

TURKISH JOURNAL OF PHARMACEUTICAL SCIENCES



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Turkish Journal of Pharmaceutical Sciences journal is regularly published 6 times in a year (February, April, June, August, October, December). The issuing body of the journal is Galenos Yayınevi/Publishing House level.

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 professionals in all fields of pharmaceutical sciences.

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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).

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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).

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Various *In Vitro* Bioactivities of Secondary Metabolites Isolated from the Sponge *Hyrtios aff. Erectus* from the Red Sea Coast of Egypt

Mısır'ın Kızıl Deniz Kıyısındaki Sünger *Hyrtios aff. Erectus*'tan İzole Edilen Farklı Sekonder Metabolitlerin Biyoaktiviteleri

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ABSTRACT

Objectives: The present study revealed the presence of bioactive constituents in *Hyrtios aff. erectus* sponge (HES) extract collected from the Red Sea using skin and scuba diving.

Materials and Methods: Cytotoxicity was tested against hepatocellular carcinoma cell lines as a prescreening test.

Results: The HES extract had high contents of total phenolic compounds (0.061 mg/g), flavonoids (0.2839 mg/g), and carotenoids (1.976 mg/g). Moreover, the HES extract showed high antioxidant capacity with 93.0% and 99% at 1 mg using 2,2'-Diphenyl- α -picrylhydrazyl and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), respectively. Cytotoxic activity against cancerous cell lines showed that the HES extract could inhibit cell growth effectively with IC_{50} =47.5 μ g/mL. Furthermore, anticancer activity using protein tyrosine kinase and sphingosine kinase 1 inhibitor screening assays resulted in 71.66% and 85.21% inhibition activity, respectively. The anti-inflammatory assays showed that the inhibition activity against cyclooxygenase (COX₁), COX₂, interleukin-6, and tumor necrosis factor- α was 71.82%, 81.13%, 80.89%, and 59.74%, respectively. At the same time, the anti-Alzheimer results using acetylcholine inhibition assay showed high activity at 1 mg with 83.51%. Additionally, the antiviral activity using the reverse transcriptase inhibition assay was 91.70%.

Conclusion: This marine sponge isolated from the Red Sea showed tremendous activity against many diseases and it is considered an excellent source for bioactive pharmaceutical compounds.

Key words: Red Sea, cytotoxic, antioxidant, anti-Alzheimer, anticancer, anti-inflammatory, antiviral

ÖZ

Amaç: Bu çalışma normal ve tüplü dalışlar ile Kızıl Deniz'den toplanan *Hyrtios aff. erectus* süngerindeki (HES) biyoaktif bileşiklerin varlığını göstermeyi amaçlamıştır.

Gereç ve Yöntemler: Sitotoksikite hepatosellüler karsinoma hücre hatlarında ön izleme testleriyle belirlenmiştir.

Bulgular: HES ekstresi yüksek derecede fenolik bileşikler, (0,061 mg/g), flavonoidler (0,2839 mg/g) ve karotenoidleri (1,976 mg/g) içermektedir. Ayrıca, 1 mg HES ekstresi 2'-difenil- α -pikrilhidrazil ve 2,2'-azino-bis (3-etilbenzotiyazolin-6-sulfonik asit) ile sırasıyla %93,0 ve %99 ile yüksek antioksidan kapasite göstermiştir. HES ekstresi kanseröz hücre hatlarına karşı sitotoksik bulunmuştur ve hücre büyümesini inhibe edebilmektedir (IC_{50} =47,5 μ g/mL). Dahası, protein tirozin kinaz ve sfingosin kinaz 1 inhibitör izleme testleri kullanılarak belirlenen antikanser aktivitesi, sırasıyla %71,66 ve %85,21 inhibitör aktiviteyle sonuçlanmıştır. Antienflamatuvar testler siklooksijenaz 1 (COX₁), siklooksijenaz 2 (COX₂), interlökin-6 ve tümör nekroz faktör- α inhibitör aktivitesinin sırasıyla %71,82, %81,13, %80,89 ve %59,74 olduğunu göstermiştir. Aynı zamanda, asetil kolin esteraz inhibisyon teti kullanılarak elde edilen anti-Alzheimer sonuçlar 1 mg dozda yüksek aktiviteyi (%83,51) belirlemiştir. Ek olarak, geri transkriptaz inhibisyon yöntemi kullanılarak bakılan antiviral aktivitesi %91,70'dir.

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Sonuç: Kızıl Deniz'den elde edilen bu deniz süngeri birçok hastalığa karşı büyük bir aktivite göstermiştir ve biyoaktif farmasötik bileşikler için mükemmel bir kaynak kabul edilebilir.

Anahtar kelimeler: Kızıl Deniz, sitotoksiste, antioksidan, anti-Alzheimer, antikanser, anti-enflamatuvar, antiviral

INTRODUCTION

Marine habitats contain a broad range of different organisms having a variety of biochemical and physiological characteristics and ability to adapt to their environment. Marine organisms such as sponges, tunicates, fishes, soft corals, nudibranchs, sea hares, mollusks, echinoderms, bryozoans, prawns, shells, sea slugs, and marine microorganisms are sources of bioactive compounds.^{1,2} Marine sponges belonging to the phylum Porifera (Metazoa), evolutionarily the oldest animals, are the single best source of marine natural products. Very recently, marine sponges of the Red Sea have been recognized as a rich source of bioactive secondary metabolites.³⁻⁸ A great number of biologically active compounds with potential antitumor, anticancer, antimicrotubule, antiproliferative, cytotoxic, and photoprotective, as well as antibiotic and antifouling properties have been isolated.

The main objective of the present study was to investigate the tremendous activities of sponge secondary metabolites collected from the Red Sea as antioxidant, cytotoxic, anti-Alzheimer, anticancer, anti-inflammatory, and antiviral agents.

MATERIALS AND METHODS

Area of study

The Red Sea (Figure 1a) comprises a wide range of tropical marine habitats, many of which are internationally recognized for their conservation, scientific, economic, or recreational value.¹⁻⁸ It attracts many human activities, which in turn impact its environment⁹⁻¹⁸ and are likely to affect biological life and disturb the Red Sea's natural ecosystems.¹⁹⁻²⁶

Sampling, identification, and prescreening bioassays of the sponge *Hyrtios aff. erectus*

Hyrtios aff. erectus samples were collected from Hurglada on the Egyptian Red Sea coastline during spring 2014 (Figure 1b). The samples were collected using skin and scuba diving, processed, washed with freshwater, and transferred directly to the laboratory in sterile polyethylene bags under reduced temperature (0°C). Identification of the sponge species was kindly performed by Dr. Nicole Voogd, at the Naturalis Biodiversity Center, Department of Marine Zoology, RA Leiden, the Netherlands. The voucher specimen is incorporated in the collections of the Zoological Museum of the University of Amsterdam under registration number RMNH POR.8633.

Chemicals and solvents

Potassium ferricyanide, ferric chloride, NaOH, chloroform, glacial acetic acid, ferric chloride solution, H₂SO₄, Folin-Ciocalteu reagent, vanillin, methanol, HCl, *n*-hexane, H₂O₂, HNO₃, Se standard, Mn standard, β-carotene, catechin, (+)-quercetin, sodium nitrite, aluminum chloride, and gallic acid were purchased from Sigma Aldrich.

Instruments

Atomic absorption spectrophotometry [AAS and Graphite Furnace Atomizer (GFA) Shimadzu] and GC-MS (Thermo, USA) were applied.

Preliminary bioactive screening of *Hyrtios aff. erectus* sponge extract (HES)

The ethyl acetate extract of *Hyrtios aff. erectus* was subjected to different chemical tests for the detection of different phytoconstituents, i.e. tannins, phlobatannins, saponins, alkaloids, flavonoids, quinines, coumarin, terpenoids, and cardiac glycosides.²⁷

Quantitative chemotaxonomy profiling

Determination of total phenolic content in HES extract

Total phenolic compounds in *Hyrtios aff. erectus* extract were determined as described by Taga et al.²⁸

Determination of total flavonoid content in HES extract

Total flavonoid content was determined by a colorimetric method reported by Zhishen et al.²⁹

Determination of total tannins in HES extract

Tannins (proanthocyanidins) were determined according to the method described by Sun et al.³⁰

Determination of total carotenoids in HES extracts

Total carotenoid content was measured according to Thaipong et al.²⁷

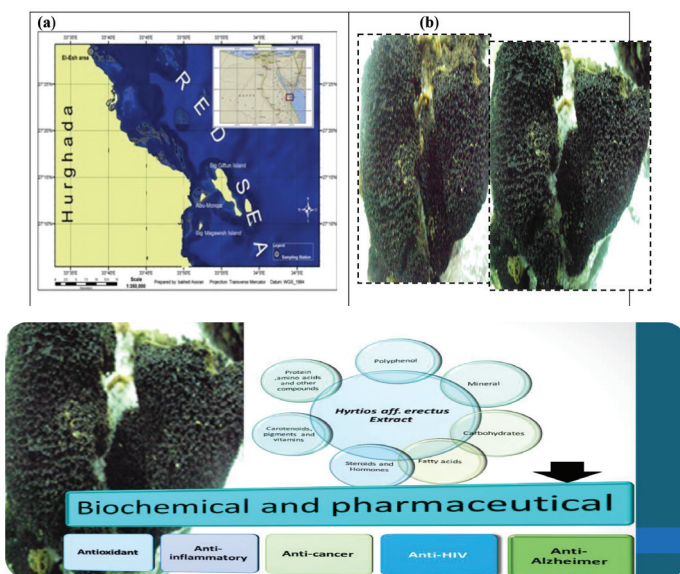


Figure 1. Location of sampling stations at the Red Sea (a) and sponge sample *Hyrtios aff. erectus* RMNH POR.8633 sample (b) and (c) represent the all steps of the present study (c) The schematic diagram of sponge extract constituents and multimedicinal effect

Preparation and extraction for mineral and metal assessment (Fe, Zn, Co, Mn, Cu, and Se) of HES extract

A 0.5 g dried sample of HES marine extract was digested using 5 mL of concentrated HNO₃, the mixture was heated using a hot plate for 1 h and when semidried 5 mL of concentrated HNO₃ and 2 mL of H₂O₂ were added and it was kept on the hot plate for 1 h. The semidried cooled residue was filtered with the help of Whatman filter paper and the residue volume was made up to 25 mL with 2N HNO₃. Analysis was carried out using an AAS (GFA Shimadzu atomic absorption spectrophotometer AA-6800) according to the Official Methods of Analysis³¹ for the determination of Fe, Zn, Co, Mn, Cu, and Se.

Elemental analysis of HES extract

The total carbon and hydrogen contents of marine HES extract were determined using a CHNO Elemental Analyzer.

Prescreening bioassays using in vitro cytotoxicity with cell lines

Different concentrations of HES extract (µg/mL) from all samples were tested for each cell line. Samples were dissolved in dimethyl sulfoxide (DMSO) and further diluted with cell culture medium. The final DMSO concentration used was 1% of total volume of the medium in all treatments, including the control group. Cells with no treatment were examined as negative and positive controls.³²

Primary screening assay

2,2'-Diphenyl-α-picrylhydrazyl radical scavenging effect of HES extract

A 2,2'-Diphenyl-α-picrylhydrazyl (DPPH) radical scavenging assay of the total *Hyrtios aff. erectus* extract was performed using a modified previously established methodology by Blois³³ and Amarowicz et al.³⁴ The scavenging ratio of the DPPH assay was calculated as follows:

$$\% \text{ scavenging} = [(A \text{ control} - A \text{ sample}) / A \text{ control}] \times 100$$

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity assay of HES extract

The 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS⁺) free radical decolorization assay was developed according to Chkraborty et al.³⁵ The percentage scavenging of ABTS⁺ was calculated by the following formula:

$$\text{Scavenging activity (\%)} = (A_0 - A_x) / A_0 \times 100$$

A_x and A₀ were the absorbance at 734 nm of samples with and without extract, respectively.

Specialized screening assays

Acetylcholinesterase inhibition (AChEI) assay of HES extract

Inhibition of acetylcholinesterase inhibition (AChE) by *Hyrtios aff. erectus* extract was evaluated as described by Moyo et al.³⁶ Percentage inhibition by extracts was calculated using the following equation:

$$\text{Inhibition (\%)} = (1 - \text{sample reaction rate}) / (\text{blank reaction rate}) \times 100$$

Determination of protein tyrosine kinase inhibitory activity of HES extract

Sample preparation

The dimethylsulfoxide (DMSO) sample solution of the appropriate extract was diluted with H₂O (1:1 v/v) to yield corresponding sample solutions (1 mg/mL). Tyrosine kinase (TK) inhibitory activity was determined using a commercial test kit (TK assay kit, nonradioactively, Takara Cat. #MK410). Protein tyrosine kinase (PTK) activity of the samples was calculated based on the prepared standard curve. The color intensity is stable for 1 h after addition of stop solution at room temperature in a light room.

$$\% \text{ inhibition} = \frac{[\text{Initial activity} - \text{inhibitor}]}{\text{Initial activity}}$$

Determination of sphingosine kinase 1 inhibitor screening assay (SHK1) of HES extract

Sphingosine kinase inhibitory activity of the crude extract was determined by using the colorimetric sphingosine kinase 1 (SK1) inhibitor screening assay kit from Cayman. The plate was covered and the fluorescence was measured using an excitation wavelength between 530 and 540 nm and an emission wavelength between 580 and 590 nm.

$$\% \text{ inhibition} = \frac{[\text{Initial activity} - \text{inhibitor}]}{\text{Initial activity}}$$

Determination of cyclooxygenase 1 (COX₁) and cyclooxygenase 2 (COX₂) inhibitor screening assay of HES extract

The COX inhibitory activity of the crude extract was determined using the colorimetric COX (ovine) inhibitor screening assay kit from Cayman. The absorbance was measured at 590 nm using a plate reader.

$$\% \text{ inhibition} = \frac{[\text{Initial activity} - \text{inhibitor}]}{\text{Initial activity}}$$

Determination of tumor necrosis factor alpha (TNF-α) assay of HES extract

The TNF-α inhibitory activity of the crude extract was determined using a KOMA BIOTECH colorimetric kit. The absorbance was measured at 450 nm.

Determination of interleukin 6 (IL-6) assays of HES extract

The IL-6 inhibitory activity of the crude extract was also determined using a KOMA BIOTECH colorimetric kit. The absorbance was also measured at 450 nm.

Determination of reverse transcriptase (RT) enzyme inhibitor screening assay of HES extract

The RT inhibitory activity of the crude extract against a purified recombinant, human immunodeficiency virus (HIV-1)-RT, was determined using a Roche colorimetric kit. The assay was performed according to Fonteh et al.³⁷ with HIV-1 protease enzyme and the substrate, which is a synthetic peptide that

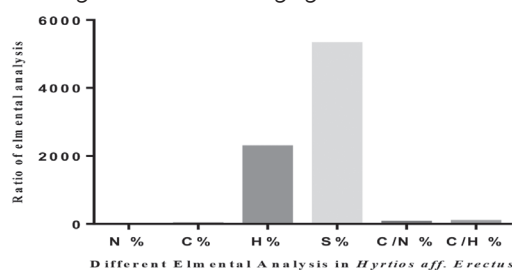
contains a cleavage site Tyr-Pro for HIV protease, as well as two covalently modified amino acids for the detection of cleavage. Acetyl pepstatin was used as a positive control for HIV-1 PR inhibition. The blank treatment consists of an assay buffer with only the substrate; untreated control of enzyme and substrate was also included. The absorbance was measured at 450 nm.

Statistical analysis

All results were analyzed by ANOVA using Prism.

RESULTS AND DISCUSSION

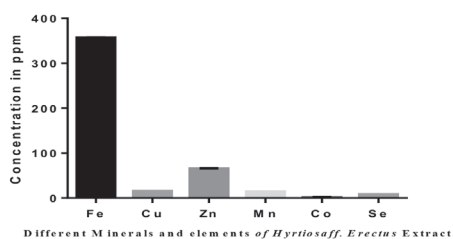
The secondary metabolites isolated from HES extract showed high contents of sulfur compounds (Figure 2). The mineral results showed high iron and zinc contents (Figure 3), in addition to polyphenol contents, which reflected high tannins and flavonoids. The crude extract of the sponge showed also high carotenoids contents (Figure 4). The bioactive profiling and diversity of natural compounds produced by sponge showed the presence of certain chemical classes of steroids, chromones, quinones, alkaloids, fatty acids; diketopiperazine, steroid, lactone, quinolone, anthraquinone, trisindole, phenol, and dihydropyridine benzoic acid derivatives; terpenoids; macrolactam; ethers; carboxylic acid; and terpenes, which are responsible for antioxidant, anti-inflammatory, antimicrobial, anti-HIV, anticancer, or antitumor activity. The quinolone derivatives are responsible for anti-HIV activity, fatty acid esters and fatty acids are responsible for anti-inflammatory activity, and pentaketides and alkaloids are responsible for neuroprotective activity.³⁸ The present study revealed that this sponge has cytotoxicity against hepatocellular carcinoma (Table 1) (Figure 5). This finding agrees well with other research



	N %	C %	H %	S %	C/N %	C/H %
Mean	3.490	7.680	2269	5301	48.73	74.25
Std. Deviation	0.01000	0.1200	0.06006	0.01001	0.2750	0.05508

Figure 2. The elemental analysis result of *Hyrtios aff. erectus*

Std: Standard

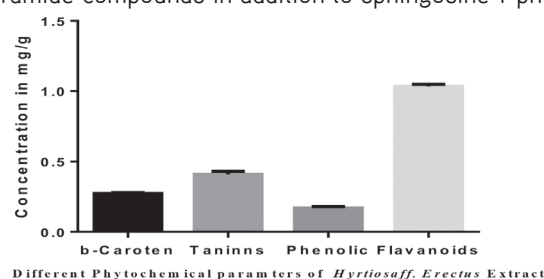


	Fe	Cu	Zn	Mn	Co	Se
Mean	356.8	15.54	65.55	14.10	1.990	7.886
Std. Deviation	0.2050	0.06506	0.4500	0.005774	0.01000	0.01445

Figure 3. The mineral profiling for *Hyrtios aff. erectus*

Std: Standard

papers.^{4-8,39} Cytotoxic activity is considered the first parameter in screening for anticancer agents,⁴⁻⁸ while the cytotoxicity assay needs to be followed by other experiments to confirm their potential activity as an anticancer agent and to determine the mechanism. It has been reported that cell death can be induced through three mechanisms: apoptosis, autophagy, and oncosis.⁴⁰ In the present study the anticancer activity was determined through two different experimental models using TK and SK1 as anticancer targets.⁴¹ Sphingolipid-metabolizing enzymes have an important role in controlling the balance of the cellular levels of some important bioactive lipids, for example proliferative compound as well as apoptotic and ceramide compounds in addition to sphingosine 1-phosphate.⁴²



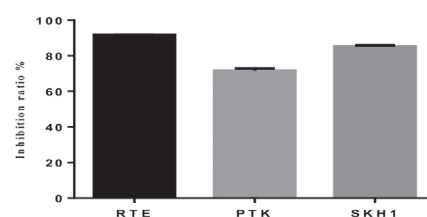
	b-Caroten	Taninns	Phenolic	Flavanoids
Mean	0.2733	0.4100	0.1700	1.035
Std. Deviation	0.005773	0.02000	0.01000	0.01500

Figure 4. The polyphenol and carotenoid profiling for *Hyrtios aff. erectus* sponge extract

Std: Standard

Table 1. Inhibitory activities of *Hyrtios aff. erectus* sponge extract against hepatocellular carcinoma cells

Sample concentration (μ g)	Viability %
50.00	47.83
25.00	69.17
12.50	80.24
6.25	93.62
3.125	97.89
1.56	100
0.00	100.00



	RTE	PTK	SKH1
Mean	91.70	71.66	85.21
Std. Deviation	0.1250	1.190	0.7050

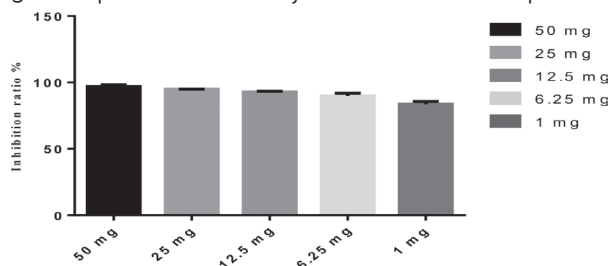
Figure 5. The anti-human immunodeficiency virus and anticancer profiling of *Hyrtios aff. erectus* sponge extract

Std: Standard, PTK: Protein tyrosine kinase, SKH: Sphingosine kinase RTE: Reverse transcriptase enzyme

The discovery of new chemotherapeutic resistance is an urgent and important challenge in oncology. Increased level of SK1 is considered a poor prognosis, and overexpression of SK1 means resistance to chemotherapeutics. Sphingosine kinase is involved in the development of different cancers and in chemotherapeutic resistance to drugs. Thus, SK1 represents an important target for anticancer drug therapy. Receptor tyrosine kinases are cell surface transmembrane proteins responsible for intracellular signal transduction. They are expressed in several cell types and, after activation by growth factor binding, trigger a series of intracellular pathways, leading to a wide variety of cell responses such as differentiation, proliferation, migration, invasion, angiogenesis, and survival. The overexpression of protein kinase members is associated with cancers and tumor cells. Therefore, tyrosine kinases are pivotal targets in drug therapy for cancer. The flavonoids, which are remarkably nontoxic⁴³ and could inhibit PTK and SKH activity, appear to have promising bioactivity as anticancer agents and are worthy of further investigation.⁴⁴ Phenolic compounds, especially flavonoids, exhibit anti-inflammatory and anticancer effects by inhibiting PTKs through several mechanisms. The first one is as an antioxidant and as being competitive inhibitors for the ATP binding sites on a variety of kinase enzymes.^{45,46} Agullo et al.⁴⁷ reported that the effectiveness of flavonoids depends mainly on the position, number, and substitution of the hydroxyl group of the β -ring. The saturation of the C_2 - C_3 bond is also an important factor that affects flavonoids inhibition of phosphatidylinositol 3-kinase. This can be easily found in marine natural products as more rings and chiral centers are there compared to synthetic compounds and drugs. Moreover, marine natural products provide molecules with larger molecular weight than synthetic compounds. While on average natural products contain fewer nitrogen, sulfur, and halogen atoms, they have higher ratios of these constituents compared to synthetic compounds and drugs.⁴⁸ Another explanation is that the *pp60src* gene product is a PTK, the activity of which has been shown to be inhibited by phenolic compounds, especially flavonoids.⁴⁹ In the present study, the total polyphenolic assay, i.e. total phenolic and flavonoids, showed that the *Hyrtios aff. erectus* extract had high polyphenolic contents (Figure 4). Flavonoids are naturally occurring polyphenolic compounds that are present in a variety of natural products, and are the most abundant antioxidants in the human diet.⁵⁰⁻⁵² While there has been a major focus on their antioxidant properties, there is an emerging view that flavonoids and their *in vivo* metabolites do not act only as conventional hydrogen-donating antioxidants, but also to modulate cell function through actions at protein kinase and lipid kinase signaling pathways (PTK and SHK). These findings are in agreement with many other previous studies.⁵³ In fact, flavonoids, and their metabolites, have been reported to act at PI 3-kinase, Akt/protein kinase B, tyrosine kinases, protein kinase C, and mitogen-activated protein kinase signaling cascades. Inhibitory or stimulatory actions at these pathways are likely to affect cellular function profoundly by altering the phosphorylation state of target molecules and by modulating gene expression.⁵³

An understanding of the mechanism of action of flavonoids, either as antioxidants or as modulators of cell signaling, is key to evaluating the potency of biomolecules as inhibitors of oxidative stress in general and in neurodegeneration.⁵⁴ The flavonoid compounds are characterized by their inhibitory effect on tyrosine kinase. Accordingly, the *Hyrtios aff. erectus* extract revealed the highest inhibition activity in PTK and SHK assays. Saponara et al.⁵⁵ reported that the activity of *pp60src* gene product, which is a PTK, has been shown to be inhibited by flavonoids. Two major types of HIV have been identified so far, HIV-1 and HIV-2. HIV-1 is the cause of the worldwide epidemic and is most commonly referred to as HIV. The basic biological processes in the HIV-1 life cycle are now well established, and natural compounds targeting specific steps in this life cycle can be found.⁵⁶ HIV RT inhibitors include nucleotide RT inhibitors and non-nucleotide RT inhibitors. Most clinical anti-HIV drugs are HIV RT.⁵⁷ In the last decade (2002-2011), 132 anti-HIV natural products were obtained from marine organisms. Of the anti-HIV bioactive marine natural products, before or after 2002, more than half were derived from marine sponges.⁵⁷ The present study indicated that the highest activities were from *Hyrtios aff. erectus* by 91.7, in agreement with previous studies.⁵⁸ Moreover, Simmons et al.⁵⁹ concluded that sessile marine organisms (sponge and seaweeds) contain substances capable of potent biological activity, which has also been demonstrated against different types of cancer and HIV/Acquired Immune Deficiency syndrome. Restoring acetylcholine levels by inhibiting AChE has become the primary treatment for the cognitive deficits of Alzheimer disease (AD).^{60,61} The inhibition of AChE is beneficial not only to the enhancement of cholinergic transmission in the brain, but also to reduce the aggregation of β -amyloid and the formation of the neurotoxic fibrils in AD. In recent decades, researchers have attempted to develop new AChE inhibitors, especially the so-called "multifunctional AChE inhibitors" with additional efficacy in vascular dementia treatment.⁶⁰ There have been plenty of phytochemicals found to be effective in inhibiting AChE, which mainly consist of alkaloids, cannabinoids, curcumins, stilbenes, and flavonoids.⁶⁰ Among them, flavonoids have attracted more and more interest for their high inhibitory activity and low toxicity.⁶² Moreover, their diverse activities such as antioxidation, inhibition of advanced glycation products, and cardio-cerebrovascular protection give them extra advantages as potential multifunctional therapeutic agents for aging-related diseases.⁶³ The anti-Alzheimer results of the present study (Figure 6) using different extracts showed the highest inhibition ratio by *Hyrtios aff. erectus*, which produces secondary metabolites for defense against other microorganisms and these secondary metabolites serve as a source of bioactive compounds for use in human therapies as they thrive in harsh oceanic climates.^{52,53,64} Many studies confirm the high activity of secondary metabolites isolated from marine *Hyrtios aff. erectus* including alkaloids, esters, fatty acids, glycosides, ketones, lipids, macrolides, alcohols, peptides, peroxides, polyketides, quinones, steroids, sterols, terpenes, and terpenoids.^{52,53,65} Chronic inflammation is thought to play crucial roles in the pathogenesis of various

diseases. Several types of drugs are used to treat inflammatory disorders, but they cause adverse side effects. Natural products offer a great hope for the discovery of bioactive lead compounds.

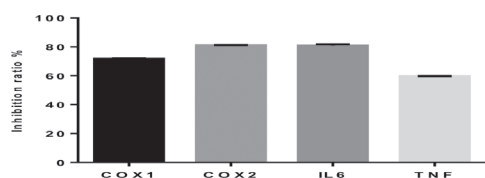


Different concentrations of *Hyrtios aff. Erectus* in Acetylcholinesterase inhibitor assay

	50 mg	25 mg	12.5 mg	6.25 mg	1 mg
Mean	96.99	95.02	92.89	89.82	83.51
Std. Deviation	1.365	0.02828	0.5515	2.001	2.135

Figure 6. The acetylcholinesterase inhibitor activity of *Hyrtios aff. erectus* extract

Std: Standard

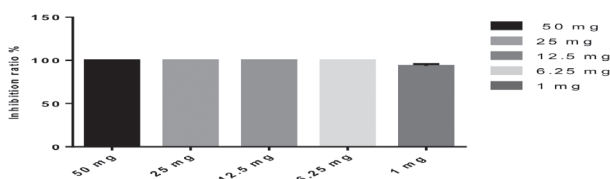


Different anti-inflammatory biochemical parameter of *Hyrtios aff. Erectus* Extract

	COX1	COX2	IL6	TNF
Mean	71.82	81.13	80.89	59.74
Std. Deviation	0.1850	0.1250	0.8850	0.06000

Figure 7. The anti-inflammatory profiling of *Hyrtios aff. erectus* extract

Std: Standard, COX: Cyclooxygenase, IL-6: Interleukin-6, TNF: Tumor necrosis factor- α

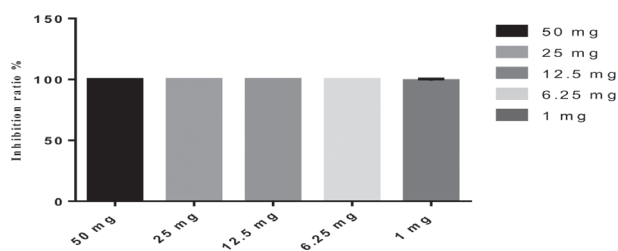


Different concentrations of *Hyrtios aff. Erectus* in DPPH

	50 mg	25 mg	12.5 mg	6.25 mg	1 mg
Mean	100.0	100.0	100.0	100.0	93.50
Std. Deviation	0.0	0.0	0.0	0.0	2.121

Figure 8. The total antioxidant capacity using scavenging (%) of 2,2'-Diphenyl- α -picrylhydrazyl assay

Std: Standard



Different concentrations of *Hyrtios aff. Erectus* in ABTS

	50 mg	25 mg	12.5 mg	6.25 mg	1 mg
Mean	100.0	100.0	100.0	100.0	99.00
Std. Deviation	0.0	0.0	0.0	0.0	1.414

Figure 9. The total antioxidant capacity using scavenging (%) 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay

Std: Standard

These compounds can be developed into drugs for treatment of inflammatory disorders. The biological and chemical diversity of marine habitats constitutes a sizeable reservoir of novel compounds. Some of them, like sesquiterpenoids, diterpenes, steroids, polysaccharides, alkaloids, fatty acids, proteins, and other chemical compounds, isolated from marine organisms are found to exhibit anti-inflammatory activity⁶⁶ in agreement with our results as shown in Figure 7. Recently different compounds from sponge have been shown to have potent anti-inflammatory activity.^{52,53} The natural products from marine sponge with different structures such as diterpenes, alkaloids, sulfated polysaccharides, and polyphenols inhibit different types of pro-inflammatory biomarkers, IL-6, TNF, NF- κ B, IL-1 β , COX₁, and COX₂, through different pathways:

1. The antioxidant effect by inhibition of the production of ROS compounds, which stimulate the pro-inflammatory biomarkers.⁶⁷
2. The direct effect by inhibition of prostaglandin and in sequence inhibition of the NF- κ B cascaded stimuli also the TNF and IL-6.⁶⁸

When the human body faces a lot of stress, ROS are produced as a result.⁶⁹ Deficiency of antioxidant agents leads to different degenerative diseases,⁶⁹ for example cardiovascular, Alzheimer, and various inflammatory diseases.⁷⁰ Consumption of antioxidants from natural sources reduces oxidative stress. Many studies showed that flavonoids and phenolic constituents have attributed to the antioxidant activities of natural compounds. Furthermore, many studies cited that minerals, for example Cu, Zn, Mg, Mn, and Se, played a significant role as antioxidants.⁷¹ Additionally, dietary antioxidants including tocopherols, carotenoids, and ascorbic acid have been investigated.⁷² Although many synthetic antioxidants have been shown to remediate oxidative stress, their lack of availability, high cost, and side effects remain the main challenge in dealing with oxidative stress, making the need to discover new antioxidant agents urgent. The sponge extract exhibits a potent antioxidant effect as shown in Figures 8 and 9, and the marine extract contains a variety of bioactive compounds known by their effect as antioxidants such as polyphenol (tannins, phenolic compounds, and flavonoids), carotenoids, and different minerals (Cu, Fe, Se, Zn, and Mn).

CONCLUSION

The secondary metabolites isolated from the sponge *Hyrtios aff. erectus* collected from the Red Sea in Egypt have been confirmed to have multimedicinal effects as anticancer, antiviral, anti-inflammation, and anti-Alzheimer agents. Further investigations should be performed to purify the pure compounds.

Conflict of interest: The authors declare that there is no conflict of interest.

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In Vivo Antioxidant Activity of Different Fractions of *Indigofera Barberi* Against Paracetamol-induced Toxicity in Rats

Sıçanlarda Parasetamol ile İndüklenen Toksikiteye Karşı *Indigofera Barberi*'nin Farklı Fraksiyonlarının *In Vivo* Antioksidan Aktivitesi

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ABSTRACT

Objectives: To evaluate the *in vivo* antioxidant activity of chloroform extract fractions of *Indigofera barberi* (whole plant) against paracetamol-induced toxicity in rats.

Materials and Methods: For 7 days, rats were treated with different chloroform extract fractions and toxicity was induced with a single dose of paracetamol by intraperitoneal injection. The group of animals pretreated with 100 mg/kg p.o. of fraction D of *Indigofera barberi* improved significantly in terms of hepatic superoxide dismutase (SOD), catalase and peroxidase activities, and glutathione levels compared to the control group.

Results: The hepatic SOD, catalase, peroxidase activities, and glutathione levels in the animal groups treated with paracetamol were 33.6±0.09 µ/mg protein, 5.5±0.23 µ/mg protein, 0.131±0.15 µ/mL, and 46.1±5.81 µM, respectively. Hepatic SOD, catalase, peroxidase, and glutathione in the fraction D treated group were 61.8±0.07 µ/mg protein, 10.6±0.16 µ/mg protein, 0.913±0.23 µ/mL, and 87.6±1.4 micro molar, respectively. Therefore, the present study revealed that fraction D of *Indigofera barberi* has significant *in vivo* antioxidant activity and can be used to protect tissue from oxidative stress.

Conclusion: From the results, fraction D of *Indigofera barberi* at a dose of 100 mg/kg, p.o., improved the SOD, catalase and peroxidase activities, and glutathione levels significantly. Based on this study, we can conclude that fraction D of *Indigofera barberi* possesses *in vivo* antioxidant activity and can be employed in protecting tissue from oxidative stress.

Key words: *Indigofera barberi*, paracetamol, silymarin, radical scavenging

ÖZ

Amaç: Sıçanlarda parasetamol ile indüklenen toksisiteye karşı *Indigofera barberi*'nin (tüm bitki) kloroform ekstre fraksiyonlarının *in vivo* antioksidan aktivitesinin belirlenmesi.

Gereç ve Yöntemler: Yedi gün boyunca sıçanlara farklı kloroform ekstraktları uygulanmıştır ve toksisite intraperitoneal tek doz parasetamol uygulaması ile indüklenmiştir. 100 mg/kg p.o. fraksiyon D ile ön uygulaması alan hayvanlar hepatik süperoksit dismutaz (SOD), katalaz ve peroksidaz aktiviteleri ve glutatyon düzeyleri açısından kontrol grubuna göre belirgin bir şekilde iyileşmişlerdir.

Bulgular: Parasetamol uygulanan grupta hepatik SOD, katalaz, ve peroksidaz aktiviteleri ve glutatyon düzeyleri sırasıyla 33,6±0,09 µ/mg protein, 5,5±0,23 µ/mg protein, 0,131±0,15 µ/mg protein ve 46,1±5,81 µM olarak bulunmuştur. Fraksiyon D uygulanan grupta hepatik SOD, katalaz ve peroksidaz aktiviteleri ve glutatyon düzeyleri sırasıyla 61,8±0,07 U/mg protein, 10,6±0,16 µ/mg protein, 0,913±0,23 µ/mg protein ve 87,6±1,4 µM bulunmuştur. Bu nedenle, bu çalışma *Indigofera barberi*'de elde edilen fraksiyon D'nin belirgin bir *in vivo* antioksidan aktivitesi olduğunu ortaya koymuştur ve dokuyu oksidatif stresten korumak için kullanılabilir.

Sonuç: Bu sonuçlar, 100 mg/kg, p.o. dozda *Indigofera barberi*'den elde edilen fraksiyon D, SOD, atalaz ve peroksidaz aktiviteleri ve glutatyon düzeylerini belirgin bir şekilde düzeltmiştir. Bu çalışmaya dayanarak, *Indigofera barberi*'den elde edilen fraksiyon D'nin *in vivo* antioksidan aktivitesinin olduğu sonucuna varabiliriz ve dokuyu oksidatif stresten korumak için kullanılabileceği söylenebilir.

Anahtar kelimeler: *Indigofera barberi*, parasetamol, silimarin, radikal süpürücü

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INTRODUCTION

Today 80% (approximately) of the total world population rely purely on plants for their health and welfare. Recently, there was an increase in interest in the therapeutic prospective of curative plants as antioxidants in minimizing free radical instigated tissue damage. In reaction to this increased acceptance and significant demand for curative and herbal plants, several supervising organizations and groups have mentioned that indigenous curative plants have been introduced into agriculture.¹ Many plants generate various bioactive molecules and this makes them a principle and plentiful source of distinct novel kinds of medicines. A huge heritage of scrutiny and knowledge regarding prophylactic and remedial medicines was accessible in ancient scholastic efforts included in the Charaka, Atharva veda, Sushruta etc.² Above 50% of all current clinical medicines are of natural product source³ and many natural products play a vital part in the medicine evolution process in pharmaceutical production.⁴ Herbal medicines and products have acquired significance in modern years because of their capability and financial value.

Therefore, there was an increasing fascination in the quantification and utilization of plant antioxidants for systematic investigation as well as commercial (cosmetic, pharmaceutical, and dietary) purposes. The different antioxidant responses include many steps involving the initiation, propagation, dividing, hindrance, and cessation of free radicals. Oxidative free radicals are produced when cells utilize oxygen for different physiological procedures. Generally by-products are reactive oxygen species (ROS) like hypochlorous acid, superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical that evolve by the cellular redox action. At modest concentrations but at high levels, ROS have favorable effects on cellular operations and immune reactions and are pivotal for life; ROS produce oxidative stress that causes cell structure and function impairment, involving DNA, lipids, sugars, and proteins.⁵ Oxidative pressure plays a crucial part in the evolution of degenerative and chronic ailments like skin diseases, cancer, autoimmune disorders, altitude sickness, cataracts, rheumatoid arthritis, osteoarthritis, coronary heart disease, aging, and various neurodegenerative diseases.⁶ Antioxidants, either *in situ* (naturally produced) or externally supplied via diet, hinder oxidative pressure in the human body. These antioxidants efficient in scavenging free radicals react by prohibiting injury caused by ROS, and thereafter enhance the cellular response and immune defense, and lower the risk of degenerative diseases and cancer.⁵

Recently, many herbal medicines have been gaining much attention as alternative drugs⁷ applicable as prophylactics for lifestyle-associated disorders but comparatively very little knowledge is accessible regarding their mechanism of action. There was extended interest in the investigation of plant outcomes, which has prompted enormous research on their possible health benefits. Traditional usages (Ayurveda) of plants are most familiar in aqueous extracts form. Recently, some papers have explored the focus for examining these plants in aqueous or ethanolic extracts and some have described activity in petroleum ether, chloroform, and benzene extracts.⁸⁻¹⁰

Indigofera barberi (Fabaceae) of the Tirumala Hills is a commonly assessed endemic herb. Vernacularly it is known as Adavineelimanadu mokka. It grows up to 1 m tall (under shrub). Branchlets are faintly angled. Leaves are 3 and they are foliolate. Leaflets are pubescent, ovate-oblong, mucronate, obtuse. Flowers (pink) are organized in axillary congested racemes. Pods are appressed, deflexed, sub-terete, sharply pointed, white-tomentose. Seeds are 2 to 4 in number. September to December is the flowering and fruiting season.¹¹

Orally, leaf powder (5 g) is administered with butter milk for controlling diabetes. Leaves (50 g), pepper (1 g), and garlic (1 g) are made into paste and formulated into pills of peanut size, and for 5 days 5 pills are administered once a day to cure jaundice as authorized by Nakkala and many tribal physicians. Whole plant powder (5 g) is administered through rice washed water once a day for 10-15 days to remove intestinal worms and as a remedy for various types of peptic ulcers and skin diseases.¹² It is also used as a coloring agent and dye. Its leaf juice is utilized as an antiseptic to cure burns, cuts, wounds, and boils. Keeping these considerations in mind, the aim of the current study was to fabricate a scientific base for the use of the fractions of chloroform extract of *Indigofera barberi* as an antioxidant agent.

MATERIALS AND METHODS

Collection of materials

The *Indigofera barberi* (whole plant, Herbarium number: VVIPS/PCL/011) was collected from the evergreen forest of the Tirumala Hills in Andhra Pradesh state, India. Samples were authenticated and certified by Dr. K. Madhava Chetty, Plant Taxonomist, Department of Botany, Sri Venkateswara University, Tirupati, India. For one week, the *Indigofera barberi* (whole plant) was sorted, ranked, cleaned, and air-dried at room temperature. By utilizing a laboratory hammer mill the plant was finely ground into powder. Finely powdered samples were gathered and tightly stored in water- and air-proof containers shielded from heat and direct sunlight until needed for extraction.

Preparation of extracts

After the *Indigofera barberi* (whole plant) was completely ground into fine powder, it was successively extracted for 18 h with various solvents of increasing polarity, i.e. with petroleum ether, ethyl acetate, chloroform, ethanol, and distilled water in a Soxhlet apparatus. The obtained extracts were concentrated to dryness in a rotary evaporator until free of the solvents.

Isolation of fractions

Thin-layer chromatography (TLC) was carried out using silica gel aluminum plates, 60F-254, 0.5 mm (TLC plates, Merck). The obtained spots were visualized in ultraviolet light and 10% H_2SO_4 in methanol. More spots were seen with chloroform extract. Thus, for further purification, the chloroform extract was subjected to column chromatography using silica gel of pore size 60-100. The silica gel column was equilibrated and counterbalanced for 1 h with petroleum ether at flow rate 5 mL/min. The chloroform extract (1 g dissolved in methanol) was

loaded onto the column and 11 fractions were collected using different solvents of varying concentrations like petroleum ether (100%), petroleum ether: ethyl acetate (4:1), petroleum ether: ethyl acetate (2:3), petroleum ether: ethyl acetate (3:2), ethyl acetate (100%), chloroform: methanol (2:3), and chloroform: methanol (3:2).

Phytochemical analysis

After isolation of fractions from chloroform extract, phytochemical analysis¹³ of fractions was carried out for the presence of alkaloids, tannins, saponins, glycosides, terpenoids, carbohydrates, flavonoids, proteins, amino acids, fixed oils, steroids, and sterols by different methods.

Animals

Albino Wistar rats of both sexes weighing 180-200 g were procured from the National Institute of Nutrition, Hyderabad, Telangana, India. The animals were kept in polypropylene cages (5 in each cage) at a relative humidity of 55-65% and medium temperature of 25±2°C. A 12 h light and dark cycle was retained in the air conditioned animal house. After arrival, all the rats were nourished with a common diet and distilled water for at least 1 week and then they were equally divided into categories with free access to food and distilled water.

Acute toxicity studies

Acute toxicity studies were performed according to the Organization for Economic Co-operation and Development guidelines.¹⁴ The animals were divided in groups and each group contained 5 animals. These grouped animals were fasted for 4 h with free access to distilled water only. The fractions were administered orally in doses of 100, 300, 1500, and 3000 mg/kg to different groups of rats and they were observed over 14 days for mortality and physical/behavioral changes. All these experimental studies on the animals were conducted after permission was obtained from the IAEC (Ref: P2/IAEC/2/2017/VVIPS/SAB/Rats).

Experimental

Group I animals served as normal controls; they receive only vehicle (gum acacia 3% solution)

Group II animals served as toxic controls, treated with paracetamol in a single dose of 2 g/kg orally to produce acute toxicity

Group III served as the standard group and was treated with silymarin (100 mg/kg)

Group IV was treated with fraction A 50 mg/kg

Group V was treated with fraction A 100 mg/kg

Group VI was treated with fraction B 50 mg/kg

Group VII was treated with fraction B 100 mg/kg

Group VIII was treated with fraction C 50 mg/kg

Group IX was treated with fraction C 100 mg/kg

Group X was treated with fraction D 50 mg/kg

Group XI was treated with fraction D 100 mg/kg

The animals in groups III to XI were treated with a single dose of paracetamol 2 g/kg, orally, 6 h after the last treatment. On day 8 the rats were sacrificed by carotid bleeding and the liver was rapidly excised, rinsed in ice-cold saline, and a 10% w/v homogenate was prepared using 0.15 M KCl. Centrifugation was conducted at 800 rpm for 10 min at 4°C. The supernatant obtained was used for the estimation of catalase and peroxidase and other enzymes. Furthermore, the homogenate was centrifuged at 1000 rpm for 20 min at 4°C and the supernatant was used for biochemical estimation.

Biochemical estimation

Estimation of superoxide dismutase (SOD)

The assay of SOD was based on the reduction of nitro blue tetrazolium (NBT) to water insoluble blue formazan, per the method of Beauchamp and Fridovich¹⁵ Liver homogenate (0.5 mL) was taken and 1 mL of 50 mM sodium carbonate, 0.4 mL of 24 mM NBT, and 0.2 mL of 0.1 mM ethylene diamine tetra acetic acid (EDTA) were added. The reaction was initiated by adding 0.4 mL of 1 mM hydroxylamine hydrochloride. Zero time absorbance was taken at 560 nm followed by recording the absorbance after 5 min at 25°C. The control was simultaneously run without liver homogenate. Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50%. The specific activity was expressed in terms of units per milligram of proteins.

Estimation of catalase

Catalase activity was measured as described by Aebi.¹⁶ Supernatant liquid (0.1 mL) was added to a cuvette containing 1.9 mL of 50 mM phosphate buffer (pH 7.0). The reaction was started by the addition of 1.0 mL of freshly prepared 30 mM H₂O₂. The rate of decomposition of H₂O₂ was measured spectrophotometrically from changes in absorbance at 240 nm. Activity of catalase was expressed as units/mg protein.

Estimation of peroxidase

The peroxidase assay was carried out per the method reported by Nicholos¹⁷ Liver homogenate (0.5 mL) was taken, and to this were added 1 mL of 10 mM KI solution and 1 mL of 40 mM sodium acetate solution. The absorbance of potassium periodide was read at 353 nm, which indicates the amount of peroxidase. Twenty microliters of H₂O₂ (15 mM) was added and the change in the absorbance in 5 min was recorded. Units of peroxidase activity were expressed as the amount of enzyme required to change the OD by 1 unit per minute. The specific activity was expressed in terms of units per milligram of proteins.

Estimation of glutathione (GSH)

The procedure to estimate the reduced GSH level followed the method described by Ellman¹⁸ The homogenate (in 0.1 M phosphate buffer, pH 7.4) was added with an equal volume of 20% trichloroacetic acid containing 1 mM EDTA to precipitate the tissue proteins. The mixture was allowed to stand for 5 min prior to centrifugation for 10 min at 200 rpm. The supernatant liquid (200 µL) was then transferred to a new set of test tubes and 1.8 mL of Ellman's reagent added (5.5'-dithio-(bis-2-nitrobenzoic acid) (0.1 mM) prepared in 0.3 M phosphate buffer

with 1% of sodium citrate solution). Then all the test tubes were made up to the volume of 2 mL. After completion of the total reaction, solutions were measured at 412 nm against a blank. Absorbance values were compared with a standard curve generated from known GSH. The GSH level in the liver was calculated as micromole/g liver.

RESULTS

Acute toxicity studies

Acute toxicity studies were carried out by the up-and-down regulation method. It was found that the extract at a limit dose from 1500 to 3000 mg/kg is safe and does not show any mortality.

Isolation of fractions

TLC was carried out using silica gel aluminum plates, 60F-254, 0.5 mm (TLC plates, Merck). Eleven fractions were collected. The yielded products were pooled into four fractions based on TLC. The yield and appearance of the four fractions are given in Table 1.

Preliminary phytochemical screening

Phytochemical screening revealed the presence of alkaloids and carbohydrates in fraction A, saponins in fraction B, glycosides in fraction C, and flavonoids in fraction D (Table 2).

Table 1. Appearance and yield of the 4 fractions of *Indigofera barberi*

Fraction	Appearance	Yield
Fraction A	Yellow	150 mg/g
Fraction B	Dark brown greenish	200 mg/g
Fraction C	Light green	150 mg/g
Fraction D	Saffron	300 mg/g

Table 2. Preliminary phytochemical screening of fractions of chloroform extract of *Indigofera barberi*

S. no.	Phytochemicals	Fraction A	Fraction B	Fraction C	Fraction D
1	Alkaloids	+	-	-	-
2	Tannins	-	-	-	-
3	Saponins	-	+	-	-
4	Glycosides	-	-	+	-
5	Terpenoids	-	-	-	-
6	Carbohydrates	+	-	-	-
7	Flavonoids	-	-	-	+
8	Proteins	-	-	-	-
9	Amino acids	-	-	-	-
10	Fixed oils	-	-	-	-
11	Steroids and sterols	-	-	-	-

In vivo antioxidant activity

Phytochemical screening of the plant showed the presence of flavonoids in fraction D. The present study was undertaken to assess the *in vivo* antioxidant effect of different fractions of chloroform extract of *Indigofera barberi* whole plant on paracetamol-induced toxicity in rats. The results showed that the levels of SOD, catalase, peroxidase, and GSH levels in the control group were 65.2 ± 0.11 , 14.31 ± 0.97 , 0.967 ± 0.13 , and 98.2 ± 1.14 , and in the paracetamol treated group were 33.6 ± 0.09 , 5.5 ± 0.23 , 0.131 ± 0.15 , and 46.1 ± 5.81 , respectively. The levels of SOD, catalase, peroxidase, and GSH in the paracetamol-treated group were significantly lower than those in the normal group. With co-administration of fraction D of *Indigofera barberi* at a dose of 100 mg/kg, the levels of SOD, catalase, peroxidase, and GSH were 61.8 ± 0.07 , 10.6 ± 0.16 , 0.913 ± 0.23 , and 87.6 ± 1.4 , respectively. This markedly prevented paracetamol-induced alterations and maintained enzyme levels near their normal values (Table 3). The standard treated group also had significantly increased levels of SOD, catalase, peroxidase, and GSH (63.9 ± 4.8 , 12.1 ± 0.81 , 0.938 ± 0.32 , and 91.6 ± 1.6 , respectively).

The results are expressed as mean \pm standard error of the mean for each group. The data were analyzed by one-way analysis of variance (ANOVA); $p < 0.01$, $p < 0.05$ indicated statistical significance.

Statistical analysis

All analyses were run in triplicate. The statistical analysis was performed by Student's t-test and ANOVA.

DISCUSSION

In paracetamol-induced toxicity, fraction D of chloroform extract of *Indigofera barberi* treatment increased the depleted levels of cellular GSH significantly in rats. Fraction D of chloroform extract of *Indigofera barberi* also restored the levels of antioxidant enzymes such as SOD and catalase almost back to their normal levels. SOD plays a vital role in the depletion and elimination of ROS and protects cells against the deleterious effects of the O_2^- derived from the peroxidative process in liver and kidney tissues¹⁹ and the observed increase in SOD activity suggests that fraction D chloroform extract of *Indigofera barberi* has an efficient protective mechanism in response to ROS. Catalase is considered the most important H_2O_2 removing enzyme and is a key component of the antioxidative defense system.²⁰ Here catalase activity was increased and then restored to normal levels on administration of fraction D of chloroform extract of *Indigofera barberi*. Peroxidase is an enzyme that catalyzes the reduction of hydroperoxides, including H_2O_2 , and functions to protect the cell from peroxidative damage.²¹ We propose that the additive and synergistic antioxidant activity of phytochemicals such as flavonoids present in *Indigofera barberi* is responsible for its potent antioxidant activity.

CONCLUSION

Phytochemical screening of the fractions showed the presence of flavonoids in fraction D. In our investigation on *Indigofera barberi*, enzymatic oxidants such as GSH, SOD, catalase, and peroxidase were improved in the drug-treated group

Table 3. Radical scavenging activity of fractions of chloroform extract of *Indigofera barberi*

Group	Treatment	Dose	SOD (U/mg protein)	Catalase (U/mg protein)	Peroxidase (U/mL)	Glutathione (μM)
I	Control	2 mL/kg	65.2 \pm 0.11	14.31 \pm 0.97	0.967 \pm 0.13	98.2 \pm 1.14
II	Paracetamol treated	2 g/kg	33.6 \pm 0.09	5.5 \pm 0.23	0.131 \pm 0.15	46.1 \pm 5.81
III	Silymarin	100 mg/kg	63.9 \pm 4.8	12.1 \pm 0.81	0.938 \pm 0.32	91.6 \pm 1.6
IV	Fraction A	50 mg/kg	42.4 \pm 0.31	5.67 \pm 0.15	0.551 \pm 0.24	77.1 \pm 0.9
V	Fraction A	100 mg/kg	51.6 \pm 0.13	6.51 \pm 0.19	0.71 \pm 0.41	82.1 \pm 0.11
VI	Fraction B	50 mg/kg	41.2 \pm 0.07	6.5 \pm 0.27	0.416 \pm 0.27	73.6 \pm 0.61
VII	Fraction B	100 mg/kg	44.8 \pm 0.09	6.8 \pm 0.29	0.519 \pm 0.23	79.1 \pm 0.74
VIII	Fraction C	50 mg/kg	33.6 \pm 0.08	6.32 \pm 0.27	0.321 \pm 0.17	58.4 \pm 1.7
IX	Fraction C	100 mg/kg	38.1 \pm 0.06	6.50 \pm 0.2	0.481 \pm 0.19	61.3 \pm 1.8
X	Fraction D	50 mg/kg	55.9 \pm 0.11	9.6 \pm 0.13	0.851 \pm 0.14	83.5 \pm 0.6
XI	Fraction D	100 mg/kg	61.8 \pm 0.07	10.6 \pm 0.16	0.913 \pm 0.23	87.6 \pm 1.4

SOD: Superoxide dismutase

as compared to the control. Based on this we conclude that fraction D of chloroform extract of *Indigofera barberi* possesses *in vivo* antioxidant activity and may be employed in protecting tissues from oxidative stress.

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Development and Validation of a Stability Indicating RP-HPLC Method for Simultaneous Estimation of Teneligliptin and Metformin

Teneligliptin ve Metformin Eş Zamanlı Tahmininde RP-HPLC Yöntemini Gösteren Stabilitenin Gelişimi ve Doğrulanması

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ABSTRACT

Objectives: The main objective of the present work is to develop a simple, precise, specific and stability method indicating reverse phase high performance liquid chromatography method for simultaneous estimation of teneligliptin and metformin in bulk and tablet dosage form.

Materials and Methods: The analysis was performed with a Kromasil C18 column (250×4.6 mm, 5 µm) at 30°C using buffer: acetonitrile: methanol (65:25:10, v/v/v) as mobile phase. The detection was carried out with a flow rate of 1.0 mL/min at 254 nm.

Results: The retention time of teneligliptin and metformin was 2.842 min and 2.017 min, respectively. The linearity range was 5-30 µg/mL for teneligliptin and 125-750 µg/mL for metformin. The forced degradation studies were performed as per the guidelines of the The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use under acidic, alkaline, oxidative, thermal, photostability, and neutral conditions.

Conclusion: This method was successfully validated for all the parameters and could detect the the correct amounts of active drug substance in formulations that are available in the market. This developed method in the present study could be successfully employed for the simultaneous estimation of teneligliptin and metformin in bulk and tablet dosage form.

Key words: Teneligliptin, metformin, RP-HPLC, validation, stability studies

ÖZ

Amaç: Bu çalışmanın temel amacı, teneligliptin ve metformini bulk ve tablet dozaj formunda eş zamanlı belirlemek için kolay, kesin, özgün ve kararlı bir ters faz yüksek performanslı sıvı kromatografisi yöntemi geliştirmektir.

Gereç ve Yöntemler: Analiz, hareketli faz olarak tampon: asetonytril: metanol (65:25:10, h/h/h) kullanılarak 30°C'de Kromasil C18 kolonu (250×4,6 mm, 5 µm) kullanılarak gerçekleştirilmiştir. Saptama 1,0 mL/dak akış hızında 254 nm'de gerçekleştirilmiştir.

Bulgular: Teneligliptin ve metformin alıkonma süresi sırasıyla 2,842 dk ve 2,017 dk olarak bulunmuştur. Doğrusallık aralığı, teneligliptin için 5-30 µg/mL ve metformin için 125-750 µg/mL'dir. Zorunlu bozunma çalışmaları asit, alkali, oksidatif, termal, fotostabilite ve nötr koşullar altında Beşeri İlaçlar için Teknik Gereksinimlerin Uyumlaştırılması Uluslararası Konseyi'nin kılavuzlarına göre yapılmıştır.

Sonuç: Bu yöntemdeki tüm parametreler başarıyla doğrulanmıştır ve yöntem piyasadaki formülasyonlardaki etkin maddelerin doğru miktarlarını belirleyebilir bulunmuştur. Bu çalışmada geliştirilen yöntem, teneligliptin ve metforminin hammadde ve tablet dozaj formunda eş zamanlı tahmini için başarıyla kullanılabilir.

Anahtar kelimeler: Teneligliptin, metformin, RP-HPLC, validasyon, stabilite çalışmaları

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INTRODUCTION

Teneiglipitin (TEN) (Figure 1) is chemically [(2S, 4S)-4-[4-(5-methyl-2-phenylpyrazol-3-yl)piperazin-1-yl]pyrrolidin-2-yl]-(1,3-thiazolidin-3-yl) methanone. It is highly effective in lowering blood glucose levels. This drug inhibits the enzyme dipeptidyl peptidase-4, which degrades incretin, a hormone adjusting blood glucose control. It is effectively used to treat type-2 diabetes mellitus.^{1,2}

Metformin (MET) (Figure 2) is chemically 1-carbamimidamido-N,N-dimethylmethanimidamide. It belongs to the biguanide class of antidiabetic drugs. It is the first line drug of choice for the treatment of type-2 diabetes. It activates adenosine monophosphate activated protein kinase, a liver enzyme that plays an important role in insulin signaling, whole body energy balance, and metabolism of glucose and fats.³⁻⁵

A literature survey reveals a good number of analytical methods for the estimation of TEN and MET individually or in combination with other drugs using ultraviolet (UV) spectrophotometry,⁶⁻⁸ high performance liquid chromatography (HPLC),⁹⁻¹⁹ HPTLC,²⁰ and LC-MS/MS.²¹ Moreover, methods were reported for the estimation of the selected drugs in their combinations using UV spectrophotometry,^{22,23} and HPLC.²⁴⁻²⁸ To the best knowledge of the authors, no stability indicating RP-HPLC method has been reported so far for the simultaneous estimation of TEN and MET. Hence, we tried to develop a simple stability indicating HPLC method for the estimation of the selected drugs. The developed method has been validated as per the guidelines of the ICH.²⁹ To establish the stability indicating nature of the method forced degradation studies were planned for the proposed method under acidic, alkaline, oxidative, thermal, photostability, and neutral conditions.³⁰

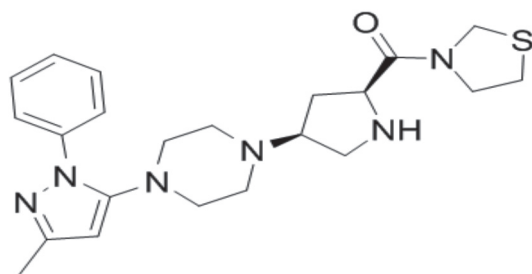


Figure 1. Chemical structure of TEN

TEN: Teneiglipitin

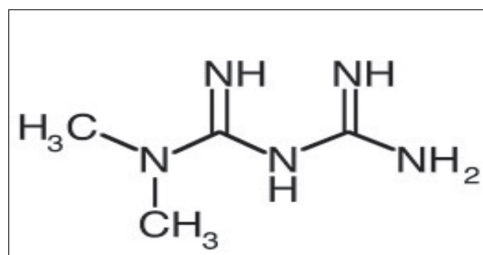


Figure 2. Chemical structure of MET

MET: Metformin

MATERIALS AND METHODS

Materials and reagents

Reference standards of TEN and MET were provided as gift samples by Spectrum Labs, (Hyderabad, India). Commercially available tablet formulation Tendia M tablets for the assay studies were purchased from a local pharmacy. HPLC grade methanol, HPLC grade acetonitrile, analytical grade orthophosphoric acid, and HPLC grade water were purchased from Merck Specialities (Mumbai, India). Ethic committee approval was not required for our study.

Instrumentation

The development and validation of the method were performed on a Waters HPLC 2695 system equipped with quaternary pumps, an autosampler, and a photodiode array detector. Empower 2 software was applied for data collection and processing.

Methodology

Statistical analysis

The analytical characteristics of the tested method in HPLC were validated to ensure the suitability of the analytical requirements and reliability of the results. The statistical One Way Variance analysis treatments were performed with the statistical software GraphPad InStat.

Preparation of standard stock solutions

Standard stock solutions of 200 µg/mL for TEN and 5000 µg/mL for MET were prepared by accurately weighing and transferring 2 mg of TEN and 50 mg of MET into 10 mL volumetric flasks. About three fourths of the volume of diluent was added, followed by sonication for 10 min. Finally, the flasks were made up to the mark with diluent to obtain the mentioned concentrations. Next, 1 mL of the above solution was pipetted out and transferred into a 10 mL volumetric flask and diluted up to the mark with diluent to obtain a concentration of 20 µg/mL for TEN and 500 µg/mL for MET.

Preparation of sample solution

Twenty tablets were weighed and average weight was calculated. Then they were powdered using a mortar and pestle and the powder equivalent to 20 mg of TEN and 500 mg of MET was accurately weighed and transferred into a 100 mL volumetric flask. Next 50 mL of diluent was added and the mixture sonicated for 25 min. Further the volume was made up with diluent to obtain a concentration of 200 µg/mL for TEN and 5000 µg/mL for MET. Filters of 0.45 micron size were employed for filtration in the mentioned procedure. Next, 1 mL of the above solution was pipetted out and transferred into a 10 mL volumetric flask and diluted up to the mark with diluents to obtain a concentration of 20 µg/mL for TEN and 500 µg/mL for MET.

Preparation of buffer

One milliliter of orthophosphoric acid was diluted to 100 mL with HPLC grade water to obtain 0.1% orthophosphoric acid buffer.

Mobile phase

Buffer, acetonitrile, and methanol were taken in the ratio of 65:25:10 (v/v/v) and used as mobile phase.

Method validation

Method validation was done as per the guidelines of the ICH.^{29,30}

System suitability

System performance parameters like retention time, number of theoretical plates, tailing factor, and resolution were calculated by injecting standard solutions six times. The resultant results were compared with the standard limits as per the guidelines.

Specificity

Specificity is the ability of a method to discriminate between the analyte of interest and other components that are present in the sample. These studies are performed to check the interferences in the optimized method. To assess the method's specificity, blank and placebo were injected into the HPLC system under optimized conditions. There should not be any interfering peak in the blank or placebo chromatograms at the retention times of the selected drugs.

Linearity

The linearity of the method was obtained by preparation of the calibration standards of 6 different concentrations in 6 replicates. The calibration curve plots for TEN and MET were obtained by plotting the peak areas on the y-axis and concentrations on the X-axis over the concentration ranges of 5-30 µg/mL for TEN and 125-750 µg/mL for MET. The correlation coefficient should be greater than 0.99.

Accuracy

The accuracy of the method was assessed by recovery experiments by adding a known quantity of pure standard drug to the sample solution and recovering the same in terms of its peak areas. The sample was spiked with standard at levels of 50%, 100%, and 150% of test concentrations. The resultant spiked sample was assayed in triplicate. The % recovery for each level should be 98%-102%.

Precision

Precision is the degree of closeness of agreement between the series of measurements obtained from multiple sampling of the same homogeneous sample under prescribed conditions. It is expressed in terms of standard deviation (SD) or relative SD (RSD). Precision may be a measure of either the degree of repeatability or the reproducibility of the analytical method.

Method precision

Sample solutions were injected under optimized conditions 6 times on 6 different days and their peak areas were recorded. RSD % for the peak areas of the 6 standard injection results should not be greater than 2.

Intermediate precision

Six replicates of sample solutions were injected under optimized conditions on the same day and their peak areas were recorded. RSD % for the peak areas of the 6 replicate injection results should not be greater than 2.

Ruggedness

The ruggedness of the method was determined by carrying out the experiment on different instruments, by different operators, and using different columns of similar types.

Robustness

The robustness of the method was determined by making small deliberate changes in the method like flow rate, mobile phase ratio, and temperature. However, one should not find remarkable changes in the results and the obtained results should be within the ranges in the ICH guidelines.

Effect of variation in flow

A sample was analyzed at 0.9 mL/min and 1.1 mL/min flow rate instead of 1.0 mL/min; the remaining conditions were kept unchanged.

Effect of variation in temperature

Temperature of 25°C and 35°C was maintained instead of 30°C. Samples were injected in triplicate and chromatograms were recorded.

Limit of detection and limit of quantitation (LOD and LOQ)

LOD is the smallest concentration that can be detected but not necessarily be quantified as an exact value. It is calculated using the formula

$$\text{LOD} = 3.3 \sigma/S, \text{ where } \sigma = \text{SD}; s = \text{slope}$$

LOQ is the lowest amount of analyte in the sample that can be quantitatively determined with precision and accuracy.

$$\text{LOQ} = 10 \sigma/S, \text{ where } \sigma = \text{SD}; s = \text{slope}$$

Forced degradation studies

TEN and MET standard samples were subjected to degradation under different stress conditions like acidic, alkali, oxidative, thermal, photostability, and neutral conditions.

For acidic and alkali degradation samples were refluxed with 2 N HCl and 2 N NaOH at 60°C for 30 min. For oxidative degradation 20% v/v H₂O₂ was used and the same was refluxed at 60°C for 30 min. For thermal degradation, a sample was placed in an oven at 105°C for 6 h; for photostability degradation, the drug was exposed to UV light by keeping the sample in a UV chamber for 7 days or 200 W h/m² in a photostability chamber; for neutral degradation, the drugs were refluxed in water for 6 h at 60°C. All the samples were diluted to obtain a final concentration of 20 µg/mL of TEN and 500 µg/mL of MET. Ten microliters of the samples were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Solution stability

The stability of the drug solution was determined for short-term stability and autosampler stability. Short-term stability was tested by keeping the samples at room temperature (25°C) for 24 h. Autosampler stability was determined by storing the samples for 24 h in the autosampler. Each sample was injected 6 times into the HPLC and the results obtained were compared with the nominal values of QC samples.

RESULTS

The results for the optimized chromatographic conditions are shown in Table 1. The system suitability parameters (tailing factor, retention time, and theoretical plates) were within the acceptance criteria. A summary of the system suitability parameters is given in Table 2. We did not find any interfering peaks at the retention times of TEN or MET (Figure 3), which shows that the method is specific. The quantification was linear in the concentration range of 5-30 µg/mL for TEN with a correlation coefficient of 0.999 (Figure 4) and 125-750 µg/mL for MET with a correlation coefficient of 0.999 (Figure 5). The results for linearity are tabulated in Table 3. The recoveries of TEN and MET were in the range of 99.35-99.94% and 99.80-100.61%, respectively. The results were compared with the guidelines and expressed as percentages and are given in Table 4. The precision of the method is satisfactory as RSD % is NMT 2%. The ruggedness was determined by different analysts and on different days. The results are given in Table 5. No remarkable changes in the results were noted in the robustness studies and hence the method is robust. The results are tabulated in Table 6. The assay results were compared with the labeled claim of TEN and MET marketed formulations and the results are tabulated in Table 7. The LOD and LOQ values were calculated using slope and standard deviation values and the same are tabulated in Table 8.

Forced degradation study

The standard solutions were subjected to different stress conditions as mentioned in the procedure. Under acidic conditions, the drugs showed degradation of about 3.66% for TEN and 3.14% for MET and we noted about 3 degradation

peaks (Figure 6). Under alkali conditions, the drugs showed degradation of about 2.75% for TEN and 2.67% for MET and 2 degradation peaks were noted (Figure 7). Under oxidative conditions, the drugs showed degradation of about 1.01% for TEN and 1.62% for MET and 1 degradation peak was noted (Figure 8). Under the remaining conditions, i.e. thermal, photostability, and neutral conditions, the degradation was less than 1% for both drugs and no degradation peak was noted (Figures 9-11).

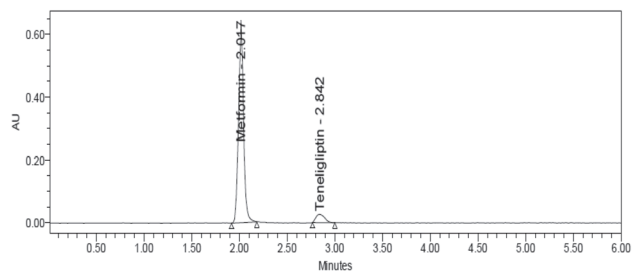


Figure 3. Chromatogram showing resolved peaks of TEN and MET
TEN: Teneligliptin, MET: Metformin

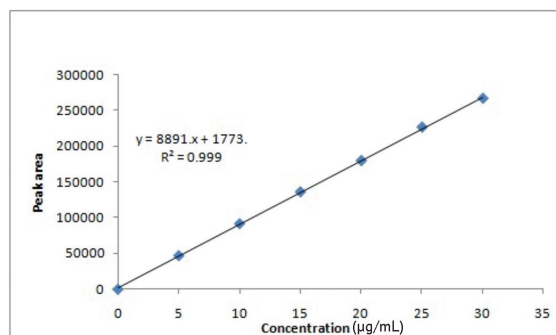


Figure 4. Linearity plot of TEN

TEN: Teneligliptin

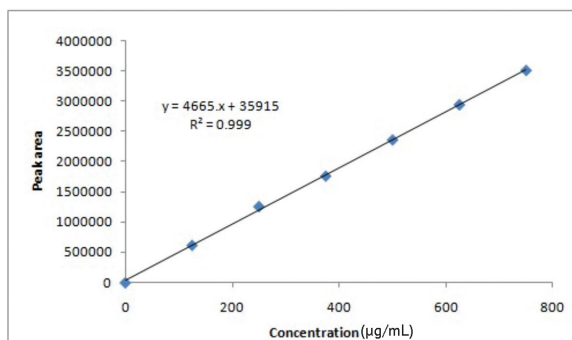


Figure 5. Linearity plot of MET

MET: Metformin

Table 1. Optimized chromatographic conditions

Parameter	Condition
Column	Kromasil C18 (250×4.6 mm, 5 µm)
Mobile phase	Buffer: acetonitrile: methanol (65:25:10, v/v/v)
Diluent	Acetonitrile: water (50:50, v/v)
Column temperature	30°C
Wavelength	254 nm
Flow rate	1 mL/min
Run time	6 minute
Injection volume	10 µL

Table 2. Summary of system suitability parameters

Parameter	TEN	MET	Acceptance criteria
Tailing factor	1.30	1.06	≤2
Retention time	2.842	2.017	≥2
Theoretical plates	4463	6783	≥2000
RSD % of area	0.72	1.08	≤2

TEN: Teneligliptin, MET: Metformin, RSD: Standard deviation relative

Table 3. Linearity values of TEN and MET

Parameter	TEN	MET
Linearity range (µg/mL)	5-30	125-750
Regression coefficient ± SD	0.999±0.0003	0.999±0.0005
Slope ± SD	8891±4.358	4665±8.386
Intercept ± SD	1773±58.66	35.915±2654.363

TEN: Teneligliptin, MET: Metformin, SD: Standard deviation

The results of the forced degradation studies are tabulated in Table 9.

Table 4. Recovery values of TEN and MET

Drug	Level	Analyte amount (mg)	Recovery amount (mg)	Mean % recovery	RSD %
TEN	50%	10	9.94	99.43	0.20
	100%	20	19.98	99.91	0.60
	150%	30	29.97	99.92	0.67
MET	50%	250	249.50	99.80	0.90
	100%	500	503.07	100.61	0.40
	150%	750	752.82	100.37	0.50

TEN: Tenoeligiptin, MET: Metformin, RSD: Standard deviation relative

Table 5. Ruggedness values of TEN and MET

Drug	Analyst-1 (Peak area)*	Analyst-2 (Peak area)*	SD	RSD %
TEN	172.792	173.167	1248	0.72
MET	2,363.854	2,320.575	12.026	0.50

TEN: Tenoeligiptin, MET: Metformin, RSD: Standard deviation relative, SD: Standard deviation

*Average of six readings

Table 6. Robustness values of TEN and MET

Condition	TEN			MET		
	Retention time	Area	Tailing factor	Retention time	Area	Tailing factor
Initial conditions	2.837	268.209	1.14	2.027	3,521.349	0.98
More flow (+0.1 mL/min)	2.827	269.207	1.13	2.012	3,540.846	0.95
Less flow (-0.1 mL/min)	2.851	267.902	1.10	2.041	3,536.801	1.00
At 35°C	2.841	267.189	1.13	2.028	3,520.932	0.99
At 25°C	2.840	269,218	1.09	2.029	3,519,867	1.11

TEN: Tenoeligiptin, MET: Metformin

Table 7. Assay results of marketed formulation (Tendia M tablets)

Drug	Label claim	Amount found	%Assay
TEN	20 mg	19.98 mg	99.90
MET	500 mg	498.85 mg	99.77

TEN: Tenoeligiptin, MET: Metformin

Table 8. LOD and LOQ values of TEN and MET

Drug	LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)
TEN	0.02	0.07
MET	1.88	5.69

TEN: Tenoeligiptin, MET: Metformin, LOD: Limit of detection, LOQ: Limit of quantitation

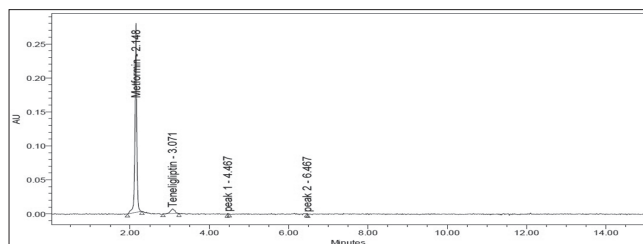


Figure 6. Chromatogram showing degraded peaks under acidic conditions

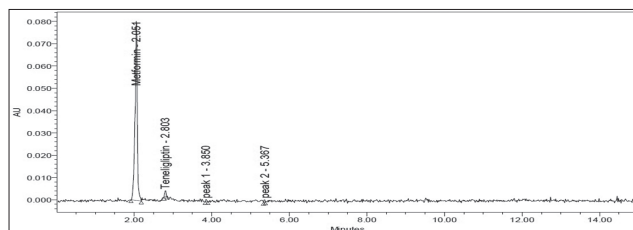


Figure 7. Chromatogram showing degraded peaks under alkali conditions

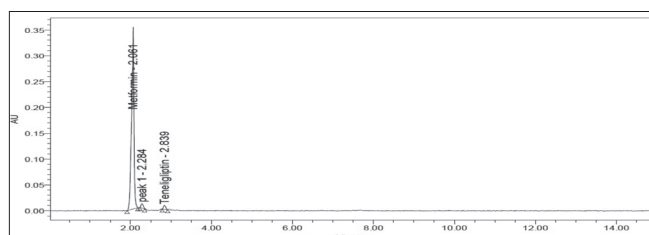


Figure 8. Chromatogram showing degraded peaks under oxidative conditions

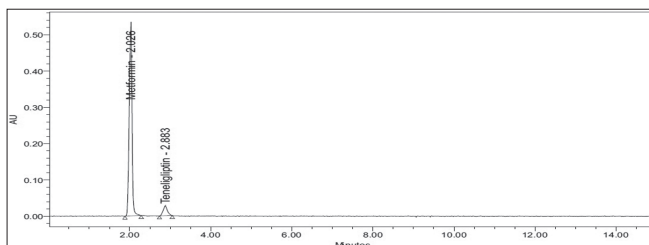


Figure 9. Chromatogram showing degraded peaks under thermal conditions

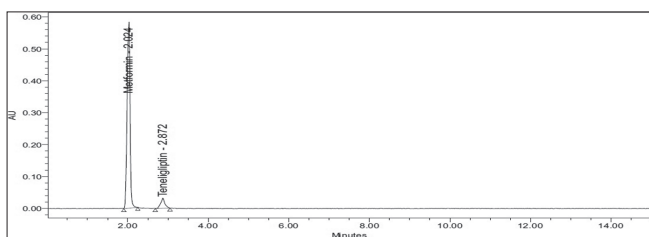


Figure 10. Chromatogram showing degraded peaks under photostability conditions

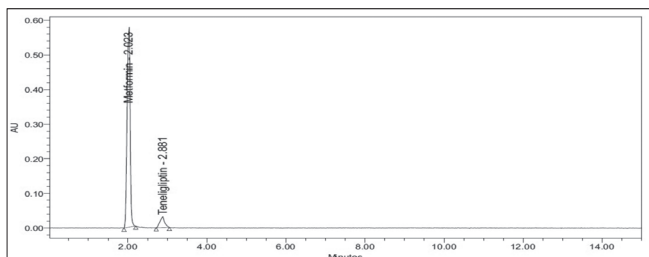


Figure 11. Chromatogram showing degraded peaks under neutral conditions

Table 9. Forced degradation data* (\pm SD) of the method

Stress condition	Amount of TEN degraded (%)	Amount of TEN recovered (%)	Amount of MET degraded (%)	Amount of MET recovered (%)
Acidic	3.67 \pm 0.89	96.33 \pm 3.12	3.15 \pm 0.62	96.85 \pm 2.19
Alkali	2.76 \pm 0.58	97.24 \pm 2.98	2.68 \pm 0.85	97.32 \pm 2.81
Oxidative	1.02 \pm 0.69	98.98 \pm 1.98	1.63 \pm 0.98	98.37 \pm 2.06
Thermal	0.75 \pm 0.08	99.25 \pm 1.87	0.62 \pm 0.10	99.38 \pm 0.91
Photostability	0.63 \pm 0.06	99.37 \pm 1.39	0.59 \pm 0.09	99.41 \pm 1.92
Neutral	0.53 \pm 0.08	99.47 \pm 2.01	0.05 \pm 0.08	99.95 \pm 1.79

TEN: Teneiglipitin, MET: Metformin, SD: Standard deviation,
*Average of three determinations (each condition)

Stability studies

The drug solutions were found to be stable for 24 h at 25°C for short-term stability and 24 h for autosampler stability.

DISCUSSION

For method optimization different ratios of acetonitrile and buffer were tried but peak resolution was not achieved. Hence, methanol was used in the mobile phase. Different ratios of orthophosphoric acid buffer, acetonitrile, and methanol were tried, i.e. 65:15:20, v/v/v; 60:20:20, v/v/v; 65:20:15, v/v/v. Finally, it was found that buffer: acetonitrile: methanol in the ratio of 65:25:10, v/v/v, gave good peaks and hence were fixed as the mobile phase. A Kromasil C18 (250 \times 4.6 mm, 5 μ m) column, 1 mL/min flow rate, 10 μ L injection volume, 30°C column oven temperature, and 254 nm wavelength were fixed as optimized conditions, which were found to be suitable for the separation of peaks. These optimized conditions gave a retention time of 2.842 min and 2.017 min for TEN and MET. All the validation results were as per the limits of the ICH and hence showed the method to be reliable and economical for the estimation of drugs. The effectiveness of the method to separate the degraded peaks from analyte shows its stability indicating nature. The degradation on the lower side, i.e. the degradation percent under all conditions, is in the range of 0.05% to 3.66%, showing the stability of the selected drugs. The RSD % values were less than 2.

CONCLUSION

The method developed possesses all the qualities to be a reliable, rapid, sensitive, specific, and economical method according to the above discussed results and data. The study showed the stability indicating nature of the method with the possible short runtime. Hence, the developed method could be conveniently and effectively used for routine simultaneous estimation of TEN and MET in bulk and pharmaceutical dosage form.

Conflicts of interest: No conflict of interest was declared by the authors. The authors alone are responsible for the content and writing of this article.

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A High Performance Thin Layer Chromatographic Method Using a Design of Experiment Approach for Estimation of Phytochemicals in Extracts of *Moringa Oleifera* Leaves

Yüksek Performanslı İnce Tabaka Kromatografi Yöntemi ile *Moringa Oleifera* Yaprak Ekstrelerindeki Fitokimyasalların Deney Tasarımı Yaklaşımı ile Tayini

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ABSTRACT

Objectives: A systematic design of experiment (DoE) based sensitive, robust high performance thin layer chromatographic (HPTLC) method was established for simultaneous estimation of gallic acid (GA), quercetin (QT), and rutin (RT) from ethanolic and aqueous leaf extracts of *Moringa oleifera*.

Materials and Methods: The chromatographic separation was carried on Merck TLC aluminum sheets of silica gel 60 F254 (10×10 cm) with mobile phase of toluene: ethyl acetate: methanol: formic acid (4.9:4.1:2:0.5, v/v/v/v) with densitometric scanning at 300 nm. The critical method parameters were initially identified by regular two level factorial design and further systematically optimized using a central composite design, evaluating the effect on selected critical analytical attributes, retention factor (RF), and peak area.

Results: The Pareto charts, 3D response surface plots, and polynomial equations for the generated models suggested significant influence of the selected factors on responses of QT, GA, and RT. The desirability and overlay plots employed provided appropriate solutions that were experimentally validated. Under the optimized conditions, the biomarkers were suitably resolved with RF values of 0.64±0.02, 0.80±0.03, and 0.22±0.02 for GA, QT, and RT, respectively, with wide linear dynamic range (200-1200 ng/band each), high accuracy (98.1-99.4%), and intra- and interday precision (%RSD <2%). When employed for quantification of these biomarkers in *Moringa oleifera* extracts, the ethanolic and aqueous extracts exhibited higher content of QT (993.5 µg/g and 832 µg/g, respectively). The ethanolic extract showed a larger amount of RT (701 µg/g). In contrast, aqueous extract exhibited a higher proportion of GA (591.1 µg/g) compared to ethanolic extract (150 µg/g).

Conclusion: This validated HPTLC method developed through a DoE approach was successfully employed for quantification of GA, QT, and RT from *Moringa oleifera* extracts and may also be extended for their simultaneous estimation in other herbal extracts, thereby reducing time, and may serve as a cost effective tool for analysis.

Key words: DoE, gallic acid, HPTLC, *Moringa oleifera*, quercetin, rutin

ÖZ

Amaç: *Moringa oleifera* yapraklarının etanol ve su ile hazırlanan ekstrelerinde gallik asit (GA), kersetin (QT) ve rutin (RT) miktarlarının eşzamanlı olarak tayin edilebilmesi amacıyla sistematik deney tasarımına (DoE) dayalı hassas ve güçlü yüksek performanslı ince tabaka kromatografisi (HPTLC) yöntemi geliştirilmiştir.

Gereç ve Yöntemler: Kromatografik ayırım Merck TLC Silika Jel Plakada 60 F254 (10×10 cm) hareketli faz olarak tolüen: etil asetat: metanol: formik asit (4,9:4,1:2:0,5; h/h/h/h) kullanılarak ve 300 nm'de yoğunluk taraması yapılarak gerçekleştirilmiştir. Kritik yöntem parametreleri ilk olarak iki faktöryelli deney tasarımı ile belirlenmiş daha sonra bu parametreler seçilen kritik analitik özellikler, alıkonma faktörü ve pik alanı üzerindeki etkileri değerlendirilerek merkez kompozit tasarım kullanılarak sistematik olarak optimize edilmiştir.

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Bulgular: Üretilen modeller için oluşturulan Pareto çizelgeleri, 3B yanıt yüzey grafikleri ve polinom denklemleri, seçilen faktörlerin QT, GA ve RT yanıtları üzerine anlamlı etkileri olduğunu göstermiştir. Uygulanan istenebilirlik ve bindirme grafikleri ile deneysel olarak doğrulanmış uygun çözümler sağlanmıştır. Optimize edilen koşullarda, biyobelirteçler; GA, QT ve RT, sırasıyla $0,64\pm 0,02$, $0,80\pm 0,03$ ve $0,22\pm 0,02$ alıkonma zamanları, geniş doğrusal dinamik aralık (herbiri 200-1200 ng/bant), yüksek doğruluk (%98,1-99,4) ve gün içi ve günler arası kesinlik (%RSD <2) ile uygun şekilde ayrılmıştır. Bu biyobelirteçlerin *Moringa oleifera* ekstrelerindeki miktar tayininde etanol ve su ile hazırlanan ekstrelerde daha yüksek QT içeriği saptanmıştır (sırasıyla $993,5 \mu\text{g/g}$ ve $832 \mu\text{g/g}$). Etanol ile hazırlanan ekstrede daha fazla RT ($701 \mu\text{g/g}$) olduğu görülmüştür. Bunun tersine, su ile hazırlanan ekstrede gözlenen GA ($591,1 \mu\text{g/g}$) oranının, etanol ile hazırlanan ekstrede ($150 \mu\text{g/g}$) göre daha yüksek olduğu tespit edilmiştir.

Sonuç: DoE yaklaşımı ile geliştirilen bu geçerli HPTLC yöntemi, *Moringa oleifera* ekstrelerinden GA, QT ve RT miktar tayinleri için başarıyla kullanılmıştır ve bu yaklaşımın diğer bitkisel ekstrelerde de bu biyobelirteçlerin miktarlarının eşzamanlı olarak tayin edilebilmesini sağlayarak analiz süresini kısaltacağı ve maliyet tasarrufu sağlayacağı düşünülmüştür.

Anahtar kelimeler: DoE, galik asit, HPTLC, *Moringa oleifera*, kersetin, rutin

INTRODUCTION

In recent years people have been consuming large quantities of herbal medicines for various therapeutic and prophylactic purposes due to their implied safety, efficacy, cultural acceptability, and lesser side effects. Herbs are a rich source of various phytoconstituents, among which phenolic acids and flavonoids are present in major proportions. Around 300 flavonoids have been isolated and their pharmacological activities have been extensively studied to date. Most of them are reported to be less toxic to humans and therefore are widely used in herbal medicine.¹

Flavonoids like quercetin (QT), rutin (RT), and phenolics like gallic acid (GA) are present in a large number of herbs and herbal preparations. QT is a natural polyphenolic present in vegetables, fruits, and juices and has been extensively studied for numerous biological activities. Chemically, QT is an aglycone of RT and other glycosides and is a powerful antioxidant and free radical scavenger. RT is used in the treatment and prevention of small varicose veins. This substance is also used in mesotherapy or intradermotherapy to stimulate circulation in treatment against cellulite. It has been used for preparing patients with jaundice for surgery. GA is a polyphenolic compound with antioxidant properties and is used to treat the common cold and fever and as a diuretic, laxative, liver tonic, restorative, antipyretic, and anti-inflammatory agent.² Figure 1 represents the chemical structures of these biomarkers.

Currently, the literature indicates that there are very few methods reported for the quantitative estimation of these biomarkers present in herbs/herbal preparations. Recently, Amir et al.³ reported an high performance thin layer chromatographic (HPTLC) method for the simultaneous estimation of QT and RT in herbs. Hussain et al.⁴ reported an HPTLC method employing

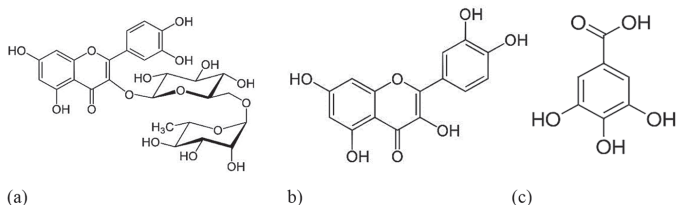


Figure 1. Chemical structure of RT (a), QT (b) and GA (c)
QT: Quercetin, RT: Rutin, GA: Gallic acid

toluene: ethyl acetate: formic acid (5:4:1) as the mobile phase for determination of QT and GA in *Abutilon indicum*. Alam et al.⁵ also reported normal phase-HPTLC for estimation of RT, GA, QT, and naringenin in extracts of *Guiera senegalensis*. However, individual mobile phases consisting of acetonitrile: water (4:6) were employed for estimation of RT and QT, while a mixture of toluene: ethyl acetate and formic acid (6:4:8) was used for determination of GA and naringenin.

Seal reported a reversed phase-High performance liquid chromatography (HPLC) method employing acetonitrile and 1% aqueous acetic acid solution as mobile phase in gradient mode with photodiode array detection at 272, 280, and 310 nm for simultaneous quantitation of flavonoids (catechin, RT, QT, myricetin, apigenin, and kaempferol) in wild edible leaves of *Sonchus arvensis* and *Oenanthe linearis*.⁶ A sensitive ultra performance liquid chromatography-ESI-MS/MS method employing protein precipitation is reported for estimation of phytoconstituents in *Polygonum capitatum* extract in rat plasma, namely phenolic acids and flavonoids like GA, quercitrin, and QT.⁷ Alam et al.⁸ also reported a HPLC method for the estimation of RT, QT, and GA in *Moringa oleifera* plants native to Saudi Arabia. A preliminary thin layer chromatography study using 0.2% 2,2-diphenyl-1-picrylhydrazyl as the spraying reagent and HPLC on a C18 reverse-phase column was employed for quantitation of GA and RT in extracts of *C. alata* and *Andrographis paniculata*.⁹ Sajeeth et al.¹⁰ reported a HPTLC method on precoated HPTLC silica gel 60 F254 plates employing toluene: ethyl acetate: formic acid (7:5:1 v/v/v/v) as mobile phase for quantitative estimation of GA, RT, and QT from *Eruca sativa* extract. Another HPTLC method on precoated silica gel GF 254 plates using toluene: acetone: glacial acetic acid (3:1:2 v/v/v/v) as mobile phase with ultraviolet (UV) detection at 254 nm for GA [retention factor (RF) 0.30] and a mixture of ethyl acetate: dichloromethane: formic acid: glacial acetic acid: water (10:2.5:1:1: 0.1, v/v/v/v/v) at 366 nm for RT and QT at an RF value of 0.13 and 0.93, respectively, is reported.¹¹ While the literature reports suggest that there are few HPTLC methods for estimation of QT, RT, and GA, these methods have employed different mobile phase compositions/different wavelengths of detection for estimation of these biomarkers. To date, there is no reported HPTLC method employing a design of experiments (DoE) approach for the simultaneous estimation of these three biomarkers in combination.

Moringa oleifera, native to India or Sub-Himalayan areas and widely spread throughout the tropical and subtropical areas, is a miracle tree and an unbelievable source of all nutrients, with various pharmacological effects in several disease conditions for its antibacterial, antidiabetic, and cardiovascular effects, and also for the treatments of stomach aches, sprains, and fever. *Moringa oleifera* contains GA, QT, and RT in considerable proportions and its anti-atherosclerotic, antioxidative, and antidiabetic activities have been reported.¹²

Recently HPTLC has been introduced in the USP as an official tool for analysis, mainly for the quantitative and qualitative analysis of herbal extracts. HPTLC, because of its highly sensitive detection ability and other advantages like low operating cost, high sample throughput, and minimum sample clean-up requirement, is now adopted for analysis as an alternative to HPLC.

DoE as per (ICH) Q2 (R1), Q8 (R2), and Q9 guidelines is a systematic approach for analytical method development and validation. Various designs can be adopted for screening and optimization of method variables that can influence the method responses. Regular two-level factorial screening is an excellent design for initial screening of variables that can affect the responses. Furthermore, for optimization of analytical method parameters, central composite design (CCD) is one of the most widely used designs, allowing better understanding of not only the main effect (effect of each individual variable selected through screening design) but also their interaction effects. A DoE approach helps to reduce the number of experiments to be performed, thereby proving to be a simple, economic, less time consuming, and robust strategy for method development.¹³

The present study reports for the first time the quantitative estimation of GA, QT, and RT by HPTLC method developed through a DoE approach and its application for estimation of these bioactive agents in *Moringa oleifera* leaf extracts.

MATERIALS AND METHODS

Plant collection and identification

Fresh leaves of *Moringa oleifera* were collected from the area around Pune. The collected parts of *Moringa oleifera* were authenticated at the Botanical Survey of India, Pune.

Chemicals and reagents

GA (99%), RT (98%), and QT (99%) were purchased from Hi Media Laboratories, Mumbai, India. All chemicals and reagents were of AR grade and aluminum-backed TLC plates precoated with a 0.2 mm layer of silica gel 60 F254 (10×10 cm) were purchased from E. Merck (Germany).

Standard preparation

A suitable quantity (5 mg) each of GA, QT, and RT was weighed accurately and transferred to separate 10 mL volumetric flasks, 5 mL of methanol was added followed by sonication for 10 min, and the volume was made up to 10 mL with methanol. The resulting solutions were filtered through Whatman filter paper and suitable volumes were applied to TLC plates for further analysis.

Sample preparation

Preparation of ethanolic and aqueous extracts of Moringa oleifera

Fresh leaves of *Moringa oleifera* were ground into small pieces. For the preparation of ethanolic extract, the powdered plant material was macerated with 70% ethanol (1:40 w/v) for 72 hour at room temperature (28±2°C) with occasional shaking.

Aqueous extract was prepared by maceration of powdered plant leaves with distilled water for 24 hour at room temperature (28±2°C) with occasional shaking.¹⁴

The extracts were filtered through Whatman filter paper and the resulting marcs were re-macerated with the same solvent until complete extraction. The residual solvents were removed using rotary evaporation and then dried using a vacuum oven (Lab-line) under pressure at 40°C to obtain dry extracts.

HPTLC instrumentation and chromatographic conditions

A Camag HPTLC system equipped with a TLC scanner 3 and win CATS 1.2.2 software (Camag, Muttens, Switzerland), a UV chamber (Camag, Muttens, Switzerland), a twin trough chamber (10×20 cm or 20×20 cm; Camag, Muttens, Switzerland), and a saturation pad (Camag, Muttens, Switzerland) was used. The standards and samples were spotted in the form of bands of width 6 mm with a Camag microliter syringe on aluminum plates precoated with silica gel 60 F254 (10×10 cm with 0.2 mm thickness, E. Merck, Germany) using a Camag Linomat V (Hamilton, Broadus, Switzerland) sample applicator. The slit dimension was kept constant at 5 mm×0.45 mm and the scanning speed was maintained at 20 mm/s. Linear ascending development was carried out in the twin trough glass chamber and the chromatograms were developed up to a length of 80 mm. The developed TLC plates were dried with the help of an air dryer.

Preliminary HPTLC analysis

Initial HPTLC trials were carried out employing solvents like toluene, isopropanol, n-butanol, methanol, ethyl acetate, formic acid, dioxane, and acetic acid in varying proportions as mobile phase. However, problems like low RF values for RT (<0.05), overlapping of the peaks of QT and GA, and large RF values for QT (>0.90) were observed. The addition of methanol resulted in improvement in the RF values of RT and QT. However, change in the proportion of methanol (>3 and <2) resulted in a considerable effect on the RF value of QT and RT. Taking this into consideration and after several permutations and combinations, a mixture of toluene, ethyl acetate, methanol, and formic acid (4:3:2:0.5 v/v/v/v) was selected as the mobile phase as it gave relatively fair separation of GA, QT, and RT. The trials suggested that there was a major influence of chromatographic method conditions on the RF value and peak area of the three biomarkers.

Method development and optimization using DoE

Further to the initial trials, a DoE approach was employed in the present study to identify and understand the influence of the method conditions on the analytical output through a thorough

understanding of the process. The predetermined objective of the present study was to identify the best/optimum conditions for effective separation of the selected biomarkers and study the influence of the method parameters on the identified critical analytical attributes (CAAs) (RF value and peak area).

Factor screening studies

Initially, based on the literature data, experimental trial results, and elaborate analysis using Ishikawa fishbone diagrams, six factors, i.e. method parameters (mobile phase ratio, time from spotting to chromatography, time from chromatography to scanning, wavelength, activation time, and saturation time) were selected for the study. The regular two-level factorial screening design using Design Expert software version 11 (Stat-Ease, Minneapolis, MN, USA) was initially employed for selection of critical method parameters (CMPs) that will influence the (CAAs). A design matrix comprising 8 experimental runs was suggested, considering two levels (low and high) for each selected method parameter. The levels selected were mobile phase ratio (4:5); saturation time (10 min: 20 min); time from spotting to chromatography (10 min: 30 min); time from chromatography to scanning (10 min: 30 min); wavelength (254 nm: 300 nm); activation time (5 min: 15 min). The Pareto charts were employed to evaluate the influence of each factor (CMPs) on selected CAAs. The polynomial equations were generated for each model as given below:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \epsilon,$$

where Y is the measured CAA associated with each factor level combination; mobile phase composition (toluene content) (X_1) and time from spotting to chromatography (X_2). The composition of the mobile phase refers to the volume of toluene with respect to the total volume of the mobile phase. The low, medium (nominal value), and high levels of dependent and independent variables were selected based on the results from preliminary experimentation. The nominal value for two factors, toluene content (X_1) and time from spotting to chromatography (X_2), were 4 mL and 10 min, respectively. Accordingly, the toluene content (X_1) was maintained between 4 mL and 5 mL. Similarly, the low and high values of the time from spotting to chromatography (X_2) were fixed at 15 min and 25 min, respectively.

Optimization of HPTLC method parameters using a central composite design

Central composite response surface design was employed to optimize the CMPs as selected through their initial screening design. The screening design was used to optimize the compositional parameters and to evaluate interaction effects and quadratic effects of the selected method parameters, i.e. the mobile phase ratio and time from spotting to chromatography. The design was specifically selected since it requires fewer

runs than a Box-Behnken design in the case of two variables. A design matrix comprising 14 experimental runs was constructed (Table 1).

Validation of the proposed HPTLC method

The proposed HPTLC method for simultaneous estimation of QT, RT, and GA was validated as per ICH guidelines.

Linearity (calibration curve)

Standard solutions of GA, QT, and RT were prepared in methanol to obtain a concentration of 0.5 mg/mL. Different volumes of standard solutions were spotted on the TLC plates in triplicate using a Camag Linomat V sample applicator to obtain bands in the concentration range of 200-1200 ng/band for GA, QT, and RT. The plates were then developed and the data of peak areas versus drug concentrations were treated by linear least squares regression analysis to obtain the regression equations.

Accuracy (recovery %)

The accuracy of the method was determined by calculating recoveries of GA, QT, and RT by the standard addition method. Known amounts of standard solutions of GA, QT, and RT were added at 80%, 100%, and 120% level to prequantified sample solution (extracts). The amounts of GA, QT, and RT were estimated by applying obtained values to the respective regression line equations.

Precision

The precision of the system was determined by measuring repeatability of sample application and measurement of peak areas for three replicates at each concentration level. To evaluate intraday precision, three mixed standards were prepared. Suitable volumes (0.4 μ L, 1.2 μ L, 2 μ L) were applied to HPTLC plates to obtain standard bands corresponding to three concentrations (200, 600, and 1000 ng) in triplicate on the same day. For the intraday precision (intermediate precision), the assays was performed on three consecutive days and the peak areas were recorded. The precision of the system and method was expressed as relative standard deviation (RSD) % of peak area.

Statistical analysis

All the data analysis was carried out in replicates and standard deviation and RSD values were computed.

The present study does not require ethics committee approval or patient informed consent.

RESULTS AND DISCUSSION

Factor screening studies

The analysis of data obtained using the regular two-level factorial design for screening of CMPs suggested that the

Table 1. Optimization trials of central composite design (where X_1 toluene content, X_2 time from spotting to chromatography)

Run	1	2	3	4	5	6	7	8	9	10	11	12	13	14
X_1	4.5	4.5	4.5	6	6	3	3	4.5	4.5	2.78	6.62	4.5	4.5	4.5
X_2	20	20	20	10	30	10	30	20	34.14	20	20	5.85	20	20

composition of the mobile phase ratio had a significant negative impact on the retention factor of QT (-83.70%) and GA (-89.51%). In the case of RT, the method parameter of time from spotting to chromatography had a major effect on the RF value (-38.82%). For the CAA of peak area, the wavelength of detection and time from chromatography to scanning contributed significantly in the case of QT, RT, and GA. However, mobile phase ratio and time from chromatography to spotting were critical for RT (Figure 2).

Optimization of CMPs using a central composite design

Based on the results of the preliminary screening design, it was thought appropriate to further optimize the effect of the identified CMPs (mobile phase ratio and time from spotting to chromatography) on selected analytical attributes (RF and peak area) using a CCD.

The selected CMPs, namely mobile phase ratio and time from spotting to chromatography, were studied at five levels ($-\alpha, -1, 0, 1, +\alpha$). The design matrix comprised a total of 14 experimental

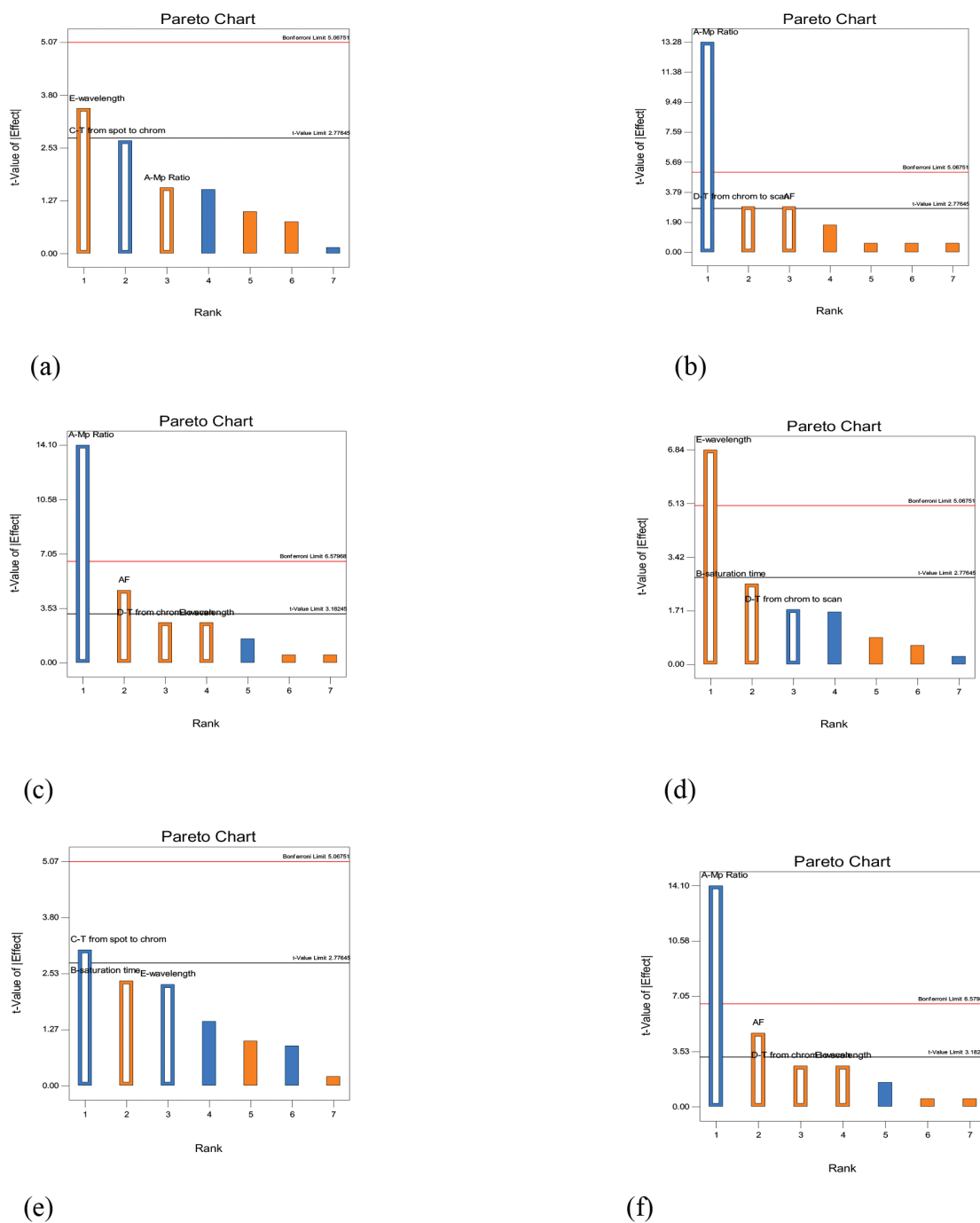


Figure 2. Pareto charts depicting the influence of CMP's on method CAA, (a) Effect on area of QT, (b) Effect on Rf of QT, (c) Effect on area of GA, (d) Effect on Rf of GA, (e) Effect on area of RT, (f) Effect on Rf of RT

CMP's: Critical method parameters, CAA: Critical analytical attributes, QT: Quercetin, GA: Gallic acid, Rf: Retardation factor, RT: Rutin

runs with 6 runs at the center point (0, 0). Standard RT, GA, and QT were prepared at the concentration of 400 ng/band and used for all experimental runs. Design Expert 10 software was employed for the data analysis.

Data validation was performed by one way analysis of variance (ANOVA) combined with the F test. Coefficients that were found to be significant ($p < 0.05$) were considered in framing the

polynomial equations. Lack of fit and correlation coefficients (r^2) were employed further to evaluate the appropriateness of model fitting (Tables 2 and 3). 2D contour plots and 3D response surface plots (Figure 3) were employed for response surface analysis. The entire model's diagnostic plots like the normal plot of probability, run plot, residual plots, and histogram plots were also employed to evaluate the degree of fitness of the data

Table 2. Summary of statistical ANOVA for response (Y_1 retardation factor)

Source	Sum of squares			Degree of freedom			Mean square			F value			p value		
	QT	RT	GA	QT	RT	GA	QT	RT	GA	QT	RT	GA	QT	RT	GA
Model	0.044	0.000	0.06	2	0	2	0.022	-	0.03	8.97	-	4.97	0.006	-	0.032
X_1	0.043	-	0.06	1	-	1	0.043	-	0.06	17.39	-	9.06	0.002	-	0.013
X_2	1.337 E-003	-	5.386 E-003	1	-	1	1.337 E-003	-	5.386 E-003	0.54	-	0.88	0.4793	-	0.3706
Residual	0.025	0.037	0.061	10	12	10	2.476 E-003	3.069 E-003	6.128 E-003	-	-	-	-	-	-
Lack of fit	0.018	0.011	0.041	6	8	6	3.083 E-003	1.320 E-003	6.869 E-003	1.97	0.20	1.37	0.2669	0.97	0.3969

ANOVA: One way analysis of variance, QT: Quercetin, RT: Rutin, GA: Gallic acid

Table 3. Summary of statistical ANOVA for response (Y_2 area)

Source	Sum of squares			Degree of freedom			Mean square			F value			p value		
	QT	RT	GA	QT	RT	GA	QT	RT	GA	QT	RT	GA	QT	RT	GA
Model	98.9	185.3	937.6	3	3	5	32.95	61.76	187.5	1.00	5.82	5.72	0.435	0.017	0.020
X_1	11.6	122.3	252.8	1	1	1	11.64	122.2	252.8	0.35	11.5	7.71	0.566	0.008	0.027
X_2	51.2	14.01	394.6	1	1	1	51.22	14.01	394.6	1.56	1.32	12.0	0.243	0.280	0.010
Residual	295.7	95.58	229.3	9	9	7	32.9	10.62	32.76	-	-	-	-	-	-
Lack of fit	258.4	52.91	202.5	5	5	3	51.7	10.58	67.51	5.54	0.99	10.0	0.061	0.5175	0.025

ANOVA: One way analysis of variance, QT: Quercetin, RT: Rutin, GA: Gallic acid

Table 4. Predicted/observed results of solutions suggested by Design Expert software

Solution results	Mob. phase (T:EA:M:FA)	Time from spotting to chromatography (min)	RF			% Content		
			GA	QT	RT	GA	QT	RT
Predicted results								
Solution 1	4.9:4.1:2:0.5	26.2	0.63	0.77	0.23	98	103	102
Solution 2	4.8: 4.2:2:0.5	21.6	0.63	0.75	0.23	97	102	102
Solution 3	5.2: 3.8:2: 0.5	26.1	0.62	0.75	0.23	97	102	101
Solution 4	5.3: 3.7:2:0.5	26.1	0.62	0.75	0.23	96.8	102	101
Observed results								
Solution 1	4.9: 4.1:2:0.5	26.2	0.64	0.77	0.23	99.56	100.1	101.8
Solution 2	4.8: 4.2:2:0.5	21.6	0.65	0.78	0.24	99.5	99.5	102
Solution 3	5.2: 3.8:2:0.5	26.1	0.57	0.73	0.19	97	99.4	98.4
Solution 4	5.3: 3.7:2:0.5	26.1	0.65	0.78	0.24	98	99	98.11

RF: Retention factor, QT: Quercetin, RT: Rutin, GA: Gallic acid, T: Toluene, EA: Ethyl acetate, M: Methanol FA: Formic acid

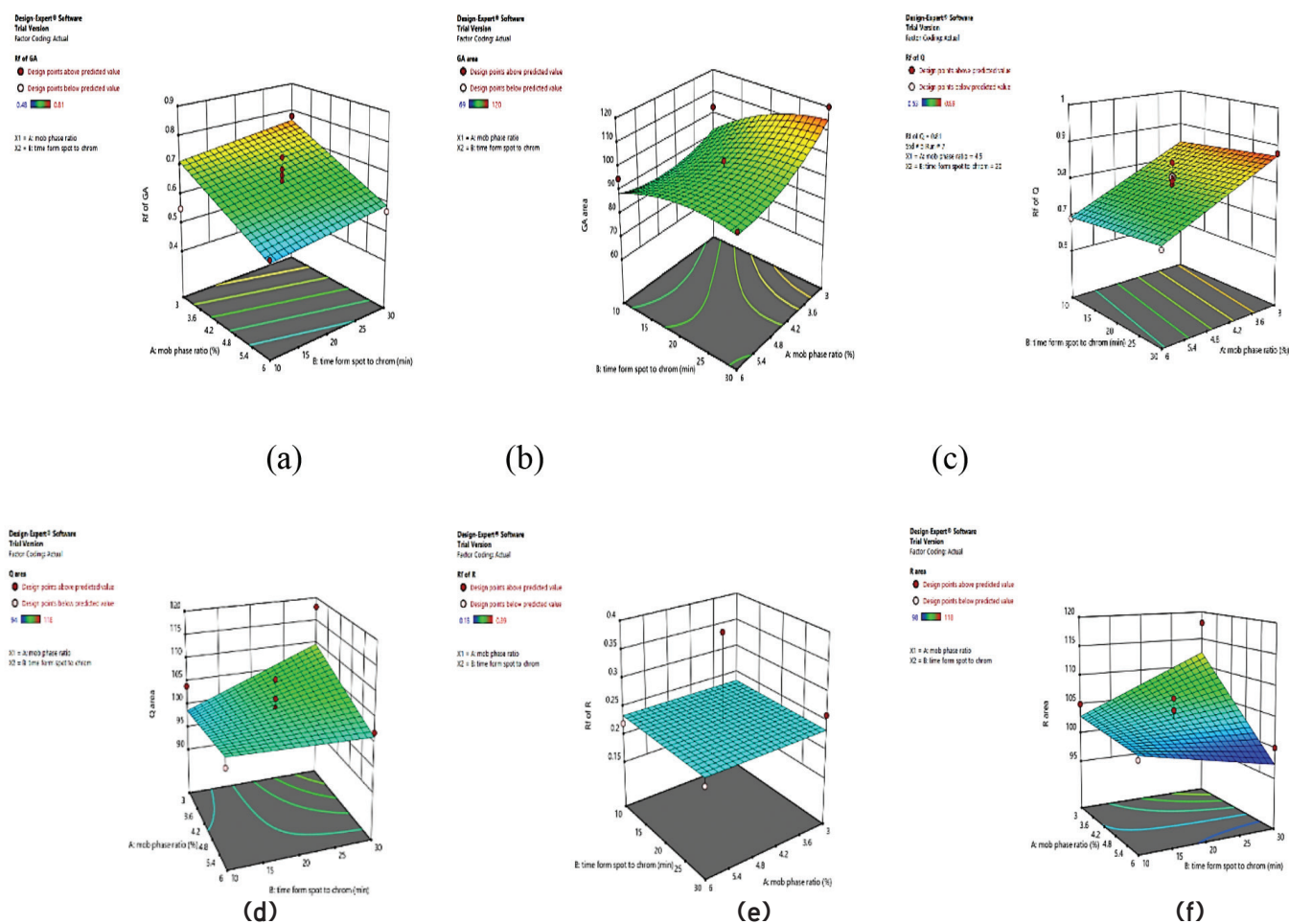


Figure 3. 3D Response surface plots showing the effect of mobile phase composition and time from spotting to chromatography on RF and area of biomarkers (a) Effect on the area of QT, (b) effect on RF of QT, (c) effect on the area of GA, (d) effect on RF of GA, (e) effect on the area of RT (f) effect on RF of RT. QT: Quercetin, RF: Retention factor, GA: Gallic acid, RT: Rutin

obtained. All the experimental runs were carried out in random order to avoid any bias in measurement.

Effect on retention factor and peak area of gallic acid

The results of the ANOVA of the model to represent the effect of selected CMPs (X_1 mobile phase ratio and X_2 time from spotting to chromatography) on responses (selected CAAs) Y_1 (RF) and Y_2 (area) are summarized in Tables 2 and 3, respectively. The 3D response surface plots were also analyzed.

For GA, the polynomial equation model generated suggested that factors X_1 and X_2 were statistically significant ($p < 0.05$). It was observed that as the mobile phase ratio varied (amount of toluene increased), it had a small negative impact on the RF value as indicated by a negative coefficient (-0.058). For the response Y_2 (peak area), the factor X_1 (mobile phase ratio) had a significant negative influence (-17.80). However, as the time from spotting to chromatography increased, the area under the curve of GA increased up to a certain point, above which it further decreased. An interaction effect of selected factors was also observed.

Effect on retention factor and peak area of quercetin

In the second model generated for the influence of factors X_1 and X_2 on responses Y_1 and Y_2 of QT, the polynomial equation and model developed were also statistically significant ($p < 0.05$). Here the factor X_1 (mobile phase ratio) had a negative impact on the RF value (Y_1) as demonstrated through the response surface plot. However, the effect of X_2 on response Y_2 (peak area) was nonsignificant ($p > 0.05$), indicating that the selected method parameters were robust and did not have any significant impact on the area of QT.

Effect on retention factor and peak area of rutin

For RT, the generated model was nonsignificant ($p > 0.05$) for X_1 and X_2 on selected response Y_1 (RF) of RT, indicating that there was no significant difference in the RF value of RT under the selected method conditions. However, it was observed that the polynomial equation for factor X_1 and X_2 had a significant positive impact ($p < 0.05$) on the peak area of RT as indicated by the positive coefficient (+1.93 and +1.18, respectively). An interaction effect of X_1 and X_2 was also observed.

In order to obtain the best chromatographic performance, the multicriteria methodology was employed by means of Derringer's desirability function. Individual desirability functions ranging from 0 (undesired response) to 1 (fully desired response) were selected. A value of D close to 1 indicates that combination of different criteria is globally optimal. The red area in the desirability plot indicates that the prediction at all

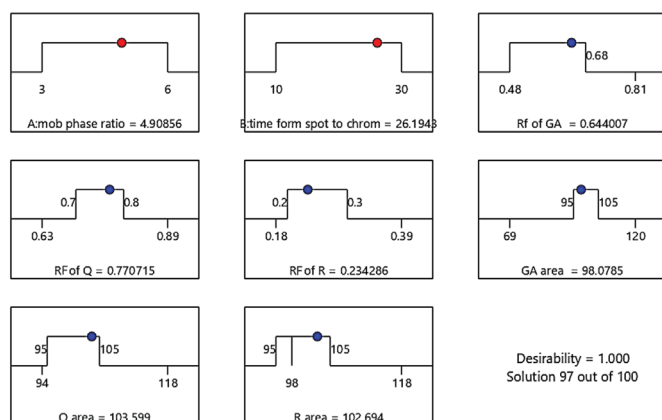


Figure 4. Optimized conditions on response basis: Desirability=1

RF: Retention factor, GA: Gallic acid

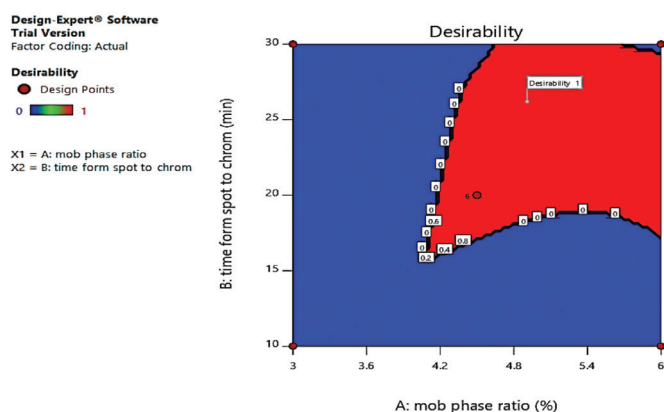


Figure 5. Desirability showing the effect of mobile phase composition and time from spotting to chromatography on Rf and area of GA, QT and RT

QT: Quercetin, RF: Retention factor, GA: Gallic acid, RT: Rutin

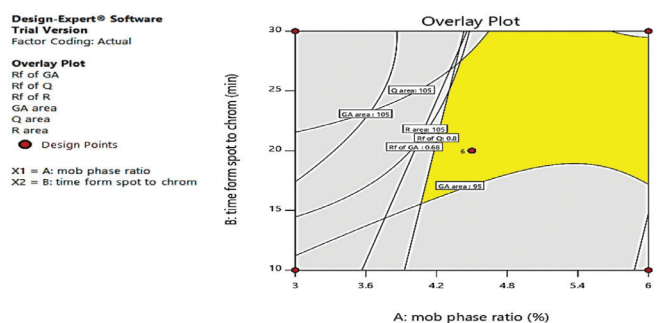


Figure 6. Overlay plot showing the effect of mobile phase composition and time from spotting to chromatography on retardation factor and peak area on GA, QT and RT

QT: Quercetin, GA: Gallic acid, RT: Rutin, RF: Retention factor

points in this region is one. The yellow area in the overlay plot indicates that all the constraints are satisfied in this region. Desirability (Figures 4 and 5) and overlay plots (Figure 6) were obtained from the models for the selected responses. The desirability and overlay plots gave the design space within which variations in CPPs did not affect the CAAs selected. However, four solutions as suggested by the software were selected such that they satisfied the desirability function of 1 and were also observed in the yellow zone in the overlay plot.

The four proposed solutions were experimentally run under the stated conditions and the resulting densitograms were evaluated to observe any deviations in RF and peak areas from the predicted values (Figure 7). The agreement between the experimental and predicted responses was assessed by calculating the percentage of prediction error using the following formula: $\text{Predicted error} = \frac{\text{Experimental response} - \text{Predicted response}}{\text{Predicted response}} \times 100$.

The results of the same are summarized in Table 4. From the data generated and prediction error calculations, it was observed that the % prediction error calculated for RF and % content of the three biomarkers were minimal in the case of solution 1. The % error for RF and % content of GA were 1.58 and 1.59, respectively, where the amount of toluene in the mobile phase was high ($X_1=4.9$ mL) and time from spotting to chromatography was also large ($X_2=26.2$ min). However, the RF values of QT and RT were found to exactly match the predicted values, while % error for % content was significantly low (-2.81% for QT and -0.19% for RT). The desirability study indicated that solution 1 gave more accurate results and therefore these optimized conditions [mobile phase composition: toluene: ethyl acetate: menthol: formic acid solution (4.9:4.1:2:0.5 v/v/v/v) and time from spotting to chromatography: 26 min] were selected for further validation studies.

Validation of the proposed HPTLC method

Linearity (calibration curve)

The linear regression data obtained for the calibration curves ($n=6$) showed an excellent linear relationship over a wide concentration range of 200-1200 ng/band for GA, QT, and RT (Table 5).

Precision

The measurement of peak area in the interday and intraday precision studies showed low % RSD (<2%), which suggested precision of the method (Table 5).

Recovery

The accuracy of the proposed HPTLC method demonstrated through recovery studies performed by spiking sample with pure drugs at 80%, 100%, and 120% indicated good recovery of the three biomarkers with % recovery in the range of 98.1-99.4% (Table 5).

Determination of GA, QT, and RT in leaf extracts of *Moringa oleifera*

The densitograms obtained on analysis of the ethanolic and aqueous extracts of *Moringa oleifera* showed three well

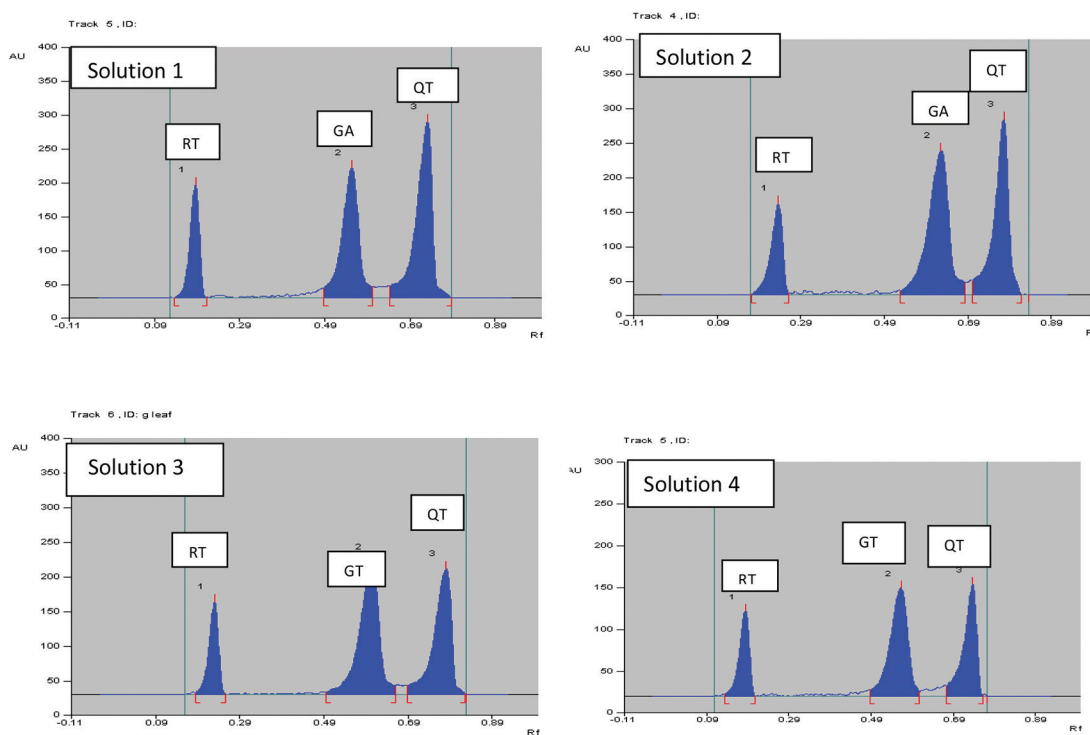


Figure 7. Densitograms of predicted solutions as per design expert software
 QT: Quercetin, GA: Gallic acid, RT: Rutin

Table 5. Validation data for gallic acid, quercetin, and rutin

Validation parameter	QT	RT	GA
Linearity			
Range (ng/band)	200-1200	200-1200	200-1200
Regression equation	$y=6.6659x+1199.1$	$y=5.0043x+1443.7$	$y=13.46x+1362.1$
r^2	0.9982	0.9958	0.9951
*Interday precision (Mean % RSD)	0.143	0.264	0.097
*Intraday precision (Mean % RSD)	0.370	0.182	0.161
Recovery			
**Mean % recovery	98.75	98.66	99.16
Mean % RSD	1.01	0.595	0.70

*n=6 at three concentration levels i.e. 200, 600, and 1000 ng/band, **n=3 at three levels i.e. 80%, 100%, and 120% of the test concentration, RSD: Relative standard deviation, QT: Quercetin, RT: Rutin, GA: Gallic acid

resolved peaks at Rf 0.80, 0.64, and 0.22 for QT, GA, and RT, respectively (Figure 8). The PDA spectral scan of the separated bands at 300 nm and the UV spectra generated exactly superimposed with the standard spectra indicating that there was no interference from other components present in the extracts. The photo documentation of the HPTLC plates also displayed distinct bands for the biomarkers when scanned in UV chamber at short wavelength (254 nm) (Figure 8c). It was observed that both the ethanolic and aqueous extracts showed higher content of QT (993.5 $\mu\text{g/g}$ and 832 $\mu\text{g/g}$, respectively). However, the ethanolic extract showed a larger amount of RT (701 $\mu\text{g/g}$) when compared to the aqueous extract (232.2 $\mu\text{g/g}$).

Table 6. Estimated content of GA, QT, and RT in aqueous and ethanolic extracts of *Moringa oleifera*

Biomarker	Aqueous extract	Ethanolic extract
GA	591.1 $\mu\text{g/g}$	150 $\mu\text{g/g}$
QT	832 $\mu\text{g/g}$	993.5 $\mu\text{g/g}$
RT	232.2 $\mu\text{g/g}$	701 $\mu\text{g/g}$

QT: Quercetin, RT: Rutin, GA: Gallic acid

In contrast, the aqueous extract exhibited a higher proportion of GA (591.1 $\mu\text{g/g}$) as compared to the ethanolic extract (150 $\mu\text{g/g}$) (Table 6). The proposed HPTLC method was successfully

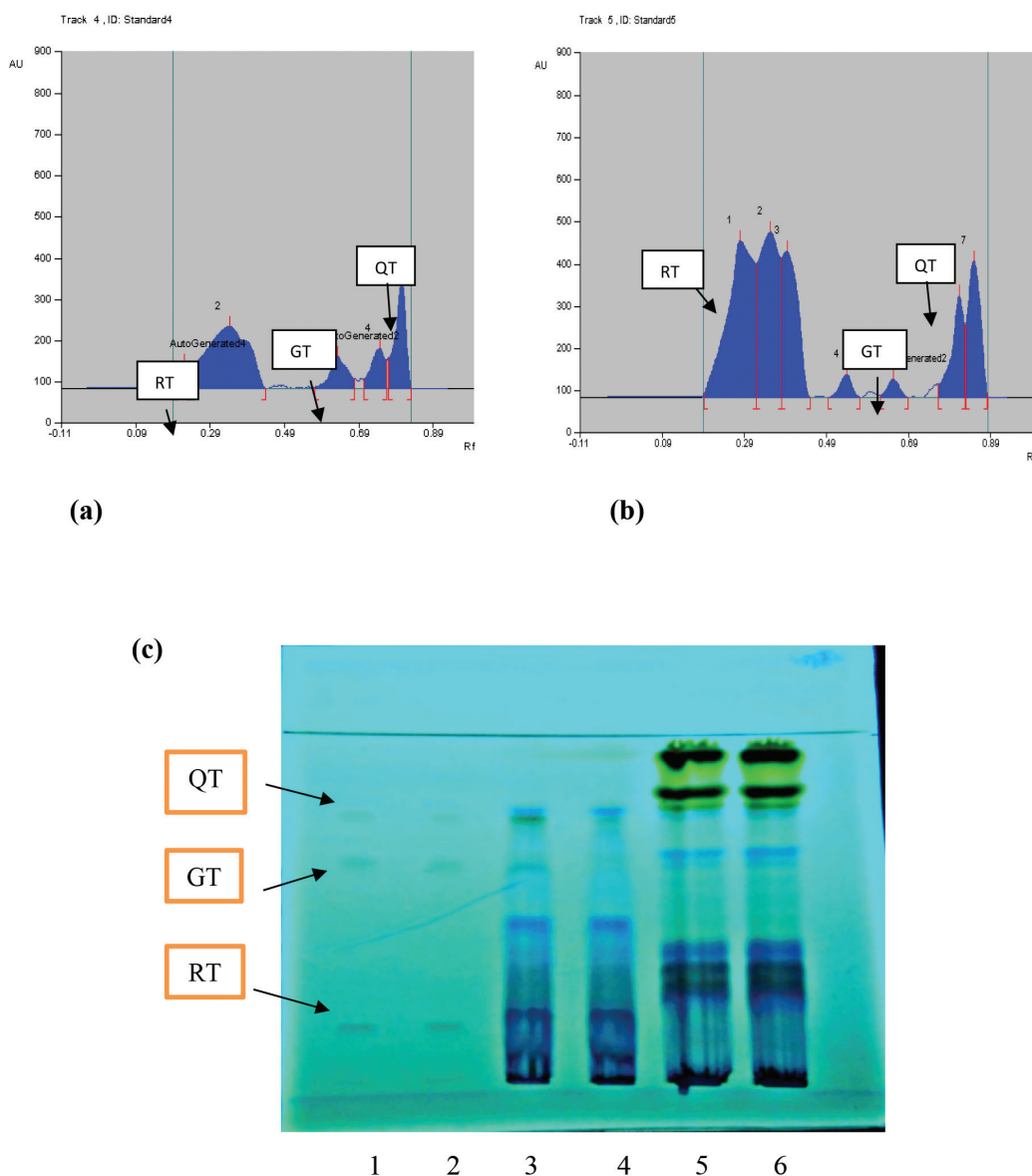


Figure 8. HPTLC densitograms of extracts of *Moringa oleifera*

(a) For aqueous extract [peak 1, 3 and 5; RT (0.24), GA (0.62) and QT (0.76)]

(b) For ethanolic extract [peak 1, 5 and 6; RT (0.23), GA (0.62) and QT (0.76)]

(c) Photo documentation of developed HPTLC plate Spots of standards (track 1 and 2), aqueous extract (track 3 and 4) and ethanolic extract (track 5 and 6)
 QT: Quercetin, GA: Gallic acid, RT: Rutin, HPTLC: High performance thin layer chromatography

employed for the estimation of these biomarkers in extracts of *Moringa oleifera*.

Although the literature reports an HPLC method employing a gradient of methanol and acetonitrile for the estimation of these biomarkers in *Moringa oleifera* plants native to Saudi Arabia by Alam et al.⁸, the RT of these biomarkers are very close to each other (0.98, 0.99, and 1.04 min for RT, GA, and QU, respectively) and their simultaneous estimation is not possible.

A recent study reports an HPTLC method for estimation of these three biomarkers in Syrian *Capparis spinosa* L. leaves carried out on precoated silica gel GF254 plates employing a four solvent composition of mobile phase [ethyl acetate-

glacial acetic acid-formic acid-distilled water (100:11:11:25)]. Moreover, densitometric scanning was performed at three different wavelengths [366 nm for RT (RF: 0.39), 280 nm for QT (RF: 0.79), and 254 nm for GA (RF: 0.81)].¹⁵

Some of the more recently reported methods employ separate mobile phase systems for estimation of these three biomarkers [for gallic acid, toluene: formic acid: ethyl acetate: methanol (3:3:8:2, v/v/v/v); for RT and QT, ethyl acetate: formic acid: glacial acetic acid: water (10:0.5:0.5:1.3, v/v/v/v)]. It was also observed that the RF values of QT were very high, which may lead to inadequate quantification as it may overlap with the solvent band.¹⁶

In comparison, the HPTLC method developed by us employs a fixed composition of mobile with quantitative measurement of the three biomarkers at 300 nm with effective separation leading to distinct bands for the three biomarkers with sufficient differences in their RF values. Moreover, the mobile phase optimized through a DoE approach and method validated as per standard guidelines make it a robust method for their simultaneous quantification.

CONCLUSION

A sensitive, accurate, and robust HPTLC method was developed for estimation of QT, RT, and GA in ethanolic and aqueous extracts of *Moringa oleifera* using a fixed composition of mobile phase [(toluene: ethyl acetate: menthol: formic acid solution (4.9:4.1:2:0.5 v/v/v/v)] with densitometric analysis at 300 nm. The chromatographic conditions were optimized using a DoE approach and involved use of a regular two level factorial screening design for initial screening of method parameters followed by a CCD for optimization of selected CMPs using Design Expert software. The present study reports for the first time a constant composition of mobile phase for effective separation of QT, RT, and GA and was employed successfully for estimation of these biomarkers in *Moringa oleifera* extracts. This method may also be extended to estimation of these biomarkers in other herbal extracts, thereby reducing time, and may serve as a cost effective tool for analysis.

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

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Floating Microspheres of Enalapril Maleate as a Developed Controlled Release Dosage Form: Investigation of the Effect of an Ionotropic Gelation Technique

Geliştirilmiş Kontrollü Salım Dozaj Formu Olarak Enalapril Maleat Yüzen Mikroküreleri: İyonotropik Jelleşme Tekniğinin Etkisinin Araştırılması

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ABSTRACT

Objectives: The purpose of this study was to provide a control drug delivery system through a newly approved work to enhance the absorption and bioavailability of enalapril maleate loaded floating microspheres by ionotropic gelation technique using a hydrophilic carrier.

Materials and Methods: Eleven developed formulations of floating microspheres were prepared by ionotropic gelation using different concentrations of sodium alginate, iota-carrageenan, sodium bicarbonate, calcium chloride, and the drug. These microspheres were characterized using a diversity of parameters like micrometric properties, percentage yield, entrapment efficiency, *in vitro* buoyancy, *in vitro* drug release, and kinetics of drug release. The optimum formula was evaluated and identified for drug-excipients compatibility using fourier transform-infrared spectroscopy (FT-IR), surface morphology, powder X-ray diffraction (XRD), and differential scanning calorimetry (DSC).

Results: From the results, F4 was selected as the optimum formula since it provides a faster and premium release of drug from the matrix (91.4%). Kinetics of drug release was found to depend on both diffusion and erosion mechanisms, as the correlation coefficient (R²) was best fitted with Korsmeyer's model and the release exponent (*n*) was shown to be between 0.43 and 0.84. Scanning electron microscopy images demonstrated spherical, discrete, and freely flowing microspheres with a particle size of 199.4±0.04 µm. Optimum buoyancy properties, percentage yield, and drug entrapment efficiency were achieved. FT-IR showed no interaction between enalapril and the polymers. DSC and XRD showed the miscibility of the drug with the polymers while maintaining the stable crystalline properties of enalapril loaded in the prepared microspheres.

Conclusion: The developed floating microspheres of enalapril maleate can be considered a promising controlled drug delivery system, thereby improving patient compliance.

Key words: Enalapril maleate, floating microspheres, gastroretentive system, iota-carrageenan, sodium alginate

ÖZ

Amaç: Bu çalışmanın amacı, bir hidrofilik taşıyıcı kullanılarak iyonotropik jelleştirme tekniği ile enalapril maleat yüklü yüzen mikro kürelerin emilimini ve biyoyararlanımını arttırmak için yeni onaylanmış bir çalışma ile kontrollü ilaç salım sistemi geliştirmektir.

Gereç ve Yöntemler: Bu çalışma kapsamında, yüzen mikrokürelerin onbir gelişmiş formülasyonu, sodyum aljinat, iota-carrageenan, sodyum bikarbonat, balmumu klorür ve ilacın farklı konsantrasyonları kullanılarak iyonotropik jelasyon yöntemi ile hazırlanmıştır. Bu mikrokürelerin karakterizasyonu, mikrometrik özellikler, yüzde verim, yükleme etkinliği, *in vitro* yüzme özelliği, *in vitro* ilaç salımı ve ilaç salım kinetiği gibi çeşitli parametreler kullanılarak yapıldı. Optimum formül, Fourier dönüşümü kızılötesi spektroskopisi (FT-IR), yüzey morfolojisi, toz X-ışını difraksiyonu (XRD) ve diferansiyel taramalı kalorimetri (DSC) kullanılarak ilaç-yardımcı madde uyumluluğu açısından değerlendirildi ve tanımlandı.

Bulgular: Elde edilen sonuçlardan, F4 matristen ilacın daha hızlı salımını sağladığı için optimum formül olarak seçildi (%91,4). Korelasyon katsayısının en iyi Korsmeyer'nin modeline uyması ve salım üs sayısının (*n*) 0,43-0,84 arasında olması nedeniyle ilaç salım kinetiğinin hem difüzyon hem de erozyon mekanizmalarına bağlı olduğu tespit edilmiştir. Taramalı elektron mikroskobu görüntüleri, parçacık boyutu 199,4±0,04 µm olan küresel, keskin ve serbestçe dönen mikroküreleri göstermektedir. Uygun yüzme özellikleri, yüzde verim ve ilaç yükleme etkinliği elde edilmiştir. FT-IR

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analizi, enalapril ve polimerler arasında hiçbir etkileşim olmadığını gösterdi. DSC ve XRD, hazırlanan enalapril yüklü mikrokürelerin stabil kristalin özelliklerini korurken, polimerler ile ilacın karışabilirliğini de ortaya koymuştur.

Sonuç: Enalapril maleat yüklü geliştirilmiş yüzen mikrokürelerinin hasta uyuncunu iyileştirebilir nitelikte ümit vadeden kontrollü ilaç salım sistemi olarak kabul edilebileceği sonucuna varılabilir.

Anahtar kelimeler: Enalapril maleat, yüzen mikroküre, gastroretentif yüzen ilaç taşıyıcı sistem, iota-carrageenan, sodyum aljinat

INTRODUCTION

Conventional drug delivery systems have insufficient control over drug release and concentration at the target site because of amendments in the concentration of the bioactive product.¹ Moreover, drug absorption in traditional dosing is dependent on the body's capability to assimilate the therapeutic molecule.² Thus, the development of modern administration techniques to maintain steady-state plasma concentration can be achieved through controlled drug release that supports unceasing drug delivery for a programmed period with foreseeable, reproducible kinetics and a drug release mechanism.³ Once achieved, the effectiveness of the drug and patient compliance are enhanced by reducing the frequency of administration.⁴

Microspheres have played a key role in the progress of controlled release systems, as they can encapsulate miscellaneous types of drugs and small molecules, nucleic acids, and proteins.⁵ They are biocompatible, can deliver superior bioavailability, and are able to release over longer periods.⁶ In addition, microspheres have been technologically advanced by numerous techniques comprising combinations of phase separations or precipitations, emulsion or solvent evaporation, and spraying methods.⁷

Floating microspheres are one of the most promising buoyant gastroretentive drug delivery systems. These are free-flowing spherical empty particles without a core, with size varying from 1 to 1000 μm .⁸ The gastrointestinal transit-controlled preparations are intended to float on gastric juice with a specific density of less than one, and due to this property a delayed transit through the stomach occurs.⁹ The slowly released drug at a preferred rate results in enhanced gastric retention with abridged fluctuations in plasma drug concentration.¹⁰

Ionotropic gelation is used mainly in our study; however, natural hydrophilic polymers (polyelectrolytes) are used to prepare drug carriers due to their ability to cross-link in the existence of counter ions to form microspheres.¹¹ These polymers, including sodium alginate, gellan gum, and hydroxypropyl methylcellulose, are used extensively for the encapsulation of drugs and act as a release rate retardant.¹² For instance, this technique has the virtue of not using organic solvents.¹³

Sodium alginate is, in fact, a water-soluble polymer that becomes a gel in the incidence of polyvalent cations such as calcium chloride, though it is built on the transition of the polymer from a liquid state to a gel.¹⁴ These gels are constituted by dropping a drug-loaded polymeric solution into an aqueous solution of multivalent cations. The cations diffuse into the drug-loaded polymeric drops, creating a three-dimensional lattice of the ionically cross-linked moiety.¹⁵

Enalapril maleate is a pro-drug employed in the treatment of hypertension. After oral administration, it becomes hydrolyzed

in the liver to release the enalaprilat, which acts as an ACE inhibitor. The extent of absorption of enalapril maleate after oral administration is approximately 60% and due to the high hepatic first-pass metabolism of the prodrug (enalapril maleate) to the active form of the drug (enalaprilat) in the gastrointestinal tract before absorption the bioavailability of enalaprilat becomes approximately 40%.¹⁶

The objective of the present study was to overcome the reduction in bioavailability as a result of the lack of absorption. An attempt is needed to provide a control drug delivery system through this newly approved work for enhancing the absorption and bioavailability of enalapril maleate loaded floating microspheres by ionotropic gelation technique using a hydrophilic carrier.

MATERIALS AND METHODS

Materials

Enalapril maleate (Baoji Guokang Bio-Technology, China), sodium alginate (Avonchem, UK), iota-carrageenan (Provizer Pharma, India), and calcium chloride (Gainland Chemical Company, UK) were obtained. All other materials used were of pharmaceutical grade.

Methods

Preparation of floating microspheres

Floating microspheres were prepared through ionotropic gelation by dissolving the primary polymer (sodium alginate) in distilled water at different concentrations and heating to 60°C while stirring. To this dispersion was added dissolved iota-carrageenan in different concentrations while stirring and the heating was continued. The required amount of sodium bicarbonate was added to the above solution in a suitable proportion and mixing continued. To this successive solution was added the drug after cooling. The drug and polymer solution was added dropwise through a syringe with a 31-gauge needle into 100 mL of calcium chloride solution and stirred at 200 rpm. The microspheres formed were kept suspended in the solution for 1 h to improve their mechanical strength and then collected by filtration. After that, the floated microspheres were washed with 100 mL of distilled water 3 times and then dried in a hot air oven for 2 h at 50°C to be stored in a desiccator.¹⁷ The composition of the floating microspheres is given in Table 1.

Characterization of floating microspheres

Micrometric properties

The floating microspheres were characterized by numerous tests to detect their properties that obey USP standards.

Particle size analysis

The floating microspheres were separated into different size fractions by sieving for 10 min through a series of standard sieves, #40, #60, #80, and #100, and the particle size of 50 floating microspheres was calculated using an optical microscope (Novel, China) and the mean particle size was calculated.¹⁸

Bulk density

A weighed quantity of floating microspheres was poured into a graduated cylinder (10 mL). Bulk density was established by a ratio of the mass of floating microspheres to bulk volume.¹⁹

Bulk density=Mass/Bulk volume

Tapped density

A weighed quantity of floating microspheres was introduced into a graduated cylinder (10 mL) and the cylinder was tapped from a height of 2 cm for 100 standard taps until there was no more diminution in the density and the volume of the microspheres was calculated.²⁰

Tapped density=Mass/Tapped volume

Carr's (compressibility) index

The compressibility index of microparticles has been anticipated to be a subsidiary measure of the bulk density, size, and shape, surface area, moisture content, and cohesiveness of materials.²¹

% Compressibility index=(Tapped density-Bulk density)×100/Tapped density

Hausner ratio

The Hausner ratio of the floating microspheres was confirmed by associating the tapped density with the bulk density as shown in the following equation.²²

Hausner ratio=Tapped density/Bulk density

Table 1. Formulation of enalapril maleate floating microspheres

Formulations	Drug (mg)	Sodium alginate (mg)	Iota-carrageenan (mg)	Sodium bicarbonate	CaCl ₂ (%)
1	20	500	100	200	2
2	20	1000	100	200	2
3	20	1500	100	200	2
4	20	1000	200	200	2
5	20	1000	400	200	2
6	20	1000	200	400	2
7	20	1000	200	800	2
8	10	1000	200	200	2
9	5	1000	200	200	2
10	20	1000	200	200	4
11	20	1000	200	200	1

CaCl₂: Calcium chloride

Angle of repose

The flow of floating microspheres was measured by assessing the angle of repose using the funnel method. Prepared microspheres were poured via a funnel fixed 1 cm above a flat surface until the apex of the microsphere pile touched the tip of the funnel.²³ The angle of repose is calculated by the following equation:

$\theta = \tan^{-1}(h/r)$,

where θ =angle of repose, h=height of pile, and r=radius of the pile.

Determination of percentage yield

The calculation of floating microsphere percentage yield was premeditated using the weight of a dried final product regarding the total weight of the drug and polymer measured initially and used for the preparation of microspheres.²⁴ The percentage yields were calculated as per the formula

Percentage yield=[Weight of microspheres obtained/(Weight of drug+polymer)]×100

Determination of entrapment efficiency

Floating microspheres can be assessed for their drug content and it can be approved by dissolving weighed amounts of crushed microspheres (through a hammer mill) in 100 mL of 0.1 N HCl. An aliquot of 1 mL was taken and diluted to 10 mL; after that the mixture was shaken and filtered through a 0.45 μ m filter and then analyzed using an ultraviolet (UV) spectrophotometer (Shimadzu 8400S, Japan) at 219 nm using the calibration curve.²⁵ Each batch should be examined for drug content in triplicate.

% Entrapment efficiency=(Actual drug content)/(Theoretical drug content)×100

Determination of in vitro buoyancy

In vitro buoyancy was determined to study the floatation behavior of microspheres in the prepared formulations. First 50 mg of microspheres was spread in 0.1 N HCl (pH 1.2; 100 mL). The mixture was then stirred at 100 rpm in a magnetic stirrer. After 8 h, the buoyant microparticles layer was collected by pipette and separated by filtration. The particulate sinking layer particles were separated by filtration. Particles of both types were dried in a desiccator until a constant weight was obtained.²⁶ The buoyancy percentage was calculated by the following equation:

% Buoyancy=(Weight of floating microspheres)/(Total weight of floating and settled microspheres)×100

All the determinations were conducted in triplicate.

In vitro drug release study

The *in vitro* drug release rate from the floating microspheres was affirmed using a paddle type six-station dissolution test apparatus (Copley, UK). An accurate amount of floating microspheres equivalent to 5, 10, and 20 mg of drug was kept in 0.1 N HCl (1.2 pH) and the dissolution fluid was maintained at 37±0.5°C at a speed of rotation of 50 rpm. Sink conditions prevailed during the *in vitro* drug release study. A 4- μ L sample

was withdrawn and filtered through a 0.45- μm membrane filter at 5, 10, 15, 20, 30, 60, 120, 180, 240, 300, 360, 480, 600, 720, and 1440 min. The initial volume of the dissolution fluid was maintained by adding 4 mL of fresh dissolution fluid after each withdrawal. The samples were analyzed by UV spectrophotometer at 219 nm to determine the concentration of enalapril maleate present in the medium.²⁷ All experiments were performed in triplicate.

Kinetics of drug release

To determine the mechanism and kinetics of drug release, the results of the *in vitro* dissolution study of enalapril microspheres were obtained for various kinetic equations. The kinetics models used were zero order, first order, Higuchi's, and Korsmeyer-Peppas. Correlation coefficient (R^2) values were calculated for the linear curves obtained by regression analysis.¹⁶

Drug-excipients compatibility study and identification

Fourier transform-infrared spectroscopy (FT-IR)

The FT-IR spectra of pure drug, polymers (sodium alginate and iota-carrageenan), and the drug with the polymer (F4 as microspheres) were recorded on a spectrophotometer (Shimadzu 8400S, Japan) using the KBr pellet technique and reported as wave number (cm^{-1}). The scanning range was from 4000 to 450 cm^{-1} . The FT-IR spectra support the identification of the functional groups present in the compound. The FT-IR spectra are also used in comparing with a standard FT-IR spectrum of the pure drug to detect any physicochemical incompatibility between the drug and different excipients.²⁸

Surface morphology

Scanning electron microscopy (SEM) was used to characterize the surface and cross-sectional morphology of the designed floating microspheres. SEM samples were mounted directly by scattering the powder lightly on dual adhesive tape fixed to an aluminum stub. Then gold/palladium coating of stubs was conducted to about 20 nm thickness under an argon atmosphere by a gold sputter module in a high vacuum evaporator. The coated samples were then scanned haphazardly, and photographs were taken with SEM (TESCAN, VEGA 3-Czech Republic).²⁹

Powder X-ray diffraction (PXRD)

The PXRD patterns of enalapril alone, sodium alginate, iota-carrageenan, and F4 were recorded using XRD (Shimadzu 6000, Japan) with a $\text{CuK}\alpha$ line as the source of radiation. Standard runs were executed with a current of 30 mA, a voltage of 40 KV, and a scanning rate of 8 deg/min over a θ range of 5-80° using a step size of 0.02° per second. It was therefore used to determine the nature of the pure drug, whether it was crystalline or amorphous, and to examine the nature of the drug as to whether it was changed or not by using a combination of polymers.³⁰

Differential scanning calorimetry (DSC)

Thermal analysis was achieved by differential scanning calorimeter (STA PT-1000, LINSEIS-Germany) equipped with argon as an inert gas to study the drug and the microspheres'

crystalline changeability. The accurate weights of enalapril alone, sodium alginate, iota-carrageenan, and F4 were recorded. Weighed samples were put into aluminum pans and hermetically sealed. The samples were heated from 20°C to 200°C at a rate of 5°C per minute under an argon atmosphere with a gas flow rate of 100 mL/min. A covered, empty pan was used as a reference. The results obtained from the heating were recorded.³¹

Statistical analysis

The results were stated as an average value \pm SD and were analyzed using single factor analysis of variance (ANOVA) to compare the sample means and to determine the statistical significance, at which $p < 0.05$ was considered significant.

The study did not require ethics committee approval or patient informed consent.

RESULTS AND DISCUSSION

Characterization of floating microspheres

Micrometric properties

The floating microsphere formulations were evaluated to detect their micrometric properties as detailed below.

Particle size analysis

The mean particle size of the floating microsphere formulations (F1-F11) was in the range of 196.55 \pm 0.28 to 520.2 \pm 0.09 μm as shown in Table 2. Formulations representing an increase in sodium alginate concentration (F1-F3) showed an increase in particle size. This could be ascribed to an increase in relative viscosity at higher concentration of sodium alginate, which requires high energy for breaking of droplets, and is more difficult to disperse due to enhancement of interfacial tension and diminished shearing efficiency, leading to the formation of large droplets of floating microspheres during the addition of polymer solution to the gelling agent.³²

In addition, the particle diameter in formulations F4 and F5 increased gradually as the concentration of iota-carrageenan increased. This may be attributed to the increase in gel strength and the formation of strong bridges between anionic iota-carrageenan molecules and cationic CaCl_2 salts, leading to helix-helix aggregation of the adjacent spiral chains that contain sulfate groups and the formation of a stable three-dimensional network.³³

Furthermore, formulations F2 and F6 showed that the particle size of floating microspheres increased as the sodium bicarbonate content increased. On the other hand, a further increase in sodium bicarbonate concentration caused a decrease in the particle size of microspheres as shown in F7. The suggested mechanism demonstrates that as the concentration of sodium bicarbonate increases the microspheres expand and increase in size. An additional increase in sodium bicarbonate concentration will make them burst and decrease in size.³⁴

The effect of drug concentration on microsphere size was studied using F8 and F9, which showed an increase in the particle size of microspheres as the drug concentration decreased. This factor was related to the solubility of microspheres, which decreased with decreasing drug concentration, thus making these microspheres more rigid and larger in terms of particle size.³⁵

The effect of the crosslinking agent (CaCl_2) on particle size was detected in F10 and F11; as the concentration of calcium chloride increased, the mean particle size of microspheres increased. This is related to the availability of a high amount of Ca^{2+} , which crosslinked with sodium alginate and iota-carrageenan polymers, thereby leading to the formation of larger microspheres.³⁶

Rheological parameters of floating microspheres

Rheological studies of enalapril maleate floating microspheres involved bulk density, tapped density, compressibility index or Carr's index, the Hausner ratio, and angle of repose. The formulations were studied for all rheological properties as shown in Table 2.

Bulk and tapped density

The bulk density and tapped density of formulations F1, F2, and F3 may be triggered by a small difference in flow properties as shown in Table 2. This is due to the use of low polymer dispersion concentrations in F1; thus the microspheres did not have as good a spherical shape as F2 and had a flattened base at the points of contact with the drying vessels; however, an increase in the concentration of sodium alginate dispersion in F2 tended to make the particles more spherical. This indicates that at low alginate concentrations the particles were composed of a loose network structure that collapsed during drying. On the other hand, a higher sodium alginate concentration formed a dense matrix structure, which prevented the collapse of microspheres. As the concentration of sodium alginate in the aqueous dispersion increased in F3 the relative viscosity of dispersion increased and it was difficult to transfer polymer dispersion through the needle into the cross-linking agent

solution and an increase in concentration moreover caused a small tail at one end of the microspheres, which significantly affected the flow properties and particle size distribution.³⁷

Floating microspheres prepared with an increased concentration of iota-carrageenan (F4 and F5) showed a decrease in bulk and tap density. The reason behind this was the swelling property of the material, which absorbs fluid from the surrounding environment in a controlled manner, making it float above the gastric contents and remain unaffected by the gastric emptying time.³⁸

Upon an increment in sodium bicarbonate concentration in F4, F6, and F7, the microspheres became more floated as the density dropped below 1.0 g/cm^3 . This indicated that when the spheres come in contact with the medium (0.1 N HCl, pH 1.2), they start to react and generate CO_2 gas with upward force and become entrapped within the matrix of sodium alginate and iota-carrageenan. This entrapment of CO_2 leads to a decrease in microsphere density; thereby they become buoyant.³⁹

Formulations of floating microspheres representing a decrease in enalapril maleate content (F8 and F9) showed a high level of bulk and tapped density. This is related to the high porosity in the spheres' matrix, which increases medium flowability directly into these floating microspheres, making them denser.⁴⁰

It was evident that bulk and tapped density increased in F4, F10, and F11 as the concentration of CaCl_2 increased. This is due to the higher concentration of crosslinking agent, which will lead to an increment in viscosity, thereby increasing the density of microspheres.⁴¹

Compressibility index or Carr's index

Carr's index is an indicator of the tendency to form bridges between microspheres. Thus, the values for all formulations were in the range of $2.36 \pm 0.1\%$ and $12.79 \pm 0.29\%$ as shown in Table 2, which displayed an excellent flow of microspheres and excellent compressibility.⁴²

Hausner ratio

The Hausner ratio was measured to indicate the cohesion between microsphere particles. The values of all formulations

Table 2. Micrometric properties of enalapril maleate floating microspheres

Formula code	Particle size (μm)	Bulk density (g/cc)	Tapped density (g/cc)	Compressibility index (%)	Hausner ratio	Angle of repose
F1	196.55 \pm 0.28	0.463 \pm 0.12	0.493 \pm 0.09	6.08 \pm 0.05	1.06 \pm 0.03	13.52 \pm 0.34
F2	242.10 \pm 0.17	0.497 \pm 0.04	0.537 \pm 0.11	7.44 \pm 0.03	1.08 \pm 0.07	15.90 \pm 0.22
F3	520.2 \pm 0.09	0.908 \pm 0.23	0.99 \pm 0.17	8.28 \pm 0.02	1.09 \pm 0.09	18.4 \pm 0.16
F4	199.4 \pm 0.04	0.812 \pm 0.02	0.860 \pm 0.07	5.58 \pm 0.01	1.06 \pm 0.05	17.64 \pm 0.11
F5	429.3 \pm 0.09	0.785 \pm 0.14	0.837 \pm 0.12	6.21 \pm 0.09	1.07 \pm 0.11	18.9 \pm 0.24
F6	403.2 \pm 0.13	0.777 \pm 0.08	0.891 \pm 0.32	12.79 \pm 0.29	1.14 \pm 0.15	16.74 \pm 0.09
F7	372.6 \pm 0.18	0.763 \pm 0.16	0.871 \pm 0.26	12.39 \pm 0.33	1.14 \pm 0.08	13.21 \pm 0.31
F8	347.4 \pm 0.21	0.812 \pm 0.25	0.840 \pm 0.27	3.33 \pm 0.17	1.03 \pm 0.04	18.1 \pm 0.17
F9	421.2 \pm 0.06	0.799 \pm 0.22	0.820 \pm 0.21	2.56 \pm 0.11	1.02 \pm 0.06	19.32 \pm 0.13
F10	458.4 \pm 0.14	0.880 \pm 0.31	0.900 \pm 0.05	6.38 \pm 0.08	1.07 \pm 0.12	20.22 \pm 0.25
F11	298.8 \pm 0.15	0.704 \pm 0.09	0.721 \pm 0.03	2.36 \pm 0.1	1.02 \pm 0.06	16.5 \pm 0.17

were below 1.25, as shown in Table 2, thus indicating good flow properties with easy handling during processing.⁴³

Angle of repose

Values of the angle of repose of all formulations were below 21°, as shown in Table 2, indicating free-flow properties of microspheres. The better flow of microspheres indicates that the floating microspheres produced were nonaggregated. Similar findings were reported for novel floating microspheres of metronidazole.⁴⁴

Percentage yield

The percentage yield of floating microsphere was examined to determine the polymer effect (sodium alginate) on the formulations. The results showed that the percentage yields of formulas F1 to F3 range from 54.5±0.925 to 72.88±0.672, as shown in Table 3. It is obvious that the increment in polymer concentration led to an increase in percentage yield. This effect can be explained by the fact that as the concentration of alginate increases the quantity of polymer becomes adequate to cover enalapril maleate particles completely. In addition, the microspheres become well distributed, discrete, and spherical and have no clumping, thus giving a good percentage of yield.⁴⁵

The increment in iota-carrageenan concentration will instantaneously lead to interfacial cross-linking taking place, followed by a more gradual gelation of the interior, which results in an increase in the percentage yield as shown in F4. From the results, it was noted that the viscosity increased dramatically with the further increase in iota-carrageenan concentration, which may retard the penetration of the enalapril into the matrix and hence decrease percentage yield, as shown in F5.⁴⁶

Formulations prepared with an increased concentration of sodium bicarbonate as a gas forming agent (F6 and F7) were observed to have a decrease in the percentage of yield. The microspheres with a small amount of sodium bicarbonate will have a highly dense internal structure of the matrix and they will be able to retain enalapril more effectively as shown in

F4. The porous microspheres with an increment in the amount of sodium bicarbonate, having a less dense internal structure, resulted in a decrease in the percentage of yield of the drug.⁴⁷

The high enalapril percentage of yield in F4 might have been due to the higher water solubility of a freely soluble drug, which is always entrapped in a higher ratio, making diffusion out of the microspheres from the gel surface difficult during the hardening of the carrageenan gel matrix. This percentage of yield decreased when the drug content decreased, as seen in F8 and F9.⁴⁸

The effect of increasing CaCl₂ concentration in F10 appears in the degree of cross-linking, which will be increased, and so the percentage yield decreased due to the difficulty of drug penetration into the microspheres. Moreover, the percentage of yield in F11 increased with decreasing CaCl₂ concentration. The reason for the high percentage yield may have been the high solubility of the drug, which will be more entrapped within the matrix of microspheres.⁴⁹

Entrapment efficiency

Drug entrapment was related to the permeation characteristics of polymers used, which could simplify the diffusion of a part of the drug that was entrapped in the medium during the preparation of floating microspheres. Drug entrapment efficiency increased with the increment in polymer concentration (F1-F3) as shown in Table 3. This is due to the increase in polymer content and so more particles of enalapril would be coated, leading to higher encapsulation efficiency.¹¹

The entrapment efficiency of iota-carrageenan in F4 and F5 decreased with an increment in polymer concentration in the gastric medium. This is because of the increment in the viscosity with the further increase in iota-carrageenan concentration, which may retard the penetration of the enalapril into the microspheres and hence decrease entrapment efficiency.⁵⁰

Table 3 showed that as the amount of sodium bicarbonate (as gas forming agent) increased (F4, F6, and F7), a decrease in entrapment efficiency was observed. Microspheres with low gas forming agents showed high entrapment efficiency as compared to those with high gas-forming agents. This result was attributed to the fact that microspheres with a low gas forming agent have an excessively compact internal structure able to keep the drug within its matrix, as compared to the less dense internal structure of the other microspheres, which consisted of a high amount of gas forming agents that cause a decrease in drug entrapment. As a result, the formation of more pores on the microspheres networks with an increased amount of sodium bicarbonate will make them have lower drug entrapment efficiency.⁵¹

Microspheres for formulations of F8 and F9 were designed with different drug concentrations. The decrease in entrapment efficiency was achieved by decreasing drug concentration. This results from a lower concentration gradient in which the drug may diffuse out of the microsphere matrix to the external medium during preparation, which tends to decrease encapsulation efficiency.⁵²

Table 3. Percentage yield, drug entrapment efficiency, and *in vitro* buoyancy

Formula code	Percentage yield	Entrapment efficiency	Buoyancy (%)
F1	54.5±0.92	73.4±0.07	80.22±0.21
F2	67.07±0.87	81.5±0.05	76.12±0.53
F3	72.88±0.67	84.3±0.15	68.89±0.44
F4	91.18±0.24	92.3±0.04	92.41±0.21
F5	84.2±0.36	88.7±0.09	70.17±0.37
F6	69.3± 0.46	76.15±0.11	72.21±0.77
F7	68.8±0.21	72.15±0.08	71.43±0.55
F8	75.9± 0.23	78.2±0.06	82.95±0.41
F9	82.7± 0.35	72.8±0.12	74.10±0.34
F10	72.38±0.23	80.5±0.17	50.92±0.74
F11	85.4±0.27	83.5±0.03	78.54±0.82

The effect of CaCl_2 on encapsulation efficiency was observed in formulations F10 and F11. The percentage encapsulation efficiency decreases with the increase in the concentration of calcium chloride as shown in F10. For microspheres cross-linked with a low level of CaCl_2 (F11) a higher drug encapsulation efficiency was seen in comparison to high levels of calcium chloride. This may be as a result of the immediate gelling of polymer (sodium alginate) on the addition of CaCl_2 and thrust out of the aqueous phase from the gel lattice.⁵³

In vitro buoyancy

The buoyancy percentage was calculated for all the formulations and it was found that all formulations were able to float on the dissolution medium (0.1 N HCl, pH 1.2) for 24 h. The buoyancy percentage of the microspheres was found to decrease with an increase in sodium alginate concentration represented by F1-F3, as shown in Table 3. This is because of the elevated viscosity of the polymer solution, which in turn is the reason for more dense microspheres and less formation of pores in addition to cavities during preparation.⁵⁴

Moreover, the increasing iota-carrageenan concentration in F2 and F4 resulted in an increment in microsphere buoyancy. This was due to the immediate crosslinking of the microsphere matrix as an outcome of the strongly acidic sulfate groups in the iota-carrageenan molecule, which allow a certain degree of polymer ionization in 0.1 N HCl (pH 1.2), leading to the formation of an insoluble gel-like layer of aggregated double-helical segments that form a three-dimensional network by complexation, and consequently slower solvent penetration into the matrices, and more controlled CO_2 diffusion was achieved, thus inducing the microspheres to float rapidly.⁵⁵ A further increment in iota-carrageenan concentration, as shown in F5, will lead to a decrease in the buoyancy effect. This was related to the increment in iota-carrageenan viscosity as the concentration rose. Thus, more entrapment of CO_2 gas and less gastric medium penetration into the matrices will lead to a decrease in buoyancy of enalapril microspheres.

The effect of increasing NaHCO_3 concentration on buoyancy was shown to be nonsignificant and is represented in F6 and F7. The reason behind this was the properties of sodium alginate, causing strong crosslinking in the polymer matrix, and addition of NaHCO_3 decreased the elasticity of the matrix without affecting viscosity, and so the buoyancy was not affected.⁵⁶

The effect of drug loading of microspheres was shown in F8 and F9. From the results, as the quantity of drug increased, more drug molecules were available at the surface of microspheres. In addition, more solid drug particles will begin to form continuous pores or channels within the matrix. Under these circumstances, the path of least resistance for drug molecules will be diffusion within the channels formed from areas where the drug has previously leached out from the matrix. Therefore, as the amount of drug content is increased and drug leaches out from the polymer, the matrix becomes more porous and higher buoyancy occurs. Lower drug contents create fewer pores within the polymeric network; hence a lower rate of drug diffusion was observed and lower buoyancy was achieved.⁵⁷

Buoyancy was found to decrease with increasing CaCl_2 , as shown in F10 and F11. CaCl_2 might be responsible for producing a more viscous matrix, which may block the pores on the surface of microspheres. Thus, a higher concentration of CaCl_2 can produce a high degree of cross-linking and thereby decreasing buoyancy from enalapril microspheres.⁵⁸

In vitro drug release

The effect of different concentrations of sodium alginate on drug release in F1-F3 was significant ($p < 0.05$), as shown in Figure 1. The release of enalapril from the prepared alginate microspheres was distinguished by an initial phase of high release (burst effect) followed by the second phase of moderate release. This biphasic manner of release is a distinctive feature of matrix diffusion kinetics.⁵⁹ A significant decrease in drug release was noted with an increment in the drug/polymer ratio in the prepared microspheres and is related to an increment in the density of the polymer matrix and in the diffusional path length that the drug molecules must traverse.⁶⁰

The release profile in Figure 2 represents a significant effect ($p < 0.05$) between formulations F2, F4, and F5. These formulations determine the influence of different concentrations of iota-carrageenan on the drug released. Two distinctive release steps were detected, where the initial rapid release was due to diffusion, while the second step was due to erosion of the matrix. A faster release in F4 in comparison to F2 was observed because of the presence of high-water content molecules in the matrix. The existence of a higher concentration of iota-carrageenan leads to higher release of the drug. It was suggested that the pore size in F4 was higher than in F2, resulting in higher release.⁶¹ A further increase in iota-carrageenan concentration, as shown in F5, caused

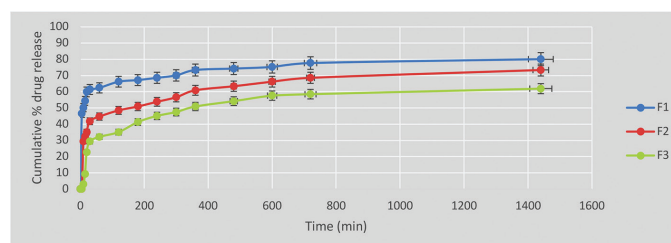


Figure 1. Dissolution profile of enalapril from floating microspheres containing different concentrations of sodium alginate (F1-F3), data given in mean \pm SD, $n=3$

SD: Standard deviation

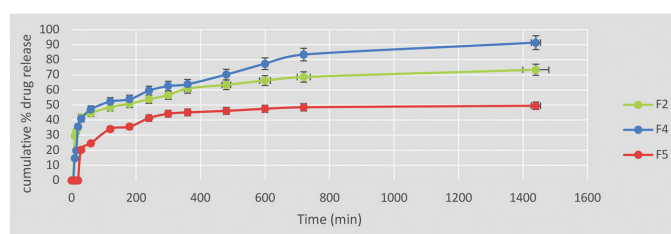


Figure 2. Dissolution profile of enalapril from floating microspheres containing different concentrations of iota-carrageenan (F2, F4, and F5), data given in mean \pm SD, $n=3$

SD: Standard deviation

a decrease in drug release. This was ascribed to the gelling property of the polymer, which could sustain the drug release from its matrix as well as the ability to wet. The gel matrix will swell and withstand erosion under acidic conditions to maintain a constant diffusion path length, forming a highly crosslinked matrix with minimum porosity.⁵⁵

Moreover, it was noted from the *in vitro* release study that the drug release rate increased nonsignificantly ($p > 0.05$) with an increase in the proportion of NaHCO_3 (F4, F6, and F7), as shown in Figure 3. This was ascribed to the low concentration of NaHCO_3 ; the alginate produces a highly dense internal structure and so a minimal amount of drug is released, but in the presence of higher concentrations of NaHCO_3 the formulations become more porous and the drug is released in a faster manner.

The effect of enalapril concentration on drug release profile in F4, F8, and F9 represents a nonsignificant effect ($p > 0.05$), as shown in Figure 4. It was noted that at a higher drug concentration in F4 less than 92% of the drug was released in 24 h as compared to 100% drug release from microspheres with lower drug concentrations in F8 and F9. In contrast to the low enalapril-loaded microspheres that exhibited smooth surfaces, the highly loaded enalapril microspheres showed rippled and rough surfaces. No enalapril crystals were observed embedded or attached firmly to the surface of the low enalapril loaded microspheres. Thus, this surface structure should be attributed to possible molecular interactions between the coating polymer and the enalapril rather than to an excess of incorporated drug, which might result in recrystallization of enalapril within the microspheres as was the case with the high enalapril loaded microspheres.⁶²

The results of the *in vitro* drug release study indicated that

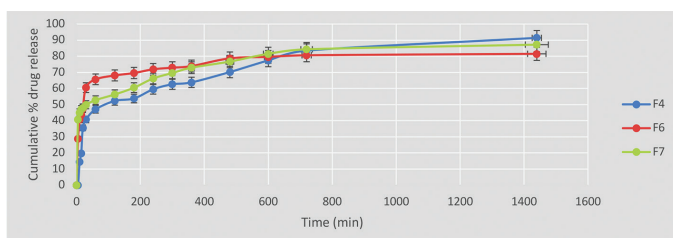


Figure 3. Dissolution profile of enalapril from floating microspheres containing different concentrations of sodium bicarbonate (F4, F6, and F7), data given in mean \pm SD, $n=3$

SD: Standard deviation

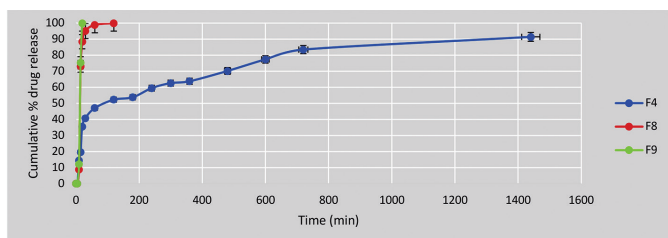


Figure 4. Dissolution profile of enalapril from floating microspheres containing different concentrations of enalapril (F4, F8, and F9), data given in mean \pm SD, $n=3$

SD: Standard deviation

CaCl_2 concentration affected drug release (F4, F10, and F11) significantly ($p < 0.05$), as shown in Figure 5. It was shown that the drug release decreased with an increase in the concentration of calcium chloride. This is attributed to the formation of a tight junction between the MM/GG residues of sodium alginate with calcium ion, which in turn decreases the swelling capacity of the microspheres. Therefore, enalapril cannot be readily released from the microspheres, as the surface roughness and porosity increase and the steric entanglements comprise a strong barrier; thus poor entry of dissolution medium into the polymer matrix may delay drug release.⁶³

Kinetic assessment of dissolution data

The release pattern of enalapril in gastric fluid (0.1 N HCl, pH 1.2) from all formulations of floating microspheres (F1 to F11) followed the Higuchi matrix model as shown in Table 4. The effervescent floating systems obeyed the Higuchi model, indicating drug release via a diffusion mechanism. In addition, formulations F1, F6, and F7 were observed to have n values of 0.43 or less, with the release mechanism following Fickian diffusion.²⁷ Formulations F5, F8, F9, and F11 were observed to have a high n value of > 0.84 ; thus the mechanism of drug release is regarded as super case II transport. The value of the release rate exponent (n) of the Korsmeyer-Peppas release model for F2, F3, F4, and F10 was $0.43 < n < 0.84$ for mass transport, which follows a non-Fickian model (anomalous transport). Therefore, it can be concluded that F4 was the optimum formula and the drug release was mainly following anomalous transport, which corresponds to a diffusion, erosion, and swelling mechanism or mixed-order kinetics.⁶⁴

Interaction study and identification of drug excipients

FT-IR

FT-IR spectroscopy was carried out to establish the compatibility of enalapril with polymers after the preparation of microspheres. Individual FT-IR scanning of the pure drug powder (Figure 6A) and polymers (sodium alginate, iota-carrageenan) is shown in Figures 6B and 6C, respectively, in addition to enalapril microspheres (F4) in Figure 5D.

The FT-IR spectrum for pure enalapril was distinguished by the principal absorption bands at 1753/cm due to C=O stretching (ester), at 1649/cm due to N-H bending, and at 1444/cm due to C-H (alkanes) bending.⁶⁵ The FT-IR spectrum of sodium alginate was characterized by absorption bands at 2933, 1610, 1421, and 1033/cm due to stretching of -CH, -COOH, -CH,

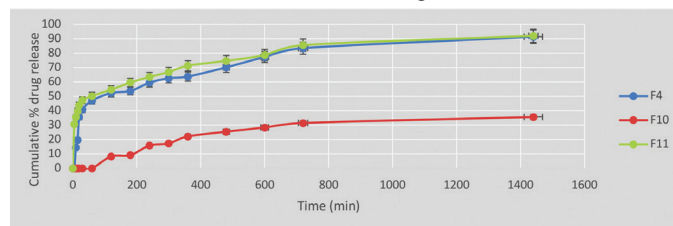


Figure 5. Dissolution profile of enalapril from floating microspheres containing different concentrations of CaCl_2 (F4, F10, and F11), data given in mean \pm SD, $n=3$

SD: Standard deviation

and -C-O-C, respectively.⁶⁶ In addition, the FT-IR spectrum of iota-carrageenan was observed with characteristic bands at 1228/cm for the ester sulfate group, 918/cm for 3,6-anhydrogalactose, 846/cm for galactose-4-sulfate, and 771/cm for 3,6-anhydrogalactose-2-sulfate.⁶⁷

The IR spectra patterns for microspheres (F4) were compared with the IR spectrum of the pure drug for confirmation of the major functional groups. All the characterized bands of enalapril with polymers appeared and indicated no significant variation in the peaks, suggesting that the drug and excipients were compatible. Moreover, it shows that there was no interaction between pure enalapril powder and the polymers used. Subsequently, it can be decided that the drug is chemically

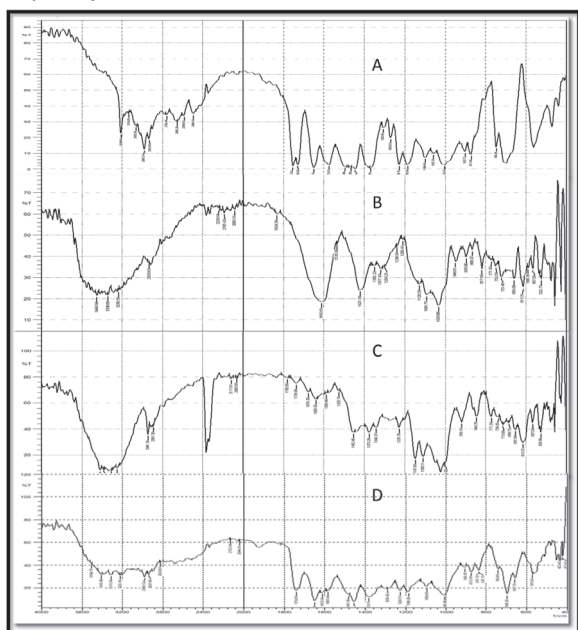


Figure 6. The FT-IR spectra for (A) pure enalapril; (B) sodium alginate; (C) iota-carrageenan; (D) enalapril microspheres (F4)
 FT-IR: Fourier transform-infrared spectroscopy

stable in the polymer matrix and can be released with ease from microspheres.

Surface morphology

The surface morphology of microspheres represented by the particle size and a characteristic shape was determined by SEM. The SEM images of microspheres taken at different magnifications are shown in Figure 7. It was noted in the SEM images that the microspheres were spherical, discrete, and freely flowing. In addition, the surfaces were slightly rough and drug crystals were also present on the surface of the microspheres. These drug crystals were responsible for the burst release of drug from the microspheres.⁶⁸

PXRD

The X-ray diffraction of pure enalapril, sodium alginate, iota-carrageenan, and F4 is shown in Figure 8. It was observed

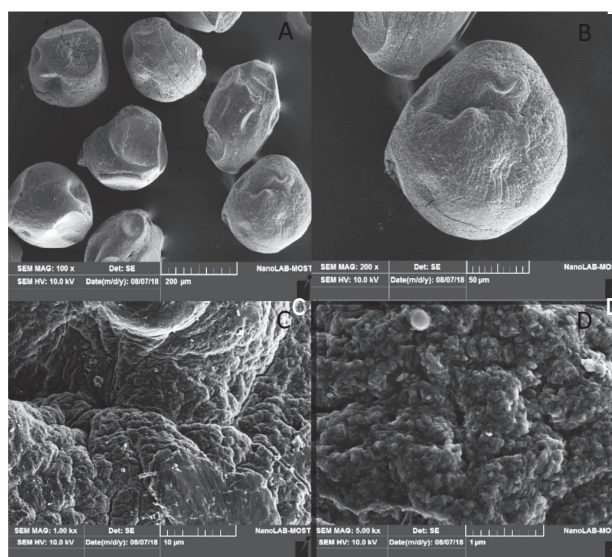


Figure 7. Scanning electron microscopy for F4 represents different magnifications A) 100x; B) 200x; C) 1K; D) 5K

Table 4. Kinetic data models for the prepared enalapril microspheres

Formulation	Zero-order		First-order		Higuchi-order		Korsmeyer-Peppas		
	K_0 (mg h ⁻¹)	R ²	K_1 (h ⁻¹)	R ²	K_H (h ^{-1/2})	R ²	n	K_{kp} (h ^{-1/3})	R ²
F1	0.0203	0.6177	-0.0003	0.7375	0.8844	0.8443	0.3509	1.0278	0.4676
F2	0.0359	0.5419	-0.0003	0.7234	1.5826	0.7563	0.5372	0.4572	0.6573
F3	0.0392	0.5623	-0.0003	0.671	1.7546	0.8089	0.6158	0.1383	0.7955
F4	0.0983	0.7108	-0.0009	0.8214	2.7776	0.8462	0.5789	0.4298	0.6399
F5	0.0357	0.4806	-0.0002	0.5361	1.6643	0.7502	0.8606	-0.5975	0.7932
F6	0.0545	0.6268	-0.0007	0.7741	1.7369	0.7963	0.176	1.4368	0.8926
F7	0.072	0.9510	-0.0008	0.985	1.72	0.9887	0.13	1.5117	0.9627
F8	1.6325	0.5322	-0.057	0.803	42.927	0.7805	1.2111	-0.0982	0.7582
F9	7.264	0.9373	-0.0607	0.8185	44.375	0.8114	1.6255	-0.3657	0.7534
F10	0.0302	0.7975	-0.0002	0.8338	1.2281	0.9486	0.8423	-0.9491	0.8966
F11	0.0731	0.8792	-0.0008	0.9601	1.9878	0.9703	0.1827	1.3785	0.9859

that the pure enalapril powder exhibited highly intense, sharply distinctive, and narrow diffraction peaks at 2θ of 10.48, 20.92, 24.89, and 31.57, indicating that the drug was a highly stable crystalline. However, when the drug was incorporated into the polymer matrix, the principal peaks of the drug diffractogram were observed with lower intensity. This could be ascribed to the crystalline state of the drug in the microsphere.⁶⁹

DSC

DSC studies were performed to investigate the physical state of the drug in the microspheres because this aspect could influence the *in vitro* and *in vivo* release of the drug from the systems. Figure 9 shows the DSC thermogram of pure enalapril powder, sodium enalapril powder, sodium alginate, iota-carrageenan, and F4

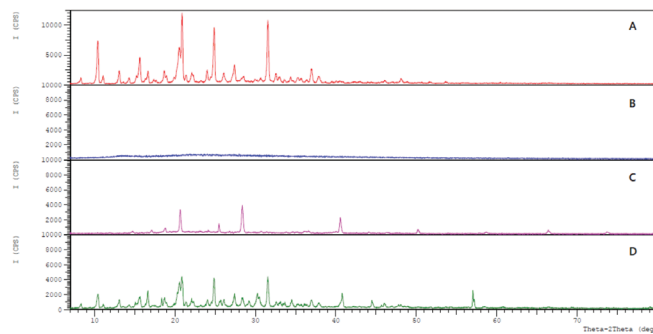


Figure 8. X-ray diffraction patterns of A) pure enalapril powder; B) sodium alginate; C) iota-carrageenan; D) F4

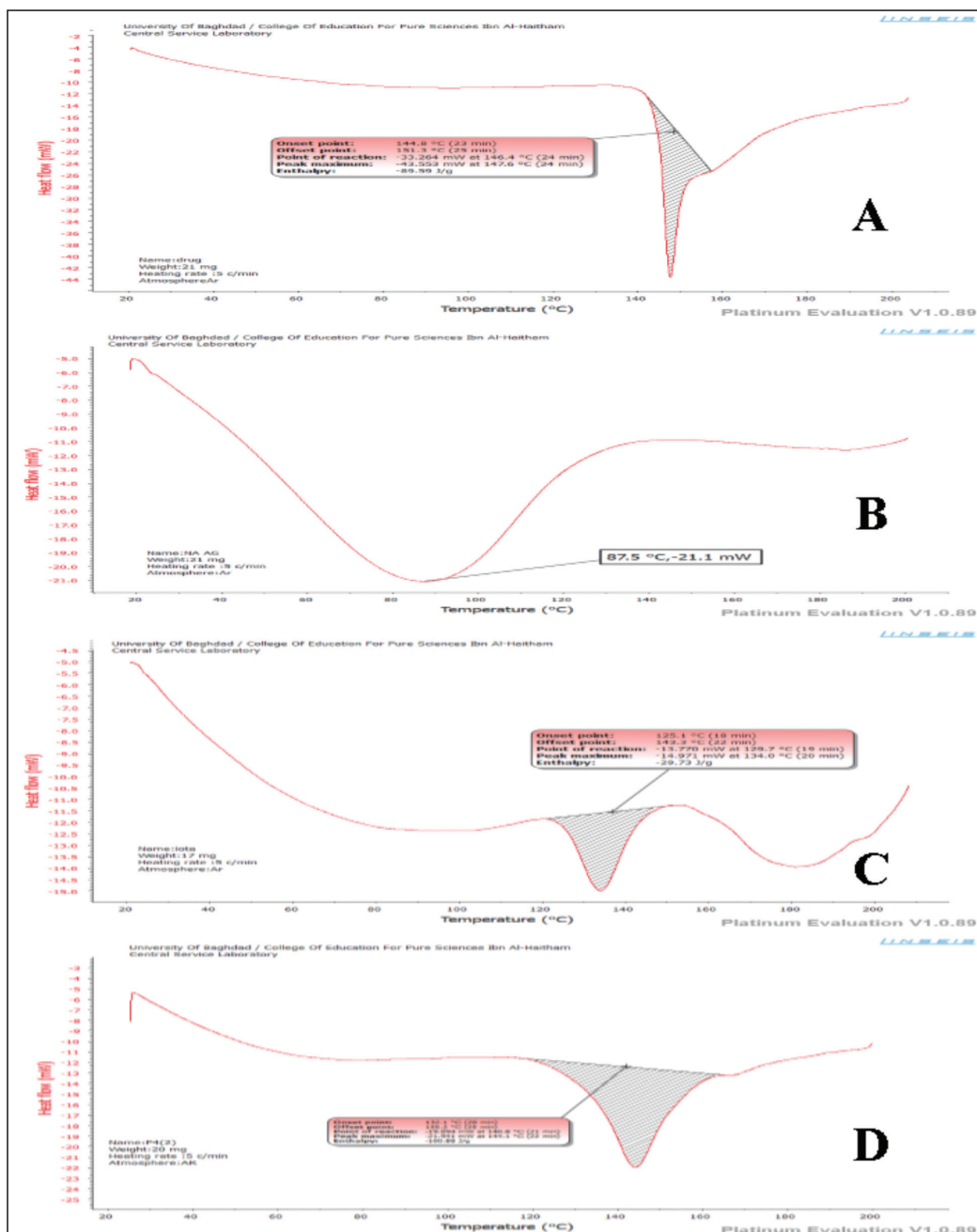


Figure 9. Differential scanning calorimetry patterns of A) pure enalapril powder; B) sodium alginate; C) iota-carrageenan; D) F4

as microspheres. Pure powder of enalapril showed a sharp endothermic peak at 147.6°C corresponding to its melting point, while sodium alginate showed a broad endothermic peak at around 87°C, which was mainly due to loss of water. The endothermic peak of the iota-carrageenan was observed at 134°C; that has a narrower peak compared with sodium alginate, which may be attributed to the lower numbers of hydrophilic OH groups (anhydride bridge). The appearance of a broader peak in F4 with minimum shifting in position indicates the presence of the crystalline drug in the microsphere samples at least at particle surface level. Therefore, it could be concluded that F4 has shown the miscibility of the drug with the polymers while maintaining stable characteristic properties of enalapril loaded in the prepared microspheres.⁷⁰⁻⁷²

CONCLUSION

The present study established the prepared enalapril maleate microspheres through ionotropic gelation to provide better therapeutic efficacy, as a result of the continuous availability of the drug. *In vitro* release studies showed a significant decrease in drug release with an increment in sodium alginate concentration and calcium chloride concentration and higher concentrations of iota-carrageenan. At the same time, the effects of sodium bicarbonate and drug concentration increment on drug release were nonsignificant. FTIR spectra suggested that the drug and excipients were compatible. Surface morphology showed a spherical shape with rough surface microspheres. X-ray diffraction showed the crystalline state of the drug. The DSC studies displayed miscibility of the drug with the polymers. The overall result indicates promising pharmaceutical dosage form of enalapril maleate loaded floating microspheres.

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Comparison of Lipid and Lipoprotein Values of Wrestlers and Soccer Players

Güreşçiler ve Futbolcuların Lipit ve Lipoprotein Değerlerinin Karşılaştırılması

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ABSTRACT

Objectives: The aim of this study was to compare the lipid and lipoprotein values of wrestlers and soccer players.

Materials and Methods: A total of 35 subjects, 17 male wrestlers who are sporting for 11.5 years and 18 male soccer player students who are sporting for 11.9 years, participated in this study. Triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) levels were determined by Hitachi 717 autoanalyzer. To determine the differences between the wrestlers and the soccer players the independent t-test was performed.

Results: There was a significant difference in body weight and body mass index between the wrestlers and the soccer players ($p < 0.05$). Moreover, there were significant differences in plasma TC, LDL-C, and HDL-C values between the wrestlers and soccer players (all, $p < 0.05$). However, there was no significant difference in plasma TG values between the wrestlers and the soccer players ($p > 0.05$). On the other hand, TC and LDL-C values of the wrestlers were significantly higher than soccer players ($p < 0.05$). The HDL-C values of the soccer players were significantly higher the wrestlers ($p < 0.05$). The ratio TC/HDL-C of the wrestlers was markedly higher than soccer players ($p < 0.05$).

Conclusion: TC, TG, HDL-C, and LDL-C values of the soccer players were in better ranges than wrestlers. This situation can be caused by the effect of different sports branches as well as the training differences. The lipid and lipoprotein values of the wrestlers and soccer players showed that they do not carry a risk of cardiovascular disease. In addition, it can be recommended that wrestlers should do jogging or aerobic training in their daily regular training.

Key words: Wrestler, soccer players, exercise, blood

ÖZ

Amaç: Bu çalışmanın amacı güreşçilerin ve futbolcuların lipit ve lipoprotein değerlerinin karşılaştırılmasıdır.

Gereç ve Yöntemler: Bu çalışmaya 11,5 yıl spor yapan 17 erkek güreşçi ve 11,9 yıl spor yapan 18 erkek futbolcu öğrenci olmak üzere toplam 35 kişi katılmıştır. Trigliserit (TG), total kolesterol (TC), yüksek dansiteli lipoprotein kolesterol (HDL-C) ve düşük dansiteli lipoprotein kolesterol (LDL-C) düzeyleri Hitachi 717 otoanalizörü ile belirlenmiştir. Güreşçi ve futbolcu arasındaki farkları belirlemek için "bağımsız t" testi yapılmıştır.

Bulgular: Güreşçiler ile futbolcular arasında vücut ağırlığı ve vücut kitle indeksinde anlamlı fark bulunmuştur ($p < 0,05$). Ayrıca, güreşçiler ve futbolcular arasında plazma TG değerlerinde anlamlı bir fark bulunmamıştır ($p > 0,05$). Güreşçilerin TC ve LDL-C değerleri futbolculara göre anlamlı derecede yüksektir ($p < 0,05$). Futbolcuların ise HDL-C değerleri güreşçilerden anlamlı derecede yüksek bulunmuştur ($p < 0,05$). Güreşçilerin TC/HDL-C oranının futbolculara göre daha yüksektir ($p < 0,05$).

Sonuç: Futbolcuların TC, TG, HDL-C ve LDL-C değerleri güreşçilerle karşılaştırıldığında, daha iyi sınırlar içerisinde bulunmuştur. Bu duruma spor branşındaki ve antrenmandaki farklılıklar neden olabilir. Güreşçilerin ve futbolcuların lipit ve lipoprotein değerleri, kardiyovasküler hastalık tehlikesi taşımadıklarını göstermiştir. Bununla birlikte güreşçilerin günlük düzenli antrenmanlarında koşuya veya aerobik antrenmana daha fazla yer vermeleri tavsiye edilebilir.

Anahtar kelimeler: Güreşçi, futbolcular, egzersiz, kan

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INTRODUCTION

Physically inactive lifestyle and low levels of cardiorespiratory fitness lead to an increase in the risk of developing numerous chronic diseases as well as all-cause mortality.¹ In the middle-aged and older periods health problems occur more commonly such as high blood pressure, obesity, muscular weakness, postural disorders, diabetes, and risk factors for coronary artery disease. Studies have shown that increasing risk factors for coronary heart disease (CHD) are high cholesterol, triglycerides (TGs), and low-density lipoprotein cholesterol (LDL-C) levels and low high-density lipoprotein cholesterol (HDL-C) levels in blood lipids.² Both aerobic and anaerobic exercises can decrease total cholesterol (TC), raise HDL-C levels, and lower the TC/HDL-C ratio. The effect of exercise on LDL-C generally has been inconsistent and is regarded to be minor in magnitude. Among elite athletes, exercise continues to promote favorable lipoprotein profiles. Elevation in body mass index (BMI) has been associated with less favorable lipoprotein profiles, with an increased relative risk of CHD, as well as increased mortality due to cardiovascular diseases.³ Studies support a significant incremental effect of exercise on blood lipids and lipoproteins in men. It has been reported that changes in HDL-C levels with exercise training were inversely related to baseline HDL-C levels. These findings suggested that individuals with the lowest HDL-C levels would exhibit the greatest increases in HDL-C with exercise.⁴ The effects of physical activity on lipid and lipoprotein metabolism, TC, LDL-C, TG, and TC/HDL-C ratio significantly decreased after exercises. Therefore, in order to become fit and to keep healthy, activities such as fitness, aerobics, and jogging are important.⁵

There is substantial, consistent, and strong evidence that physical activity is a deterrent for developing many forms of cardiovascular disease (CVD). Many studies have shown that the cholesterol is related to CHD. LDL-/HDL-C ratio can show an increased rate of arteriosclerosis. Low blood levels of HDL-C are an independent risk factor for CVD.^{6,7} Positive effects of applied long regular exercises on physical, physiological, psychological, and motoric features have been reported and one of the most important positive effects of regular exercise is on blood biochemistry. Regular and well-tuned intensity aerobic exercise reduces TC, LDL-C, TGs, and blood lipid levels, while increases HDL-C levels when estimated.⁸ Wrestling and soccer involve very vigorous physical activity. It has also been pointed out that hypercholesterolemia and low levels of HDL-C were more pronounced in power sports (i.e. weight lifting, boxing, wrestling, and judo) and anaerobic sports (i.e. tennis, sprints and jumps, gymnastics, and ice skating).^{9,10} Physical activity has a beneficial effect on the serum lipid profile. The recognition of the cardiovascular risk in a sedentary lifestyle and of the benefits of regular exercise has led to the promotion of sport as a means to improve health and prevent certain diseases. However, the response of the lipid profile to an exercise session or training program is different depending on the type of exercise undertaken, its intensity and frequency, the duration of each session, and the time spent in such a program.¹¹ A large number of epidemiological studies have revealed a relationship between dyslipidemia and the prevalence of atherosclerosis

and CHD. Increased physical activity is associated with a reduction in the risk of CVD, but there is conflicting information about the optimal intensity and the amount of exercise necessary for this reduction.¹² Epidemiological studies suggest that individually measured and programmed physical activity and the implementation of primarily aerobic physical activity lead to increased concentrations of HDL-C and lowered TG, TC, and LDL-C. When the intensity of the workout is well controlled, the power consumption is a major factor affecting lipids and lipoproteins. Training leads to a series of adaptation, morphological, and functional changes at the level of the cardiovascular system and neuromuscular system, as well as lipids. Recent studies have shown that dosed individually and programmed physical activity leads to increases in the concentration of HDL-C, and decreasing TG, TC, and LDL-C.¹³

The aim of the present study was to compare lipid and lipoprotein values of wrestlers and soccer players and to investigate CVD risks. It is thought that there is a difference between the lipid and lipoprotein values of soccer players and wrestlers because they have differences in terms of branches and training. Soccer players do aerobic training in their regular training more than wrestlers do. This study is important in terms of comparing lipid and lipoprotein values of wrestlers and soccer players.

MATERIALS AND METHODS

Subjects

Seventeen male wrestlers who had done sports for 11.5 years and 18 male soccer players who had done sports for at least 11.9 years, a total of 35 subjects, participated in the present study. The wrestlers were university students and national athletes. The soccer players were college students playing in different leagues. Written consent was obtained from all the participants, who volunteered to participate in the present study.

Lipid and lipoprotein measurement

Blood samples were obtained from the antecubital vein of the subjects 48 h before exercise sessions. Fasting blood samples were taken in the morning. The samples were analyzed for TG, TC, HDL-C, and LDL-C. These levels were determined by Hitachi 717 autoanalyzer. Blood samples were taken within the scope of the ethic committee report of Ondokuz Mayıs University (report no: B.30.2.ODM.O.20.08/255).

$BMI = \text{Body weight (kg)} / \text{Height (m)}^2 = (\text{kg}/\text{m}^2)$

Statistical analysis

The analysis was performed using SPSS version 22. The Kolmogorov-Smirnov test was used to evaluate the normality of parameters. Comparisons of age and height were used and to determine the differences between the groups independent t-tests were performed. Statistical significance was set at $p < 0.05$.

RESULTS

The physical anthropometric and motoric characteristics of the wrestlers and soccer players are given in Table 1. The

serum lipid values are compared in Table 2. Table 3 shows the cardiovascular risk status of the participants.

DISCUSSION

Previous epidemiologic studies have demonstrated an increased risk of CHD and cardiovascular death with an increase in BMI.³ Stevens et al.¹⁴ found among healthy men that increasing BMI was associated with an increased relative risk of cardiovascular death, particularly among younger subjects. The Canadian Heart Health Surveys Research group reported an increased prevalence of dyslipidemia associated with an elevated BMI; a positive association between BMI and TC, LDL-C, and TG levels; and an inverse relationship with HDL-C levels.¹⁵ In another study, Garry and McShane³ found comparing mean lipid values among

BMI categories demonstrated lower HDL-C ($p < 0.01$), higher TGs ($p < 0.05$), and higher TC/HDL-C ratios ($p < 0.001$) with an increasing BMI in soccer players. In the present study, BMI for the wrestlers was higher than that for the soccer players. BMI was 25.14 kg/m² for the wrestlers and 22.95 kg/m² for the soccer players. There was a significant difference in body weight and BMI between the wrestlers and the soccer players ($p < 0.05$). There was no significant difference between them in terms of age, body height, or years of training ($p > 0.05$). The majority of studies showed that decreased TG, cholesterol, and LDL-C levels and increased HDL were the result of applied training when the severity, duration, and frequency of exercise were approved.¹⁶ A study involving soccer training found decreases in LDL, cholesterol, and LDH levels and a statistically significant result was found.¹⁷ The most important effect of exercise on the human body is on the metabolic system, especially lipids. Lipids and lipoproteins are risk factors for CHD.¹³ Most cross-sectional studies indicate smaller, nonsignificant differences in TC and LDL-C levels between exercise-trained and sedentary individuals.^{18,19} Aydoğan¹⁶ found TG and HDL-C levels did not differ between wrestling groups. Imamoglu et al.⁴ stated that there were no significant differences in plasma TC and TG values between their groups of wrestlers and students. No significant differences were found in HDL-C and LDL-C values between wrestlers and male students. In the present study, there were significant differences in plasma TC, LDL-C, and HDL-C values between the wrestlers and the soccer players ($p < 0.05$, $p < 0.001$). There were no significant differences in plasma TG values between the wrestlers and the soccer players ($p > 0.05$). TC and LDL-C values of the wrestlers were significantly higher than those of the soccer players ($p < 0.05$). HDL-C values of the soccer players were significantly higher than those of the wrestlers ($p < 0.05$).

Koc⁸ found significant reductions in exercise-induced TC, cholesterol, and LDL-C and increases in HDL levels. Labović et al.¹³ reported that athletes had lower TC, LDL-C, and TG levels and higher serum HDL than nonathletes. Friedmann and Kindermann¹⁹ found HDL-C levels higher in an endurance training male group than in an inactive group. A lot of studies report that aerobic exercises increase HDL-C levels. Exercise is a potential factor that may modify lipid profiles and therefore reduce the risk for CHD.²⁰ Imamoglu et al.⁴ did not find HDL-C to be significantly different between their groups. Because male wrestlers perform mostly nonaerobic exercises and strength training, exercises like these did not increase the HDL-C levels. At the end of their study, which investigated the effects of aerobic exercise on blood lipids, it was found that the exercise had no effect on TG or cholesterol parameters, but caused an increase in HDL-C level and a reduction in LDL-C level.²¹ In a previous study examining the effect of 6 weeks of wrestling and wrestling-technique-based circuit exercise on the plasma lipoprotein profile, it was shown that cholesterol and HDL decreased significantly.²² Some research reports a lower HDL-C in power-anaerobic athletes.^{4,23} When the literature was reviewed about the effects of exercise on plasma lipids and lipoproteins, the results indicated that moderate and low

Table 1. Physical characteristics of the wrestlers and soccer players

Parameters	Wrestlers	Soccer players	t
Age (year)	23.72±1.87	24.10±1.75	0.07
Body height (cm)	174.43±6.72	174.16±6.81	0.10
Body height (kg)	75.80±11.3	69.49±9.6	2.96*
BMI (kg/m ²)	25.04 ±3.64	22.95±3.62	2.88*
Years of training	11.5±5.4	11.9±5.5	0.29

* $p < 0.05$, BMI: Body mass index

Table 2. Comparison of serum lipid values of the wrestlers and soccer players

mg/100 cc	Groups	Mean	SD	t
TC	Wrestlers	177.69	12.34	3.67**
	Soccer players	163.34	12.60	
TG	Wrestlers	96.65	14.95	0.92
	Soccer players	94.25	12.14	
HDL-C	Wrestlers	54.72	3.82	2.16*
	Soccer players	57.45	3.39	
LDL-C	Wrestlers	132.63	15.43	2.68*
	Soccer players	119.47	15.33	

* $p < 0.05$, ** $p < 0.001$, SD: Standard deviation, TC: Total cholesterol, TG: Triglyceride, HDL-C: High-density lipoprotein cholesterol, LDL-C: L-density lipoprotein cholesterol

Table 3. Risk of cardiovascular diseases of the wrestler and soccer players

mg/100 cc	Groups	Mean	SD	t
TC/HDL-C	Wrestlers	3.25	0.34	2.28*
	Soccer players	2.84	0.35	
LDL-C/HDL-C	Wrestlers	2.42	0.27	1.16
	Soccer players	2.08	0.30	

* $p < 0.05$, SD: Standard deviation, TC: Total cholesterol, HDL-C: High-density lipoprotein cholesterol, LDL-C: L-density lipoprotein cholesterol

intensity exercises are of great importance. These events show an increase in HDL-C, decrease in LDL-C, and increased protective effects against arteriosclerosis.⁴ It has been identified that regular aerobic exercise reduces total blood cholesterol, serum TGs, and LDL-C and increases HDL-C.^{23,24} In the present study, the soccer players were found to have better HDL-C levels than the wrestlers. This may be the result of intensive training and nutrition. It is suggested that wrestlers should do more running and aerobic training.

People who have HDL-C cholesterol levels under 40 mg/100 cc have more than three times the risk of cardiovascular diseases than people who have high HDL-C levels.¹⁸ The periodic risk of heart disease can be estimated by dividing TC by HDL-C. As a result of the estimation 4.5-5 levels show important CVD risk, while 3.8-4 levels show low CVD risk.²⁵ Another study reported that the risk factor is high if the TC/HDL-C ratio is higher than 5 and low if the ratio is lower than 3.5.²⁴ Imamoglu et al.⁴ mentioned that the lipid and lipoprotein values of their four groups indicated that individuals in the exercise groups would not be exposed to the danger of cardiovascular diseases. The cardiovascular risk ratio of wrestlers is higher than that of other groups. Athletes engaging in aerobic sports at a high level are particularly favored with respect to their low overall risk of CHD and, in particular, to their highly favorable plasma lipoprotein pattern.²⁶ High-intensity aerobic training results in improvement in HDL-C.^{2,27} Studies of large populations of men have shown that those who exercise at a moderate or strenuous level have a lower incidence of CHD. The effect of exercise has been shown in controlled studies to raise HDL-C levels, lower TG, and lower the TC/HDL-C ratio.³ A training program that emphasizes strength, power, speed ability, resistance, explosive movements, and interval sprints can result in undesirable health and fitness consequences for the participants in power-anaerobic based sports.²² Garry and McShane³ demonstrated that the strongest relationship between BMI and lipoprotein levels occurred between BMI and the TC/HDL-C ratio. Wrestling is categorized as a power-anaerobic based sport on the basis of its nature of practice and competition times.²² In the present study the cardiovascular risk ratio (TC/HDL-C) was 3.25 mg/100 cc for the wrestlers and 2.84 mg/100 cc for the soccer players. The LDL-C/HDL-C was 2.42 mg/100 cc for the wrestlers and 2.08 mg/100 cc for the soccer players. In the present study, it is meaningful that the TC/HDL-C ratio of the wrestlers was higher than that of the soccer players ($p < 0.05$). There was no significant difference in the LDL-C/HDL-C ratio between the wrestlers and the soccer players ($p > 0.05$). The TC/HDL-C and LDL-C/HDL-C ratios for the wrestlers were higher than those for the soccer players. This can be attributed to the fact that the training form of the wrestlers is more anaerobic than that of the soccer players. Wrestlers and soccer player with the highest BMIs and elevated TC/HDL-C ratios may be those at greatest risk for future CVD, regardless of their TC or LDL-C values.

CONCLUSION

The TC, TG, HDL-C, and LDL-C of the soccer players were better than those of the wrestlers. This situation can be caused

by branches and training differences. This result shows that between wrestlers and soccer players there were differences in lipid and lipoprotein levels. The lipid and lipoprotein values of the wrestlers and soccer players showed that they are not at risk of cardiovascular disease. In addition, it is recommended that wrestlers should do jogging or aerobic training in their daily regular training.

Conflicts of interest: No conflict of interest was declared by the authors. The authors alone are responsible for the content and writing of the paper.

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Theoretical Study on Ionization of Boric Acid in Aqueous Solution by *Ab Initio* and DFT Methods at T=298.15 K

T=298,15 K'da *Ab Initio* ve DFT Yöntemleri ile Borik Asidin Sulu Çözeltisinin İyonlaştırılması Üzerine Teorik Bir Çalışma

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ABSTRACT

Objectives: The aim of this research work was to theoretically calculate the pK_a value of boric acid in aqueous solution by theoretical methods at T=298.15 K.

Materials and Methods: Boric acid has antifungal and antiviral properties. It is used in various prescription pharmaceutical products. The *ab initio* and density functional theory (DFT) methods were used in this research work.

Results: To explain the determined acidic dissociation constant, the various molecular conformations and solute-solvent interactions of the species of boric acid were considered. The basis set at the B3LYP/6-31+G (d) level of theory was selected for DFT calculations. We analyzed the formation of intermolecular hydrogen bonds between several species of boric acid and water molecules through Tomasi's method.

Conclusion: The result showed that there was comparable agreement between the experimentally and theoretically determined pK_a values for boric acid.

Key words: Boric acid, acidic dissociation constant, DFT, *ab initio*

ÖZ

Amaç: Bu araştırmanın amacı, borik asidin sulu çözeltisinin pK_a değerini teorik olarak T= 298,15 K'da hesaplamaktır.

Gereç ve Yöntemler: Borik asit antifungal ve antiviral özelliklere sahiptir. Çeşitli reçeteli farmasötik ürünlerde kullanılır. Bu araştırma çalışmasında *ab initio* ve yoğunluk fonksiyonel teorisi (DFT) yöntemleri kullanılmıştır.

Bulgular: Belirlenen asit disosiyasyon sabitini açıklamak için, borik asit türlerinin çeşitli moleküler konformasyonları ve çözünen-çözücü etkileşimleri göz önünde bulunduruldu. B3LYP / 6-31 + G (d) teori düzeyindeki temel set DFT hesaplamaları için seçilmiştir. Tomasi metodu ile çeşitli borik asit türleri ve su molekülleri arasında intermoleküller hidrojen bağlarının oluşumu analiz edildi.

Sonuç: Çalışmanın sonucu, borik asit için deneysel ve teorik olarak belirlenen pK_a değerleri arasında karşılaştırılabilir bir uyum olduğunu göstermiştir.

Anahtar kelimeler: Borik asit, asit disosiyasyon sabiti, DFT, *ab initio*

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INTRODUCTION

Boric acid is a weak acid that forms a white and water-soluble powder.¹ It can be naturally found in seawater, many plants, and most fruits. Boric acid has been used as a mild antiseptic or bacteriostat in eyewashes and mouthwashes. Aqueous solutions of boric acid are typically used for ophthalmic irrigation to cleanse, refresh, and soothe irritated eyes and used for removal of loose foreign material, air pollutants, or chlorinated water.²

Boric acid is predominantly eliminated unchanged by the kidney; small amounts are also excreted in sweat, saliva, and feces. Boric acid is concentrated in the brain and liver.³ Boric acid and its derivatives have been shown to promote riboflavinuria in both animals and humans.⁴

Metabolism of inorganic borates by biological systems is not feasible because excessive energy is required to break the boron-oxygen bond. Inorganic borates, in low concentrations, convert to boric acid at physiological pH in the aqueous layer overlying mucosal surfaces prior to absorption.⁵

Studies of the acidity of organic compounds are important and play a very significant role in the evaluation of the activity, reaction mechanisms, and structures of organic compounds.

Equilibrium constants for ionization reactions are usually called ionization constants or acidic dissociation constants (pK_a). pK_a is an important physico-chemical parameter in drug absorption. Many drug compounds include at least one acid and/or basic group, and the ionization state of these groups plays an important role in determining the physico-chemical properties of compounds. Information about the pK_a value of compounds plays a major role in the expansion of drug formulations.⁶⁻⁸

Reliable and accurate methods for calculating relative and absolute pK_a values are important for understanding of the effective pK_a values in molecules. Some studies detailing the acid-base properties of compounds in aqueous solutions and in the gas phase are also available.⁹ Different experimental procedures are frequently used for the determination of acidity constants. These methods are high-pressure liquid chromatography, liquid-liquid partitioning chromatography, and methods that involve potentiometric titrations or spectrophotometric determination in water or in mixtures of solvents. Manov et al.¹⁰ determined the ionization constant of boric acid and the pH of certain borax-chloride buffer solutions from 0 to 60°C. Arcis et al.¹¹ determined the ionization of boric acid in water from 298 K to 623 K by AC conductivity and Raman spectroscopy. Dickson carried out emf measurements using the cell: Pt | H₂ (g, 101.325 kPa) | borax in synthetic seawater | AgCl; Ag over the temperature range 273.15-318.15 K, and at five salinities from 5 to 45. The obtained results of that research work were used to calculate the stoichiometric (ionic medium) dissociation constant for boric acid in seawater media on the "total" hydrogen ion scale.¹²

For boric acid, the values of pK_a can be calculated using *ab initio* and density functional theory (DFT) methods.¹³⁻¹⁸ These computational methods have an important advantage. In these methods, the important structural properties of molecules, in

solution, such as the dihedral angle between the indicated atoms (D); total atomic charge (Mulliken) (q); bond lengths between the indicated atoms (d); and bond angles (A) are calculated. These structural properties can be used in research works as well as in various industries.

In the DFT method, the calculation of electronic structure was performed with DFT and the electrostatic features were modeled through external charge distributions and continuum dielectrics. The polarizable continuum model (PCM) using the integral equation formalism variant PCM is the default self-consistent reaction field (SCRf) method. This method creates the solute cavity via a set of overlapping spheres. It was initially devised by Tomasi and coworkers and Pascual-Ahuir and coworkers. Tomasi's method allowed us to prove that cations, neutral molecules, and anions form intermolecular hydrogen bonds (IHBs) with some molecules of water.¹⁹

The present paper deals with the influence of factors such as the SCRf model applied, choice of a particular thermodynamic equation, atomic radii used to build a cavity in the solvent (water), optimization of geometry in water, inclusion of electron correlation, and the dimension of the basis set on the solvation free energies and on the calculated pK_a values. The pK_a value of boric acid was calculated in aqueous solution by *ab initio* and DFT methods and temperature of 25°C. We investigated the molecular conformations and solute-solvent interactions of the cation, anion, and neutral species of boric acid to explain the obtained acidic dissociation constants.

MATERIALS AND METHODS

Initially, the structure of species of boric acid was optimized by semiempirical PM3 method in the program HyperChem (CS Chem 3D version 5.0). All calculations about the geometries of the initial and solvated molecules in water were done using the software package Gaussian 09. The DFT calculations were carried out using the hybrid exchange-correlation functional of Becke, Lee, Yang, and Parr (B3LYP) and the Gaussian 6-31G (d) basis set was used.²⁰

To analyze the solvent effects on all species involved in the selected ionization reaction, the PCM of Miertus and Tomasi.²¹ was used. In this method, the solvent is represented as a structureless polarizable medium characterized by its dielectric constant. Finally, we selected the solvation of the species by means of IHB_s that involve one molecule of the mentioned species and some molecules of water.

RESULTS AND DISCUSSION

The trend of a molecule to lose its H⁺ is quantified as pK_a . Boric acid is a weak acid and it has three acid groups. A proton can separate from the hydroxyl group to give an ionized species (Figure 1). This concept of microscopic ionization constant is shown in Equation 1:

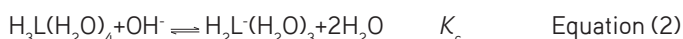
$$k = \frac{[H^+][B(OH)_2O^-]}{[B(OH)_3]} \quad \text{Equation (1)}$$

The total free energies (in Hartree and $\text{kJ}\cdot\text{mol}^{-1}$) for the single and solvated species of boric acid, in water, were calculated at the B3LYP/6-31+G (d) level of the theory, using Tomasi's method, at $T=298.15$ K and the results are shown in Table 1. This table shows that the total free energy for various species of boric acid increases with increasing number of water molecules. It shows that the solvation process causes an increase in the total free energy for various species of boric acid. In other words, solvation of the boric acid is an endothermic process. The values of total free energy for various species of boric acid (Table 1) were applied to calculate the $\text{p}K_a$ value of boric acid. In addition, these data help us to suggest an appropriate reaction regarding the deprotonation process of boric acid.

Various reactions including the neutral and anion species of boric acid were considered in the program Excel and some of these reactions were not considered further because their equilibrium constants were not comparable with the experimental ones. The selected equation for the deprotonation process of boric acid as well as the experimentally determined and theoretically calculated $\text{p}K_a$ is shown in Table 2.

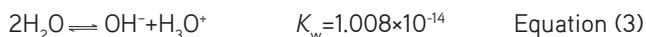
Ionization constant of boric acid

In aqueous solutions, the molecule of boric acid can undergo the below reaction:



In the above reaction, $\text{H}_3\text{L}(\text{H}_2\text{O})_4$ (Figure 2A) is the neutral species of boric acid solvated with four molecules of water and $\text{H}_2\text{L}^-(\text{H}_2\text{O})_3$ (Figure 2B) represents the anion species of boric acid solvated with three water molecules.

During the reaction of Equation 2, the autoprotolysis of two water molecules, in pure water, can occur as shown below:



The very low amount of K_w shows that a few water molecules are ionized in pure liquid water.

The reaction of Equation 4 can be obtained by combining Equation 2 and 3:

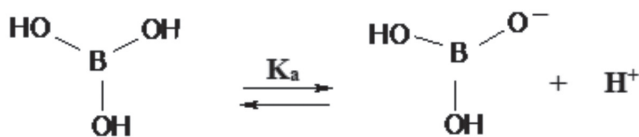


Figure 1. The scheme of deprotonation of boric acid

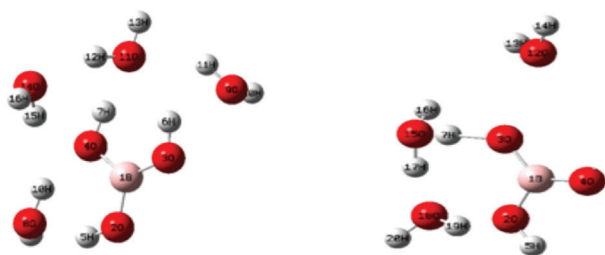
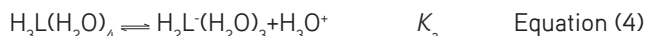


Figure 2. The calculated structure for the neutral (A) and cation (B) species of boric acid solvated with four and three water molecules, respectively, obtained at the B3LYP/6-31+G(d) level of theory and using Tomasi's method at 298.15 K



It is clear that the value of K_a can be calculated using K_c and K_w as below:

$$K_a = K_c \times K_w \quad \text{Equation (5)}$$

Equation 5 was applied to calculate the values of the ionization constant of boric acid, K_a , in water at $T=298.15$ K. The theoretically calculated value of $\text{p}K_a$ for boric acid at $T=298.15$ K is shown in Table 2. As can be seen in this table, there is good agreement between the experimentally determined ($\text{p}K_a=9.237$)²² and theoretically calculated $\text{p}K_a$ values of boric acid at this temperature.

Table 3 shows the optimized values of structural properties for the anion and neutral species of boric acid, in water, obtained at the B3LYP/6-31+G (d) level of theory with Tomasi's method at $T=298.15$ K.

As can be seen in Table 3, for boric acid, the values of q_{O4} for $\text{HL}^-(\text{H}_2\text{O})_3$ and $\text{H}_2\text{L}^-(\text{H}_2\text{O})_4$ are -1.104481 and -0.907847, respectively. It shows that the absolute value of electrical charge around the O_4 atom in $\text{HL}^-(\text{H}_2\text{O})_3$, compared to that in $\text{H}_2\text{L}^-(\text{H}_2\text{O})_4$, increases and it can imply H^+ separates from the O_4 atom during the deprotonation process of boric acid in water.

Table 1. The calculated total free energy (G_{sol}°) using Tomasi's method at the B3LYP/6-31+G (d) level of theory for neutral and cationic species of boric acid at 298.15 K

No	Species	G_{sol}° (Hartree)	$G_{\text{sol/molecule}}^\circ$ ($\text{kJ}\cdot\text{mol}^{-1}$)
0	H_2L^-	-252.913276	-664023.7424
1	$\text{H}_2\text{L}^-(\text{H}_2\text{O})$	-329.373889	-432385.5313
2	$\text{H}_2\text{L}^-(\text{H}_2\text{O})_2$	-405.824119	-355163.7074
3	$\text{H}_2\text{L}^-(\text{H}_2\text{O})_3$	-482.272605	-316551.6507
4	$\text{H}_2\text{L}^-(\text{H}_2\text{O})_4$	-558.723888	-293385.8854
0	HL	-252.517526	-662984.7009
1	$\text{H}_3\text{L}(\text{H}_2\text{O})$	-328.959459	-431841.4884
2	$\text{H}_3\text{L}(\text{H}_2\text{O})_2$	-405.403531	-354795.6228
3	$\text{H}_3\text{L}(\text{H}_2\text{O})_3$	-481.843749	-316270.1604
4	$\text{H}_3\text{L}(\text{H}_2\text{O})_4$	-558.291915	-293159.0564
0	H_3O^+	-76.862	-201801.1616
0	H_2O	-76.434	-200677.4477
0	OH^-	-75.952	-199411.9569

Table 2. The value of $\text{p}K_a$ for the deprotonation of boric acid obtained using the Tomasi's method at the B3LYP/6-31+G (d) level of theory, at 298.15 K

Species	Selected equations	$\text{p}K_a$ (calculated)	$\text{p}K_a$ (experimental)
Boric acid	$\text{H}_3\text{L}(\text{H}_2\text{O})_4 \rightleftharpoons \text{H}_2\text{L}^-(\text{H}_2\text{O})_3 + \text{H}_3\text{O}^+$	9.36436	9.237

Table 3. The calculated structural properties for the neutral and cation species of boric acid using Tomasi's method at the B3LYP/6-31+G (d) level of theory at 298.15 K

Species	Calculated magnitudes	
Boric acid	$H_2L(H_2O)_4$	$HL^-(H_2O)_3$
K_c	2313964803	-
K_a	2.2956E+23	-
qB_1	1.311027	1.174291
qO_2	-1.018240	-0.947905
qO_3	-1.132647	-0.912080
qO_4	-0.907847	-1.104481
qH_7	0.650111	0.605440
qO_9	-1.093296	-
qH_{11}	0.549604	-
qH_{13}	0.562297	0.557999
qH_{16}	0.637048	0.539621
qH_{19}	-	0.599065
qH_{20}	-	0.558377
dO_2B_1	1.382044	1.357259
dO_3B_1	1.384941	1.366905
dO_4B_1	1.355540	1.412990
dH_5O_2	0.971181	0.979039
dH_6O_3	0.991077	-
dH_7O_3	-	0.977600
dH_7O_4	0.967252	-
dO_8O_4	2.788456	-
dO_9O_3	3.841056	-
$dH_{11}O_9$	0.973583	-
$dH_{13}O_{12}$	-	0.968626
dH_6O_9	2.124582	-
$dH_{19}O_2$	-	2.098563
$AO_3B_1O_2$	114.708261	119.682443
$AO_4B_1O_2$	118.806632	118.757031
$AH_5O_2B_1$	111.895234	112.464635
$AH_6O_3B_1$	121.477128	-
$AH_7O_3B_1$	-	111.480812
$AO_9O_3B_1$	144.288763	-
$AH_{10}O_9O_3$	115.173761	-
$AH_{11}O_9O_3$	139.273690	-
$AH_{13}O_{12}O_3$	-	147.569337
$AH_{16}O_{15}O_3$	-	130.127543

Table 3. Continued

$DO_4B_1O_2O_3$	-179.779914	-178.510094
$DH_5O_2B_1O_4$	3.701327	-177.469800
$DH_6O_3B_1O_4$	14.750747	-
$DH_7O_3B_1O_4$	177.137823	-171.677912
$DH_{10}O_9O_3B_1$	-18.098064	-
$DH_{13}O_{12}O_3B_1$	-	147.569337
$DH_{16}O_{15}O_3B_1$	-	130.127543

K_c : Equilibrium constant of equation, K_a : Acidic dissociation constants of species in water, D: Dihedral angle between the indicated atoms (Å), : Total atomic charge (Mulliken) (au), d: Bond lengths between the indicated atoms, A: Angles (°)

Study on H-bonding between selected species of boric acid and water

The structural properties of a species, solved in water, can help us to understand the interaction between this species and water (H-bonding). One of the most important of these structural properties is the bond length between the indicated atoms from solute and solvent (water) molecules (in Å). These data, for neutral and cation species of boric acid, are listed in Table 3. The power of hydrogen bonds can be classified as strong (bond length is between 1.2 Å and 2.2 Å and the angle is between 175° and 180°), moderate (bond length is between 1.5 Å and 2.2 Å and the angle is between 130° and 180°), and weak (bond length is between 2.2 Å and 3.2 Å and the angle is between 90° and 150°).²³ As can be seen in Table 3, for $H_2L(H_2O)_4$, the bond length between atom H_6 , from boric acid, and O_9 , from water, is 2.124582 ($dH_6O_9=2.124582$). In addition, for $H_2L^-(H_2O)_3$, the bond length between atom O_2 , from boric acid, and H_{19} , from water, is 2.098563 ($dH_{19}O_2=2.098563$). These data show that for boric acid the power of H-bonding between $H_2L(H_2O)_4$ and water and also between $H_2L^-(H_2O)_3$ and water are classified as moderate. It must be noted that IHBs data can be used in the design of benefit and help us to predict nano drugs.⁷

CONCLUSION

In this research work, we showed the feasibility of a theoretical method, DFT and *ab initio*, to calculate the ionization constants of boric acid at $T=298.15$ K. As a result, we selected various acid-base reactions that include the solvation of the hydrogen, hydroxyl ions, and other anions or neutral molecules in protic solvents such as water, which possess a high hydrogen-bond-donor capability. The calculations performed at the B3LYP/6-31+G (d) levels of theory using Tomasi's method allowed us to prove that neutral molecules and anions form IHBs with some molecules of water. In addition, the comparison between experimentally determined and theoretically calculated pK_a , for boric acid, shows that there is good agreement between them at 298.15 K.

Conflicts of interest: No conflict of interest was declared by the authors. The authors alone are responsible for the content and writing of the paper.

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Cleaning Method Validation for Estimation of Dipyrindamole Residue on the Surface of Drug Product Manufacturing Equipment Using Swab Sampling and by High Performance Liquid Chromatographic Technique

İlaç Ürün İmalat Ekipmanının Yüzeyindeki Dipiridamol Kalıntısının Sürüntü Örnekleme ve Yüksek Performanslı Sıvı Kromatografisi Tekniği Kullanılarak Tahmin Edilmesi için Temizleme Metodu Validasyonu

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ABSTRACT

Objectives: Cleaning validation is the procedure used to ensure that the cleaning process has eliminated the residues of drug substance from on the equipment surface after manufacture. A simple, sensitive, robust, and accurate high performance liquid chromatographic method was developed for the quantitative estimation of dipyrindamole in swab samples obtained from the equipment surface after the manufacture of dipyrindamole modified release capsules.

Materials and Methods: The method was developed by using a Hypersil BDS C18 (150×4.6 mm, 5 µm) column with mobile phase containing a mixture of buffer (potassium dihydrogen phosphate buffer, pH 7.0±0.05) and methanol in the ratio of 30:70 v/v. Flow rate was 1.5 mL/min, column temperature was 45°C, and injection volume was 5 µL.

Results: The method was validated and a specificity study was conducted to prove that there was no interference from blank and swab blank at the retention time of dipyrindamole. The limit of detection and limit of quantification (LOQ) were established by using a series of linearity solutions and were found to be 0.041 µg/mL and 0.124 µg/mL, respectively. The method precision at the LOQ level was 8.6% relative standard deviation (RSD), method precision was 0.2% RSD, and ruggedness was 0.3% RSD. The method was accurate from the concentration of 0.13 µg/mL to 21.80 µg/mL and the recovery results met the acceptance criteria. The linearity of the method was found from 0.12 µg/mL to 20.14 µg/mL and the r^2 value was 0.997. The robustness for the flow rate, wavelength, column temperature, buffer pH, and mobile phase ratio variations was tested, and all the system suitability parameters were met.

Conclusion: The method validation was performed as per the regulatory requirements and guidelines. The validation parameters met the acceptance criteria and the proposed method can be applied for the intended routine swab analysis.

Key words: Dipyrindamole, swab, method development, validation, cleaning

ÖZ

Amaç: Temizlik validasyonu, temizleme sürecinin üretimden sonra ilaç maddesinin ekipman yüzeyinden kalıntılarını uzaklaştırdığından emin olmak için kullanılan bir prosedürdür. Dipiridamolün modifiye salım yapan kapsüllerinin üretiminden sonra ekipman yüzeyinden alınan sürüntü örneklerde dipiridamolün kantitatif tahmini için basit, hassas, kararlı ve kesin bir yüksek performanslı sıvı kromatografisi yöntemi geliştirilmiştir.

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Gereç ve Yöntemler: Yöntem, hipersil BDS C18 (150×4,6 mm, 5 µm) kolonu kullanılarak hareketli faz içeren tampon (potasyum dihidrojen fosfat tamponu, pH 7,0±0,05) ve metanolün 30:70 (h/h) oranında kullanılmasıyla geliştirilmiştir. Akış hızı 1,5 mL/min, kolon sıcaklığı 45°C ve enjeksiyon hacmi 5 µL olarak belirlenmiştir.

Bulgular: Yöntem valide edilmiştir ve özgünlük çalışması, kör ve sürüntü körünün dipiridamol alkonma zamanı ile girişim göstermediğini kanıtlamak için gerçekleştirilmiştir. Deteksiyon sınırı ve kantifikasyon sınırı bir seri doğrusallık çözeltisi kullanılarak belirlenmiştir ve sırasıyla 0,041 µg/mL ve 0,124 µg/mL olarak bulunmuştur. Yöntemin kesinliği kantifikasyon sınırında %8,6 relatif standart sapma (RSD), yöntem kesinliği %0,2 RSD ve dayanıklılığı %0,3 RSD bulunmuştur. Yöntem 0,13 µg/mL ile 21,80 µg/mL konsantrasyonu aralığında kesin bulunmuştur ve geri kazanım sonuçları kabul edilebilirlik kriterlerini karşılamaktadır. Yöntemin doğrusallığı 0,12 µg/mL ile 20,14 µg/mL arasında bulunmuştur ve bulunan r^2 değeri 0,997'dir. Akış hızı, dalga boyu, kolon sıcaklığı, tampon pH'sı ve hareketli faz oranındaki değişimler için dayanıklılık çalışması yapılmıştır ve tüm sistem uygunluk parametreleri karşılanmıştır.

Sonuç: Yöntem validasyonu, düzenleyici gereklilikler ve kurallara göre gerçekleştirilmiştir. Validasyon parametreleri kabul kriterlerini karşılamıştır ve önerilen bu yöntem amaçlanan sürüntü rutin analizi için kullanılabilir.

Anahtar kelimeler: Dipiridamol, sürüntü, yöntem geliştirme, validasyon, temizleme

INTRODUCTION

Cleaning validation should be performed to confirm the efficiency of any cleaning procedure when pharmaceutical products are in contact with equipment. In the pharmaceutical manufacturing industry it is well known that the manufacturing equipment and manufacturing area should be cleaned after every manufacturing process of drug products and this process is strictly endorsed by the regulatory authorities. Cleaning validation is a vital analytical responsibility of the quality management system in the pharmaceutical industry and this process ensures that the cleaning procedure effectively eliminates the residue from the manufacturing equipment and manufacturing area below a predetermined tolerable limit. The cleaning process ensures the product quality of different products, is a helpful tool to avoid cross-contamination, and is a requirement of European Union guidelines for Good Manufacturing Practice and the United States Food and Drug Administration (USFDA). Cleaning validation involves two different activities: one is development and validation of the cleaning process used to remove the drug from the manufacturing equipment surfaces and the other is development and validation of the methods used to measure the residues on the surfaces of the manufacturing equipment. Evaluation of the sensitivity and specificity of the analytical method used to detect residue is critical. The residue analytical method should be able to detect and quantify the drug substance at a very low level from the manufacturing equipment. The residue analytical procedure should be tested in the mixture of sampling method used to show that residue can be recovered from the equipment surface with the specified levels in the accuracy study before concluding the sampling procedure. In general, two types of sampling procedure were found acceptable by the regulatory authorities and frequently practicing pharmaceutical industries. The popular sampling method is the direct method of sampling on the surface of the manufacturing equipment and another method is to use rinse solutions from the manufacturing equipment. The positive aspect of direct sampling of the equipment surface is that the areas hardest to clean and that are reasonably available can be projected, important for finding a level of residue per given surface area. In the case of rinse samples, the two benefits of using rinse samples are that a larger surface area may be sampled and unreachable systems or ones that cannot be routinely disassembled can be sampled

and estimated. The disadvantage of rinse samples is that the residue may not be soluble or may be physically occluded in the manufacturing equipment surface area.

With direct surface sampling there is a possibility of interference from the swab sticks as they have some glue content and before finalizing the sampling procedure the specificity also should be evaluated. The selection of the extraction solvent is a critical step during the development of the cleaning method, the drug substance should be soluble and recoverable across the accuracy swab sample level, and the results should meet the acceptance criteria.

The drug product manufacturer's rationale for the residue limits established should be logical based on the manufacturer's scientific knowledge of the materials involved. It is important to describe the analytical method sensitivity of the residue method in order to fix sensible acceptable limits. According to the USFDA, the limit should be based on logical criteria, involving the risk associated with residues of determined products. The calculation of an acceptable limit of residues and a maximum allowable carryover for an active pharmaceutical ingredient in the production equipment should be based on therapeutic doses, toxicity, and a general limit (10 µg). Several mathematical formulas have been proposed to establish the acceptable residual limit.¹⁻¹⁷

The drug substance dipiridamole (Figure 1): chemical name: 2,2',2",2""-[4,8-di (piperidin-1-yl) pyrimido [5,4-d] pyrimidine-2,6-diyil] dinitrilo] tetraethanol, CAS Registry number: 58-32-2, molecular formula: C₂₄H₄₀N₈O₄, molecular mass: 504.6, appearance: bright yellow, crystalline powder, solubility:

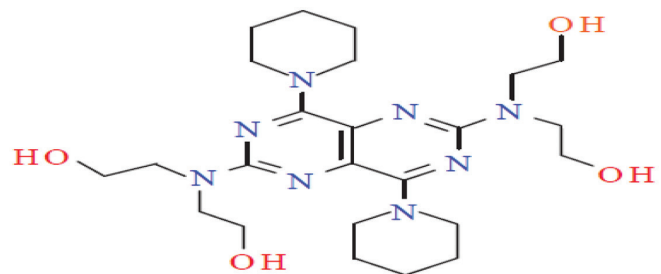


Figure 1. Structure of dipiridamole

practically insoluble in water, slightly soluble in acetone, soluble in anhydrous ethanol, and it dissolves in dilute mineral acids.

The aim of the present study was to develop a simple and fast analytical method for the estimation of the dipyrnidamole content in swab samples after the manufacture of dipyrnidamole modified release capsules on the surface of the manufacturing equipment and to meet the regulatory requirements. Hence, the developed method was subjected to analytical validation with respect to specificity, linearity, precision, accuracy, robustness, and ruggedness. The specificity studies were performed on the diluent, swab, and placebo during the analytical method validation as per International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human (ICH) guidelines.¹⁸ The developed and validated method can be used for the routine swab samples analysis.

MATERIALS AND METHODS

Chemicals and reagents

High performance liquid chromatographic (HPLC)/analytical grade water, potassium dihydrogen phosphate, methanol, and sodium hydroxide were used. The dipyrnidamole drug substance and dipyrnidamole working standard were supplied by Bluefish Pharmaceuticals Private Limited (India).

Equipment

The analytical method was developed and validated by using the HPLC from Agilent 1200 with a VWD/PDA detector. The output signal was monitored and processed using specific software. An analytical balance from Mettler Toledo, a Sartorius pH meter, and a refrigerator were used.

Chromatographic conditions

The proposed method was developed using a Hypersil BDS C18 (150×4.6 mm) 5 µm column with mobile phase containing a mixture of mobile phase (buffer: potassium dihydrogen phosphate buffer, pH 7.0±0.05) and methanol solution in the ratio of 30:70 v/v. The flow rate was 1.5 mL/min with a column temperature of 45°C, detection wavelength of 295 nm, and sample injection volume of 5 µL.

Preparation of solutions

Diluent solution

Methanol was used as diluent.

Preparation of dipyrnidamole standard solution

Weigh and transfer about 50 mg of dipyrnidamole working standard into a 50 mL volumetric flask. Add about 35 mL of diluent and sonicate for 2 to 3 min until the material is completely dissolved. Pipette out 1 mL of the above solution into a 100 mL volumetric flask, make it up to volume with diluent, and mix well. Pipette out 4 mL of the above solution into a 10 mL volumetric flask, make it up to volume with diluent, and mix well.

Preparation of test tubes and swabs

Take the clean and dry test tubes. Rinse the required number of swabs and test tubes with about 10 mL of swabbing solvent two

times. Squeeze out the swab against the side of the test tubes and discard the swabbing solvent.

Preparation of blank solution

Transfer 10 mL of swabbing solvent to the above cleaned test tube. Place a cleaned swab into the test tube and sonicate for 10 min. Squeeze the swab and take it out and mix well.

Preparation of test solution

Transfer 10 mL of swabbing solvent to the above cleaned test tube. Place a cleaned swab into the test tube to wet the swab with swabbing solvent. Squeeze the swab by pressing it against wall of the test tube. Do the swabbing at the prescribed area of equipment. After swabbing, place the swab in the above test tube containing swabbing solvent and sonicate for 10 min.

Squeeze the swab by pressing it against the wall of the test tube and take it out and filter it through a membrane filter and inject.

System suitability criteria

The present relative standard deviation of the dipyrnidamole peak area for six replicate injections should not be more than 5.0.

The tailing factor for dipyrnidamole peak in standard solution should not be more than 2.0.

The present relative standard deviation of dipyrnidamole peak retention time for six replicate injections should not be more than 1.0.

The % recovery for dipyrnidamole check standard solution should not be less than 95.0% and 105.0%.

RESULTS AND DISCUSSION

Method development

During the method development stage, the standard solution was prepared with a known concentration, blank solution was scanned in a ultraviolet spectrophotometer, and the diluent blank (Figure 2) and dipyrnidamole working standard (Figure 3) spectrums collected to check the wavelength maxima.

The dipyrnidamole peak retention time was about 2.8 min in the chromatogram and the relative standard deviation for the six replicate injections was 0.2% and this proved that the method is reproducible. The accuracy study was conducted by spiking a known concentration of dipyrnidamole solution in an SS plate of 0.4 µg/mL, 4 µg/mL, and 6 µg/mL and the % recovery for all the levels was calculated and the results were found in the range of 99% to 100% and this proved that the method is accurate. The linearity study was conducted by spiking a known concentration of dipyrnidamole solution of about 0.4 µg/mL, 1 µg/mL, 2 µg/mL, 4 µg/mL, 5 µg/mL, and 6 µg/mL, the square of correlation coefficient was calculated and found to be 0.999, and this proved that the method is linear from 0.4 µg/mL to 6 µg/mL. Based on the above-mentioned satisfactory results, the below-mentioned chromatographic conditions were finalized for the quantitative estimation of dipyrnidamole in the swab samples from drug product manufacturing of the dipyrnidamole modified release capsules equipment surface after manufacturing. The

chromatographic conditions are a Hypersil BDS C18 (150×4.6 mm) 5 μ m column with mobile phase containing a mixture of mobile phase (buffer: potassium dihydrogen phosphate buffer, pH 7.0±0.05) and methanol in the ratio of 30:70 v/v. The flow rate was 1.5 mL/min with column temperature of 45°C and detection wavelength of 295 nm. The injection volume was 5 μ L with isocratic flow. Hence, this method can be introduced for routine swab analysis.

Method validation

The proposed analytical method for the quantitative estimation of dipyrnidamole in swab samples from drug product manufacturing of the dipyrnidamole modified release capsules equipment surface area after manufacturing was validated as per the ICH.¹⁸ The validation characteristics specificity, precision, accuracy, linearity, range, ruggedness, and robustness were determined.

System suitability

To check the system suitability criteria, solutions were prepared and injected as per the test method of analysis. All the system suitability parameters were found well within the acceptance criteria. A summary of the system suitability is given in Table 1.

Table 1. System suitability criteria and results

Parameter	Acceptance criteria	Result
The present relative standard deviation of dipyrnidamole peak area for six replicate injections	Not more than 5.0	2.0%
The tailing factor for dipyrnidamole peak in standard solution	Not more than 2.0	1.2
The present relative standard deviation of dipyrnidamole peak retention time for six replicate injections	Not more than 1.0	0.2%
The % recovery for dipyrnidamole check standard solution	Not less than 95.0 and not more than 105.0	96.7%

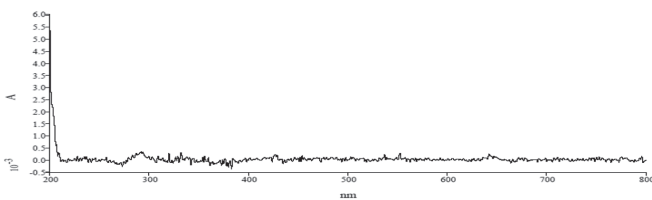


Figure 2. Spectrum of diluent blank

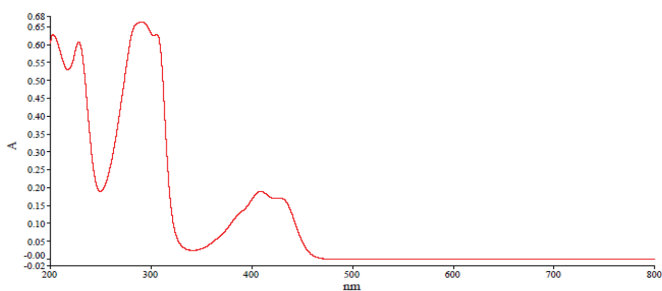


Figure 3. Spectrum of standard

Specificity

To study the specificity, required solutions like diluent as blank, swab blank, and standard solution were prepared and injected as per the test method. It was observed that there was no peak interference at the retention time of dipyrnidamole from blank and swab blank solutions in the chromatogram. Specimen chromatograms of diluent as blank are shown in Figure 4, swab blank in Figure 5, and standard in Figure 6, and overlaid chromatograms of diluent as blank, swab blank, and standard in Figure 7.

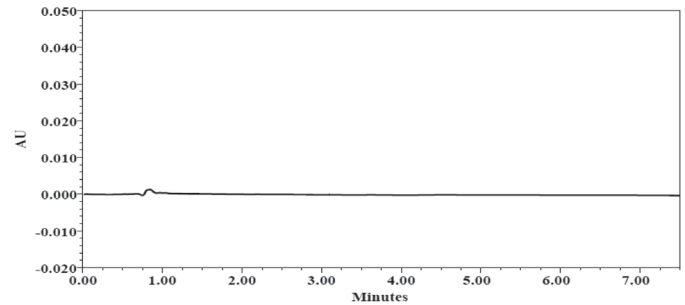


Figure 4. Specimen diluent blank chromatograms

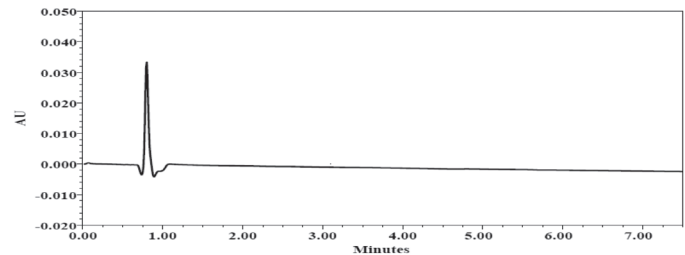


Figure 5. Specimen swab blank chromatograms

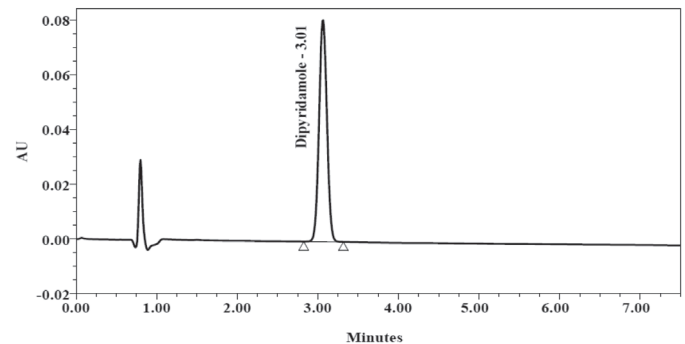


Figure 6. Specimen standard chromatograms

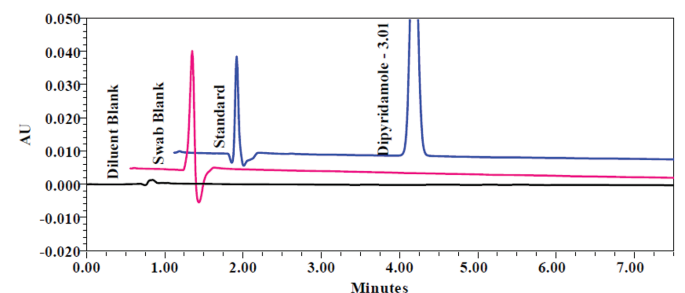


Figure 7. Specimen overlaid chromatograms

Estimation of limit of detection (LOD) and limit of quantification (LOQ)

To evaluate the concentration limits as LOD and LOQ, a series of linearity solutions were prepared ranging in concentration from about 0.1 µg/mL to 2.0 µg/mL and the square of correlation coefficient, slope of the curve, and y-intercept were determined. The LOQ was calculated based on the standard deviation of the response and the slope as mentioned in the formula below. A summary of the results of the LOD and LOQ estimation study is given in Table 2 and Figure 8.

$$LOQ = \frac{10 \times \sigma}{S}$$

where

σ = the standard deviation of the response

S = the slope of the calibration curve

Method precision at LOQ level

The method precision at LOQ concentration was determined by preparing six replicate test preparations (n=6) of dipyrindamole stock solution and samples were analyzed as per the test method. The % relative standard deviation (RSD) for response of dipyrindamole six replicate injections was calculated and found within the acceptance criteria. A summary of the method precision for the LOQ level study results is given in Figure 9.

Method precision (repeatability)

Precision was determined by preparing six replicate test preparations (n=6) of dipyrindamole stock solution spiked onto an SS plate (4x4 inch) and samples were analyzed as per the test method. The % recovery for replicate injections and % RSD for response of six replicate injections of dipyrindamole were calculated and found within the acceptance criteria. A summary of the method precision study results is given in Figure 10.

Table 2. Estimation of LOD and LOQ

Description	Dipyrindamole
Square of correlation coefficient (r ²)	0.999
Slope	11073.25
Y-intercept	50.4388
Limit of detection (µg/mL)	0.041
Limit of quantification (µg/mL)	0.124

LOD: Limit of detection, LOQ: Limit of quantification

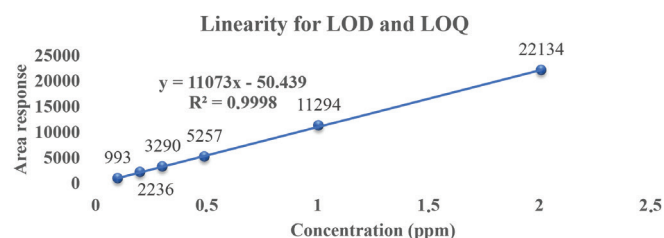


Figure 8. Linearity for LOD and LOQ

LOD: Limit of detection, LOQ: Limit of quantification

Accuracy

In the accuracy study, a series of sample solutions were prepared in triplicate six preparations for lower level (LOQ) and higher level (500%) by spiking the dipyrindamole drug substance stock onto an SS plate (4x4 inch) at LOQ 50%, 100%, 200%, 300%, and 500% and analyzed as per the test method. The spiked concentrations of dipyrindamole were 0.12 µg/mL, 2.01 µg/mL, 4.03 µg/mL, 8.05 µg/mL, 12.08 µg/mL, and 21.14 µg/mL. Individual % recovery, mean % recovery, % RSD, and squared correlation coefficient for linearity of the test method were calculated and the results were found within the predefined acceptance criteria. A summary of the accuracy study results is given in Table 3 and Figure 11.

Linearity

The linearity was studied by analyzing the standard solutions. A series of solutions of dipyrindamole standard solutions were prepared in the range of LOQ to about 500% and injected into the HPLC system. Linearity of detector response was established by plotting a graph of concentration vs. response of dipyrindamole. The detector response was found to be linear from about LOQ to 500% and injected into the HPLC system and analyzed as per the test method. The concentrations of

Table 3. Accuracy data of dipyrindamole

Spike level	% Mean recovery of dipyrindamole	Average amount added (µg/mL)	Average amount found (µg/mL)
Level-1	101.5	0.12	0.13
Level-2	97.5	2.01	1.97
Level-3	98.7	4.03	3.98
Level-4	99.7	8.05	8.03
Level-5	105.1	12.08	12.70
Level-6	103.1	21.14	21.80

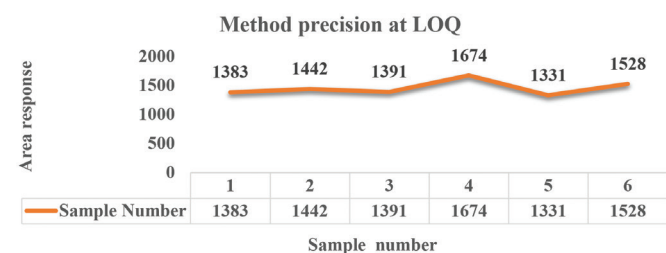


Figure 9. Method precision at LOQ level

LOQ: Limit of quantification

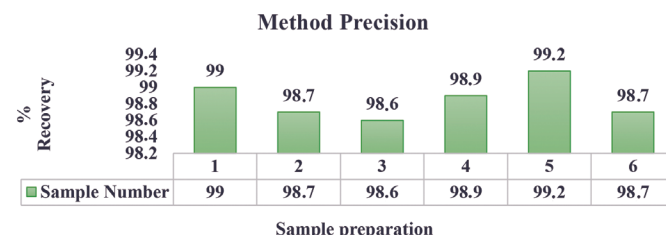


Figure 10. Method precision

dipyridamole were 0.1208 $\mu\text{g/mL}$, 2.0137 $\mu\text{g/mL}$, 4.0274 $\mu\text{g/mL}$, 8.0548 $\mu\text{g/mL}$, 12.0821 $\mu\text{g/mL}$, and 20.1369 $\mu\text{g/mL}$.

The square of correlation coefficient, slope, and % y-intercept at 100% level, and intercept and residual sum of squares were calculated and the results met the acceptance criteria. A summary of the linearity study results is given in Table 4 and Figure 12.

Ruggedness

The intermediate precision was determined by preparing six replicate test preparations (n=6) of dipyridamole stock solution spiked onto an SS plate (4x4 inch) and samples were analyzed as per the test method using a different HPLC system and a different column of the same make by a different analyst on a different day. The % recovery for replicate injections and % RSD for response of six replicate dipyridamole injections were

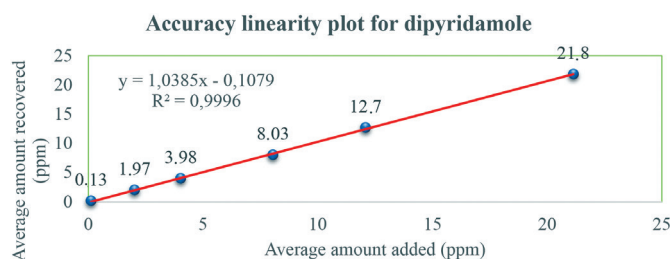


Figure 11. Accuracy linearity plot for dipyridamole

Table 4. Linearity data of dipyridamole

Linearity level	% Linearity	Concentration ($\mu\text{g/mL}$)	Area response
Level-1	LOQ	0.1208	1514
Level-2	50%	2.0137	24558
Level-3	100%	4.0274	49035
Level-4	200%	8.0548	97506
Level-5	300%	12.0821	152086
Level-6	500%	20.1369	231128
Square of correlation coefficient (r^2)			0.997
Slope			11622.7
Y-intercept			2686.68
Residual sum of squares			122556978.290215

LOQ: Limit of quantification

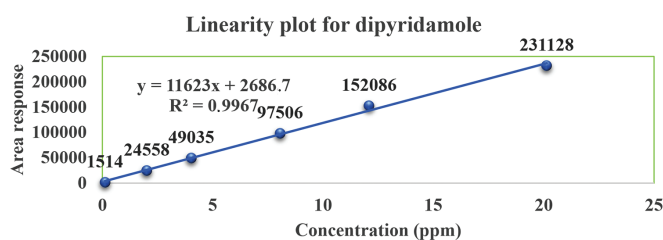


Figure 12. Linearity plot for dipyridamole

calculated and met the acceptance criteria. A summary of the ruggedness study results is given in Table 5 and Figure 13.

Solution stability and mobile phase stability

The solution stability of dipyridamole was determined by keeping swab sample solution and standard solutions in tightly capped volumetric flasks at room temperature for 1 day and 2 days and measuring against freshly prepared standard solution. The standard solution and swab sample solutions was found stable for 2 days at room temperature.

The stability of the mobile phase was also determined by freshly prepared solutions of dipyridamole at 1 day and 2 days. The mobile phase was found stable for 2 days at room temperature.

Robustness

Robustness of the proposed method was performed by keeping the chromatographic conditions constant with the following deliberate variations:

- Change in flow rate
- Change in wavelength
- Change in mobile phase buffer pH
- Change in HPLC column temperature
- Change in mobile phase composition

The standard solution was injected in replicate for each above mentioned change. The system suitability parameters were recorded for dipyridamole peak and the system suitability

Table 5. Ruggedness data

Sample no.	Method precision % recovery	Intermediate precision % recovery
1	99.0	102.6
2	98.7	103.0
3	98.6	102.5
4	98.9	102.3
5	99.2	102.8
6	98.7	102.3
Mean	98.9	102.6
% RSD	0.2	0.3
Overall % RSD	0.3	

RSD: Relative standard deviation

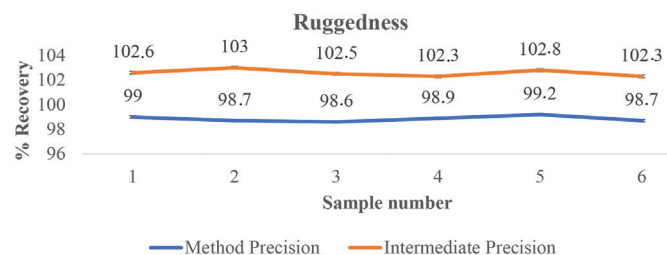


Figure 13. Ruggedness

Table 6. Robustness data

Parameter variation	The present relative standard deviation of dipyrindamole peak area for six replicate injections should not be more than 5.0	The tailing factor for dipyrindamole peak in standard solution should not be more than 2.0	The present relative standard deviation of dipyrindamole peak retention time for six replicate injections should not be more than 1.0	The % recovery for dipyrindamole check standard solution should not be less than 95.0% and 105.0%
Flow 1.30 mL/min	0.3	1.2	0.1	98.0
Flow 1.70 mL/min	0.2	1.2	0.2	98.0
Wavelength 293 nm	0.2	1.2	0.2	97.4
Wavelength 297 nm	0.3	1.2	0.2	97.1
Column temp 40°C	0.2	1.2	0.0	99.5
Column temp 50°C	0.2	1.2	0.0	99.4
Buffer pH 6.8	0.3	1.2	0.0	97.1
Buffer pH 7.2	0.7	1.2	0.3	96.5
Mobile phase ratio 35:65% v/v	0.2	1.2	0.0	99.9
Mobile phase ratio 20:80% v/v	0.2	1.2	0.0	99.7

found well within the acceptance criteria. A summary of the robustness study results is given in Table 6.

The authors declare that no experiments were conducted using human subjects and no ethics committee approval is required for this publication.

APPLICATION OF THE PROPOSED METHOD

The developed analytical method can be applied for the analysis of swab samples from the dipyrindamole drug product manufacturing unit. All the analytical validation parameters met the predefined acceptance criteria and the method was proven to be suitable for analysis of swab samples from drug product manufacturing of the dipyrindamole modified release capsules equipment surface area after manufacturing.

CONCLUSIONS

The developed method was validated as per the ICH guidelines and can be used for the quantitative estimation of dipyrindamole in swab samples from drug product manufacturing of the dipyrindamole modified release capsules equipment surface area after manufacturing. The method was precise, accurate, linear, robust, rugged, and specific and there was no interference found during the specificity study at the retention time of dipyrindamole peak. The validated method can be applied for swab samples from drug product manufacturing of the dipyrindamole modified release capsules equipment surface area after manufacturing.

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Inhibitory Effect of Roselle Aqueous Extracts- HPMC 6000 Gel on the Growth of *Staphylococcus Aureus* ATCC 25923

Hibiscus Sabdariffa L Sulu Ekstrelerini İçeren HPMC 6000 Jel Formülasyonunun *Staphylococcus Aureus* ATCC 25923 Büyümesi Üzerine İnhibitör Etkisi

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ABSTRACT

Objectives: Roselle (*Hibiscus sabdariffa* L.) is a medicinal plant commonly used as a beverage and herbal medicine. Complex compounds in the aqueous extracts have provided good antibacterial activity by which the growth of gram-negative and -positive bacteria is inhibited. The aims of this research were to formulate hydroxypropyl methylcellulose (HPMC) 6000 gel containing the extract and investigate the inhibitory activity of the extract and its gel formula against *Staphylococcus aureus* ATCC 25923.

Materials and Methods: Thin layer chromatography (TLC) on silica gel GF254 was used for analyzing flavonoids and polyphenols using butanol: acetic acid: water (4:1:5) and chloroforms: ethyl acetate: formic acid (0.5:9:0.5) as eluent, respectively. A serial dilution of aqueous extract powder in citrate buffer was made to obtain 0.50, 0.25, 0.10, 0.05, and 0.02 mg/mL solution. The roselle aqueous extract (3%) was formulated as a component of gel containing HPMC 6000 in various concentrations (2%, 3%, and 4%). A diffusion agar method on two layers of nutrient agar media was applied using *Staphylococcus aureus* ATCC 25923 and gentamicin 25 ppm as bacterial test and standard, respectively. After incubation for 24 h at 37°C, the inhibitory effect was denoted by a clear zone around the hole and the inhibitory activity was measured as minimum inhibitory concentration (MIC).

Results: The aqueous extract of *Hibiscus sabdariffa* L. contained flavonoid and polyphenol compounds based on the TLC chromatogram profile. It was found that the gel formula containing 3% HPMC 6000 and 3% aqueous extract gave a good physical characteristic and the lowest MIC (6.0 mg/mL), equivalent to 7.58 ppm of gentamicin standard at 12.0 mg/mL concentration.

Conclusion: The HPMC 6000 at 3% (w/w) concentration in roselle aqueous extract gel preparation gave good physical characteristics. The gel preparation exhibited inhibitory activity against *Staphylococcus aureus* ATCC 25923 shown by MIC 6.0 mg/mL. Formula 2 is recommended and should be further investigated for implementation in topical preparations.

Key words: Inhibitory effect, *Hibiscus sabdariffa*, HPMC 6000, *Staphylococcus aureus*

ÖZ

Amaç: *Hibiscus sabdariffa* L. yaygın olarak içecek ve bitkisel ilaç olarak kullanılan tıbbi bir bitkidir. Sulu ekstrelerindeki kompleks bileşikler, gram negatif ve pozitif bakterilerin büyümesini inhibe ederek antibakteriyel aktivite göstermiştir. Bu araştırmanın amacı, ekstre içeren hidroksipropil metilselüloz (HPMC) 6000 jelini formüle etmek, ekstrelin inhibe edici etkisini ve jel formülünün *Staphylococcus aureus* ATCC 25923'e karşı gösterdiği inhibitör etkiyi araştırmaktır.

Gereç ve Yöntemler: Elüent olarak butanol: asetik asit: su (4:1:5) ve kloroform:etil asetat: formik asit (0,5:9:0,5) kullanılarak sırasıyla flavonoidleri ve polifenollerini analiz etmek amacıyla silika jel GF254 üzerinde ince tabaka kromatografisi gerçekleştirildi. 0,50, 0,25, 0,10, 0,05 ve 0,02 mg/mL

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konsantrasyonda çözelti elde etmek için sitrat tamponu içinde sulu ekstrenin seri seyreltilmesi yapıldı. *Hibiscus sabdariffa*'nın sulu ekstresinin (%3), çeşitli konsantrasyonlarda (%2, %3 ve %4) HPMC 6000 içeren jelleri formüle edildi. Bakteriyel test ve standart olarak sırasıyla *Staphylococcus aureus* ATCC 25923 ve gentamisin 25 ppm kullanılarak agar ortamına difüzyon agar yöntemi uygulandı. 37°C'de 24 saat inkübasyondan sonra, inhibitör aktivite, minimum inhibitör konsantrasyon (MIC) olarak ölçüldü.

Bulgular: *Hibiscus sabdariffa* L.'nin sulu ekstresinin, flavonoid ve polifenol bileşikleri içerdiği ince tabaka kromatografisi-kromatogramı ile belirlendi. %3 HPMC 6000 ve %3 sulu ekstre içeren jel formülünün, 12,0 mg/mL konsantrasyonda 7,58 ppm gentamisin standardına eşdeğer olacak şekilde en düşük MIC değerine (6,0 mg/mL) sahip olduğu ve fiziksel özelliklerinin iyi olduğu bulunmuştur.

Sonuç: *Hibiscus sabdariffa* sulu ekstresinin %3 (a/a) konsantrasyonda HPMC 6000 içeren jel formülasyonunda iyi fiziksel özellikler gösterdiği tespit edildi. Jel formülü, *Staphylococcus aureus* ATCC 25923'e karşı MIC 6,0 mg/mL değeri ile inhibitör aktivite gösterdi. En iyi formül olarak belirlenen formül 2'nin topikal preparatlarda kullanılabilmesi için ileri araştırmalara ihtiyaç vardır.

Anahtar kelimeler: Inhibitör etki, *Hibiscus sabdariffa*, HPMC 6000, *Staphylococcus aureus*

INTRODUCTION

Roselle (*Hibiscus sabdariffa* L.) is a medicinal plant commonly produced as a beverage and herbal medicine. It has multiple activities, one of which is antibacterial activity.¹ The aqueous extracts of roselle calyces contain saponins, alkaloids, tannins, polyphenols, flavonoids, and their glycosides. The saponins and flavonoids make up the largest content.^{2,3} These compounds indicate synergistic effects. Complex compounds in the extracts have provided good antibacterial activity.⁴ Proto-catechuic acid is a polyphenolic compound found in roselle calyces. It inhibited the bacterial growth of methicillin resistant *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* at 5 mg/mL.⁵⁻⁷

In terms of antimicrobial activity, roselle aqueous extract was used at a concentration above its minimum inhibitory concentration (MIC) 3%. The low pH values of roselle aqueous extract (2.42±0.01) led to hydroxypropyl methylcellulose (HPMC) 6000 being chosen as a gelling agent at concentrations of 2%, 3%, and 4%, because this matrix is stable and indicates good swelling ability in acidic conditions. An effort to discover a new topical dosage form containing roselle extract as active ingredient against infectious diseases was the main target of the present research.

MATERIALS AND METHODS

Chemicals

The materials were pharmaceutical grade. Dried aqueous extract of roselle was purchased from PT ASIMAS; HPMC 6000, citric acid monohydrate, sodium citrate dihydrate, propylene glycol, sodium benzoate, gentamicin sulfate, and nutrient agar from Oxoid; sodium chloride from Merck; and distilled water from PD Surabaya Air Suling. *Staphylococcus aureus* ATCC 25923 was obtained from the Department of Microbiology, Faculty of Medicine, Airlangga University.

Qualitative analysis of roselle aqueous extracts

Analysis of the extract included an organoleptic examination (shape, odor, and color) and pH, while the chromatogram pattern of flavonoids and polyphenols was analyzed by thin layer chromatography (TLC) on Kiesel Gel GF₂₅₄ plates. The chromatographic profile of the flavonoids was evaluated by shaking 1 g of the extract with *n*-hexane repeatedly until it was colorless and the residue was dissolved in 5 mL of ethanol. Then

the solution was spotted and developed in butanol: acetic acid: water (4:1:5, v/v). The presence of flavonoids was denoted by intensive yellow spots on the plate after contact with ammonia fumes. The polyphenols' chromatogram pattern of the extracts was obtained by mixing 1 g of extract and 10 mL of hot distilled water at room temperature. The solution was spotted on a TLC plate after filtering and developed in chloroforms:ethyl acetate: formic acid (0.5:9:0.5, v/v) and sprayed with FeCl₃ solution for indicating the presence of polyphenols by the appearance of black spots.^{1,2}

Qualitative analysis of HPMC 6000

The qualitative examination of HPMC 6000 including pH value and viscosity was analyzed using a pH-meter and Brookfield viscometer, respectively.^{6,8}

Viscosity was measured according to the Brookfield viscometer manual. The spindle was lowered and centered in the test material (600 mL in beaker) to meet the "meniscus" of the fluid at the center position of the immersion groove. The viscosity measurement was performed by turning of the switch "ON". Time was allowed for the indicated reading to stabilize. The reading was noted and multiplied by the factor appropriate to the viscometer model/spindle/speed combination being used. The available table or the FACTOR FINDER was referred to for calculating viscosity. Readings below 10.0% torque (dial reading) should be avoided.

Determining the MIC of roselle aqueous extracts

The MIC of roselle aqueous extracts was determined by agar diffusion method and molding hole against *Staphylococcus aureus* ATCC 25923. The bacterial test was cultured on nutrient agar medium slants in glass tubes and incubated for 24 h at 37°C. The inoculum suspension was prepared by adding sterile 0.9% NaCl solution to fresh culture, shaking, and measuring the optical density at 580 nm adjusted until 25% transmittance of inoculum was obtained. The extracts weighed 100 mg and were dissolved in citrate buffer until 10 mL. The solution was diluted to 0.50, 0.25, 0.10, 0.05, and 0.02 mg/mL to obtain a concentration higher than the MIC. Two layers of test media were prepared and applied. The agar was perforated with 6 sterile holders. Samples and a positive control (gentamicin 25 ppm) were put into each of the holes, incubated at 37°C for 24 h, and observed. The growth inhibitory zone diameter was measured and the smallest concentration that still inhibited the growth of the test bacterium (MIC) was determined.

Formulation of roselle aqueous extracts gel

Based on the MIC of the roselle extract, the gel formula was examined using the extract at higher concentration than the MIC. Some 7.5 g of the extracts and 250 mg of sodium benzoate were dissolved in warmed citrate buffer (70-80°C) and then poured into HPMC 6000 dispersion with 10 g of propylene glycol. The solution was stirred until gel mass formed and stopped at 35°C. The composition of the gel formulation is shown in Table 1.

Physical examination of the gel preparation

Physical examination of the gel preparation included viscosity, pH, and dispersive analysis. The analysis of the dispersive power was carried out using two calibration slides. Approximately 1 g of gel was put in the middle of the slide and covered with the other slide. Weights were orderly added starting from 5 g on the upper slide. The weight was continuously added until the preparation no longer spread (approximately 5 min) and the diameter was recorded. Afterwards, a curve of the relationship between the dispersion diameter (cm) and the weight (g) was observed. The dispersion ability was determined from the slope of the regression equation of the dispersion diameter and the weight.⁹⁻¹¹ The experiment was replicated three times.

Determining the MIC of the selected formula

Gel solution of 12.0 mg/mL was diluted to obtain solution at 6.0, 3.0, 1.5, 0.8, 0.4, 0.2, 0.1, and 0.05 mg/mL concentrations. The determination of MIC was carried out the same as for the extract. The agar was perforated with 19 sterile holders. Approximately 50 µL of the positive control (gentamicin), negative control (gel base), and sample were put into each hole. The disk was incubated at 37°C for 24 h and the growth inhibitory zone and its diameter (mm) were observed and measured. Gentamicin solution at 100 ppm was made and diluted to obtain solution at 25, 20, 15, 10, and 5 ppm concentrations. A logarithmic test of gentamicin concentration vs. the inhibitory zone diameter (mm) curve was performed and the regression equation obtained was used to calculate the inhibitory activity of the sample solution equally to the gentamicin standard by plotting inhibitory diameter.

Statistical analysis

The significant difference of inhibitory activity among roselle aqueous extract formulas was determined by one-way variance analysis (ANOVA). Furthermore, the significant differences

Materials	Formula					
	Preparation (%)			Base (%)		
	1	2	3	1	2	3
HPMC 6000	2	3	4	2	3	4
Roselle aqueous extracts	3	3	3	-	-	-
Propylene glycol	5	5	5	5	5	5
Sodium benzoate	0.1	0.1	0.1	0.1	0.1	0.1
Citrate buffer (pH 4.505)	89.9	88.9	87.9	92.9	91.9	90.9

HPMC: Hydroxypropyl methylcellulose

were determined by the honestly significant difference test with the reliability value of 0.95 ($\alpha=0.05$). If the value is >0.05 then there is no significant difference between the tested formulas. This study does not require ethics committee approval or patient informed consent.

RESULTS

Screening of the extract contents was carried out according to Marlina et al.¹² and Villani et al.¹³ Based on the profile of the TLC chromatogram, roselle aqueous extracts contained polyphenols and flavonoids as reported in previous research.¹⁴ The pH value of the extract was 2.54 ± 0.004 , close to the literature (2.42 ± 0.01).^{2,3}

Regardless of HPMC qualification, its viscosity was >100 cPss at 2% concentration.^{15,16} The viscosity criterion was accepted if the measured result was not less than 75.0% and not more than 140.0%.

The MIC of the roselle aqueous extracts was 0.1 mg/mL against *Staphylococcus aureus* ATCC 25923 (Table 2, Figures 1 and 2). This value is higher than that in previous research; the MIC of the aqueous extracts of roselle calyces against *Staphylococcus aureus* and *Streptococcus faecalis* was reported as 0.5 mg/mL. Furthermore, *Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella typhi* were inhibited by the MIC value of 1.0 mg/mL.^{7,16} Despite these effects, roselle extracts have a therapeutic effect for gastrointestinal infection, diarrhea, and skin diseases.⁷

The viscosity and pH values of the gel base and its preparation are depicted in Figures 3 and 4. It was found that viscosity of

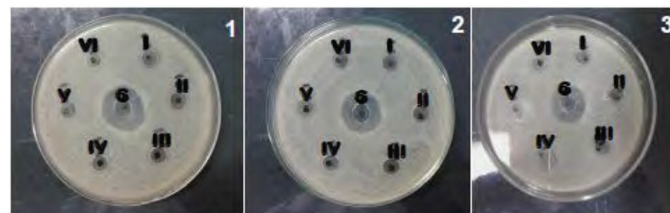


Figure 1. The result of MIC determination of roselle aqueous extracts (1, 2, 3=triple replication; I=0.50 mg/mL; II=0.25 mg/mL; III=0.12 mg/mL; IV=0.10 mg/mL; V=0.05 mg/mL; VI=0.02 mg/mL; G=gentamicin 25 ppm) MIC: Minimum inhibitory concentration

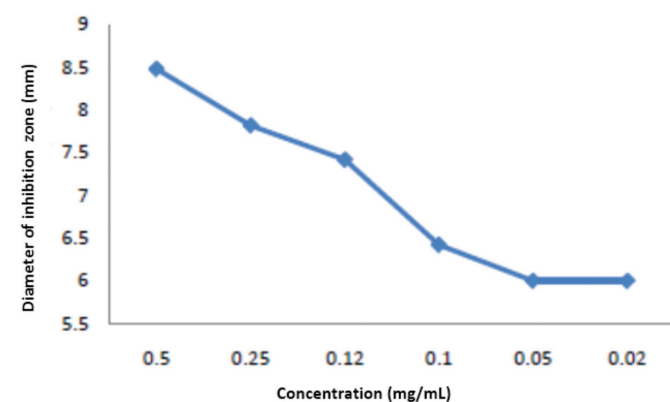


Figure 2. Graph of inhibitory activity of roselle aqueous extracts

the gel preparation containing aqueous roselle extracts was higher than that of the gel base (without the roselle extract). On the other hand, the pH value of the gel preparation was lower than that of the gel base.

The dispersive power analysis of the gel base and gel preparation depicted in Figure 5 showed that both the gel base and preparations of 1st and 2nd formulas reached the maximum dispersion capacity at 10 g and 35 g loading load (the weight of the load placed on the gel base and gel preparation), respectively, while the 3rd formula reached maximum dispersion capacity at 65 g loading load.

The bacterial inhibitory activity of the gel preparations indicated that the greater concentration of HPMC, the lower inhibition

Table 2. The result of MIC determination of roselle aqueous extracts

Rep Conc. (mg/mL)	Inhibitory diameter (mm)			
	1	2	3	Average
0.50	8.80	8.65	8.00	8.48±0.42
0.25	8.20	7.70	7.55	7.82±0.34
0.12	7.70	7.35	7.20	7.42±0.26
0.10	6.65	-	6.60	6.62±0.05
0.05	-	-	-	-
0.02	-	-	-	-

Diameter of reservoir: 6.00 mm, MIC: Minimum inhibitory concentration

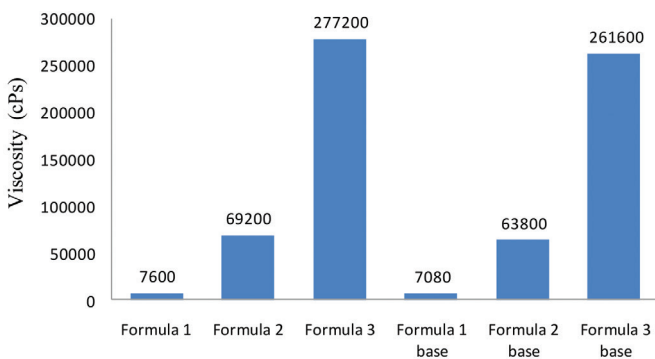


Figure 3. Graph of the viscosity of the gel preparation of roselle aqueous extracts and gel/formula base

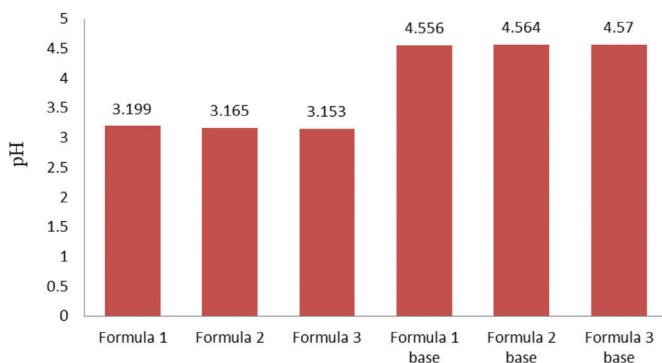


Figure 4. Graph of the pH of the gel preparation and gel/formula base of roselle aqueous extracts

activity was obtained (Figure 6 and Table 3). The greater the viscosity of the gel preparation, the lower capacity of active material to be released.⁹⁻¹¹

Based on the physical evaluation, formula 2 was chosen, because its viscosity was close to the specification (30,000 cPs). The result of the MIC determination of formula 2 (Figure 7), the inhibitory diameter (Table 4), and the inhibitory graph of formula 2 (Figure 8) were analyzed statistically. Gentamicin 25 ppm was chosen as the positive control to ensure that the bacterial test used in this research was sensitive against the antibiotic. A serial concentration of gentamicin was used as the standard curve for evaluation of the extract potency relative to the standard.

Based on one-way ANOVA, there was a significant difference between the inhibitory activity of 12.0 and 6.0 mg/mL, and there was no significant difference between 3.0, 1.5, and 0.8 mg/mL of the gel preparation. In conclusion, formula 2 exhibited MIC at 6.0 mg/mL against *Staphylococcus aureus* ATCC 25923.

The inhibitory activity of gentamicin at serial dilution against the test bacterium was evaluated by a regression equation, where Y and X were the diameter of the inhibitory zone (mm) and log of concentrations (ppm), respectively. The log concentration of formula 2 with diameter of inhibitory zone 9.75 mm was calculated by the regression equation. Equivalent to this growth inhibitory diameter (x), 7.58 ppm of gentamicin concentration was obtained. Furthermore, the inhibitory potency of the roselle aqueous extracts gel at 12.0 mg/mL (roselle concentration in gel 3% w/w) against *Staphylococcus aureus* ATCC 25923 was equal to 7.58 ppm of the gentamicin sulfate standard solution.

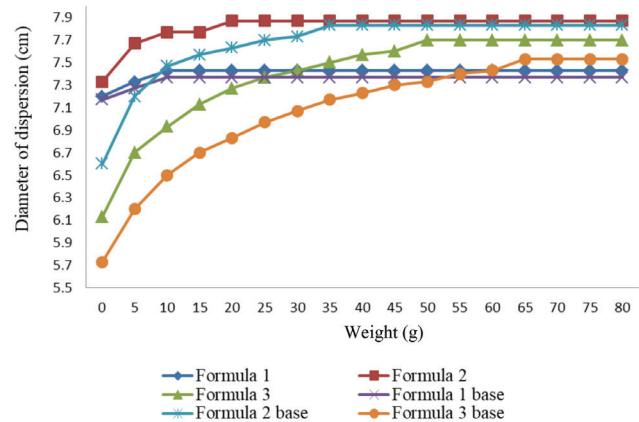


Figure 5. Graph of dispersive power of the gel preparation and gel/formula base of roselle aqueous extracts

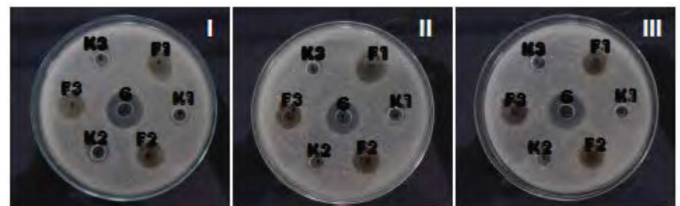


Figure 6. The antibacterial activities of formula 1, 2 and 3 at 12.0 mg/mL (I, II, and III=replication; F1=formula 1; F2=formula 2; F3=formula 3; K1=formula base 1; K2=formula base 2; K3=formula base 3; G=gentamicin 25 ppm)

DISCUSSION

Identification of polyphenols and flavonoids in the chromatogram pattern showed that they play an important role in antibacterial activity.^{4,12,14} The pH of 1% solution of roselle aqueous extracts was highly acidic due to high organic acid contents, such as malic acid and ascorbic acid. The acidity of roselle also plays an important role in its antibacterial activity.^{1,2,14} The qualification of the HPMC 6000 indicated that the matrix had viscosity

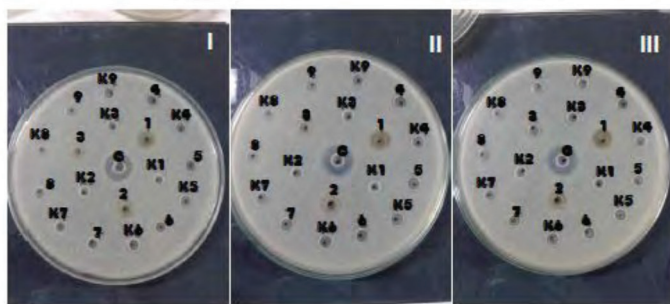


Figure 7. The result of the MIC determination of formula 2 (I, II, III=replication 3; 1=12.0 mg/mL; 2=6.0 mg/mL; 3=3.0 mg/mL; 4=1.5 mg/mL; 5=0.8 mg/mL; 6=0.4 mg/mL; 7=0.2 mg/mL; 8=0.1 mg/mL; 9=0.05 mg/mL; K1=dilution base 1; K2=dilution base 2; K3=dilution base 3; K4=dilution base 4; K5=dilution base 5; K6=dilution base 6; K7=dilution base 7; K8=dilution base 8; K9=dilution base 9; G=gentamicin at 25 ppm), MIC: Minimum inhibitory concentration

satisfactory for a gelling agent. The pH value of 2% w/w solution of HPMC 6000 in water was 4.445 ± 0.053 stabilized by the acidic properties of the extract. The pH value was different from the literature (5.0-8.0)¹⁵ possibly because of the different producers, the quality, and the storage condition of the raw materials.

It was found that the MIC of the roselle aqueous extracts against *Staphylococcus aureus* ATCC 25923 was 0.1 mg/mL. This value was used as the concentration of the formula, to which 3% w/w

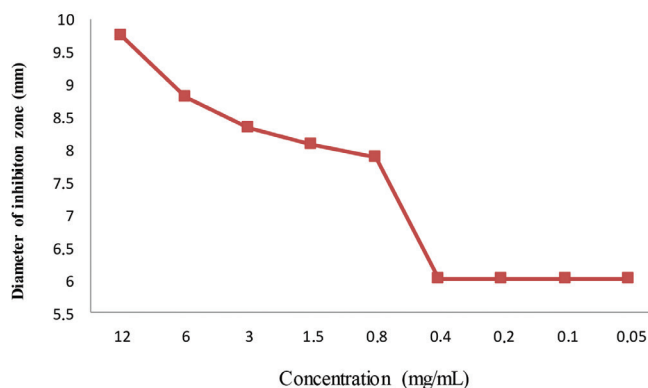


Figure 8. Inhibitory activity of the formula 2 gel preparation of roselle aqueous extracts

Table 3. Inhibitory diameter of formulas 1, 2, and 3 gel preparation of roselle aqueous extracts

Replication	Inhibitory diameter (mm)					
	Formula			Formula base		
	1	2	3	1	2	3
1	10.70	10.10	9.45	7.30	7.05	6.80
2	11.00	9.80	9.70	6.70	6.20	6.05
3	10.70	9.50	9.00	6.50	6.05	6.10
Average	10.80±0.17	9.80±0.30	9.38±0.36	6.83±0.42	6.43±0.54	6.32±0.42

Diameter of reservoir: 6.00 mm

Table 4. The result of the MIC determination of formula 2 gel preparation of roselle aqueous extracts

Rep Conc. (mg/mL)	Inhibitory diameter (mm)							
	Preparation				Base			
	1	2	3	Average	1	2	3	Average
12.00	9.10	9.90	10.25	9.75±0.59	8.45	8.60	9.00	8.68±0.28
6.00	8.60	8.70	9.10	8.80±0.26	8.40	-	8.55	8.47±0.15
3.00	8.35	8.35	8.30	8.33±0.03	8.30	8.30	8.00	8.20±0.17
1.50	8.30	8.25	7.70	8.08±0.33	8.20	8.25	7.60	8.02±0.36
0.80	8.10	8.00	7.50	7.87±0.32	8.00	8.00	7.30	7.77±0.40
0.40	-	-	-	-	-	-	-	-
0.20	-	-	-	-	-	-	-	-
0.10	-	-	-	-	-	-	-	-
0.05	-	-	-	-	-	-	-	-

Diameter of reservoir: 6.00 mm, MIC: Minimum inhibitory concentration

was set as the extract concentration based on the preliminary optimization.

The gel formula was tested using three concentrations of HPMC 6000 (2%, 3%, and 4%, w/w). The ingredients of the preparation formula were propylene glycol as a humectant, roselle aqueous extracts as an active material, sodium benzoate as a preservative, and citrate acid and sodium citrate as buffer. The gel base preparation without the extracts was formulated to identify the effect of roselle aqueous extracts on the physical characteristics of the gel preparation. The gel preparation was made of 250 g with citrate buffer dissolved with pH of 4.505 and each formula was made for one dosage. Replication was not performed due to the limited number of roselle aqueous extracts. It was found that the viscosity of formulas 1, 2, and 3 was 7600, 69.200 and 277.200 cPs, respectively. On the other hand, the viscosity of the gel base of formulas 1, 2, and 3 was 7080, 63.800 and 261.600 cPs, respectively. The presence of roselle aqueous extracts 3% w/w increased the viscosity.

The pH value of formulas 1, 2, and 3 was 3.199 ± 0.003 , 3.165 ± 0.002 , and 3.153 ± 0.006 , respectively. The pH value of the base gel formulas 1, 2, and 3 was 4.556 ± 0.006 , 4.564 ± 0.006 , and 4.570 ± 0.006 , respectively. It can be concluded that the pH of the preparation was much lower than the pH of the gel base even though they were treated by citrate with 0.02 of buffer capacity. This occurred because the buffer capacity failed to hold the pH of the preparation containing 3% (w/w) quite acidic extract of roselle aqueous extracts. The statistical test using one-way ANOVA ($p=0.05$) showed that there was a significant difference among the pH of formulas 1, 2, and 3, as well as the pH of the gel base formulas 1, 2, and 3.

The slope calculation of the regression equation of the dispersion diameter vs. weight of loads to evaluate the dispersal ability of the gel preparation and the base gel of formulas 1, 2, and 3 as depicted in Figure 5 was performed statistically by one-way ANOVA ($p=0.05$). It was found that there was no significant difference in the slope between formulas 1 and 2, but a significant difference was found between formulas 1 and 3 and between formulas 2 and 3. The significant difference in the slope no found between formula 1 and formula gel base; formula 2 and gel base 2; but no significant difference between formula 3 and gel base 3. The capacity of dispersion was denoted by the diameter of maximum dispersion on the adding of certain loads, by which the gel preparation was not dispersed anymore.

According to the slope value and the loads to reach maximum dispersion capacity, it can be concluded that the gel preparation formula dispersed more easily than the gel base, because the viscosity of the gel base is lower than that of the preparation. Since the pH value of the gel preparation was close to 3 and the analysis of the dispersive power was conducted 30 days after the preparation was made, this might have caused the gel preparation to become unstable. The viscosity of the HPMC solution was stable at pH 3-11, but the stability might be disturbed if there is an active material that possesses strong acidity.¹⁵ In the present research, the active material was acid solution of the roselle aqueous extracts.

The inhibitory activity test of the gel base was performed to minimize the effects of the gel component. The activity test aimed to ensure that the growth inhibitory responses were derived from the gel preparation. The bioassay indicated that the gel preparation exhibited higher inhibitory activity than the gel base. The gel preparation of formulas 1, 2, and 3 exhibited growth inhibitory diameter of 10.80 ± 0.17 mm, 9.80 ± 0.30 mm, and 9.38 ± 0.36 mm, respectively. The one-way ANOVA ($p=0.05$) showed that there was a significant difference between formulas 1 and 2, as well as between formulas 1 and 3. There was no significant difference between formulas 2 and 3. The viscosity of the gel preparation might affect the release of the active materials. The higher the viscosity, the more difficult the active materials are released, because of the difficult mobility of the active materials.⁹⁻¹¹

Based on the physical characterization, the selected gel preparation was formula 2, the one containing HPMC 6000 concentration of 3% (w/w) with specification of acid gel preparation with viscosity of 30.000 cPs. The three formulas had pH values that did not meet the specification. Therefore, the formula was selected in accordance with the viscosity value that was close to the specification, namely formula 2. Then the MIC of formula 2 was determined. The preparation was diluted until it reached a concentration of 0.05 mg/mL. The growth inhibitory activity appeared at a dilution of 12.0-0.8 mg/mL. However, the zone was higher than that of the gel base. The statistical test using one-way ANOVA indicated that there was a significant difference between the activity of the gel preparation at 12.0 and 6.0 mg/mL and the gel base. In addition, a significant difference was not found between the inhibitory activity of the gel preparation with concentration of 3.0, 1.5, and 0.8 mg/mL and the gel base. The nonsignificant difference between the gel preparation and the gel base indicated that the inhibitory activity was not caused by the roselle aqueous extracts, but was affected by other components in the formula, such as propylene glycol and sodium benzoate. The smallest concentration showed the existence of a significant difference between the inhibitory activity of the preparation and the gel base at 6.0 mg/mL. In conclusion, the concentration of the roselle aqueous extracts of formula 2 might be recommended for its antibacterial activity toward *Staphylococcus aureus* ATCC 25923. The MIC of the gel preparation was higher than that of the roselle aqueous extracts, because the gelling agent/polymer of the gel preparation might have affected the release of the roselle aqueous extracts from the three preparation formulas.

The potential ratio of formula 2 that inhibited the test bacterium was determined using gentamicin sulfate standard. Correlation between the growth inhibitory diameter of the gentamicin solution at 5-25 ppm against *Staphylococcus aureus* ATCC 25923 and the concentration log of the gentamicin standard was used to determine the potency of the gel preparation through the regression equation: $y=10.2584x+0.5479$ with $r=0.9837$. Formula 2 exhibited growth inhibitory activity against *Staphylococcus aureus* ATCC 25923 equal to gentamicin sulfate standard solution of 7.58 ppm.

CONCLUSION

The HPMC 6000 at 3% (w/w) concentration in roselle aqueous extracts gel preparation gave good physical characteristics. The gel preparation exhibited inhibitory activity against *Staphylococcus aureus* ATCC 25923 depicted by MIC 6.0 mg/mL. Formula 2 is recommended and should be further investigated for implementation in topical preparations.

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Evaluation of the Antioxidant Potency of *Seseli* L. Species (Apiaceae)

Seseli L. Türlerinin (Apiaceae) Antioksidan Potansiyellerinin Değerlendirilmesi

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ABSTRACT

Objectives: In the present study, the antioxidant potency of ethyl acetate (AcOEt) and methanol (MeOH) extracts from the aerial parts of *Seseli* L. species was investigated for the first time.

Materials and Methods: *Seseli* species L. such as *Seseli andronakii* Woronow ex Schischk., *S. campestre* Besser, *S. corymbosum* Boiss. & Heldr., *S. gummiferum* subsp. *gummiferum* Pall. ex Sm., *S. hartvigii* Parolly & Nordt, *S. libanotis* (L.) W.Koch, *S. petraeum* M.Bieb., *S. peucedanoides* (M.Bieb.) Koso-Pol., *S. resinosum* Freyn & Sint., and *S. tortuosum* L. growing in Turkey were collected and evaluated for their antioxidant capacity by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and lipid peroxidation (LPO) inhibition methods.

Results: The highest activities as a scavenger of DPPH radicals were found in the AcOEt extracts of *S. peucedanoides* (M.Bieb.) Koso-Pol. (IC₅₀=0.49 mg/mL), and *S. libanotis* (IC₅₀=0.75 mg/mL); α -tocopherol was used as a positive control. On the other hand, in the LPO assay, the highest activities were determined in AcOEt and MeOH extracts (at 5 mg/mL) of *S. tortuosum* and *S. libanotis* (84-94%).

Conclusion: This report gives important information about the antioxidant capacity of *Seseli* L. species. This research on antioxidant capacity proves that the use of some species used in Eastern Anatolia (in salads) is correct. With this screening study performed in *Seseli* L. species growing in Turkey, in the future, it is planned to isolate antioxidant compounds from the most active strains of *Seseli* L.

Key words: Antioxidant, Apiaceae, DPPH, LPO, *Seseli*

ÖZ

Amaç: Bu çalışmada, ilk kez *Seseli* L. türlerinin toprak üstü kısımlarından elde edilen, etil asetat (AcOEt) ve metanol (MeOH) ekstrelerinin antioksidan potansiyelleri araştırılmıştır.

Gereç ve Yöntemler: Türkiye'de yetişen bazı *Seseli* L. türlerinin, *Seseli andronakii* Woronow ex Schischk., *S. campestre* Besser, *S. corymbosum* Boiss. & Heldr., *S. gummiferum* subsp. *gummiferum* Pall. ex Sm., *S. hartvigii* Parolly & Nordt, *S. libanotis* (L.) W.Koch, *S. petraeum* M.Bieb., *S. peucedanoides* (M.Bieb.) Koso-Pol., *S. resinosum* Freyn & Sint., *S. tortuosum* L., antioksidan kapasiteleri 1,1-difenil-2-pikrilhidrazil (DPPH) radikali süpürme kapasitesi ve lipid peroksidasyonu (LPO) inhibisyon yöntemleri ile değerlendirilmiştir.

Bulgular: En yüksek radikal süpürücü etkinliği *S. peucedanoides* (M.Bieb.) Koso-Pol. (IC₅₀=0,49 mg/mL) ve *S. libanotis* (IC₅₀=0,75 mg/mL) EtOAc ekstrelerinde olduğu bulunmuştur; α -tokoferol pozitif kontrol olarak kullanılmıştır. Diğer yandan, LPO deneyinde, en yüksek aktivite *S. tortuosum* ve *S. libanotis* (%84-94)'in EtOAc ve MeOH (5 mg/mL dozda) ekstrelerinde tespit edilmiştir.

Sonuç: Bu çalışmada, *Seseli* L. türlerinin antioksidan kapasitesi hakkında önemli bilgiler elde edilmiştir. Antioksidan kapasiteleri üzerine yapılan bu araştırma ile, bazı türlerin Doğu Anadolu'da gıda olarak (salatalarda) kullanımının doğruluğu bir kez daha gösterilmiştir. Türkiyede yetişen *Seseli* L. türlerinde yapılan bu tarama çalışması ile, gelecekte, antioksidan etki gösteren bileşiklerin en aktif *Seseli* L. türlerinden izole edilmesi planlanmaktadır.

Anahtar kelimeler: Antioksidan, Apiaceae, DPPH, LPO, *Seseli*

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INTRODUCTION

The Apiaceae (previously Umbelliferae) is a well-known family in the plant kingdom with aromatic plants and economically important species.¹ Some members of the family are used as foods, spices, condiments, and ornaments.²⁻⁴ The genus *Seseli* L. belongs to the family Apiaceae and is distributed in Asia and Europe, comprising more than 12 taxa in Turkey, of which 4 are native to the region.⁵⁻⁸ In addition, new species have recently been discovered.⁹⁻¹² Moreover, the latest taxonomy of the type section of the genus *Seseli* has been given based on the molecular data with recently updated names.¹³ *Seseli* is an ancient Greek name given to some individual members of the family Apiaceae by Hippocrates.¹⁴ *Seseli* species are mainly rich in coumarins as well as terpenoids, essential oils, etc.^{15,16} and have many important pharmacological activities with healing effects such as in inflammation, swelling, rheumatism, pain, and the common cold.¹⁷ On the other hand, the fruit of *S. indicum* has been reported to have anthelmintic, carminative, stomachic, and stimulant properties.¹⁸ *S. sibiricum* is used for blending beverages and as a medicine for livestock in Kashmir.¹⁹ In addition, the fruit of *S. libanotis* is a local remedy for blood pressure control in Pakistan, and its essential oil from the fruit has potent antimicrobial activity.²⁰ While *S. indicum* exhibited strong insect repellent activity²¹ and fungitoxicity,²² the fruit of *S. tortuosum* is recorded to have emmenagogic and antifatulent effects.²³ Moreover, the leaves of *S. libanotis* (Kelemkeşir or Kelemenkeşir in Turkish) are consumed as a vegetable in salads in Eastern Turkey.²⁴

In Turkey, there are limited studies on *Seseli* species based on coumarins²⁵⁻²⁹ and essential oils.³⁰⁻³⁴ Previously, antimicrobial,³⁵ anti-inflammatory, and antinociceptive³⁶⁻³⁸ effects have been examined in Turkish *Seseli* species.

The plant kingdom presents secondary plant metabolites (especially polyphenols) as a wide range of natural antioxidants.³⁹⁻⁴² The natural antioxidants in plants are of great interest in natural product science and many herbs have significant antioxidant potency.⁴³ Antioxidants decrease

oxidative stress in cells and are therefore very useful in the treatment of major degenerative diseases.⁴⁴ The physiological role of antioxidant agents is to scavenge for free radicals^{45,46} in the case of overproduction of these reactive species.⁴⁷

Therefore, in the present study, we aimed to investigate the antioxidant potential of the aerial parts of Turkish *Seseli* species. The species were screened using *in vitro* 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and lipid peroxidation (LPO) inhibition assays.

MATERIALS AND METHODS

Plant material

Plant materials were collected from different localities in Turkey. All of the *Seseli* L. species were identified by Prof. H. Duman from the Department of Biology, Faculty of Science and Arts, Gazi University, Ankara, Turkey. Voucher specimens were deposited at the Herbarium of the Faculty of Pharmacy of Ankara University and the Herbarium of Gazi University, Ankara, Turkey. The species are listed in Table 1 (ethical committee approval and patient consent were not required).

Extraction of the plants

The extraction method in Fenglin et al.⁴⁸ and Báthori et al.⁴⁹ was used with some modifications. The aerial parts of each plant material, which were dried and powdered, were prepared according to the procedures described below:

-*The ethyl acetate (AcOEt) extract*: The plant material (10 g) was extracted with AcOEt at room temperature by a magnetic stirrer (x200 mL) for 24 hour. The extract was evaporated to dryness in a vacuum to give a crude AcOEt extract.

-*The methanol (MeOH) extract*: After the AcOEt extraction, the plant material (10 g) was extracted with MeOH (80%) at room temperature by a magnetic stirrer (x200 mL) for 24 hour. The extract was evaporated to dryness *in vacuo* to give a crude methanolic extract. The yields of all extracts are given in Table 2.

Table 1. Plant names and collection sites of Turkish *Seseli* L. species

Species	Location	Herbarium no
<i>S. andronakii</i> Woronow ex Schischk	Erzurum, Oltu-Sarıkayalar, 1450-1750 m	ED 1617
<i>S. campestre</i> Besser	İstanbul, Sultanbeyli, Paşaköy c. 500 m	ED 1656
<i>S. corymbosum</i> Boiss. and Heldr.	Antalya-Akseki, Pınarbaşı village 1650-1900 m	AEF 21701
<i>S. gummiferum</i> subsp. <i>gummiferum</i> Pall. ex Sm.	Ankara-Hasanoğlan, İdris mountain 1600-1700 m	AEF 21999
<i>S. hartvigii</i> Parolly and Nordt	Antalya-Saklıkent, Bakırlar mountain, 2300-2500 m	AEF 21700
<i>S. libanotis</i> (L.) W.Koch	Ardahan-Posof, 1900 m	ED 1622
<i>S. petraeum</i> M.Bieb.	Gümüşhane, The road to Alemdar village, 1400 m	ED 1644
<i>S. peucedanooides</i> (M.Bieb.) Koso-Polo	Ankara-Hasanoğlan, İdris mountain, 1600-1700 m	AEF 23158
<i>S. resinosum</i> Freyn and Sint.	Bartın-Çakraz, 0-5 m	AEF 21696
<i>S. tortuosum</i> L.	Ankara, Beynam forest, 1400 m	ED 1612

AEF: Herbarium of the Faculty of Pharmacy of Ankara University

Chemicals

Ascorbic acid, thiobarbituric acid (TBA), DPPH, and α -tocopherol were purchased from Sigma Chemical Co (St. Louis, MO, USA).

Antioxidant capacity of the extracts

Radical scavenging capacity (DPPH)

The model of scavenging stable DPPH radicals is a widely used method to evaluate antioxidant activities in a relatively short time compared with other methods. The effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen donating ability.⁵⁰ The reaction mixture contained 100 μ M DPPH in MeOH and different concentrations of the crude extract. Absorbance at 517 nm was measured on a Shimadzu UV-1601 UV-VIS spectrometer at various concentrations (30 min after starting the reaction) at room temperature and the scavenging activity was calculated as the percentage of radical reduction. In our study, samples were dissolved in MeOH (80%) and AcOEt to 10 mg/mL and diluted to various concentrations. The scavenging activity was calculated as the percentage of radical reduction. The values of IC₅₀ were determined from a calibration curve for each plant extract. Each experiment was performed in triplicate. IC₅₀ values were determined from a calibration curve for each plant extract and α -tocopherol was used as the reference compound.

Assay of lipid peroxidation (LPO)

LPO was determined by a modified version of the method described by Mihara et al.⁵¹ It was measured spectrophotometrically by estimation of the TBA reactant substances (TBARS). Amounts of TBARS were expressed in nmol malondialdehyde/g tissue. A typical optimized assay mixture containing 0.5 mL of liver homogenate, 0.1 mL of Tris-HCl buffer (pH 7.2), 0.05 mL of 0.1 mM ascorbic acid, and 0.05 mL of 4 mM FeCl₂ and 0.05 mL of various concentrations of crude extract or α -tocopherol were incubated for 1 h at 37°C. After incubation, 3.0 mL of H₃PO₄

Table 2. The yield of extracts from Turkish *Seseli* L. species

Species	AcOEt extract (w/w % mg)	MeOH extract (w/w % mg)
SA	370	154
SA	390	154
SCa	1030	108
SGG	870	128
SH	330	119
SL	270	200
SP	750	118
SPeu	310	100
SR	420	120
ST	570	163

SA: *S. andronakii*, SH: *S. hartvigii*, ST: *S. tortuosum*, SL: *S. libanotis*, SGG: *S. gummiferum* subsp. *gummiferum*, SPeu: *S. peucedanoides*, SR: *S. resinosum*, SC: *S. corymbosum*, SCa: *S. campestre*, SP: *S. petraeum*, AcOEt: Ethyl acetate, MeOH: Methanol

and 1 mL of 0.6% TBA were added and the resulting mixture was shaken vigorously. The mixture was boiled for 30 minute. After cooling, *n*-butanol was added and the mixture was shaken vigorously. Then the *n*-butanol phase was separated by centrifugation at 3000 rpm for 10 minute. The absorbance of the supernatant was measured at 532 nm against a blank, which contained all reagents except the liver homogenate.

Statistical analysis

Values of experimental results were considered as the mean of at least three determinations (\pm standard deviation).

RESULTS AND DISCUSSION

The present study deals with the radical scavenging activity (Table 3) and LPO (Table 4) of the AcOEt and MeOH extracts obtained from *Seseli* L. species growing in Turkey such as *Seseli andronakii*, *S. campestre*, *S. corymbosum*, *S. gummiferum* subsp. *gummiferum*, *S. hartvigii*, *S. libanotis*, *S. petraeum*, *S. peucedanoides* (M.Bieb.) Koso-Pol, *S. resinosum*, and *S. tortuosum*. The antioxidant activities of AcOEt and MeOH extracts obtained from the *Seseli* species were investigated by the DPPH scavenging and nonenzymatic rat hepatic microsomal LPO methods. In addition, their antioxidant activities were compared with those of the standard antioxidant α -tocopherol. The DPPH free radical scavenger assay is a simple and basic screening method for the discovery of bioactive substances. Free radicals are species that damage all the components of the body (lipids, proteins, DNA, etc.) and take part in mutations. In this case, antioxidants are important for body protection, helping reduce oxidative damage in the human body, and prevent LPO in foods.^{52,53}

Table 3. Inhibitory effects of *Seseli* extracts on DPPH stable radicals

Samples	AcOEt extracts	MeOH extracts
	IC ₅₀ (mg/mL)	IC ₅₀ (mg/mL)
Control		
SA	1.91 \pm 0.04	0.125 \pm 0.003
SH	1.94 \pm 0.03	0.225 \pm 0.002
ST	1.65 \pm 0.02	0.205 \pm 0.05
SL	0.75 \pm 0.07	0.187 \pm 0.002
SGG	3.07 \pm 0.04	0.088 \pm 0.001
SPeu	0.49 \pm 0.1	0.091 \pm 0.004
SR	1.18 \pm 0.15	0.086 \pm 0.001
SC	2.47 \pm 0.06	0.253 \pm 0.005
SCa	4.27 \pm 0.14	0.185 \pm 0.008
SP		0.172 \pm 0.006
α -Tocopherol	0.013 \pm 0.001	

SA: *S. andronakii*, SH: *S. hartvigii*, ST: *S. tortuosum*, SL: *S. libanotis*, SGG: *S. gummiferum* subsp. *gummiferum*, SPeu: *S. peucedanoides*, SR: *S. resinosum*, SC: *S. corymbosum*, SCa: *S. campestre*, SP: *S. petraeum*, AcOEt: Ethyl acetate, MeOH: Methanol

In our experiments, the results indicated that the extracts of some Turkish *Seseli* species have considerable effects on scavenging DPPH radicals (Figure 1). The AcOEt extract of *S. peucedanoides* ($IC_{50}=0.49$ mg/mL) and *S. libanotis* ($IC_{50}=0.75$ mg/mL) showed the most potent radical scavenging capacity (Table 3). These extracts were followed by *S. resinosum* ($IC_{50}=1.18$ mg/mL), *S. tortuosum* ($IC_{50}=1.65$ mg/mL), *S. andronakii* ($IC_{50}=1.91$ mg/mL), *S. hartvigii* ($IC_{50}=1.94$ mg/mL), *S. corymbosum* ($IC_{50}=2.47$ mg/mL), *S. gummiferum* subsp. *gummiferum* ($IC_{50}=3.07$ mg/mL), and *S. campestre* ($IC_{50}=4.27$ mg/mL) extracts.

The MeOH extracts of *Seseli* species have a higher DPPH radical scavenging effect than AcOEt extracts. The results showed that MeOH extracts of *S. resinosum*, *S. gummiferum* subsp. *gummiferum*, and *S. peucedanoides* have the highest scavenging capacity ($IC_{50}=0.086$, $IC_{50}=0.088$, and $IC_{50}=0.091$, respectively).

The TBA test results showed that MeOH extracts of *Seseli* spp. exhibited potent antioxidant effects (81-96% inhibition at 5 and 10 mg/mL concentrations) when compared to α -tocopherol. The AcOEt and MeOH extracts of *S. tortuosum* have the strongest anti-LPO activity (84-96% inhibition at a dose of 10 mg). The AcOEt and MeOH extracts of *S. campestre*, *S. andronakii*, and *S. gummiferum* subsp. *gummiferum* also exhibited a high anti-LPO effect in the LPO assay (Table 4).

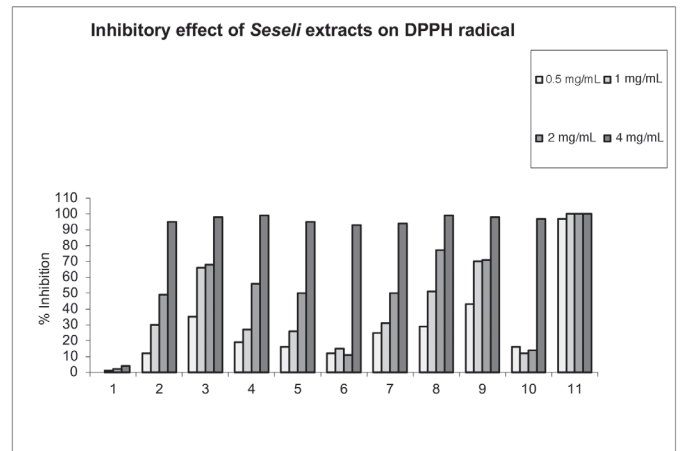


Figure 1. Ethyl acetate extracts of *Seseli* species (1-10) and (11) α -tocopherol at various concentrations

(1) *S. andronakii*, (2) *S. hartvigii*, (3) *S. tortuosum*, (4) *S. libanotis*, (5) *S. gummiferum* subsp. *gummiferum*, (6) *S. peucedanoides*, (7) *S. resinosum*, (8) *S. corymbosum*, (9) *S. campestre*, (10) *S. petraeum*

Table 4. Antilipid peroxidation effects of *Seseli* extracts^a

	Concentrations mg/mL			Concentrations mg/mL		
	nmol MDA/g tissue	% Inhibition		nmol MDA/g tissue	% Inhibition	
Control	AcOEt extracts			MeOH extracts		
b	NE^c			NE^c		
SA	2.5	0.148	34	5	0.027	88
	5	0.045	80	10	0.024	89
SH	2.5	0.084	63	5	0.026	88
	5	0.052	77	10	0.025	89
ST	2.5	0.102	55	5	0.011	95
	5	0.036	84	10	0.009	96
SL	2.5	0.222	1.2	5	0.037	83
	5	0.085	45	10	0.014	94
SGG	2.5	0.085	62	5	0.042	81
	5	0.039	82	10	0.035	84
SPeu	2.5	0.195	13	5	0.021	91
	5	0.129	43	10	0.022	90
SR	2.5	0.144	36	5	0.043	81
	5	0.049	78	10	0.026	88
SC	2.5	0.151	33	5	0.025	89
	5	0.067	70	10	0.018	92
SCa	2.5	0.088	61	5	0.025	89
	5	0.043	81	10	0.02	91
SP	2.5	0.156	31	5	0.028	87
	5	0.058	74	10	0.026	81
α -Tocopherol	0.22	0.009	96	0.22	0.009	96
	0.44	0.003	99	0.44	0.003	99

^aEach value represents the mean \pm standard deviation of 2-4 independent experiments, ^bAcOEt or MeOH only, control for extracts, ^cNE: No effect

SA: *S. andronakii*, SH: *S. hartvigii*, ST: *S. tortuosum*, SL: *S. libanotis*, SGG: *S. gummiferum* subsp. *gummiferum*, SPeu: *S. peucedanoides*, SR: *S. resinosum*, SC: *S. corymbosum*, SCa: *S. campestre*, SP: *S. petraeum*

In previous studies, the antioxidant potency of MeOH extract of *S. pallasii*, *S. libanotis* subsp. *libanotis*, and *S. libanotis* subsp. *intermedium* (aerial parts and fruits) was determined. *S. libanotis* subsp. *libanotis* showed the strongest antioxidant activity in the DPPH assay.⁵⁴ Various extracts in different polarities from the roots, leaves, flowers, and fruit of *S. rigidum* were also studied, and the hexane extract of the root had the best effect among the other plant parts in the DPPH assay.^{55,56} In another study, the antioxidant activity of *Seseli rigidum* was evaluated in five extracts in different polarities (water, MeOH, acetone, ethyl acetate, and petroleum ether). The antioxidant effect of the aerial parts of the species was determined *in vitro* using DPPH reagent, and the highest antioxidant activity was expressed in water extract (46.15 µg/mL).⁵⁷ Moreover, some of the compounds isolated from the methanolic extracts (80%) of *Seseli diffusum* have been found to have a strong antioxidant effect.⁵⁸

It is known that *Seseli* species contain phenolic compounds consisting mainly of coumarins,¹⁶ which have notable antioxidant potency.⁵⁹⁻⁶¹ In addition, mostly oxygenated coumarins are accumulated in the AcOEt fractions, and the glycosides are present in the MeOH extract. The MeOH extract exhibits higher antioxidant activity, which may be explained by the presence of coumarin glucosides as highly polar compounds in the extract. The results show that there seemed to be a good match between the content of the extracts and the antioxidant capacity. Finally, the activity might be due to the polar coumarins of the active *Seseli* species.^{52,62}

CONCLUSION

Natural products are generally known to be a good source of active compounds that have potential for the development of new therapeutic agents. The antioxidant properties of the AcOEt and MeOH extracts of *Seseli* species expressed as α -tocopherol equivalent antioxidant capacity were studied using DPPH and LPO assays. These results indicate that plant extracts prevent oxidative damage in normal cells due to their antioxidant properties. The best part of our research was that *Seseli* species growing in Turkey were screened for the first time for their antioxidant capacity. In addition, this research provides a scientific basis for the medicinal use of these plant materials. Therefore, we can conclude from the results of the present study that *Seseli* species may be a potential source of natural antioxidant compounds for the treatment of oxidative degeneration.

Conflicts of interest: No conflict of interest was declared by the authors.

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Comparative *In Vitro* and *In Vivo* Evaluation of Fenofibric Acid as an Antihyperlipidemic Drug

Antihiperlipidemik İlaç Olarak Fenofibrik Asidin Karşılaştırmalı *In Vitro* ve *In Vivo* Değerlendirilmesi

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ABSTRACT

Objectives: Fenofibric acid (FA) is antihyperlipidemic agent and commercially available as a tablet formulation that weighs 840 mg for 105 mg of active substance. A new formulation with less inactive substance was developed as an alternative to the conventional formulation. The purpose of this study was to evaluate the dissolution and the relative bioavailability of the surface solid dispersion (SSD) and conventional formulations of FA by comparing them with the reference formulation in its commercial tablets. The *in vitro-in vivo* correlation among these tablet formulations was also evaluated.

Materials and Methods: The dissolution study was performed in phosphate buffer pH 6.8 and biorelevant fasted state simulated intestinal fluid. Dissolution efficiency and mean dissolution time (MDT) were used to compare the dissolution profiles. The bioavailability study, using nine healthy volunteers, was conducted based on a single-dose, fasted, randomized, crossover design. The *in vivo* performance was compared using the pharmacokinetic parameters C_{max} , T_{max} , AUC_{0-72} , and $AUC_{0-\infty}$. A linear correlation model was tested using MDT and mean residence time (MRT).

Results: The results indicated that there were significant differences in the dissolution performances but no significant differences among the mean C_{max} , T_{max} , AUC_{0-72} , or $AUC_{0-\infty}$ estimated from the SSD, conventional, and reference formulations. A poor correlation was found between MRT and MDT of the three formulations.

Conclusion: The SSD formulation led to an instantaneous dissolution of the drug due to the presence of the polymer and the physical structure of the SSD. The conventional formulation could not achieve rapid dissolution despite its satisfying the requirement for immediate drug release dosage form. Both formulations could be considered bioequivalent with the reference. The *in vitro* dissolution behavior of FA using a single medium did not reflect their *in vivo* properties in the fasted condition. There was no correlation between the *in vitro* dissolution and the *in vivo* bioavailability of FA in this condition.

Key words: Fenofibric acid, surface solid dispersion, dissolution, bioavailability, correlation

ÖZ

Amaç: Fenofibrik asit (FA) antihiperlipidemik bir ajandır ve 105 mg aktif madde içeren 840 mg ağırlığında bir tablet formülasyonu şeklinde ticari olarak temin edilebilir. Konvansiyonel formülasyona alternatif olarak inaktif madde miktarı daha az yeni bir formülasyon geliştirildi. Bu çalışmanın amacı, yüzey katı dispersiyonunun (SSD) ve konvansiyonel FA formülasyonlarının disolüsyonunu ve göreceli biyoyararlanımını ticari tabletlerdeki referans formülasyon ile karşılaştırmaktır. Bu tablet formülasyonları arasındaki *in vitro-in vivo* korelasyon da değerlendirildi.

Gereç ve Yöntemler: Disolüsyon deneyleri fosfat tamponu pH 6,8 ve açlık durumu yapay bağırsak sıvısı içinde yapıldı. Disolüsyon profillerini karşılaştırmak için disolüsyon verimliliği ve ortalama disolüsyon süresi (MDT) kullanıldı. Dokuz sağlıklı gönüllü üzerinde gerçekleştirilen biyoyararlanım çalışması, tek doz, aç karna, randomize, çapraz bir tasarım kullanılarak gerçekleştirildi. *In vivo* performans, C_{max} , T_{max} , AUC_{0-72} ve $AUC_{0-\infty}$ farmakokinetik parametreleri kullanılarak karşılaştırıldı. MDT ve ortalama kalış süresi (MRT) kullanılarak doğrusal korelasyon modeli test edilmiştir.

Bulgular: Sonuçlar, çözünme performanslarında önemli farklılıklar olduğunu, ancak SSD, geleneksel ve referans formülasyonlardan tahmin edilen ortalama C_{max} , T_{max} , AUC_{0-72} veya $AUC_{0-\infty}$ arasında önemli farklılıklar olmadığını gösterdi. Üç formülasyonun MRT ve MDT değerleri arasında zayıf bir korelasyon bulundu.

Sonuç: SSD formülasyonu, polimerin varlığı ve SSD'nin fiziki yapısı nedeniyle ilacın ani disolüsyonuna yol açtı. Konvansiyonel formülasyon, hızlı salım dozaj formu gereksinimini karşılamasına rağmen disolüsyonu hızlı olmadı. Her iki formülasyon da referansın biyoşdeğeri olarak değerlendirilebilir.

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FA'nın bir ortamdaki *in vitro* disolüsyon profili, açlık durumunda *in vivo* özelliklerini yansıtmamıştır. Bu durumda fenofibrik asidin *in vitro* disolüsyonu ile *in vivo* biyoyararlanımı arasında korelasyon yoktu.

Anahtar kelimeler: Fenofibrik asit, yüzey katı dispersiyonu, disolüsyon, biyoyararlanım, korelasyon

INTRODUCTION

Fenofibric acid (FA), the active moiety of fenofibrate, is an antihyperlipidemic agent because it is the synthetic ligand that binds to nuclear peroxisome proliferator-activated receptors alpha.¹⁻³ FA is a carboxylic acid moiety, while fenofibrate is an ester moiety.⁴ Figure 1 shows the chemical structures of both FA and fenofibrate. In its marketed form, fenofibrate is insoluble and recommended to be taken with food, and it typically includes nonmicronized tablets, micronized capsules, microcoated micronized tablets, and hard gelatin capsules. The nanocrystal formulation of fenofibrate and the conventional formulation of FA currently available on the market can be taken with or without food.³ A single 105 mg dose of FA is bioequivalent to a single 145 mg dose of fenofibrate in both fed and fasted states.⁵ Not only is the production of the nanocrystal formulation of fenofibrate inflexible, but the high cost also has to be taken into account. As a result, FA has been chosen and developed as an alternative to fenofibrate for oral administration.

Like fenofibrate, FA is mainly absorbed from the gastrointestinal (GI) tract. However, it does better than fenofibrate, causing its bioavailability to be higher than that of fenofibrate in all GI regions.⁶ The absolute oral bioavailability of FA in rats stands at 40%.⁷ Physicochemically, FA is characterized as a poorly soluble weak acid drug. The pKa of FA is 4 and the log P is 3.85.⁸ FA has relatively poor solubility at gastric pH (the pH is lower than its pKa), but it has fairly good solubility at intestinal pH.¹ The solubility of FA is 162.5 µg/mL in water and 1156 µg/mL at pH 6.8.⁹ Due to its adequate permeability, FA is classified as a class II drug in the Biopharmaceutical Classification system (BCS) subclass (a) for weak acids.¹⁰ The poor solubility of FA in water may cause its dissolution to be reasonably slow and its bioavailability to be unpredictable.

Recently, FA has become commercially available as a tablet formulation, namely Fibricor® (the brand for 105 mg FA). The weight of this formulation is 840 mg and it consists of many ingredients for the active substance of 105 mg. The dosage form of FA with increased dissolution is developed to examine other possible platforms. The dissolution rate of BCS class II drugs is the limiting step for their oral bioavailability. The surface solid dispersion (SSD) formulation is regarded as a method to improve the dissolution rate and bioavailability of

poorly soluble drugs. The distribution of drug particles on the carrier surface can enhance the wettability, dissolution rate, and consequently bioavailability of drugs.^{11,12} The FA SSD has been investigated. In the simulated intestinal fluid, the data showed that the dissolution of FA increased more than that of the pure drug.¹³ In the present study, a new FA formulation with enhanced dissolution and less inactive ingredients was developed and evaluated for its *in vitro* and *in vivo* performance. It has never appeared in any publication.

The drug dissolution rate and bioavailability are influenced by the manufacturing process and the changes happening during formulation. Therefore, bioavailability issues are frequently used to assess the safety and efficacy of drug products. Only two studies have been reported so far to enhance the dissolution and bioavailability of FA. The FA loaded pellet is prepared with magnesium carbonate and k-carrageenan employing the extrusion/spheronizing technique followed by coating with ethyl cellulose. The pellet is bioequivalent to the commercial product in beagle dogs.¹⁴ Additionally, a mixture of FA and magnesium carbonate at a weight ratio of 2/1 can improve the solubility, dissolution, and oral bioavailability of FA.⁹ No information about the *in vitro-in vivo* correlation (IVIVC) of this drug is available. One of the challenges of biopharmaceutics research is to figure out the correlation of the *in vitro* drug release information of various drug formulations with the *in vivo* drug profiles. In relation to FA, the correlation between the dissolution rate and the *in vivo* performance is likely to be predicted.

The present study examined the results of both the bioavailability and the dissolution of the two tested formulations and the immediate-release reference formulation. The formulations tested herein are the SSD and conventional methods. The *in vitro* dissolution characteristics of these tablets exhibited different release patterns, meaning the correlation between the *in vitro* dissolution and the *in vivo* bioavailability of these tablet formulations is also under investigation.

MATERIALS AND METHODS

Materials

The FA and the standard FA used in this study were purchased from BOC Science and Sigma, both of which are based in the USA. Other materials such as croscarmellose sodium (CS), Avicel PH 101, lactose monohydrate, Manihot starch, magnesium stearate, and talc were obtained from the local supplier in Indonesia. The 105 mg FA® tablets, the generic version of Fibricor® reference tablets (Mutual Pharmaceutical), were bought from International Pharmacy, USA. The biorelevant medium fasted state simulated intestinal fluid (FaSSIF) was purchased from Biorelevant.com Ltd (Croydon, UK). Then a number of materials were acquired from the Merck Group of

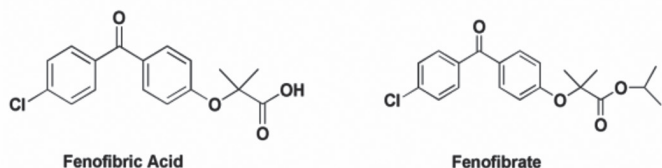


Figure 1. Chemical structures of fenofibric acid and fenofibrate

Germany, including sodium hydroxide, potassium dihydrogen phosphate, sodium dihydrogen phosphate monohydrate, and sodium chloride. In addition, the distilled water used for all dissolution experiments and all other reagents were of analytical grade. The IS of 4'-chloro-5-fluoro-2-hydroxyl benzophenone (CFHB) was obtained from Apollo Sci (UK), the blank plasma from the Indonesia Red Cross, Bandung (Indonesia), and the rest (methanol, ethyl acetate, hydrochloric acid, and acetonitrile) from JT Baker (USA). All reagents used herein were of analytical grade, with the exception of acetonitrile of high performance liquid chromatography (HPLC) grade.

Methods

Preparation of surface solid dispersion and conventional formulations

The SSD formulation of FA with CS (1:1 w/w) was prepared by the solvent evaporation method. First, the drug was dissolved in ethanol to obtain a clear solution. The carrier CS was then dispersed in the drug solution, and the solvent was removed using a rotary evaporator. The viscous residues produced were dried in an oven at 40°C to allow complete evaporation of ethanol in order to obtain constant weight of powder. Subsequently, the mass was passed through a 40 mesh sieve to get dry free-flowing powder ready for compression into tablets by the direct compression method. Avicel PH 101 and magnesium stearate (1% w/w) were later added as a diluent and lubricant. This mixture was checked for flowability and compressibility before the compression of this mass into tablets. The blend was compressed by a single punch tablet press with punch size 10 mm into 300 mg tablets with an FA concentration of 105 mg.

The conventional formulation was prepared by the wet granulation method. The drug was mixed thoroughly with lactose monohydrate as a diluent and then granulated with starch paste (10% w/w). The dried granules were incorporated with dried starch (10% w/w), magnesium stearate (1% w/w), and talc (2% w/w). The same procedures for flowability, compressibility, and compression were also applied to this mixture with the same tablet press, punch size, and FA concentration.

Besides the above formulations, the reference formulation of FA was also used in the present study. FA® itself is actually a generic version of Fibracor®, whose formulation contains FA, copovidone, crospovidone, magnesium stearate, and microcrystalline cellulose in its 840 mg tablet weight.

Drug content uniformity in tablet formulations

In each formulation, the tablet samples were weighed accurately and transferred into a 100 mL volumetric flask. The solvent mixture of 2 M urea and 1 M sodium citrate (5 mL each) was added, and the mixture was heated for 15 min. This procedure was performed for the solubilization of FA, and the solvent mixture was used as a hydrotropic agent.¹⁵ The solution was eventually filtered through Whatman filter paper, while the remaining filtrate was diluted with distilled water and analyzed using a ultraviolet/visible (UV/Vis) spectrophotometer (Shimadzu 1800A) at 299 nm. The FA concentration was determined based on the calibration curve previously built. The

experiment for drug content was repeated three times, and the results were expressed as the mean ± standard deviation.

Dissolution studies

The release characteristics of the tested formulations and the reference formulation were evaluated for the dissolution rate in a type 2 (paddle) dissolution apparatus (Electrolab TDT-08L, USP), using 500 mL of phosphate buffer pH 6.8, and the biorelevant medium FaSSIF was maintained at 37±0.5°C. The paddle rotation speed was set at 50, 75, and 100 rpm. The samples were taken at specified time intervals and replaced with equal volumes of fresh dissolution media to maintain constant volumes in the flasks. The samples were filtered through a 0.45 µm membrane. The filtered samples were diluted with the dissolution medium, and the FA concentration was determined by a UV/Vis spectrophotometer at the wavelengths of 298.7 nm for phosphate buffer and 299 nm for FaSSIF. The FA concentration was determined based on the calibration curve previously built. The dissolution experiment was conducted three times, and the results were expressed as the mean values of the dissolution efficiency (DE)₆₀ parameter (%).

Bioavailability studies

This study was approved by the Health Research Ethics Committee of the Faculty of Medicine Universitas Padjadjaran Bandung (897/UN6.C.10/PN/2017) in accordance with the Declaration of Helsinki and International Conference on Harmonisation-Good Clinical Practice guidelines. There were nine eligible subjects included in the present study. The subjects were all healthy and male. Their age varied from 22 to 48 years, weight from 47 to 68 kg, and height from 155 to 175 cm. These values give the standard body mass index 18-25 kg/m². Furthermore, they were required not to have any significant medical history and evidence of hepatic, renal, GI, or hematological disorders; acute or chronic diseases; clinically significant abnormalities; or drug abuse or allergy. Moreover, they were instructed to abstain from taking any concomitant medication, food supplement, or herbal medicine for at least 14 days prior to and during the study. Subjects were excluded if they had participated in any clinical study or used the investigational drugs within the past 30 days prior to starting the present study. In addition, caffeine-containing beverages were not allowed while the study was being conducted. All chosen participants were given written informed consent forms after the nature and purpose of the study were explained.

The protocol applied a randomized, three-way crossover design with nine subjects in each period. In the first period, after overnight fasting and predose blood sampling, every subject was given a single dose of any formulation in a random way along with 250 mL of water. Food and drinks (other than water 2 h after dosing) were not allowed until 4 h after dosing. Standard meals for both lunch and dinner were served at 4 and 10 h, respectively, while a snack was given 8 h after drug administration. Blood pressure, heart rate, respiration rate, and adverse events were monitored during blood sampling. Approximately 5 mL of the serial venous blood samples were drawn using 22G drawing needles into VACUETTE® tubes

containing 100 μL of sodium citrate 0.485 M as the anticoagulant predose (0 h) and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, 16, and 24 h postdose.⁵ The blood samples were centrifuged with Germany's EBA 20 Hettich at 5000 $\times g$ for 15 min, and the plasma samples were separated and kept frozen at -20°C in three Eppendorf tubes with distinct codes until the analysis was done. The participants returned on a nonconfined basis for continued pharmacokinetic blood sampling at 36, 48, and 72 h after drug administration in each period. After one-week washout, they were requested to return to the laboratory for the same blood sample analysis so as to complete the crossover design.

HPLC assay

The concentration of FA in plasma was determined using the HPLC method, developed and validated by Shah et al.¹⁶ Corpus Fontium Historiae Byzantinae (CFHB) was used as the internal standard (IS). The method was verified before being used in the study. Stock solutions of 1 mg/mL were prepared for FA and CFHB and were diluted in methanol to obtain seven FA containing standard solutions of 0.05–20 $\mu\text{g}/\text{mL}$ and one IS containing solution of 250 $\mu\text{g}/\text{mL}$. All of these solutions were then stored at -20°C . The calibration curve was established by spiking the working standard solutions (50 μL) and the IS solution (50 μL) into drug-free human plasmas (450 μL). In relation to the concentration, matrix-matched FA solutions were prepared in plasmas at various concentrations of 0.05, 0.1, 0.5, 1, 5, 10, and 20 $\mu\text{g}/\text{mL}$, whereas IS solution was at concentration 250 $\mu\text{g}/\text{mL}$. A similar method was employed to prepare quality control (QC) samples in human plasmas. Four additional QC samples were of 0.05, 0.5, 10, and 15 $\mu\text{g}/\text{mL}$.

The analytical separation was performed on an Inertsil® C18 (4.6 \times 150 mm, Waters) column, and the mobile phase was a gradient of acetonitrile and 0.01 M phosphate buffer pH 2.8 (75:25), with a flow rate of 1 mL/min that runs for 7 min. The samples were detected at 287 nm (Waters 2487 dual λ absorbance detector). The retention times for both FA and CFHB as the IS were 3.5 and 5.5 min, respectively. No interfering peaks were observed at either retention time. A typical chromatogram is shown in Figure 2. The limit of quantification for FA was 0.05 $\mu\text{g}/\text{mL}$. Plasma concentrations of FA were obtained from standard curves linear over a range of 0.05–20 $\mu\text{g}/\text{mL}$.

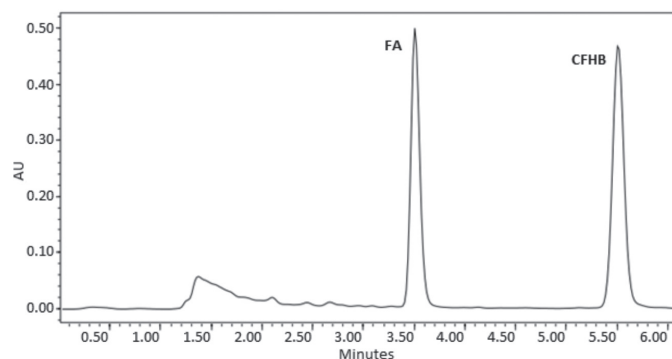


Figure 2. Chromatographic profiles of FA and CFHB as the internal standard in extracted human plasma

FA: Fenofibric acid, CFHB: 4'-chloro-5-fluoro-2-hydroxyl benzophenone

Plasma sample and preparation

Samples were prepared using the liquid-liquid extraction technique. Into 500 μL plasma sample were added 50 μL of IS solution (250 $\mu\text{g}/\text{mL}$) and 1 mL of 1 N HCl followed by mixing for 30 s in a vortex mixer. Then 3 mL of ethyl acetate was added and the mixture was mixed in a roller mixer for 30 min, followed by centrifugation for 15 min at 5000 $\times g$. The top organic layer was separated and evaporated for drying at 40°C using a stream of nitrogen. The residue was reconstituted in 100 μL of the mobile phase and 60 μL was injected into the HPLC system (Waters 1525 binary pump).

Dissolution data analysis

DE was used for comparison of dissolution rates, calculated from the area under the dissolution curves at 60 min, and expressed as a percentage of the rectangle area described by 100% dissolution within the same time. Analysis of variance (ANOVA) was used to compare the DE of test and reference tablets profiles at 60 min ($\alpha=0.05$).

Pharmacokinetic analysis

The pharmacokinetic parameters were calculated by a noncompartmental method. The elimination rate constant (K_{el}) was obtained from the least-square regression log linear portion (the last 3–5 points) of the plasma concentration/time profile. The area under the curve to the last measurable concentration (AUC_{0-t}) was calculated by the linear trapezoidal rule. The area under the curve extrapolated to infinity ($AUC_{0-\infty}$) was estimated with the equation $AUC_{0-t} + C_t/K_{el}$, where C_t is the last measured concentration. The peak plasma concentration (C_{max}) and the corresponding time to peak (T_{max}) were estimated by inspecting the individual drug plasma concentration/time profiles.

Statistical analysis

For the parameters of AUC_{0-t} , $AUC_{0-\infty}$, C_{max} , T_{max} , and $t_{1/2}$, ANOVA was applied for untransformed data. The level of significance was $\alpha=0.05$ and a p value of <0.05 was considered statistically significant.

Correlation development

The principle of statistical moment analysis was utilized to assess the correlation of mean FA plasma concentration versus time in connection with ingestion of the three formulations. Mean dissolution time (MDT) was used to determine the correlation with *in vivo* mean residence time (MRT).

RESULTS

In vitro studies

All products fulfilled the general pharmaceutical requirements for weight variation, content assay, and content uniformity assay. The prepared tablets complied with the official specifications for disintegration time, hardness, and friability. The *in vitro* dissolutions were conducted in two different media (phosphate buffer pH 6.8 and biorelevant FaSSIF) and each at three different rotation speeds to determine their dissolution profile under various conditions.

The *in vitro* dissolution profiles of the SSD (F1) and conventional formulations (F2) are presented in Figure 3, and a summary of the mean DE_{60} of all FA tablets is given in Table 1. Significant differences existed between F1:F2 and F2:FA[®] in all conditions, whereas no significant difference arose from F1:FA[®] in 5 conditions.

In vivo studies

The concentration/time profiles of oral administration of both the SSD and conventional formulations and the reference formulation are depicted in Figure 4. All formulations resulted

in an identical curve of plasma drug concentration versus time. The mean pharmacokinetic parameters of all FA tablets are summarized in Table 2. No significant difference was seen for any of the pharmacokinetic parameters from those formulations.

In vitro-in vivo relationship

Statistical moment analysis has been suggested as a better method to examine the IVIVC. A poor correlation between *in vivo* MRT and *in vitro* MDT for the three formulations was found in the present study (Figure 5).

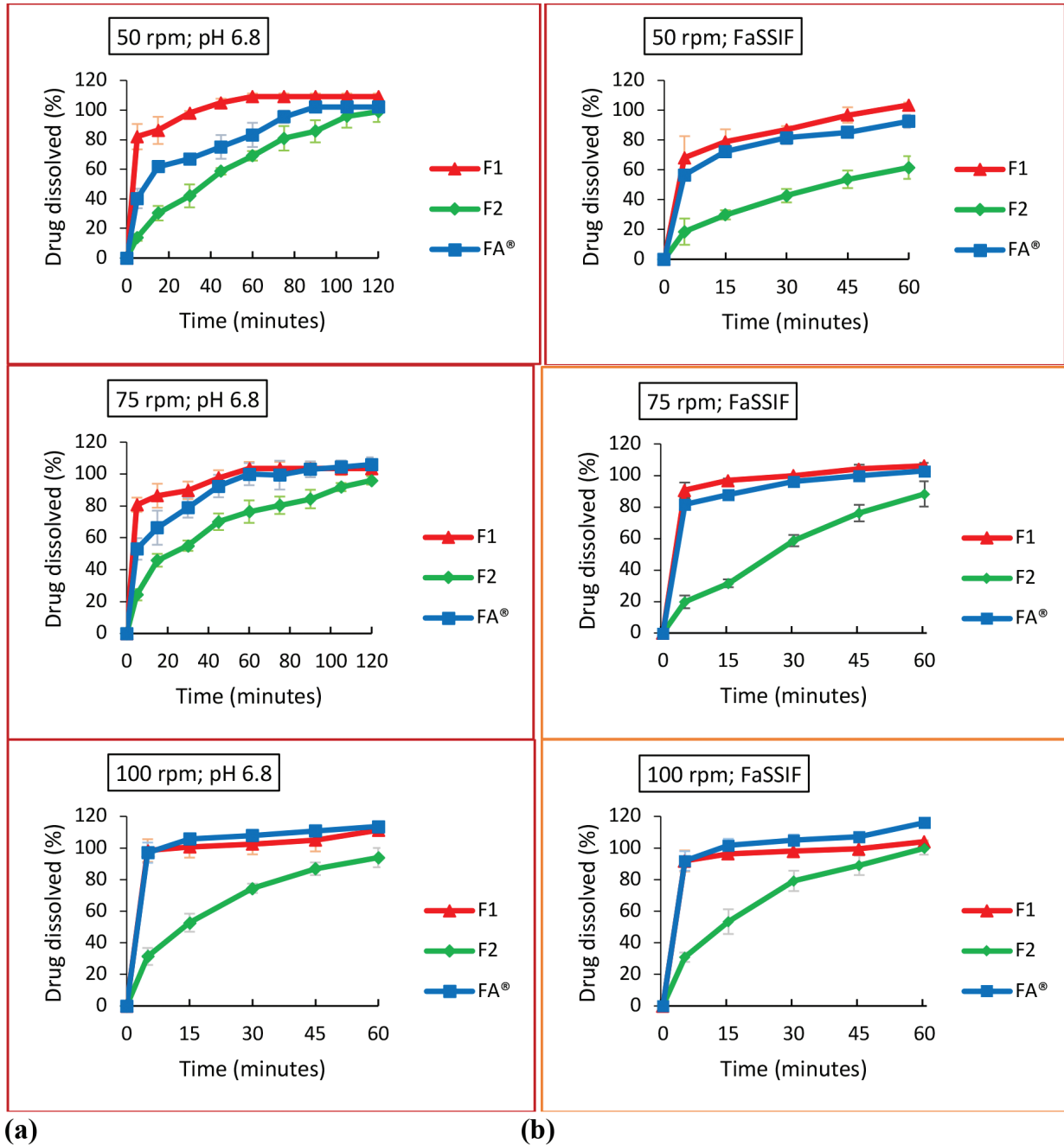


Figure 3. Dissolution profiles of SSD formulation (F1), conventional formulation (F2), and reference formulation (FA[®]) in phosphate buffer pH 6.8 (a) and FaSSIF biorelevant medium (b) at 50, 75, and 100 rpm

SSD: Surface solid dispersion, FA: Fenofibric acid, FaSSIF: Fasted state simulated intestinal fluid

DISCUSSION

The new formulation of FA, a BCS Class II drug, was developed in the present study and selected as an alternative to fenofibrate for oral administration. The SSD formulation was prepared by the solvent evaporation method to increase the dissolution of FA and compared to conventional and reference formulations. A conventional formulation of FA was prepared using wet granulation. All of these formulations met the general pharmaceutical requirements for physicochemical properties. However, significant differences were observed between them. The SSD formulation (F1) led to an instantaneous dissolution of the drug, releasing approximately 90% within the first 5 min in 75 and 100 rpm conditions. In contrast, the conventional formulation (F2) released nearly 80% of the drug within 45 min. Meanwhile, the reference formulation (FA®) yielded the same dissolution as F1. The FA dissolution from F1 increased due to the presence of the polymer and the physical structure of the SSD. In this case, FA was dispersed well on the CS surface, and the fine particles were able to increase its surface area for solubilization. When the CS came into contact with the dissolution medium, it caused swelling and made it possible for FA to be wet to dissolve in the media. The swelling of the CS caused cluster deaggregation of the drug particles and facilitated the dissolution process. Meanwhile, F2 could not achieve rapid dissolution despite the fact that around 80% of the drug dissolved within 45 min, and it satisfied the requirement for immediate drug release dosage form. Based on the data of the *in vitro* dissolution, there were significant differences found in the dissolution performances and therefore included in the development of the IVIVC.

The mean of all pharmacokinetic parameters from each product were not significantly different ($p > 0.05$), suggesting that the plasma profiles generated by FA® were comparable to those produced by F1 and F2. The intrasubject CV was relatively small. Based on this analysis, F1 and F2 could be considered bioequivalent with FA®.

An appropriate condition of the dissolution study based on *in vivo* performance was adapted for routine and in process control for the FA formulation. The condition of dissolution in this study was similar to that proposed by the FDA (in pH 6.8 and 75 rpm) and correlated with the plasma profiles already obtained by performing bioavailability studies. Four correlation levels were defined in the IVIVC. It has been suggested to employ statistical moment analysis as a better method for examining the IVIVC. A level B correlation used all *in vitro* and *in vivo* data

and it was therefore employed between MRT and MDT. There was no correlation ($R^2=0.028$) between MRT and MDT of the three formulations found in this study. Since the dissolution of the drug from F2 was slower than that of FA®, the IVIVC could not be achieved.

The *in vitro* dissolution behavior of FA did not reflect their *in vivo* properties in the fasted condition. The use of single medium dissolution for FA in the present study failed to create *in vivo* correlation. Moreover, a relatively significant difference was observed between the dissolution properties of both F1:F2 and F2:FA®. These formulations as *in vivo* bioequivalences are shown in Figure 4 and Table 2. Apparently, the dissolution media

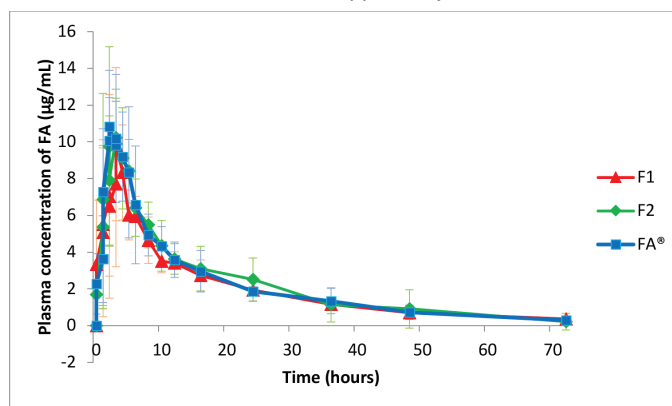


Figure 4. Average plasma concentration vs. time profiles of FA after oral administration (105 mg doses) of SSD formulation (F1), conventional formulation (F2), and reference formulation (FA®) in nine healthy male subjects. Data are shown as mean \pm SD

FA: Fenofibric acid, SSD: Surface solid dispersion, SD: Standard deviation

Table 2. Pharmacokinetic parameters of fenofibric acid after single dose oral administration of three different formulations in nine healthy male subjects

Parameters	F1 (Mean \pm SD)	F2 (Mean \pm SD)	FA® (Mean \pm SD)
AUC ₀₋₇₂ ($\mu\text{g}\cdot\text{h}/\text{mL}$)	136.94 \pm 30.85	157.57 \pm 55.81	150.57 \pm 40.49
AUC _{0-∞} ($\mu\text{g}\cdot\text{h}/\text{mL}$)	148.45 \pm 34.62	171.09 \pm 62.95	158.22 \pm 42.14
C _{max} ($\mu\text{g}/\text{mL}$)	11.79 \pm 3.72	12.94 \pm 3.95	14.12 \pm 2.68
T _{max} (h)	2.99 \pm 0.39	2.67 \pm 0.41	2.63 \pm 0.35
T _{1/2} (h)	20.97 \pm 3.36	19.72 \pm 6.65	17.25 \pm 4.12

SD: standard deviation, FA: Fenofibric acid

Table 1. Dissolution parameters (DE₆₀) of fenofibric acid from three formulations at six conditions

Code	DE ₆₀ (%) \pm SD					
	50 rpm		75 rpm		100 rpm	
	pH 6.8	FaSSIF	pH 6.8	FaSSIF	pH 6.8	FaSSIF
F1	92.55 \pm 3.50	85.05 \pm 1.53	87.88 \pm 4.96	95.78 \pm 1.79	99.11 \pm 6.07	93.89 \pm 3.26
F2	41.55 \pm 1.64	40.29 \pm 4.31	53.44 \pm 3.89	53.77 \pm 2.57	66.97 \pm 3.29	69.50 \pm 4.38
FA®	63.79 \pm 3.71	75.35 \pm 1.34	75.81 \pm 5.91	90.43 \pm 1.21	93.11 \pm 0.73	96.23 \pm 3.03

DE: Dissolution efficiency, SD: Standard deviation, FaSSIF: Fasted state simulated intestinal fluid, FA: Fenofibric acid

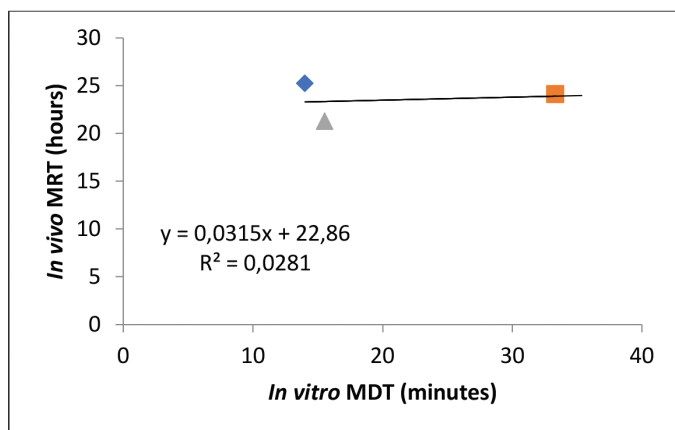


Figure 5. Correlation between MDT of *in vitro* dissolution (in pH 6.8; 75 rpm) and MRT of plasma drug concentration from three formulations F1 (◆), F2 (■), and FA[®] (▲)

MDT: Mean dissolution time, MRT: Mean residence time, FA: Fenofibric acid

in this study did not completely simulate the conditions of the GI tract. It is reported that a biorelevant dissolution medium has the ability to predict well the *in vivo* performances of insoluble drugs. However, that purpose was not achieved in the present study. Further studies are suggested using biorelevant pH gradient methods to obtain a strong IVIVC.

In most cases, statistically significant differences of *in vivo* MRT among various formulations were not significant enough to produce a strong correlation between MRT and MDT. For a 105 mg dose of FA and aqueous solubility of 0.162 mg/mL, 650 mL of fluid was required to dissolve a single dose. Therefore, the volume of water taken initially not only dissolved the drug to a great extent but also decreased the dependency of drug absorption on drug dissolution.¹⁷ This phenomenon led to a nil correlation in the present study. The fact that the *in vitro* differences in the early dissolution were not realized in the *in vivo* differences was attributed to the continuous excretion of bile that happened in the GI tract.¹⁸ There was still a possibility that FA was absorbed with the help of a transporter (facilitated transport) and/or energy (active transport). However, the amount was likely to be limited, even if much was dissolved.

Study limitation

The present study was limited by its use of a single medium method for dissolution testing. Further studies are suggested to use biorelevant pH gradient methods to obtain a strong IVIVC.

CONCLUSION

The *in vitro* dissolution behavior of FA using a single medium did not reflect its *in vivo* properties in the fasted condition. There was no correlation between the *in vitro* dissolution and the *in vivo* bioavailability of FA in this condition.

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Ethnobotanical Study of Medicinal Plants in Aziziye District (Erzurum, Turkey)

Aziziye (Erzurum, Türkiye) İlçesindeki Tıbbi Bitkilerin Etnobotanik Çalışması

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ABSTRACT

Objectives: The present research was conducted to document the usage of medicinal plants, plant parts utilized, and methods of preparation by the people living in Aziziye district, situated in the western part of Erzurum.

Materials and Methods: The medicinal plant species utilized by local public for remedial aims were collected and identified. The related knowledge about conventional herbal medicine was collected, herbarium materials were prepared, and they were deposited in the Herbarium of the Faculty of Science, Atatürk University.

Results: A total of 77 medical plants pertaining to 30 families were defined in this research. Amongst these, 62 species grew naturally and 15 species were cultivated. The most widespread medicinal plant families were Asteraceae (14), Rosaceae (7), Lamiaceae (5), and Apiaceae (5). The most widespread preparation was decoction.

Conclusion: The ethnobotanical outcomes documented in this study provide practical evidence about the use of medicinal plants among the inhabitants of Aziziye District. Furthermore, the results revealed that the medicinal plants of the region are a major source of herbal drugs for primary healthcare utilized among the rural communities. This study can be utilized as baseline knowledge for further scientific research to improve new plant-based commercial drugs, and may transfer the traditional information as regards usage of medicinal herbs to new generation.

Key words: Aziziye, ethnobotany, Erzurum, medicinal plants, Turkey

ÖZ

Amaç: Sunulan bu araştırma, Erzurum'un batı kesiminde yer alan Aziziye ilçesinde yaşayan insanların kullandıkları tıbbi bitkilerin kullanımı, kullanılan bitki kısımları ve hazırlama yöntemlerini belgelemek amacıyla yapılmıştır.

Gereç ve Yöntemler: Yerel halkın tedavi amaçları için kullandığı tıbbi bitkiler toplanıp, tanımlandı. Geleneksel bitkisel ilaçlarla ilgili bilgiler toplandı; herbaryum materyalleri hazırlandı, Atatürk Üniversitesi Fen Fakültesi Herbaryumu'na konuldu.

Bulgular: Araştırmada 30 familyaya ait toplam 77 tıbbi bitki tanımlanmıştır. Bu türlerin 62'si doğal olarak yetişmekte, 15 tür ise ekilmektedir. En yaygın tıbbi bitkiler Asteraceae (14), Rosaceae (7), Lamiaceae (5) ve Apiaceae (5) familyalarına aittir. En yaygın hazırlama şekli dekoksiyondur.

Sonuç: Bu çalışmada elde edilen etnobotanik sonuçlar, tıbbi bitkilerin Aziziye ilçesi sakinleri arasında kullanımı hakkında pratik veriler sunmaktadır. Dahası, bu sonuçlar kırsal topluluklar arasında kullanılan, bölgedeki tıbbi bitkilerin, birinci basamak sağlık hizmetleri için önemli bitkisel ilaç kaynağı olduğunu ortaya koymaktadır. Bu araştırma, bitki esaslı yeni ticari ilaçların iyileştirilmesinde daha fazla bilimsel araştırma için temel bilgi kaynağı olarak kullanılabilir ve genç nesillerde tıbbi bitkilerin geleneksel kullanımı ile ilgili bilgi aktarılmasına olanak sağlayacaktır.

Anahtar kelimeler: Aziziye, etnobotanik, Erzurum, tıbbi bitkiler, Türkiye

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INTRODUCTION

Herbs have been invariable sources of both protective and therapeutic traditional medicine preparations for people since ancient times.¹ The World Health Organization forecasted that about 60% of the worlds inhabitants in developing countries trust herbs for curing a variety of illnesses, owing to the lack of modern healthcare resources.²

Turkey's flora is very rich, comprising about 11.000 species, 33% of which are endemic and Turkish people have utilized these herbs for diversified aims. Along with its rich flora, a wide diversity of habitats also exist in Turkey.^{3,4} The flora of Turkey is rich owing to its different ecological zones, geographical variations, and diversified climates. This variation in flora has contributed a rich source of medicinal herbs, which has long been utilized by Anatolian people, and therefore there has been an accumulation of valuable folk medicinal information in the district.⁵

In Turkey ethnobotanical research has been performed since the Republican period began in 1923 and the effects and names of plants have been documented; these studies have increased in recent years in particular.⁵

The flora of East Anatolia in Turkey is also rich owing to its different ecological zones, geographical variations, and diversified climates. Erzurum is a medium-size city in eastern Turkey. The province is located in the upper basin of Karasu, the source of the River Euphrates, around the edge of Mount Ereğli in the Palandöken mountain range in the southeast of Erzurum plain, and situated on a curved plateau 1850 to 1980 m above sea level. The province of Erzurum is a local center in whose zone of effect there are all of the provinces of Erzurum, Kars, Iğdır, Ardahan, and Ağrı; but not Refahiye, İliç, and Kemaliye districts, all in Erzincan Province; Bayburt and Yusufeli district; Varto, Bulanık, and Malazgirt districts of Muş Province; Karlıova District of Bingöl Province; and Pulumur District of Tunceli Province.⁶

The purpose of the present research was to introduce information about the utilization of conventional herbal medicine and other uses of the plants in these districts and was conducted to document the usage of medicinal plants, plant parts utilized, and methods of preparation by the people living in Aziziye, situated in western Erzurum.

MATERIALS AND METHODS

Investigation region

The largest geographical area of Turkey is Eastern Anatolia and it is far from the effect of the sea owing to its being surrounded by coastal mountain ranges.⁷ Erzurum is established in the Upper Euphrates section of the Eastern Anatolian region. It is the largest city in Eastern Anatolia, with a population of 780.847 and an area of 25.066 km², and it is an old settlement. It lies between 40°15' and 42°35' eastern longitudes and 40°57' and 39°10' northern latitudes (Figure 1). Erzurum neighbors Rize, Artvin, and Ardahan in the north, Kars and Ağrı in the east, Bingöl and Muş in the south, and Erzincan and Bayburt in

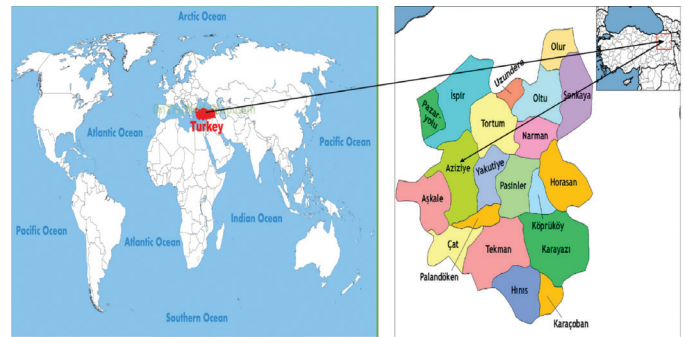


Figure 1. Geographical location of the investigation region

the west. Mean daily temperature is 19.6 in summer and -8.6°C in winter. Annual rainfall is 453 mm and the count of days on which it snows is 50. The duration of snow cover is 114 days.⁸

Data collection

The field research was conducted through collecting ethnobotanical knowledge using structured and semistructured interviews with all knowledgeable people native to 5 villages, namely Söğütlü (1), Çıkıklı (2), Sorkunlu (3), Kapılı (4), and Beypınarı (5). This study is a project of the Ministry of Forestry and so they decided to study these villages. Midwives, shepherds, woodsmen, farmers, healers, beekeepers, housewives, teachers, mukhtars, and people collecting plants, a total of 98 people, were interviewed face to face. While 56 of the informants were women (57.14%), the remaining 42 were men. For each recorded plant one questionnaire was filled out during the conversations and videos, photos, and records were obtained from these people with their permission. The interviews were conducted in a diversity of places (tea houses, farms, mosques, houses, gardens, fields, etc.). Conversant adults, patients, and local healers were the resources of knowledge and data (local names, therapeutic effects, part (s) of plants utilized, and methods of preparation and administration). Patient consent was not required for the study.

Plant materials

The plants were collected in 2017 and 2018 from the villages. The collected herbs were pressed and described by the author Özkan Aksakal using *Flora of Turkey and the East Aegean Islands* and *Türkiye Bitkiler Listesi (Damarlı Bitkiler)*.⁹⁻¹¹ The plant family names were organized in alphabetical order. The scientific names of the herb species were given with reference to the plant list.¹² Voucher specimens were stored at the Herbarium of the Faculty of Science, Atatürk University.

Statistical analysis

The data are presented as mean \pm standard error and variation analysis was performed through one-way ANOVA determined via Bonferroni complementary analysis, which was conceived to represent statistical significance.

RESULTS

The demographic characteristics of the research participants were recorded through face-to-face interviews. A total of 98 participants (56 female, 42 male) were interviewed (9 persons

aged between 27 and 36 years, 18 persons aged between 37 and 46, 26 persons aged between 47 and 56, 23 persons aged between 57 and 66, and 22 persons aged over 66). All of the informants were native and they were living in the villages. Forty-three of the participants had never received education (Table 1).

A total of 77 medicinal plant taxa were collected in Aziziye District (Erzurum, Turkey) and they belong to 30 plant families. Amongst them, 62 species are wild and 15 species are cultivated plants. The 77 herbs defined in the area prepared in alphabetical order of their family and botanical names are presented in Table 2. *Anthemis calcarea*, *Scorzonera tomentosa*, *Tragopogon aureus*, *Cephalaria anatolica*, and *Quercus macranthera* are endemic species and therapeutic (Table 2). The most widespread medicinal plant families were Asteraceae (14), Rosaceae (7), Lamiaceae (5), and Apiaceae (5).

The most widely utilized plant organs to prepare remedies were the aerial parts (27), leaves (16), fruits (13), flowers (12), roots (11), seeds (9), and barks (6), although branches, bulbs, stems, and tubers were also utilized in some remedies. On occasion, local people also utilized other components, such as butter, lemon, soap, olive oil, beeswax, egg, or honey to prepare remedies.

Table 1. Demographic characteristics of the participants

Demographic characteristics	Number
Age	
27-36	9
37-46	18
47-56	26
57-66	23
Above 66	22
Sex	
Female	56
Male	42
Educational level	
Illiterate	43
Primary school	38
Secondary school	12
High school	4
University	1
Employment status	
Housewife	56
Farmer	35
Pensioner	4
Shepherd	1
Other jobs	2
Total	98

The major methods for preparing remedies were decoction, infusion, fresh, chewing, boiling, crushing, and cooking. Decoction (34), crushing (28), infusion (6), and cooking (6) are the methods generally utilized for the preparation of remedies (Table 2).

DISCUSSION

Plant sources have a long history of being utilized as medicinal necessities. It is frequently mentioned that 80% of the world's population still relies on conventional medicines to meet their primary healthcare needs and almost 25% of modern medicines are derived from nature, many of which were derived from traditional utilizations. The utilization of traditional medicines is usually affected by the accessibility, availability, and admissibility of healthcare services. Especially in distant regions of developing countries, medicinal plants may form the only existing source of healthcare.

It was seen that some medicinal plant taxa were widely utilized for commercial aims owing to the research conducted in study regions. A large part of the people in the villages of the area mentioned that *Cephalaria* spp. have been utilized as a hemostatic and for wound healing. Moreover, *Alkanna orientalis*, *Plantago* spp., and *Malva* spp. have been utilized for wounds as an antiinflammatory.

As a result of the study of the plant names, it was determined that most of them were derived from Turkish. Gümüşhane, Erzincan, Kars, Bingöl, Muş, and Ağrı are close to our research area. However, the names of some local plants utilized in these areas varied, such as *Plantago major* (pel hewes, omulwaş, sinirli ot, sinirotu), *Malva neglecta* (dolik, tolluk), *Rosa canina* (gül tonik, şilan), *Urtica dioica* (gezgezok, gerzinik), *Gundelia tournefortii* (kinger, kereng), *Eremurus spectabilis* (yelig, gulik), *Alkanna orientalis* (gelzun, havajo), and *Rheum ribes* (ribes, riwes, rewas).¹³⁻¹⁸

The informants utilized medical plants mainly for the treatment of wounds and skin conditions, digestive system diseases, respiratory diseases, kidney and urinary system disorders, and diabetes mellitus. It has been determined that the number of plants used for cardiovascular problems is the lowest.

The species *Plantago* spp., *Malva neglecta*, *Rheum ribes*, and *Rumex crispus* were the most widely utilized medicinal plants and were recorded in Erzurum in the literature. With respect to that literature, *Prangos ferulacea* (diabetes), *Achillea biebersteinii* (wounds), *A. millefolium* (wounds), *Anthemis* spp. (stomachache), *Cichorium intybus* (wounds), *Alkanna* spp. (wounds), *Cephalaria* spp. (wounds), *Malva* spp. (wounds), *Rheum ribes* (diabetes), *Ranunculus* spp. (rheumatism), and *Rosa pimpinellifolia* (hemorrhoids) have similar uses.¹¹⁻¹⁶

Usages of members of the families Acanthaceae, Amaryllidaceae, Aristolochiaceae, Capparaceae, Caryophyllaceae, Cistaceae, Corylaceae, Crassulaceae, Cuscutaceae, Ephedraceae, Ericaceae, Gentianaceae, Geraniaceae, Illecebraceae, Loranaceae, Onagraceae, Orchidaceae, Paeoniaceae, Papaveraceae, Plumbaginaceae, Polygalaceae, Portulacaceae, Primulaceae, Resedaceae, Thymelaeaceae, Tiliaceae,

Table 2. Traditional uses of medicinal plants in Aziziye (west of Erzurum, Turkey)

No.	Family	Plant species, voucher specimen, endemism, and location	Local name	Plant part (s) used ^a	Preparation ^b	Adm. ^c	Use
	Amaryllidaceae	<i>Asphodelus aestivus</i> Brot., ATA 10097, 2	Çiriş, ciriş	Aer	Raw	Eat	Digestive, constipation
	Amaryllidaceae	<i>Eremurus spectabilis</i> M.Bieb., ATA 10098, 3	Çiriş, ciriş	Aer	Raw	Eat	Digestive
	Amaryllidaceae	<i>*Allium cepa</i> L., ATA 10100, 1-5	Soğan	Bul	Coo	Ext	Antiinflammatory, scar, wounds
Raw					Eat	Galactagogue	
Boi					Ext	Toothache, gingivitis	
Cru					Ext	Ecchymosis	
	Amaryllidaceae	<i>*Allium sativum</i> L., ATA 10101, 1-5	Sarımsak	Bul	Cru mix with honey	Int	Cardiac disorders, antihypertensive, antiinflammatory
	Apiaceae	<i>Eryngium campestre</i> L., ATA 10019, 1, 2	Boğa dikeneni	Roo	Cru with onion and add green soap, milk	Ext Ps	Antiinflammatory, furuncle
	Apiaceae	<i>Prangos ferulacea</i> (L.) Lindl., ATA 10021, 1, 3	Çaşır, çağşır, çakşır	Roo	Dec	Int	Diabetes
	Apiaceae	<i>Anthriscus nemorosa</i> (M.Bieb.) Spreng., ATA 10023, 2	Hırhindik, hrhındok	Aer	Dec	Ext	Carminative
	Apiaceae	<i>Ferula orientalis</i> L., ATA 10025,1-5	At çaşırı, çağşır, çakşır	Roo	Dec	Int	diabetes
	Apiaceae	<i>Zosima absinthifolia</i> Link, ATA 10026, 3	Peynir otu	Aer with Flo	Inf	Int	hemorrhoid
	Asteraceae	<i>Achillea millefolium</i> var <i>millefolium</i> L., ATA 10028, 1-5	Civanperçemi, kılıç otu, sarı çiçek	Lea	Cru	Ext	Wounds, hemostatic
Flo				Dec	Int	Menstrual pain, menstrual irregularity	
				Cru and mix with honey	Eat	Antitussive	
	Asteraceae	<i>Cichorium intybus</i> L., ATA 10030, 2, 4	Çatlangoz çatlangos, çatlankuş, çatlankuz, çatlangaz	Aer with flo	Ps, burnt and mix with butter	Ext	Wounds
Flo				Burnt butter	Ext	Wounds, scar	
Roo				Burnt and mix with butter	Ext	Eczema	
	Asteraceae	<i>Achillea biebersteinii</i> Hub.-Mor., ATA 10031, 1-5	Kılıç otu, sarı civan perçemi, kırk kilit	Lea	Cru	Ext	Wounds, hemostatic
Aer				Boi Ps	Ext	Hemostatic, eczema	
	Asteraceae	<i>Anthemis cretica</i> L., ATA 10032, 3	Papatya	Aer with flo	Dec	Int	Sore throat, expectorant, antiinflammatory
	Asteraceae	<i>**Anthemis calcarea</i> Sosn., ATA 10034, 4	Papatya	Flo	Inf	Int	Stomachache
					Dec	Int	Sore throat, expectorant, antiinflammatory

Asteraceae	<i>Helichrysum plicatum</i> DC., ATA 10035, 1-5	Altın otu, sarı çiçek	Flo	Inf	Int	Kidney stone, diuretic
Asteraceae	<i>Gundelia tournefortii</i> L., ATA 10039, 1-5	Kenger, kelenk	Roo	Raw	Ext chewing gum	Stomach disorders, against nausea
Asteraceae	<i>Scorzonera latifolia</i> (Fisch. & C.A.Mey.) DC., ATA 10040, 1-5	Yakıotu, sakız	Lea	Raw	Ext juice of roots used as gum	Plaster, against nausea
Asteraceae	** <i>Scorzonera tomentosa</i> L., ATA 10041, 1-5	Yakıotu, sakız	Roo	Raw	Eat	Hemostatic
Asteraceae	<i>Tragopogon reticulatus</i> Boiss. et Huet, ATA 10042, 1-5	Yemlik	Aer	Cru	Ext	Plaster, wounds, hemostatic
Asteraceae	<i>Tragopogon buphthalmoides</i> (DC.) Boiss., ATA 10044, 1-5	Yemlik	Aer	Cru	Ext	Plaster, wounds, hemostatic
Asteraceae	** <i>Tragopogon aureus</i> Boiss., ATA 10045, 4	Yemlik	Aer	Cru	Ext	Plaster, wounds, hemostatic
Asteraceae	<i>Artemisia absinthium</i> L., ATA 10047, 4, 5	Acı yavşan otu	Aer with flo	Raw	Ext Chewing	Stomachache
Asteraceae	<i>Artemisia campestris</i> L., ATA 10049, 5	Yavşan	Aer	Cru	Ext Chewing	Stomachache
Asteraceae	<i>Artemisia santonicum</i> L., ATA 10050, 5	Yavşan, süpürge otu	Aer with Fru	Cru	Int only juice	Stomachache
			Aer	Dec	Int	Shortness of breath
Amaranthaceae	<i>Beta lomatogona</i> Fisch. & C.A.Mey., ATA 10061, 1, 4	Kızılca	Aer	Dec	Int	Constipation, digestive
Amaranthaceae	<i>Beta trigyna</i> Waldst. & Kit., ATA 10062, 4	Kızılca	Aer	Dec	Int	Constipation, digestive
Berberidaceae	<i>Berberis crataegina</i> DC., ATA 10051, 3, 4	Kızambuk, karambuk	Roo	Boi	Ext Bathing with yellow juice	Jaundice in children
Betulaceae	<i>Betula alba</i> L., ATA 10002, 3, 5	Huş ağacı, kayın	Bar	Dec	Ext, Gar	Sore throat, antiseptic
Boraginaceae	<i>Alkanna orientalis</i> (L.) Boiss., ATA 10054, 1-5	Havaciva, havajo, hevajo	Roo	Cru coo with butter	Ext	Wounds, burns, scar, antiinflammatory
				Cru with olive oil, added beeswax	Ext	Wounds, burns, scar, antiinflammatory, ulcer
				Dec	Ext	Wounds, scar, antiinflammatory
				Boi and add butter	Int before breakfast	Asthma, bronchitis, shortness of breath, ulcer
Brassicaceae	* <i>Brassica napus</i> L., ATA 10058, 3, 4	Şalgam	Roo	Raw, mix with egg and lemon	Int	Kidney stone, flu

Caprifoliaceae	<i>Cephalaria tchihatchewii</i> Boiss., ATA 10072, 1-5	Gevreik, gevreyik, gevrek	Aer	Raw Cru	Ext	Hemostatic, wounds, scar
Caprifoliaceae	** <i>Cephalaria anatolica</i> Shkhiyan, ATA 10073, 1-5	Gevreik, gevreyik, gevrek	Aer	Raw Cru	Ext	Hemostatic, wounds
Cornaceae	* <i>Cornus mas</i> L., ATA 10066, 2	Kızılıçık	Fru	Dec Raw	Int, Eat Eat	Diarrhea Diarrhea
Cucurbitaceae	* <i>Cucurbita pepo</i> L., ATA 10067, 1-5	Kabak	See	Cru mix with honey	Eat 1 tablespoon before breakfast	Anthelmintic
Cucurbitaceae	* <i>Cucumis sativus</i> L., ATA 10070, 1-5	Salatalık	Per	Raw	Ext	Headache
Cupressaceae	<i>Juniperus communis</i> L., ATA 10071, 1-5	Ardıç	Ste, Bar	Tar	Ext	Skin disorders, eczema, wounds
Elaeagnaceae	<i>Hippophae rhamnoides</i> L., ATA 10075, 3	Ekşi, yabani iğde	Lea Fru	Inf Dec	Int Int	Diabetes Diabetes
Elaeagnaceae	<i>Elaeagnus angustifolia</i> L., ATA 10076, 4	iğde	Lea	Dec	Int	Diabetes
Euphorbiaceae	<i>Euphorbia stricta</i> ATA 10078, 1, 2	Sütlücan	Lat	Ps	Ext	Antihemorrhagic
Euphorbiaceae	<i>Euphorbia oblongifolia</i> (K.Koch) K.Koch, ATA 10079, 3	Sütlücan, sütleğen	Lat	Ps	Ext	Antihemorrhagic
Fabaceae	* <i>Lens culinaris</i> Medik. ATA 10081, 1-5	Yeşil mercimek	See	Coo	Int before breakfast	Anthelmintic
Fabaceae	* <i>Lathyrus sativus</i> L., ATA 10107, 3	Küşne	See	Boi with salt and sugar	Eat for 10 days before breakfast	Anthelmintic
Fabaceae	<i>Astragalus microcephalus</i> Willd., ATA 10082, 1-5	Geven	Roo	Gum	Ext	Hand cracks, emollient
Fagaceae	** <i>Quercus macranthera</i> Fisch. & C.A.Mey. ex Hohen., ATA 10085, 3	Palut, pelit	Ped	Burnt and mix with butter	Ext	Wounds, edema
Lamiaceae	<i>Mentha longifolia</i> (L.) L., ATA 10088, 3,4	Yarpuz	Aer	Dec	Ext Ps	Headache
Lamiaceae	<i>Mentha aquatica</i> L., ATA 10089, 2,3	Su nanesi	Lea	Dec	Int	Sore throat, against nausea
Lamiaceae	<i>Salvia verticillata</i> subsp. <i>amasiaca</i> (Frey & Bornm.) Bornm., ATA 10090, 5	Adaçayı	Aer	Dec	Ext Gar	Toothache
Lamiaceae	<i>Origanum rotundifolium</i> Boiss., ATA 10095, 3	Dağ kekiği, anık	Aer	Inf	Int	Cough, sedative, stomachache

Lamiaceae	<i>Micromeria fruticosa</i> (L.) Druce, ATA 10096, 2	Çemen, dağ kekiği	Aer	Dec	Int	Cough, stomachache
Linaceae	* <i>Linum usitatissimum</i> L. ATA 10103, 4	Zegerek	See	Coo	Ext	Wounds, scar
Juglandaceae	* <i>Juglans regia</i> L., ATA 10105, 3	Ceviz	Bar, Per	Dec	Int	Diarrhea, hair loss
Malvaceae	<i>Malva neglecta</i> Wallr., ATA 10106, 1-5	Ebegümeci, ebekömeci, ebegümeci	Aer with flo	Boi	Int	Expectorant, bronchitis, asthma, sore throat
			Lea	Boi	Ext use pulp	Wound healing, antiinflammatory, stomachache, prostate
			Aer	Raw Cru	Ext	Rheumatism
				Dec	Int before breakfast	Cold, expectorant, bronchitis, asthma, urinary system disorders
			Lea	Dec with leaf of <i>Plantago major</i>	Int	Antiinflammatory, edema
				Coo with flour	Ext	Edema
Malvaceae	<i>Malva sylvestris</i> L., ATA 10107, 1-5	Ebegümeci, ebekömeci, ebegümeci	Aer with flo	Dec	Inh	Cold, expectorant, bronchitis, asthma, mouth sore
			Lea	Dec	Ext	Wounds, scar, antiinflammatory, edema
Moraceae	* <i>Morus alba</i> L., ATA 10111, 1-5	Dut	Dried Fru	Dec	Int	Sore throat, expectorant, stomachache
Moraceae	* <i>Morus nigra</i> L., ATA 10112, 3	Kara Dut	Fru	Cru	Ext	Eczema
Pinaceae	<i>Pinus sylvestris</i> L., ATA 10116, 1-5	Çam, sarı çam	Bra, Ste	Res	Ext	Hand cracks, emollient, skin disorders
				Dry distillation Tar Boi with butter	Ext Ps	Ecchymosis, tubercle, crack, wounds, emollient
				Dry distillation Tar	Ext	Eczema, skin disorders, wounds
Poaceae	* <i>Triticum vulgare</i> Vill., ATA 10117, 1-5	Den, buğday	See	Cru mix with egg white	Ext	Fracture, tubercle, paronychia
Poaceae	* <i>Hordeum vulgare</i> L., ATA 10120, 1-5	Arpa	Tes	Cru mix with olive oil	Ext	Wounds, hand cracks, emollient
			See	Dec	Int	Kidney stone, urinary system diseases, diuretic, prostate ailments

Polygonaceae	<i>Rumex crispus</i> L., ATA 10121, 1-5	Evelik	Lea	Boi	Ext Use pulp	Sore throat, stomachache
				Dec	Int	kidney stone, urinary system diseases, diuretic, hemorrhoid, constipation
Polygonaceae	<i>Rheum ribes</i> L., ATA 10123, 1-5	Eşgin, ışgın	Roo	Dec	Int	Diabetes
			See	Cru mix honey	Int	Hemorrhoids, constipation
Plantaginaceae	<i>Plantago major</i> L., ATA 10125, 1-5	Bağa yaprağı, bağa otu	Lea	Raw	Ext	mastitis, mammalgia, slipped disc, furuncle, wounds
				Dec	Ext	Sore throat, urinary system diseases, wounds, hemostatic
Plantaginaceae	<i>Plantago lanceolata</i> L., ATA 10126, 1-5	Bağa yaprağı, bağa otu, pelheves	Lea	Raw	Ext	Mastitis, mammalgia, slipped disc, furuncle, wounds, edema
				Dec	Int	Hemorrhoid
Ranunculaceae	<i>Ranunculus kotschyi</i> Boiss., ATA 10128, 1-5	Katır tırnağı, mayıs çiçeği, düğün çiçeği	Flo or Lea	Cru	Ext Applied only 2-3 minutes	Rheumatism
			Aer with flo	Cru mix with honey	Ext Applied on knees for 1-2 minutes	Rheumatism
Rosaceae	<i>Rosa canina</i> L., ATA 10131, 1-5	Kuşburnu	Fru	Dec	Int	Diuretic, urinary system diseases, cold, flu
Rosaceae	<i>Cotoneaster integerrimus</i> Medik., ATA 10134, 5	Girgit, gıvgıt	Fru	Dec	Int	Antidiarrheal
Rosaceae	<i>Rosa pimpinellifolia</i> Bunge, ATA 10133, 1-5	Karakara, koyun gözü	Fru	Dec	Int	Hemorrhoids
Rosaceae	<i>Malus sylvestris</i> (L.) Mill., ATA 10137, 1-5	Ekşi elma, yabani elma	Fru	Dec	Int Before breakfast	Diabetes
				Coo, wrapped in a cloth	Ext	Earache
Rosaceae	<i>Crataegus azarolus</i> var. <i>pontica</i> (K.Koch) K.I.Chr., ATA 10140, 3	Alıç, aluç	Fru	Raw, mix with lemon and honey	Int	Cardiac diseases, hypertension
Rosaceae	<i>Crataegus orientalis</i> Pall. ex M.Bieb., ATA 10141, 2	Alıç, aluç	Fru	Raw	Int	Cardiac diseases, hypertension
Rosaceae	<i>Pyrus elaeagnifolia</i> Pall., ATA 10144, 1-5	Yabani armut, ahlat	Fru	Raw	Int Eat	Diarrhea

Salicaceae	<i>Populus nigra</i> L., ATA 10146, 1-5	Kara kavak	Bar	Cru mix with egg and soap	Ext	Fracture, dislocation, wounds
Salicaceae	<i>Populus alba</i> L., ATA 10147, 1-5	Ak kavak	Bar	Cru, mix with white and soap	Ext	Fracture, dislocation, wounds
Salicaceae	<i>Salix alba</i> subsp. <i>alba</i> L., ATA 10148, 1-5	Söğüt	Bar	Dri and Cru	Ext	Antiinflammatory, wounds
			Lea	Cru	Ext	Antipyretic, heat prostration
Scrophulariaceae	<i>Verbascum oreophilum</i> C. Koch, ATA 10151, 1	Siğir kuyruğu, girç	Lea	Boi	Inh	Hemorrhoids
Solanaceae	* <i>Solanum tuberosum</i> L., ATA 10153, 1-5	Kartol	Tub	Raw, Cut into small pieces, add salt then applied the head and foot	Ext	Headache
Solanaceae	<i>Hyoscyamus niger</i> L., ATA 10154, 1-5	Batbat, patpat, deli batbat	See	Hea	Inh into mouth	Toothache
Urticaceae	<i>Urtica dioica</i> subsp. <i>dioica</i> L., ATA 10156, 1-5	Isırgan	Aer	Dec	Int	Diuretic, urinary system diseases, shortness of breath
			See	Raw, mix with honey	Int Before breakfast	Arthritis, asthma

*Cultivated plants, **Endemic plants, *Plant part (s) used: Aer: Aerial parts, Bar: Bark, Bra: Branches, Bul: Bulb, Flo: Flowers, Fru: Fruits, Lat: Latex, Lea: Leaves, Ped: Peduncle, Res: Resin, Roo: Roots, Ste: Stem, See: Seeds, Per: Pericarp, Tub: Tuber, Who: Whole plant, *Preparations: Boi: Boiled, Cooked: Coo, Cru: Crushed, Dec: Decoction, Hea: Heated, Inf: Infusion, Mixed: Mix, Ps: Paste, Adm: Administration, Int: Internal use, Ext: External use, Eat: Eaten as meal, Gar: Gargle, Inh: Inhalation, ATA: Atatürk University

Typhaceae, Valerianaceae, and Violaceae were found in other studies but were not recorded in the nearby areas.

The informants stated that *Ranunculus* spp. should be utilized with care owing to their serious side effects such as edema, irritation, and redness and so these species must not be held on the skin for more than 1-2 min.

Furthermore, during this research we detected that some medicinal plants are utilized as spices and this is more prevalent in rural areas. *Mentha longifolia*, *Mentha aquatica*, *Origanum rotundifolium*, and *Micromeria fruticosa* are consumed as spices. Especially members of the family Lamiaceae are utilized as spices. In the area, some of the wild edible plants such as *Anthemis cretica*, *Anthemis calcarea*, *Mentha longifolia*, *Mentha aquatica*, *Salvia verticillata* subsp. *amasiaca*, *Origanum rotundifolium*, *Micromeria fruticosa*, *Rosa canina*, *Rosa pimpinellifolia*, *Crataegus pontica*, and *Crataegus orientalis* are utilized as herbal tea.

CONCLUSION

With the rapid improvement in mobile communication tools, deforestation through anthropogenic activities, and migration of the younger generations to urban areas leaving a gap in the cultural faiths and practices of indigenous society, ethnic values are being diminished from day to day. Furthermore, the younger generations are not interested in folkloric values including traditional medicines. Additionally, the improvement in the health system and easy access to doctors reduced the

utilization of medicinal herbs. These factors increase the risk of losing valuable ethnomedicinal knowledge. Hereby, conducting ethnobotanical research is becoming more important as gathering ancient knowledge from people is very difficult.

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Statistical Design and Optimization of Sustained Release Formulations of Pravastatin

Pravastatinin Uzatılmış Salım Formülasyonlarının İstatistiksel Tasarım Kullanılarak Geliştirilmesi ve Optimizasyonu

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ABSTRACT

Objectives: The objective of the current study was to formulate a sustained release (SR) formulation for pravastatin. Pravastatin is a lipid lowering, biopharmaceutical classification class-III agent.

Materials and Methods: SR tablets of pravastatin were prepared using variable amounts of hydroxy methyl propyl cellulose (HPMC) K4M and sodium carboxy methyl cellulose in various proportions by direct compression in a 3² factorial design. The amounts of the polymers HPMC K4M and sodium carboxy methyl cellulose required to obtain prolonged release of drug were chosen as independent variables, X₁ and X₂, respectively, whereas times taken for 10%, 50%, 75%, and 90% drug release were chosen as dependent variables.

Results: Nine formulations were developed and were checked using pharmacopoeial tests. The results showed that all the factorial batches were within the standard limits. The dissolution parameters of all formulations were subjected to kinetic fitting and various statistical parameters were determined. Polynomial equations were developed and verified for dependent variables. Formulation F₅, containing 25 mg of HPMC K4M and 25 mg of sodium carboxy methyl cellulose, was the formulation most similar (similarity factor f₂=89.559, difference factor f₁=1.546) to the marketed product (Pravachol).

Conclusion: The best formulation (F₅) follows Higuchi's kinetics and non-Fickian diffusion zero order kinetics (n=1.083).

Key words: Pravastatin, sustained release tablet, HPMC K4M, 3² factorial design, zero order kinetics, non-Fickian diffusion mechanism

ÖZ

Amaç: Bu çalışmanın amacı, biyofarmasötik sınıflandırma sistemi III grubunda yer alan lipit düşürücü etkili pravastatinin sürekli salım yapan formülasyonunun geliştirilmesidir.

Gereç ve Yöntemler: Pravastatin'in sürekli salım tabletleri, direkt kompresyonla 3² faktöryel tasarıma göre çeşitli oranlarda hidroksi metil propil selüloz (HPMC) K4M ve sodyum karboksil metil selüloz kullanılarak hazırlanmıştır. Pravastatin'in uzun süreli salınımını elde etmek için bağımsız değişkenler olarak HPMC K4M (X₁) ve sodyum karboksil metil selüloz (X₂) miktarları; %10, %50, %75 ve %90 ilaç salımı için geçen süreler ise bağımlı değişkenler olarak seçilmiştir.

Bulgular: Dokuz formülasyon geliştirilmiş ve bu formülasyonlara farmakopede belirtilen kontrol testleri uygulanmıştır. Sonuçlar geliştirilen tüm formülasyonların standart limit değerler arasında olduğunu göstermiştir. Tüm formülasyonların disolüsyon parametreleri kinetik modellere uygulanarak çeşitli istatistiksel parametreler belirlenmiştir.

Polinomial denklemler bağımlı değişkenler için geliştirilmiş ve doğrulanmıştır. 25 mg HPMC K4M ve 25 mg sodyum karboksil metil selüloz içeren formülasyondan (F₅) pravastatin salımı, ticari preparatı (Pravachol) ile benzer bulunmuştur (benzerlik faktörü f₂=89,559; fark faktörü f₁=1,546).

Sonuç: En iyi formülasyonunun (F₅), Higuchi kinetiği, Fick kanununa uymayan difüzyonun sıfırıncı derece kinetiğine (n=0,864) uygun davranış gösterdiği bulunmuştur.

Anahtar kelimeler: Pravastatinin, sürekli salım tableti, HPMC K4M, 3² faktöryel tasarım, sıfırıncı derece kinetik, Fick kanununa uymayan difüzyon mekanizması

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INTRODUCTION

Enteral delivery is an effective, popularly used mode of administration for both immediate and new drug delivery systems. In the case of chronic therapy, immediate release dosage forms are administered in a repetitive manner, resulting in more problems. The majority of these drugs undergo the first pass effect or presystemic elimination, which results in poor bioavailability and shorter activity.

Sustained release (SR) formulations show constant C_{ss} levels for a prolonged period, decreased dosing frequency, and patient compliance.² Zero-order drug release from the formulation will aid the C_{ss} constantly for a longer period. Zero-order kinetics is one of the aims of SR forms.^{2,3}

Polymers were utilized for achieving sustained drug release. The literature reveals that utilization of polymers plays a key role in pharmaceutical product development.⁴

Natural polymers remain preferred due to their numerous advantages. Extensively used natural gums include xanthan gum, guar gum, tragacanth gum, and alginates. Cellulosics like hydroxy methyl propyl cellulose (HPMC), hydroxy propyl cellulose, carboxy methyl cellulose (CMC), and sodium (S) CMC belong to the semisynthetic category and have been extensively studied in SR tablet formulations.⁵

Direct compression is a widely used manufacturing method for the preparation of tablets.⁶ The current research experimentation focuses on the design of a SR formulation for pravastatin.

Pravastatin, a potent hypolipidemic agent, belongs to biopharmaceutical classification class-III. It is a specific inhibitor (competitive) of HMG CoA. Pravastatin is useful for the effective management of atherosclerotic vascular disease. It undergoes an extensive first pass effect in the liver. Its bioavailable fraction is 0.17, about 50% of protein binding (plasma proteins). The elimination half-life for pravastatin is 1.5-2 h and it is eliminated from the body via feces and urine. Hence, research work was planned to formulate and evaluate SR tablets for pravastatin as a model drug and had the objective that the optimized formulation trial should show desired SR of the drug by means of an enhanced dissolution rate.⁷⁻¹⁴

Response surface methodology (RSM) with a polynomial equation has been extensively applied in the design and development of pharmaceutical products. Variations of RSM include 3^2 factorial design, central composite design, and Box-Behnken design. RSM is applied when only a few significant factors are involved in the optimization procedure. The advantage of this method is less experimentation and time, the results are more effective, and it is more cost effective than tradition experimentation models.¹⁵⁻¹⁸

Hence an attempt was made in the present research work to formulate SR tablets of pravastatin using HPMC K4M and SCMC. Instead of a heuristic method, a standard statistical tool design of experiments was used to study the effect of formulation variables on the release properties.

A 3^2 factorial design was used to study the effect of polymers on the drug release profile (effect of independent variables

or factors), i.e. the quantity of HPMC K4M and SCMC, on the dependent variables ($t_{10\%}$, $t_{50\%}$, $t_{75\%}$, $t_{90\%}$).¹⁹

MATERIALS AND METHODS

The materials used in the research were procured from various sources. Pravastatin was a gift sample from Konis Pharma Ltd, Baddi, India. HPMC K4M, SCMC, and lactose were obtained from Meditech Pharma Ltd, Solan. Magnesium stearate, talc, and lactose obtained from Loba Chemie Pvt. Ltd, Bombay.

Formulation and development of SR pravastatin tablets

Quantities required for the HPMC K4M and SCMC for the preparation of SR pravastatin tablets were selected as independent variables. $t_{10\%}$, $t_{50\%}$, $t_{75\%}$, and $t_{90\%}$ were selected as dependent variables. Polynomial equations were developed for dependent variables as per backward stepwise linear regression analysis.^{20,21}

The 3 levels of X_1 (HPMC K4M) were 7.5%, 12.5%, and 17.5%. The 3 levels of X_2 (SCMC) were 7.5%, 12.5%, and 17.5% (% with respect to average weight of tablet). Nine SR pravastatin tablet formulations were designed using selected combinations of X_1 and X_2 and checked for the selection of the optimum composition required to meet the primary objective of the study.

Preparation of SR pravastatin tablets

All the ingredients were procured and weighed accurately. They were mixed uniformly in a poly bag for 10-15 min. The resulting mix was subjected to screening (#44). Lubricant was added, followed by mixing well and then compression using a tablet compressor. The resulting tablets were checked in terms of pharmacopoeial limits. The tablets were packed in well-closed air-tight containers.

Experimental design

The experimental design used in the current research was a 3^2 factorial design; the quantity of HPMC K4M was labeled X_1 and the quantity of SCMC was labeled X_2 and they are presented in Table 1. The 3 levels chosen for both X_1 and X_2 were coded as -1=7.5%, 0=12.5%, and +1=17.5%. The formulations for the factorial trials are presented in Table 2.

Evaluation of SR pravastatin tablets

Hardness

This test was performed with the help of a Monsanto hardness tester.

Friability

This test was carried out in a Roche friabilator. The initial weight (W_0) of 20 tablets was noted and then they were dedusted in a drum with a speed of 25 rpm for 4 min and weighed (W) again. Percentage friability was calculated using the following equation. The weight loss should not be more than 0.8%.

$$\text{Friability (\%)} = \frac{(w_0 - w)}{w} \times 100$$

Assay

This test was carried out by taking a fixed number of samples (20) and subjecting them to pulverization. From that above resultant

mixture powder equivalent to 100 mg was dissolved in 100 mL of solvent (6.8 buffer) and sonicated if necessary followed by filtration. The absorbance of the resultant solution was measured using a ultraviolet (UV)-Visible spectrophotometer at 239 nm.¹⁵

Thickness

This test was performed with the help of vernier calipers.

In vitro dissolution study

Dissolution tests were performed using the USP Apparatus 2. The specifications were followed as per official methods such as dissolution medium for initial 2 h is 900 mL of pH 1.2 buffer followed by pH 6.8, at 50 rpm and 37±0.5°C. Samples were collected at fixed time intervals by a pre-filter connected syringe and replacement of fresh fluid was done simultaneously. The absorbance of samples was measured at 239 nm using a Labindia UV-3200 UV-Visible spectrophotometer (n=3).^{9,12,14}

Kinetic modeling of drug release

The kinetic data were subjected to statistical modeling, i.e. zero order, first order, Higuchi, and Korsmeyer-Peppas kinetics.^{22,23}

The study did not require ethics committee approval or patient informed consent because it did not focus on any clinical parameter and did not utilize any humans/animals for the processing of work.

RESULTS AND DISCUSSION

SR tablets of pravastatin were formulated with the help of a 3² factorial design for identifying the optimized composition of polymers (HPMC K4M and SCMC) and to obtain prolonged/sustained drug release from the formulation. The experimental design is presented in Table 1. The 2 factors involved in the design of formulations are quantity of HPMC K4M and SCMC, which were labeled as independent variables (X_1 , X_2), while kinetic parameters were labeled as dependent variables ($t_{10\%}$, $t_{50\%}$, $t_{75\%}$, $t_{90\%}$). Nine factorial batches were designed and all trials had 40 mg of pravastatin as a SR tablet dosage form by direct compression technique as per the formulae given in Table 2.

All final batches were subjected to various final product quality assurance tests like mean hardness, mean thickness, friability, weight variation, and drug content, and the results are summarized in Table 3. Hardness for finished batches was in the range of 3.47±0.3-4.10±0.5 kg/cm². Thickness for finished batches was in the range of 2.45±0.15-2.86±0.14 mm. Results for the friability test were less than 0.51%. Drug content for finished batches met the acceptance criterion. Drug release studies were performed for finished batches using pH 1.2 buffer for an initial 2 hour followed by phosphate buffer pH 6.8 as operated under a standard set of conditions at 50 rpm (paddle), 37±0.5°C. Dissolution plots are presented in Figures 1-4 (kinetic plots) and the statistical parameters are summarized in Table 4. % percentage cumulative drug release for finished batches F_1 - F_9 at 12 hour was 88.88-99.61%. The result revealed that the release rate of drug was inversely proportional to the quantity of polymers. Hence the desired drug release was achieved by

manipulating values of independent variables. A difference was seen in dependent variables due to change in proportions of X_1 and X_2 . Formulation coded F_5 containing 25 mg of HPMC K4M and 25 mg of SCMC produced desirable release characteristics ($t_{10\%}$ =0.459 h, $t_{50\%}$ =3.025 h, $t_{75\%}$ =6.040 h, $t_{90\%}$ =10.045 h), which was probably due to variation in the viscosity of the polymer matrix. An increase in the viscosity of the stagnant layer results in a corresponding decrease in drug release (due to thicker gel layer formation).²⁴ The dissolution profiles of SR pravastatin tablets were subjected to kinetic modeling. The results are presented in Table 4 and Figures 1-4. The results reveal that all formulation batches best fitted zero order kinetics and r^2 was in the range of 0.995-0.999. They also fitted Higuchi's kinetics; r^2 was in the range of 0.941-0.968. The Peppas treatment revealed that all batches follow a non-Fickian diffusion path (n values 1.046-1.397). Polynomial equations were developed for all dependent variables by linear stepwise backward regression analysis with the help of PCP Disso software and response morphological plots were constructed using SigmaPlot V13. The response morphological plots are presented in Figures 5-8 for $t_{10\%}$, $t_{50\%}$, $t_{75\%}$, and $t_{90\%}$ using X_1 and X_2 on both axes to show the effects of independent variables on the dependent variables. Kinetic parameters for the trials (F_1 - F_9) are presented in Table 5.

The polynomial equation for the 3² full factorial design was as follows:

$$Y = b_0 + b_1 X_1 + b_2 X_2 + b_{12} X_1 X_2 + b_{11} X_1^2 + b_{22} X_2^2 \dots$$

Y- dependent variable, b_0 - mean response of 9 trials, b_1 - estimated coefficient for X_1 , b_2 - estimated coefficient for X_2 , b_{12} - interaction term, X_1^2 and X_2^2 - coefficients for nonlinearity. Validity of the derived equations was evaluated by formulating 2 counter check batches of intermediate quantities (C_1 , C_2).

Table 1. Experimental design layout

Name of ingredients	Experimental design										
	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	C_1	C_2
X_1	1	1	1	0	0	0	-1	-1	-1	-0.5	+0.5
X_2	1	0	-1	1	0	-1	1	0	-1	-0.5	+0.5

Table 2. Formulation of SR pravastatin tablets

Name of ingredients	Quantity of ingredients per tablet (mg)								
	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9
Pravastatin	40	40	40	40	40	40	40	40	40
HPMC K4M	35	35	35	25	25	25	15	15	15
SCMC	35	25	15	35	25	15	35	25	15
Lactose	82	92	102	92	102	112	102	112	122
Talc	4	4	4	4	4	4	4	4	4
Magnesium stearate	4	4	4	4	4	4	4	4	4
Total weight	200	200	200	200	200	200	200	200	200

SR: Sustained release, HPMC: Hydroxy methyl propyl cellulose, SCMC: Sodium carboxy methyl cellulose

The equations for dependant variables developed as mentioned below,

$$Y_1 = 0.514 - 0.012 X_1 - 0.094 X_2 - 0.038 X_1 X_2 + 0.055 X_1^2 + 0.0171 X_2^2 \text{ (for } t_{10\%})$$

$$Y_2 = 3.393 - 0.078 X_1 - 0.612 X_2 - 0.250 X_1 X_2 + 0.363 X_1^2 + 0.112 X_2^2 \text{ (for } t_{50\%})$$

$$Y_3 = 6.79 - 0.155 X_1 - 1.222 X_2 - 0.507 X_1 X_2 + 0.722 X_1^2 - 0.225 X_2^2 \text{ (for } t_{75\%})$$

$$Y_4 = 11.280 - 0.260 X_1 - 2.01 X_2 - 0.840 X_1 X_2 + 1.21 X_1^2 + 0.371 X_2^2 \text{ (for } t_{90\%})$$

Batch (F₅) is the identical product

The +ve sign for the coefficient of X₁ in Y₁, Y₂, Y₃, and Y₄ signifies that as the amount of X₁ increases all independent variable values also increase. In other words the data demonstrate that both X₁ and X₂ affect t_{10%}, t_{50%}, t_{75%}, and t_{90%}. From the results it can be concluded that an increase in the amount of polymer leads to a decrease in release rate of the drug and the drug release pattern may be altered by changing the quantities of X₁ and X₂ to appropriate levels. The dissolution parameters predicted from the polynomial equations and those actually observed from the experimental results are summarized in Table 6. Closeness of results was seen between actual values and predicted values. This proves that the polynomial equation developed was valid and confirms the validity of the derived

equations. The response surface/surface morphological plots were presented to show the effects of X₁ and X₂ on dependent variables. The final best (optimized, based on desirability factor above 0.999) formulation (F₅) is an identical product showing a similarity factor (f₂) of 89.559, difference factor (f₁) of 1.546, and t_{cal} is <0.05 when compared with the marketed product (Pravachol).

CONCLUSION

The current research work focused on the utility of macromolecules (polymers) such as HPMC K4M and SCMC in the formulation of SR tablets for pravastatin using a 3² factorial design. The results revealed that the amount of polymers was inversely proportional to the rate of drug release from the formulation. Utilization of polymers in the formulation was beneficial for obtaining prolonged release of the active moiety. Formulation F₅ follows zero order release and a non-Fickian diffusion mechanism. F₅ may be administered for the effective management of hypercholesterolemia and atherosclerotic vascular disease and to reduce the risk of cardiovascular disease. The best formulation shows good retaining

Table 3. Final product quality assurance parameters

S.no.	Formulation code	Hardness (kg/cm ²)	Thickness (mm)	Friability (%)	% Weight variation	Drug content (%)
1	F ₁	3.52±0.1	2.76±0.12	0.28±0.02	200.3±0.12	99.23±0.27
2	F ₂	3.47±0.3	2.86±0.14	0.25±0.022	199.72±0.28	98.36±0.64
3	F ₃	4.10±0.5	2.76±0.12	0.41±0.04	199.2±0.31	98.53±0.37
4	F ₄	3.79±0.2	2.64±0.16	0.38±0.022	199.51±0.45	99.46±0.44
5	F ₅	4.05±0.5	2.68±0.12	0.35±0.05	201.0±0.19	99.40±0.300
6	F ₆	3.88±0.20	2.54±0.26	0.22±0.027	202.1±0.14	99.65±0.35
7	F ₇	3.50±0.40	2.56±0.14	0.51±0.04	200.6±0.14	99.23±0.32
8	F ₈	4.05±0.20	2.54±0.16	0.48±0.02	201.1±0.19	99.59±0.31
9	F ₉	3.85±0.5	2.45±0.15	0.23±0.027	199.6±0.28	98.47±0.43

Table 4. Regression analysis for factorial trials

S.no.	Formulation code	Kinetic parameters											
		Zero order			First order			Higuchi			Korsmeyer-Peppas		
		a	b	r	a	b	r	a	b	r	a	b	r
1	F ₁	5.626	8.461	0.998	2.231	0.106	0.878	24.13	30.78	0.941	0.566	1.352	0.995
2	F ₂	3.711	8.25	0.999	2.175	0.093	0.929	22.35	30.260	0.953	0.659	1.258	0.999
3	F ₃	1.123	7.550	0.999	2.103	0.071	0.972	18.85	28.020	0.964	0.739	1.147	0.997
4	F ₄	1.384	8.337	0.999	2.278	0.133	0.892	18.58	31.11	0.968	0.961	0.966	0.999
5	F ₅	2.222	8.354	0.997	2.185	0.100	0.932	21.36	30.76	0.951	0.830	1.083	0.996
6	F ₆	2.571	7.513	0.999	2.078	0.072	0.991	16.32	28.42	0.974	0.864	1.046	0.992
7	F ₇	3.976	8.231	0.997	2.164	0.090	0.925	22.57	30.21	0.952	0.631	1.282	0.999
8	F ₈	1.165	7.850	0.999	2.130	0.082	0.949	19.71	29.17	0.961	0.618	1.317	0.974
9	F ₉	4.121	8.125	0.995	2.137	0.081	0.968	22.66	29.91	0.957	0.525	1.397	0.992

a: Intercept, b: Slope, r: Correlation coefficient

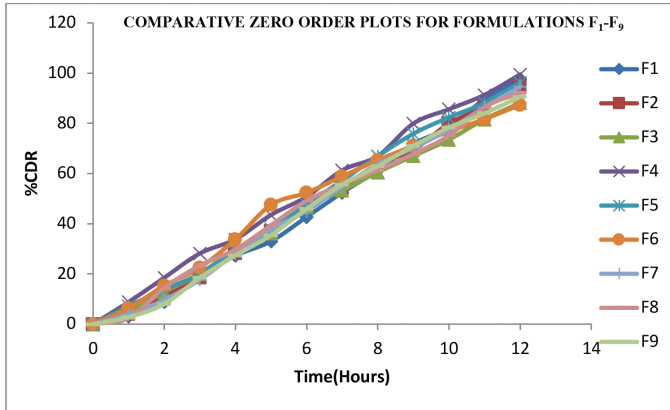


Figure 1. Comparative zero order plots

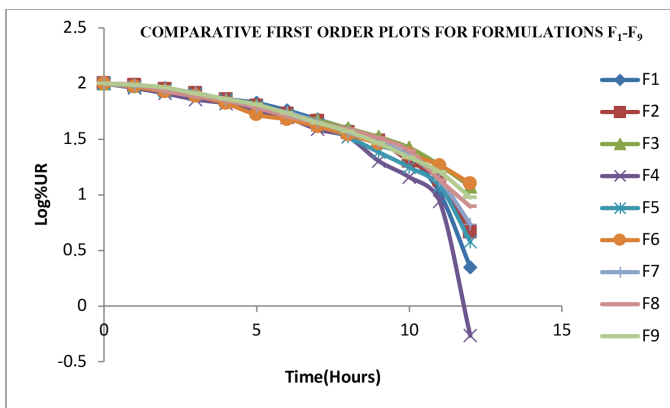


Figure 2. Comparative first order plots

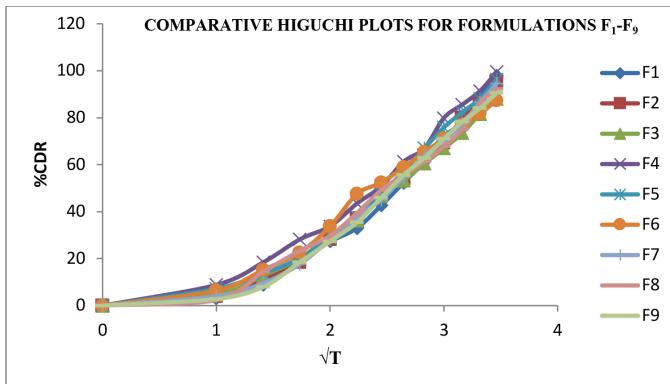


Figure 3. Comparative Higuchi plots

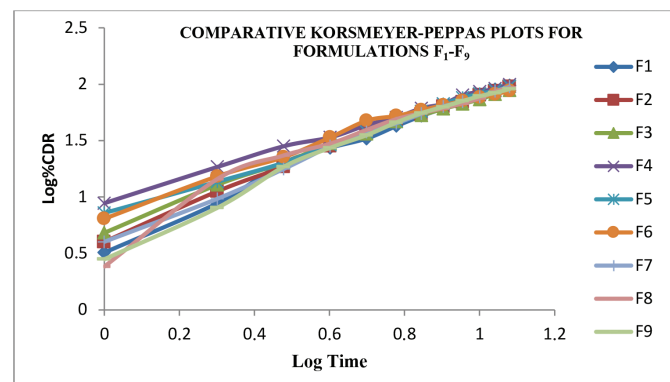


Figure 4. Comparative Korsmeyer-Peppas plots

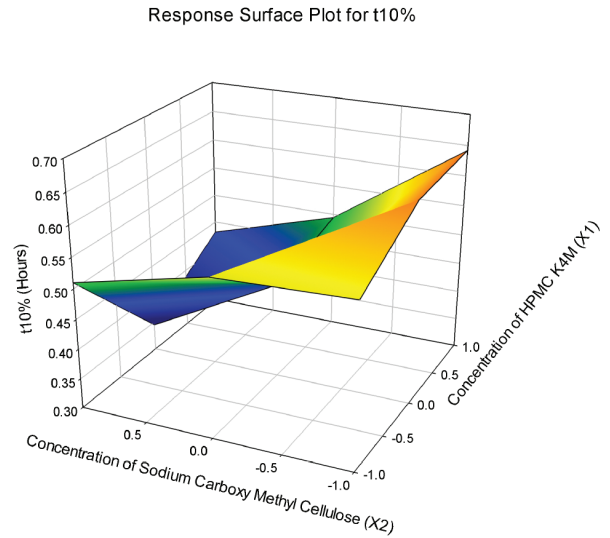


Figure 5. Response surface plots for t10%

t: Time taken to release

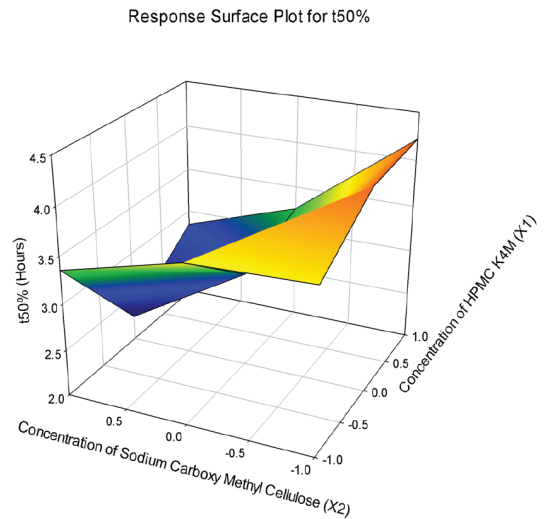


Figure 6. Response surface plots for t50%

HPMC: Hydroxy methyl propyl cellulose

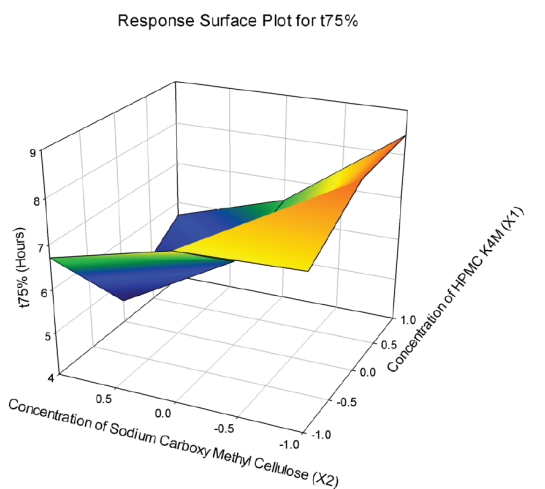


Figure 7. Response surface plots for t75%

t: Time taken to release

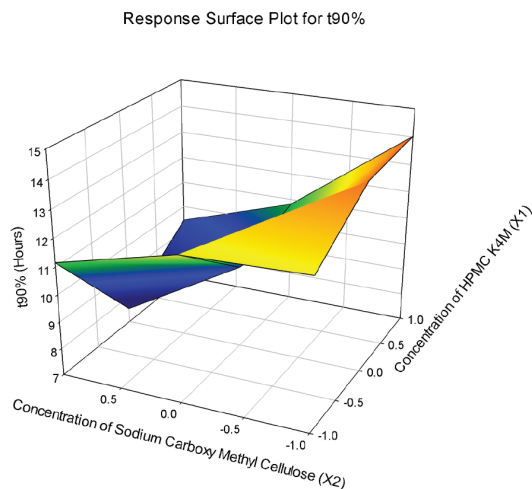


Figure 8. Response surface plots for $t_{90\%}$

Table 5. Dissolution parameters of SR pravastatin tablets

S.no.	Formulation code	Kinetic parameters			
		$t_{10\%}$ (h)	$t_{50\%}$ (h)	$t_{75\%}$ (h)	$t_{90\%}$ (h)
1	F ₁	0.431	2.837	5.671	9.423
2	F ₂	0.493	3.253	6.521	10.81
3	F ₃	0.646	4.225	8.449	14.01
4	F ₄	0.352	2.267	4.539	7.529
5	F ₅	0.459	3.025	6.040	10.045
6	F ₆	0.637	4.181	8.341	13.859
7	F ₇	0.511	3.361	6.718	11.120
8	F ₈	0.564	3.694	7.383	12.252
9	F ₉	0.561	3.735	7.469	12.411

t: Time taken to release, SR: Sustained release

Table 6. Kinetic parameters for counter check formulations

Formulation code	Predicted value				Actual observed value			
	$t_{10\%}$ (h)	$t_{50\%}$ (h)	$t_{75\%}$ (h)	$t_{90\%}$ (h)	$t_{10\%}$ (h)	$t_{50\%}$ (h)	$t_{75\%}$ (h)	$t_{90\%}$ (h)
C ₁	0.579	3.790	7.591	12.610	0.582	3.785	7.597	12.585
C ₂	0.473	3.101	6.212	10.317	0.477	3.107	6.215	10.313

characteristics. It also avoids the first pass effect, which will ultimately improve the clinical response.

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Extended Hildebrand Solubility Approach: Prediction and Correlation of the Solubility of Itraconazole in Triacetin: Water Mixtures at 298.15°K

Geniřletilmiş Hildebrand Çözünürlük Yaklaşımı: 298,15°K'da İtrakonazolün Triasetin: Su Karışımlarında Çözünürlüğünün Belirlenmesi ve Korelasyonu

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ABSTRACT

Objectives: The aim of the study is to explore the suitability of an empirical approach for the extended Hildebrand solubility approach (EHSA) to predict and correlate the solubility of the crystalline drug itraconazole (ITRA) in triacetin: water mixtures.

Materials and Methods: The physicochemical properties of ITRA like fusion enthalpy, solubility parameter, and ideal mole fraction solubility were estimated. The solubilities of ITRA in mixed solvent blends comprising triacetin: water were determined at 298.15°K. Theoretical solubilities were back calculated using a polynomial regression equation of the interaction energy parameter W as a function of the solubility parameter (δ_1) of the solvent mixture. Similarly, the solubilities were predicted by direct method based on the use of logarithmic experimental solubilities ($\log X_2$) against the solubility parameter (δ_1) of the solvent mixture. The predictive capabilities of both EHSA and the direct method were compared using mean percent deviations.

Results: The solubility of ITRA was increased in all the triacetin: water blends and was highest in the blend in which the solubility parameter of ITRA equaled that of the solvent mixture. The prediction capacities of the direct method (mean % deviation was -1.89%) were better than those of EHSA (mean % deviation was 9.76%) in the fifth order polynomial.

Conclusion: The results indicated that the solubility of any crystalline solute can be adequately predicted and correlated with the mere knowledge of physicochemical properties and EHSA. The information could be of help in process and formulation development.

Key words: Itraconazole, extended Hildebrand solubility approach, interaction energy, solubility parameter, prediction, correlation of solubilities

ÖZ

Amaç: Triasetin: su karışımlarında kristal formdaki itrakonazol (ITRA)'nın çözünürlüğünün genişletilmiş Hildebrand çözünürlük yaklaşımı (EHSA) için uygunluğunun deneysel bir yaklaşımla tahmin ve korele edilmesi bu araştırmanın amacıdır.

Gereç ve Yöntemler: ITRA'nın füzyon entalpisi, Hildebrand çözünürlük yaklaşımı çözünürlük parametresi ve ideal mol oranı gibi fizikokimyasal özellikleri tahmin edilmiştir. ITRA'nın triasetin: sudan oluşan karışım halindeki çözeltilerdeki çözünürlükleri 298,15°K'da belirlenmiştir. Teorik çözünürlükleri çözelti karışımındaki çözünürlük parametresi (δ_1)'nin bir fonksiyonu olarak etkileşim enerji parametresi W kullanılarak polinomial regresyon denklemi ile hesaplanmıştır.

Bulgular: Tüm triasetin: su karışımlarında ITRA'nın çözünürlüğü artmıştır ve çözünürlüğün en yüksek olduğu karışım ITRA'nın çözünürlük parametresinin çözelti karışımına eşit olduğu karışımdır. Doğrudan yöntemin tahmin kapasitesi (ortalama % sapması -%1,89) beşinci polinomial sırada EHSA'dan (ortalama % sapması %9,76) daha iyi bulunmuştur.

Sonuç: Bu sonuçlar çözünen kristalin çözünürlüğünün tek başına fizikokimyasal özellikler ve EHSA bilgileriyle yeterince öngörülebileceğini ve ilişkilendirilebileceğini göstermiştir. Bu bilgi süreç ve formülasyon geliştirmede yardımcı olabilir.

Anahtar kelimeler: İtrakonazol, genişletilmiş Hildebrand çözünürlük yaklaşımı, etkileşim enerjisi, çözünürlük parametresi, tahmin, çözünürlüklerin korelasyonu

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INTRODUCTION

Many pharmaceutically important processes like synthesis, extraction, recrystallization, purification, and dosage form development require the solubilization of an active pharmaceutical ingredient (API) in neat and/or solvent blends. Many times these APIs are complex organic substances and are ideal theoretical candidates for understanding solubility behavior with the use of predictive methods. Itraconazole (ITRA) (Figure 1) is one such triazole compound widely used because of its antifungal activity. However, its use is limited due to its poor solubility.^{1,2} Knowledge about its solubility behavior and solubility improvement is much needed for the development of better formulations with increased effectiveness. Thus, ITRA is an ideal candidate for estimating its solubility and understanding its solubility behavior in solvent mixtures with the help of empirical predictive models.

The Hildebrand-Scatchard equation is an empirical approach used to predict the solubility of poorly soluble compounds in a variety of solvents ranging from nonpolar to polar according to regular solution theory.³ However, in pharmaceuticals many irregular solutions are observed due to self-association of solute or solvent molecules and complexation.

The extended Hildebrand solubility approach (EHSA) is an adaptation of the Hildebrand-Scatchard equation that allows the estimation of solubilities of polar as well as nonpolar moieties in a variety of solvents with different polarities like water, alcohols, sulfoxides, glycols, and acetates.⁴ The EHSA has been considered as an adventitious empirical model for prediction of solubility due to numerous reasons. They include ability of solubility prediction of a wide variety of solutes in irregular solutions, ability of predicting the solubility of any solute in pure or mixed solvents using fundamental physicochemical properties, it is applicable not only for crystalline solids in liquid solutions but also for liquid-liquid and gas-liquid systems, and it gives more accurate prediction of mole fraction solubilities compared to other empirical methods. The solubility parameter, an intrinsic physicochemical property of solute and solvent, is the square root of cohesive energy density and could be used to understand the solution behavior of regular and irregular solutions. To date, some work has been done to study the solubility behavior of pharmaceutically important substances

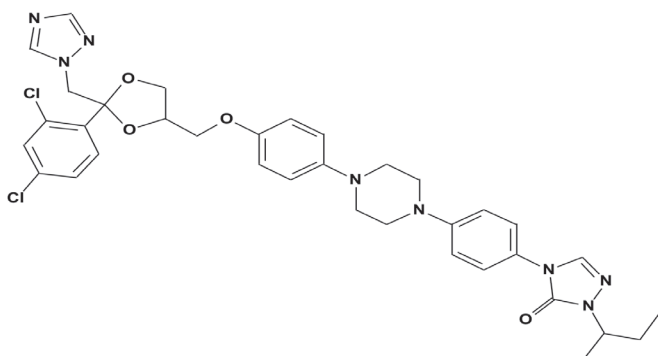


Figure 1. Structure of ITRA

ITRA: Itraconazole

with the use of EHSA by Martin et al.,⁵ Bustamante et al.,⁶ Rathi and Mourya,⁷ Sotomayor et al.,⁸ and Delgado et al.⁹

The solubility of crystalline solids in a variety of solutions can be described with the EHSA expression as⁵

$$-\log_{10} X_2 = -\log_{10} X_2^i + A (\delta_1 - \delta_2)^2 \quad \text{Equation (1)}$$

The solubility of crystalline solid solute in irregular solutions may be estimated using the following equation:¹⁰

$$-\log_{10} X_2 = -\log_{10} X_2^i + A (\delta_1^2 + \delta_2^2 - 2W), \quad \text{Equation (2)}$$

Where X_2^i are the ideal mole fraction and experimental mole fraction solubility of the solute, respectively. The terms A and W are the solubility parameters of the respective solvent mixtures and the solute, respectively. Furthermore, W represents the interaction energy parameter for the solute-solvent blend interaction in irregular solutions.

The term A in Equations 1 and 2 can be expressed as

$$A = \frac{V_2 \phi_1^2}{2.303RT} \quad \text{Equation (3)}$$

where V_2 represents the molar volume of the solute, i.e. ITRA, R represents universal gas constant, and ϕ_1 expresses the volume fraction of the solvent mixture.

The volume fraction ϕ_1 can be calculated as

$$\phi_1 = \frac{V_1 (1 - X_2)}{V_1 (1 - X_2) + V_2 X_2}, \quad \text{Equation (4)}$$

where V_1 is the reflected molar volume of the triacetin: water solvent mixture. X_1 and X_2 represent moles of the solute and solvent, respectively.

The ideal mole fraction solubility can be expressed as a negative logarithm and can be given by the following equation:

$$-\log X_2^i = \frac{\Delta H_f (T_m - T)}{2.303RT_m T} \quad \text{Equation (5)}$$

Here ΔH_f gives fusion enthalpy of solid crystalline ITRA. T_m reflects melting temperature and T represents the absolute temperature (298.15 K).

The logarithmic value of the activity coefficient could be expressed by the equation

$$\log \gamma_2 = A (\delta_1^2 + \delta_2^2 - 2W) = \frac{V_2 \phi_1^2}{2.303RT} (\delta_1^2 + \delta_2^2 - 2w) \quad \text{Equation (6)}$$

As we could not confine the interaction term W , the other approach will be an experimental estimation of interaction energy by determining ITRA solubility in solvent mixtures using Equation 2. A realistic equation for the determination of W has not been reported to date. Hence, it is evaluated using Equation 6 by back calculations. Then these values of W can be used further for the prediction of the solubility of a solute in any other solvent system as a function of the solubility parameter of the respective solvent mixture.

$$w = C_0 + C_1 \delta_1 + C_2 \delta_1^2 + C_3 \delta_1^3 + C_4 \delta_1^4 + \dots + C_n \delta_1^n \quad \text{Equation (7)}$$

Triacetin is a pharmaceutically important chemical substance used as a solvent¹¹ for the solubilization of various drugs and polymers because of its biocompatibility in topical and

injectable preparations.^{12,13} It is also capable of affecting the film-forming properties¹⁴ as well as adhesive properties in topical preparations and is capable of forming stable depots in the case of injectable preparations.¹⁵

Thus, the present work was carried out to establish the suitability of EHSA to study the solute-solvent interaction and solution behavior and to predict the solubility of ITRA in triacetin: water mixtures as a function of the solubility parameter.

MATERIALS AND METHODS

Materials

A gift sample of ITRA was obtained from USV (Mumbai, India). The solvents like triacetin were received from Loba Chemie (India). The double distilled water used in the study was prepared in the laboratory. Other chemicals and reagents used in the study were of analytical grade.

Determination of itraconazole solubility

The saturation solubility study method was employed for the determination of ITRA solubility.¹⁶ Double distilled water was used to prepare solvent mixtures. Binary compositions of triacetin: water were used from 0% to 100% by the mass fraction of triacetin. Then 10 g of binary solvents blends were taken in screw cap vials and were saturated by the addition of excess of drug. These vials were mounted in an orbital shaker (Remi, India) at 298.15 K and 100 rpm for 72 h. The saturation time of 72 h was established through the preliminary studies. After 72 h, these vials were removed and the solutions were filtered carefully with the help of micro filters of 0.45 μm . The filtrate was collected, diluted suitably, and was subjected to spectrophotometric analysis using a double beam ultraviolet spectrophotometer (Shimadzu, Japan) at 255 nm. All the experiments were performed in triplicate. The densities of pure solvent blends and filtered saturated blends were determined

and used for the estimation of saturated solubilities in terms of mole fraction.

Differential scanning calorimetric (DSC) study

The melting temperature and melting fusion enthalpy of ITRA were determined by performing DSC analysis. The DSC thermogram was produced using a differential scanning calorimeter (DSC-1, Mettler Toledo, Switzerland). An ITRA sample weighing 5.0 mg was kept in an aluminum pan and then it was sealed with the lid. These pans were subjected to heating from 313.15 K to 573.154 K at a rate of 10 K.min⁻¹ under nitrogen purging.

Statistical analysis

Statistical analysis of the solubility data was performed using the EHSA. The polynomial regression analysis and statistical evaluation of the data were performed using Minitab statistical software (Version 18) and MS Excel.

RESULTS AND DISCUSSION

The melting temperature (T_m) of ITRA was 443.5 K and the melting fusion enthalpy (ΔH_m) was 65.32 kJ.mol⁻¹ at 298.15 K. From these values, the ideal mole fraction solubility of the drug was estimated to be 3.04×10^{-7} and the value of $\log_{10} X_{2,obs}$ was found to be 3.04 expressed in mole fraction using Equation 5. The investigated mole fraction solubilities of ITRA in triacetin: water mixtures with a wide range of polarity described in terms of the solubility parameter of the solvent mixture from 10.77 to 23.40 H are given in Table 1.

The uncertainties in the solubility investigation were <2% in all cases. Table 1 also expresses the mass fractions, volume fractions, and solubility parameter of the solvent blend with respect to its composition. These volume fractions and solubility parameter were determined using the additive property

Table 1. Triacetin: water solvent mixture composition, Hildebrand solubility parameter, solubilities of ITRA expressed as molarity and mole fractions. Activity coefficients for ITRA in triacetin: water mixtures are expressed as logarithmic values at 298.15 K

TA mass fraction	ϕ_{TA}	δ_1	Itraconazole solubility			
			Mol.L ⁻¹	$X_{2,obs}$	Standard deviation	$\log_{10} X_{2,obs}$
0.0000	0.0000	23.40	7.41E-06	1.34E-07	1.95E-03	3.8320
0.1000	0.0986	22.14	1.15E-05	4.31E-07	2.08E-03	3.3233
0.2000	0.1943	20.87	4.69E-05	2.65E-06	2.56E-04	2.5355
0.3000	0.2971	19.61	2.24E-04	1.68E-05	2.21E-03	1.7338
0.4000	0.3871	18.35	4.02E-04	3.72E-05	2.37E-04	1.3879
0.5000	0.4845	17.09	1.59E-03	1.75E-04	2.25E-03	0.7161
0.6000	0.5893	15.82	3.39E-03	4.28E-04	2.40E-04	0.3264
0.7000	0.6818	14.56	7.34E-03	1.05E-03	3.25E-03	-0.06165
0.8000	0.7820	13.30	1.13E-02	1.80E-03	3.14E-03	-0.2961
0.9000	0.8975	12.03	3.66E-02	6.32E-03	2.45E-03	-0.8429
1.0000	0.1000	10.77	1.63E-02	3.06E-03	2.15E-04	-0.5279

ϕ_{TA} - volume fractions of solvent triacetin, ITRA: Itraconazole

phenomenon. The solubilities of ITRA in terms of molarity and mole fractions are also presented in Table 1.

The ideal, experimental, and calculated solubilities of ITRA with respect to the solubility parameter of the solvent blend for regular solutions at 298.15 K are presented in Figure 2. These calculated solubilities were estimated using molar volume and solubility parameters. The values were obtained from the literature for solvents. For ITRA, these values were calculated using Fedors' group contribution method.¹⁷ According to regular solutions theory, the peak in solubility would be attained where the solubility parameter of the solvent mixture matches that of the solute (Figure 2). In the present study, it was observed that the peak in solubility of ITRA was achieved in a solvent blend of 0.9 mass fractions of triacetin where the solubility parameter of ITRA nearly corresponded with that of the solvent mixture

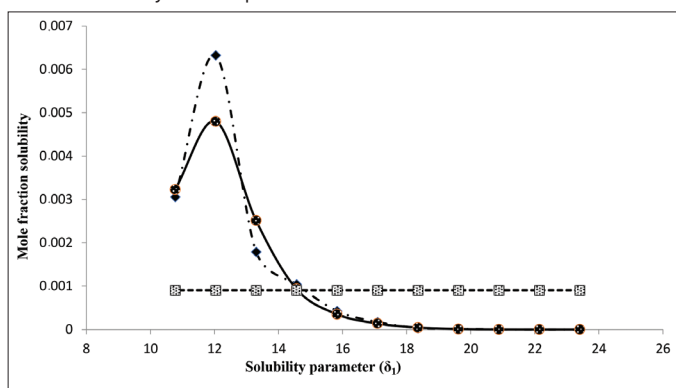


Figure 2. Experimental solubilities (dotted line joined by filled circles) and solubilities calculated by fifth order polynomial regression equation (continuous line joined by filled diamonds) for irregular solution of ITRA developed using the empirical model of Hildebrand as a function of the solubility parameter of the solvent mixtures at 298.15 K. The discontinuous line (long dash joined by crosses) represents the ideal solubility calculated using Equation 1

ITRA: Itraconazole

($\delta_1=12.03$ H). This was attributed to the matching of polarities of ITRA and 0.9 mass fraction of solvent mixture of triacetin: water. From the results it could be inferred that the ITRA has the same polarity as that of the 0.9 mass fraction of the solvent mixture of triacetin: water. The molar volume and solubility parameter of ITRA were derived using Fedors' contribution method and were found to be $457.5 \text{ cm}^3 \cdot \text{mol}^{-1}$ and $24.3987 \text{ (J/cm}^3)^{1/2}$ or 11.93 H, respectively.

Volume fractions (ϕ_1) of the solvent mixtures were estimated using Equation 4. The values were nearly equal to unity due to the smaller values of solubility of ITRA in all the solvent mixtures. The values of activity coefficients are also given in Table 1. These values were greater than one in solvent mixtures where the proportion of water was higher. Table 2 summarizes experimental parameters like volume fractions of solvent mixture (ϕ_1), A , K , W_{obs} , W_{cal} and ITRA in triacetin: water mixtures at 298.15 K. It was observed that the values of the Walker parameter were greater than one, indicating a rise in solubilities due to increased solute-solvent interactions. The variation in interaction energy parameter W with respect to the solubility parameter of the solvent blend is shown in Figure 3. The graph shows the deviation from linearity as the value of W was estimated using the squares of two terms (δ_1 and δ_2) and a variable term consisting of $(-\log \gamma_2/A)$ as reflected in following equation:

$$W = 0.5 \times (\delta_1^2 + \delta_2^2 - \frac{\log \gamma_2}{A}) \text{ Equation (8)}$$

The values of W were estimated using a regular polynomial equation as a function of solubility parameter of solvent blend in order 5 (Equation 7). The following polynomial regression equation of order 5 was produced as a function of the solubility parameter to back calculate the values of :

Table 2. Experimental parameters like volume fractions of solvent mixture (ϕ_1), A , K , W_{obs} and W_{cal} for ITRA in triacetin: water mixtures at 298.15 K

d_1 (H)	ϕ_1	$10A \text{ (cm}^3 \cdot \text{J}^{-1})$	$K \text{ (J} \cdot \text{cm}^{-3})^a$	$W_{obs} \text{ (J} \cdot \text{cm}^{-3})$	$W_{cal} \text{ (J} \cdot \text{cm}^{-3})$
23.40	0.99999	3.3532	1.2152	339.2284	338.9677
22.14	0.99998	3.3532	1.1785	311.2304	311.0476
20.87	0.99993	3.3531	1.1454	285.2436	285.0894
19.61	0.99989	3.3528	1.1150	260.8722	260.6502
18.35	0.99980	3.3499	1.0846	237.4166	237.5737
17.09	0.99923	3.3491	1.0599	216.0417	215.8906
15.82	0.99838	3.3486	1.0375	195.8421	195.7189
14.56	0.99653	3.3448	1.0204	177.2373	177.1646
13.30	0.99471	3.3330	1.0087	160.0004	160.2219
12.03	0.98333	3.2825	1.0091	144.8587	144.6741
10.77	0.99261	3.3118	1.0115	129.9579	129.9934

^a1 J.cm⁻³=1 MPa, ITRA: Itraconazole

$$w_{cal} = (-265.2679) + (104.3851) \delta_1 + (-12.1646) \delta_1^2 + (0.7556) \delta_1^3 + (-0.0222) \delta_1^4 + (0.0002) \delta_1^5 \quad \text{Equation (9)}$$

These back calculated values of W_{cal} were used to calculate the solubilities of ITRA (Table 3). Such theoretically estimated solubilities were then compared with experimental ones and the mean percent deviation was obtained. It was found to be 9.76% for the EHSA method. The worth of the EHSA method for the correlation and estimation of solubilities with the use of the EHSA equation could be established by performing the calculations using an equation consisting of other variables. Therefore, the theoretical solubility values were calculated using the direct method based upon polynomial equation of $\log_{10} X_2$ as a function of the solubility parameter of solvent blend δ_1 of order 5 (Equation 10).

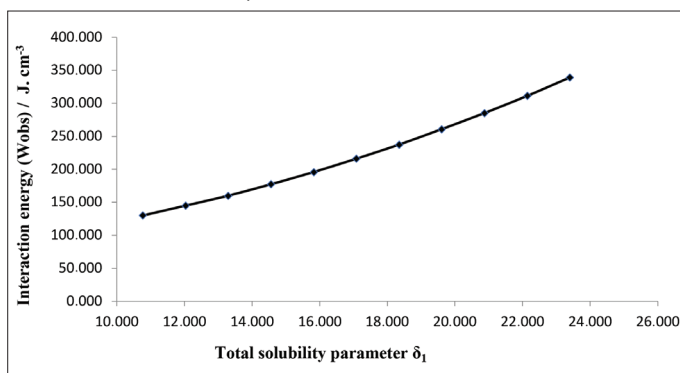


Figure 3. Variation in interaction energy W of ITRA in triacetin: water mixtures as a function of the solubility parameter of the binary solvent mixture at 298.15 K

ITRA: Itraconazole

$$\log_{10} X_2 = B_0 + B_1 \delta_1 + B_2 \delta_1^2 + B_3 \delta_1^3 + B_4 \delta_1^4 + B_n \delta_1^n \quad \text{Equation (10)}$$

Here calculated solubilities were again compared with experimental ones and the mean percent deviation was obtained. It was -1.89% (Table 3). The solubility prediction capabilities of both methods were compared using these mean percent deviation values. Similarly, solubility prediction behavior was obtained with the use of polynomial regression equations of order 5 for EHSA and the direct method for drugs like phenacetin,¹⁸ meloxicam,¹⁹ and piroxicam.⁸ In the present study, the solubility correlation and prediction were better by the direct method as compared to that of EHSA with a polynomial of order 5. Nonetheless, it must be remembered that these methods were based upon some of the physico-chemical properties. There is a need for a method for the exact determination of the Walker parameter for the estimation of

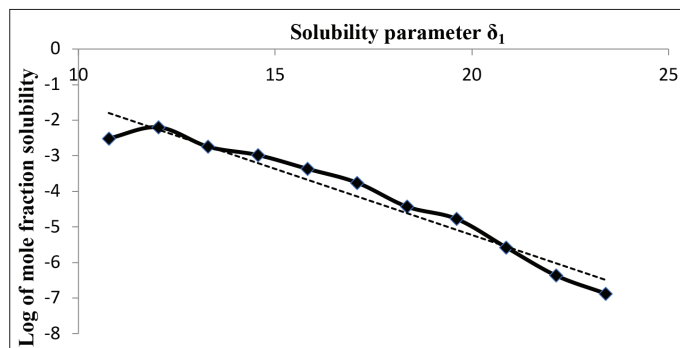


Figure 4. Log of mole fraction solubility ($\log X_2$) as a function of the solubility parameter of ITRA in triacetin: water mixtures at 298.15 K

ITRA: Itraconazole

Table 3. Calculated solubilities of ITRA in triacetin: water mixtures using calculated values estimated by polynomial regression equations of order 5 (by EHSA method) and using $\log X_2$ values determined as a function of the solubility parameter with the use of a polynomial regression equation of order 5 (by direct method). Percentage differences with respect to experimental solubilities are also indicated at 298.15 K

d_1 (H)	X_{2cal}		% deviation ^a	
	EHSA method	Direct method	EHSA method	Direct method
23.40	8.94E-08	1.33E-07	3.31E+01	4.42E-01
22.14	3.25E-07	4.44E-07	2.46E+01	-2.93E+00
20.87	2.09E-06	2.61E-06	2.12E+01	1.26E+00
19.61	1.19E-05	1.40E-05	2.90E+01	1.67E+01
18.35	4.74E-05	5.33E-05	-2.74E+01	-4.35E+01
17.09	1.38E-04	1.51E-04	2.08E+01	1.34E+01
15.82	3.54E-04	3.78E-04	1.73E+01	1.17E+01
14.56	9.36E-04	9.71E-04	1.05E+01	7.25E+00
13.30	2.52E-03	2.54E-03	-4.03E+01	-4.14E+01
12.03	4.80E-03	4.92E-03	2.41E+01	2.22E+01
10.77	3.23E-03	3.25E-03	-5.56E+00	-6.03E+00
Mean value^b			9.76	-1.89

^aMean value^b was calculated using values obtained in neat solvents-triacetin, water and nine binary solvent mixtures
ITRA: Itraconazole, EHSA: Hildebrand solubility approach

interaction energy parameter. Already it has been proved that the EHSA method could be used to calculate drug solubilities as it depended upon some simpler physicochemical properties like solubility parameter, molar volume, and experimental solubilities. Thus, EHSA could have potential applications in various pharmaceutical science processes.

ITRA showed both positive and negative deviations in solubility as reported previously (Figure 4).^{20,21} The reason for such deviation from ideal solubilization could be the predominance of interactions between cosolvent and water over the solute-solvent interactions.²² Similar types of observations were reported by Gómez et al.,²³ Kharwade et al.,²⁴ Thimmasetty et al.,²⁵ Rathi and Deshpande,²⁶ and Cárdenas et al.²⁷ The major force behind the solubilization in water-rich mixtures could be entropy. It might have resulted in loss in structure of water surrounding the nonpolar ITRA by triacetin molecules. At the higher proportions of triacetin, the solubilization could be enthalpy driven. At these higher proportions of triacetin water molecules might have lost their three dimensional structure completely and they might have become available for interaction with ITRA molecules.¹⁹ The other reason for the positive deviation from the log linear model could be the drug-drug molecule interactions in the saturated solution. This could be further confirmed with spectral studies.

CONCLUSION

The present study showed the application of EHSA to the ITRA solubility data in triacetin: water mixtures at 298.15 K with the help of physicochemical properties like fusion enthalpy, molar volume, and Hildebrand solubility parameter obtained by Fedors' group contribution method. The peak in experimental solubility was observed at a point where the solubility parameter of ITRA matched that of the solvent mixture. Better prediction of solubilities was achieved with the help of a polynomial regression equation as a function of the solubility parameter of order 5 for both EHSA and the direct method. The direct method exhibited better prediction capacities (mean percent deviations -1.89%) as compared to EHSA (mean percent deviations 9.76%). Furthermore, it could be asserted based on the study that EHSA should be used to understand the solubility behavior of solutes of different polarities in a variety of solvents and their mixtures. The information obtained could be useful for the process and formulation development of such drugs.

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Psychoactive Bath Salts and Neurotoxicity Risk

Psikoaktif Banyo Tuzları ve Nörotoksisite Riski

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ABSTRACT

Synthetic cathinones are new designer drugs that possess hallucinogenic and psychostimulant properties, and are designed to mimic the effects of illegal substances such as cocaine, amphetamines, and 3,4-methylenedioxymethamphetamine (ecstasy) and to produce rewarding effects, circumventing existing laws and penalties. Synthetic cathinones, also referred to as 'bath salts', have become popular particularly among young people since the mid-2000s. Similar to other psychomotor stimulants, synthetic cathinones have the potential to increase monoamine concentration in the synaptic cleft by targeting the plasma membrane transporters of dopamine, norepinephrine, and serotonin. Because of their structural similarities to amphetamines, it has been suggested that synthetic cathinones may have a neurotoxicity profile similar to that of their amphetamine congeners. Therefore, it has been hypothesized that synthetic cathinones may induce neurotoxicity on monoamine nerve endings in the striatum, hippocampus, and cortex. To date, with regard to synthetic cathinone neurotoxicity, parameters such as monoamine depletion, biosynthetic enzyme inhibition, cytotoxicity, generation of reactive oxygen species, pro-oxidation status, and the ability to induce neuroinflammation were investigated in both *in vitro* and *in vivo* experimental studies. Compared with amphetamines, synthetic cathinones appear to have more moderate effects than their amphetamine congeners in terms of neurotoxic effects. However, many synthetic cathinone users take these substances simultaneously with other substances such as benzodiazepines, amphetamines, ecstasy, tetrahydrocannabinol, and ethanol and this abuse can modify their neurotoxic effects. Hence, it is important to understand the underlying mechanism of early neurotoxic effects in case of polysubstance use. In this review, we aimed to present up-to-date information on the abuse potential of synthetic cathinones, their legal status, mechanism of action, and particularly their neurotoxic effects.

Key words: Psychoactive, hallucinogen, bath salts, synthetic cathinones, neurotoxicity

ÖZ

Sentetik katinonlar, mevcut yasa ve cezaları atlatılmak amacıyla, kokain, amfetamin ve 3,4-metilendioksümetamfetamin (ekstazi) gibi yasadışı maddelerin etkilerini taklit etmek ve benzer ödüllendirici etkiler yaratmak üzere geliştirilmiş halüsinojenik ve psikostimülan özellikte yeni tasarlanmış yasadışı maddelerdir. Banyo tuzları olarak da bilinen sentetik katinonlar, 2000'li yılların ortalarından itibaren özellikle genç bireyler arasında popüler hale gelmeye başlamıştır. Diğer psikomotor uyarıcılara benzer şekilde, sentetik katinonlarda, dopamin, norepinefrin ve serotoninin plazma membran taşıyıcılarını hedef alarak sinaptik aralıktaki monoamin konsantrasyonunu arttırmaktadır. Amfetaminlere olan yapısal benzerlikleri nedeniyle, sentetik katinonların amfetamin homologları ile benzer nörotoksisite profiline sahip olabileceği düşünülmüştür. Bu nedenle, sentetik katinonların striatum, hipokampus ve kortekste monoamin sinir uçları üzerinde nörotoksisiteyi indükleyebileceği hipotezi öne sürülmüştür. Şimdiye dek sentetik katinonların nörotoksisitesi ile ilgili olarak yapılan *in vitro-in vivo* deneysel çalışmalarda, monoamin depleyonu, biyosentetik enzim inhibisyonu, sitotoksisite, reaktif oksijen türlerinin oluşumu, pro-oksidasyon durumu ve nöroinflamasyon indükleme yeteneği gibi parametreler incelenmiştir. Sentetik katinonların nörotoksik etkiler açısından amfetamin homologlarından daha ilımlı olduğu görülmektedir. Ancak, pek çok sentetik katinon kullanıcısının bu maddeleri benzodiazepinler, amfetaminler, ekstazi, tetrahidrokannabinol ve etanol gibi diğer yasal olmayan ilaç veya bağımlılık yapıcı maddelerle birlikte almaktadır. Çünkü bu maddelerin nörotoksik etkileri değil, bu maddeler, sentetik katinonların nörotoksik etkilerini modifiye edebilmektedir. Bu nedenle, çoklu madde maruziyeti durumundaki erken nörotoksik etkilerin altında yatan mekanizmaların anlaşılması önemlidir. Bu derlemede, sentetik katinonların suistimal potansiyeli, yasal durumları, etki mekanizmaları ve özellikle nörotoksik etkileri hakkında güncel bilgi sunulması amaçlanmıştır.

Anahtar kelimeler: Psikoaktif, halüsinojen, banyo tuzları, sentetik katinonlar, nörotoksisite

INTRODUCTION

Synthetic cathinones are a subgroup of new psychoactive substances (NPSs) that possess hallucinogenic and psychostimulant properties and are designed to mimic the effects of illegal substances such as cocaine, 3,4-methylenedioxymethamphetamine (MDMA), and other amphetamines, circumventing existing laws.¹ NPSs, or to use

the other term 'designer drugs', are intentionally mislabeled and marketed as bath salts, fertilizers (although they have no such purpose), plant food, laboratory chemicals, or reagents and are marked as 'not for human consumption' or 'not tested for danger/toxicity' to avoid criminal liability.^{2,3} Synthetic cathinones are one of the most commonly found psychoactive substances in these designer drug mixtures.¹

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Bath salts contain one or more synthetic cathinone derivatives. The most popular bath salt constituents are 4-methylcathinone (mephedrone), 3,4-methylenedioxymethcathinone (methylone), and 3,4-methylenedioxypropylvalerone (MDPV). In point of fact, these compounds have no legitimate use as a bath additive in these products.⁴

Synthetic cathinones are usually a white, amorphous or crystalline powder, used by oral, rectal routes, injection, or inhalation.⁵ Some synthetic cathinone derivatives also can be smoked or used as 'vapor' in e-cigarette-like devices.⁶ Synthetic cathinones, also referred to as 'bath salts', have become popular particularly among young individuals since the mid-2000s.⁴ In recent years, synthetic cathinones have increased in both supply and demand in every corner of the world. Because they are known as 'legal alternatives' to illicit drugs, there has been a dramatic increase in sales. Another reason that has made bath salts popular and widespread is the fact that they are easily accessible on the web.⁷ Drug trade through the cryptomarket on the web, called the Darknet, allows users to anonymously buy drugs. According to the United Nations Office on Drugs and Crime, while drug trafficking over the Darknet remains small, there was an increase in drug transactions of some 50% annually between 2013 and 2016.⁸

Low doses of synthetic cathinones cause euphoria and alertness; however, high doses or chronic use can cause serious adverse effects such as hallucination, psychosis, delirium, hyperthermia, tachycardia, renal failure, and ischemia. Similar to other psychomotor stimulants, synthetic cathinones increase monoamine concentration in the synaptic cleft by targeting the plasma membrane transporters of dopamine (DAT), norepinephrine (NET), and serotonin (SERT). Induction of dopaminergic transmission suggests that they have a high potential for addiction. Because of their structural similarities with amphetamines, attempts have been made to develop many synthetic cathinone derivatives as anorectics, central nervous system stimulants, or antidepressants, but the problem of addiction has hindered their clinical use.^{9,10}

Synthetic cathinones could physically harm users, which could also pose public health threats such as violence and irresponsible driving. Due to the health risks posed by bath salts, certain synthetic cathinones such as mephedrone, methylone, and MDPV have been made illegal in many countries. Even though they are seen as a legal alternative by users, their legal situation rapidly changes and differs among countries.¹¹ The illegal availability of popular synthetic cathinones in many countries has led to the synthesis of new cathinone derivatives with minor modifications of their functional groups. This leads to a great diversity of product composition even within the same brand.¹² The replacement of synthetic cathinones with newly developed cathinone derivatives causes a major problem in terms of the drug policy. The major problem is theoretically many structural variants of the cathinone are possible.¹³ The number of synthetic cathinones reported in 2008 was 8, but this had increased to 68 by the end of 2014. The cumulative number reported to the European Monitoring Center for Drugs

and Drug Addiction between 2005 and 2016 was 118. The total number reached 128 by 2017.¹⁴

The gradual increase in the number of new derivatives makes it difficult to detect synthetic cathinones in biological specimens with routine toxicological tests. Since synthetic cathinones are a new chemical class, they cannot be determined by conventional techniques. The low specificity of immunoserological tests causes false-positive results. Qualitative and quantitative analyses of synthetic cathinones require more sensitive techniques and equipment such as gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS). Due to the challenges with routine imaging techniques, the rate of use in subpopulations such as military personnel is still increasing in some countries.¹⁵ No global data are available, but the prevalence of mephedrone abuse among European adults aged 16-59 was estimated to have reached 0.5% in 2012-2013.¹⁴ In 2010 and 2011, there were a total of 362 calls made to the Texas Poison Center due to synthetic cathinone intoxication; 84.5% were over 20 years of age, 74% were male patients, 47.8% were exposed via inhalation, and 28.7% only by oral route.¹⁵

The most commonly seized synthetic cathinones in Europe were α -pyrrolidinovalerophenone (α -PVP), 3-methylmethcathinone, ethylone, 4-chloromethcathinone, and pentadrone in 2015.¹⁶ In recent years, a second-generation synthetic cathinone derivative, α -PVP, has rapidly gained popularity. α -PVP, or 'flakka' to give it its street name, is a synthetic cathinone derivative bearing a pyrrolidine ring, colloquially called the 'zombie drug' due to the unpredictably bizarre and erratic behavior seen in its users. Recently, flakka abuse has been seen in Turkey, where it is classified as an illegal substance.^{17,18} A great handicap regarding α -PVP abuse is its legal/under control status in some European and United Nations member states, e.g., Belgium, Denmark, Spain, Bulgaria, and Japan.¹⁹

The underlying mechanisms of the behavioral effects of cathinone analogues are not the same in all synthetic cathinone compounds; they depend on the chemical composition, function, and their selectivity on DAT/NET/SERT. The growing body of information on the neurotoxicity of synthetic cathinones justifies a review on the neurotoxicity of the frequently used synthetic cathinones.

CHEMICAL STRUCTURE AND PROPERTIES

Synthetic cathinones are the structural analogues of amphetamines and are chemically referred to as β -ketone analogues because of the carbonyl (=O) group in β carbon. The common pharmacophore group responsible for the biological effect seen in amphetamine, MDMA (ecstasy), and cathinones is phenethylamine, represented in Figure 1.²⁰

The substitutional regions of synthetic cathinones are α and β carbon. The length of the substitution at the N-terminus varies within itself. MDPV and its derivatives differ from other derivatives by the presence of the nitrogen-containing pyrrolidine ring.⁴ There is no scientific classification of

synthetic cathinones; however, substituted cathinones can be classified as pyrrolidine-bearing/not pyrrolidine-bearing derivatives (Figure 2).

MDPV is structurally similar to pyrovalerone, the psychoactive drug used in the treatment of chronic lethargy.³ Pyrovalerone cathinones (MDPV and α -PVP) are highly lipophilic compared with other derivatives, thus having high blood-brain barrier (BBB) penetration/transition and a high volume of distribution. This results in longer plasma and tissue half-lives.²¹ The presence of electrophilic groups such as fluorine and the length of carbon substitution at the N-terminus increase the lipophilic nature of synthetic cathinone analogues.

MECHANISM OF ACTION

The underlying mechanism of the behavioral and physiological effects of synthetic cathinone derivatives is not fully understood yet. In general, synthetic cathinones increase the monoamine concentration in the synaptic cleft. The monoamine increase in the synaptic region mediates the stimulatory and hallucinogenic effects of synthetic cathinones.^{4,10,22}

Similar to other psychomotor stimulants, synthetic cathinones target plasma membrane DAT, NET, and SERT.⁴ Two distinct mechanisms mediate the increase in monoamine concentration in the synaptic cleft. In the first mechanism, as in amphetamines, it stimulates non-exocytic neurotransmitter release (secretory agents/substrates) by inhibiting the vesicular monoamine transporter-2 (VMAT2) and reversing the transporter influx. Substrates can stimulate neurotransmitter release from the cytosolic pool or synaptic vesicles. Monoamine neurotransmitters are packed into synaptic vesicles with the VMAT2. Since VMAT2 functions as a proton-monoamine antiporter, the change in the intravesicular pH value disrupts

the ability of VMAT2 to carry monoamines, thereby inhibiting vesicular storage of monoamines. Substrates such as MDMA alter the pH gradient required for VMAT2-mediated monoamine accumulation. In the second mechanism, as in cocaine, they inhibit the uptake of neurotransmitters from the synaptic cleft by inhibiting plasma membrane transporters, which are responsible for the uptake of NE and 5-HT as well as DA (reuptake inhibitors/blockers).^{22,23} Substrates and blockers differ in terms of their acute and chronic effects.⁴ Substrates can cause permanent damage to monoamine neurons by the loss of functional transporters and neurotransmitter depletion.²⁴ Synthetic cathinones may act as an inhibitor or as a substrate (as an inhibitor on single transporter/more than one transporters or as a substrate on single transporter/more than one transporters) on DAT, NET, and/or SERT.¹³ The underlying mechanism of the physiological and behavioral effects of synthetic cathinones differs from agent to agent depending on chemical composition, function (secretory agent or reuptake inhibitor), and DAT/NET/SERT selectivity. This shows that synthetic cathinone analogues have a complex mechanism. Mephedrone is a nonselective substrate and stimulates the non-exocytotic release of NE, 5-HT, and DA. MDPV is a potent blocker of the DAT and NET, and also has a slight effect on the SERT.^{4,25} Therefore, mephedrone and MDPV act via different mechanisms. When administered in combination, while MDPV blocks their re-uptake, mephedrone increases the concentration of neurotransmitters in the synaptic cleft. MDPV is 30-50 times more potent as a DA reuptake inhibitor than cocaine. Therefore, the combined use of mephedrone and MDPV causes effects resembling those of methamphetamine following cocaine intake, but with a more potent trend.¹³

To date, a few synthetic cathinone compounds' mechanisms of

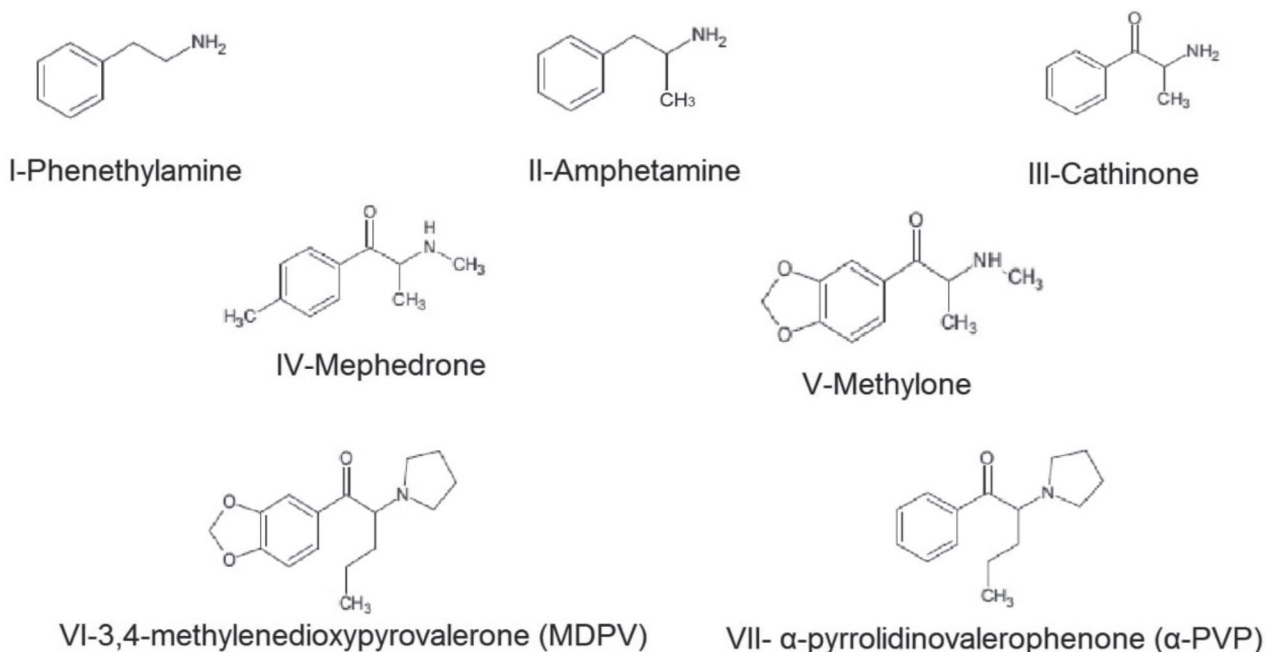


Figure 1. Chemical structure of phenethylamine, amphetamine, cathinone, and other synthetic cathinone derivatives²⁰

action have been defined in detail. Many synthetic cathinone derivatives are still in the process of being researched. Although researchers have made some progress on the structure-activity relationship of synthetic cathinones, further research is needed to predict the behavioral effects of new derivatives.

NEUROTOXICITY

Due to synthetic cathinones differing in terms of their mechanism and potential for causing changes in the extracellular concentrations of DA, NE, and 5-HT, their mood swing effects, toxicity, and dependence potentials are also divergent. Therefore, the toxicity of each synthetic cathinone should be assessed separately.^{9,26}

The neurotoxicity induced by amphetamines has been studied in detail in humans and animals. In these studies, several parameters such as neuroinflammation-inducing ability, oxidative stress, cytotoxicity, neurotransmitter system dysregulation, and changes in monoamine transporter levels and receptors were evaluated.²⁷

Methamphetamine is one of the best-known compounds for its neurotoxic effects at the dopamine nerve endings in the striatum. It has been demonstrated that MDMA causes long-term 5-HT depletion at monoamine nerve endings. Amphetamine neurotoxicity includes inhibition of biosynthetic enzymes of monoamines, i.e. tryptophan hydroxylase (TPH-2) and tyrosine hydroxylase (TH), DAT and SERT inactivation, diminished VMAT2 function, degeneration and apoptosis in unmyelinated axons at the nerve endings, prolonged DA, and 5-HT depletion.²⁸

Because of their structural similarities to amphetamines, it has been suggested that synthetic cathinones may have

a neurotoxicity profile similar to that of their amphetamine congeners. Hence, synthetic cathinones may cause neurotoxicity at the monoamine nerve endings in the striatum, hippocampus, and cortex.²⁹ Parameters such as monoamine depletion, biosynthetic enzyme inhibition, cytotoxicity, generation of reactive oxygen species (ROS), and ability to induce neuroinflammation of synthetic cathinones were compared with amphetamines. Researchers have reached the conclusion that synthetic cathinones are more moderate than their amphetamine congeners in terms of these toxic effects.²⁷

The neurotoxicity studies of synthetic cathinone compounds are generally directed to mephedrone toxicity.³⁰⁻³⁶ The neurotoxicity induced by mephedrone has not been fully elucidated yet. Despite the considerable evidence that mephedrone is dangerous, how it plays a role in the central nervous system is controversial. While no damage to dopaminergic nerve endings or no change in monoamine levels in the synaptic cleft was stated in parts of studies with animal models,^{31,32} in some other studies decreases in DAT and SERT function and damage to dopaminergic/serotonergic nerve endings were observed.^{30,33}

Many synthetic cathinone users take these substances simultaneously with other drugs such as benzodiazepines, other amphetamines, ecstasy, tetrahydrocannabinol, and ethanol.³⁷ It is known that 95% of synthetic cathinone users are also alcohol consumers at the same time. How ethanol modifies the neurotoxicity induced by mephedrone remains unknown but researchers found that ethanol exacerbated the decrease in DAT and TH levels in the frontal cortex as well as the reduction in SERT and TPH-2 levels by 2-fold in CD1 mice. Moreover, this decrease was correlated with a 2-fold increase in lipid peroxidation levels.³⁵

In another study with pregnant rats, repeated exposure to

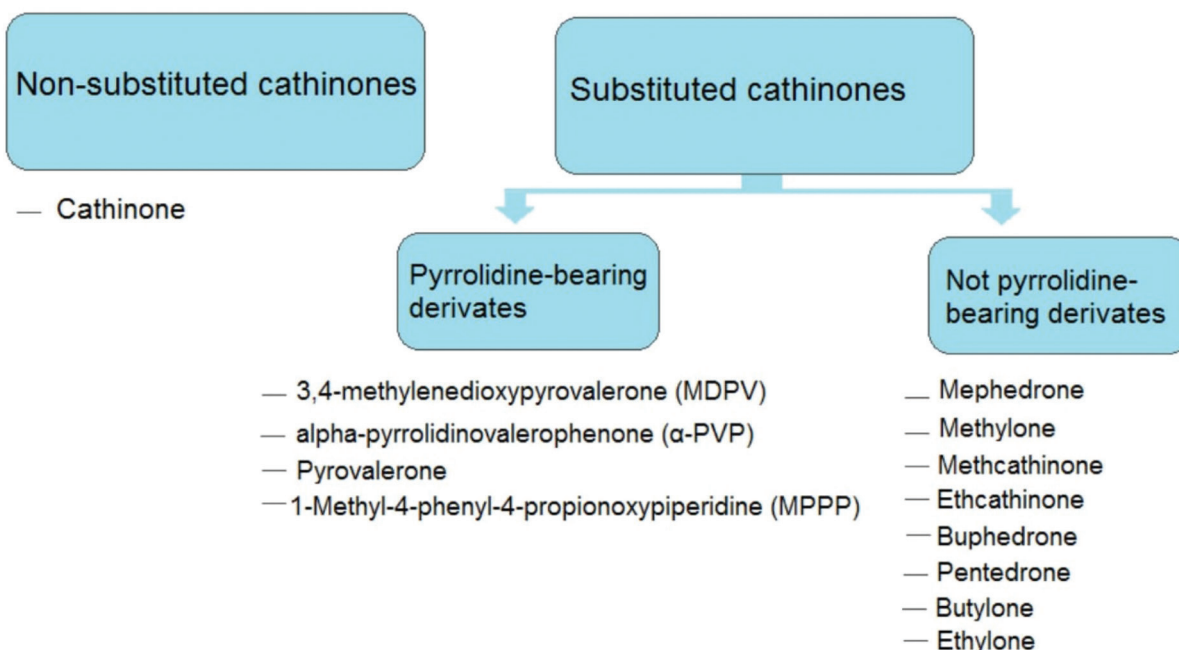


Figure 2. Classification of synthetic cathinone analogues

mephedrone during pregnancy caused low birth weight and increased risk of stillbirth. Moreover, it has been shown that repetitive use of mephedrone is associated with hippocampal damage and disrupts learning and memory processes.³⁶

An increase in cytotoxicity and lipid peroxidation parameters by inducing oxidative stress in the frontal cortex was observed after mephedrone application in rats.³⁴ In addition to brain tissue, mephedrone induces oxidative stress also in the kidneys, heart, liver, and spleen of mice.³⁸

There are also studies about MDPV and methylone neurotoxicity.^{29,33,39-43} In a study comparing the neurotoxicity of methylone and MDPV with that of MDMA, a concentration-dependent decrease in cell viability was observed in differentiated and undifferentiated dopaminergic cells (MDPV-MDMA>methylone). Differentiated cells were found to be more vulnerable than undifferentiated cells. As a mechanism of neurotoxicity, findings of increased intracellular ROS and NO levels, glutathione depletion, elevated levels of oxidative glutathione, deterioration of mitochondrial membrane potential, and intracellular ATP depletion indicate oxidative stress and mitochondrial dysfunction, resulting in cell death. Condensed chromatin, pyknotic cell formation, and caspase -3, -8, and -9 activations suggest that apoptosis is the mechanism of cell death induced by methylone and MDPV.³⁹

Microglia and astrocytes are primary inflammation modulators in the central nervous system. Increased glial-based cytokines in chronic neuroinflammation cause toxic effects in vulnerable neurons. The studies on the neuroinflammation-inducing ability of synthetic cathinones are controversial. Some *in vivo* studies have not found any evidence for astroglial activation in the striatal or cortical regions after mephedrone administration.^{33,34,44} However, increased glial-specific marker (glial fibrillary acidic protein) immunoreactivity in the hippocampus 7 days after mephedrone administration at recreational dose was observed.⁴⁵

In a recent study, it has been found that methylone neurotoxicity is related to posology. Furthermore, increased immobility in animals indicates depression-like effects.⁴⁰ Neurochemical/enzymatic changes in rats following methylone administration at the recreational dose (20 mg/kg) were tested and hyperthermia developed 30 min after dosing. In addition, serotonergic impairment accompanied by astrogliosis in the frontal cortex was observed, but no significant neurotoxicity was found in the dopaminergic system. The study was conducted at a high ambient temperature (26±1°C) to simulate the hot conditions in dance clubs where methylone was used.⁴¹ Time- and concentration-dependent ROS formation, reduction in cellular proliferation rate (only at high concentrations), apoptosis induction, and necrosis have been observed in human dopaminergic SH-SY5Y cells regarding the cytotoxic effects of MDPV.⁴² Induction of ROS formation may be the underlying mechanism of the early toxic effects of MDPV.

Various studies have reported adverse effects of methamphetamine and MDMA on the BBB. When comparing the toxic effects of MDPV on the BBB with those of

methamphetamine and MDMA, MDPV was found to be more toxic than its amphetamine homologues. MDPV showed cytotoxic effects at all concentrations. There was a decrease in cellular proliferation at 1 mM concentration, an increase in reactive oxygen and nitrogen species at all concentrations, and change in endothelial morphology at ≥0.5 mM concentrations.⁴³ These data suggest that synthetic psychostimulants may be toxic to the monoaminergic system as well as to the BBB through different mechanisms.

Another derivative that has attracted attention in recent years is α-PVP. Awareness of the case reports associated with α-PVP ingestion is increasingly important due to severe toxic effects and fatal intoxications.^{6,46-48} Although the exact mechanism of α-PVP remains unclear, it is known that α-PVP is designed to cause the brain to become flooded with dopamine. Due to its structural similarity to MDPV, α-PVP was suggested to be a NE-dopamine reuptake inhibitor and dopamine releaser, therefore causing an increase in locomotor activity.⁴⁹ However, studies investigating the potential neurotoxicity of α-PVP in the literature are very few. We came across one molecular mechanistic study about α-PVP. α-PVP modulated immediate early gene expression such as Arc/Arg3 and c-fos in the frontal cortex, striatum, and hippocampus in that study, thus indicating this drug has an impact on brain homeostasis.⁵⁰ Still, more research is needed with regard to the neurotoxic effects of α-PVP in different brain regions.

DISCUSSION AND CONCLUSION

Synthetic cathinones represent a broad class of pharmacologically active compounds that can cause numerous effects via different mechanisms. Synthetic cathinones are a global threat with rapidly increasing abuse rates. Due to these substances differing in terms of their mechanism and potency, their mood-altering effect, toxicity, and dependence potentials are also divergent. Therefore, each synthetic cathinone compounds should be evaluated separately.

In the literature, studies are generally directed to synthetic cathinones' psychostimulant and locomotor stimulant effects, neurocognitive performance, and memory, while neurotoxicity studies are limited. The neurotoxicity of synthetic cathinones has not been fully elucidated yet. The neurotoxic effects of synthetic cathinones on the serotonergic and dopaminergic system are controversial. Differences in experimental design, such as temperature and animal species, make it difficult to compare the results. In some animal model studies, no damage or changes in monoamine levels were reported at the dopaminergic nerve endings in the striatum, whereas in some other studies decreased DAT and SERT function and damage to dopaminergic/serotonergic nerve endings was observed. Preclinical findings indicate that the adverse effects of synthetic cathinones can often be exacerbated by high temperature and crowded conditions, as in dance club conditions where the drug is used. For this reason, in some neurotoxicity studies, this high ambient temperature (>26°C) has been imitated. It might be said that the neurotoxicity induced by synthetic cathinones is more

frequently observed in studies conducted at high temperatures. Since synthetic cathinones are often combined with other cathinone derivatives or noncathinone compounds such as alcohol, amphetamines, ecstasy, and cocaine, it is important to understand the neurotoxic effects in case of polysubstance use. Further research is needed from the toxicological point of view in order to address the health problems associated with bath salts.

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