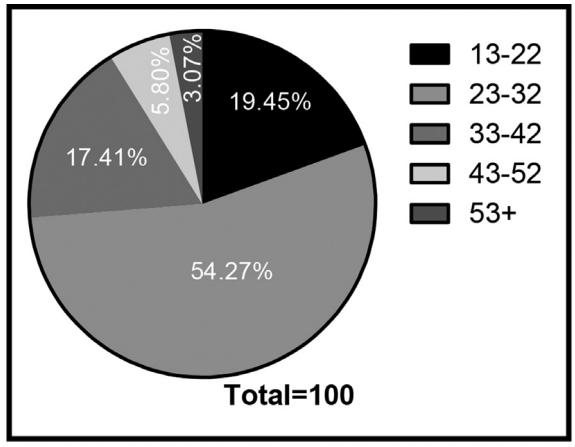


Figure 4. Two hundred nineteen three patients' percentage distribution according to age

Table 4. Substance abuse profile of 293 patients according to sex

Substance	Male (n=260)		Female (n=33)		Statistical results
Alcohol					
Non-users	176	67.69%	20	60.61%	χ^2=0.664, p=0.289
Users	84	32.31%	13	39.39%	
Cocaine					
Non-users	199	76.54%	26	78.79%	χ^2=0.773, p=0.773
Users	61	23.46%	7	21.21%	
Morphine					
Non-users	193	74.23%	28	84.85%	χ^2=1.781, p=0.182
Users	67	25.77%	5	15.15%	
Amphetamine					
Non-users	247	95.00%	30	90.91%	χ^2=0.949, p=0.330
Users	13	5.00%	3	9.09%	
MDMA					
Non-users	240	92.31%	30	90.91%	χ^2=0.079, p=0.778
Users	20	7.69%	3	9.09%	
Cannabis					
Non-users	170	65.38%	22	66.67%	χ^2=0.021, p=0.884
Users	90	34.62%	11	33.33%	
Synthetic cannabinoids					
Non-users	229	88.08%	32	96.97%	χ^2=2.38, p=0.123
Users	31	11.92%	1	3.03%	

n: analyzed urine sample; %: percentage of substance abuse; α: 0.05

synthetic cannabinoids. As a result, a total of 59 synthetic cannabinoid molecules were detected to have been used by 32 of 293 patients: 25.42% UR144 N-pentanoic acid, 20.34% UR144 N-5-hydroxypentyl, 18.64% JWH-18 N-pentanoic acid, 8.47% JWH-018 5-hydroxypentyl, 5.08% JWH-018 5-hydroxyindole, 8.47% AM-2201 6-hydroxyindole, 5.08% AM-2201 4-hydroxyindole, 3.39% JWH-250 5-hydroxyindole, 1.69% JWH-073 N-butanoic acid, 1.69% JWH-073 3-hydroxybutyl, and 1.69% BB22 3-carboxyindole existed in the 59 synthetic cannabinoid molecules detected. JWH019 N-6-hydroxyhexyl,

JWH081 N-4-hydroxypentyl, JWH122 N-5-hydroxypentyl, JWH210 N-4-hydroxypentyl, JWH-250 5-carboxypentyl, JWH-250 4-hydroxypentyl, MAM2201 N-pentanoic acid, XLR11 N-4-hydroxypentyl molecules were not identified in the urine samples (Figure 6).

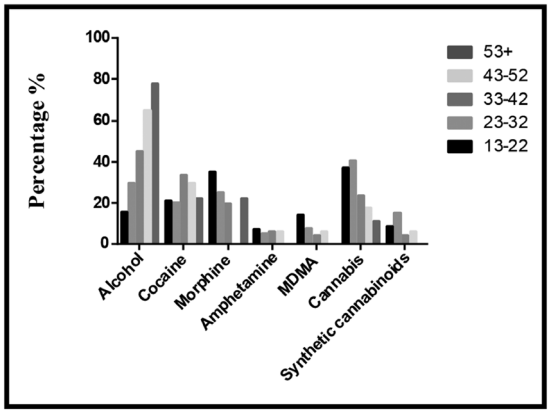


Figure 5. Substance abuse profile of 293 patients according to age groups

When the multiple substance abuse of 293 patients was assessed, abuse of two or more substances was identified in 97 patients. In the 97 patients, use of two substances was identified in 76 (78.35%) patients, use of three in 16 (17.53%), and use of four substances was identified in 4 patients (4.12%) (Figure 7). The distribution of multiple-substance abuser patients' results is given in Table 6.

DISCUSSION

Alcohol and substance addiction is amongst the leading factors causing significant problems in Turkey as it does around the world.[8] According to UNODC data, 1.6 million drug seizure cases took place around the world in 2006. Sixty-five percent of the drugs seizes was cannabis, 14% was opium and derivatives, 9% was coca plant and derivatives, 2% ecstasy, and 5% amphetamines.[5] In the UNODC 2015 world drug report, it stated that the most commonly used drugs of patients aged 15-64 years were cannabis, cocaine, and amphetamine derivatives.[1,2] Turkey is affected by world drug marketing both as a transit and target country. Cannabis, opiates, cocaine, amphetamine

Table 5. Substance abuse profile of 293 patients according to age groups

Substance	Age (among all users) (years)										
	13-22 (n=57)		23-32 (n=159)		33-42 (n=51)		43-52 (n=17)		53+ (n=9)		Statistical results
Alcohol											
Non-users	48	84.21%	112	70.44%	28	54.90%	6	35.29%	2	22.22%	χ^2=27.708, p<0.001
Users	9	15.79%	47	29.56%	23	45.10%	11	64.71%	7	77.78%	
Cocaine											
Non-users	45	78.95%	127	79.87%	34	66.67%	12	70.59%	7	77.78%	χ^2=4.302, p=0.367
Users	12	21.05%	32	20.13%	17	33.33%	5	29.41%	2	22.22%	
Morphine											
Non-users	37	64.91%	119	74.84%	41	80.39%	17	100.00%	7	77.78%	χ^2=9.673, p=0.046
Users	20	35.09%	40	25.16%	10	19.61%	0.00	0.00 %	2	22.22 %	
Amphetamine											
Non-users	53	92.98%	151	94.97%	48	94.12%	16	94.12%	9	100.00%	χ^2=0.868, p=0.929
Users	4	7.02	8	5.03%	3	5.88%	1	5.88%	0	0.00%	
MDMA											
Non-users	49	85.96%	147	92.45%	49	96.08%	16	94.12%	9	100.00%	χ^2=4.98, p=0.289
Users	8	14.04%	12	7.55%	2	3.92%	1	5.88%	0	0.00%	
Cannabis											
Non-users	36	63.16%	95	59.75%	39	76.47%	14	82.35%	8	88.89%	χ^2=9.501, p=0.05
Users	21	36.84%	64	40.25%	12	23.53%	3	17.65%	1	11.11%	
Synthetic cannabinoids											
Non-users	52	91.23%	135	84.91%	49	96.08%	16	94.12%	9	100.00%	χ^2=7.23, p=0.124
Users	5	8.77%	24	15.09%	2	3.92%	1	5.88%	0	0.00%	

n: analyzed urine sample; %: percentage of substance abuse; α: 0.05

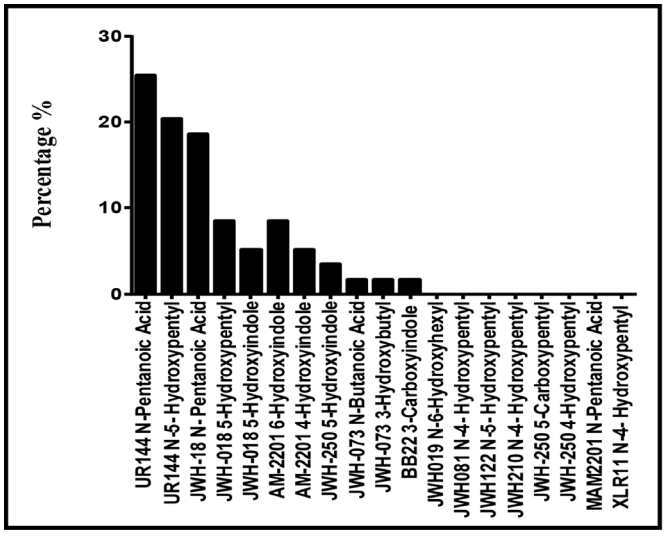


Figure 6. Percentage distribution of 19 types of Synthetic cannabinoids

The percentage distribution of 25.42% UR144 N-Pentanoic acid, 20.34% UR144 N-5-Hydroxypentyl, 18.64% JWH-18 N-Pentanoic acid, 8.47% JWH-018 5-Hydroxypentyl, 5.08% JWH-018 5-Hydroxyindole, 8.47% AM-2201 6-Hydroxyindole, 5.08% AM-2201 4-Hydroxyindole, 3.39% JWH-250 5-Hydroxyindole, 1.69% JWH-073 N-Butanoic acid, 1.69% JWH-073 3-Hydroxybutyl, 1.69% BB22 3-Carboxyindole, 0.00% JWH019 N-6-Hydroxyhexyl, 0.00% JWH081 N-4-Hydroxypentyl, 0.00% JWH122 N-5-Hydroxypentyl, 0.00% JWH210 N-4-Hydroxypentyl, 0.00% JWH-250 5-Carboxypentyl, 0.00% JWH-250 4-Hydroxypentyl, 0.00% MAM2201N-Pentanoic acid, 0.00% XLR11 N-4-Hydroxypentyl molecules in the patients diagnosed with Synthetic cannabinoids in their urine samples

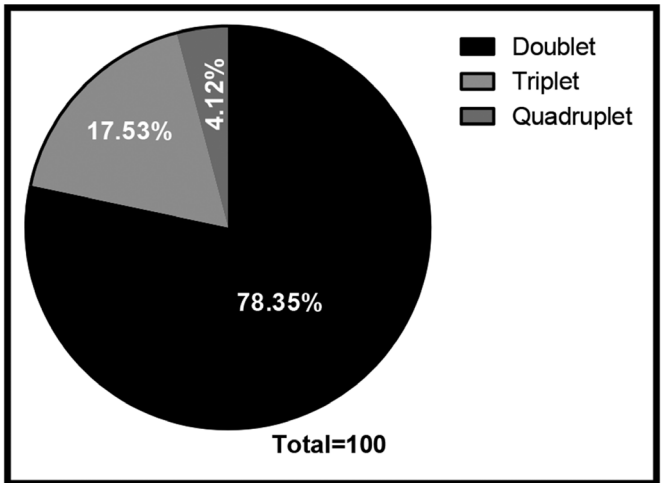


Figure 7. Percentage distribution of the multiple substances identified in the urine of the patients

The percentage distribution of the double, triple, and quadruple substance use of 97 patients who were identified to have used more than one substance with the analysis of their urine samples

derivatives, and synthetic cannabinoids are present amongst the drugs mainly seized in Turkey.[8]

Alcohol consumption is on the rise all around the world, most particularly in developing countries and countries in transition. To illustrate, alcohol consumption increase is faster in Eastern Europe, Russia, and the West Pacific Region.[9] Erhan Deveci et

Table 6. Percentage distribution of the substance abuse of patients who were identified to have used multiple substances

Substance	n	%
Alcohol (n=35)		
Alcohol + Cannabis	22	62.86
Alcohol + Cocaine	13	37.14
Alcohol + Morphine	9	25.71
Alcohol + MDMA	3	8.57
Alcohol + Synthetic cannabinoids	1	2.86
Cocaine (n=45)		
Cocaine + Alcohol	13	28.89
Cocaine + Morphine	16	35.56
Cocaine + Amphetamine	3	2.86
Cocaine + MDMA	5	11.11
Cocaine + Cannabis	25	55.56
Cocaine + Synthetic cannabinoids	1	2.22
Cannabis (n=70)		
Cannabis + Alcohol	22	31.43
Cannabis + Cocaine	25	37.71
Cannabis + Morphine	16	22.86
Cannabis + Amphetamine	2	2.76
Cannabis + MDMA	16	22.86
Cannabis + Synthetic cannabinoid	11	15.71
Morphine (n=31)		
Morphine + Alcohol	9	29.03
Morphine + Cocaine	16	51.61
Morphine + MDMA	2	6.45
Morphine + Cannabis	16	51.61
Morphine + Synthetic cannabinoids	1	3.23
Amphetamine (n=6)		
Amphetamine + Cocaine	3	50.00
Amphetamine + MDMA	2	33.33
Amphetamine + Cannabis	2	33.33
MDMA (n=19)		
MDMA + Alcohol	3	15.79
MDMA + Cocaine	5	26.33
MDMA + Morphine	2	10.53
MDMA + Amphetamine	2	10.53
MDMA + Cannabis	16	84.21
MDMA + Synthetic cannabinoids	2	10.53
Synthetic Cannabinoids (n=12)		
Synthetic cannabinoids + Alcohol	1	8.33
Synthetic cannabinoids + Cocaine	1	8.33
Synthetic cannabinoids + Morphine	1	8.33
Synthetic cannabinoids + MDMA	2	16.67
Synthetic cannabinoids + Cannabis	11	91.67

n: analyzed urine sample; %: percentage of substance abuse

al.[10] conducted a study with 2258 Turkish university students, of which 70.3% were males and 29.7% were females. They reported that 30.25% of the males and 19.1% of the females used alcohol.[10] In another study conducted with 396 Turkish university students, 56.6% were reported to have used alcohol at least once throughout their lives.[11]

In our study, the determination of alcohol and drug use was performed through the assessment of the report models obtained by analyzing urine samples, rather than by a survey or an oral interview.

The determination of ethyl glucuronide, which is a metabolite of alcohol, facilitated this aspect of the research.[12] Ninety-seven (33.11%) of 293 patients used alcohol; 32.31% of males and 39.39% of females (Table 3). We concluded that sex has no significance with regard to alcohol consumption because no significant difference was identified between the sexes with regards alcohol consumption (Table 4). Although men were stated to use more alcohol in the Erhan Deveci et al.[10] interviews, in our study, we determined no significant correlation between sex and alcohol use. The difference between the two studies could be a result of the use of two different research methods.

In a study Asan et al.[13] on the sociodemographic attributes of patients treated at an AMATEM unit, the age average of the patients treated for alcohol addiction was determined as higher than the age average of the those treated for substance abuse. In our study, a difference between age and alcohol consumption was identified to exist when the patients who were detected to have used substances were divided into age groups. Alcohol consumption was observed to increase in percentage as age increased (Table 5). The findings in our study show a resemblance to those in the literature.

In 2003, a French study by Mura et al.[14] aimed to determine the prevalence of alcohol, cannabinoids, opiates, cocaine metabolites, amphetamines, and therapeutic psychoactive drugs in blood samples from drivers injured in road accidents; the substances they determined most frequently were, in order, alcohol, cannabinoids, benzodiazepines, and opiates. Similarly, in our study, we saw alcohol, cannabis, and morphine most commonly, and this shows similarity with the literature.

Benzoylecgonine, which can be detected for 2-3 days in the urine as a metabolite of cocaine, was tested for in the present study.[7,15] Sixty-eight of the 293 patients (23.21%) used cocaine (Table 3). There was no statistical correlation between cocaine consumption and sex (Table 4). No correlation between age and cocaine could be found (Table 5). In a study on the substance use profile of the public of Iran, cocaine and the other stimulants were reported as negligible.[16] In our study, we determined cocaine in 23.21% of our patients' urine samples. The fact that this average is higher in our country could be the result of our country's location as a transition point in world drug market.[8]

The use of opiates was determined in line with resulting free morphine levels. By converting into free morphine, morphine glucuronide, which can be detected for up to 48 hours in the urine as a metabolite of heroine, codeine, and morphine, was considered in the analysis in our study to determine opiate-based substances.[17] When the results of the analysis were taken into consideration, free morphine was detected in the urine samples of 72 of 293 patients (24.57%), who were thereby determined to have used opiates (Table 3). In a study on the substance use profile of Iranian public, morphine was in first place.[16] Likewise, in our study, we determined a high incidence of morphine use in the urine samples. However, no correlation between sex and morphine could be found (Table 4). When the age groups were assessed, a significant difference between age and morphine use was observed. Morphine was determined to be mostly used in the age groups of 13-22 years and 23-32 years (Table 5).

Amphetamine can be detected for 1-3 days in the urine.[7] In our study, amphetamine was detected in the urine samples of 16 of 293 (5.46%) patients (Table 3). No difference was observed when the use of amphetamine was evaluated with regard to sex and age groups (Table 4, 5). However, surprisingly, use of amphetamine was not be detected in patients aged 53 and above. MDMA can be detected for 1-3 days in the urine.[7] In our study, MDMA was detected in the urine samples of 23 of 293 (7.85%) patients (Table 3). No difference was observed when the use of MDMA was evaluated with regard to sex and age groups (Table 4, 5). Similarly, use of MDMA was not detected in patients aged 53 and above.

Cannabis is amongst the most frequently used drugs in many countries such as the United Kingdom and the United States of America. It has been reported that the 20% of the youth living in these countries use cannabis at least once a week.[8] Cannabis is used widely by young people throughout the world.[18,19]

In a study, it was concluded that young people aged between 12-17 years purchased cannabis more than those aged between 50 and 64 years, and the use of cannabis was greater in amongst the young. In the same study, the use of cannabis was reported stated to be higher amongst males compared with females.[20] A metabolite of cannabis, 11-nor-THC-9-carboxylic acid, can be detected for long periods in the urine.[21] In our study, cannabis was detected in the urine samples of 101 of 293 (34.47%) patients (Table 3). Although no difference between sex and use of cannabis was observed in our study (Table 4), a difference was determined to exist between the age groups and use of cannabis. The use of cannabis in the age groups of 13-22 years and 23-32 years, which are regarded as the young population, was determined to be higher than that of the other age groups (Table 5). The findings in our study resemble rates of cannabis use by young people throughout the world.

Drugs that containing synthetic cannabinoids in Turkey are known as Bonsai, and K2, spice, aroma, and dream in the global market have been widely used since 2006.[4] In July 2012, the United States Drug Enforcement Agency classified JWH-018, JWH-019, JWH-073, JWH-081, JWH-122, JWH-200, JWH-203, JWH-250, JWH-398, AM694, AM2201, RCS-4, RCS-8, HU-210, CP 47, 497-C7, CP 47, 497-C8 and their analogs as Schedule I controlled substances. Recently, UR-144, XLR11, and AKB48 were added to the Schedule I controlled substance list. Many countries use the same list.[22] In a study, synthetic cannabinoids

were detected in the blood samples of 29 patients aged 14 to 30 years who were admitted to the emergency service between 2008 and 2011, 25 of whom were males and 4 were females. During 2008-2009, JWH-018 was popular, in 2010, it was JWH-122, and in 2011, JWH-210 was widely detected.[23] In our study, synthetic cannabinoids were detected in the urine samples of 32 patients out of 293 (10.92%) (Table 3). No difference was observed when the use of synthetic cannabinoids was evaluated with regard to sex and age groups (Table 4, 5). However, similar to MDMA and amphetamines, use of synthetic cannabinoids was not detected in patients aged 53 years and above.

In our study, 19 types of synthetic cannabinoid molecules were analyzed in urine samples in order to detect the use of synthetic cannabinoids. As a result of the analysis, a total of 59 synthetic cannabinoid molecules were detected to be used by 32 of 293 patients (Figure 6). The top ranking metabolites of the 19 different synthetic cannabinoid molecules were respectively: UR144 N-pentanoic acid, UR144 N-5-hydroxypentyl, JWH-18 N-pentanoic acid, JWH-018 5-hydroxypentyl, AM-2201 6-hydroxyindole, JWH-018 5-hydroxyindole, AM-2201 4-hydroxyindole, JWH-250 5-hydroxyindole, JWH-073 N-butanoic acid, JWH-073 3-hydroxybutyl, and BB22 3-carboxyindole.

UR144 N-pentanoic acid, UR144 N-5-hydroxypentyl, and JWH-18 N-pentanoic acid were most frequently detected in the urine samples. The findings in our study and literature show differences in consumption of synthetic cannabinoids because the variety of synthetic cannabinoids changes rapidly around the world each year.

When the percentage distribution of the double, triple, and quadruple substance use of 97 patients who were identified to have used more than one substance was taken into consideration (Figure 7), it was determined that the use of cannabis (62.86%) was most common with alcohol, the use of cannabis (55.56%) was the most common with cocaine, the use of cannabis (51.61%) and cocaine (51.61%) were most common with morphine, the use of cocaine (50.00%) was the most common with amphetamines, the use of cannabis (84.21%) was the most common with MDMA, the use of cocaine (37.71%) was the most common with cannabis, and the use of cannabis (91.67%) was most common with synthetic cannabinoids (Table 6).When the results of the study are taken into consideration, the use of cannabis was widely observed in patients who used more than one drug.

To summarize our study, the drugs mostly detected in the urine samples of 293 patients were cannabis, alcohol, morphine, cocaine, synthetic cannabinoids, MDMA, and amphetamine, respectively. Cannabis took first place, and amphetamine was the least popular. Sex appeared to play no significant role in the preference of substance use. The rate of substance abuse according to age groups showed variations for alcohol, morphine, and cannabis. The use of alcohol increased in percentage as age increased. Use of morphine and cannabis was common amongst the young population. The most commonly detected molecules of the 19 synthetic cannabinoids that were subjected to analysis were: UR144 N-pentanoic acid, UR144 N-5-hydroxypentyl, JWH-18 N-pentanoic acid, JWH-018 5-hydroxypentyl, JWH-018 5-hydroxyindole, AM-2201 6-hydroxyindole, and AM-22014-hydroxyindole. The use of cannabis was widely observed in patients who used more than one drug.

CONCLUSION

The prevalence of alcohol and drug use in Turkey is considered to be at substantial levels and does not qualify as negligible. The data reflecting the dimensions of substance dependence in Turkey is known to heavily rely on arrests by Narcotics Police and some survey data, and statistical assessments of data obtained through analysis of biologic materials are limited. More reliable assessments are considered to be achieved by integrating analytically obtained data such as those in our study, together with data of surveys used in the determination of substance abuse profiles around Turkey and the world, or information received from national departments that investigate smuggling.

Conflict of Interest: No conflict of interest was declared by the authors.

REFERENCES

1. Smart RG, Ogborne AC. Drug use and drinking among students in 36 countries. Addict Behav. 2000;25:455-460.
2. UNODC. World Drug Report 2015, 2015.
3. UNODC. World Drug Report 2011, 2011.
4. Every-Palmer S. Warning: legal synthetic cannabinoid-receptor agonists such as JWH-018 may precipitate psychosis in vulnerable individuals. Addiction. 2010;105:1859-1860.
5. The Grand National Assembly of Turkey The Parliamentary Inquiry Commission Established to Search for Problems Regarding Substances SD, Dependence, Drug Trafficking and to Discern the Necessary Precautions. The problems determined in the area of substance use and dependence, in the prevention of drug trafficking and recommendations offered, 2009.
6. TUBIM. Turkish drug report 2012, 2012.
7. Verstraete AG. Detection times of drugs of abuse in blood, urine, and oral fluid. Ther Drug Monit. 2004;26:200-205.
8. TUBIM. Turkish drug report 2014, 2014.
9. Monteiro MG. A World Health Organization perspective on alcohol and illicit drug use and health. Eur Addict Res. 2001;7:98-103.
10. Erhan Deveci S, Açik Y, Ferdane Oguzöncül A, Deveci F. Prevalence and factors affecting the use of tobacco, alcohol and addictive substance among university students in eastern Turkey. Southeast Asian J Trop Med Public Health. 2010;41:996-1007.
11. Turhan E, İandia T, Özer C, Akoğlu S. Substance use, violence among university students and their some psychological characteristics. Turkish Journal of Public Health. 2011;9:33-44.
12. Keten A, Zeren C, Arslan MM, Daglıoglu N, Karanfil R, Şen BB. Determination of ethyl glucuronide in fingernails by LC/MS-MS. Rom J Leg Med. 2013;21:67-72.

13. Asan Ö, Tıkır B, Okay İH, Göka E. Sociodemographic and Clinical Features of Patients with Alcohol and Substance Use Disorders in a Specialized Unit. Journal of Dependence. 2005;16:1-8.

14. Mura P, Kintz P, Ludes B, Gaulier JM, Marquet P, Martin-Dupont S, Vincent F, Kaddour A, Goullé JP, Nouveau J, Moulsma M, Tilhet-Coartet S, Pourrat O. Comparison of the prevalence of alcohol, cannabis and other drugs between 900 injured drivers and 900 control subjects: results of a French collaborative study. Forensic Sci Int. 2003;133:79-85.

15. Cengiz G, Cecen SS, Soylemezoglu T. Determination of cocaine in urine by GC/MS method. Journal of Faculty of Pharmacy of Ankara University. 2004;33:125-149.

16. Mokri A. Brief overview of the status of drug abuse in Iran. Arch Iranian Med. 2002;5:184-190.

17. Duydu Y. Simultaneous determination of morphine, codeine and 6-monoacetyl morphine in urine by GC/MS method. Journal of Faculty of Pharmacy of Ankara University. 1998;27:101-113.

18. Moore TH, Zammit S, Lingford-Hughes A, Barnes TR, Jones PB, Burke M, Lewis G. Cannabis use and risk of psychotic or affective mental health outcomes: a systematic review. Lancet. 2007;370:319-328.

19. Silins E, Fergusson DM, Patton GC, Horwood LJ, Olsson CA, Hutchinson DM, Degenhardt L, Tait RJ, Borschmann R, Coffey C, Toumbourou JW, Najman JM, Mattick RP; Cannabis Cohorts Research Consortium. Adolescent substance use and educational attainment: An integrative data analysis comparing cannabis and alcohol from three Australasian cohorts. Drug Alcohol Depend. 2015;156:90-96.

20. Burns RM, Caulkins JP, Everingham SS, Kilmer B. Statistics on cannabis users skew perceptions of cannabis use. Front Psychiatry. 2013;4:138.

21. Grauwiler SB, Scholer A, Drewe J. Development of a LC/MS/MS method for the analysis of cannabinoids in human EDTA-plasma and urine after small doses of *Cannabis sativa* extracts. J Chromatogr B Analyt Technol Biomed Life Sci. 2007;850:515-522.

22. Scheidweiler KB, Huestis MA. Simultaneous quantification of 20 synthetic cannabinoids and 21 metabolites, and semi-quantification of 12 alkyl hydroxy metabolites in human urine by liquid chromatography-tandem mass spectrometry. J Chromatogr A. 2014;1327:105-117.

23. Hermanns-Clausen M, Kneisel S, Szabo B, Auwärter V. Acute toxicity due to the confirmed consumption of synthetic cannabinoids: clinical and laboratory findings. Addiction. 2013;108:534-544.

Turk J Pharm Sci 2017;14(3):304-310
DOI: 10.4274/tjps.25238

ORIGINAL ARTICLE

A Cost Saving and Waste Minimization Study About Handling of the Antineoplastic Agents

Antineoplastik İlaç Hazırlamada Tıbbi Malzeme Tasarrufu ve Atık İlaç Miktarının Azaltılması

Metin Deniz KARAKOÇ*

Denizli State Hospital, Zafer Gökşin Oncology Centre, Denizli, Turkey

ABSTRACT

Objectives: As a cancer treatment option, chemotherapy costs make up a large part of the budgets of social insurance foundations and related expenditures are increasing continuously annually. Cost saving and waste minimizing strategies are required to reduce the expenditures in the field of oncology. The study aimed to reduce the amount of wasted antineoplastic drugs and medical supply consumption.

Materials and Methods: The study explains why vials with a larger size and drugs in liquid form should be preferred over various smaller sizes and powder forms of antineoplastic preparations.

Results: Amounts of drug wastage, vial adaptor, and transfer set consumption data were recorded regularly for a period of seven months. The average vial adaptor consumption per patient in the last three months decreased from 5 to 3.3. The preference of liquid forms as much as possible instead of powder forms, which has a shorter stability time after dilution, and the choice of larger package sizes of frequently used drugs decreased vial adaptor consumption. Potential savings were calculated as around 31.660 USD annually. Costs of total wasted doses were 8.699.87 USD, and the whole antineoplastic drug consumption was 515.500 USD during the study. A decrease of 0.58 USD was observed per capita when the first and last three-month periods were compared in terms of waste costs.

Conclusion: These values indicate that the reduction of wasted drugs have potential annual savings of 3.375 USD. It is shown that total potential savings of 35.000 USD could be made per year. By implementing the same principles in all hospitals in Turkey, approximately 2.8 million USD could be made annually. The pharmaceutical industry and hospital pharmacists have important responsibilities in this issue.

Key words: Antineoplastic, cost saving, chemotherapy, drug waste, pharmacoeconomy

ÖZ

Amaç: Günümüzde kanser kemoterapisi giderleri sosyal güvenlik kurumlarının bütçelerinden önemli bir pay almakta ve harcamalar yıldan yıla sürekli olarak artmaya devam etmektedir. Onkolojideki giderleri azaltmak için tasarruf sağlayıcı ve atık ilaç miktarını azaltıcı stratejilere ihtiyaç vardır. Çalışma, hem tedaviden arta kalan yarım doz antineoplastik ilaçların hem de tedavide kullanılan tıbbi malzeme sarfiyatının azaltılmasını amaçlamaktadır.

Gereç ve Yöntemler: Çalışmada çeşitli ambalaj boyları ve toz formdaki flakonlar yerine mümkün olduğunca büyük boy flakonlar ve konsantre sıvı formdaki ilaçlar tercih edilmiştir. İmha edilen ilaç miktarı ile flakon adaptörü ve transfer seti sarfiyatları yedi ay müddetince düzenli olarak kaydedilmiştir.

Bulgular: Çalışmanın son üç ayında ortalama olarak hasta başına harcanan flakon adaptörü sayısının 5'ten 3.3'e düştüğü belirlenmiştir. Sulandırıldıktan sonraki stabilite süresi daha kısa olan toz formdaki antineoplastiklerin mümkün olduğunca konsantre sıvı form ilaçlarla değiştirilmesi ve sık kullanılan ilaçlarda daha büyük boy flakonlar kullanılmasıyla flakon adaptörü sarfiyatı azaltılarak tasarruf sağlanmıştır. Yıllık potansiyel tasarrufun 31.660 $ civarında olduğu hesaplanmıştır. Çalışma sırasında kullanılan antineoplastik ilaçların maliyeti 515.500 $ olurken bunun 8.699.87 $ tutarındaki kullanılmayan kısmının imha edildiği belirlenmiştir. Çalışmanın ilk üç aylık dönemi ile son üç aylık dönemi karşılaştırıldığında imha edilen ilaç miktarında hasta başına ortalama 0.58 $ azalma olduğu görülmüştür.

Sonuç: Çalışmada imha edilen ilaç miktarının azaltıldığı ve yılda 3.375 $ potansiyel tasarruf sağlamanın mümkün olduğu gösterilmiştir. Toplamda ise yılda 35.000 $ potansiyel tasarruf sağlanabileceği belirlenmiştir. Türkiye çapında bütün hastanelerde aynı prensipler uygulanarak yıllık 2.8 milyon $ tasarruf sağlanması mümkündür. Bu konuda ilaç endüstrisi ve hastane eczacılarına önemli sorumluluklar düşmektedir.

Anahtar kelimeler: Antineoplastik, maliyet tasarrufu, kemoterapi, ilaç imhası, farmakoekonomi

*Correspondence: **E-mail:** metindeniz.karakoc@saglik.gov.tr, **Phone:** +90 258 263 93 11 **ORCID-ID:** orcid.org/0000-0003-3188-8738
Received: 11.08.2016, **Accepted:** 09.02.2017

INTRODUCTION

Cancer is one of the leading causes of human deaths all over the world. According to the latest data reported from the International Agency for Research on Cancer, the estimated incidence will continue to increase in coming years. Therefore, cancer treatment has gained importance rapidly in the medical world, especially in recent years. Surgery, radiotherapy, and chemotherapy are commonly applied methods for cancer treatment. These treatments can be administered to patients with cancer individually, sequentially or in combination. Among these treatments, chemotherapy is the most frequently performed method, it is used all over the world as in our country, and stands out as the costliest treatment option.

Chemotherapy is the major treatment for most patients with cancer, even when other treatment options can not be applicable. There are many different chemotherapy administering protocols for several cancer types. According to the selected protocol, administered antineoplastic drug numbers and administration frequencies vary among patients. The number of administered antineoplastic drugs, existing dose forms on the market, and the administering frequency affects both the total cost of medical supplies and the amounts of wasted drugs during preparation. Antineoplastic drug doses are calculated for each patient by physicians using many criteria such as body surface area, renal and hepatic function, age, and sex.[1] Therefore, drug doses vary between patients. Generally, it is not possible for the pharmaceutical industry to determine ideal doses that would be sufficient for a patient in a single vial. Consequently, there is an amount of unavoidable drug waste if it is not possible to use the remaining dose for another patient within the stability period.[2,3]

The costs of anticancer drug developments and licensing are increasing rapidly, approximately 1 billion-1.8 billion USD in the United States of America.[4,5] In recent years, the expenditure for cancer treatment has increased, largely due to the increase in cancer prevalence, demographic changes, and the incorporation of new and expensive drugs into clinical practice. It was reported in a study that a seven-fold increase was seen in anticancer drug expenditure in Australia between 2000 and 2012.[6] This result is consistent with the results of another study conducted in Europe between 1993 and 2004.[7] It is a huge burden on government budgets and also individual cancer patients' expenditure. The current status brings responsibilities for health workers in relation to rational use of drugs and medical supplies.

The study aimed to reduce the amount of drug waste and medical supplies consumption by preferring larger size vials and liquid form drugs instead of various sizes and formulations of antineoplastic preparations and to achieve savings.

EXPERIMENTAL

The study was conducted in Denizli State Hospital Oncology Center. First, written permission (24.12.2015 - 16661972) was obtained from the hospital management for the study. In the study, we classified patients in Denizli State Hospital in terms of administered antineoplastic solution numbers as single, double, and three or more for a period of seven months (January - July, 2016) to determine the most economical chemotherapy administering sets. Each vial adaptor and transfer set that was used in the drug preparation was purchased for 3.2 USD before the study. Vial adaptor and transfer set consumption data per patient were recorded daily and regularly. Furthermore, total medical supply consumption and amount of drug waste was calculated in US dollars. All antineoplastic drug preparations in the study were performed in a bio-safety cabinet inside a validated negative pressure clean room using closed-system vial adaptors and transfer sets. Exposure to antineoplastic drugs during preparation and administration can present a health risk to medical staff.[8] According to the National Institute for Occupational Safety and Health, a closed-system transfer device is defined for use in compounding and administering sterile doses of chemotherapy and other hazardous drugs, as a drug transfer device that mechanically prohibits the transfer of environmental contaminants into the system, and the escape of hazardous drugs or vapor concentrations outside the system. The benefits of using closed-system transfer devices has been described in several previous studies.[8-13]

It is only possible to use a drug inside an opened vial to maintain both its microbiologic and physicochemical stability. No solution is added to the unused amount of concentrated liquid form drug vials. Thus, the drug's physicochemical stability continues as long as it is microbiologically stable. Manufacturing companies that provide medical supplies ensure that their products maintain a microbiologic barrier for seven days and support it with literature.[14] Therefore, concentrated liquid form drugs are accepted as microbiologically stable for seven days after setting the adaptors to the vials, and unused drugs at the end of seventh day are wasted. On the other hand, the physicochemical stability of lyophilized powder form drugs generally deteriorates rapidly after reconstitution. According to the manufacturer's instructions of drugs used in the study, the maximum stability period varied between 8 to 24 hours. Therefore, in compliance with the manufacturer's instructions, unused powder form drugs were wasted at the end of their maximum stability period.

A total of 32 different drugs were used throughout the study. Various dose form changes were made for 14 drugs. Larger sized and concentrated liquid forms were preferred instead of lyophilized powder forms that required dilution before use for 3 of 14 drugs (epirubicin, gemcitabine, and oxaliplatin). The use of docetaxel preparations that required ethanol as solvent stopped and 'ready to use' solutions were preferred that needed no dilution. For the other 10 of 14 drugs (bevacizumab, carboplatin, cisplatin, doxorubicin, etoposide, ifosfamide, irinotecan, paclitaxel, pemetrexed, and panitumumab), it larger sized vials with no formulation change were preferred. Concentrated liquid form drugs and larger sized packages were preferred in order to reduce vial adaptor consumption, achieve the greatest possible economic saving, and also shorten the drug preparation time by reducing the workload of medical staff. On the other hand, no dose form changes were made for 18 of 32 antineoplastic drugs used in the study (5-fluorouracil, azacitidine, bortezomib, dacarbazine, eribulin, liposomal doxorubicin, nab-paclitaxel,

raltitrexed, topotecan, trastuzumab, vinblastine, vincristine, fludarabine, cetuximab, cyclophosphamide, methotrexate, rituximab, and vinorelbine). The first thirteen drugs were devoid of alternative dose forms on the market, and the last five are rarely used and lesser consumed drugs compared with the others.

Each drug's consumption rate was taken into account separately. Purchasing procedures for the designed saving scheme started in January 2016, but the new drugs came to the hospital in late March 2016. The list of used intravenous antineoplastic drugs during the study, dose forms, and frequency scores (Table 1) are given below. The differences between the groups were

Table 1. List of used intravenous antineoplastic drugs and dosage forms during the study

	Drug name	Using frequency score*	First dose forms	Modified dose forms
1	5-Fluorouracil	5	1 g	1 g
2	Azacitidine	1	100 mg	100 mg
3	Bevacizumab	3	100 mg	100 and 400 mg
4	Bortezomib	1	3.5 mg	3.5 mg
5	Carboplatin	4	50 and 150 mg	450 mg
6	Cetuximab	2	100 mg	100 mg
7	Cisplatin	4	10 and 50 mg	100 mg
8	Cyclophosphamide	3	0.5 g	0.5 g
9	Dacarbazine	1	100 mg	100 mg
10	Docetaxel	4	20 and 80 mg	80 mg (ready to use)
11	Doxorubicin	3	10 mg	10 and 50 mg
12	Epirubicin	4	10 and 50 mg	50 mg
13	Eribulin	1	0.88 mg	0.88 mg
14	Etoposide	3	50 mg	100 mg
15	Fludarabine	1	50 mg	50 mg
16	Gemcitabine	4	200 mg and 1 g	1 g
17	Ifosfamide	1	0.5 g	1 g
18	Irınotecan	3	40 mg and 100 mg	300 mg
19	Liposomal doxorubicin	1	20 mg	20 mg
20	Methotrexate	1	50 mg	50 mg
21	Nab-paclitaxel	1	100 mg	100 mg
22	Oxaliplatin	3	50 mg	200 mg
23	Paclitaxel	4	30 and 100 mg	300 mg
24	Panitumumab	3	100 mg	100 and 400 mg
25	Pemetrexed	2	100 mg	100 and 500 mg
26	Raltitrexed	1	2 mg	2 mg
27	Rituximab	2	100 and 500 mg	100 and 500 mg
28	Topotecan	1	4 mg	4 mg
29	Trastuzumab	5	150 mg	150 mg
30	Vinblastine	1	10 mg	10 mg
31	Vincristine	2	1 mg	1 mg
32	Vinorelbine	1	50 mg	50 mg

*Scale of using frequency; 5: Every day, 4: At least 3 days per week, 3: At least 2 days per week, 2: Average 1-2 patient per week, 1: Average 1-3 patient per month

investigated using chi-square (χ^2) and the Kruskal-Wallis tests in the Statistical Package for the Social Sciences (SPSS) version 22.

RESULTS

At the beginning of the study, drugs were prepared according to the present inventory (Table 1- first dose forms) in January, February, and March 2016. The purchasing processes of new dose forms were completed in late March. In early April, newly purchased drugs and previous inventory drugs were used together. At the end of April, it was observed that a number of savings had been achieved despite the use of old and new type drugs together. Vial adaptor savings were observed (Table 2) in the months following April when compared with the previous months. The decrease in vial adaptor consumption in May, June, and July showed statistically significance when compared with January ($p<0.05$) and February ($p<0.001$).

It was determined that 51.94% of patients were administered single drugs, 34.07% were administered double, and 13.99% of patients were administered three or more antineoplastic drugs. The average consumption of transfer sets per patient was calculated as 1.63. Neither changes in treatment regimens nor numbers of patients caused a statistically significant difference ($p>0.05$) in average transfer set consumption. The preference of liquid forms as much as possible over powder forms and larger package size drugs decreased vial adaptor consumption. The average consumption of vial adaptors reduced to 3.3 from 5 in the last three months of the study compared with the first three months. During the study, an average of 485 patients were administered chemotherapy monthly. Considering that each vial adaptor bought for 3.2 USD, the potential annual savings of the hospital was calculated as 31.660 USD.

Beside the monetary savings, although it could not be shown by the numeric data, it was determined that using larger size packages and liquid form drugs significantly shortened the preparation time. Shortening of preparation time reduces the risk of exposure to cytotoxic agents on medical staff and also accelerates the services offered to patients.

Active ingredients of bortezomib, topotecan, bleomycin, cyclophosphamide, and ifosfamide were wasted in every month of the study. Although whole doses of 5-fluorouracil, cisplatin, trastuzumab, carboplatin, cetuximab, bevacizumab, docetaxel, etoposide, irinotecan, paclitaxel, panitumumab, raltitrexed, vinblastine, and vincristine agents were all used, none was wasted throughout the study. Using concentrated liquid form drugs that do not require dilution, enables longer storage for remaining doses and reduces the amount of wasted drugs. There were no wasted doses of epirubicin, oxaliplatin, and gemcitabine after we began to use liquid forms instead of powder forms. It was determined that there was a reduction in the cost of total wasted drugs (Figure 1) in the last four months of the study when compared with previous months.

The total cost of wasted doses was 8.699.87 USD, and the total antineoplastic drug consumption was 515.500 USD during the study. It was determined that 1.69% of the antineoplastic drugs that were prepared in the unit could not be used for other patients and wasted. A 0.58 USD decrease was observed

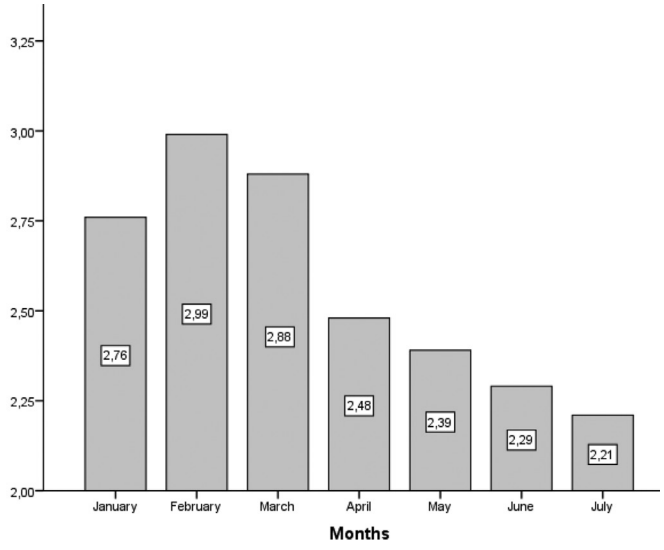


Figure 1. Monthly average of wasted drug costs ($) per chemotherapy

Table 2. Administered parenteral antineoplastic drug numbers and distributions with medical supply consumption and amounts of wasted drugs

Month	1 agent	2 agent	3 and 3+ agent	Total patient number	Vial adaptor consumption per patient	Average vial adaptor consumption per patient	Average transfer set consumption per patient	Wasted drug amount ($)	Wasted drug amount per patient ($)
January	248	145	68	461	2.306	5.00	1.64	1.272.36	2.76
February	253	130	80	463	2.320	5.01	1.62	1.384.37	2.99
March	262	142	75	479	2.402	5.01	1.59	1.379.52	2.88
April	239	183	63	485	2.086	4.30	1.64	1.202.80	2.48
May	246	182	59	487	1.554	3.19	1.61	1.163.93	2.39
June	256	187	63	506	1.786	3.53	1.62	1.158.74	2.29
July	260	188	67	515	1.637	3.18	1.69	1.138.15	2.21
Total	**1764**	**1157**	**475**	**3.396**	**14.091**			**8.699.87**	

per capita when the first and last three-month periods were compared in terms of waste costs. It was determined that the amount of wasted drugs could be reduced and 3.375 USD per year could potentially be saved when considering the monthly average number of patients administered chemotherapy during the study.

DISCUSSION

In recent years, many researchers aimed at cost saving and minimizing waste in oncology by using different methods. One of these was the dose rounding method, which may be an alternative way to reduce waste. It was reported in a study that 15.922 USD cost savings were achieved in a period of three months by dose rounding of biologic anticancer agents to an amount within 10% of the ordered dose.[15] In addition, it was shown in a recent study that using a computer for storing data on amount and stability of unused chemotherapy drugs could contribute to reducing total anticancer drug expenditure by about 5%.[16]

It was reported in a study that daily monitoring of antineoplastic drug consumption and an internal waste minimization protocol were able to achieve savings of 15.700 Euros every month. The projection of an annual cost-saving result of 188.000 Euros corresponds to a recovery of 4% on the spending for oncologic drugs.[17] These savings are relatively higher when compared with ours. The difference is probably based on the patient numbers and chemotherapy protocol differences between the studies. Similarly, in an another study, it was reported that 8.3% of antineoplastic drugs were wasted annually before applying a planned cost-saving protocol, and the authors observed a 45% reduction in drug waste expenditure.[18] Despite gaining great success as the monetary amount, the rate of wasted drug costs were much higher even in the final status than the average of our study (1.69%). Savings can be maximized by applying the dose rounding method together with the model that we applied in the study. However, we did not apply dose rounding, which is one of the shortcomings of the present study. Besides, administering rarely used and quite expensive specific medicines (e.g., bortezomib, eribulin, liposomal doxorubicin, nab-paclitaxel, pemetrexed) only in certain hospitals in a city can contribute to achieving savings by reducing the total amount of unused doses after opening vials instead of administering these drugs in every hospital.

The average cost of wasted drugs per month was calculated as 1.242.84 USD in the present study. However, a Turkish study performed about unused chemotherapy drugs reported a cost of 6.406.93 USD for wasted drugs within two months.[19] The disparity between the studies is probably due to different patient numbers or short storage times because of using needles in the other study instead of closed-system transfer devices.

There are three types of antineoplastic drug administering sets on the market; single, double, and four inlets. Increasing the inlet number raises the price of administering sets, which leads to elevated costs in chemotherapy. Therefore, it is not rational to administer single anticancer agents to patients with double or four inlet administration sets. Classifying patients according to the administered antineoplastic agent numbers and determining the use ratios enables more rational purchasing processes of the antineoplastic drug administering sets.

In coming years, it is possible that we will see a decrease in the use of intravenous antineoplastic agents because of the increasing administration of oral formulations. It has been shown in some studies that oral antineoplastic drugs improve patient treatment adherence and reduces waste.[20-22] On the other hand, an amount of oral antineoplastic drugs also become waste because of the inability to read and understand complex instructions; compliance risks, which may reflect inadequate treatment adherence; over adherence or reduced persistency; unanticipated drug interactions with food and other medications; and apparent non-responsiveness to the drug regimen.[23] Despite some of the disadvantages described above, the increase in oral antineoplastic drug use can make savings in health expenditures by reducing the waste when compared with intravenous antineoplastics.

Some drugs are marketed only in one package size, which leads to heavy spending on vial adaptors during preparation in some situations. Similarly, 5-fluorouracil and cisplatin are frequently used drugs for chemotherapy and the maximum marketed vials are 1 g and 100 mg, respectively. Solutions for the market such as 10 g 5-fluorouracil and 500 mg cisplatin vials would provide lower vial adaptor consumption and shorter preparation times.

Remaining doses in an opened vial can only be used if it is stable both microbiologically and physicochemically. In the study, the manufacturer's instructions were strictly followed regarding the maximum stability period for dilution-requiring drugs. However, there are conflicting results between manufacturer's instructions and some scientific studies for many antineoplastic drugs. For instance, the manufacturer of bortezomib advises a maximum stability period of 8 hours after dilution; however, a 2014 study reported that bortezomib diluted with isotonic sodium chloride maintained its physicochemical stability for up to 21 days.[24] Another study reported that bortezomib remained stable for five days in the vial after dilution.[25] Conflictingly, another study showed that bortezomib maintained its stability for up to 33 days at 2.5 mg/mL concentration at room temperature.[26] Moreover, there is an ongoing debate about the stability of topotecan. The manufacturer recommends using topotecan within 24 hours after dilution. However, it has been shown that topotecan remained stable for 28 days when diluted with sterile water.[27] The results of a different study were consistent with these results, although the authors tested at a different temperature.[28] On the other hand, in another study conducted by the same research team, topotecan hydrochloride was stable for up to 24 hours at room temperature and for up to 7 days at 5˚C in PVC and polyolefin infusion bags and glass bottles containing either 5% dextrose injection or 0.9% NaCl injection.[29]

There are numerous similar conflicts for most antineoplastic drug stability periods. It is observed that drug companies generally indicate in the instruction manuals that drugs maintain their stability for 8 to 24 hours after opening the

vials. Nevertheless, stability studies that have been conducted by many researchers revealed that the stability period of drugs were longer than those notified in the manufacturer's instruction manuals. The drug companies probably do not want to take risk about the patient's health and therefore indicate deliberately short-term stability periods for the opened vials. This subject merits further studies.

In Turkey, bortezomib and topotecan are marketed only in single dose forms as 3.5 mg and 4 mg vials, respectively. These expensive drugs are less consumed in terms of number when compared with the other drugs used in the study, and it is observed that the total doses of these drugs in a single vial are usually too much for one patient. Due to the lower circulation of patients and shorter storage time after dilution, the remaining doses of topotecan and bortezomib are constantly being wasted. It is thought that launching 2 mg and 0.5 mg for bortezomib and 1 mg for topotecan would reduce the amount of wasted drugs in chemotherapy units. Similarly, in a study that was published in 2011, it was reported that the average bortezomib dose per patient was 2.1 mg and the average amount of waste was 39.5%. Therefore, in the same study, it was recommended that the most convenient vial doses of bortezomib were 2.5 mg and 0.5 mg.[30]

There are very few studies about antineoplastic drug preparation and waste minimization in Turkey. In one of these studies, it was reported that preparing antineoplastic solutions was generally the responsibility of nurses.[19] However, in the last few years, this duty has been performed by hospital pharmacists in Turkey. The performance of this task by hospital pharmacists will increase the amount of savings by allowing more efficient evaluation of unused doses of antineoplastic drugs. Pharmacists are more competent about storage conditions and the maximum stability periods of opened antineoplastic drug vials. This situation can contribute to obtaining better results both economically and with patient health.

In the present study, it was shown how the application of a simple waste minimization model could reduce drug waste expenditure. Moreover, classifying patients according to the administered antineoplastic agent numbers gives an idea about selecting the most convenient chemotherapy administering sets for the future. Larger size and liquid form drug selections provided a cost saving via enabling more storage time and reduced vial adaptor consumption. Marketing larger sized vials for 5-fluorouracil and cisplatin would improve savings by reducing vial adaptor consumption and shortening the drug preparation period. Moreover, it should be more economical to launch smaller dose forms for bortezomib and topotecan in terms of reducing drug waste. Despite showing the reduction in vial adaptor consumption and amounts of wasted drugs, this is a single-center study and it is limited by its short duration. There is need for multi-center, long-term studies in the future.

CONCLUSION

High costs in cancer chemotherapy can be reduced with pharmacoeconomic approaches and rational use of drugs. It was shown that with 3.375 USD savings of waste drug reduction and 31.660 savings from medical supply consumption, a total of 35.000 USD potential savings could be made per year in our hospital. By applying the same principles countrywide in all hospitals that administer chemotherapy, it is possible to save approximately 2.8 million USD annually in Turkey.

The pharmaceutical industry and hospital pharmacists have important responsibilities in this issue. The medical industry has to redefine the dose forms of rarely used and expensive antineoplastic medicines considering the average application doses. Launching smaller dose forms of such medicines on the market would have a positive effect for the country's budget. On the other hand, as explained above, frequently used drugs in chemotherapy such as cisplatin and fluorouracil should be available in larger dose forms. This situation will reduce the number of vials used and lead to lesser consumption of medical supplies.

Drug preparation staff must be strictly controlled by pharmacists so as to ensure use of unused doses. Moreover, pharmacists should observe the usage frequency of all antineoplastic drugs to determine the most convenient dose forms for their hospital; keeping all forms of antineoplastic medicines in pharmacy stocks burdens hospital's financial balance and increases the workload of drug preparation staff. Preference of larger dose forms for frequently used liquid form drugs reduces vial adaptor consumption. Furthermore, for rarely used drugs, preferring smaller dose forms over larger forms will provide less unused medicine disposal and provide cost savings.

Conflict of Interest: No conflict of interest was declared by the authors.

REFERENCES

1. Gao B, Klumpen HJ, Gurney H. Dose calculation of anticancer drugs. Expert Opin Drug Metab Toxicol. 2008;4:1307-1319.
2. Paci A, Veal G, Bardin C, Levêque D, Widmer N, Beijnen J, Astier A, Chatelut E. Review of therapeutic drug monitoring of anticancer drugs part 1 cytotoxics. Eur J Cancer. 2014;50:2010-2019.
3. Vigneron J, Astier A, Trittler R, Hecq JD, Daouphars M, Larsson I, Pourroy B, Pinguet F; French Society of Hospital Pharmacists; European Society of Oncology Pharmacists. SFPO and ESOP recommendations for the practical stability of anticancer drugs: an update. Ann Pharm Fr. 2013;71:376-389.
4. Gupta SC, Sung B, Prasad S, Webb LJ, Aggarwal BB. Cancer drug discovery by repurposing: teaching new tricks to old dogs. Trends Pharmacol Sci. 2013;34:508-517.
5. Ruggeri BA, Camp F, Miknyoczki S. Animal models of disease: pre-clinical animal models of cancer and their applications and utility in drug discovery. Biochem Pharmacol. 2014;87:150-161.
6. Karikios DJ, Schofield D, Salkeld G, Mann KP, Trotman J, Stockler MR. Rising cost of anticancer drugs in Australia. Intern Med J. 2014;44:458-463.
7. Dranitsaris G, Ortega A, Lubbe MS, Truter I. A pharmacoeconomic modeling approach to estimate a value-based price for new oncology drugs in Europe. J Oncol Pharm Pract. 2012;18:57-67.

8. Vyas N, Yiannakis D, Turner A, Sewell GJ. Occupational exposure to anti-cancer drugs: A review of effects of new technology. J Oncol Pharm Pract. 2014;20:278-287.
9. Silver SR, Steege AL, Boiano JM. Predictors of adherence to safe handling practices for antineoplastic drugs: A survey of hospital nurses. J Occup Environ Hyg. 2016;13:203-212.
10. Edwards MS, Solimando DA Jr, Grollman FR, Pang JL, Chasick AH, Hightman CM, Johnson AD, Mickens MG, Preston LM. Cost savings realized by use of the PhaSeal® closed-system transfer device for preparation of antineoplastic agents. J Oncol Pharm Pract. 2013;19:338-347.
11. Vyas N, Turner A, Clark JM, Sewell GJ. Evaluation of a closed-system cytotoxic transfer device in a pharmaceutical isolator. J Oncol Pharm Pract. 2016;22:10-19.
12. Simon N, Vasseur M, Pinturaud M, Soichot M, Richeval C, Humbert L, Lebecque M, Sidikou O, Barthelemy C, Bonnabry P, Allorge D, Décaudin B, Odou P. Effectiveness of a Closed-System Transfer Device in Reducing Surface Contamination in a New Antineoplastic Drug-Compounding Unit: A Prospective, Controlled, Parallel Study. PLoS One. 2016;11:e0159052.
13. Massoomi FF, Neff B, Pick A, Danekas P. Implementation of a safety program for handling hazardous drugs in a community hospital. Am J Health Syst Pharm. 2008;65:861-865.
14. Bouza E, Muñoz P, López-Rodríguez J, Jesús Pérez M, Rincón C, Martín Rabadán P, Sánchez C, Bastida E. A needleless closed system device (CLAVE) protects from intravascular catheter tip and hub colonization: a prospective randomized study. J Hosp Infect. 2003;54:279-287.
15. Winger BJ, Clements EA, DeYoung JL, O'Rourke TJ, Claypool DL, Vachon S, VanDyke TH, Zimmer-Young J, Kintzel PE. Cost savings from dose rounding of biologic anticancer agents in adults. J Oncol Pharm Pract. 2011;17:246-251.
16. Respaud R, Tournamille JF, Saintenoy G, Linassier C, Elfakir C, Viaud-Massuard MC, Antier D. Computer-assisted management of unconsumed drugs as a cost-containment strategy in oncology. Int J Clin Pharm. 2014;36:892-895.
17. Mordenti P, Vecchia S, Damonti E, Riva A, Muroni M, Cordani MR, Cremona G, Cavanna L. An Anticancer Drug Unit for the whole provincial oncologic network of Piacenza: improving safety and savings. Med Oncol. 2015;32:457.
18. Fasola G, Aprile G, Marini L, Follador A, Mansutti M, Miscoria M. Drug waste minimization as an effective strategy of cost-containment in oncology. BMC Health Serv Res. 2014;14:57.
19. Ata A, Abali H, Yengel E, Arican A. It is not only the empty vials that go into the garbage can during chemotherapy drugs preparation: a cost analysis of unused chemotherapy drugs in cancer treatment. J BUON. 2012;17:781-784.
20. Khandelwal N, Duncan I, Ahmed T, Rubinstein E, Pegus C. Oral chemotherapy program improves adherence and reduces medication wastage and hospital admissions. Natl Compr Canc Netw. 2012;10:618-625.
21. Benjamin L, Buthion V, Iskedjian M, Farah B, Rioufol C, Vidal-Trécan G. Budget impact analysis of the use of oral and intravenous anti-cancer drugs for the treatment of HER2-positive metastatic breast cancer. J Med Econ. 2013;16:96-107.
22. Greer JA, Amoyal N, Nisotel L, Fishbein JN, MacDonald J, Stagl J, Lennes I, Temel JS, Safren SA, Pirl WF. A Systematic Review of Adherence to Oral Antineoplastic Therapies. Oncologist. 2016;21:354-376.
23. Khandelwal N, Duncan I, Ahmed T, Rubinstein E, Pegus C. Impact of clinical oral chemotherapy program on wastage and hospitalizations. Am J Manag Care. 2011;17:169-173.
24. Walker SE, Charbonneau LF, Law S. Stability of Bortezomib 2.5 mg/mL in Vials and Syringes Stored at 4°C and Room Temperature (23°C). Can J Hosp Pharm. 2014;67:102-107.
25. André P, Cisternino S, Chiadmi F, Toledano A, Schlatter J, Fain O, Fontan JE. Stability of bortezomib 1-mg/mL solution in plastic syringe and glass vial. Ann Pharmacother. 2005;39:1462-1466.
26. Berruezo garcia J, Espinosa Bosch M, Sanchez Rojas F, Bosch Ojeda C. Chemical stability of bortezomib solutions in original manufacturer vial at room temperature and in syringe at 4°C. Int J Pharm Bio Sci 2013;3:449-458.
27. Patel K, Craig SB, McBride MG, Palepu NR. Microbial inhibitory properties and stability of topotecan hydrochloride injection. Am J Health Syst Pharm. 1998;55:1584-1587.
28. Krämer I, Thiesen J. Stability of topotecan infusion solutions in polyvinylchloride bags and elastomeric portable infusion devices. J Oncol Pharm Practice. 1999;5:75-82.
29. Craig SB, Bhatt UH, Patel K. Stability and compatibility of topotecan hydrochloride for injection with common infusion solutions and containers. J Pharm Biomed Anal. 1997;16:199-205.
30. Clark L, Castro AP, Fortes AF, Santos F, Clark O, Engel T, Pegoretti B, Teich V, Vianna D, Puty F. Ideal vial size for bortezomib: real-world data on waste and cost reduction in treatment of multiple myeloma in Brazil. Value Health. 2011;14(5 Suppl 1):S82-84.

Turk J Pharm Sci 2017;14(3):311-318
DOI: 10.4274/tjps.43043

REVIEW

Lycopene: Is it Beneficial to Human Health as an Antioxidant?

Likopen: Antioksidan Olarak İnsan Sağlığına Faydalı mı?

Merve BACANLI[1]*, Nurşen BAŞARAN[1], A. Ahmet BAŞARAN[2]

[1]Hacettepe University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology, Ankara, Turkey
[2]Hacettepe University, Faculty of Pharmacy, Department of Pharmacognosy, Ankara, Turkey

ABSTRACT

It is well known that free oxygen radicals play an important role in the pathogenesis of several chronic disorders. Antioxidants are known as potential scavengers of reactive oxygen species that can protect biologic membranes against oxidative damage. Recent interest in phytochemicals has increased because of their protective effects against free oxygen radicals. Lycopene, which belongs to the carotenoid family, is the most effective singlet oxygen scavenger *in vitro* of all the carotenoids. Foods that contain lycopene and related supplements have been reported to prevent chronic diseases including cancer, asthma, and cardiovascular disorders. The aim of the article was to give a brief review of the antioxidant properties and beneficial health effects of lycopene.

Key words: Lycopene, antioxidant, health effects

ÖZ

Birçok kronik hastalığın patogenezinde serbest oksijen radikallerinin önemli rol oynadığı bilinmektedir. Antioksidanlar reaktif oksijen türevlerinin olası süpürücüleri olarak bilinir, bu nedenle biyolojik membranları oksidatif hasara karşı koruyabilirler. Serbest oksijen radikallerine karşı koruyucu etkileri nedeniyle fitokimyasallara ilgi giderek artmaktadır. Karetonoid ailesinde yer alan likopen tüm karotenoidler içerisinde *in vitro* şartlarda en etkili tekli oksijen süpürücüsüdür. Likopen içeren gıdalar ve likopen desteklerinin kanser, astım ve kardiyovasküler hastalıklar gibi kronik hastalıklara karşı koruyucu etkilerinin bulunduğu rapor edilmiştir. Bu makalede likopenin antioksidan özellikleri ve sağlığa yararlı etkilerine ilişkin bir özet verilmesi amaçlanmıştır.

Anahtar Kelimeler: Likopen, antioksidan, sağlık etkileri

INTRODUCTION

It is well known that free oxygen radicals play important roles in the pathogenesis of several chronic disorders such as cancer, diabetes, and cardiovascular and neurologic diseases.[1,2]

Antioxidants are known as potential scavengers of reactive oxygen species (ROS) that protect biologic membranes against oxidative damage. If the balance between free radicals and antioxidant defense is destroyed by chemicals, the imbalance may lead to damage to DNA, lipids, and proteins.[3] The role of oxidative stress induced by ROS and the oxidative damage of important biomolecules are the main focuses of research related to human diseases.[4]

Recent interest in phytochemicals, especially plant phenolics, has increased because of their protective effects against free oxygen radicals. Phenolic compounds are secondary metabolites that are derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways in plants.[5,6] They contribute to the sensory qualities of fruits and vegetables: color, astringency, bitterness, and aroma.[7]

It is estimated that approximately 50% of cancer cases and 35% of cancer deaths in the United States can be attributed to poor diet.[8] Many studies showed that fruit and vegetable-rich diets were associated with a decreased risk of chronic diseases.[9]

Carotenoids, which produce colors ranging from yellow to red, are synthesized by plants and microorganisms.[10] More than 700 carotenoids have been identified, about 40 of which are present in the human diet, and about 20 have been identified in blood and tissues.[11,12] β-carotene, α-carotene, lycopene, α-cryptoxanthine, lutein and zeoxanthin are the main carotenoids in the human diet.[12,13]

Lycopene (Figure 1), a noncyclic carotenoid found in tomatoes (*Solanum lycopersicum,* Solanaceae), has received considerable scientific interest in recent years. Red-colored fruits and vegetables are the main sources of lycopene in the human diet,

*Correspondence: E-mail: mervebacanli@gmail.com, Phone: +90 505 259 20 39 ORCID-ID: orcid.org/ 0000-0001-0757-0572
Received: 07.10.2016, Accepted: 01.12.2016

although not all red-colored plants contain lycopene. Its main function is to absorb light during photosynthesis and to protect plants against photosensitization.[10]

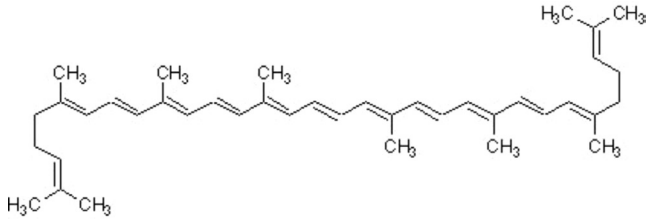


Figure 1. Lycopene

This article mainly focuses on the protective effects of lycopene against chronic diseases, and the chemistry and sources, dietary intake, mechanism of action, bioavailability, and pharmacokinetics of lycopene will also be discussed.

DATA COLLECTION

Information about lycopene was obtained from a literature search of electronic databases such as Google Scholar, PubMed, and Scopus for publications on health beneficial effects of lycopene. *Lycopene*, *antioxidant*, *tomato lycopene*, and *lycopene and health* were used as key terms.

Chemistry and sources of lycopene

Lycopene is found in processed tomato products, watermelon, pink grapefruits, papaya, and apricot.[19] Its chemical structure is an open chain hydrocarbon containing 11 conjugated and 2 non-conjugated double bonds arranged in a linear array. During chemical reactions, light or thermoenergy, these bonds can undergo isomerization from trans to mono or poly *cis*-isomers. It lacks a β-ionene ring structure and has no provitamin A activity, but exhibits a physical quenching rate constant with singlet oxygen ($^1O_2$) almost twice as high as that of β-carotene. Its molecular formula is $C_{40}H_{56}$ and it has all-*trans*, 5-*cis*, 9-*cis*, 13-*cis* and 15-*cis* isoforms.[19] The *trans* isoform is commonly found in the human diet and the *cis* isoform is found in human blood, plasma, breast milk, and human tissues.[22-25] The color of lycopene is related to its isomeric form.[22] Antioxidant potential of lycopene isomers are 5-*cis*>9-*cis*>7-*cis*>13-*cis*>15-*cis*>11-*cis*>all-trans, respectively.[26]

Dietary intake levels of lycopene

The United States Food and Drug Administration in America granted Generally Recognized as Safe status to lycopene as a nutritional supplement.[10] Dietary intake of lycopene varies due to populations. Lycopene intake in Italy with an average intake of 7.4 mg/day is greater than in other countries.[14] The average intake of lycopene is 6.6-10.5 mg/day for men and 5.7-10.4 mg/day for women in the United States, 1.1 mg/day in the United Kingdom, 1.6 mg/day in Spain, 3.8 mg/day in Austria, 4.8 mg/day in France, and 4.9 mg/day in the Netherlands.[15,16]

For many years, lycopene containing foods and lycopene supplements have been used without any safety problems. Rao and Agarwal[17] (1998) demonstrated that lycopene intake levels varied from 5 to 75 mg/day in tomato juice, tomato sauce, and nutritional supplements in healthy humans. No adverse effects due to lycopene consumption were observed. Similarly, no evidence of toxic effects of lycopene were seen with two synthetic crystalline lycopene (BASF lycopene 10 CWD and Lyco Vit 10%, each containing approximately 10% lycopene)[18] and lycopene derived from a fungal biomass of *Blakeslea trispos*, suspended in sunflower oil in rats.[19] No teratogenic effects were also observed in a two-generation rat study.[20]

Bioavailability and kinetics of lycopene

Lycopene bioavailability can be affected by many factors such as food processing and dietary composition.[21] Food processing in particular and thermal processing may improve lycopene bioavailability by breaking down cell walls, which weakens the bonding forces between lycopene and the tissue matrix, thus making lycopene more accessible and enhancing the *cis*-isomerization. The bioavailability of *cis*-isomers in food is higher than that of all-*trans* isomers.

The amount of lycopene present in processed tomato products is often much higher in fresh tomatoes given that processing often involves concentrations via water loss. Multiple studies have shown that lycopene from thermally processed tomato products was more bioavailable than lycopene from fresh tomatoes.[22] Ketchup contains 9.9-13.44 mg lycopene/100 g, whereas fresh tomatoes contain 0.88-7.44 mg lycopene/100 g wet weight.[23,24]

Lycopene bioavailability is also greatly affected by dietary composition. Consuming lycopene with fat increases its bioavailability because lycopene is a lipid-soluble compound. For example, consuming salads with full-fat dressing results in higher blood carotenoid levels than eating salads with reduced fat dressing. When salads were consumed without fat in the same study, no measurable lycopene uptake occurred.[25] The consumption of tomato salsa with avocado (as lipid source) led to a 4.4-fold increase in lycopene absorption as compared with salsa without avocado.[26]

It is believed that lycopene was absorbed by passive diffusion as lipids. Many studies suggested that lycopene absorption may be facilitated by other transporters, but this has not yet been confirmed.[27,28] Competition by other carotenoids or cholesterol may also influence lycopene absorption.[16] Age, sex, hormonal status, body mass and composition, blood lipid levels, smoking and alcohol may influence the absorption of lycopene.[29] Lycopene is found in the highest concentrations in the liver, testes, adrenal glands, and adipose tissues. It is found in lower concentrations in the kidney, ovary, lung, and prostate.[30]

Very little is known about the *in vivo* metabolism of lycopene. Only a few metabolites, such as 5,6-dihydroxy-5,6-dihydro lycopene, have been detected in human plasma. It is suggested that lycopene may undergo *in vivo* oxidation to form epoxides, which then may be converted to the polar 5,6-dihydoxy-5,6-dihydrolycopene through metabolic reduction.[31] In humans,

lycopene absorption is in the range of 10-30%, with the remainder being excreted.[17,29]

Health protective effects of lycopene

The biologic activities of carotenoids depend on their provitamin A activity, but lycopene does not have provitamin activity due to the lack of a β-ionic ring structure.[32] The main biologic activity of lycopene is thought be its antioxidant properties. It is the most effective $^1O_2$ scavenger *in vitro* of all the carotenoids.[33]

Increasing clinical evidence supports the role of lycopene as a micronutrient with important health benefits because it is suggested to play a role in disorders related to oxidative stress and cancer. It is a highly efficient antioxidant with a $^1O_2$ and has free radical scavenging capacity. Lycopene can scavenge oxygen radicals, reduce oxidative stress, and prevent ROS generation. It may protect against the *in vivo* oxidation of lipids, proteins, and DNA.[34,35]

Inhibition of insulin-like growth factor-I and androgen signaling, interleukin-6 expression, improving immune responses and gap junction communication (GJC), induction of phase II drug metabolizing enzymes and oxidative defense genes are the important suggested non-oxidative mechanisms of action of lycopene.[36]

Human tumors are generally deficient in GJC and its upregulation is associated with the decreased proliferation of tumor cells. Thereby, improving GJC is a suggested mechanism in the prevention of cancers. With this effect, lycopene could be an anticarcinogenic compound.[10,37] Suppression of the carcinogen-induced phosphorylation of regulatory proteins such as p53 and Rb antioncogenes by lycopene may also play an important role in the suppression of cell division at the G-G1 cell cycle phase.[38]

Lycopene acts as a hypocholesterolemic agent by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A reductase. This mechanism may be associated with the reduction of risk of cardiovascular diseases.[39] In experimental animals, lycopene induced immunoenhancement.[40] The increase in the phenotypic and functional maturation of dendritic cells by lycopene was also reported.[41]

Cancer

The consumption of tomatoes and tomato products has been associated with a reduced risk of a number of different types of cancers.[42] A study of older Americans indicated that a high intake of tomatoes was associated with a significant reduction in mortality from cancers of all sites.[43,44] In the Mediterranean, the incidence of cancer is lower due to the rich diet in tomatoes and tomato products.[45]

It has been shown that lycopene reduced the oxidative injury by stimulating the levels and activities of antioxidant enzymes including glutathione (GSH), glutathione-S-transferases, and glutathione peroxidase in animals with gastric cancer.[46] Lycopene prevents the oxidative damage of DNA, lipids, and proteins.[47] It modulates immune function, and induces apoptotic cell death.[10] It is also suggested to inhibit ROS production and decrease the phosphorylation of extracellular signal-regulated kinase (ERK), which results in the inhibition of cancer cell growth.[34,48,49] Lycopene inhibited phosphorylation of ERK, which is a major regulator of cell proliferation, apoptosis, and differentiation in gastric cells, as well as hepatocarcinoma cells.

Lycopene also decreased Bcl-2 and increased levels of Bax, which induce the release of cytochrome C and other pro-apoptotic factors from mitochondria, leading to apoptosis. Bcl-2 is an important anti-apoptotic protein that regulates cell death. Bcl-2 inhibits apoptosis by reducing caspase activation such as caspase 3 and 8. Bax protein, a member of the Bcl-2 family of proteins, is a regulator of apoptosis and promotes apoptosis.[50,51] Lycopene treatment inhibited cancer cell proliferation by increasing cell cycle arrest in the G0-G1 phase.[52]

Lycopene supplementation prevented changes in p53 expression in gastric mucosa of ferrets and it was suggested that lycopene may protect against the development of gastric cancer by inhibiting p53 dependent apoptosis and providing the balance of apoptosis and cell proliferation. Lycopene also prevented changes in p53 overexpression in gastric mucosa exposed to cigarette smoke.[53] The main evidence in support of the role of lycopene in the prevention of these cancers comes from cell culture, animal, and epidemiologic studies.[10]

Of all cancers, the role of lycopene in the prevention of prostate cancer has been studied the most.[10] Hall[54] (1996) and Kotake-Nara et al.[55] (2001) demonstrated that lycopene inhibited the growth of DU145 prostate cancer cells. Similar to these studies, Kim et al.[56] (2002) showed the protective effects of lycopene as an antioxidant on LNCaP prostate cancer cells growth. The effect of whole tomato powder (13 mg lycopene per kg diet), lycopene beadlets (161 mg lycopene per kg diet), and control beadlets (0 mg lycopene per kg diet) were evaluated for their effect on prostate cancer in a rat model. The study showed that the consumption of tomato powder, but not lycopene, inhibited prostate carcinogenesis.[57] In a cohort study, Seventh-Day Adventist men who consumed high levels of tomato products more than five times per week had significantly decreased risk of prostate cancer compared with men who consumed lower amounts of tomato products less than once per week.[58] In another study, lycopene induced the apoptosis of pancreatic cancer cells by suppressing the expression of survivin, cIAP1, and cIAP2. Lycopene may be a promising therapeutic agent for human pancreatic cancer.[59]

Gastric (stomach) cancer remains as one of the major causes of cancer death in the world.[60,61] Dietary factors are believed to play an important role in the prevention of gastric cancer, among which dietary carotenoids have received considerable interest.[62-64] Inverse associations between tomato or lycopene consumption (intake) and gastric cancer risk were observed in a variety of populations.[44,65-76] A suggestive, but not statistically significant, inverse association was observed in a study conducted in Belgium with 449 subjects even though the study population had a low consumption of tomatoes.[74] In an ecologic study, plasma levels of various nutrients in samples of Japanese populations in various regions were evaluated and the lowest gastric cancer rates were found in regions higher

in plasma lycopene, whereas regions low in lycopene had the highest rates.[77] Ito et al.[78] (2005) examined cancer mortality and serum levels of carotenoids, retinol and tocopherol, in the inhabitants of a rural area of Japan and found that serum levels of lycopene were associated with a reduced risk of death from stomach cancer. They suggested that lycopene may be a promising biomarker to predict mortality related with stomach cancer.

It has been concluded that consumption of lycopene-containing foods may decrease for risk of breast cancers. Cui et al.[79] (2008) found that lycopene consumption was inversely associated with estrogen and progesterone receptor positive breast cancer risk in postmenopausal women. Lung cancer is the leading cause of cancer death for both men and women. Epidemiological studies suggest that higher intake of lycopene is associated with either a reduced risk of lung cancer[80], or no change in lung cancer risk, as compared with lower intake levels.[81] The protective effects against ovarian cancer, colorectal cancer, and pancreatic cancer were demonstrated in different epidemiologic studies.[16]

Cardiovascular diseases

Cardiovascular disease is a leading cause of death in America and the Western world.[82] Oxidation of low-density lipoprotein (LDL) is the main mechanism of cardiovascular diseases. Thus, antioxidants may have an effect in reducing LDL oxidation. Due to this effect, lycopene may be beneficial in cardiovascular diseases as an antioxidant.[83]

Low plasma lycopene levels were reported by many researchers in hypertension, myocardial infarction, stroke, and atherosclerosis.[84] Lycopene was decreased in carotid artery intima-media thickness.[85] Some clinical trials have also supported a relationship between cardiovascular disease and lycopene intake. In a study, 19 healthy subjects consumed placebo (0 mg lycopene), tomato juice (50.4 mg lycopene), spaghetti sauce (39.2 mg lycopene), and tomato oleoresin (75 mg lycopene) treatment daily for one week and went through a one-week washout period between each treatment week. The serum lycopene concentration doubled in subjects on lycopene-containing treatments and also a significant decrease in serum lipid peroxidation and LDL oxidation was observed after subjects consumed any one of the three lycopene-containing treatments.[86] In an another study, healthy individuals received one of three tomato treatments for 15 days (condensed tomato soup, ready-to-serve tomato soup, or V8® vegetable juice). Blood samples were taken at baseline and after treatment. A measure of protection against oxidative stress was significantly increased in all three treatment groups.[87] Shen et al.[88] (2007) treated 24 subjects with either fresh tomato, tomato juice, or a lycopene drink (all delivering 40 mg lycopene/day) for six weeks. It was found that triglyceride levels and LDL were decreased, and high-density lipoprotein (HDL) increased in subjects who consumed fresh tomato and tomato juice.[88] Bohn et al.[89] (2009) demonstrated that soy tomato beverage consumption significantly reduced LDL+VLDL levels and increased HDL levels in 18 healthy men and women.

Neurodegenerative diseases

The high lipid content of the nervous system, low antioxidant capacity, and the presence of iron, coupled with its high aerobic metabolic activity, make it particularly susceptible to oxidative damage.[90] Most studies have shown the effects of antioxidants on nervous system disorders including Alzheimer's disease, Huntington's disease, and Parkinson disease.[91] In an animal study, a beneficial effect of lycopene supplementation in rotenone-induced Parkinson's disease was demonstrated.[92] Lycopene showed protective effects against myeloid β-induced neurotoxicity in cultured rat cortical neurons[93], 3-nitropropionic acid-induced mitochondrial oxidative stress, and dysfunctions in nervous system[94], and trimethyltin-induced neurotoxicity in primary cultured rat hippocampal neurons.[95]

Gastrointestinal diseases

A significant increase has been seen in peptic ulcer incidence worldwide. The generation of ROS is the major cause of stress-induced ulcers.[96] Therefore, it is suggested that powerful antioxidants may be useful in the treatment of ulcers.[97] Accordingly, to overcome the adverse effects of drugs and provide efficacious and safe therapy, herbal antioxidants may be useful due to their antioxidant effects.[98] The anti-ulcer activity of lycopene can be attributed to different mechanisms, including inhibition of gastric acid secretion, reinforcement of the gastric mucosal barrier, and its free radical scavenging activity.[99] It has been shown that lycopene (2 mg/kg) and hesperidin (100 mg/kg) decreased gastric secretions and total acidity as well as increased gastric pH due to the restoration of normal gastric conditions in ulcer-induced rats.[98] Similarly, Boyacioglu et al.[13] (2016) demonstrated that lycopene treatment exhibited protection against indomethacin-induced gastric ulcer in rats in a dose-dependent manner. In the same study, it was demonstrated that superoxide dismutase (SOD) activity and GSH levels were higher in the lycopene-treated group, and catalase (CAT) activity and malondialdehyde (MDA) levels were lower in the lycopene-treated group when compared with controls. These results suggest that lycopene had antioxidant effects on the treatment of ulcers.[13]

Helicobacter pylori is an important risk factor for chronic gastritis, peptic ulcer, and gastric carcinoma. In *H. pylori*-induced gastric injury, ROS is the major toxic factor.[100] Lycopene is reported to have a significant inhibitory effect on gastric acid secretion followed by efficacy against *H. pylori* infections.[101-103] Jang et al.[100] (2012) showed that lycopene rescued the *H. pylori*-infected cells from DNA damage and apoptosis in gastric epithelial cells. It also inhibited *H. pylori*-induced increases in ROS production and alterations in the cell cycle.[100]

Gastroesophageal reflux disease (GERD) commences due to reflux of gastric content into the esophagus, which results in mucosal devastation.[104] ROS play an important role in the pathogenesis of GERD.[105] In rats, lycopene (50 and 100 mg/kg) showed significant protection against experimental esophagitis. The gastric content is a major factor in the pathogenesis of GERD and it is claimed that lycopene decreased the acidity (total and free) and gastric volume, and thereby subsequently

increased the pH. Modulation in pH and acidity of gastric content after lycopene treatment was also reflected through a decrease in esophagitis indices. In the same study, a positive modulation in GERD by lycopene was seen in physiologic changes. Similar to other studies, it was shown that SOD and CAT enzyme activities and GSH levels were higher in the lycopene group and MDA levels were lower when compared with controls.[106]

Bone health

Oxidative stress, which has been shown to control the function of both osteoclasts and osteoblasts, may contribute to the pathogenesis of the skeletal system including the most prevalent metabolic disease, osteoporosis. A number of studies revealed that ROS increase bone resorption. Others suggested that ROS may be involved in the regulation of osteoclast formation and osteoclast motility. Limited studies demonstrated that lycopene had beneficial effects on bone health.[107]

Kim et al.[108] (2002) showed that lycopene stimulated the proliferation of osteoblast-like SaOS-2 cells. On the other hand, Park et al.[109] (1997) reported that lycopene had an inhibitory effect on the cell proliferation of MC3T3 cells, which are osteoblastic cells of lower species. However, both studies reported a stimulation of alkaline phosphatase activity. Rao et al.[110] (2003) cultured cells from bone marrow prepared from rat femurs in 16-well calcium phosphate-coated OsteologicTM multi-test slides (Millenium Biologix Inc.). Varying concentrations of lycopene in the absence or presence of the resorbing agent PTH-(1-34) were added at the start of culture and at each medium change every 48 hours. Lycopene inhibited TRAP+ multinucleated cell formation in both vehicle- and PTH-treated cultures. The number of cells stained with the NBT reduction product formazan was decreased by treatment with 10^{-5} M lycopene, indicating that lycopene inhibited the formation of ROS-secreting osteoclasts.[110] Ishimi et al.[111] (1999) in murine osteoclasts formed in coculture with calvarial osteoblasts. Furthermore, they could not demonstrate any effect of lycopene on bone resorption.[111]

Other diseases

Oxidative stress has been suggested as an important contributory factor in male infertility.[112] Antioxidants for male infertility could potentially have a great impact on the management of couples with infertility. To date, a small number of studies have evaluated the role of vitamins and antioxidants (mostly as single agents) in male infertility, but additional studies are needed.[10] Dawson et al.[113] (1993) demonstrated the activity of vitamin C on semen quality, and Geva et al.[114] (1996) and Suleiman et al.[115] (1996) showed the activity of vitamin E on semen quality. Men with antibody-mediated infertility were found to have lower semen lycopene levels than fertile controls.[116]

Low serum levels of lycopene have also been associated with increased risk of psychiatric disorders.[117]

A study with 17 adults with asthma treated with placebo, tomato extract (Lyc-o-Mato®, 45 mg/day lycopene), and tomato juice (45 mg/day of lycopene) for 7 days showed reduced airway neutrophil influx and reduced sputum neutrophil elastase activity after the tomato extract and tomato juice treatment. During placebo treatment, plasma lycopene concentrations decreased, the percentage of neutrophils increased, and neutrophil elastase levels increased.[118]

CONCLUSION

Diets rich in fruits and vegetables are associated with a lower risk of numerous diseases including cancers. Intake of fruits and vegetables that contain high levels of vitamins C and A, phenolics, and carotenoids for reducing cancer risk have been made by several researchers.

Lycopene is one of the major carotenoids in Western diets, accounting for more than 50% of carotenoids in humans. A brief summary of the literature relating to lycopene and its role in health is presented in this article. Promising data from epidemiologic as well as cell culture and animal studies suggest that lycopene and the consumption of lycopene-containing foods may affect several chronic disorders. Nevertheless, more clinical data are needed to support this hypothesis. In addition, further detailed research is required to understand other beneficial health effects of lycopene and its mechanisms.

Conflict of Interest: No conflict of interest was declared by the authors.

REFERENCES

1. Rao YK, Geethangili M, Fang SH, Tzeng YM. Antioxidant and cytotoxic activities of naturally occurring phenolic and related compounds: a comparative study. Food Chem Toxicol. 2007;45:1770-1776.
2. Lima CF, Fernandes-Ferreira M, Pereira-Wilson C. Phenolic compounds protect HepG2 cells from oxidative damage: relevance of glutathione levels. Life Sci. 2006;79:2056-2068.
3. Halliwell B, Chirico S. Lipid peroxidation: its mechanism, measurement, and significance. Am J Clin Nutr. 1993;57(Suppl 5):715S-724S.
4. Rao A, Rao L. Lycopene and human health. Curr Top Nutraceutical Res. 2004;2:127-136.
5. Balasundram N, Sundram K, Samman S. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. Food Chem. 2006;99:191-203.
6. Bravo L. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutr Rev. 1998;56:317-333.
7. Alasalvar C, Grigor JM, Zhang D, Quantick PC, Shahidi F. Comparison of volatiles, phenolics, sugars, antioxidant vitamins, and sensory quality of different colored carrot varieties. J Agricul Food Chem. 2001;49:1410-1416.
8. Williams GM, Williams CL, Weisburger JH. Diet and cancer prevention: the fiber first diet. Toxicol Sci. 1999;52(Suppl 2):72-86.
9. Ignarro LJ, Balestrieri ML, Napoli C. Nutrition, physical activity, and cardiovascular disease: an update. Cardiovasc Res. 2007;73:326-340.
10. Rao AV, Ray MR, Rao LG. Lycopene. Adv Food Nutr Res. 2006;51:99-164.
11. Agarwal S, Rao AV. Carotenoids and chronic diseases. Drug Met Drug Interact. 2000;17:189-210.
12. Britton G, Liaaen-Jensen S, Pfander H. In: Carotenoids volume 5: nutrition and health, Ed(s): Springer Science & Business Media, 2009.

13. Boyacioglu M, Kum C, Sekkin S, Yalinkilinc HS, Avci H, Epikmen ET, Karademir U. The effects of lycopene on DNA damage and oxidative stress on indomethacin-induced gastric ulcer in rats. Clin Nutr. 2016;35:428-435.

14. Lucarini M, Lanzi S, D'Evoli L, Aguzzi A, Lombardi-Boccia G. Intake of vitamin A and carotenoids from the Italian population results of an Italian total diet study. Int J Vit Nutr Res. 2006;76:103-109.

15. Porrini M, Riso P. What are typical lycopene intakes? J Nutr. 2005;135:2042S-2045S.

16. Story EN, Kopec RE, Schwartz SJ, Harris GK. An update on the health effects of tomato lycopene. Annu Rev Food Sci Technol. 2010;1:189-210.

17. Rao AV, Agarwal S. Bioavailability and *in vivo* antioxidant properties of lycopene from tomato products and their possible role in the prevention of cancer. Nutr Cancer. 1998;31:199-203.

18. Mellert W, Deckardt K, Gembardt C, Schulte S, Van Ravenzwaay B, Slesinski R. Thirteen-week oral toxicity study of synthetic lycopene products in rats. Food Chem Toxicol. 2002;40:1581-1588.

19. Jonker D, Kuper CF, Fraile N, Estrella A, Rodríguez Otero C. Ninety-day oral toxicity study of lycopene from Blakeslea trispora in rats. Regul Toxicol Pharmacol. 2003;37:396-406.

20. Michael McClain R, Bausch J. Summary of safety studies conducted with synthetic lycopene. Regul Toxicol Pharmacol. 2003;37:274-285.

21. Xianquan S, Shi J, Kakuda Y, Yueming J. Stability of lycopene during food processing and storage. J Med Food. 2005;8:413-422.

22. Gärtner C, Stahl W, Sies H. Lycopene is more bioavailable from tomato paste than from fresh tomatoes. Am J Clin Nutr. 1997;66:116-122.

23. Nguyen ML, Schwartz SJ. Lycopene: chemical and biological properties. Food technology (USA). 1999.

24. Rao AV, Waseem Z, Agarwal S. Lycopene content of tomatoes and tomato products and their contribution to dietary lycopene. Food Res Int. 1998;31:737-741.

25. Brown MJ, Ferruzzi MG, Nguyen ML, Cooper DA, Eldridge AL, Schwartz SJ, White WS. Carotenoid bioavailability is higher from salads ingested with full-fat than with fat-reduced salad dressings as measured with electrochemical detection. Am J Clin Nutr. 2004;80:396-403.

26. Unlu NZ, Bohn T, Clinton SK, Schwartz SJ. Carotenoid absorption from salad and salsa by humans is enhanced by the addition of avocado or avocado oil. J Nutr. 2005;135:431-436.

27. During A, Dawson HD, Harrison EH. Carotenoid transport is decreased and expression of the lipid transporters SR-BI, NPC1L1, and ABCA1 is downregulated in Caco-2 cells treated with ezetimibe. J Nutr. 2005;135:2305-2312.

28. Moussa M, Landrier JF, Reboul E, Ghiringhelli O, Coméra C, Collet X, Fröhlich K, Böhm V, Borel P. Lycopene absorption in human intestinal cells and in mice involves scavenger receptor class B type I but not Niemann-Pick C1-like 1. J Nutr. 2008;138:1432-1436.

29. Stahl W, Sies H. Lycopene: a biologically important carotenoid for humans? Arch Biochem Biophys. 1996;336:1-9.

30. Kun Y, Ssonko Lule U, Xiao-Lin D. Lycopene: Its Properties and Relationship to Human Health. Food Rev Int. 2006;22:309-333.

31. Khachik F, Carvalho L, Bernstein PS, Muir GJ, Zhao DY, Katz NB. Chemistry, distribution, and metabolism of tomato carotenoids and their impact on human health. Exp Biol Med (Maywood). 2002;227:845-851.

32. Clinton SK. Lycopene: chemistry, biology, and implications for human health and disease. Nutr Rev. 1998;56:35-51.

33. Sies H, Stahl W. Vitamins E and C, beta-carotene, and other carotenoids as antioxidants. Am J Clin Nutr. 1995,62(6 Suppl):1315S-1321S.

34. Agarwal S, Rao AV. Tomato lycopene and its role in human health and chronic diseases. CMAJ. 2000;163:739-744.

35. Tapiero H, Townsend DM, Tew KD. The role of carotenoids in the prevention of human pathologies. Biomed Pharmacother. 2004;58:100-110.

36. Wertz K, Siler U, Goralczyk R. Lycopene: modes of action to promote prostate health. Arch Biochem Biophys. 2004;430:127-134.

37. Zhang LX, Cooney RV, Bertram JS. Carotenoids enhance gap junctional communication and inhibit lipid peroxidation in C3H/10T1/2 cells: relationship to their cancer chemopreventive action. Carcinogenesis. 1991;12:2109-2114.

38. Matsushima-Nishiwaki R, Shidoji Y, Nishiwaki S, Yamada T, Moriwaki H, Muto Y. Suppression by carotenoids of microcystin-induced morphological changes in mouse hepatocytes. Lipids. 1995;30:1029-1034.

39. Fuhrman B, Elis A, Aviram M. Hypocholesterolemic effect of lycopene and beta-carotene is related to suppression of cholesterol synthesis and augmentation of LDL receptor activity in macrophages. Biochem Biophys Res Commun. 1997;233:658-662.

40. Chew BP, Park JS. Carotenoid action on the immune response. J Nutr. 2004;134:257S-261S.

41. Kim GY, Kim JH, Ahn SC, Lee HJ, Moon DO, Lee CM, Park YM. Lycopene suppresses the lipopolysaccharide-induced phenotypic and functional maturation of murine dendritic cells through inhibition of mitogen-activated protein kinases and nuclear factor-kappaB. Immunology. 2004;113:203-211.

42. Giovannucci E, Rimm EB, Liu Y, Stampfer MJ, Willett WC. A prospective study of tomato products, lycopene, and prostate cancer risk. J Natl Cancer Inst. 2002;94:391-398.

43. Colditz GA, Branch LG, Lipnick RJ, Willett WC, Rosner B, Posner BM, Hennekens CH. Increased green and yellow vegetable intake and lowered cancer deaths in an elderly population. Am J Clin Nutr. 1985;41:32-36.

44. Franceschi S, Bidoli E, La Vecchia C, Talamini R, D'Avanzo B, Negri E. Tomatoes and risk of digestive-tract cancers. Int J Cancer. 1994;59:181-184.

45. La Vecchia C. Mediterranean epidemiological evidence on tomatoes and the prevention of digestive-tract cancers. Proc Soc Exp Biol Med. 1998;218:125-128.

46. Velmurugan B, Bhuvaneswari V, Burra UK, Nagini S. Prevention of N-methyl-N'-nitro-N-nitrosoguanidine and saturated sodium chloride-induced gastric carcinogenesis in Wistar rats by lycopene. Eur J Cancer Preve. 2002;11:19-26.

47. Palozza P, Simone R, Catalano A, Boninsegna A, Böhm V, Fröhlich K, Mele MC, Monego G, Ranelletti FO. Lycopene prevents 7-ketocholesterol-induced oxidative stress, cell cycle arrest and apoptosis in human macrophages. J Nutr Biochem. 2010;21:34-46.

48. Rao LG, Mackinnon ES, Josse RG, Murray TM, Strauss A, Rao AV. Lycopene consumption decreases oxidative stress and bone resorption markers in postmenopausal women. Osteoporosis Int. 2007;18:109-115.

49. Palozza P, Colangelo M, Simone R, Catalano A, Boninsegna A, Lanza P, Monego G, Ranelletti FO. Lycopene induces cell growth inhibition by altering mevalonate pathway and Ras signaling in cancer cell lines. Carcinogenesis. 2010;31:1813-1821.

50. Velmurugan B, Mani A, Nagini S. Combination of S-allylcysteine and lycopene induces apoptosis by modulating Bcl-2, Bax, Bim and caspases during experimental gastric carcinogenesis. Eur J Cancer Prev. 2005;14:387-393.

51. Khan N, Afaq F, Mukhtar H. Apoptosis by dietary factors: the suicide solution for delaying cancer growth. Carcinogenesis. 2006;28:233-239.

52. Zhang B, Gu Y. Low expression of ERK signaling pathway affecting proliferation, cell cycle arrest and apoptosis of human gastric HGC-27 cells line. Mol Biol Rep. 2014;41:3659-3669.

53. Liu C, Russell RM, Wang XD. Lycopene supplementation prevents smoke-induced changes in p53, p53 phosphorylation, cell proliferation, and apoptosis in the gastric mucosa of ferrets. J Nutr. 2006;136:106-111.

54. Hall AK. Liarozole amplifies retinoid-induced apoptosis in human prostate cancer cells. Anticancer Drugs. 1996;7:312-320.

55. Kotake-Nara E, Kushiro M, Zhang H, Sugawara T, Miyashita K, Nagao A. Carotenoids affect proliferation of human prostate cancer cells. J Nutr. 2001;131:3303-3306.

56. Kim L, Rao AV, Rao LG. Effect of lycopene on prostate LNCaP cancer cells in culture. J Med Food. 2002;5:181-187.

57. Boileau TW, Liao Z, Kim S, Lemeshow S, Erdman JW Jr, Clinton SK. Prostate carcinogenesis in N-methyl-N-nitrosourea (NMU)-testosterone-treated rats fed tomato powder, lycopene, or energy-restricted diets. J Nat Cancer Inst. 2003;95:1578-1586.

58. Mills PK, Beeson WL, Phillips RL, Fraser GE. Cohort study of diet, lifestyle, and prostate cancer in Adventist men. Cancer. 1989;64:598-604.

59. Jeong Y, Lim JW, Kim H. Lycopene Induces Apoptosis in Pancreatic Cancer Cells. The FASEB Journal 30 (1 Supplement), 691.623-691.623, 2016.

60. Crew KD, Neugut AI. Epidemiology of gastric cancer. World J Gastroenterol. 2006;12:354-362.

61. Tsugane S, Sasazuki S. Diet and the risk of gastric cancer: review of epidemiological evidence. Gastric Cancer. 2007;10:75-83.

62. Persson C, Sasazuki S, Inoue M, Kurahashi N, Iwasaki M, Miura T, Ye W, Tsugane S; JPHC Study Group. Plasma levels of carotenoids, retinol and tocopherol and the risk of gastric cancer in Japan: a nested case-control study. Carcinogenesis. 2008;29:1042-1048.

63. Jenab M, Riboli E, Ferrari P, Friesen M, Sabate J, Norat T, Slimani N, Tjønneland A, Olsen A, Overvad K, Boutron-Ruault MC, Clavel-Chapelon F, Boeing H, Schulz M, Linseisen J, Nagel G, Trichopoulou A, Naska A, Oikonomou E, Berrino F, Panico S, Palli D, Sacerdote C, Tumino R, Peeters PH, Numans ME, Bueno-de-Mesquita HB, Büchner FL, Lund E, Pera G, Chirlaque MD, Sánchez MJ, Arriola L, Barricarte A, Quirós JR, Johansson I, Johansson A, Berglund G, Bingham S, Khaw KT, Allen N, Key T, Carneiro F, Save V, Del Giudice G, Plebani M, Kaaks R, Gonzalez CA. Plasma and dietary carotenoid, retinol and tocopherol levels and the risk of gastric adenocarcinomas in the European prospective investigation into cancer and nutrition. Br J Cancer. 2006;95:406-415.

64. Nomura AM, Hankin JH, Kolonel LN, Wilkens LR, Goodman MT, Stemmermann GN. Case-control study of diet and other risk factors for gastric cancer in Hawaii (United States). Cancer Causes Control. 2003;14:547-558.

65. Graham S, Haughey B, Marshall J, Brasure J, Zielezny M, Freudenheim J, West D, Nolan J, Wilkinson G. Diet in the epidemiology of gastric cancer. Nutr Cancer. 1990;13:19-34.

66. Correa P, Fontham E, Pickle LW, Chen V, Lin YP, Haenszel W. Dietary determinants of gastric cancer in south Louisiana inhabitants. J Nat Cancer Inst. 1985;75:645-654.

67. Haenszel W, Kurihara M, Segi M, Lee RK. Stomach cancer among Japanese in Hawaii. J Nat Cancer Inst. 1972;49:969-988.

68. Tajima K, Tomina S. Dietary habits and gastro-intestinal cancers: a comparative case-control study of stomach and large intestinal cancers in Nagoya, Japan. Jpn J Cancer Res. 1985;76:705-716.

69. Modan B, Cuckle H, Lubin F. A note on the role of dietary retinol and carotene in human gastro-intestinal cancer. Int J Cancer. 1981;28:421-424.

70. Buiatti E, Palli D, Decarli A, Amadori D, Avellini C, Bianchi S, Biserni R, Cipriani F, Cocco P, Giacosa A, Marubini M, Puntoni R, Vindigni C, Fraumeni Jr J, Blot W. A case-control study of gastric cancer and diet in Italy. Int J Cancer. 1989;44:611-616.

71. Ramón JM, Serra L, Cerdó C, Oromí J. Dietary factors and gastric cancer risk. A case-control study in Spain. Cancer. 1993;71:1731-1735.

72. González CA, Sanz JM, Marcos G, Pita S, Brullet E, Saigi E, Badia A, Riboli E. Dietary factors and stomach cancer in Spain: a multi-centre case-control study. Int J Cancer. 1991;49:513-519.

73. Boeing H, Jedrychowski W, Wahrendorf J, Popiela T, Tobiasz-Adamczyk B, Kulig A. Dietary risk factors in intestinal and diffuse types of stomach cancer: a multicenter case-control study in Poland. Cancer Causes Control. 1991;2:227-233.

74. Tuyns AJ, Kaaks R, Haelterman M, Riboli E. Diet and gastric cancer. A case-control study in Belgium. Int J Cancer. 1992;51:1-6.

75. Hansson LE, Nyrén O, Bergström R, Wolk A, Lindgren A, Baron J, Adami HO. Diet and risk of gastric cancer. A population-based case-control study in Sweden. Int J Cancer. 1993;55:181-189.

76. La Vecchia C, Negri E, Decarli A, D'Avanzo B, Franceschi S. A case-control study of diet and gastric cancer in northern Italy. Int J Cancer. 1987;40:484-489.

77. Tsugane S, Tsuda M, Gey F, Watanabe S. Cross-sectional study with multiple measurements of biological markers for assessing stomach cancer risks at the population level. Environ Health Perspect. 1992;98:207-210.

78. Ito Y, Kurata M, Hioki R, Suzuki K, Ochiai J, Aoki K. Cancer mortality and serum levels of carotenoids, retinol, and tocopherol: a population-based follow-up study of inhabitants of a rural area of Japan. Asian Pac J Cancer Prev. 2005;6:10-15.

79. Cui Y, Shikany JM, Liu S, Shagufta Y, Rohan TE. Selected antioxidants and risk of hormone receptor-defined invasive breast cancers among postmenopausal women in the Women's Health Initiative Observational Study. Am J Clin Nutr. 2008;87:1009-1018.

80. Ito Y, Wakai K, Suzuki K, Ozasa K, Watanabe Y, Seki N, Ando M, Nishino Y, Kondo T, Ohno Y, Tamakoshi A; JACC Study Group. Lung cancer mortality and serum levels of carotenoids, retinol, tocopherols, and folic acid in men and women: a case-control study nested in the JACC Study. J Epidemiol. 2005;15(Suppl 2):S140-S149.

81. Satia JA, Littman A, Slatore CG, Galanko JA, White E. Long-term use of beta-carotene, retinol, lycopene, and lutein supplements and lung cancer risk: results from the VITamins And Lifestyle (VITAL) study. Am J Epidemiol. 2009;169:815-828.

82. Kochanek KD, Murphy SL, Anderson RN, Scott C. Deaths: final data for 2002. Natl Vital Stat Rep. 2004;53:1-115.

83. Rao AV. Lycopene, tomatoes, and the prevention of coronary heart disease. Exp Biol Med (Maywood). 2002;227:908-913.

84. Wolak T, Paran E. Can carotenoids attenuate vascular aging? Vascul Pharmacol. 2013;59:63-66.

85. Zou ZY, Xu XR, Lin XM, Zhang HB, Xiao X, Ouyang L, Huang YM, Wang X, Liu YQ. Effects of lutein and lycopene on carotid intima-media thickness in Chinese subjects with subclinical atherosclerosis: a randomised, double-blind, placebo-controlled trial. Brit J Nutr. 2014;111:474-480.

86. Agarwal S, Rao AV. Tomato lycopene and low density lipoprotein oxidation: a human dietary intervention study. Lipids. 1998;33:981-984.

87. Hadley CW, Clinton SK, Schwartz SJ. The consumption of processed tomato products enhances plasma lycopene concentrations in association with a reduced lipoprotein sensitivity to oxidative damage. J Nutr. 2003;133:727-732.

88. Shen YC, Chen SL, Wang CK. Contribution of tomato phenolics to antioxidation and down-regulation of blood lipids. J Agricul Food Chem. 2007;55:6475-6481.

89. Bohn T, Blackwood M, Francis D, Tian Q, Schwartz SJ, Clinton SK. Bioavailability of phytochemical constituents from a novel soy fortified lycopene rich tomato juice developed for targeted cancer prevention trials. Nutr Cancer. 2013;65:919-929.

90. Halliwell B. Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. Drugs Aging. 2001;18:685-716.

91. Retz W, Gsell W, Münch G, Rösler M, Riederer P. In: Free radicals in Alzheimer's disease, Ed(s): Springer, 1998.

92. Kaur H, Chauhan S, Sandhir R. Protective effect of lycopene on oxidative stress and cognitive decline in rotenone induced model of Parkinson's disease. Neurochem Res. 2011;36:1435-1443.

93. Qu M, Li L, Chen C, Li M, Pei L, Chu F, Yang J, Yu Z, Wang D, Zhou Z. Protective effects of lycopene against amyloid β-induced neurotoxicity in cultured rat cortical neurons. Neurosci Lett. 2011;505:286-290.

94. Sandhir R, Mehrotra A, Kamboj SS. Lycopene prevents 3-nitropropionic acid-induced mitochondrial oxidative stress and dysfunctions in nervous system. Neurochem Int. 2010;57:579-587.

95. Qu M, Zhou Z, Chen C, Li M, Pei L, Chu F, Yang J, Wang Y, Li L, Liu C, Zhang L, Zhang G, Yu Z, Wang D. Lycopene protects against trimethyltin-induced neurotoxicity in primary cultured rat hippocampal neurons by inhibiting the mitochondrial apoptotic pathway. Neurochem Int. 2011;59:1095-1103.

96. Panda V, Sonkamble M. Anti-ulcer activity of Ipomoea batatas tubers (sweet potato). Func Foods Health Disease. 2012;2:48-61.

97. Han RM, Zhang JP, Skibsted LH. Reaction dynamics of flavonoids and carotenoids as antioxidants. Molecules. 2012;17:2140-2160.

98. Jain D, Katti N. Combination treatment of lycopene and hesperidin protect experimentally induced ulcer in laboratory rats. J Intercul Ethnopharmacol. 2015;4:143-146.

99. Conn PF, Schalch W, Truscott TG. The singlet oxygen and carotenoid interaction. J Photochem Photobiol B. 1991;11:41-47.

100. Jang SH, Lim JW, Morio T, Kim H. Lycopene inhibits *Helicobacter pylori*-induced ATM/ATR-dependent DNA damage response in gastric epithelial AGS cells. Free Radic Biol Med. 2012;52:607-615.

101. Karemore TV, Motwani M. Evaluation of the effect of newer antioxidant lycopene in the treatment of oral submucous fibrosis. Indian J Dent Res. 2012;23:524-528.

102. Shidfar F, Agah S, Ekhlasi G, Salehpour A, Ghourchian S. Lycopene an adjunctive therapy for *Helicobacter pylori* eradication: a quasi-control trial. J Complement Integr Med. 2012;9:14.

103. Milani C, Maccari M, Mosconi P. Action of lycopene in the experimental gastric ulcer. Pharmacology. 1970;4:334-340.

104. Locke GR 3rd, Talley NJ, Fett SL, Zinsmeister AR, Melton LJ 3rd. Prevalence and clinical spectrum of gastroesophageal reflux: a population-based study in Olmsted County, Minnesota. Gastroenterology. 1997;112:1448-1456.

105. Jiménez P, Piazuelo E, Sánchez MT, Ortego J, Soteras F, Lanas A. Free radicals and antioxidant systems in reflux esophagitis and Barrett's esophagus. World J Gastroenterol. 2005;11:2697-2703.

106. Giri AK, Rawat JK, Singh M, Gautam S, Kaithwas G. Effect of lycopene against gastroesophageal reflux disease in experimental animals. BMC Complement Altern Med. 2015;15:110.

107. Rao LG, Guns E, Rao AV. Lycopene: its role in human health and disease. Agro Food. 2003;7:25-30.

108. Kim L, Rao AV, Rao LG. Lycopene II effect on osteoblasts: the carotenoid lycopene stimulates cell proliferation and alkaline phosphatase activity of SaOS-2 cells. J Med Food. 2003;6:79-86.

109. Park CK, Ishimi Y, Ohmura M, Yamaguchi M, Ikegami S. Vitamin A and carotenoids stimulate differentiation of mouse osteoblastic cells. J Nutr Sci Vitaminol (Tokyo). 1997;43:281-296.

110. Rao LG, Krishnadev N, Banasikowska K, Rao AV. Lycopene I effect on osteoclasts: lycopene inhibits basal and parathyroid hormone-stimulated osteoclast formation and mineral resorption mediated by reactive oxygen species in rat bone marrow cultures. J Med Food. 2003;6:69-78.

111. Ishimi Y, Ohmura M, Wang X, Yamaguchi M, Ikegami S. Inhibition by carotenoids and retinoic acid of osteoclast-like cell formation induced by bone-resorbing agents *in vitro*. J Clin Biochem Nutr. 1999;27:113-122.

112. Zini A, de Lamirande E, Gagnon C. Reactive oxygen species in semen of infertile patients: levels of superoxide dismutase- and catalase-like activities in seminal plasma and spermatozoa. Int J Androl. 1993;16:183-188.

113. Dawson VL, Dawson TM, Bartley DA, Uhl GR, Snyder SH. Mechanisms of nitric oxide-mediated neurotoxicity in primary brain cultures. J Neurosci. 1993;13:2651-2661.

114. Geva E, Bartoov B, Zabludovsky N, Lessing JB, Lerner-Geva L, Amit A. The effect of antioxidant treatment on human spermatozoa and fertilization rate in an *in vitro* fertilization program. Fertil Steril. 1996;66:430-434.

115. Suleiman SA, Ali ME, Zaki ZM, el-Malik EM, Nasr MA. Lipid peroxidation and human sperm motility: protective role of vitamin E. J Androl. 1996;17:530-537.

116. Palan P, Naz R. Changes in various antioxidant levels in human seminal plasma related to immunoinfertility. Arch Androl. 1996;36:139-143.

117. Li Y, Zhang J. Serum concentrations of antioxidant vitamins and carotenoids are low in individuals with a history of attempted suicide. Nutr Neurosci. 2007;10:51-58.

118. Wood LG, Garg ML, Powell H, Gibson PG. Lycopene-rich treatments modify noneosinophilic airway inflammation in asthma: proof of concept. Free Radical Res. 2008;42:94-102.

Turk J Pharm Sci 2017;14(3):319-323
DOI: 10.4274/tjps.28291

REVIEW

Cytochrome P-450 Polymorphisms and Clinical Outcome in Patients with Non-Small Cell Lung Cancer

Küçük Hücreli Dışı Akciğer Kanseri Hastalarında Sitokrom P-450 Polimorfizmleri ve Klinik Sonuçları

Mümtaz İŞCAN*, Ahmet Oğuz ADA

Ankara University, Faculty of Pharmacy, Department of Toxicology, Ankara, Turkey

ABSTRACT

Lung cancer is an increasing worldwide public health problem. Most patients with lung cancer have non-small cell lung cancer (NSCLC). These patients are mainly treated with standard platinum-based chemotherapy. Poor response and great inter-individual variety in treatment response occurs among these patients. There is accumulating evidence to support the hypothesis that genetic polymorphisms alter the drug response and survival. Cytochrome P450 (CYP) enzymes metabolize antineoplastic drugs and are involved in drug resistance. Polymorphic CYPs have altered enzyme activities and thus they may influence the response to chemotherapy and survival in patients with lung cancer. In the current review, recent findings with respect to the role of mainly *CYP1A1, CYP1B1, CYP2D6, CYP2E1* and *CYP3A4* gene polymorphisms in response to chemotherapy and survival in patients with NSCLC have been provided, which could be useful for clinicians in the prognosis of these patients who are mainly treated with platinum-based chemotherapy.

Key words: CYP, polymorphisms, response to chemotherapy, survival, lung cancer

ÖZ

Akciğer kanseri dünyada artan bir sağlık sorunudur. Akciğer kanseri hastalarının çoğu küçük hücreli dışı akciğer kanseridir (KHDAK). Bu hastalar genelde ilk basamakta standart platin bazlı kemoterapi ile tedavi olmaktadır. Bu hastalarda kemoterapiye yanıt ise düşük düzeyde olmakta ve tedaviye yanıtta bireyler arasında büyük farklılıklar görülmektedir. Genetik polimorfizmlerin ilaca karşı alınan yanıt ile sağkalım üzerinde etkili olduğu hipotezini destekleyen kanıtlar giderek artmaktadır. Sitokrom P450 (CYP) enzimleri antineoplastik ilaçları metabolize etmekte ve ilaç direncine neden olabilmektedir. Polimorfik CYP'lerin enzim aktiviteleri değişkenlik göstermekte ve dolayısıyla akciğer kanseri hastalarının kemoterapiye yanıtlarında ve sağkalım süreleri üzerinde etkileri olabilmektedirler. Bu derlemede, özellikle platin bazlı kemoterapi alan KHDAK hastalarının prognozunda klinisyenlere yararlı olabilecek, *CYP1A1, CYP1B1, CYP2D6, CYP2E1* ve *CYP3A4* gen polimorfizmlerinin bu tedaviyi alan KHDAK hastalarının kemoterapiye yanıt ve sağkalım süreleri üzerindeki etkileri ile ilgili olarak son yıllarda elde edilen bulgular ortaya konmaktadır.

Anahtar kelimeler: CYP, polimorfizm, kemoterapiye yanıt, sağkalım, akciğer kanseri

INTRODUCTION

Lung cancer is an increasing worldwide public health problem, particularly in men.[1] It is responsible for the majority of the deaths arising from cancer.[2] However, non-small cell lung cancer (NSCLC) is the most common type among patients with lung cancer and standard platinum chemotherapy has poor efficiency in these patients.[3,4] Thus, the investigation of the reasons behind this failure of chemotherapy and thus possibly poorer survival in these patients is very important. There are various mechanisms involved in the resistance of tumors against antineoplastic agents.[5] There is accumulating evidence to support the hypothesis that genetic polymorphisms alter drug response and survival.[6-11]

Cytochrome P450 (CYP) is a superfamily of phase I oxidation enzymes, the first 3 families of which metabolize xenobiotics such as environmental carcinogens and various drugs (Table 1) including antineoplastic drugs (Table 2).[12-16] They are also involved in drug resistance.[14,15] These *CYP* genes are polymorphic, and the most common alleles of these CYP polymorphisms have been shown to alter enzyme activities (Table 3).[17]

The therapeutic efficiency and survival period may vary among patients with lung cancer because CYPs metabolize several chemotherapeutic agents depending on CYP activity.[16,18,19] Therefore, the relationship between *CYP* gene polymorphisms and response to chemotherapy and survival in patients with cancer is currently a major area of research. All these kinds of

*Correspondence: E-mail: iscan@pharmacy.ankara.edu.tr, Phone: +90 533 343 48 24 ORCID-ID: orcid.org/
Received: 09.12.2016, Accepted: 19.01.2017

Table 1. Examples of xenobiotics/drugs metabolized by CYP enzymes[12,13,15]

Enzyme	Xenobiotics/Drugs
CYP1A1	PAHs, chemical epoxides, aromatic and halogenic amines, heterocyclic hydrocarbons
CYP1B1	PAHs, chemical epoxides, and diol epoxide, halogenic and heterocyclic hydrocarbons
CYP2D6	Carvedilol, paroxetine, haloperidol, propranolol, nitrosamines (e.g. NNK)
CYP2E1	N-nitroso dimethylamine, halothane, acetaminophene, benzene, ethanol, chloroform, vinylchloride
CYP3A4	Cyclosporine, tacrolimus, clarithromycin, diazepam, Aflatoxin B_1&G_1, 1-nitropyrene, benzo(a)pyrene 7,8-dihydrodiol

PAHs: Polycyclic aromatic hydrocarbons, NNK: Nitrozamine 4-(methylnitrosamino)-1-(3-pridyl)-1-butanone

Table 2. Role of CYP enzymes in antineoplastic drug metabolism[14,16]

Enzyme	Antineoplastic drugs
*CYP1A1**	Flutamide[1], Ifosfamide[1], Imatinib, Tamoxifen[1], Toremifene[1],
*CYP1B1**	Docetaxel[1], Flutamide[1], Tegafur[1]
CYP2D6	Gefinitib[1], Tamoxifen[2], Vincristine[1], Vinorelbine[1], Imatinib[1]
CYP2E1	Etoposide[1], Vinorelbine[1]
CYP3A4	Cyclophosphamide[2], Docetaxel[2], Etoposide[2], Gefinitib[2], Ifosfamide[2], Imatinib[2], Tamoxifen[2], Teniposide[2], Vincristine[2]

[1]Minor metabolism by corresponding CYP, [2]Major metabolism by corresponding CYP, *Mainly extrahepatic (Lung, Gastrointestinal tract, Kidney)

Table 3. Polymorphisms of CYPs and changes in corresponding enzyme activities[17]

Subfamily	Chromosome	Allele	Nucleotide change	Enzyme activity
CYP1A	15q22	*CYP1A1*2A*	3798T>C	Increase
		*CYP1A1*2C*	2454A>G	Increase
CYP1B	2	*CYP1B1*2*	142C>G; 355G>T	No change
		*CYP1B1*3*	4326 C>G	Increase
		*CYP1B1*4*	4390A>G	Decrease
CYP2D	22	*CYP2D6*10A*	100 C>T	Decrease
CYP2E	10	*CYP2E1*5B*	-1293G>C; -1053C>T	Decrease
		*CYP2E1*6*	7632 T>A	Decrease
		*CYP2E1*7B*	-71G>T; -333T>A	Not known
CYP3A	7	*CYP3A4*1B*	-392A>G	Increase
		*CYP3A4*3*	23171T>C	Not known
		*CYP3A4*18*	20070T>C	Decrease

information obtained from studies are necessary and important to lead physicians toward the new era of precision medicine in lung cancer therapy.

Several previous reviews described the potential of CYP polymorphisms in cancer therapy.[16,20,21] The research area is rapidly developing, with many studies and insights being published, bringing the possibility of individualized cancer therapy closer. However, this review provides a different aspect of CYP polymorphisms compared with previous ones, particularly focusing on recent findings with respect to the role of *CYP1A1*, *CYP1B1*, *CYP2E1*, *CYP2D6,* and *CYP3A4* gene polymorphisms in response to chemotherapy and survival in patients with NSCLC.

The associations between CYP polymorphisms and response to chemotherapy and survival in patients with lung cancer

Studies investigating the relationship between *CYP* gene polymorphisms and response to platinum-based chemotherapy and survival in patients with lung cancer have mainly focused on *CYP1A1*, *CYP1B1*, *CYP2E1*, *CYP2D6,* and *CYP3A4* (Table 4).

As seen in Table 4, a couple of studies exist with respect to the *CYP1A1* polymorphism and survival in lung cancer, and their results are contradictory.[7,18,22,23] Goto et al.[22] found that the *CYP1A1*2A* mutant allele significantly shortened survival compared with those of wild-type genotypes in patients with NSCLC. However, Li et al.[18] found no such association in

Table 4. CYP polymorphisms, response to platinum-based chemotherapya and survival in patients with lung cancer

Allele	Survival	Response to chemotherapy	Reference
*CYP1A1*2A*	Mutant allele (Shorter)	ND	(22)
	No effect	wild type allele (better response)[b]	(18)
*CYP1A1*2C*	No effect	ND	(23)
	No effect	No effect	(7)
*CYP1B1*3*	Mutant allele (Shorter)	mutant allele (worse response)[c]	(19)
*CYP1B1*4*	No effect	No effect	(7)
	No effect	No effect[c]	(19)
*CYP2D6*10A*	No effect	No effect	(18)
*CYP2E1*5B*	Mutant allele (Longer)	ND	(24)
	Mutant allele (Shorter)	ND	(25)
	No effect	No effect	(18)
	No effect	No effect	(9)
*CYP2E1*6*	No effect	ND	(18)
	No effect	No effect	(9)
*CYP2E1*7B*	No effect	No effect	(9)
*CYP3A4*1B*	ND	No effect[c]	(26)[d]
*CYP3A4*3*	ND	No effect	(26)
*CYP3A4*18*	ND	No effect	(26)

ND: Not determined, [a]Cisplatin+Etoposide, Cisplatin+Gemcitabine, Cisplatin+Docetaxel, Cisplatin+Vinoralbine, Cisplatin+Paclitaxel, Carboplatin+Paclitaxel, [b]Non-platinum-based chemotherapy, [c]Mainly docetaxel-based chemotherapy, [d]Histologic type was not provided

this regard for the *CYP1A1*2A* polymorphism. Pryzgodzki et al.[23] observed no association between the *CYP1A1*2C* polymorphism and survival in patients with NSCLC. Likewise, a recent study also demonstrated the lack of association between the *CYP1A1*2C* polymorphism and survival in patients with NSCLC.[7]

There are only two studies in regard to the influences of *CYP1B1* polymorphisms on survival in lung cancer (Table 4). Recently, Vasile et al.[19] demonstrated that *CYP1B1*3* mutant allele carriers had shorter survival compared with wild-type allele carriers. These investigators, however, observed no association between *CYP1B1*4* polymorphisms and survival in patients with NSCLC. In line with Vasile et al.[19], Ada et al.[7] previously showed that the *CYP1B1*4* polymorphism was not associated with survival in patients with NSCLC, although a notable trend towards worsening of survival in *CYP1B1*4* mutant allele carriers was determined (Table 4).

On the other hand, in recent years, data about the associations between the *CYP1A1*2A*, *CYP1A1*2C*, *CYP1B1*3*, and *CYP1B1*4* polymorphisms and responses to mainly platinum-based chemotherapy have been provided by researchers (Table 4).[7,18,19] Li et al.[18] found that wild-type allele carriers of the *CYP1A1* gene had a better response to non-platinum drug therapy only, but not to platinum-based chemotherapy, than those of variant allele carriers of the gene. There was no influence of the *CYP1A1*2C* polymorphism on the response to platinum-based chemotherapy in patients with NSCLC.[7] The response to chemotherapy of *CYP1B1*3* variant allele carriers was worse than wild-type allele carriers of the *CYP1B1* gene in patients with NSCLC who were treated with docetaxel after platinum-based chemotherapy.[19] However, no association was found between the *CYP1B1*4* polymorphism and response to chemotherapy.[7,19]

A study investigating the association between the *CYP2D6*10A* polymorphism and therapeutic response or survival in patients with NSCLC has recently been reported (Table 4).[18] These investigators observed no influence of this polymorphism on response to either platinum-based chemotherapy or non-platinum-based chemotherapy and survival in patients with advanced NSCLC.

Studies concerning the relationship between the *CYP2E1* polymorphism and survival in patients with lung cancer are also rather limited and the results are not conclusive (Table 4). For example, studies on the *CYP2E1*5B* polymorphism are rather conflicting. Oyama et al.[24] found an increase in survival in mutant allele carriers, whereas Haque et al.[25] observed shorter survival in mutant carriers, and Li et al.[18] found no association between this *CYP* gene polymorphism and survival in patients with NSCLC. Przygodzki et al.[23] reported no significant association between the *CYP2E1*6* polymorphism and survival in patients with NSCLC. Moreover, almost no information is available with

respect to the relationship between these polymorphisms and response to chemotherapy in patients with NSCLC. The first data in this regard were provided by Li et al.[18] who observed no significant association between the *CYP2E1*5B* polymorphism and response to chemotherapy in NSCLC. Likewise, the recent results of Karacaoğlan et al.[9] on the *CYP2E1*5B* polymorphism are also in line with the findings of Li et al.[18], both in regard to response to chemotherapy and survival. However, their findings are in contrast to those of Oyama et al.[24] and Haque et al.[25] in respect to survival. The findings of Karacaoğlan et al.[9] in regard to the effect of *CYP2E1*6* polymorphism on survival also coincided with the results of Przygodzki et al.[23] The only study for the *CYP2E1*7B* polymorphism on this issue was reported by Karacaoğlan et al.[9] who observed no association between the *CYP2E1*7B* polymorphism and response to chemotherapy and survival in patients with NSCLC.

Choi et al.[26] investigated the influences of *CYP3A4* polymorphisms on response to chemotherapy in patients who had various type of cancer, including lung cancer (Table 4). The authors genotyped *CYP3A4* polymorphisms, namely *CYP3A4*1B*, *CYP3A4*3*, and *CYP3A4*18*, but could find no association between these polymorphisms and response to chemotherapy in patients with lung cancer treated with docetaxel concomitantly with cisplatin, doxorubicin, capecitabine, cisplatin-cetuximab, and ifosfamide.

Multigene polymorphisms in response to chemotherapy and survival in lung cancer

Recent studies have demonstrated that the simultaneous analysis of gene polymorphisms may correlate well with the clinical outcome better than the single polymorphism studies. This seems to be valid for CYPs as well. For example, the combined *CYP1A1*2A* mutant allele and *GSTM1* null genotype were associated with better response to chemotherapy in NSCLC patients.[18] The study of Ada et al.[7] in NSCLC patients also revealed that the combined *CYP1A1*2C* and *GSTP1* (Ile105Val) mutant alleles or *CYP1B1*4* and *GSTP1* (Ile105Val) mutant alleles had notable trends toward worsening of survival. Recently, Karacaoğlan et al.[9] demonstrated that combined *CYP1A1*2C* and *TP53* (Arg72Pro) mutant genotypes were associated with the worsening of the survival in NSCLC patients. In addition, they observed that the combined *CYP2E1*7B* and *TP53* (Arg72Pro) mutant alleles had notable trends toward worsening survival. Hence, the analysis of more than one gene polymorphisms of CYPs with other gene polymorphisms as shown with other xenobiotic/drug metabolizing enzymes and *TP53* gene polymorphisms could provide more important information of their involvement in the clinical outcome in NSCLC.

CONCLUSION

Based on current data, the role of polymorphisms of genes that encode CYP enzymes in response to chemotherapy and survival in patients with NSCLC seems to depend on the individual *CYP* gene and to a certain extent, the treatment regimen.

The altered enzyme activities due to gene mutations appear to have no significant impact in patients with NSCLC treated with distinct chemotherapy regimens, namely platinum-based chemotherapy, non-platinum-based chemotherapy or docetaxel-based chemotherapy because there is a lack of associations between *CYP1A1*2C*, *CYP1B1*4*, *CYP2D6*10A*, *CYP2E1*6*, *CYP2E1*7B*, *CYP3A4*1B*, *CYP3A4*3,* and *CYP3A4*18* polymorphisms and response to chemotherapy. These findings are also likely to show that these CYP polymorphisms are not functioning as a predictor of response to the distinct chemotherapy regimens mentioned above. However, wild-type allele carriers of *CYP1A1* gene seems to benefit more from non-platinum-based treatment than platinum-based chemotherapy. On the other hand, *CYP1B1*3* mutant allele carriers had a poor response, mainly to docetaxel-based chemotherapy. However, further studies are required to confirm these findings.

Conflicting results are also noted with respect to the influence of *CYP1A1*2A* and *CYP2E1*5B* polymorphisms in particular on the survival of patients with NSCLC. These inconsistencies need to be clarified through further studies. On the other hand, the lack of association between the *CYP1A1*2C*, *CYP1B1*4*, and *CYP2E1*6* polymorphisms and survival in patients with NSCLC appears to be conclusive. The *CYP2D6*10A* and *CYP2E1*7B* polymorphisms are also likely to have no influence on survival in patients with NSCLC. However, these findings need to be verified in further studies.

It is noteworthy that other important factors should also be considered when evaluating the role of these genes in the clinical outcome of cancer therapy. For example, besides single gene polymorphisms, multiple function *CYP* gene polymorphisms with other gene polymorphism analyses have also shown to provide important information about their involvement in clinical outcomes in NSCLC.

Overall, current studies have shown that the effects of each individual *CYP* gene polymorphism on clinical outcomes in patients with NSCLC are likely to be modest, which suggests that more comprehensive information is necessary to predict more accurately the role of CYP polymorphisms in selecting the most appropriate chemotherapy regimen. Recent efforts in this regard are promising.[27]

Thus, future studies that incorporate multigene polymorphisms, and phenotypic, epigenetic, and clinical variables will hopefully provide a better understanding of the role of CYP polymorphisms in precision medicine for patients with NSCLC.

ACKNOWLEDGEMENT

The authors' research was supported by the Research Fund of Ankara University; grants: 20060803002HPD, 20070803005HPD and 10A3336002.

Conflict of Interest: No conflict of interest was declared by the authors.

REFERENCES

1. Firat D, Celik I. Cancer Statistics in Turkey and in the World 1993-1995. Turkish Association for the Cancer Research and Control. Ankara; 1998.
2. Howe HL, Wingo PA, Thun MJ, Ries LA, Rosenberg HM, Feigal EG, Edwards BK. Annual report to the nation on the status of cancer (1973 through 1998), featuring cancers with recent increasing trends. J Natl Cancer Inst. 2001;93:824-842.
3. Bunn PA Jr. Chemotherapy for advanced non-small-cell lung cancer: who, what, when, why? J Clin Oncol. 2002:20(18 Suppl):23S-33S.
4. Goksel T, Akkoclu A; Turkish Thoracic Society, Lung and Pleural Malignancies Study Group. Pattern of lung cancer in Turkey, 1994-1998. Respiration. 2002;69:207-210.
5. Stewart DJ. Tumor and host factors that may limit efficacy of chemotherapy in non-small cell and small cell lung cancer. Crit Rev Oncol Hematol 2010;75:173-234.
6. Booton R, Ward T, Heighway J, Ashcroft L, Morris J, Thatcher N. Glutathione-S-transferase P1 isoenzyme polymorphisms, platinum-based chemotherapy, and non-small cell lung cancer. J Thorac Oncol. 2006;1:679-683.
7. Ada AO, C Kunak S, Hancer F, Bilgen S, Suzen SH, Alpar S, Gulhan M, Kurt B, Iscan M. CYP and GST polymorphisms and survival in advanced non-small cell lung cancer patients. Neoplasma. 2010;57:512-521.
8. Yin JY, Huang Q, Zhao YC, Zhou HH, Liu ZQ. Meta-analysis on pharmacogenetics of platinum-based chemotherapy in non small cell lung cancer (NSCLC) patients. PLoS One. 2012;7:e38150.
9. Karacaoğlan V, Ada AO, Bilgen S, Çetinkaya GT, Soydaş E, Kunak CS, Alpar SM, Gülhan M, Işcan M. Xenobiotic/drug metabolizing enzyme and TP53 polymorphisms and clinical outcome in advanced nonsmall cell lung cancer patients. Turk J Med Sci. 2017;47:554-562.
10. Chen J, Wang Z, Zou T, Cui J, Yin J, Zheng W, Jiang W, Zhou H, Liu Z. Pharmacogenomics of platinum-based chemotherapy response in NSCLC: a genotyping study and a pooled analysis. Oncotarget. 2016;7:55741-55756.
11. Yin JY, Li X, Zhou HH, Liu ZQ. Pharmacogenomics of platinum-based chemotherapy sensitivity in NSCLC: toward precision medicine. Pharmacogenomics. 2016;17:1365-1378.
12. Guengerich FP, Wu ZL, Bartleson CJ. Function of human cytochrome P450s: characterization of the orphans. Biochem Biophys Res Commun. 2005;338:465-469.
13. Pelkonen O, Turpeinen M, Hakkola J, Honkakoski P, Hukkanen J, Raunio H. Inhibition and induction of human cytochrome P450 enzymes: current status. Arch Toxicol. 2008;82:667-715.
14. Michael M, Doherty MM. Drug metabolism by tumours: its nature, relevance and therapeutic implications. Expert Opin Drug Metab Toxicol. 2007;3:783-803.
15. Zhou SF, Liu JP, Chowbay B. Polymorphism of human cytochrome P450 enzymes and its clinical impact. Drug Metab Rev. 2009;41:89-295.
16. van Schaik RH. CYP450 pharmacogenetics for personalizing cancer therapy. Drug Resist Updat. 2008;11:77-98.
17. The Human Cytochrome P450 (CYP) Allele Nomenclature Database. Accessed 29 September 2016. Available: http://cypalleles.ki.se/
18. Li W, Yue W, Zhang L, Zhao X, Ma L, Yang X, Zhang C, Wang Y, Gu M. Polymorphisms in GSTM1, CYP1A1, CYP2E1, and CYP2D6 are associated with susceptibility and chemotherapy response in non-small-cell lung cancer patients. Lung. 2012;190:91-98.
19. Vasile E, Tibaldi C, Leon GL, D'Incecco A, Giovannetti E. Cytochrome P450 1B1 (CYP1B1) polymorphisms are associated with clinical outcome of docetaxel in non-small cell lung cancer (NSCLC) patients. J Cancer Res Clin Oncol. 2015;141:1189-1194.
20. Ingelman-Sundberg M, Sim SC, Gomez A, Rodriguez-Antona C. Influence of cytochrome P450 polymorphisms on drug therapies: pharmacogenetic, pharmacoepigenetic and clinical aspects. Pharmacol Ther. 2007;116:496-526.
21. Preissner SC, Hoffmann MF, Preissner R, Dunkel M, Gewiess A, Preissner S. Polymorphic cytochrome P450 enzymes (CYPs) and their role in personalized therapy. PLoS One. 2013;8:e82562.
22. Goto I, Yoneda S, Yamamoto M, Kawajiri K. Prognostic significance of germ line polymorphisms of the CYP1A1 and glutathione S-transferase genes in patients with non-small cell lung cancer. Cancer Res. 1996;56:3725-3730.
23. Przygodzki RM, Bennett WP, Guinee DG Jr, Khan MA, Freedman A, Shields PG, Travis WD, Jett JR, Tazelaar H, Pairolero P, Trastek V, Liotta LA, Harris CC, Caporaso NE. p53 mutation spectrum in relation to GSTM1, CYP1A1 and CYP2E1 in surgically treated patients with non-small cell lung cancer. Pharmacogenetics. 1998;8:503-511.
24. Oyama T, Matsumoto A, Isse T, Kim YD, Ozaki S, Osaki T, Sugio K, Yasumoto K, Kawamoto T. Evidence-based prevention (EBP): approach to lung cancer prevention based on cytochrome 1A1 and cytochrome 2E1 polymorphism. Anticancer Res. 2003;23:1731-1737.
25. Haque AK, Au W, Cajas-Salazar N, Khan S, Ginzel AW, Jones DV, Zwischenberger JB, Xie J. CYP2E1 polymorphism, cigarette smoking, p53 expression, and survival in non-small cell lung cancer: a long term follow-up study. Appl Immunohistochem Mol Morphol. 2004;12:315-322.
26. Choi JR, Kim JO, Kang DR, Shin JY, Zhang XH, Oh JE, Park JY, Kim KA, Kang JH. Genetic Variations of Drug Transporters Can Influence on Drug Response in Patients Treated with Docetaxel Chemotherapy. Cancer Res Treat. 2015;47:509-517.
27. Yin JY, Li X, Li XP, Xiao L, Zheng W, Chen J, Mao CX, Fang C, Cui JJ, Guo CX, Zhang W, Gao Y, Zhang CF, Chen ZH, Zhou H, Zhou HH, Liu ZQ. Prediction models for platinum-based chemotherapy response and toxicity in advanced NSCLC patients. Cancer Lett. 2016;377:65-73.

Turk J Pharm Sci 2017;14(3):324-334
DOI: 10.4274/tjps.49368

REVIEW

The Role of Secondary Metabolites on Gynecologic Cancer Therapy: Some Pathways and Mechanisms

Jinekolojik Kanser Tedavisinde Sekonder Metabolitlerin Rolü: Bazı Yolaklar ve Mekanizmalar

Mürşide Ayşe DEMİREL[1], İpek SÜNTAR[2]*

[1]Gazi University, Faculty of Pharmacy, Laboratory Animals Breeding and Experimental Research Center, Ankara, Turkey
[2]Gazi University, Faculty of Pharmacy, Department of Pharmacognosy, Ankara, Turkey

ABSTRACT

Gynecologic cancers are among the most common cancers in humans and animals. Treatment success depends on several factors including stage at diagnosis, tumor type, origin and metastasis. Currently, surgery, chemotherapy, and radiotherapy are preferred in the treatment of these cancers. However, many anticarcinogenic drugs can cause severe adverse effects and also the expected response to treatment may not be obtained. In recent studies, the importance of the relationship between cancer and inflammation has been emphasized. Therefore, several phytochemicals that exhibit beneficial bioactive effects towards inflammatory pathways were proven to have anticarcinogenic potential for gynecologic cancer therapy. This review summarizes the role of inflammatory pathways in gynecologic cancers and effective secondary metabolites for cancer therapy.

Key words: Gynecologic cancers, pathway, secondary metabolites, phytoconstituents, inflammation

ÖZ

Jinekolojik kanserler insanlarda ve hayvanlarda en yaygın görülen kanserler arasındadır. Tedavi başarısı tanıdaki evre, tümör tipi, orijini ve metastazı içeren birçok faktöre bağlıdır. Günümüzde bu kanserlerin tedavisinde, cerrahi müdahale, kemoterapi ve radyoterapi uygulanmaktadır. Ancak, birçok anti-karsinojenik ilaç ciddi yan etkilere neden olabilir ve ayrıca tedaviye beklenen yanıt alınamayabilir. Son yıllarda yapılan çalışmalarda kanser ve inflamasyon arasındaki ilişkinin önemi vurgulanmıştır. Ve bununla birlikte, inflamatuvar yolaklara karşı yararlı biyoaktif etkiler gösteren birçok fitokimyasalın jinekolojik kanser tedavisi için antikarsinojenik potansiyele sahip olduğu kanıtlanmıştır. Bu derlemede, jinekolojik kanserlerdeki inflamatuvar yolaklar ve bu yolaklarda etkili sekonder metabolitlerin tedavideki rolü özetlenmektedir.

Anahtar kelimeler: Jinekolojik kanserler, yolak, sekonder metabolitler, bitkisel bileşenler, inflamasyon

INTRODUCTION

Cancer is a complex disease in which cells in a specific tissue are no longer fully responsive to the signals within the tissue that regulate cellular differentiation. The disease is characterized by abnormal cell growth spread through the blood and lymph systems to other tissues in the body. It is globally the second leading cause of death for both men and women[1,2]; approximately 1 in 6 deaths is due to cancer.[2] Cancer is commonly diagnosed in domestic animals as it is in humans.[3] Mammary gland tumors, skin tumors, osteosarcomas, and hemopoietic tumors are the most prevalent malignant tumors which cause mortality in dogs and cats.[4,5]

It has been demonstrated that the activation of the inflammatory pathways including cytokines, nuclear factor kappa B, (NF-κB) prostaglandins, cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF), free radicals, inducible nitric oxide synthase (iNOS), and signal transducers and activators of transcription (STAT)-3 lead to the development of various malignant tumors.[6,7] As chronic inflammation has been recognized as a potential risk factor for cancer progression, targeting inflammatory pathways could be beneficial for preventing the development of gynecologic cancers.

The goal of cancer therapy includes both prolonging survival and preserving a high quality of life.[8] However, many drugs used in the treatment of cancer can cause adverse effects such as fatigue, nausea, vomiting, malaise, diarrhea, and headaches. Most novel drugs are still under research because gynecologic cancers are common and show a low survival rate, as in ovarian and breast cancer especially. Due to the adverse effects of many synthetic drugs, secondary metabolites of medicinal plants have attracted attention for scientific research such that they may be used as proven beneficial anticancer agents.[9,10] In this study,

*Correspondence: E-mail: ipesin@gazi.edu.tr, ORCID-ID: orcid.org/0000-0003-4201-1325
Received: 15.12.2016, Accepted: 19.07.2017

we aimed to review the inflammatory pathways related to gynecologic cancers and effective plant secondary metabolites as anti-inflammatory agents for cancer management in both humans and animals.

Overview on gynecological cancers in humans and animals

Gynecologic cancers, the fourth most common type of cancers in women, affect the tissue and organs of the female reproductive system including the ovaries, uterus, cervix, and vulva-vagina.[11-13] Vulvar and vaginal cancers include only about 2% of malignant neoplasms of the genital tract in women. Vaginal cancers are caused by primary tumors of the vagina or metastasis of other gynecologic cancers. Primary vaginal cancer is rare, seen in approximately 1 in every 1100 women. Treatment success depends on the origin of the tumor.[14] Because endometrial and cervical cancers can generally be diagnosed at the preinvasive stage, treatment success is higher. However, ovarian cancer is one of the deadliest cancer types because diagnosis is frequently in the late stage due to the lack of obvious symptoms.[13]

Gynecologic cancers in animals occur as a consequence of many carcinogenic factors such as genetic, immune, and hormonal changes due to endogenous or exogenous factors, ionized radiation, chemical agents, and oncogenic viruses.[15] Primary ovarian tumors and uterine tumors are rarely observed in domestic animals.[16-18] The clinical signs of these tumors are generally less obvious than those of other cancers and are realized incidentally during laparatomy or ultrasonographic examination. These tumors mainly have benign character and can be treated by removing the relevant organ along with the tumor.[4,17]

Canine transmissible venereal tumor, also known as infectious sarcoma, venereal granuloma, and transmissible lymphosarcoma Sticker tumor, is a benign reticuloendothelial tumor that effects the external genitalia in both sexes, but is occasionally observed in the internal genitalia, other organs, conjunctiva mucosae, the oral and nasal cavities. Transmissible venereal tumor is usually transmitted to genital organs during coitus. Some treatment protocols including surgical resection, radiotherapy, immunotherapy, biotherapy, and chemotherapy have been administered for transmissible venereal tumor. However, chemotherapy without surgical intervention has been determined to be the most effective and practical therapy with vincristine sulfate, vinblastine, doxorubicin, and cyclophosphamide as single agents or in combination.[19,20]

Mammary tumors, the frequency of which varies according to the animal species, is recognized as one of the gynecologic cancer types in veterinary medicine. Dogs are the most frequently affected by mammary tumors among domestic species.[21,22] On the other hand, mammary tumors are rare in livestock.[23] Steroid hormones play an important role in the hyperplasia and neoplasia of mammary gland tissue. There are estrogen and/or progesterone receptors in mammary tumor cells in animals; these receptors may influence the pathogenesis of tumor and response to hormone therapy. The treatment of malignant mammary tumors should include surgery and chemotherapy.[4,24]

Breast cancer is the most frequent cancer in women and diagnosed in approximately 25% of all cancer types.[25] Inflammatory breast cancer, a subtype of breast cancer, is rare (2-5%). The 5-year survival rate is low. Appropriate therapy including chemotherapy, mastectomy, and radiation therapy improve prognosis of breast cancer. Despite improvements in treatment modalities, high-grade or metastatic breast cancer cannot generally be treated. The main purpose of treatment is to improve the quality of life and prolong survival.[26,27]

Inflammatory response in cancer

Several inflammatory mediators are responsible for the formation of cancer. Various anticancer drugs exhibit action directly on pro-inflammatory cytokines such as interleukin (IL)-6 or *tumor necrosis factor* (TNF)-α. Furthermore, reactive oxygen species (ROS) and reactive nitrogen species lead to carcinogenesis by causing a cellular redox imbalance in miscellaneous cancer cells. In addition to STAT3, the Ras protein can also be activated in response to IL-6.[28]

Acute inflammation is the protective response of organisms against tissue destruction. Inflammation heals spontaneously after improving the tissue. However, continuation of the infection and immun system deficiency could result in chronic inflammation, which may lead to tissue damage and finally carcinogenesis. It was first mentioned in 1863 by Rudolf Virchow that leucocytes in neoplastic tissues serve the possible relationship between inflammation and cancer. He observed that the "lymphoreticular infiltrate" exibited the origin of cancer in the chronic inflammatory region.[29] It was hypothesized that angiogenesis was one of the molecular actions that provided a connection between chronic inflammation and cancer, and tumors have been called "wounds that do not heal." Chronic inflammation has been demonstrated to be a key factor in the pathogenesis of malignant tumors such as with human papilloma virus infection, which causing cervical cancer.[6]

Apoptosis is a process of programmed cell death that appears in multicellular organisms. Therefore, inadequate apoptosis causes uncontrolled cell proliferation. Chemotherapeutic agents prevent tumor cell proliferation and even kill tumor cells through apoptotic pathways. Therefore, apoptosis plays an important role in chemotherapy. The process of apoptosis includes contraction and membrane blebbing and nuclear fragmentation. The execution of apoptosis involves the signal transmission pathway.[30,31] TNF is a cell-signaling protein produced chiefly by effective macrophages, which are involved in systemic inflammation. It is the main mediator of binary hipaloptic apoptosis. Inflammatory responses are initiated by the binding of TNF to its receptor. Fas ligand (FasL) is a cytotoxic type II transmembrane protein of the TNF family. The engagement of FasL with its receptor (apoptosis antigen 1 or cluster of differentiation 95) initiates death-inducing signaling complex formation, which includes accessory molecules, the Fas-associated death domain protein, caspase-8, and -10.[32]

COX enzymes are bi-functional membrane-bound enzymes that are responsible for the formation of prostanoids, including thromboxane and prostaglandins. COX-1, which is

stably expressed in cells and tissues, is generally involved in housekeeping functions. COX-3 is only expressed from spesific tissue such as brain and spinal cord. COX-2 is generally found at low levels in cells, whereas it significantly increases in tissue with tumor cells. It has been considered that this situation could be due to the cross-talk between inflammatory mediators such as ILs and cytokines (i.e., IL-1, IL-6, and TNF-α). The association between COX-2 expression in cancers and tumor size has been reported. The prevention of COX-2 expression may inhibit cancer formation because COX-2 is a pro-inflammatory mediator that can be stimulated even in the very early stages of carcinogenesis.[7,33,34] COX-2 transcriptional activation is mediated by transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), specificity protein 1 transcription factor, and activator protein (AP)-1. COX-2 over-expression is related to high grade of tumor, metastasis, recurrence, and survival rates in canine mammary carcinomas. Furthermore, the highest levels of COX-2 were reported to be expressed in inflammatory mammary carcinoma.[35]

The transcription factor NF-κB is a nuclear factor that binds to the enhancer element of the immunoglobulin kappa light-chain of activated B cells. Negative regulators of some signaling pathways of NF-κB are up-regulated by NF-κB. This situation generally results in the deactivation of NF-κB in acute inflammation. The persistent stimulus of NF-κB in chronic inflammation appears to exceed inhibitory feedback circuits, which leads to an increased constitutive activity of NF-κB. There is a two-way relationship between inflammation and NF-κB in cancer. NF-κB is a part of the immune defense that eliminates transformed cells. Therefore, activation of NF-κB is contributed by effectiveness of cytotoxic immune cells against tumor cells. Accordingly, NF–κB, which has a variety of pro-tumorigenic functions, is mainly activated in tissue with tumor.[36,37] The anti-tumorigenic function of the immune system with NF-κB has been known as tumor-immunosurveillance. The immune defense against cancer cells is not adequate to eliminate abnormal cells; these may proceed onto "escape phase" and "equilibrium phase" in which the immune system has the ability to control tumor progression. These phases are characterized by chronic inflammation with increased levels of NF-κB. It has been considered that the activity of NF-κB with a pro-tumorigenic effect is similar in immune-suppressed patients and patients with chronic inflammatory diseases. The antiapoptotic genes that provide cell survival mechanisms are up-regulated by NF-κB activation.[38] NF-κB stimulates cytokines such as TNF-α, IL-1, IL-6, and IL-8, which regulate the immune response, as well as induce adhesion molecules, which provide migration of leukocytes to sites of inflammation.[39] Generally, the contribution of inflammation and NF-κB to cancer induction and progression is complicated. NF-κB signaling has been reported to cause cancer progression by epithelial-mesenchymal transition because NF-κB is associated with an up-regulation of matrix metalloproteinases and VEGF and its receptors.[40]

Ovarian cancer originates mainly from the ovarian surface epithelium. Ovarian epithelial cells are exposed to several pro-inflammatory mediators such as cytokines, ILs, growth factors, prostaglandins and eicosanoids. Therefore, the ovulation process can be considered as a potential inflammation period due to the rise of pro-inflammatory mediator production, which leads to oxidative stress.[41] Moreover, ovarian cancer occurs due to the activation of collagenase, an enzyme that destroys the extracellular matrix. The recurring period of cellular damage and repair in high oxidation conditions disrupt DNA replication.[6]

Therapeutic approach to gynecologic cancers

For the determination of cancer stage and therefore appropriate treatment strategies, histopathologic evaluation should be carried out as the initial step to detect the differentiation of neoplastic lesions. On the treatment procedure, extirpation of the tumor and application of chemotherapeutic agents, particularly including paclitaxel and cisplatin-based derivatives, are generally preferred. Endometrial cancers are successfully treated by hysterectomy. Contrary to expectations, high-grade endometrial tumors could only be appropriately removed in 44-72% of patients. Nevertheless, neoadjuvant chemotherapy can sometimes be effective after removing the mass. Likewise, the effect of radiotherapy has not been fully identified. Cervical cancers are frequently squamous cell carcinomas originating from the epithelial cells lining the cervix. Radical therapy on cervical cancers includes surgery and application of chemotherapeutics such as a combination of histone deacetylase inhibitor (vorinostat) and proteasome inhibitor (bortezomib) as well as radiotherapy. However, treatment success is dependent on clinical factors such as age, histologic type and grade. Radiotherapy is regarded as a beacon of hope in high-grade cervical cancer. On the other hand, as the first-line therapy in ovarian cancer, cisplatin and its derivatives are applied following surgical removal tumors. However, in some cases, ovarian cancer can progress or recur despite chemotherapy, which is known as chemoresistance and has a poor prognosis. Chemoresistance occurs due to the dysregulation of signaling factors which are responsible for the induction of cell death. In additon, these chemotherapeutic agents can cause infertility and serious side effects. For this reason, new therapeutic approaches to cure cancer with fewer adverse effects, as well as to overcome chemoresistance, are needed in all gynecologic cancer types.

Bioactive secondary metabolites derived from botanical sources may represent a promising therapeutical strategy for both cancer management and chemosensitivity enhancement. Recently, several studies revealed that different types of phytoconstituents had diverse potential applications in signaling pathways associated with cancer[42] by inhibiting factors that are dysregulated in malignant cells, either individually or by enhancing the effects of conventional therapy. One of the most important advantages of phytochemicals over synthetic drugs is their high tolerability. Moreover, plant extracts, which contain thousands of phytochemicals, have been shown to be potentially active on multiple targets within various oncogenic signaling pathways.[43]

Effects of phytochemicals against gyecological cancers

High risk of cancers is associated with environmental factors

and unhealthy lifestyle behaviors. According to epidemiologic evidence, dietary behavior notably affects cancer prevalence.[44] It is known that diets rich in fruits and vegetables provide a reduction in cancer risk, which is attributable to the effects of phytochemicals.[45] A number of natural compounds that have been reported to exhibit beneficial biologic effects in gynecologic cancer therapy are presented in Figure 1.

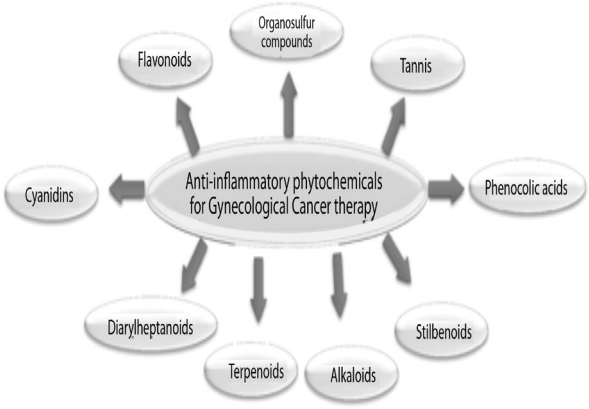


Figure 1. Secondary metabolite groups exhibiting beneficial effects in gynecologic cancer therapy

According to previous epidemiologic research, a diet rich in flavonoid-containing foods is associated with a decreased risk of cancers including breast, digestive system, skin, and prostate.[46] These compounds have the capacity to inhibit cancer development via different biologic activity mechanisms including suppression of inflammation and angiogenesis, regulation of the cellular response to oxidative stress and DNA damage, retardation of cell proliferation, and induction of apoptosis.[47] Studies have demonstrated that flavonoids can induce apoptosis in human breast cancer cells by inhibiting the activity of fatty acid synthase, which catalyzes the synthesis of long-chain fatty acids.[46,48] Apigenin, a flavone-type compound widely found in fruits and vegetables, is a bioactive constituent with anti-inflammatory, antioxidant, and anticancer activities.[47] The anti-inflammatory action of apigenin was shown to supress downstream events by binding to COX-2, and to regulate mitogen-activated protein kinase (MAPK) pathways in endometrial cancer cells through its selective effect on AP-1.[13,49] Supression of procarcinogen-activating enzyme over-expression, which causes DNA mutations and induction of apoptosis through the p53-related pathway, as well as inhibition of tumor cell growth by acting as as a cytochrome P450 (CYP)-1 family enzyme inhibitor, were previously shown for apigenin treatment.[47,50] A previous study reported that apigenin showed cytotoxic action on Michigan Cancer Foundation (MCF)-7 breast cancer cell lines.[7] In addition, the anti-proliferative action of apigenin in human epidermal growth factor receptor 2 over-expressed breast cancer cells by inhibition of phosphatidylinositide 3-kinase (PI3K) activity and Akt kinase activity was also demonstrated.[51] Another study showed that intrinsic and extrinsic apoptotic pathways were involved in the induction of apoptosis by apigenin in malondialdehyde (MDA)-MB-453 human breast cancer cells.[52] Apigenin was demonstrated to down-regulate cyclin D1, D3, and cyclin-dependent kinase (CDK)4 levels, and increased p27 protein levels in breast cancer cells.[53] Through suppression of AP-1 activity, apigenin inhibited phorbol 12-myristate 13-acetate-mediated cell survival and tumor cell invasion in the estrogen-insensitive breast tumor cell line MDA-MB-231.[46,54] By CDK1 and cyclin-dependent kinase inhibitor 1 p21 (Cip1) pathway regulation, apigenin treatment was shown to induce G(2)/M phase cell cycle arrest in SK-BR-3 cells. Moreover, apigenin was reported to cause activation in MDA-MB-468 cells with extracellular signal-regulated kinase (*ERK*)/MAPK phosphorylation[55], and to inhibit E2-induced DNA synthesis in MCF-7 cells.[56,57] Anti-estrogenic effects mediated through estrogen receptor (ER)-binding dependent and independent mechanisms were also revealed.[58,59] Apigenin inhibited the growth of human cervical carcinoma HeLa cells through an apoptotic pathway induced by a p53-dependent increase in p21/waf1 protein expression[60], and exerted an anti-proliferative effect against SiHa human cervical cancer cells.[61] Apigenin was shown to play a beneficial role in the treatment of endometrial cancer in postmenopausal women.[46]

Luteolin, a flavonoid-type compound, possesses multiple biologic characteristics including anti-inflammatory and antioxidant properties, and displays cancer chemopreventive effect.[13] The anticancer potential of luteolin could be related to its anticancer activity, which is also provided by inhibition of cell proliferation, metastasis and angiogenesis, and induction of apoptosis. In addition, luteolin suppresses cell survival pathways including PI3K/Akt, NF-κB, and the X-linked inhibitor of apoptosis protein (XIAP).[62] It was shown to have the ability to inhibit protein kinase Cε and Src kinase in the oncogenic signaling pathway.[13,63] Luteolin was demonstrated to have a notable cytotoxic activity in human papillomavirus (HPV)-positive cervical cancer cells in a dose-dependent manner. HPV E6 and E7 oncogene expressions were suppressed and caspase cascades were activated. Luteolin also inhibited the expression of Bcl-2 and Bcl-xL.[64]

Fisetin, 3,3',4',7-tetrahydroxyflavone, from Fabaceae plants such as *Acacia greggii* A. Gray, and *Acacia berlandieri* Benth., was shown to possess anti-proliferative activity and display anticarcinogenic potential by inducing apoptosis.[7,65-67] Fisetin inhibited the invasion and migration of cervical cancer cells by dose-dependently supressing the expression and activity of urokinase plasminogen activator and decreased p38 MAPK phosphorylation. Fisetin affected the nuclear translocation of NF-κB and inhibited tTPA (tetradecanoylphorbol-13-acetate)-enhanced migration and invasion.[68] Fisetin was found to display anti-inflammatory activity in lipopolysaccharide (LPS)-induced acute pulmonary inflammation, and anti-carcinogenesis action.[69,70] Fisetin demonstrated an inhibitory effect on Wnt signaling by modulating the expression of beta-catenin[71,72], and the reducing effect on NF-κB and AP-1.[7,73]

A citrus flavonoid, tangeretin, displayed inhibitory action on the growth and invasive properties of human mammary cancer cells when co-administered with tamoxifen *in vitro*. However, it was reported that tangeretin exhibit no inhibitory activity on

tumor growth, moreover, it completely neutralized tamoxifen's inhibitory action *in vivo*.[74]

A common flavonol compound, kaempferol, was reported to induce apoptosis in ovarian cancer cells through p53 activation.[75] Yang et al. [76], reported that kaempferol inhibited quinine reductase 2 by blocking NF-κB activity. Kaempferol was shown to act as a breast cancer resistance protein (Bcrp, Abcg2) inhibitor in Madin-Darby canine kidney cell monolayers.[7,77] Kaempferol inhibited VEGF expression, induced the phosphorylation of Akt, and modulated *p53, Bad, Bax* and *Bcl-xL* genes, all of which induce apoptosis in ovarian cancer cells.[50,78]

Another flavonol, myricetin, which is commonly present in fruits and vegetables, was found to exhibit an anti-angiogenic effect through the inhibition of PI3K and the suppression of matrix metalloproteinases responsible for vascular growth.[13,79] In two cisplatin-resistant ovarian cancer cell lines, namely OVCAR-3 and A2780/CP70, myricetin exerted a higher cytotoxic effect than cisplatin. On the other hand, it was found to be less cytotoxic to the normal ovarian cell line IOSE-364. Therefore, due to its potential cytotoxic effect and selectivity against cisplatin-resistant cancer cells, myricetin could be beneficial in overcoming cancer chemoresistance.[80]

According to epidemiologic studies, soy products, which are rich in isoflavonoids namely genistein, daidzein, and glycitein, have important roles in decreasing the incidence and mortality rates of breast cancer, especially by acting as natural selective ER modulators.[81-86] In ER-positive breast cancer, it has been reported that estrogen receptors are over-expressed by approximately 70%. By binding ER, estrogen induces mammary cell proliferation and cell division, as well as DNA replication, and disrupts the cell cyle, apoptosis, and DNA repair, which results in tumor formation.[7] Due to estrogen-antagonistic activities, these compounds decrease the risk of hormone-dependent tumors.[87] Genistein regulates genes related to the cell cycle and apoptosis[88], and inhibits angiogenesis. A number of studies demonstrated the protective effects of genistein against ovarian cancer.[89-91] Dose- and time-dependent growth inhibitory action was detected in HeLa cells treated with genistein. This activity was found to be mediated by apoptosis and cell cycle arrest at the G2/M phase. Moreover, genistein induced migratin inibition by regulating matrix metallopeptidase (MMP)-9 and tissue inhibitors of metalloproteinases 1 expression.[92] Genistein was shown to function as an inhibitor on tyrosine kinase by exerting its effect via DNA topoisomerase II inhibition.[93,94] Moreover, genistein was suggested to be involved in the c-Jun N-terminal kinase (JNK) pathway in inducing the effect of AP-1.[7,95]

According to several *in vitro* and *in vivo* studies, *Rosmarinus officinalis* L. (Lamiaceae) extracts were reported to have important roles as anti-inflammatory, anti-tumorogenic, and anti-proliferative agents.[7] Anticarcinogenic activities of the extracts prepared from *R. officinalis* were shown in MCF-7 and MDA-MB-231 cell lines.[96] As the main metabolite of the plant, rosmarinic acid displayed cytotoxic effects against two human breast cancer cell lines, adriamycin-resistant MCF-7/Adr and wild-type MCF-7/wt[97], and inhibited bone metastasis from breast carcinoma through the NF-κB ligand (RANKL)/RANK/osteoprotegerin pathway by the suppression of IL-8 expression.[98] Rosmarinic acid was also reported to exert DNA methyltransferase inhibition activity, which is an important potential therapeutic feature against cancer. Co-administration of rosmarinic acid with cisplatin provided sensitivity against chemoresistant-human ovarian cancer cell lines by blocking the cell cycle and resulting in inhibition of cell proliferation and apoptosis.[99,100]

Cyanidins are a group of compounds from red berries including grapes, blackberry, cranberry, raspberry and red cabbage. Cyanidin-3-glucoside was reported to block ethanol-induced ErbB2/cSrc/FAK pathway activation in breast cancer cells and prevented metastasis[7], and markedly inhibited ovarian cancer cell proliferation by downregulating the expression of Mucin4 in HO-8910PM cells.[101] Studies have shown that peonidin-3-glucoside and cyanidin-3-glucoside exhibited strong inhibitory activity on cell growth of breast cancer cells HS578T through G2/M arrest, regulated protein levels of CDKs, and induced caspase-3 activation, chromatin condensation, as well as cell death.[102]

Epigallocatechin gallate (EGCG), a major catechin found in green tea, was reported to be effective in the treatment of breast cancer through the inhibition of hypoxia-inducible factor 1α and NFκB activation, as well as VEGF expression in cultured E0771 cells. In an *in vivo* study in mice, EGCG remarkably decreased tumor weight, tumor CD and tumor VEGF expression, but displayed no apparent activity on body and heart weight, and angiogenesis and VEGF expression in the heart and skeletal muscle.[103] EGCG was also reported to be beneficial in treating cervical cancer.[104] Studies that investigated the molecular mechanisms revealed that EGCG exhibited its effect by inhibiting the anti-apoptotic protein Bcl-xl.[105,106] The inhibitory effect on MAPK, CDK, growth factor-related cell signaling, and induction of AP-1 and NF-κB, topoisomerase I, and matrix metalloproteinases are among the other pathways on which EGCG acts.[7,107]

Resveratrol is a stilbenic compound found mainly in red grape skin and peanuts. It was shown to have chemopreventive potential through the activation of LPS-induced NF-κB-luciferase activity at lower doses, but inhibition at higher doses through the reduction of LPS-induced IκB-α phosphorylation and induction of caspase-3 activation.[7,108] It was also shown to possess a potent growth-inhibitory effect against various human cancer cells. In a previous study, resveratrol supressed the *in vitro* cellular invasion of NuTu-19 ovarian cancer cells; however, the effect was not observed *in vivo*.[109]

A metabolite derived from resveratrol, piceatannol (3,3',4,5'-tetrahydroxy-trans-stilbene), was reported to be a potent cisplatin sensitivity enhancer in OvCA. Piceatannol induced the expression of p53-mediated pro-apoptotic protein NOXA, caspase-3 activation, and enhanced XIAP degradation through the ubiquitin-proteasome pathway, which is related to the induction of dynamin-related protein

(Drp) 1-dependent mitochondrial fission, which results in more effective apoptosis induction. In a xenograft mouse model, reduction in tumor size was recorded with the combination treatment of cisplatin and piceatannol.[110] Anti-invasive, anti-adhesive, and anti-migration activity mechanisms of piceatannol in MDA-MB-231 cells were found to occur through the inhibition of MMP-9 involved in PI3K/AKT and NF-κB pathways.[111]

Phenethyl isothiocyanate is an effective constituent obtained from plants from the Brassicaceae family.[112] Its chemopreventive potential against breast cancer cells[113,114] and cervical cancer[115] was investigated previously. Phenethyl isothiocyanate was found to have an apoptosis induction effect in chemotherapeutic drug-resistant cell lines. Phenethyl isothiocyanate enhanced death receptor (DR)4 and DR5 and cleaved caspase-3 expression, induced caspase-8, and supressed ERK1/2 and MEK phosphorylation in cervical cancer cells.[7,115] Phenethyl isothiocyanate was also reported to exert an inhibitory effect on the adhesion and invasion of HeLa cells by G2/M phase arrest induction and CDK1, MMP2/9, CD44, intercellular adhesion molecule 1 supression. It was considered to act via the transforming growth factor (TGF)β/Smad2 pathway, evident by increasing TGFβ, IL6, and IL8 production and Smad2 phosphorylation.[116] In another study, phenethyl isothiocyanate was demonstrated to have cytotoxic potential against OVCAR-3 cells by its anti-proliferative effect in a dose-dependent manner. Apoptosis induction was through caspase-3 and -9 activation. Activation of Akt, ERK1/2, and the expression of transcription factor c-Myc were inhibited and pro-apoptotic p38 and JNK1/2 were activated by phenethyl isothiocyanate treatment.[112]

Sulforaphane is an organosulfur component of cruciferous plants. Sulforaphane was demonstrated to enhance tumor suppression protein transcription. Sulforaphane also suppressed the Wnt/β-catenin self-renewal pathway in breast cancer stem cells.[117] It exerted potent antiproliferative activity in the human ovarian cancer cell line SKOV3, and mouse ovarian cancer cell lines C3 and T3, through down-regulation of cell cycle transition regulators cyclin D1, CDK4, and CDK6, and identifying the Akt pathway as a target.[118]

Indole-3-carbinol, a constituent from Brassica sp., and its digestion metabolite, diindolylmethane, were demonstrated to have anti-cancer activities against hormone responsive cancers such as breast and ovarian cancers.[119] It was also recently shown that diindolylmethane possessed higher activity than indole-3-carbinol in *in vitro* studies.[120] It was revealed that diindolylmethane affected the NF-κB/Wnt/Akt/mTOR pathways, modulated key cytochrome CYPs enzymes, regulated angiogenesis, invasion, and metastasis, and the epigenetic behavior of cancer cells.[88] Diindolylmethane and indole-3-carbinol induced *HO-1* and *SOD1* genes and exhibited synergistic action with isothiocyanates, such as phenethyl isothiocyanate and sulforaphane.[121]

Triterpenic compounds are a broad group of trepenoids including cucurbitanes, dammaranes, ergostanes, friedelanes, lanostanes, limonoids, lupanes, oleananes, tirucallanes, and ursanes. *In vitro* and *in vivo* studies indicated their chemopreventive and therapeutical effects on breast cancer through apoptosis, nitric oxide (NO), DR4, DR5, caspase-3/7, caspase 8, Bax, JNK, MAPK, p38 induction, and phosphor-STAT3, *poly polymerase* cleavage, COX-2, IL-1β, NF-κB, IκB kinase α/β, cyclin D1, cyclin A, cyclin B1, Erα protein and mRNA, human epidermal growth factor receptor 2 phosphorylation, caveolin-1, Akt, Janus Kinase 1, STAT3, Bcl2, c-Jun, c-Fos, JNK, the *mechanistic target of rapamycin* (mTOR) suppression, as well as cell cycle blockage.[122]

Saffron is a spice from the dry stigmas of the plant *Crocus sativus* L., which has been traditionally used as a remedy for several problems such as cancer by ancient Arabian, Indian, and Chinese cultures. Crocetin is a carotenoid-type component of saffron, and has been demonstrated to have remarkable activity as an anti-tumor agent in *in vitro* and *in vivo* studies. Crocetin inhibits the growth of cancer cells through the inhibition of nucleic acid synthesis, induction of the antioxidative system, apoptosis, and hindering growth factor signaling pathways.[123] Crocetin was shown to inhibit LPS-induced nitric oxide release, reduce the levels of TNF-α, IL-1β, and intracellular ROS, activate the NF-κB pathway, and prevent LPS-induced hippocampal cell death.[124] Crocetin and its derivatives were found to have anti-proliferative effect in MCF-7 and MDA-MB-231 breast cancer cells in a dose-dependent manner.[125] Crocetin displayed proapoptotic action in MCF-7 breast cancer cells through the caspase-dependent pathway by enhancing Bax protein expression.[126] Crocetin analogues were shown to decrease colony formation and cellular RNA and DNA synthesis[127], as well as viability of HeLa cells.[128] Besides cell growth reduction, crocetin-derived compounds including crocin, safranal, and picrocrocin, displayed apoptotic activity.[123,129]

Previous studies demonstrated that *Zingiber officinale Roscoe* (Zingiberaceae) as one of the important plant species that possessed an inhibitory effect on ovarian cancer cell growth through the inhibition of NF-κB activation, and reduced VEGF and IL-8 secretion.[130] Gingerol is the active secondary metabolite of *Z. officinale*. The anticarcinogenic potential of gingerol was also investigated against breast and ovarian cancers and was reported to exhibit antioxidant, anti-inflammatory, and antitumor activities by diminishing iNOS and TNF-α expression via suppression of IκB-α phosphorylation and NF-κB nuclear translocation.[130-132]

Curcuminoids are the main phytoconstituents of the popular Indian plant, *Curcuma longa* L., one of the species from the family Zingiberaceae.[7] An *in vivo* study revealed that curcumin prevented chemoresistance to paclitaxel treatment by downregulating NF-κB, MAPK, and Akt pathways.[133] The anticancer activity of curcumin was attributed to its capacity to induce apoptosis in cancer cells without showing cytotoxic action on healthy cells. The interaction of curcumin with NF-κB indicates a relationship between its anti-inflammatory and anticarcinogenic effects.[134,135] When co-administered with paclitaxel, curcumin exhibited a synergistic decrease in tumor volume and incidence in a xenograft model in nonobese diabetic/

severe combined immunodeficiency mice. Furthermore, pre-administration of curcumin to cervical cancer cells enhanced sensitivity to paclitaxel.[13] The inhibitory activity of curcumin was demonstrated on ovarian tumor cell (A2780) growth using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.[136] Curcumin was shown to dose-dependently inhibit Bcl-2 and P53 protein expressions, reduce NF-κB expression, and enhance caspase-3 expression.[137] The cell growth suppression effect was supported with another type of human ovarian cancer cells (Ho-8910).[138,139] Curcumin was shown to possess an mTOR inhibitory effect[140] and modulatory activity on tumor cell growth via multiple cell signaling pathway regulation including cyclin D1, c-Myc, Bcl-2, Bcl-x, cFLIP, XIAP, c-IAP1, caspase-8, 3, 9, p53, p21, DR4, DR5, JNK, Akt and 5' adenosine monophosphate-activated protein kinase.[141]

Hirsutenone, a diarylheptanoid-type component of *Alnus hirsuta* (Spach) Rupr. (Betulaceae) barks, was demonstrated to sensitize cisplatin-resistant ovarian and cervical cancer cells.[13] Hirsutenone activated p53 through phosphorylation at Ser 15 in cells with wild-type p53, and affected p53-null and p53-mutant cell lines. These actions were reported to be partially regulated by Akt, linking hirsutenone-dependent PI3K inhibition.[142]

Previous studies revealed that piperlongumine, an alkaloid-type compound from *Piper longum* L., significantly and dose-dependently induced cell apoptosis, G2/M phase arrest, and intracellular ROS accumulation. Furthermore, combination therapies of low-dose piperlongumine/cisplatin or paclitaxel provided an anti-growth effect on human ovarian cancer cells. Piperlongumine also enhanced cisplatin-induced apoptosis via increased levels of Drp 1-dependent mitochondrial fission.[13,110,142,143]

CONCLUSION

Gynecologic cancers occur as a result of the disruption of multicellular targets and survival signaling.[13] Inflammatory pathways have been found to possess important roles during this stage. According to *in vitro* and preclinical cancer prevention and treatment studies, plant extracts and their constituents have been proven to be effective such that they may be potential agents in gynecologic cancer therapy by exhibiting beneficial activities on multiple targets within various oncogenic signaling pathways including inflammation.[7,43] According to several scientific reports, combination therapy of plant-based drugs with commercially used anticarcinogenic drugs has been presented as an effective approach.[144] Flavonoids, cyanidins, tannins, phenolic acids, stilbenoids, organosulfur compounds, terpenoids, diarylheptanoids, and alkaloids can be counted among the phytoconstituents that exhibit anticarcinogenic effects by regulating inflammatory pathways, and could be further evaluated as novel drug candidates after clinical studies have been completed.

Conflict of Interest: No conflict of interest was declared by the author.

REFERENCES

1. Dalkic E, Wang X, Wright N, Chan C. Cancer-drug associations: a complex system. PLoS One. 2010;5:e10031.
2. GBD 2015 Risk Factors Collaborators. Global, regional, and national comparative risk assessment of 79 behavioural, environmental and occupational, and metabolic risks or clusters of risks, 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015. Lancet. 2016;388:1659-1724.
3. Thamm D, Dow S. How companion animals contribute to the fight against cancer in humans. Vet Ital. 2009;45:111-120.
4. Morris J, Dobson J. Small Animal Oncology. 1st ed. Blackwell Science; United Kingdom; 2001.
5. Cannon CM. Cats, cancer and comparative oncolog. Vet Sci. 2015;2:111-126.
6. Kisielewski R, Tołwińska A, Mazurek A, Laudański P. Inflammation and ovarian cancer current views. Ginekol Pol. 2013;84:293-297.
7. Wang H, Khor TO, Shu L, Su ZY, Fuentes F, Lee JH, Kong AN. Plants vs. cancer: a review on natural phytochemicals in preventing and treating cancers and their druggability. Anticancer Agents Med Chem. 2012;12:1281-1305.
8. Siegel R, DeSantis C, Virgo K, Stein K, Mariotto A, Smith T, Cooper D, Gansler T, Lerro C, Fedewa S, Lin C, Leach C, Cannady RS, Cho H, Scoppa S, Hachey M, Kirch R, Jemal A, Ward E. Cancer treatment and survivorship statistics, 2012. CA Cancer J Clin. 2012;62:220-241.
9. Prabhu A, Venkat P, Gajaraj B, Kılıngar Nadumane V. Induction of apoptosis in the cervical cancer cell line HeLa by a novel metabolite extracted from the fungus Aspergillus japonicus Saito. Turk J Biol. 2014;38:922-929.
10. Birudu RB, Naik MJ. Anticancer properties of secondary metabolites of medicinal plants in carcinoma. Br Med Bull. 2014;2:662-668.
11. Benedet JL, Bender H, Jones H 3rd, Ngan HY, Pecorelli S. FIGO staging classifications and clinical practice guidelines in the management of gynecologic cancers. FIGO Committee on Gynecologic Oncology. Int J Gynaecol Obstet. 2000;70:209-262.
12. Amant F, Van Calsteren K, Halaska MJ, Beijnen J, Lagae L, Hanssens M, Heyns L, Lannoo L, Ottevanger NP, Vanden Bogaert W, Ungar L, Vergote I, du Bois A. Gynecologic cancers in pregnancy: guidelines of an international consensus meeting. Int J Gynecol Cancer. 2009;19(Suppl 1):1-12.
13. Farrand L, Oh SW, Song YS, Tsang BK. Phytochemicals: a multitargeted approach to gynecologic cancer therapy. Biomed Res Int. 2014:2014:890141.
14. Hacker NF, Eifel PJ, van der Velden J. Cancer of the vagina. Int J Gynaecol Obstet. 2015;131(Suppl 2):84-87.
15. Lyman GH. Risk factors for cancer. Primary Care. 1992;19:465-479.
16. Gülçubuk A, Altun ED, Bozkurt ER, Sontaş BH, Haktanır D. Ovarian teratoma in a dog. Turk J Vet Anim Sci. 2012;36:573-576.
17. Demirel MA, Ergin I. Unilateral typical type serous borderline ovarian tumor in a Pointer dog. Med Weter. 2016;72:321-323.
18. Serin G, Aydogan A, Yaygingul R, Tunca R. Uterine leiomyosarcoma in a dog: a case report. Vet Med. 2010;55:405-408.
19. Mello Martins MI, Ferreira de Souza F, Gobello C. The canine transmissible venereal tumor: etiology, pathology, diagnosis and treatment. In: Concannon PW, England G, Verstegen III J, Linde-Forsberg C, eds, Recent Advances in Small Animal Reproduction, International Veterinary Information Service, Ithaca NY (www.ivis.org), 2005: A1233.0405.

20. Lopes PD, dos Santos ACAA, Silva JES. Canine transmissible venereal tumor in the genital area with subcutaneous metastases in the head - case report. RPCV. 2015;110:120-123.

21. Moulton JE. Tumours of mammary gland. In: Moulton JE. eds, Tumours in Domestic Animals. 3rd edn. Berkeley and Los Angeles; University of California Press; 1990:518-552.

22. Sleeckx N, de Rooster H, Veldhuis Kroeze EJ, Van Ginneken C, Van Brantegem L. Canine mammary tumours, an overview. Reprod Domest Anim. 2011;46:1112-1131.

23. Prpar Mihevc S, Dovč P. Mammary tumors in ruminants. Acta Agric Slov. 2013;102:83-86.

24. Port Louis LR, Varshney KC, Nair MG. An immunohistochemical study on the expression of sex steroid receptors in canine mammary tumors. ISRN Vet Sci. 2012;2012:378607.

25. Maeda S, Saimura M, Minami S, Kurashita K, Nishimura R, Kai Y, Yano H, Mashino K, Mitsuyama S, Shimokawa M, Tamura K; Kyushu Breast Cancer Study Group. Efficacy and safety of eribulin as first- to third-line treatment in patients with advanced or metastatic breast cancer previously treated with anthracyclines and taxanes. Breast. 2017;32:66-72.

26. Robertson FM, Bondy M, Yang W, Yamauchi H, Wiggins S, Kamrudin S, Krishnamurthy S, Le-Petross H, Bidaut L, Player AN, Barsky SH, Woodward WA, Buchholz T, Lucci A, Ueno NT, Cristofanilli M. Inflammatory breast cancer: the disease, the biology, the treatment. CA Cancer J Clin. 2010;60:351-375.

27. Yeh ED, Jacene HA, Bellon JR, Nakhlis F, Birdwell RL, Georgian-Smith D, Giess CS, Hirshfield-Bartek J, Overmoyer B, Van den Abbeele AD. What radiologists need to know about diagnosis and treatment of inflammatory breast cancer: a multidisciplinary approach. Radiographics. 2013;33:2003-2017.

28. Macciò A, Madeddu C. Inflammation and ovarian cancer. Cytokine. 2012;58:133-147.

29. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? Lancet. 2001;357:539-545.

30. Li-Weber M. Targeting apoptosis pathways in cancer by Chinese medicine. Cancer Lett. 2013;332:304-312.

31. Bai L, Wang S. Targeting apoptosis pathways for new cancer therapeutics. Annu Rev Med. 2014; 65:139-155.

32. Wajant H. The Fas signaling pathway: more than a paradigm. Science. 2002;296:1635-1636.

33. Maturu P, Jones D, Ruteshouser EC, Hu Q, Reynolds JM, Hicks J, Putluri N, Ekmekcioglu S, Grimm EA, Dong C, Overwijk WW. Role of Cyclooxygenase-2 Pathway in Creating an Immunosuppressive Microenvironment and in Initiation and Progression of Wilms' Tumor. Neoplasia. 2017;19:237-249.

34. Verdoodt F, Kjaer SK, Friis S. Influence of aspirin and non-aspirin NSAID use on ovarian and endometrial cancer: Summary of epidemiologic evidence of cancer risk and prognosis. Maturitas. 2017;100:1-7.

35. de M Souza CH, Toledo-Piza E, Amorin R, Barboza A, Tobias KM. Inflammatory mammary carcinoma in 12 dogs: clinical features, cyclooxygenase-2 expression, and response to piroxicam treatment. Can Vet J. 2009;50:506-510.

36. Nelson DE, Ihekwaba AE, Elliott M, Johnson JR, Gibney CA, Foreman BE, Nelson G, See V, Horton CA, Spiller DG, Edwards SW, McDowell HP, Unitt JF, Sullivan E, Grimley R, Benson N, Broomhead D, Kell DB, White MR. Oscillations in NF-kappaB signaling control the dynamics of gene expression. Science. 2004;306:704-708.

37. Escárcega RO, Fuentes-Alexandro S, García-Carrasco M, Gatica A, Zamora A. The transcription factor nuclear factor-kappa B and cancer. Clin Oncol (R Coll Radiol). 2007;19:154-161.

38. Hoesel B, Schmid JA. The complexity of NF-κB signaling in inflammation and cancer. Mol Cancer. 2013;12:86.

39. Karin M. NF-kappaB as a critical link between inflammation and cancer. Cold Spring Harb Perspect Biol. 2009;1:a000141.

40. Huber MA, Azoitei N, Baumann B, Grünert S, Sommer A, Pehamberger H, Kraut N, Beug H, Wirth T. NF-kappaB is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression. J Clin Invest. 2004;114:569-581.

41. Freedman RS, Deavers M, Liu J, Wang E. Peritoneal inflammation - A microenvironment for Epithelial Ovarian Cancer (EOC). J Transl Med. 2004;2:23.

42. Aherne SA, O'Brien NM. Dietary flavonols: chemistry, food content, and metabolism. Nutrition. 2002;18:75-81.

43. Johannot L, Somerset SM. Age-related variations in flavonoid intake and sources in the Australian Population. Public Health Nutr. 2006;9:1045-1054.

44. Cheung ZH, Leung MC, Yip HK, Wu W, Siu FK, So KF. A neuroprotective herbal mixture inhibits caspase-3-independent apoptosis in retinal ganglion cells. Cell Mol Neurobiol. 2008;28:137-155.

45. McKay DL, Blumberg JB. A review of the bioactivity and potential health benefits of chamomile tea (Matricaria recutita L.). Phytother Res. 2006; 20: 519-530.

46. Shukla S, Gupta S. Apigenin: a promising molecule for cancer prevention. Pharm Res. 2010;27:962-978.

47. Sung B, Chung HY, Kim ND. Role of Apigenin in Cancer Prevention via the Induction of Apoptosis and Autophagy. J Cancer Prev. 2016;21:216-226.

48. Brusselmans K, Vrolix R, Verhoeven G, Swinnen JV. Induction of cancer cell apoptosis by flavonoids is associated with their ability to inhibit fatty acid synthase activity. J Biol Chem. 2005;280:5636-5645.

49. Frigo DE, Duong BN, Melnik LI, Schief LS, Collins-Burow BM, Pace DK, McLachlan JA, Burow ME. Flavonoid phytochemicals regulate activator protein-1 signal transduction pathways in endometrial and kidney stable cell lines. J Nutr. 2002;132:1848-1853.

50. Jagadeeshan S, Kunnumakkara AB, Ramachandran I, Nair SA. Anticancer aktivities of fruits and vegetables against gynecological cancers. In: Kunnumakkara AB. eds. Anticancer Properties of Fruits and Vegetables: A Scientific Review. World Scientific; Singapore; 2014:131-160.

51. Way TD, Kao MC, Lin JK. Degradation of HER2/neu by apigenin induces apoptosis through cytochrome c release and caspase-3 activation in HER2/neu-overexpressing breast cancer cells. FEBS Lett. 2005;579:145-152.

52. Choi EJ, Kim GH. Apigenin induces apoptosis through a mitochondria/caspase-pathway in human breast cancer MDA-MB-453 cells. J Clin Biochem Nutr. 2009;44:260-265.

53. Way TD, Kao MC, Lin JK. Apigenin induces apoptosis through proteasomal degradation of HER2/neu in HER2/neu-overexpressing breast cancer cells via the phosphatidylinositol 3-kinase/Akt-dependent pathway. J Biol Chem. 2004;279:4479-4489.

54. Lindenmeyer F, Li H, Menashi S, Soria C, Lu H. Apigenin acts on the tumor cell invasion process and regulates protease production. Nutr Cancer. 2001;39:139-147.

55. Choi EJ, Kim GH. Apigenin causes G(2)/M arrest associated with the modulation of p21(Cip1) and Cdc2 and activates p53-dependent apoptosis pathway in human breast cancer SK-BR-3 cells. J Nutr Biochem. 2009;20:285-290.

56. Wang C, Kurzer MS. Phytoestrogen concentration determines effects on DNA synthesis in human breast cancer cells. Nutr Cancer. 1997;28:236-247.

57. Wang C, Kurzer MS. Effects of phytoestrogens on DNA synthesis in MCF-7 cells in the presence of estradiol or growth factors. Nutr Cancer. 1998;31:90-100.

58. Collins-Burow BM, Burow ME, Duong BN, McLachlan JA. Estrogenic and antiestrogenic activities of flavonoid phytochemicals through estrogen receptor binding-dependent and -independent mechanisms. Nutr Cancer. 2000;38:229-244.

59. Long X, Fan M, Bigsby RM, Nephew KP. Apigenin inhibits antiestrogen-resistant breast cancer cell growth through estrogen receptor-alpha-dependent and estrogen receptor-alpha-independent mechanisms. Mol Cancer Ther. 2008;7:2096-2108.

60. Zheng PW, Chiang LC, Lin CC. Apigenin induced apoptosis through p53-dependent pathway in human cervical carcinoma cells. Life Sci. 2005;76:1367-1379.

61. Wu C, Chen F, Rushing JW, Wang X, Kim HJ, Huang G, Haley-Zitlin V, He G. Antiproliferative activities of parthenolide and golden feverfew extract against three human cancer cell lines. J Med Food. 2006;9:55-61.

62. Lin Y, Shi R, Wang X, Shen HM. Luteolin, a flavonoid with potential for cancer prevention and therapy. Curr Cancer Drug Targets. 2008;8:634-646.

63. Byun S, Lee KW, Jung SK, Lee EJ, Hwang MK, Lim SH, Bode AM, Lee HJ, Dong Z. Luteolin inhibits protein kinase C(epsilon) and c-Src activities and UVB-induced skin cancer. Cancer Res. 2010;70:2415-2423.

64. Ham S, Kim KH, Kwon TH, Bak Y, Lee DH, Song YS, Park SH, Park YS, Kim MS, Kang JW, Hong JT, Yoon DY. Luteolin induces intrinsic apoptosis via inhibition of E6/E7 oncogenes and activation of extrinsic and intrinsic signaling pathways in HPV-18-associated cells. Oncol Rep. 2014;31:2683-2691.

65. Gábor M, Eperjessy E. Antibacterial effect of fisetin and fisetinidin. Nature. 1966;212:1273.

66. Maher P, Dargusch R, Ehren JL, Okada S, Sharma K, Schubert D. Fisetin lowers methylglyoxal dependent protein glycation and limits the complications of diabetes. PLoS One. 2011;6:e21226.

67. Arai Y, Watanabe S, Kimira M, Shimoi K, Mochizuki R, Kinae N. Dietary intakes of flavonols, flavones and isoflavones by Japanese women and the inverse correlation between quercetin intake and plasma LDL cholesterol concentration. J Nutr. 2000;130:2243-2250.

68. Chou RH, Hsieh SC, Yu YL, Huang MH, Huang YC, Hsieh YH. Fisetin inhibits migration and invasion of human cervical cancer cells by down-regulating urokinase plasminogen activator expression through suppressing the p38 MAPK-dependent NF-κB signaling pathway. PLoS One. 2013;8:e71983.

69. Geraets L, Haegens A, Brauers K, Haydock JA, Vernooy JH, Wouters EF, Bast A, Hageman GJ. Inhibition of LPS-induced pulmonary inflammation by specific flavonoids. Biochem Biophys Res Commun. 2009;382:598-603.

70. Lim DY, Park JH. Induction of p53 contributes to apoptosis of HCT-116 human colon cancer cells induced by the dietary compound fisetin. Am J Physiol Gastrointest Liver Physiol. 2009;296:1060-1068.

71. Teiten MH, Gaascht F, Dicato M, Diederich M. Targeting the wingless signaling pathway with natural compounds as chemopreventive or chemotherapeutic agents. Curr Pharm Biotechnol. 2012;13:245-254.

72. Syed DN, Afaq F, Maddodi N, Johnson JJ, Sarfaraz S, Ahmad A, Setaluri V, Mukhtar H. Inhibition of human melanoma cell growth by the dietary flavonoid fisetin is associated with disruption of Wnt/β-catenin signaling and decreased Mitf levels. J Invest Dermatol. 2011;131:1291-1299.

73. Liao YC, Shih YW, Chao CH, Lee XY, Chiang TA. Involvement of the ERK signaling pathway in fisetin reduces invasion and migration in the human lung cancer cell line A549. J Agric Food Chem. 2009;57:8933-8941.

74. Bracke ME, Depypere HT, Boterberg T, Van Marck VL, Vennekens KM, Vanluchene E, Nuytinck M, Serreyn R, Mareel MM. Influence of tangeretin on tamoxifen's therapeutic benefit in mammary cancer. J Natl Cancer Inst. 1999;91:354-359.

75. Luo H, Rankin GO, Li Z, Depriest L, Chen YC. Kaempferol induces apoptosis in ovarian cancer cells through activating p53 in the intrinsic pathway. Food Chem. 2011;128:513-519.

76. Yang JH, Kondratyuk TP, Jermihov KC, Marler LE, Qiu X, Choi Y, Cao H, Yu R, Sturdy M, Huang R, Liu Y, Wang LQ, Mesecar AD, van Breemen RB, Pezzuto JM, Fong HH, Chen YG, Zhang HJ. Bioactive compounds from the fern Lepisorus contortus. J Nat Prod. 2011;74:129-136.

77. An G, Gallegos J, Morris ME. The bioflavonoid kaempferol is an Abcg2 substrate and inhibits Abcg2-mediated quercetin efflux. Drug Metab Dispos. 2011;39:426-432.

78. Lin Z, Bazzaro M, Wang MC, Chan KC, Peng S, Roden RB. Combination of proteasome and HDAC inhibitors for uterine cervical cancer treatment. Clin Cancer Res. 2009;15:570-577.

79. Jung SK, Lee KW, Byun S, Lee EJ, Kim JE, Bode AM, Dong Z, Lee HJ. Myricetin inhibits UVB-induced angiogenesis by regulating PI-3 kinase in vivo. Carcinogenesis. 2010;31:911-917.

80. Huang H, Chen AY, Ye X, Li B, Rojanasakul Y, Rankin GO, Chen YC. Myricetin inhibits proliferation of cisplatin-resistant cancer cells through a p53-dependent apoptotic pathway. Int J Oncol. 2015;47:1494-1502.

81. Kasiske BL, O'Donnell MP, Lee H, Kim Y, Keane WF. Impact of dietary fatty acid supplementation on renal injury in obese Zucker rats. Kidney Int. 1991;39:1125-1134.

82. Lee MM, Gomez SL, Chang JS, Wey M, Wang RT, Hsing AW. Soy and isoflavone consumption in relation to prostate cancer risk in China. Cancer Epidemiol Biomarkers Prev. 2003;12:665-668.

83. Lee HP, Gourley L, Duffy SW, Estéve J, Lee J, Day NE. Dietary effects on breast-cancer risk in Singapore. Lancet. 1991;337:1197-1200.

84. Magee PJ, Rowland IR. Phyto-oestrogens, their mechanism of action: current evidence for a role in breast and prostate cancer. Br J Nutr. 2004;91:513-531.

85. Peeters PH, Keinan-Boker L, van der Schouw YT, Grobbee DE. Phytoestrogens and breast cancer risk. Review of the epidemiological evidence. Breast Cancer Res Treat. 2003;77:171-183.

86. Pike AC, Brzozowski AM, Hubbard RE, Bonn T, Thorsell AG, Engström O, Ljunggren J, Gustafsson JA, Carlquist M. Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. EMBO J. 1999;18:4608-4618.

87. Myung SK, Ju W, Choi HJ, Kim SC; Korean Meta-Analysis (KORMA) Study Group. Soy intake and risk of endocrine-related gynaecological cancer: a meta-analysis. BJOG. 2009;116:1697-1705.

88. Banerjee S, Kong D, Wang Z, Bao B, Hillman GG, Sarkar FH. Attenuation of multi-targeted proliferation-linked signaling by 3,3'-diindolylmethane (DIM): from bench to clinic. Mutat Res. 2011;728:47-66.

89. Andres S, Abraham K, Appel KE, Lampen A. Risks and benefits of dietary isoflavones for cancer. Crit Rev Toxicol. 2011;41:463-506.

90. Kim MK, Kim K, Han JY, Lim JM, Song YS. Modulation of inflammatory signaling pathways by phytochemicals in ovarian cancer. Genes Nutr. 2011;6:109-115.

91. Lee JY, Kim HS, Song YS. Genistein as a Potential Anticancer Agent against Ovarian Cancer. J Tradit Complement Med. 2012;2:96-104.

92. Hussain A, Harish G, Prabhu SA, Mohsin J, Khan MA, Rizvi TA, Sharma C. Inhibitory effect of genistein on the invasive potential of human cervical cancer cells via modulation of matrix metalloproteinase-9 and tissue inhibitors of matrix metalloproteinase-1 expression. Cancer Epidemiol. 2012;36:387-393.

93. Markovits J, Linassier C, Fossé P, Couprie J, Pierre J, Jacquemin-Sablon A, Saucier JM, Le Pecq JB, Larsen AK. Inhibitory effects of the tyrosine kinase inhibitor genistein on mammalian DNA topoisomerase II. Cancer Res. 1989;49:5111-5117.

94. López-Lazaro M, Willmore E, Austin CA. Cells lacking DNA topoisomerase II beta are resistant to genistein. J Nat Prod. 2007;70:763-767.

95. Gopalakrishnan A, Xu CJ, Nair SS, Chen C, Hebbar V, Kong AN. Modulation of activator protein-1 (AP-1) and MAPK pathway by flavonoids in human prostate cancer PC3 cells. Arch Pharm Res. 2006;29:633-644.

96. Yesil-Celiktas O, Sevimli C, Bedir E, Vardar-Sukan F. Inhibitory effects of rosemary extracts, carnosic acid and rosmarinic acid on the growth of various human cancer cell lines. Plant Foods Hum Nutr. 2010;65:158-163.

97. Berdowska I, Zieliński B, Fecka I, Kulbacka J, Saczko J, Gamian A. Cytotoxic impact of phenolics from Lamiaceae species on human breast cancer cells. Food Chem. 2013;141:1313-1321.

98. Xu Y, Jiang Z, Ji G, Liu J. Inhibition of bone metastasis from breast carcinoma by rosmarinic acid. Planta Med. 2010;76:956-962.

99. Tai J, Cheung S, Wu M, Hasman D. Antiproliferation effect of Rosemary (Rosmarinus officinalis) on human ovarian cancer cells in vitro. Phytomedicine. 2012;19:436-443.

100. Hossan MS, Rahman S, Bashar ABMA, Jahan R, Al-Nahain A, Rahamatullah M. Rosmarinic acid: A review of its anticancer action. World J Pharm Pharm Sci. 2014;3:57-70.

101. Zeng L, Gao J, Zhang R. [Study on anti-tumor effect of cyanidin-3-glucoside on ovarian cancer]. Zhongguo Zhong Yao Za Zhi. 2012;37:1651-1654.

102. Chen PN, Chu SC, Chiou HL, Chiang CL, Yang SF, Hsieh YS. Cyanidin 3-glucoside and peonidin 3-glucoside inhibit tumor cell growth and induce apoptosis in vitro and suppress tumor growth in vivo. Nutr Cancer. 2005;53:232-243.

103. Gu JW, Makey KL, Tucker KB, Chinchar E, Mao X, Pei I, Thomas EY, Miele L. EGCG, a major green tea catechin suppresses breast tumor angiogenesis and growth via inhibiting the activation of HIF-1α and NFκB, and VEGF expression. Vasc Cell. 2013;5:9.

104. Qiao Y, Cao J, Xie L, Shi X. Cell growth inhibition and gene expression regulation by (-)-epigallocatechin-3-gallate in human cervical cancer cells. Arch Pharm Res. 2009;32:1309-1315.

105. Leone M, Zhai D, Sareth S, Kitada S, Reed JC, Pellecchia M. Cancer prevention by tea polyphenols is linked to their direct inhibition of antiapoptotic Bcl-2-family proteins. Cancer Res. 2003;63:8118-8121.

106. Cherbonnel-Lasserre C, Dosanjh MK. Suppression of apoptosis by overexpression of Bcl-2 or Bcl-xL promotes survival and mutagenesis after oxidative damage. Biochimie. 1997;79:613-617.

107. Lambert JD, Yang CS. Mechanisms of cancer prevention by tea constituents. J Nutr. 2003;133:3262-3267.

108. Jeong WS, Kim IW, Hu R, Kong AN. Modulatory properties of various natural chemopreventive agents on the activation of NF-kappaB signaling pathway. Pharm Res. 2004;21:661-670.

109. Stakleff KS, Sloan T, Blanco D, Marcanthony S, Booth TD, Bishayee A. Resveratrol exerts differential effects in vitro and in vivo against ovarian cancer cells. Asian Pac J Cancer Prev. 2012;13:1333-1340.

110. Farrand L, Byun S, Kim JY, Im-Aram A, Lee J, Lim S, Lee KW, Suh JY, Lee HJ, Tsang BK. Piceatannol enhances cisplatin sensitivity in ovarian cancer via modulation of p53, X-linked inhibitor of apoptosis protein (XIAP), and mitochondrial fission. J Biol Chem. 2013;288:23740-23750.

111. Ko HS, Lee HJ, Kim SH, Lee EO. Piceatannol suppresses breast cancer cell invasion through the inhibition of MMP-9: involvement of PI3K/AKT and NF-κB pathways. J Agric Food Chem. 2012;60:4083-4089.

112. Satyan KS, Swamy N, Dizon DS, Singh R, Granai CO, Brard L. Phenethyl isothiocyanate (PEITC) inhibits growth of ovarian cancer cells by inducing apoptosis: role of caspase and MAPK activation. Gynecol Oncol. 2006;103:261-270.

113. Hahm ER, Singh SV. Bim contributes to phenethyl isothiocyanate-induced apoptosis in breast cancer cells. Mol Carcinog. 2012;51:465-474.

114. Moon YJ, Brazeau DA, Morris ME. Dietary phenethyl isothiocyanate alters gene expression in human breast cancer cells. Evid Based Complement Alternat Med. 2011;2011:462525.

115. Huong le D, Shim JH, Choi KH, Shin JA, Choi ES, Kim HS, Lee SJ, Kim SJ, Cho NP, Cho SD. Effect of β-phenylethyl isothiocyanate from cruciferous vegetables on growth inhibition and apoptosis of cervical cancer cells through the induction of death receptors 4 and 5. J Agric Food Chem. 2011;59:8124-8131.

116. Zhang L, Hao Q, Bao L, Liu W, Fu X, Chen Y, Wu H. Phenethyl isothiocyanate suppresses cervical carcinoma metastasis potential and its molecular mechanism. Mol Med Rep. 2014;10:2675-2680.

117. Li Y, Zhang T, Korkaya H, Liu S, Lee HF, Newman B, Yu Y, Clouthier SG, Schwartz SJ, Wicha MS, Sun D. Sulforaphane, a dietary component of broccoli/broccoli sprouts, inhibits breast cancer stem cells. 2010;16:2580-2590.

118. Chaudhuri D, Orsulic S, Ashok BT. Antiproliferative activity of sulforaphane in Akt-overexpressing ovarian cancer cells. Mol Cancer Ther. 2007;6:334-345.

119. Acharya A, Das I, Singh S, Saha T. Chemopreventive properties of indole-3-carbinol, diindolylmethane and other constituents of cardamom against carcinogenesis. Recent Pat Food Nutr Agric. 2010;2:166-177.

120. Bradlow HL, Zeligs MA. Diindolylmethane (DIM) spontaneously forms from indole-3-carbinol (I3C) during cell culture experiments. In Vivo. 2010;24:387-391.

121. Saw CL, Cintrón M, Wu TY, Guo Y, Huang Y, Jeong WS, Kong AN. Pharmacodynamics of dietary phytochemical indoles I3C and DIM: Induction of Nrf2-mediated phase II drug metabolizing and antioxidant

genes and synergism with isothiocyanates. Biopharm Drug Dispos. 2011;32:289-300.

122. Bishayee A, Ahmed S, Brankov N, Perloff M. Triterpenoids as potential agents for the chemoprevention and therapy of breast cancer. Front Biosci (Landmark Ed). 2011;16:980-996.
123. Gutheil WG, Reed G, Ray A, Anant S, Dhar A. Crocetin: an agent derived from saffron for prevention and therapy for cancer. Curr Pharm Biotechnol. 2012;13:173-179.
124. Nam KN, Park YM, Jung HJ, Lee JY, Min BD, Park SU, Jung WS, Cho KH, Park JH, Kang I, Hong JW, Lee EH. Anti-inflammatory effects of crocin and crocetin in rat brain microglial cells. Eur J Pharmacol. 2010;648:110-116.
125. Chryssanthi DG, Lamari FN, Iatrou G, Pylara A, Karamanos NK, Cordopatis P. Inhibition of breast cancer cell proliferation by style constituents of different Crocus species. Anticancer Res. 2007;27:357-362.
126. Mousavi SH, Tavakkol-Afshari J, Brook A, Jafari-Anarkooli I. Role of caspases and Bax protein in saffron-induced apoptosis in MCF-7 cells. Food Chem Toxicol. 2009;47:1909-1913.
127. Abdullaev FI, Frenkel GD. Effect of saffron on cell colony formation and cellular nucleic acid and protein synthesis. Biofactors. 1992;3:201-204.
128. Tavakkol-Afshari J, Brook A, Mousavi SH. Study of cytotoxic and apoptogenic properties of saffron extract in human cancer cell lines. Food Chem Toxicol. 2008;46:3443-3447.
129. Escribano J, Alonso GL, Coca-Prados M, Fernandez JA. Crocin, safranal and picrocrocin from saffron (Crocus sativus L.) inhibit the growth of human cancer cells in vitro. Cancer Lett. 1996;100:23-30.
130. Rhode J, Fogoros S, Zick S, Wahl H, Griffith KA, Huang J, Liu JR. Ginger inhibits cell growth and modulates angiogenic factors in ovarian cancer cells. BMC Complement Altern Med. 2007;7:44.
131. Lee HS, Seo EY, Kang NE, Kim WK. [6]-Gingerol inhibits metastasis of MDA-MB-231 human breast cancer cells. J Nutr Biochem. 2008;19:313-319.
132. Oyagbemi AA, Saba AB, Azeez OI. Molecular targets of [6]-gingerol: Its potential roles in cancer chemoprevention. Biofactors. 2010;36:169-178.
133. Sreekanth CN, Bava SV, Sreekumar E, Anto RJ. Molecular evidences for the chemosensitizing efficacy of liposomal curcumin in paclitaxel chemotherapy in mouse models of cervical cancer. Oncogene. 2011;30:3139-3152.
134. Bachmeier BE, Mohrenz IV, Mirisola V, Schleicher E, Romeo F, Höhneke C, Jochum M, Nerlich AG, Pfeffer U. Curcumin downregulates the inflammatory cytokines CXCL1 and -2 in breast cancer cells via NFkappaB. Carcinogenesis. 2008;29:779-789.
135. Aggarwal BB, Shishodia S. Suppression of the nuclear factor-kappaB activation pathway by spice-derived phytochemicals: reasoning for seasoning. Ann NY Acad Sci. 2004;1030:434-441.
136. Kumar SS, Surianarayanan M, Vijayaraghavan R, Mandal AB, MacFarlane DR. Curcumin loaded poly(2-hydroxyethyl methacrylate) nanoparticles from gelled ionic liquid in vitro cytotoxicity and anti-cancer activity in SKOV-3 cells. Eur J Pharm Sci. 2014;51:34-44.
137. Ganta S, Amiji M. Coadministration of Paclitaxel and curcumin in nanoemulsion formulations to overcome multidrug resistance in tumor cells. Mol Pharm. 2009; 6:928-939.
138. Crist KA, Zhang Z, You M, Gunning WT, Conran PB, Steele VE, Lubet RA. Characterization of rat ovarian adenocarcinomas developed in response to direct instillation of 7,12-dimethylbenz[a]anthracene (DMBA) coated suture. Carcinogenesis. 2005;26:951-957.
139. Hamam F. Curcumin: New weapon against cancer. Food and Nutrition Sciences. 2014;5:2257-2264.
140. Beevers CS, Chen L, Liu L, Luo Y, Webster NJ, Huang S. Curcumin disrupts the Mammalian target of rapamycin-raptor complex. Cancer Res. 2009;69:1000-1008.
141. Ravindran J, Prasad S, Aggarwal BB. Curcumin and cancer cells: how many ways can curry kill tumor cells selectively? Aaps J. 2009;11:495-510.
142. Farrand L, Kim JY, Byun S, Im-aram A, Lee J, Suh JY, Lee KW, Lee HJ, Tsang BK. The diarylheptanoid hirsutenone sensitizes chemoresistant ovarian cancer cells to cisplatin via modulation of apoptosis-inducing factor and X-linked inhibitor of apoptosis. J Biol Chem. 2014;289:1723-1731.
143. Farrand L, Kim JY, Im-Aram A, Suh JY, Lee HJ, Tsang BK. An improved quantitative approach for the assessment of mitochondrial fragmentation in chemoresistant ovarian cancer cells. PLoS ONE. 2013;8:e74008.
144. Kma L. Roles of plant extracts and constituents in cervical cancer therapy. Asian Pacific J Cancer Prev. 2013;14:3429-3436.

2017 Author Index - 2017 Yazar Dizini

2017 Author Index - 2017 Yazar Dizini

2017 Subject Index

2017 Konu Dizini