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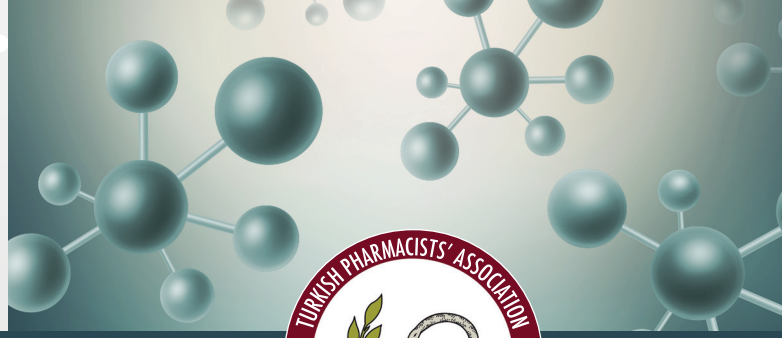

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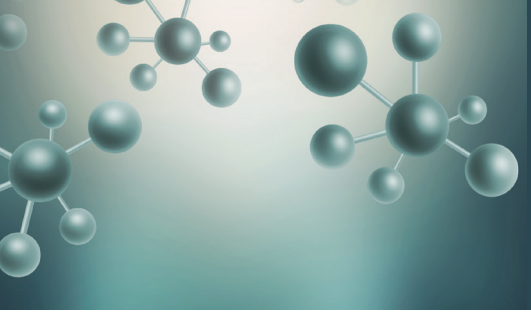

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PRISMA statement of preferred reporting items for systematic reviews and meta-analyses (Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group. Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med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

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acknowledgement sections. Title, abstract and key words should be written in both Turkish and English. The manuscript should be formatted in accordance with the above-mentioned guidelines and should not exceed 16 A4 pages.

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

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

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

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

Original research articles should have the following sections:

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CONTENTS

Turk J Pharm Sci 2023;20(6):347-351
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ORIGINAL ARTICLE

Evaluation of SARS-CoV-2 Antibody Levels in Pharmacists and Pharmacy Staff Following CoronaVac Vaccination

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ABSTRACT

Objectives: The aim of this study was to determine the seropositivity rate of pharmacists and pharmacy staff after the administration of two doses of the CoronaVac-SinoVac vaccine and to assess changes in their antibody levels according to sociodemographic characteristics.

Materials and Methods: This descriptive study was conducted between June 04, 2021 and September 30, 2021 in pharmacies located in Istanbul, Türkiye. The results of self-initiated immunoglobulin (Ig) G testing of the pharmacists and pharmacy staff, conducted at diagnostic laboratories contracted by the Istanbul Chamber of Pharmacists, were obtained using an online data collection tool. IgG measurements taken from 15 days up to 120 days after the two vaccine doses were included in the study. Participants were asked whether they smoked, had any chronic diseases (hypertension, chronic obstructive pulmonary disease, asthma, diabetes, *etc.*), or took any medications. Subgroup analyses were performed for each method used to measure antibody levels.

Results: The study included 329 pharmacists/pharmacy staff (298 pharmacists and 31 pharmacy staff). The mean age of the participants was 49.7 ± 13.7 years, and 71.4% were female. The antibody positivity of the 329 participants was 94.9% following the two doses. The positivity rate was 95.4% in participants under 65 years of age, whereas it was 91.8% in those aged 65 years and over. There was no significant difference in the mean age between those with positive and negative antibody results ($p > 0.05$). Although antibody levels were lower older people, smokers, and those with chronic diseases, this difference was not statistically significant ($p > 0.05$).

Conclusion: Seropositivity developed following the administration of two doses of CoronaVac-Sinovac vaccines. IgG antibody levels were lower in older adults, smokers, and those with chronic diseases, although not to a statistically significant extent. Further studies are needed to better understand the reasons for the different immunological responses to COVID-19.

Key words: COVID-19, pharmacist, CoronaVac-Sinovac, seropositivity, vaccines, antibodies

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is caused by a virus belonging to the Coronavirus family (HCoV-229E, HCoV-OC43, HCoV-NL63, HKU1-CoV), a family that often causes epidemics in the winter months and is responsible for one-third of common cold cases.[1]

Vaccines play a crucial role in increasing herd immunity, preventing severe diseases, and controlling ongoing health crises. CoronaVac (Sinovac Life Sciences, Beijing, China) is an inactivated vaccine against COVID-19 that shows good immunogenicity in animal trials and can neutralize severe acute respiratory syndrome (SARS-CoV-2) with vaccine-derived neutralizing antibodies.[2] The first vaccine used within the scope of the COVID-19 immunization program in Türkiye was CoronaVac. Phase III studies were completed in Türkiye, Brazil, Chile, and Indonesia. The positive neutralizing antibody rate was reported to be higher than 90%.[3] In addition, the results of phase III clinical trials conducted in Brazil showed that two doses of the Sinovac vaccine protected 50.7% of symptomatic COVID-19 patients and all moderate-to-severe cases.[4] The

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vaccine was approved for emergency use by World Health Organization (WHO) on 01.06.2021.

Both pre-clinical and clinical studies have revealed that the vaccine provides adequate protection for a certain period and prevents hospital admissions to a great extent.[1,5,6] The Turkish Ministry of Health approved the use of CoronaVac (Sinovac) on 13.01.2021, after which the vaccine was first administered to healthcare workers (HCW).[7]

CoronaVac has been demonstrated to be well tolerated in healthy adults aged 60 years and above and is immunogenic with neutralizing antibody responses against live SARS-CoV-2 having been shown to not decrease in said healthy adults over 60 years. Further studies are needed on the efficacy of this vaccine in preventing COVID-19 in older adults.[8]

HCWs, older adults, and those with underlying health problems are especially at higher risk.[9,10] HCWs have continued to work on the front line to care for their patients throughout the COVID-19 pandemic, and despite changes in routine practices, community pharmacists have delivered uninterrupted services to their patients. Moreover, the vaccine has been administered to community pharmacists and pharmacy staff in Türkiye, and they have, to a large degree, taken part in vaccination campaigns. Although there are studies in the literature on the changes in antibody levels in society and, more specifically, health workers created by COVID-19 vaccines and the factors affecting these changes, for these changes to be better understood, more studies need to be conducted with pharmacists and pharmacy staff. The aim of the present study was to measure the presence of antibodies produced following vaccination in pharmacists and pharmacy staff and to investigate the association between quantitative antibody values and age, underlying chronic diseases, smoking, and use of medications.

MATERIALS AND METHODS

Study design and participants

This descriptive study was conducted between June 04, 2021 and September 30, 2021 in pharmacies located in Istanbul, Türkiye. After informing the pharmacists and pharmacy staff about the study, all individuals aged over 18 years were invited to participate in the study (5,234 pharmacists and pharmacy staff in these pharmacies). A stratified sampling method was used with the target of accessing data from 500 participants. For data collection, an e-mail was sent to all members of the Istanbul Chamber of Pharmacists. The results of the tests (for which the pharmacists and pharmacy staff volunteered and which occurred in diagnostic laboratories contracted by the Istanbul Chamber of Pharmacists) were collected through an online data collection tool developed by the researchers according to the literature.[11]

The study was based on the results of COVID-19 spike antibody [immunoglobulin (IgG)] tests performed at the following centers contracted by the Istanbul Chamber of Pharmacists: Biruni Laboratories, Datalab Laboratory, Gelişim Tıp Laboratories, Türk Kızılay Sağlık A.Ş. (Turkish Red Crescent Health Co.), and Yaşar Hospital. IgG measurements taken from 15 days up to 120 days after the two vaccine doses were included in the study.

Participants were asked whether they smoked, had any chronic diseases (hypertension, chronic obstructive pulmonary disease, asthma, diabetes, *etc.*), or took any medications. Subgroup analyses were performed for each method used to measure antibody levels.

In accordance with the central limit theorem, parametric tests were used without testing for normality. For data analysis, the mean and standard deviation along with minimum and maximum values of characteristics were used for statistics of the continuous variables, while frequency and percentage values were used to describe the categorical variables. Student's *t*-test was used to compare the means of two independent groups, and the chi-square test was used to evaluate the categorical variables. Statistical significance was set at $p < 0.05$. For data analysis, the www.e-picos.com New York software and MedCalc statistical package program were used.[12]

The study received ethical approval from the Institute of Health Sciences Ethical Committee of Marmara University (approval number: 17.05.2021-82). Permission for the study was obtained from the Ministry of Health of the Republic of Türkiye. Online informed consent was obtained from all participants.

RESULTS

Three hundred and seventy-four pharmacists/pharmacy staff participated in the study; however, 45 of them were excluded due to COVID-19 diagnoses, and analyses continued with 329 participants (298 pharmacists and 31 pharmacy staff). Of these, 312 had positive antibody levels, while 17 were measured negative. In the evaluation of the relationship between antibody levels and sociodemographic parameters, only positive patients were considered, the results of laboratories with small sample numbers were not included, and the antibody levels of 222 individuals were evaluated in the two laboratories, where most of the tests were performed (Figure 1).

The antibody results of 62 individuals under the other category were not further evaluated for descriptive characteristics

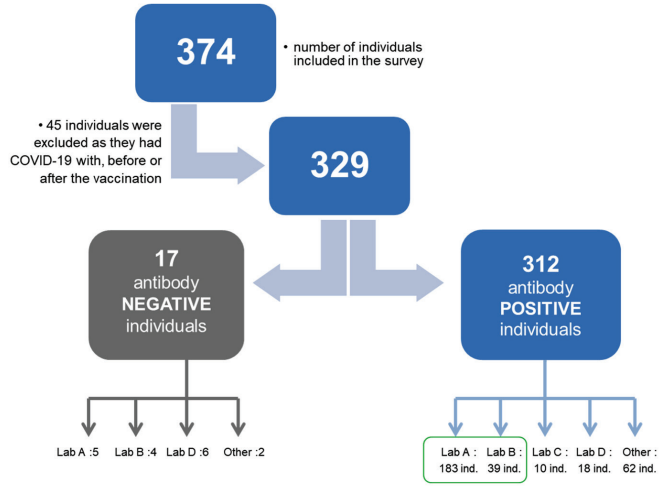

Figure 1. Flow chart of the study

(Because of the small number of individuals, the results of Lab C and Lab D were not further evaluated for descriptive characteristics)

because they were obtained from different institutions laboratories.

The mean age of the 329 participants was 49.7 ± 13.7 years; 71.4% were female, and 94.9% had positive antibody levels following two doses of the CoronaVac. The rate of positivity was 95.4% in participants aged < 65 years and 91.8% in those aged ≥ 65 years. Fifty percent of the participants were tested within 45 days following the second dose of Sinovac to determine antibody response. The participants with negative and positive antibody test results had no statistically significant differences in the descriptive parameters listed in Table 1 ($p > 0.05$).

Comparison of antibody levels according to sociodemographic characteristics of individuals with a positive antibody response after vaccination is provided in Table 2. These results were analyzed only internally without comparing the two laboratories. Although antibody levels were lower in smokers, older people, and the ones with chronic diseases, this difference was not statistically significant.

DISCUSSION

Following the vaccination during the COVID-19 pandemic, many studies have assessed the antibody levels of HCWs worldwide including Türkiye. Most of these studies included doctors and healthcare staff working in hospitals and clinics. The present study is the first to investigate community pharmacists and pharmacy staff's post-vaccine antibody levels and their association with age, smoking status, and chronic diseases.

We found antibody positivity to be approximately 95% after two doses of the CoronaVac. In other studies conducted with healthcare professionals in Türkiye, seropositivity rates of 99.6%, 99.4%, and 97%, respectively, were observed after four weeks in individuals vaccinated with two doses of the CoronaVac.[13] The slightly lower positivity rate in our study may be related to antibody level measurements being made over a wider time interval after two doses of the vaccine.

A subject we investigated was the possible factors affecting the vaccine antibody response. Although it is well known in other vaccines that the antibody response is affected by decreasing T-cell-derived antibody production and B lymphocyte production with age, more data are needed on the COVID-19 vaccine. In this study, the antibody level in older participants was lower than that in younger individuals; however, the difference was not statistically significant. In a study conducted with HCWs vaccinated with the CoronaVac in Türkiye, the antibody level was found to be highest in the group aged 30-39 years, but this finding was not statistically significant.[13] In Xia et al.'s[14] study, neutralizing antibody titers were lower in the group aged ≥ 60 years compared with the group aged 18-59 years. Similarly, in a study performed in Türkiye by Şenol Akar et al.,[15] the antibody response was significantly higher in participants aged 18-64 years.

The association with age was shown not only in studies of the CoronaVac-SinoVac but also in studies of other COVID-19 vaccines produced using different methods. To compare the antibody responses against the first and second doses of Biontech/Pfizer BNT162b2, a cohort study was conducted with two age groups: < 60 years and > 80 years. Although most participants produced titers of specific IgG antibodies in response to the SARS-CoV-2 spike protein, titers were found to be significantly lower in older individuals.[16] It was observed that antibody levels measured three months after the second dose of the Biontech/Pfizer BNT162b2 vaccine in HCWs in Japan were higher in individuals aged 20-29 years than in those aged 60-79 years.[6]

Table 1. Comparison of antibody test results by descriptive characteristics (n: 329)

Descriptive characteristics		Antibody test status after vaccination			
		Total (n: 329)	Negative (n: 17)	Positive (n: 312)	*P* value
Sex, n (%)	Female	235 (71.4)	11 (4.7)	224 (95.3)	0.58
	Male	94 (28.6)	6 (6.4)	88 (93.6)	
Disease status, n: 212, n (%)	Yes	139 (65.6)	8 (5.8)	131 (94.2)	0.56
	No	73 (34.4)	6 (8.2)	67 (91.2)	
Medication use, n: 104, n (%)	Yes	81 (77.9)	4 (4.9)	77 (95.1)	0.61
	No	23 (22.1)	2 (8.7)	21 (91.3)	
Smoking status, n: 208, n (%)	Yes	63 (30.3)	6 (9.5)	57 (90.5)	0.20
	No	145 (69.7)	7 (4.8)	138 (95.2)	
Age, n: 311, n (%)	< 65 years	262 (84.2)	12 (4.6)	250 (95.4)	0.30
	≥ 65 years	49 (15.8)	4 (8.2)	45 (91.8)	
Time from the second vaccine to antibody, mean ± SD (days)		68.7 ± 51.4	80.8 ± 54.2	68.1 ± 51.2	0.32

SD: Standard deviation

Table 2. Comparison of antibody levels according to sociodemographic characteristics of participants with a positive antibody response

Descriptive characteristics	Antibody level (AU/mL) after vaccination		
		Lab A results (n: 183)	Lab B results (n: 39)
	Groups	Mean ± SD	Mean ± SD
Sex	Female	575.12 ± 528.65	31.25 ± 52.17
	Male	580.57 ± 502.17	15.98 ± 34.1
	p	0.56	0.40
Age	< 65 years	595.37 ± 552.93	32.91 ± 23.84
	≥ 65 years	577.33 ± 434.54	9.42 ± 8.21
	p	0.86	0.26
Disease status	Yes	527.13 ± 469.41	32.78 ± 39.8
	No	550.67 ± 507.82	42.36 ± 64.03
	p	0.87	0.67
Medication use	Yes	491.99 ± 423.81	62.27 ± 81.09
	No	815.19 ± 754.37	6.85 ± 62.28
	p	0.12	0.44
Smoking status	Non-smoker	583.38 ± 531.32	61.07 ± 58.92
	Smoker	446.44 ± 392.31	15.24 ± 16.86
	p	0.23	0.07

Lab A SARS-CoV-2 IgG quantitative antibody, reference value: < 50 AU/mL-negative; ≥ 50 AU/mL-positive
Lab B COVID-19 IgG antibody (against spike protein), reference value: < 1 BAU/mL negative; ≥ 1 BAU/mL positive
SD: Standard deviation

In this study, it was observed that comorbid disease and chronic medication use had no effect on antibody response. A study on HCWs vaccinated with the Biontech/Pfizer BNT162b2 vaccine in Japan found that other diseases, such as diabetes and allergic diseases, were not associated with low antibody levels.[6] However, a study by Güzel et al.[17] found that the presence of comorbidities (diabetes mellitus and cardiovascular disease) decreased COVID-19 IgG antibody levels.

Watanabe et al.[18] conducted an observational study with 86 HCWs who received Pfizer-BioNTech COVID-19 vaccines and showed that higher waist circumference, smoking, and the presence of hypertension were associated with lower antibody titers. Two studies conducted in Indonesia on HCWs vaccinated with the CoronaVac concluded that the presence of hypertension negatively affects antibody levels.[19]

In this study, lower antibody levels were observed in smokers; however, this was not statistically significant. A study conducted by Uysal et al.[13] in Türkiye found that antibody levels were lower in smokers than in non-smokers (p = 0.032) among HCWs vaccinated with the CoronaVac vaccine. In another study conducted with healthcare professionals in Türkiye, antibody levels following CoronaVac vaccination were found to be significantly higher in non-smokers.[15] Nomura et al.[6] reported that age and smoking habits were the most important factors associated with low antibody titers. They highlighted that being a smoker was associated with lower antibody titers and that quitting smoking before vaccination may improve the individual efficacy of the vaccine.[6]

A study including a group of HCWs in Italy examined the effects of smoking on the humoral response produced by the BioNTech-Pfizer COVID-19 vaccine. In the study, antibody levels were lower in smokers than in non-smokers 60 days after vaccination (p = 0.002). According to the authors, although the pathophysiological basis of the effect of smoking on the dynamics of vaccine-induced anti-SARS-CoV-2 antibodies is not yet clear, previous studies reported that antibody response decreases more rapidly in smokers who receive other vaccines, such as vaccines for hepatitis B and influenza. Exposure to smoking impairs the immune system's ability to form memory cells, which are critical for maintaining the protective immune response induced by vaccines.[8]

Study limitations

This study has several limitations. The foremost limitation was the lower-than-expected number of participants, as the participants did their antibody testing voluntarily and at their own expense. Individuals with positive and negative antibody results were not compared because this was not a purpose of this study. Because the study evaluated post-vaccination antibody levels, it is likely that some individuals with negative results did not participate. Another limitation was conducting the study on a specific occupational group. Because of this specificity, the data cannot be generalized. In this study, only the COVID-19 spike antibody (IgG) response to the vaccine was assessed, and no information on cellular immunity was provided. Therefore, it cannot be assumed that our results provide adequate evidence regarding the level of protection from the disease that this vaccine offers individuals. Age, smoking, and the presence of diseases that may affect antibody response were examined; however, due to difficulties in collecting data from patients, parameters such as obesity and distribution of existing diseases and drugs could not be evaluated.

CONCLUSION

CoronaVac, an inactive SARS-CoV-2 vaccine administered to community pharmacists and pharmacy staff, produced seropositivity within two months in approximately 95% of the participants. The antibody response was higher in participants younger than 65 years, non-smokers, and those without a chronic disease; however, the differences were not statistically significant. Further studies are needed to determine how much the vaccine protects individuals from the disease. Although the effects of smoking, old age, and the presence of chronic diseases on the immunological response to the COVID-19 vaccine have been clinically proven by other studies, there is still a need for trials with a higher number of individuals and longer follow-ups.

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Ethics

Ethics Committee Approval: The study received ethical approval from the Institute of Health Sciences Ethical Committee of Marmara University (approval number: 17.05.2021-82). Permission for the study was obtained from the Ministry of Health of the Republic of Türkiye.

Informed Consent: Online informed consent was obtained from all participants.

Peer-review: Externally peer reviewed.

Authorship Contributions

Concept: G.Ü., S.D.S., M.S., Design: G.Ü., S.D.S., M.S., Data Collection or Processing: G.Ü., S.D.S., Analysis or Interpretation: G.Ü., S.D.S., M.S., Literature Search: G.Ü., S.D.S., Writing: G.Ü., S.D.S., M.S.

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ORIGINAL ARTICLE

Taste Masking of Steroids for Oral Formulations

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ABSTRACT

Objectives: Oral steroids are commonly prescribed to children. Steroids have a strong bitter taste that limits their oral acceptance in children. The objective of this study was to formulate a pediatric-friendly and palatable oral dosage form of steroids.

Materials and Methods: Solid dispersions of dexamethasone were prepared using polyethylene glycol, pectin, and Eudragit as carrier polymers, and chocolate as a flavoring agent. Taste masking efficiency was evaluated by healthy volunteers to select the best formula. The selected formula was pressed into chewable tablets with varying amounts of sweeteners. Chewable tablets were evaluated for palatability, hardness, and chewing index. The typical application of the taste masking approach was confirmed using prednisolone.

Results: Eudragit-based solid dispersions were effective in dexamethasone taste masking. Using 40% mannitol resulted in palatable tablets with acceptable hardness and chewing difficulty. The effectiveness of the taste masking approach was successfully used to prepare prednisolone chewable tablets. However, an increase in the carrier: drug ratio and a change in the flavor to pineapple were necessary to achieve maximum palatability of prednisolone chewable tablets.

Conclusion: Eudragit solid dispersion is an effective method for the taste masking highly bitter steroids. The solid dispersion was successfully pressed into a palatable, easy-to-chew, and pediatric-friendly chewable tablet dosage form. The carrier: drug ratio and the choice of flavoring agent are crucial factors in improving tablet palatability.

Key words: Solid dispersion, taste masking, steroids, dexamethasone, eudragit

INTRODUCTION

Oral steroids are widely used in children in liquid dosage forms for the treatment of asthma, virus-associated wheezing, Crohn's disease, and others.[1] While liquid dosage forms occupy the largest share of pediatric medicines and present the most widely acceptable dosage form,[2] they come with a fair share of problems such as dose accuracy issues due to differences in measured dose and the need for elaborate patient instructions on proper dose measurements. Additionally, some children refuse to take liquid medicines.[3] In contrast, chewable tablets grant ease of administration in toddlers and young children, dose accuracy over liquid preparations, and enhanced stability.[4] Compared to medicated gummies, chewable tablets can be produced into scored tablets that can further allow dose adjustment and flexibility, and they have been suggested to be associated with less misuse.[5] As such, there is an expanding interest in formulating pediatric medicines in chewable tablet dosage form.[6]

An important consideration in drug formulations that release the drug in the mouth is palatability.[7,8] Chewable tablets mandate the release of the drug in the oral cavity, resulting in an immediate sense of the drug taste. This renders chewable tablets not suitable for medicines of non-pleasant taste, including steroids. Consequently, adequate taste masking is required to formulate medicines into chewable tablets, which have been the focus of several studies.[9-12] Although the use of different sweeteners and flavoring agents has been widely used, several medicines have a strong unpleasant taste that dominates over all other additives incorporated in the formulation. More effective taste masking approaches include solid dispersion, complexing with taste masking excipients, coating of medicine through micro- or nanoencapsulation, and the use of ion-exchange resins.[13-16]

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Solid dispersions, defined as the dispersion of one or more active ingredients in an inert carrier in the solid state, have been successfully used for taste masking purposes in the formulation of different oral dosage forms.[16,17] Different techniques have been used to prepare solid dispersions with hot melt, solvent evaporation, and solvent-melt methods being the most employed methods. The solvent evaporation method involves solubilizing the drug and the polymer in a common solvent and then evaporating the solvent.[18] This method offers several advantages, including ease of preparation and avoidance of heat that can lead to thermal instability of the drug.[19]

The objective of this study was to formulate a pediatric-friendly and palatable oral dexamethasone and prednisolone dosage form. There have been few reports of taste-masked steroid formulations. One study reported orally disintegrating tablets of dexamethasone.[20] However, the taste evaluation was not reported clearly to allow full evaluation of the taste masking efficiency. Another study reported microparticle-based orodispersible films and tablets of prednisolone.[21]

This study aimed to develop, optimize, and characterize a taste-masking formulation of dexamethasone on a chewable tablet formulation. The study employed solid dispersion using three different polymers: polyethylene glycol (PEG), film-forming pectin, and pH-dependent solubility Eudragit®. Solid dispersions were prepared using the solvent evaporation method and incorporating different flavoring agents. The prepared solid dispersion was pressed into chewable tablets that were characterized for palatability and chewing difficulty, among other attributes. The expanded applicability of the taste masking method was demonstrated using prednisolone, another steroid commonly prescribed for children.

MATERIALS AND METHODS

Materials

Dexamethasone and prednisolone from Hangzhou Hyper Chemical Ltd. (China), Eudragit E PO from Xi'an Sonwu Biotech Co. Ltd. (China) were purchased. D-Sorbitol was obtained from Thomas Baker Chemicals (India), while Avicel® PH102 was purchased from FMC, Belgium. PEG 4000 and 6000 from BDH Chemical Ltd. (England), mannitol from Gerhard Buchmann (Germany), and HMPC 15 cps (hydroxy propyl methyl cellulose) from HI Media (India) were obtained. All solvents and reagents used were of analytical grade.

Dexamethasone solid dispersion preparation and characterization

Solid dispersion preparation

A dexamethasone solid dispersion was prepared by the solvent evaporation method. In brief, predetermined amounts of dexamethasone and carrier polymer(s) were accurately weighed. For PEG/HPMC solid dispersions, PEG was dissolved in ethanol in a mortar and then HPMC was added gradually. Dexamethasone stock solution at a concentration of 20 mg/mL was added, and the mixture was poured into a petri dish to allow solvent evaporation. The solid dispersion was collected and sieved through a 40-mesh sieve. Eudragit® E PO solid dispersions were prepared as previously described.[22] Briefly, Eudragit® E PO was freshly dissolved in 96% ethanol with continuous stirring. Dexamethasone solution was added and the solution was left on a magnetic stirrer until a thick gel consistency was observed. The solidified gel was triturated and sieved through a 40-mesh sieve. For pectin-containing formulations, pectin was dissolved in water at a concentration of 40 mg/mL at 40 °C and used fresh. When indicated, chocolate (at 40-50% of formulation weight) was melted and thinned in ethanol to obtain a pourable slurry that was added to the polymer solution prior to adding dexamethasone solution. A liquid flavoring agent (caramel, grapes or hazelnut) was added to the polymer solution before adding the dexamethasone solution. When required, oven drying was employed for solid dispersion formulations containing aqueous flavoring agents. A full description of all formulations assessed in this study is summarized in Table 1.

When required, blank Eudragit® E PO solid dispersion was prepared using the same method without the addition of dexamethasone.

Percentage yield

The collected solid dispersion granules were weighed, and the weight (W_{SD}) was divided by the total weight of the drug and polymer (and chocolate when applicable), W_T. The percentage yield was calculated using equation 1.

$$percent\ yield = \left[\frac{W_{SD}}{W_T}\right] x\ 100 \quad (1)$$

Drug content

An accurately weighted amount of the solid dispersion was dissolved in 96% ethanol on a magnetic stirrer for 3 h, after which the solution was filtered using a filter paper. Drug content was determined spectrophotometrically at 241 nm. A sample of blank solid dispersion was treated in parallel.

Differential scanning calorimetry

Five milligrams of dexamethasone, Eudragit® E PO, dexamethasone: Eudragit® E PO physical mixture, dexamethasone solid dispersion, and blank Eudragit® E PO solid dispersion were placed in a sealed aluminum pan and heated at a scanning rate of 10 °C/min over a temperature range of 25-300 °C in a nitrogen atmosphere at a flow rate of 100 mL/min. Thermal analyses were performed using Shimazu DSC.

Taste masking effectiveness of solid dispersion

All seventeen dexamethasone solid dispersion formulations described in Table 1 were evaluated for their effectiveness in masking the bitter taste of the drug by healthy volunteers. Volunteers were asked to place a small amount of the formulation on their tongues and score the solid dispersion bitterness on a scale of 0-10, where 10 is very bitter and zero is not bitter. Volunteers were instructed to expel the solid dispersion and not to sallow it and to rinse their mouths after each taste evaluation. Ten volunteers evaluated each solid dispersion formulation.

Preparation and characterization of dexamethasone chewable tablets

Preparation of dexamethasone chewable tablets

Chewable tablets were prepared according to the formulations presented in Table 2. The accurately weighed ingredients were sieved and mixed geometrically. Tablet mixtures were characterized for flowability by calculating the angle of repose using the fixed funnel method.[23] Carr's index was calculated by measuring bulk and tapped densities using a Copley JV 2000 tapped density tester, UK. Carr's index was calculated according to equation 2.

Carr's index = (Tapped density-Bulk density / Tapped density) x 100 (2)

To prepare the chewable tablets, the tablet blend was compressed into tablets using an 8-mm flat punch tablet press.

Chewable tablet evaluation

Hardness test: Six randomly selected tablets were evaluated using a YD-2 hardness tester (Sinopharm, China). Hardness was expressed as kilopond (kp).

Ease of chewing: Ease of chewing was evaluated using the chewing difficulty index (CDI) and volunteers' rating of chewing difficulty. CDI was calculated according to equation 3, where F_h is the tablet breaking force under diametral compression and H is tablet thickness.[24]

$$CDI = F_h H \quad (3)$$

In addition, healthy volunteers assessed the ease of tablet chewing. Volunteers were asked to chew the tablet and rate tablet chewing as easy, medium or hard. Five to six volunteers assessed each formula. Volunteers were instructed to discard chewed tablets and rinse their mouths after expelling the tablets.

In vitro disintegration: The *in vitro* disintegration time was determined using the disintegration test apparatus as *per* USP specifications. Briefly, one tablet was placed in each of the six vessels of the apparatus basket. 0.1 N HCl (900 mL) was used as the disintegration medium, and the temperature was kept at 37 ± 2 °C. The apparatus was operated for 30 min, and the time required for complete tablet disintegration was recorded.

In vitro dissolution: The dissolution profile of the prepared dexamethasone tablets was determined using USP dissolution apparatus II (paddle method) and according to USP specifications.[25] The dissolution study was performed using 0.1 N HCl as the dissolution media at a 37 ± 0.5 °C and 100 rpm. A 5 mL sample was withdrawn at 0, 5, 15, 20, 30, and 45 min. Samples were filtered through 45 µm Millipore filter. Samples were extracted with three 15 mL portions of chloroform. Chloroform was evaporated from the combined extracts and the residue was dissolved in 20 mL of ethanol and analyzed spectrophotometrically at 241 nm. The amount released in mg was calculated using equation 4, where C is the dexamethasone concentration and V is the volume of the aliquot extracted with chloroform. Drug release was considered satisfactory, if not less than 2.8 mg (70%) dexamethasone was released in 45 min portion of dexamethasone dissolved.

Table 1. Formulation of the dexamethasone solid dispersion (expressed as *w/w*)

Formulation	Dexamethasone	PEG 6000	HMPC	Pectin	Eudragit® EPO	Flavoring agent
F1	0.1	1.5				Chocolate
F2	0.1	3.0				Chocolate
F3	0.1	1.5	0.75			/
F4	0.1	1.5	1.5			/
F5	0.1	1.5	1.5			Caramel/chocolate
F6	0.1	1.5	1.5			Grape
F7	0.1	3.0	1.5			Chocolate
F8	0.1	3.0	1.5			Hazelnut/chocolate
F9	0.1	3.0	3.0			Caramel/chocolate
F10	0.1			0.1		Hazelnut/chocolate
F11	0.1			0.2		Hazelnut/chocolate
F12	0.1			0.2		Caramel/chocolate
F13	0.1	1.5	1.5	0.1		Caramel/chocolate
F14	0.1				0.2	Chocolate
F15	0.1				0.4	Chocolate
F16	0.1				0.8	Chocolate
F17	0.1				0.4	/

Table 2. Chewable tablet formulation of the model steroids employed in this study

Ingredient	A Dexamethasone			B Prednisolone		
Notation	**D1**	**D2**	**D3**	**P1**	**P2**	**P3**
SD, weight (drug dose)	40 (4 mg)	40 (4 mg)	40 (4 mg)	95 (5 mg)	95 (5 mg)	45 (5 mg)
Flavor	Chocolate	Chocolate	Chocolate	Chocolate	Chocolate	Pineapple
Avicel, weight (%)	80 (40%)	80 (40%)	60 (30%)	150 (30%)	150 (25%)	150 (30%)
PEG 4000, weight (%)	20 (10%)	20 (10%)	20 (10%)	50 (10%)	50 (8%)	50 (10%)
Sorbitol, weight (%)	60 (30%)					
Mannitol, weight (%)		60 (30%)	80 (40%)	205 (41%)	305 (51%)	255 (51%)
Final weight (mg)	**200**	**200**	**200**	**500**	**600**	**500**

SD: Standard deviation

(in mg) = 10(C/V)(Au/*(As)* (4)

Tablet palatability: Five healthy volunteers were asked to evaluate the palatability of the tablet and report their acceptance of the chewable tablet on a scale of 1-5 with one being very unpalatable and five being very palatable. The volunteers were instructed to chew the tablet, expel it, and rinse their mouths after each taste.

Applicability of the taste masking technique for other steroids

Solid dispersion preparation

Prednisolone solid dispersion was prepared using Eudragit® E PO at drug: polymer ratios of 1:4, 1:6, and 1:8 using the same method described for dexamethasone. The flavors used included chocolate and pineapple. Healthy volunteers evaluated the taste masking efficiency of the prepared solid dispersions, similar to the procedure described for dexamethasone solid dispersion. Each formulation was assessed by five volunteers.

Chewable tablet preparation and characterization

Chewable tablets of prednisolone were prepared according to the formulations presented in Table 2B. The accurately weighed ingredients were sieved and mixed geometrically. The tablet mixture was compressed into tablets using a 12-mm flat punch tablet press for prednisolone tablets. Chewable tablets were characterized for hardness, chewing index, ease of chewing, and palatability, as described earlier for dexamethasone chewable tablets.

Statistical analysis

When indicated, data were analyzed by the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test. Differences were considered significant at an adjusted p value < 0.05. statistical analyses were conducted using GraphPad Prism 7.04.

Compliance with ethical standards

The study proposal was approved by the ethical committee of the institution (approval number: RECAUBCP-31102020B).

RESULTS AND DISCUSSION

Dexamethasone solid dispersion preparation and characterization

Seventeen solid dispersion formulations were successfully prepared for dexamethasone using three types of polymers, namely PEG, pectin, and Eudragit® E PO (Table 1). Successful preparation indicates the ability to grind the solid dispersion mass into fine particles that can be used for tablet preparation. Percent yield for all seventeen formulas ranged from 80 to 98%, with lower yield for pectin-containing formulas (F10-F13) and formulas F5-F6 and higher yield for formulas F1-F4 (PEG 6000). All seventeen formulas were characterized for taste masking efficiency, which was the goal of the solid dispersion preparation. For PEG-based solid dispersions, F1-9, volunteers reported highly variable results, with the general trend being failure of the solid dispersion to mask the bitter taste of dexamethasone (Figure 1). Similar incomplete taste masking by PEG 6000 was reported for arbidol hydrochloride.[16] PEG 6000 was selected based on its oral safety, relative ease of handling and processing in solid dispersion preparation, and low affinity for ethanol, which further enhances solid dispersion preparation.[26] Additionally, it is superior to the lower molecular weight PEG in dissolution rate enhancement.[27] Formulation F6 showed the best outcome among PEG-based formulations. This was the only formulation that used a grape flavoring agent. However, this formulation consistently had a lower yield (about 81%) and required extended oven drying due to the large volume

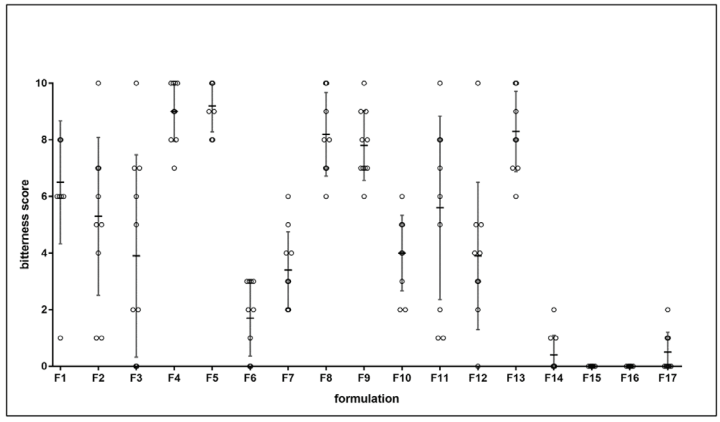

Figure 1. Taste masking efficiency of different solid dispersion formulations. Bitterness level is expressed as a scale of 0-10 with 0 is not bitter and 10 is very bitter. Data are expressed as mean ± SD, n: 10

SD: Standard deviation

of aqueous flavoring agent required. Therefore, no further modifications to improve this formulation were attempted.

The other polymer used for taste masking was pectin. Pectin is a natural polymer that is abundant and safe. Pectin has been suggested to have good taste masking properties and has been used as a carrier for solid dispersion.[28] It is hypothesized to limit drug release in the mouth because it is specifically hydrolyzed by colon bacteria,[29,30] thereby reducing or masking the bitter taste of the drug. Pectin-based solid dispersions, F10-F13, resulted in variable outcomes for taste masking efficiencies comparable to those obtained for the PEG-based formulation (Figure 1). Additionally, solid dispersion yield and processing were not highly reproducible due to the tendency of pectin to form plastic-like sheets that are difficult to pulverize.[30] Pectin has been used to limit drug release in acidic media.[31] In this study, we displayed the technical limitations of using pectin as a sole carrier for solid dispersions. In addition, the combination of PEG and pectin failed to efficiently mask the bitter taste of dexamethasone, F13 (Figure 1). Higher amounts of pectin than those used in F13 resulted in a sheet-like material, and the solid dispersion was rated as a failed formulation (data not shown).

On the other hand, all three Eudragit® E PO-based formulations showed promising outcomes even with a lower drug: polymer ratio of 1:2 in F14 (Figure 1). A further increase of the ratio to 1:4 in F15 resulted in complete taste masking of the bitter dexamethasone taste. Eudragit® are acrylic-based polymers with varying solubility properties that allow the control of the rate of drug release.[32] The Eudragit® E family are soluble in pH below 5, which is below saliva pH[32] making it suitable for taste masking applications.[8,22] To evaluate the need for chocolate for taste masking, a 1:4 solid dispersion was prepared without chocolate. Taste masking evaluation indicated a better performance of the chocolate containing solid dispersion (Figure 1), F15 *vs.* F17. These results agree with previous reports on flavor-free taste-masked microparticles that were largely rated "tasteless" but not pleasant.[33] Chocolate flavoring had been shown to be effective for taste masking of bitter drugs[34,35] and has been used to improve the palatability and acceptability of dexamethasone oral formulations.[36] Additionally, chocolate represents an excellent flavoring agent for pediatric dosage forms due to its high acceptability among children and even adults.[37,38] Besides palatability improvement, chocolate has been suggested to offer several health benefits, including antioxidant effects, cardiovascular effects, and possible cognitive effects.[39,40] As such, chocolate containing Eudragit® E PO solid dispersion with a dexamethasone:polymer ratio of 1:4 was used for further evaluation.

Selected solid dispersion formulation characterization

Before chewable tablet preparation, the chocolate containing 1:4 Eudragit® E PO-based solid dispersion was evaluated for yield, drug content, and thermal analysis. A percent yield by weight of 92.88 ± 3.50% was obtained across the different batches prepared. The dexamethasone content of the solid dispersion was found to be 97.91 ± 2.21% of the initial drug used in the preparation. These results confirm the technical suitability of the prepared solid dispersion for further formulation into a pharmaceutical dosage form. DSC analyses were conducted to confirm the formation of solid dispersions. The DSC thermogram of pure dexamethasone presented an endothermic peak at 257.41 °C and Eudragit® E PO thermogram showed two peaks one at 227.48 °C and another broad peak at 86.48 °C (Figure 2A, B). A dexamethasone peak was evident in the physical mixture

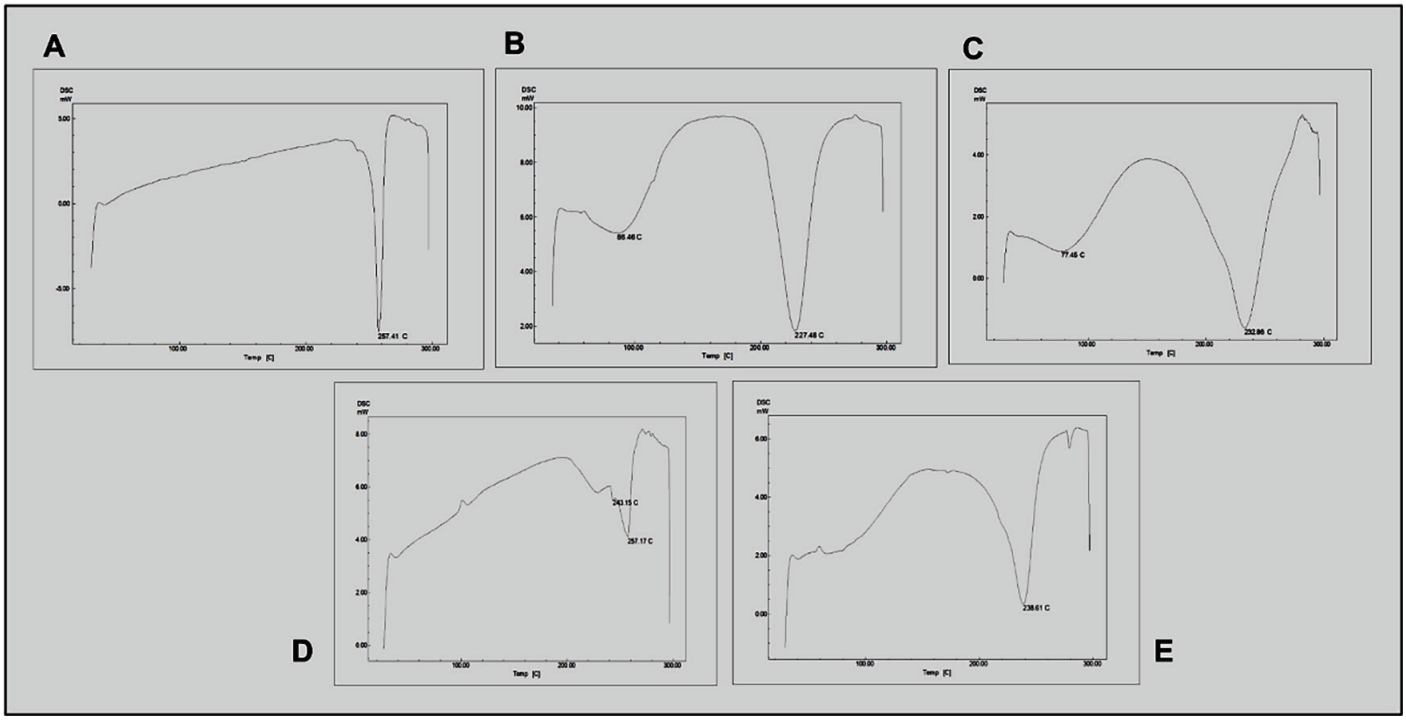

Figure 2. DSC profile of A: dexamethasone, B: Eudragit® EPO only, C: 1:4 solid dispersion, D: 1:1 physical mixture, and E: blank solid dispersion

but absent in the solid dispersion thermogram, confirming solid dispersion formation and indicating drug polymer miscibility and possible drug solubility in the polymer liquid phase (Figure 2C, D).[41,42]

Preparation and characterization of dexamethasone chewable tablets

Dexamethasone solid dispersion was developed into chewable tablets for optimal delivery to patients. Tablets were developed according to the mixtures presented in Table 2. The tablet blend contained basic constituents required for tablet compression such as binders and diluents. Because chewable tablets are intended mainly for children, the tablet blend was limited to essential excipients.[43] Sweeteners at different weight percentages were incorporated to improve tablet palatability. The change of sorbitol to mannitol resulted in a marginal non-significant reduction in tablet blend flow properties (Table 3). Such results are expected because of the poorer flow properties and compressibility of mannitol compared with sorbitol. However, tablet blend flow properties remained well. Thus, it was used without further adjustments.

Chewable tablets should have palatable taste, be easy to chew, compressed into acceptable size and shape, and readily disintegrate.[44] The tablet blend used in this study was compressed into 8-mm chewable tablets with 2-mm thickness. The use of 30% sorbitol as a sweetener resulted in a moderately palatable tablet. The use of mannitol at the same concentration resulted in an improved palatability that was significantly improved by further increasing the mannitol content in the tablet blend to 40% (Figure 3A). Sorbitol and mannitol are both naturally occurring sugar alcohols (polyols) that are used as bulk sweeteners in pharmaceutical preparations. They have comparable sweetness, and both are less sweet than sucrose. Mannitol and sorbitol are non-caloric, non-hyperglycemic, and do not cause dental decay, which makes them attractive for pharmaceutical dosage forms compared with sucrose. Mannitol specifically is commonly used in chewable tablets because of its high negative heat of solution, which boosts its cooling effect.[45,46] Even though the tablets were evaluated by adult participants, the chocolate component and sweet taste from mannitol are expected to enhance tablet acceptance by pediatric patients.

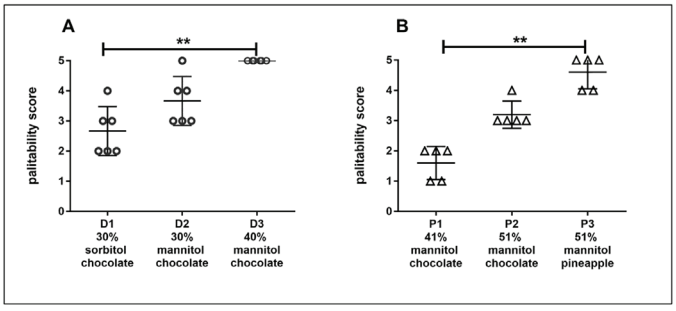

Figure 3. Palatability of steroids chewable tablets. A: Dexamethasone chewable tablets, B: Prednisolone chewable tablets. Data are expressed as mean ± SD, n: 5-6. **: $p < 0.01$

SD: Standard deviation

Table 3. Precompression characterization of the chewable tablet powder blend

		Angle of repose	Carr's index	Flow properties
Solid dispersion		17.14 ± 1.33		Excellent
Tablet blend	D1	28.57 ± 0.99	10.86 ± 0.16	Excellent and good
	D2	31.80 ± 1.96	11.58 ± 0.58	Good and good
	D3	30.78 ± 0.88	11.45 ± 1.11	Good and good

Values are expressed as mean ± SD, n: 3
SD: Standard deviation

In addition to palatability, dexamethasone chewable tablets were evaluated for hardness and chewing difficulty. Chewable tablets are intended to be chewed completely by the patients and they are largely intended for pediatric patients.[47] Excessive tablet hardness might lead to incomplete chewing by the patient which can cause serious side effects, including choking and intestinal obstruction. A CDI has been described and is recommended by the FDA in quality attribute guidance for chewable tablets.[44] The index relates tablet hardness measured under diametral loading and tablet thickness, and it provides a direct estimate of the force required to break the tablets in the mouth.[24] As *per* FDA guidance, the hardness of small chewable tablets should not exceed 12 kp to avoid serious side effects. Dexamethasone tablets for all three formulations showed comparable results in terms of hardness and CDI with no significant differences (Table 4). The hardness of the three different formulations was below 12 kp, and they were rated easy to chew by volunteers, suggesting a successful chewable tablet formulation.

A major consideration in chewable dosage forms is the need for mechanical stress provided by chewing to initiate drug release. However, it is possible that the patient will swallow the tablet or inadequately chew it. Consequently, disintegration and dissolution tests were conducted to ensure tablet performance compliance with USP specifications if tablets were swallowed by the patient without chewing. Chewable tablets from all three formulations passed the disintegration test according to the USP specifications for plain tablets. The disintegration time for all three formulations was comparable with no significant differences (Table 4). The dissolution profile for the optimal chewable tablet formulation showed more than 70% dexamethasone release within 45 min, which suggests that the chewable tablet has passed the USP tolerance limit for the dissolution test (Figure 4).

Applicability of the taste masking technique to other steroids

To demonstrate the applicability of the prepared solid dispersion for taste masking of other steroids, the same method was applied to prepare prednisolone chewable tablets. Initially, chocolate containing Eudragit® E PO solid dispersion was prepared according to the method described for dexamethasone. The use of a drug: polymer ratio of 1:2 was not sufficient to mask the bitter taste of prednisolone and an increase up to 1:8 was

required to reduce the bitter taste as rated by the volunteers. These results agree with the previously reported taste masking efficiency of Eudragit® E PO microbeads.[48] Additionally, a pineapple-flavored solid dispersion was prepared, and it was rated nearly non-bitter by the volunteers (data not shown). Chewable tablet blends were prepared like the dexamethasone optimal blend starting with 41% mannitol, which was increased to 51%. However, 51% mannitol was insufficient to produce sufficiently palatable tablets, Figure 3B. In contrast, the use of pineapple as a flavoring agent with 51% mannitol (P3) enhanced the palatability of the prednisolone pineapple-flavored chewable tablets compared with both chocolate-flavored tablet formulations (P1 and P2), Figure 3B. While chocolate flavoring was reported to be optimal for bitter drug taste masking,[49] it is possible that the taste masking effect of the chocolate-based solid dispersion was disrupted by the tablet compression force, which resulted in premature drug release, causing non-palatable tablets.[50] This speculation is based on the fact that a lower amount of mannitol was sufficient to produce palatable tablets in the pineapple flavored tablets (Table 2). Additionally, for some bitter medications, a high content of the flavoring agent is needed to mask the bitter taste,[35,49] which was not possible for the chocolate-containing chewable tablets prepared in this study to avoid a larger tablet size that renders the tablet no longer suitable for pediatric patients.

Prednisolone chewable tablets were rated easy to chew by all participants, except for the chocolate-containing tablet compressed with 51% mannitol (P2), which was rated moderate to chew by 3/6 participants. Tablet hardness and chewability index were within the accepted level, indicating successful tablet formulation (Table 5).

Study limitations

Stability studies should be conducted to confirm the long-lasting taste masking efficiency of the developed formulation.

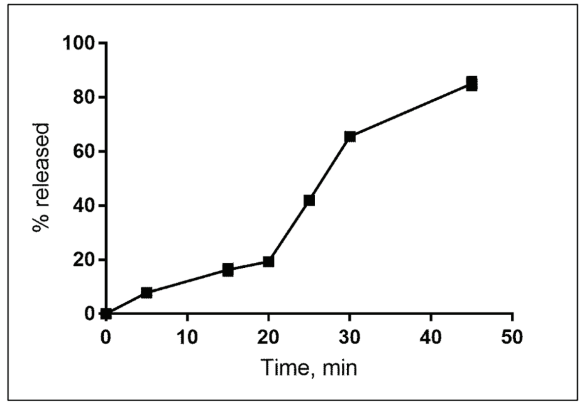

Figure 4. Dissolution profile of dexamethasone chewable tablets. Tablets were prepared according to formulation D3. The dissolution test was conducted in 0.1 N HCl. Data are plotted as mean ± SD, n: 3

SD: Standard deviation

Table 4. Characterization of the dexamethasone chewable tablets

Parameter	Formulation			Statistical differences
	D1 30% sorbitol	D2 30% mannitol	D3 40% mannitol	
Weight uniformity (mg)	199 ± 1	197 ± 2	200 ± 4	NA
Hardness (kp)	4.4 ± 0.4	4.7 ± 0.1	4.6 ± 0.3	ns
Chewing difficulty index (Nm)	0.87 ± 0.08	0.94 ± 0.03	0.92 ± 0.05	ns
Ease of chewing	Easy	Easy	Easy	NA
Disintegration time (sec)	18.6	22.1	18.9	ns

Samples are presented as mean ± SD, n: 6, NA: Not applicable, ns: Non-significant
SD: Standard deviation

Table 5. Characterization of the dexamethasone chewable tablets

Parameter	Formulation			Statistical differences
	P1 41% mannitol chocolate	P2 51% mannitol chocolate	P3 51% mannitol pineapple	
Tablet thickness (mm)	1	1.2	1	NA
Weight uniformity (mg)	493 ± 9	604 ± 12	496 ± 11	NA
Hardness (kp)	7.3 ± 0.7	6.6 ± 0.7	6.8 ± 0.6	ns
Chewing difficulty index (Nm)	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	ns
Ease of chewing	Easy	Easy/moderate	Easy	NA

Samples are presented as mean ± SD, n: 6, NA: Not applicable; ns: Non-significant
SD: Standard deviation

Evaluation of optimal taste masking in the targeted population (pediatric patients) was not feasible due to ethical considerations.

CONCLUSION

A chewable tablet formulation was developed with improved palatability and taste masking of bitter steroids. The chewable tablets contained the bitter steroid in a taste-masking flavored solid dispersion employing Eudragit® E PO polymer. The drug: carrier ratio and the flavor choice impacted the palatability of the chewable tablets. The chewable tablets were within acceptable levels of hardness and were easy to chew as rated by healthy volunteers. The chewable tablet formulation is expected to improve compliance in pediatric patients and allow dosing flexibility, when prepared as scored tablets. Further studies are required to assess the long-term stability of the prepared tablet.

Ethics

Ethics Committee Approval: The study proposal was approved by the ethical committee of the institution (approval number: RECAUBCP-31102020B).

Informed Consent: Volunteers were informed about the content of the sample and possible side effects.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: K.K.A., H.J.K., Design: K.K.A., H.J.K., Data Collection or Processing: K.K.A., H.J.K., I.J.A.R., Z.S.A., Analysis or Interpretation: K.K.A., H.J.K., Literature Search: K.K.A., Z.S.A., Writing: K.K.A., H.J.K.

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

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ORIGINAL ARTICLE

Pharmacist's Impact on Medication Adherence and Drug-Related Problems in Patients with Epilepsy

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ABSTRACT

Objectives: Drug-related problems (DRPs) and non-adherence are important barriers to ensuring optimal antiseizure drug treatment. The aim of this study was to improve medication adherence, detect and manage DRPs, and decrease the number of seizures with pharmacist-led education in patients with epilepsy.

Materials and Methods: A prospective and interventional study was conducted in collaboration with the Department of Neurology, the rational drug usage unit of a hospital pharmacy in a university hospital. The impact of pharmacist-led education on medication adherence and interventions in the management of DRPs was assessed in patients with epilepsy who were admitted to the outpatient clinic. A total of 39 patients with epilepsy were evaluated in terms of medication adherence, DRPs, and seizure control over a 2-month follow-up period and patient satisfaction with pharmacy services at the end of the study.

Results: A total of 59 DRPs were detected, and 71.2% of them were accepted and implemented both by physicians and/or patients. Pharmacist interventions solved 62.7% of DRPs. The number of patients with high-level medication adherence significantly increased from 17 to 28 after pharmacist-led education ($p < 0.001$). The number of seizures decreased in 19 patients (48.7%) during the 2-month period. Patient satisfaction was high in all patients.

Conclusion: It is shown that the contribution of pharmacists in treating patients with epilepsy is beneficial in improving medication adherence, detection and management of DRPs, and decreasing the number of seizures.

Key words: Drug-related problems, epilepsy, medication adherence, patient education, pharmacist

INTRODUCTION

Epilepsy, one of the most common neurological illnesses worldwide and with neurobiological, cognitive, psychological, and social consequences, is a chronic neurological disorder characterized by recurrent and unprovoked seizures that frequently begin in childhood or adolescence.[1,2] Therefore, epilepsy may lead to an increase in morbidity and restriction of daily activities and occupational abilities.[3] The lack of seizure control has unfavorable outcomes such as falls, injury, increased admission rate in physician office hospitalization, loss of work, and increase in healthcare cost.[4]

Antiseizure drugs (ASMs) are the principal treatment options for controlling or preventing seizures in patients with epilepsy.[5] Although appropriate use of ASMs reduces the frequency of seizures by approximately 67%, epilepsy can remain unrestrained in some patients.[5,6] However, in 70-80% of patients with new-onset epilepsy, seizures can be entirely controlled with appropriate ASM choice and medication adherence.[1] Medication adherence refers to the extent to which the recommendations given by a healthcare professional are followed by the patient. Ensuring medication adherence is important in terms of contributing to the drug selection of patients and strengthening the relationship between the healthcare provider and the patient.[7] Drug-specific adherence problems in patients with epilepsy are considered the high frequency of ASMs, polytherapy, and ASM-specific problems

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such as adverse events and low efficacy.[8] Additionally, ASMs have serious adverse effects that may lead to discontinuation of drug therapy, non-adherence, and a negative impact on the quality of life of patients with epilepsy. Therefore, optimization of treatment must ensure the safety and efficacy of ASMs.[9]

Another concern is comorbidities and co-medications with ASMs, which may enhance the frequency of drug-related problems (DRPs) and drug-drug interactions that may lead to loss of seizure control, adverse effects, and toxicity.[10] Clinical pharmacists have many significant roles in this patient group, such as therapeutic drug monitoring, interpretation of laboratory tests, determination of drug-drug interactions and adverse effects of medications, dosage adjustment, and patient education regarding medications and diseases.[11] A systematic review emphasized that clinical pharmacists may have a positive impact on medication adherence, patients' knowledge regarding medications and disease, and the quality of life of patients with epilepsy.[12] In a study, it was revealed that adherence improvement education and motivational interviews provided by the pharmacist reduced the number of seizures and improved ASM knowledge in patients with epilepsy.[13] DRPs contribute to non-adherence or low adherence rates due to inappropriate medication use and adverse effects.[14] Because medication adherence is essential to ensure the control of seizures and prevent treatment failure, pharmacist-led educational interventions may be beneficial in the management of epilepsy. In this study, we detected and manage DRPs, reduce the number of seizures, and improve medication adherence through pharmacist-led education in patients with epilepsy.

MATERIALS AND METHODS

Study design

This study was an interventional, pre-post, collaborative, and prospective study with 2 months a 2-month patient follow-up period.

Study settings

This study was conducted at the University of Health Sciences, Türkiye, Izmir Tepecik Training and Research Hospital between April 15 and October 31, 2019, in Türkiye. The hospital has a rational drug usage unit that belongs to the hospital pharmacy. This study was conducted in collaboration with the rational drug usage unit and the Department of Neurology.

Study population

All patients over 18 years-old with epilepsy, who were able to communicate, used more than four medications and were willing to sign an informed consent form were involved in the study. Patients who have cognitive impairment (such as dementia, Alzheimer's disease), speech disorder, pregnancy or decline to participate in our study were excluded.

Routinely, patients admitted to the Neurology Outpatient Clinic receive epilepsy care from the physician. Following the physician-patient interview, patients who meet the inclusion criteria are directed to the rational drug use unit by the physician. Within the scope of this study, detection and treatment of DRPs and education on disease, medication, and lifestyle changes were provided to patients by the pharmacist in the rational drug unit.

Data collection

During the first interview, the patient's demographics, routine medications, herbal medications, comorbidities, number of ASMs, and number of seizures were recorded in patient follow-up forms from the hospital information system database and patients through a face-to-face interview. To determine DRPs, the Pharmaceutical Care Network Europe (PCNE 8.01) classification was performed by the pharmacist. The pharmacist reported the interventions to the physician to resolve the identified DRPs. Additionally, we used the Morisky-Green-Levine Scale (MGLS) to assess medication adherence. After two months, when patients visited their physicians for routine follow-up, a second patient-pharmacist interview was conducted. During the second interview, MGLS and the Patient Satisfaction with Pharmacist Services Questionnaire (PSPSQ 2.0) were administered, and the number of seizures within those two months was recorded.

Tools

In the first pharmacist-patient interview, the pharmacist applied the MGLS to assess the medication adherence level. Self-reporting medication adherence scales have many advantages, such as being cost-effective, brief, patient-centered, and having good psychometric properties. Developed by Donald E. Morisky and validated by Morisky, Green, and Levine, self-reporting MGLS, which consists of 4 "yes" (0 point) and "no" (1 point) questions, is used to evaluate the medication adherence of patients with chronic diseases. The medication adherence level of patients. This study's aimedents were determined as low (0 point), medium (1-2 points), and high (3-4 points). The validity of the MGLS was validated and translated into Turkish by Yilmaz and Buzlu.[15]

PCNE classification is an instrument that is periodically updated and widely used for the classification of DRPs. In our study, PCNE was used to classify pharmacist-identified DRPs and their causes, interventions, and outcomes. PCNE version 8.01 consists of 5 main domains (P: Problems; C: Causes; I: Planned intervention; A: Acceptance; O: Status of acceptance).[16]

Developed by Sakharkar et al.[17] PSPSQ 2.0 is an instrument for evaluating the satisfaction of patients with chronic diseases in pharmaceutical services. PSPSQ comprises 22 questions, and each question is scored on a 4-point Likert scale (strongly agree, agree, disagree, and strongly disagree) ranging from 1 to 4 points. In addition, three sub-dimensions (quality of care, patient-pharmacist relationship, and overall satisfaction) are involved in the PSPSQ. The study of validation and translation into Turkish was conducted by Okuyan et al.[18] The translated form of the PSPSQ consists of 20 questions, excluding the 5th and 19th questions.

Interventions

A pharmacist-led education service was provided to patients during the face-to-face pharmacist-patient interview.

Educational interventions were determined by the physician and pharmacist. Educational interventions increase medication adherence. In this context, following the determination of the patient's medication usage pattern, the importance of medication adherence, non-adherence, and its leading to problems were learning objectives. To identify DRPs, we used patient and hospital information database notes. The pharmacist asked questions about prescribed or over-the-counter medicines, herbal drugs, and adverse effects during the patient-pharmacist interview. Additionally, drug interaction assessment was performed using web tools. If the pharmacist determined a DRP, it was classified according to PCNE. Following this process, pharmacist interventions were proposed to the physician or patients. While for the interventions at the prescriber level, the acceptance rate of interventions and status of DRPs were assessed in the first interview, for the interventions at the patient level, the acceptance rate and status of DRPs were assessed in the second interview.

Statistical analysis

Quantitative variables were described using means, standard deviations (SD), medians (minimum-maximum), and frequencies (percentage) for categorical variables. The Wilcoxon test was used to evaluate the differences in the DRP rate before and after pharmacist-led education. The significance level was defined as $p < 0.05$. SPSS version 25.0 was used to perform all statistical analyses (IBM Corporation, New York, USA).

Ethical approval

The study was approved by the University of Health Sciences Türkiye, Izmir Tepecik Training and Research Hospital Non-Interventional Research Ethics Committee (no: 2019/6-10).

RESULTS

The data from 39 patients with epilepsy (56.4% male) who met the inclusion criteria were analyzed in this study. During the study period, 78 interviews were conducted with the pharmacist. The mean (± SD) age of patients was found 43.6 ± 12.6 years. Most of the patients stated no alcohol consumption, no herbal supplement or non-prescription medication use, and no ability to manage self-care (89.7%, 76.9%, and 79.5%, respectively). The most common comorbidities were major depression (25.6%), hypertension (18%), and hypothyroidism (10.3%). The mean number of drugs *per* patient was 5.5 ± 1.1 and the mean number of ASMs was 2.7 ± 1.0. In addition, the most used ASMs were sodium valproate (71.1%), carbamazepine (56.4%), levetiracetam (38.6%), and lamotrigine (38.6%). The clinical characteristics of the patients are shown in Table 1.

Medication adherence was found to be moderate in 21 patients (53.9%) at baseline, and a significant increase in medication adherence was observed after pharmacist-led education, and the number of patients with high-level medication adherence increased from 17 to 28 ($p < 0.001$) (Table 2). When the influence of education level on medication adherence was evaluated, the number of patients with high-level medication adherence increased after education both in primary school (from 6 patients to 17 patients) and high school (from 9 patients to 11 patients) graduates ($p < 0.001$).

The number of seizures decreased in 19 patients (48.7%), increased in 3 patients (7.7%), and did not change in 17 patients (43.6%) during the 2-month period before and after pharmacist-led education. All patients were satisfied with the pharmacist's service (PSPSQ 2.0; disagree 0%, strongly disagree 0%). Higher satisfaction among patients was detected according to each part of PSPSQ 2.0 (quality of care, patient-pharmacist relationship, overall satisfaction) at the end of the study (Table 3).

A total of 59 DRPs (1.5 DRPs per patient) were detected during the study period (Table 4). The majority of DRPs were associated with treatment safety based on the adverse drug reactions of ASMs. Patient-related DRP causes were associated with inappropriate drug use. The majority of

Table 1. Clinical characteristics of patients (n: 39)

	n (%)
Gender	
Women	17 (43.6)
Men	22 (56.4)
Education	
Illiterate	4 (10.3)
Primary school	23 (59.0)
High school	12 (30.8)
Comorbidity	
No comorbidity	17 (43.5)
1	18 (46.2)
2	4 (10.3)
Number of anti-seizure drugs	
1	5 (12.8)
2	9 (23.1)
3	18 (46.2)
4	5 (12.8)
5	2 (5.1)

Table 2. Medication the medication adherence level according to MGLS before and after education (n: 39)

	Before education	After education	
Medication adherence, MGLS	**n (%)**	**n (%)**	***p***
Low	1 (2.6)	1 (2.6)	
Moderate	21 (53.9)	10 (25.6)	<0.001
High	17 (43.6)	28 (71.8)	

MGLS: Morisky-Green-Levine scale, statistical analysis: Wilcoxon

Table 3. Pharmacist service satisfaction of patients according to PSPSQ 2.0 (n: 39)

	Strongly agree	Agree	Disagree	Strongly disagree
	n (%)			
Quality of care	11 (28.6%)	28 (71.4%)	0 (0%)	0 (0%)
Patient-pharmacist relationship	17 (44.5%)	22 (55.5%)	0 (0%)	0 (0%)
Overall satisfaction	33 (85.5%)	6 (14.5%)	0 (0%)	0 (0%)

PSPSQ 2.0: Patient Satisfaction with Pharmacist Services Questionnaire-Version 2.0

Table 4. DRPs according to PCNE 8.01 classification (n: 59)

Domains			
Problems (P)	**Code**	**Definitions**	**n (%)**
Treatment effectiveness	P1.2	Effect of drug treatment not optimal	12 (20.3)
	P1.3	Untreated symptoms or indications	8 (13.6)
Treatment safety	P2.1	Adverse drug event (possibly) is occurring	31 (52.5)
Others	P3.2	Unnecessary drug-treatment	5 (8.5)
	P3.3	Unclear problem/complaint	3 (5.1)
Causes (C)	**Code**	**Definitions**	**n (%)**
Drug selection	C1.4	Inappropriate combination of drugs or drugs and herbal medication	3 (5.1)
Dispensing	C5.1	Prescribed drug not available	2 (3.4)
	C5.2	Necessary information not provided	1 (1.7)
Drug use process	C6.1	Inappropriate timing of administration and/or dosing intervals	7 (11.9)
	C6.2	Drug under-administered	1 (1.7)
The patient related	C7.1	Patient uses/takes less drug than prescribed or does not take the drug at all	8 (13.6)
	C7.2	Patient uses/takes more drug than prescribed	1 (1.7)
	C7.4	The patient uses unnecessary drugs	4 (6.8)
	C7.5	The patient takes food that interacts	4 (6.8)
	C7.7	Inappropriate timing or dosing intervals	1 (1.7)
	C7.8	Patient administers/uses the drug in the wrong way	22 (37.3)
	C7.9	Patient unable to use the drug/form as directed	3 (5.1)
Other	C8.1	No or inappropriate outcome monitoring	2 (3.4)
Planned interventions	**Code**	**Definitions**	**n (%)**
At the prescriber level	I1.3	Intervention proposed to the prescriber	2 (3.4)
	I1.4	Intervention discussed with the prescriber	13 (22.0)
At the patient level	I2.1	Patient (drug) counseling	37 (62.7)
	I2.4	Spoken to a family member orcaregiver	1 (1.7)
At the drug level	I3.4	Instructions for use changed to ...	4 (6.8)
	I3.5	Drug stopped	1 (1.7)
Other intervention or activity	I4.1	Other intervention (specify)	1 (1.7)

Table 4. Continued

Intervention acceptance	Code	Definitions	n (%)
Intervention accepted (by prescriber or patient)	A1.1	Intervention accepted and fully implemented	42 (71.2)
	A1.2	Intervention accepted, partially implemented	11 (18.6)
	A1.3	Intervention accepted but not implemented	4 (6.8)
	A1.4	Intervention accepted, implementation unknown	1 (1.7)
Other (no information on acceptance)	A3.1	Intervention proposed, acceptance unknown	1 (1.7)
Status of the DRP	**Code**	**Definitons**	**n (%)**
Not known	O0.1	Problem status unknown	3 (5.1)
Solved	O1.1	Problem solved	37 (62.7)
Partially solved	O2.1	Problem partially solved	14 (23.7)
Not solved	O3.1	Problem not solved, lack of cooperation of patient	5 (8.5)

DRPs: Drug-related problems, PCNE 8.01: Pharmaceutical Care Network Europe, version 8.01

pharmacist interventions was at the patient level such as patient counseling, especially on drug use (62.7%). Most of the interventions (71.2%) were accepted and implemented by both physicians and patients, and 62.7% of DRPs were completely solved *via* pharmacist interventions.

DISCUSSION

The fundamental components of epilepsy therapy are seizure control and DRP minimization. The emergence of DRPs in routine practice, however, is an unavoidable undesirable circumstance. Furthermore, DRPs contribute to lower medication adherence, quality of life, and treatment satisfaction. In this study, DRPs and the impact of pharmacist-led education on medication adherence rates were assessed in 39 patients with epilepsy. We found that the education provided by pharmacists positively affected the medication adherence rate and solved most DRPs.

Medication adherence requires ensuring optimal pharmacotherapy to prevent treatment failures in patients with epilepsy. In addition, pharmacists play a key role in ensuring medication adherence through educational methods and pharmaceutical care. The baseline ASM adherence rate was found 43.6%, slightly lower than other studies in Ethiopia (55.7%) and China (51.9%).[10,19] Patient characteristics (cultural, socio-demographic, and education levels, *etc.*), study methods and tools used to measure medication adherence may have caused this discrepancy. Medication adherence can be assessed in a variety of ways, including pill counting, therapeutic drug monitoring, and electronic tools, although there is no gold standard. In our study, medication adherence was measured using a self-report tool; however, the use of an additional method to assess medication adherence could have supported the results.

Many studies display the positive impact of pharmacists on medication adherence. Consistent with previous studies, our study showed that medication adherence was positively affected by pharmacist-provided educational interventions.[13] In addition, the differences in education level among patients with epilepsy may have influenced the difference in the effect of pharmacist-led education on medication adherence. Similar to our findings, higher medication adherence was detected in patients with higher educational levels compared with patients with lower educational levels in a recent study.[7] Therefore, in particular high educational status patients, pharmacist-led educational interventions may be more beneficial to improve medication adherence. Ensuring optimal medication adherence may play an important role in reducing the frequency of seizures. A positive correlation between poor medication adherence and more seizures has been found in recent studies.[3,20] Similar to the literature, the frequency of seizures was also decreased in our study because of increased medication adherence with pharmacist-led education.

DRPs are an obstacle to ensuring optimal drug therapy and high medication adherence and preventing seizures. The detection and solution of DRPs play an important role in managing the treatment appropriately. In our study, it was found that DRPs *per* patient were 1.5 on treatment effectiveness. In a study, it was found that the DRP of patients admitted to the rheumatology and internal diseases outpatient clinic was 2.4 *per* patient, and 63% of DRPs were clinically significant.[21] This discrepancy may be explained by the use of more drugs in rheumatology and internal disease, the high number of patients, and the high incidence of possible drug-drug interactions. Additionally, solutions to DRPs must be conducted together with patients and health care professionals. From the results of our study, most DRPs were solved by pharmacist intervention, consistent with a study.[22] The safety of ASMs plays a key role in maintaining treatment and preventing problems of other existing comorbidities. In a previous study, optimal ASM treatment of patients with epilepsy at a nursing home was challenged by adverse effects and drug-drug interactions.[23] Findings from our study, treatment safety (possible adverse effects) and treatment effectiveness problems were found to be major problems. Drug-drug interactions were not clinically significant, and this may be explained by the existence of relatively young patients and the low incidence of polypharmacy in our study. The major cause of detected

DRPs was patient-related problems (inappropriate drug use by patients); therefore, planned intervention was provided at the patient level in our study. In addition, the pharmacist's interventions and recommendations regarding the management of DRPs are highly accepted by physicians. In our study, most of the interventions were at the patient level, but as consistent with other studies, intervention at the prescriber level was highly accepted and applied.[21,24]

Patient satisfaction with pharmacy services may be positively affected by pharmacist-led education in patients with chronic diseases such as epilepsy. Similar to our findings, the satisfaction of patients with chronic disease was found to be high in other studies as well.[18] In a brief communication, it was shown that patient satisfaction was improved by ensuring high medication adherence in patients with epilepsy.[25]

Study limitations

This study has some limitations. The number of patients was limited because of the short study period and the distance between the locations of the neurology department and the rational drug usage unit. In addition, because of the involvement of younger patients, fewer comorbidities, and less drug use, the determination of DRPs was limited.

CONCLUSION

In conclusion, the pharmacist in the multidisciplinary team has key roles such as ensuring medication adherence, detection and management of DRPs, and contributing to optimal treatment during the follow-up period of patients with epilepsy. Because of high patient satisfaction and improved medication adherence, pharmacist-led education plays an important role in ensuring optimum therapy.

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Ethics

Ethics Committee Approval: The study was approved by the University of Health Sciences, Türkiye, Izmir Tepecik Training and Research Hospital Non-Interventional Research Ethics Committee (no: 2019/6-10).

Informed Consent: All participants involved in this study signed the informed consent form.

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Authorship Contributions

Surgical and Medical Practices: E.Ö., A.Ç.T., İ.F.U., Concept: E.Ö., K.D., Design: E.Ö., A.Ç.T., İ.F.U., U.Ş., K.D., Data Collection or Processing: E.Ö., Analysis or Interpretation: E.Ö., K.D., U.Ş., Literature Search: O.F., E.Ö., Writing: O.F., E.Ö.

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ORIGINAL ARTICLE

Anticancer Effect of Theranekron® on Androgen-Dependent Prostate Cancer Cells

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ABSTRACT

Objectives: Prostate cancer (PCa) is a significant health problem in men worldwide. Although there are numerous treatment choices for PCa, acquired resistance limits treatment success. Therefore, there is a need for new approaches as powerful resources for use in alternative or supportive therapeutic strategies for anticancer therapeutics. Theranekron® is a commercially available alcoholic extract of *Tarantula cubensis*. Recent studies have shown the potent anticancer effect of theranekron in human tumors, including PCa. Herein, we comparatively examined the antiproliferative activity of theranekron and its biochemical action on androgenic signaling and cell cycle-related cyclin proteins in androgen-dependent PCa cells, LNCaP, VCaP, and 22Rv1.

Materials and Methods: Human androgen-dependent PCa cells, LNCaP (CRL-1740TM), 22Rv1 (CRL-2505TM), and VCaP (CRL-2876TM) were used to evaluate the effect of theranekron *in vitro*. The impact of theranekron on cell viability was evaluated using a WST-1-based viability test. Its impact on AR, cyclin A2, cyclin B1, and cyclin E1 was examined by immunoblotting. To test the anti-malignant effect of theranekron on 3D tumor formation of PCa cells, soft agar assay was used.

Results: Our results indicated that theranekron treatment significantly reduced the viability of PCa cells. It remarkably decreased the protein levels of AR, cyclin A2, cyclin B1, and cyclin E1 in a dose-dependent manner. In addition, Theranekron administration strongly limited the 3D tumor formation of LNCaP, 22Rv1, and VCaP cells.

Conclusion: Our findings strongly suggest that theranekron may offer potent therapeutic efficacy against androgen-dependent PCa cells. Moreover, it may be a potent component for preventing acquired resistance to chemotherapeutics.

Key words: Androgen receptor, anti-cancer, cell-cycle, theranekron, prostate cancer, venom

INTRODUCTION

Cancer affected 17 million people in 2018 and led to the death of 9.6 million people worldwide.[1] Prostate cancer (PCa) is a significant health problem and is the second most common cancer type in men worldwide. Surgical intervention, cryotherapy, chemotherapy, and androgen deprivation therapy are frequently preferred for treating PCa.[2,3] However, both resistance to chemotherapeutics and conversion of PCa to castration-resistant PCa substantially limit treatment success.[4]

Animal venoms have a complex rich mixture of various bioactive molecules and thereby exhibit numerous pharmacological actions in the cells. In recent years, it has been recommended as a potent resource for use in an alternative or supportive therapeutic strategy for anticancer therapeutics, and its biochemical activities are being studied on a large scale by numerous research groups.[5,6]

Tarantula cubensis, also known as the Cuban tarantula, is a large arachnid from the family Theraphosidae.[7] Theranekron® is a commercially available alcoholic extract of *Tarantula clabensis* and is widely used in veterinary medicine for the treatment of numerous animal diseases such as panaritium, laminitis, foot rot, arthritis, abscesses, and several injuries.[7,8] Various studies have demonstrated that theranekron exerts various biochemical actions in mammalian human cells, including anti-inflammatory,

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wound healing, and anticancer well.[9,10] Moreover, resorptive, regenerative, antiphlogistic, and demarcative effects have been reported in proliferative and necrotic tissues.[11,12] The antitumor properties of theranekron have been reported in canine mammary tumors and in *in vitro* human cancer models.[13,14] Very recent *in vitro* studies have focused on the effects of theranekron on different cancer models, including breast, lung, osteosarcoma, and prostate.[15] Our previous study showed that the androgen-sensitive human prostate adenocarcinoma cell line LNCaP was more sensitive to Theranekron than the normal prostate cell line PNT1A.[13] In the present study, we investigated the effects of theranekron on LNCaP, VCaP, and 22Rv1 cells, known as androgen-dependent PCa cell lines.

Herein, we comparatively examined the antiproliferative activity of Theranekron® and investigated its biochemical action on androgenic signaling and cell cycle-related cyclin proteins by immunoblotting. In addition, we tested the action of Theranekron on anchorage-independent cell growth of androgen-dependent PCa cells using a 3D cell culture model. Our findings suggest that Theranekron® may offer potent therapeutic efficacy against androgen-dependent PCa cells. Moreover, it may be a potent component for preventing acquired resistance to chemotherapeutics.

MATERIALS AND METHODS

Materials

Cell culture supplements such as fetal bovine serum (FBS), L-glutamine, Dulbecco's Modified Eagle Medium (DMEM), and Roswell Park Memorial Institute (RPMI) 1640 Medium were obtained from Capricorn Scientific. Theranekron® was provided by Richter Pharma AG, Wels, Austria.

Rabbit polyclonal anti-cyclin A2 (#91500) (1:2000), anti-cyclin B1 (#12231) (1:2000), and anti-cyclin E1 (#20808) (1:2000) were purchased from Cell Signaling Technology. Polyclonal rabbit antibody anti-AR (#22089-1-AP) (1:2500) was obtained from Proteintech. Mouse monoclonal anti-beta-actin (#A5316) (1:10000) antibody was provided by Sigma-Aldrich. HRP-conjugated goat anti-rabbit (#31460) (1:5000) and anti-mouse (#31430) (1:5000) IgG (H+L) were obtained from Thermo Scientific.

Cell culture

The human androgen-sensitive prostate adenocarcinoma cell lines LNCaP (CRL-1740™), 22Rv1 (CRL-2505™), and VCaP (CRL-2876™) were obtained from American Type Tissue Culture. LNCaP and 22Rv1 cells were cultured in RPMI 1,640. VCaP cells were propagated in DMEM. All cell culture media were enriched with 10% FBS, 2 mM L-glutamine, and 5 mg mL^{-1} penicillin/streptomycin (Capricorn-Scientific). Cultured cells were maintained in a humidified atmosphere of 5% CO_2 and 95% air at a constant temperature of 37 °C.

Cell viability assay

Cells were seeded in 96-well plates (10,000 cells/well) and grown for 24 h. The cells were then treated with theranekron in various doses for 48 h. The WST-1 cell viability assay (Takara) was performed according to the manufacturer's instructions. The absorbance was determined at 450 nm with 600 nm as the reference wavelength using a microplate spectrophotometer (BioTek, Epoch 2). Average absorbance values were calculated, and viability rates are presented in the graph as a percentage fold change. IC_{50} values of Theranekron® were determined using GraphPad Prism 5 software.

Western blotting

Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer and centrifuged at 14,000 rpm for 20 min at 4 °C. The insoluble phase was removed and the supernatant was collected. The concentration of total protein was determined by the bicinchoninic acid (BCA) assay (Takara). Protein (30 µg) was used in immunoblotting studies. Samples were denatured in 4x Laemmli buffer at 70 °C for 15 min and separated on hand-cast polyacrylamide gels. Separated proteins were transferred to an Immobilon®-P polyvinylidene fluoride (PVDF) membrane (Bio-Rad). The membrane was blocked in 5% nonfat dry milk in phosphate-buffered saline (PBS) containing 0.1% tween (TBS-Tween) for 1 h at room temperature, and then primary and secondary antibodies were applied for 2 h at room temperature. Target proteins were monitored using enhanced Clarity™ chemiluminescence (ECL) solution (Bio-Rad) in ChemiDoc XRS+ (Bio-Rad). The densitometry of protein bands was calculated using Image Studio™ Lite (LI-COR®).

Soft agar assay

The soft agar colony formation assay was adapted according to Borowicz.[16] Equal volumes of 2 DMEM and 2 RPMI-1640 with 20% FBS and sterile 1.2% low-melting agar were gently mixed and added to a 12-well cell culture plate. Cell suspensions prepared in 150 µL media were mixed with 250 µL of 2x DMEM or 2x RPMI 1640 containing 20% FBS and 250 µL of 0.6% agar and, then, transferred to the top of the solidified bottom agar layer. Theranekron® was applied to the cells, and the images of growing colonies were taken using a Sunny SopTop inverted microscope and an OD400UHW camera system. Colonial growth was quantified by taking from independent areas to 5 images and counting.

Statistical analysis

Data are expressed as means ± standard deviation. Statistical significance was confirmed using paired two-tailed Student's *t*-test, and multiple comparisons of significance were analyzed by one-way ANOVA and Tukey's tests (*$p < 0.05$, #$p < 0.005$).

RESULTS

Theranekron® decreases the viability of human androgen-dependent PCa cells

To investigate the effect of theranekron on the viability of androgen-dependent LNCaP, 22Rv1, and VCaP PCa cells, we performed a WST-1-based cell viability test. Theranekron® doses used in this study were selected according to the results of our previous study with LNCaP cells.[13] For this aim, we treated PCa cells with 12.5 µg/mL and 25 µg/mL doses of theranekron for 24 h and then measured cell viability. Our findings revealed

that theranekron administration significantly decreased the cell viability of all tested PCa cells in a dose-dependent manner (Figure 1a, b). In addition, we determined that VCaP cells were more susceptible to theranekron than LNCaP and 22Rv1 cells.

Theranekron® decreases AR levels and induces cell cycle arrest in PCa cells

To evaluate the action of Theranekron® on cell cycle-related proteins of androgen-dependent PCa cells, we treated LNCaP, VCaP, and 22Rv1 cells with various doses of Theranekron®, and then protein expression levels of cyclin A2, cyclin B1, and cyclin E1 were analyzed by immunoblotting. We found that theranekron application decreased the expression levels of all tested cyclin proteins in a dose-dependent manner (Figure 2a, b). In addition, we tested androgen receptor (AR) protein levels and our data indicated that theranekron administration remarkably reduced the expression level of AR proteins in all androgen-dependent PCa cells (Figure 2a, b). In these studies, beta-actin expression was used as a loading control.

Theranekron® remarkably reduces 3D tumor formation in PCa cells

Most animal model trials fail because bioactivity tests performed in monolayer culture systems are insufficient to mimic real tumor formation and tumor environment. Therefore, we conducted anchorage-independent 3D tumor formation studies to test the effect of Theranekron® on PCa progression. Our data indicated that theranekron application significantly inhibited the tumor formation of LNCaP, VCaP, and 22Rv1 cells and strongly reduced the developing tumor volume in all three PCa cell lines (Figure 3a, b).

DISCUSSION

Currently, a few natural compounds or their synthetic analogs are clinically used against cancer.[17] In particular, spider venoms show potent effects on cancer cells because of their strong bioactive contents. Therefore, they are seen as potential drug candidates because of their anticancer and antinociceptive activities.[18] Numerous spider venoms have modes of action on cancer cells. The whole venom of *Macrothele raveni* triggers DNA fragmentation and activates several caspase enzymes in human breast carcinoma, cervical carcinoma, and hepatocellular carcinoma cells. Lycosin-1, an active compound of *Lycosa singoriensis* venom, activates mitochondrial cell death signaling in human lung adenocarcinoma, human prostate carcinoma, and colon adenocarcinoma cells.[19]

The commercially available alcoholic extract of *Tarantula cubensis* venom, Theranekron®, is often used in veterinary medicine to treat animal tumors. Therapeutically, Theranekron® has exhibited anticancer, anti-inflammatory, antiphlogistic, demarcative, and wound healing properties in clinical studies.[7]

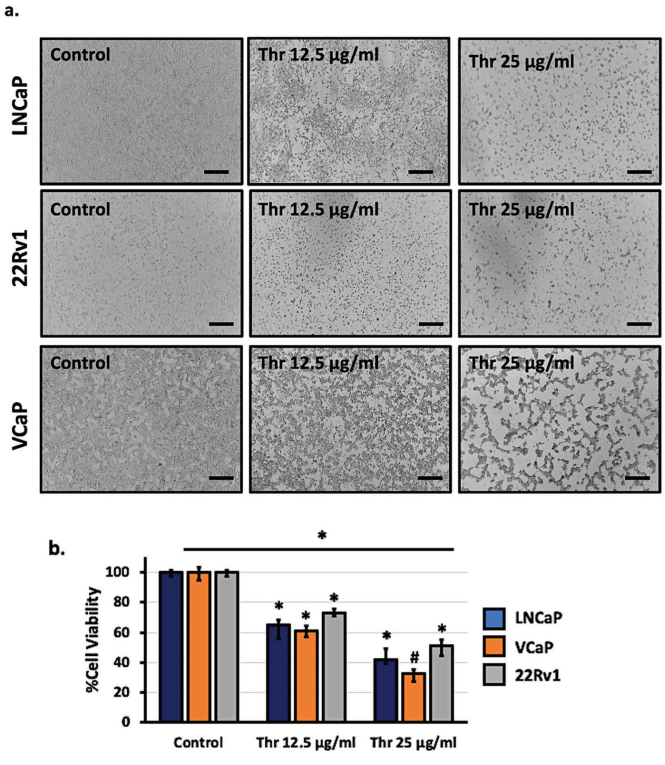

Figure 1. Theranekron® treatment decreased the viability of androgen-dependent PCa cells. a) Microscopic images of Theranekron-applied LNCaP, VCaP, and 22Rv1 cells. Scale bars indicate 5 µm. b) Cells were treated with vehicle, 12.5, and 25 µg/mL Theranekron® for 24 h, and cell viability was measured by WST-1 assay. Data are represented as mean ± SE of three independent experiments performed in three replicants (*p < 0.05, #p < 0.01).

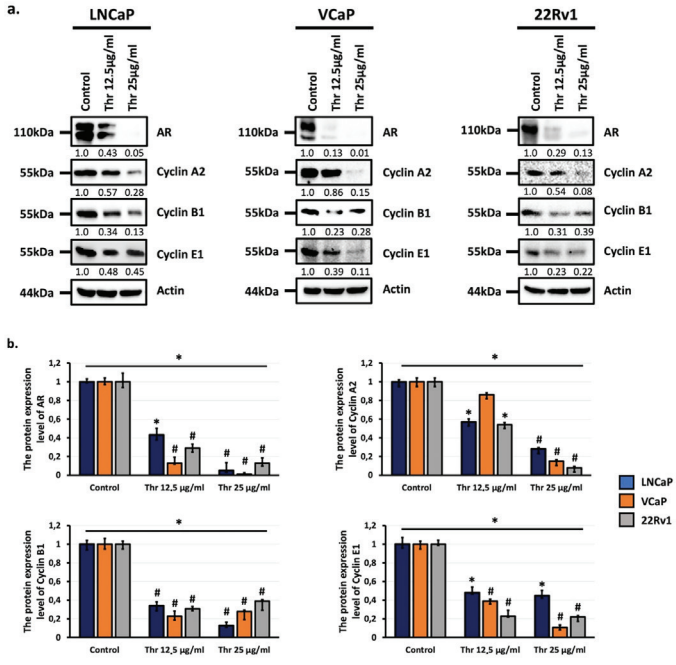

Figure 2. Evaluation of the effect of Theranekron® on AR and cyclin A2, B1, and E1 proteins. a) PCa cells were seeded in 6-well plates and treated with a vehicle, 12.5, and 25 µg/mL Theranekron for 24 h. Total protein was isolated from the cell pellets as described in the Materials and Methods section, and AR, cyclin A2, cyclin B1, and cyclin E1 proteins were examined by immunoblotting. Beta-actin was used as the loading control. The densitometry of protein bands was calculated using Image StudioTM Lite (LI-COR®), normalized against the control group, and presented under the immunoblotting results. b) Graphical representation of the densitometry results (n: 3). The control group was set to 1. (*p < 0.05, #p < 0.001)

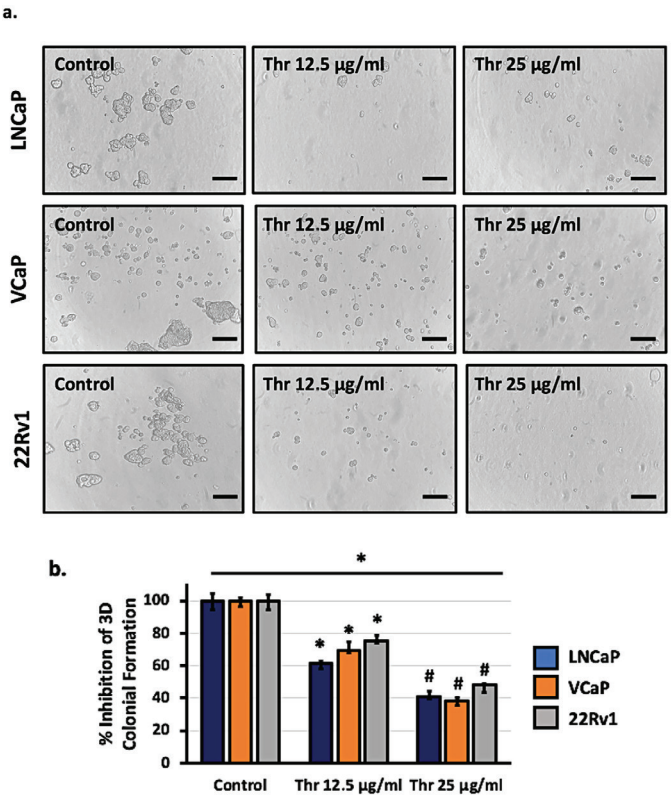

Figure 3. The effect of Theranekron® on 3D tumor formation of PCa cells. a) Anchorage-independent tumor formation of PCa cells was examined using a soft agar colony formation assay, as described in the Materials and Methods section. b) % inhibition of colonial growth was measured using ImageJ software. Three independent biological and three technical repeats *per* experiment were performed. Representative images are shown (*$p < 0.05$, #$p < 0.005$)

Table 1. Values of the effect of Theranekron on cell viability in LNCaP, VCaP, and 22Rv1 cells. Data are presented as the mean and standard deviation (±) of three independent repeats

	Cell viability	
Cell lines	**Thr 12.5 µg/mL**	**Thr 25 µg/mL**
LNCaP	65.256 ± 4.46	42.939 ± 1.96
VCaP	61.256 ± 3.82	35.931 ± 2.32
22Rv1	73.256 ± 3.81	50.691 ± 5.31

In addition, the usage of Theranekron has been reported in endometritis, cutaneous papillomatosis, pododermatitis, and foot and mouth lesions in veterinary medicine.[20-24] Recent studies have focused on the anticancer effect of Theranekron® in human cancer cells.[13,15] Erzurumlu et al.[13] reported that the androgen-dependent PCa cell line LNCaP was more susceptible to the androgen-independent metastatic PCa cell line Du145 and the healthy prostatic cell line PNT1A. Mechanistically, it affects autophagic activity and induces endoplasmic reticulum stress in androgen-dependent PCa cells. In addition, it markedly reduced the epithelial-mesenchymal transition of LNCaP cells.[13]

Herein, we focused on the impact of theranekron in androgen-dependent PCa cells and comparatively investigated the roles of androgenic signaling, cell cycle, and therapeutic impact on the 3D tumor formation of PCa cells. First, we examined the effects of Theranekron on cell viability in LNCaP, VCaP, and 22Rv1 cells. Our findings indicated that Theranekron administration more strongly decreased the viability of VCaP cells compared with LNCaP and 22Rv1 cells (Figure 1). In addition, Theranekron® doses in all applications effectively decreased the viability in all tested cell lines in a dose-dependent manner (Figure 1, Table 1).

The androgenic signal is a crucial mechanism in PCa cell progression. AR protein is induced by androgens in androgen-dependent PCa cells, and the expression of AR target genes is then stimulated through a specialized transcriptional program.[25,26] AR target genes include proto-oncogenic gene products that support prostate tumorigenesis. Therefore, suppression of AR signaling is among the major therapeutic choices developed for PCa. We evaluated the effect of Theranekron® on AR protein levels in LNCaP, VCaP, and 22Rv1 cells and found that Theranekron administration remarkably decreased the expression level of AR protein in a dose-dependent manner (Figure 2). These data suggest that Theranekron plays a potent regulatory role in AR protein levels in AR-expressing PCa cells.

In addition, we examined the changes in cell cycle-related cyclin A2, cyclin B1, and cyclin E1 protein levels by immunoblotting based on the effect of Theranekron on cell viability. Cyclin proteins regulate the transition between phases of the cell cycle by activating cyclin-dependent kinase (CDK) enzymes.[27] Cyclin A2 protein activates CDK2 kinase and promotes G1/S and G2/M phase transitions in cells.[28] Cyclin B1 regulates the transition from the G2 phase to mitosis.[29] Cyclin E1 is essential for G1 phase progression and entry into the S phase in the mammalian cell division cycle.[30,31] Our data indicated that Theranekron treatment markedly reduced cyclin A2, B1, and E1 expression in a dose-dependent manner in all tested androgen-dependent PCa cells (Figure 2). These results suggest that Theranekron® exhibits an anticancer effect by inducing cell cycle arrest in LNCaP, VCaP, and 22Rv1 cells.

Finally, we examined the effect of Theranekron® on 3D tumor formation of PCa cells. Anchorage-independent growth is a hallmark of carcinogenesis.[32] The most important limitations of studies on *in vitro* monolayer culture systems are the insufficient 3D cell interactions and the inability to mimic *in vivo* models of tumor formation exactly.[33,34] In addition, because the distribution of bioactive compounds on cells in monolayer culture systems is two-dimensional, *in vivo* test results have mostly failed. 3D culture models created with soft agar colony formation are one of the models that best mimic *in vivo* tumor forms. For this purpose, we performed 3D PCa formation for LNCaP, VCaP, and 22Rv1 cells, and then we tested the effect of Theranekron® on tumor progression and tumor volumes. Our findings showed that Theranekron® significantly reduced tumor formation in all tested PCa cells in a dose-dependent manner (Figure 3a, b). Collectively, these results suggest that Theranekron® has potent antitumorigenic activities on PCa cells by regulating androgenic signaling mechanisms and leading

to cell cycle arrest. These results revealed new biochemical effects of theranekron® on PCa cells.

Study limitations

In this study, the anticancer effect of Theranekron® on PCa cells was investigated *in vitro*. To investigate the effect of theranekron on PCa cells in more detail, further *in vivo* studies should be performed.

CONCLUSION

The present study suggests that the use of Theranekron® is not only effective on animal tumors in the veterinary field but can also offer effective therapeutic results on human tumors.

Acknowledgment

We thank Süleyman Demirel University, Innovative Technologies Application and Research Center. We thank Fahri Saatçioğlu (Department of Biosciences, University of Oslo, Norway) for providing the human prostate adenocarcinoma cell lines LNCaP, VCaP, and 22Rv1.

Ethics

Ethics Committee Approval: This study does not require any ethical permission.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Y.E., Design: Y.E., Data Collection or Processing: Y.E., Analysis or Interpretation: Y.E., Literature Search: Y.E., H.K.D., D.Ç., Writing: Y.E.

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

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ORIGINAL ARTICLE

Methyl Jasmonate Modulates Feeding Behaviors and Hypothalamic Expression of the Orexin 1 Receptor in Rats

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ABSTRACT

Objectives: Active plant ingredients have been successfully used in modern medicine to control appetite and energy hemostasis. This study was designed to evaluate the efficacy of the phytohormone methyl jasmonate (MJ) on food-related behaviors in rats.

Materials and Methods: Adult male Wistar rats were randomly divided into different groups (7 rats) and infused intracerebroventricularly (*i.c.v.*) with MJ vehicle (DMSO) or MJ (2.5, 5 and 10 µg/rat). Then, the individual rats were placed in an automated open field-like apparatus to assess a 12-h food-related activity in light and dark times. After behavioral tests, immunofluorescence staining of the orexin 1 receptor (Orx1R) was studied in the hypothalamus of rats.

Results: MJ (2.5, 5, and 10 µg/rat) administration significantly decreased food intake in the light and dark phases compared with the control group. Moreover, all the MJ-treated groups exhibited a decrease in visits to food containers at the light and dark times ($p < 0.001$). In addition, rats infused with MJ at 5 µg and 10 µg spent less time in the ports of food containers in the light and dark phases in comparison with control rats. Time in zone-related to food and locomotor activity was significantly decreased in the MJ (5 µg) groups during the light time and in all MJ-injected groups in the dark time. Moreover, hypothalamic expression of Orx1R in rats treated with MJ (5 µg) was significantly lower as compared to the control group.

Conclusion: Overall, the results indicated the potential of MJ to modulate feeding-related behavior and Orx1R expression in the hypothalamus of rats.

Key words: Methyl jasmonate, feeding behavior, Ox1R, rats

INTRODUCTION

Feeding behavior and energy consumption have been considered important health concerns in humans. In particular, plant-based diets have gained scientists' attention in recent decades. Plant ingredients have been widely used in the food industry to control appetite, body weight, metabolic disturbances, and energy intake.[1,2]

The plant hormone methyl jasmonate (MJ) was initially isolated from the floral scent of jasmine plant. The growing body of evidence shows its value in modulating neurologic processes in animals.[3-6] It is structurally similar to anti-inflammatory prostaglandins (PGE2), which could decrease the inductin of interleukin-6 (IL-6), nitric oxide, and tumor necrosis factor (TNF-α).[7] It has been shown that MJ attenuates depression-like behavior, anxiolytic responses, and learning and memory decline in rodents.[3,5,8] Moreover, MJ was able to decrease stress oxidative indices in the brains of mice.[9,10]

In addition, jasmonate is usually ingested with plant nutrients and may elicit physiological competence or toxic effects when ingested at high dosages.[11] IMJ is present in plant foods in

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different concentrations, so its intake is strongly influenced by cultural and social nutritional behaviors.[12]

In different areas of the brain, neuromodulators are also intricately involved in energy expungers and food intake.[13,14] Studies have emphasized the key role of hypothalamic orexin peptides, *i.e.* orexin-A and orexin B, in appetite and metabolic procedures. These neuropeptides act by activating two G-protein-coupled receptors, including the orrin 1 receptor (Orx1R) and orrin 2 receptor (Orx2R). Orexin-A is equal at both receptors; however, orexin-B exhibits more competence to Orx2R. It has been shown that diet intervention is related to changes in OrxR expression in the rat brain.[15,16]

Although MJ is found in most dietary plant sources,[17] there is a lack of study to report the capability of MJ to modulate feeding behavior. This study was designed to evaluate, whether central administration of MJ can modulate feeding-associated behavior in rats or not. Moreover, alteration in OrxRs expression in the hypothalamus was evaluated in MJ-infused rats.

MATERIALS AND METHODS

Animal

Adult male Wistar rats weighing 230-270 g were divided into different groups. The rats were caged under controlled light/dark cycle (12/12 h) conditions and constant temperature (22 ± 2 ˚C). The diet and water were available at all times. All rats were habituated to lab environment for 30 min *per* day in the cage for a week and then valued.

Surgery

Ketamine (60 mg/kg) and xylazine (5 mg/kg) anesthetized rats were placed in a stereotaxic device (Stoelting, USA). A 23-gauge stainless steel guide cannula was bilaterally inserted into the lateral ventricles. The stereotaxic coordinates were derived from Paxinos and Franklin[18] atlas (AP = 1.6 mm, ML = ± 0.8, and DV = 3.4 mm). The cannulas were then attached to the skull using two screws and dental acrylic. Rats were recovered for 7 days in separate cages before the initiation of experiments.

Drug

MJ (purity > 95%) was bought from Sigma-Aldrich and sodium chloride 0.9% *w/v* was diluted.

Microinjection

Microinjections were accomplished with a Hamilton syringe (1 µL) connected to a needle (27-gauge) *via* a polyethylene tube. Drug infusion was performed at a rate of 1 µL/min/rat/ side.

Immunofluorescence

Paraffin blocks through the hypothalamic nuclei were sectioned and deparaffinized. The sections were treated for 30 min for antigen retrieval by hydrochloric acid solution (2%). After 5 min at room temperature, the samples were neutralized by incubation in 0.1 M sodium borate buffer, and after 30 min, they were washed in phosphate buffer saline (PBS). The primary antibody diluted (1 in 100) with PBS was added to the samples, and they were then placed in a refrigerator at 2 to 8 ˚C for 24 h to creating a humid environment to prevent tissue drying. After 24 h, the brain tissue was removed from the refrigerator and washed 4 times with PBS for 5 min each time. The secondary antibody was then added at a dilution of 1 to 150 and incubated in a 37 ˚C incubator for 90 min in the dark. The sample was transferred after 3 washes from the incubator to a dark room, we added DAPI (Sigma-D9542). Twenty minutes later, the samples were washed with PBS. Glycerol and PBS solution were poured on the sample and Orx1R immunoreactivity in each section was observed using a fluorescence microscope. Using a digital camera the microscopic images (x40) enclosing the population of Orx1R immunoreactive neurons in each section were taken.

Rat preference meter device

A square automated device (60 x 60 cm) with 30 cm high black plexiglass was used. The floor was separated into nine equal squares. For recording and monitoring the rats' location and food and water consumption, the apparatus was equipped with underneath load sensors. The animal was released from the central square (square 5) for assaying preference behavior. The four middle squares show the preference for the content of the nearest container. Also, the corner squares have been considered for resting animals. Detailed visual cues that help the rat remember taste memory were also assimilated (Figure 1).

Experimental design

Seventy food-deprived (12 h) rats were randomly divided into ten groups (n: 7) as follows: untreated control, sham-operated that was cannulated and infused with MJ vehicle (DMSO), and MJ-treated groups that were cannulated and injected with three different doses of MJ (2.5, 5, and 10 µg/rat) in two phases of light and dark. Food-related activities were evaluated using an automated system. Total food consumption, number of visits, time spent, and distance traveled to food ports and zones were calculated using software. In the habituation trial, the animals were allowed to freely explore the device two days (15 min/day) before the test. The rats were released when one container was provided by a 30 g normal pellet and allowed food intake within a 12-h period (separately in two phases day and night). In the habituation test, the animal was discarded from the experiment, when it spent more time in a particular zone or did not show probing behavior.[19]

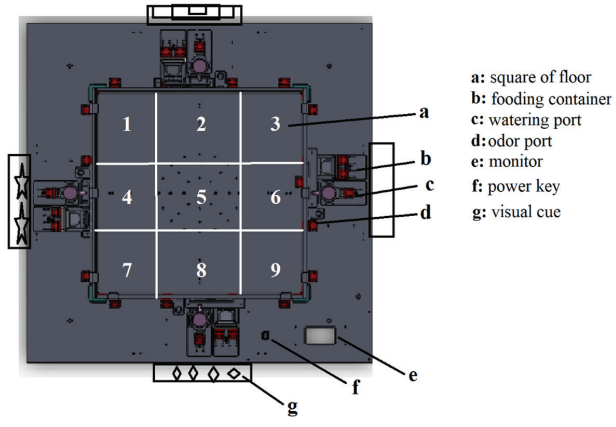

Figure 1. Overlook of the rat preference meter apparatus

Statistical analysis

All behavioral data were calculated using SPSS software. The data are expressed as means ± standard error of the mean. A two-way ANOVA was applied for analyzing the data. $P < 0.05$ was considered significant.

RESULTS

Food consumption

Figure 2 describes the amount of food intake in different groups of rats. Rats treated with MJ showed a significant decrease in food consumption in both light and dark phases compared with control and sham groups. The lowest amount of food consumption in the light and dark phases was indicated in the MJ-injected groups at 5 and 10 µg, respectively ($p < 0.001$).

Number of visits

There were significant differences between the control and MJ (2.5, 5, and 10 µg) groups in the number of total entries to the food ports and zones. The MJ groups showed a decrease in the number of visits in the light and dark phases ($p < 0.001$) as compared to the control group (Figure 3). In all groups, entries to food ports and zones were significantly increased in the dark phase compared to the light phase (Figure 3).

Time spent in the port and zone

Figure 4A presents that the time spent in the food port in the MJ-treated groups (5 and 10 µg), was significantly lower than that in the control group. The overall amount of time spent in the food zone was significantly decreased in MJ-infused rats at 5 and 10 µg in the light and dark phases (Figure 4B).

Locomotor activity

The distance traveled in the light phase in the MJ-treated group (5 µg) was significantly lower than that in the control group ($p < 0.05$). In the dark phase, there were increases in traveled distance in the groups of rats treated with MJ at 2.5, 5, and 10 µg as compared to control and sham rats. In addition, the distance traveled in the dark phase was significantly increased compared with that in the light phase ($p < 0.001$) (Figure 5).

IHC

Immunofluorescence staining of Orx1R in hypothalamic nuclei, including the ventral arterial thalamic nucleus (VA) and anterior hypothalamic arc (AHC), was achieved using antibodies directed against Orx1R combined with DAPI nucleus staining. Figure 6, panels A and B, display representative sections taken from the atlas of Paxinos and Watson, and immunofluorescence of Orx1R-positive cells in the hypothalamic nuclei, respectively, in control and MJ (5 µg) treated groups. In Figure 6, panel C, the numbers of Orx1R-positive cells in the hypothalamic nuclei VA (graph A) and AHC (graph B) were significantly attenuated in MJ-treated animals (5 µg) as compared to the control group ($p < 0.01$).

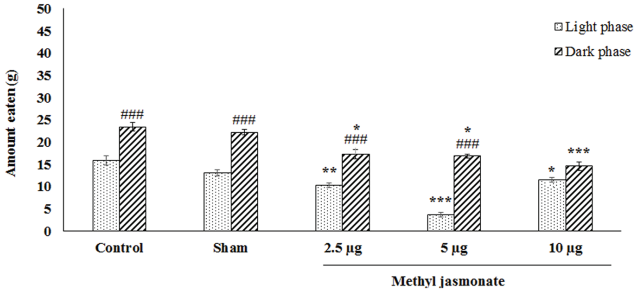

Figure 2. Effect of MJ (2.5, 5, and 10 µg/rat) on food consumption (in grams) in light and dark time (n: 7). Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs* control and sham groups in light phase ###$p < 0.001$ *vs* control and sham groups in the dark phase

DISCUSSION

The data of this study indicated that central administration of MJ can attenuate feeding-related behaviors in adult male rats. The behavioral effects were accompanied by Orx1R downregulation in the hypothalamus of rats.

Here, an automated open-field box was used to monitor the feeding behavior of rats in a 12 h light and 12 h darkness cycle.[20] The acknowledged characteristics included the amount of food consumed, time spent, number of visits, and distance each rat traveled in ports and zones of food containers.[19] In line with previous studies on nocturnal animals, the highest nurturing activities were found in the darkness time.[21]

This study was the first to report MJ intervention on feeding behavior in rats. However, previous studies have emphasized the efficiency of MJ in modulating some neuronal processes, including learning and memory, anxiety-like behavior, stress, and nociception.[8,22,23]

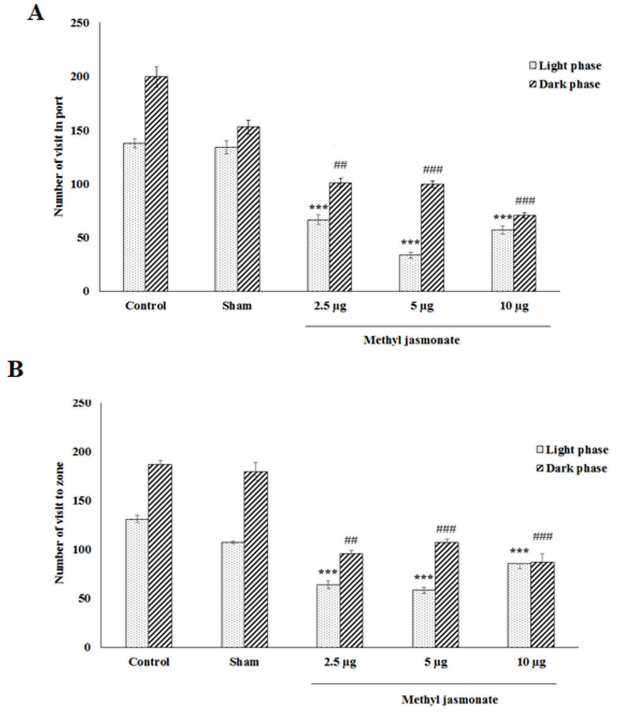

Figure 3. Effect of MJ (2.5, 5, and 10 µg/rat) on the number of visits to different ports (a) and zones (b) during light and dark time (n: 7). Data are presented as mean ± SEM. *$p < 0.05$, ***$p < 0.001$ vs control and the same groups, ##$p < 0.01$, ###$p < 0.001$

There are few data available showing MJ involvement in feeding behaviors. In rats suffering from arthritis and healthy rats, oral administration of MJ for 18 consecutive days increased the activity of mitochondrial $NADP^+$-dependent enzymes and decreased the levels of glucose flux through glycolysis in the liver. Regarding MJ effects to decrease hepatic glucokinase activity and glycolysis, it potentially might increase mitochondrial ROS production.[24,25]

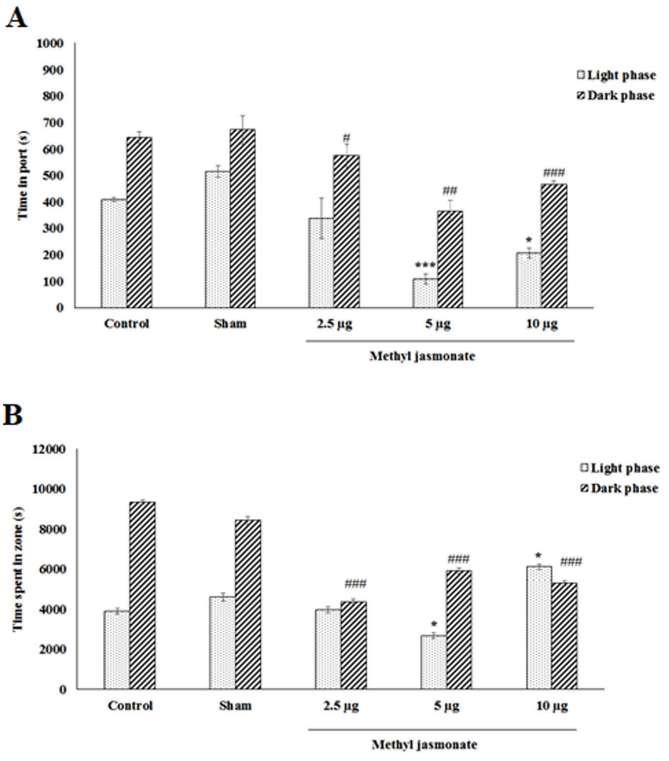

Figure 4. Effect of MJ (2.5, 5, and 10 µg/rat) on the time spent in different ports and zones during light and dark time (n: 7). Data are presented as mean ± SEM.

*p < 0.05, **p < 0.01, ***p < 0.001 *vs* control and sham groups in light phase

#p < 0.05, ###p < 0.001 *vs* control and sham groups in dark phase

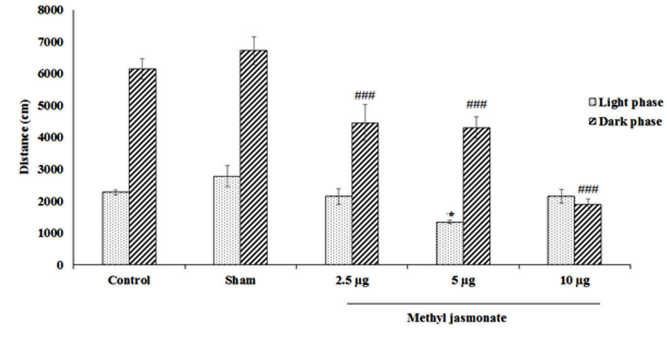

Figure 5. Effect of MJ (2.5, 5, and 10 µg/rat) on the distance traveled by rats in various food zones in light and dark time (n: 7). Data are presented as mean ± SEM

*p < 0.05, ***p < 0.001 *vs* control and sham groups in light phase

###p < 0.001 *vs* control and sham groups in the dark phase

MJ has been shown to have low toxicity in *in vivo* and *in vitro* studies. It showed selective toxicity against tumor cells with no effect on normal human cells.[25] Moreover, MJ (100-300 mg/kg/*i.p.*) treatment could not exert any acute toxic symptoms or death in mice. However, rats treated with MJ at doses of 400 and 500 mg/kg have shown abnormal behavioral changes including ataxia, sedation, and hyperventilation.[26]

MJ, as a linolenic acid-derived cyclopentanone phytohormone, bears structural similarities with prostaglandins.[24] It inhibits prostaglandin E, TNF-α, NFκB-mediated production of nitric oxide, and interleukin LPS -activated murine macrophages.[24,27] Prostaglandin-induced anorexia is associated with alteration of hypothalamic CRF and α-MSH neuronal activities.[28] Arachidonic acid as a prostaglandin precursor has an anorectic effect similar to F2α-induced anorexia in rats.[29] In this study, it is possible to assume that MJ anorexic activity was mediated by manipulation of inflammatory cytokine signaling molecules.

The activities and levels of oxidant/antioxidant agents have been emphasized in studies on metabolic challenges and energy expenders.[30] In this regard, MJ decreased oxidative stress activity in the brain.[9] In addition, it increased reactive oxygen species generation in human cells.[27,31] Furthermore, MJ decreased scopolamine pro-oxidative effects in mice.[9] In a recent study, MJ was able to suppress oxidative stress in rats' hippocampus and prefrontal cortex.[32] Therefore, in this study, it is supposed that MJ antioxidant value might be involved in modulation of feeding behavior of rats.

The localization of Orx1R neurons to the LHA shows their involvement in the central circuitry controlling energy metabolism.[33,34] Here, MJ decreased feeding behavior was associated with Orx1R downregulation in the hypothalamic nuclei including VA and VHC of rats. This indicates that MJ anorexic effects are at least partially medicated with interference on Orx1R signaling in the brain. Orexin neurons are multifunctional neurons that regulate a variety of physiological processes, primarily sleep- and feeding-related behavior.[35,36] Central infusion of an Orx1R agonist increased feeding behavior

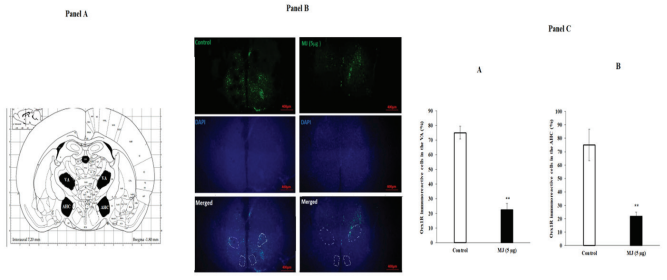

Figure 6. Immunostaining for Orx1R in the hypothalamus of rats. Panel A shows coronal sections through the ventral arterial thalamic nucleus (VA) and anterior hypothalamic arc (AHC) adapted from the atlas of Paxinos and Watson. Panel B indicates Orx1R staining in hypothalmic cells (green), DAPI staining indicates the position of the nuclei in cells (blue), and the merged image of Orx1Rand DAPI of control and MJ (5 µg) groups (n: 4). Panel C, statistical comparison of Orx1R immunoreactive cells in the section from the VA (graph A) and AHC (graph B) of the hypothalamus. Data are presented as mean ± SEM.

**p < 0.01 *vs* control

in rodents and zebrafishes,[37,38] whereas an Ox1R selective antagonist attenuated food intake in rats.[39] Increased food intake and Fos expression in the hypothalamic orexinergic neurons of rats after orexin A administration in the nucleus accumbens. Moreover, food consumption increased in rats treated with orexin.[40] On the other hand, peripheral orexin-A injection did not significantly affect daily food consumption, meal frequency, meal size, and values of total energy expenditure.[36] Notably, it may indicate that administration of orexin A in the central area produces a more powerful effect on increasing food consumption than administration in the peripheral area. Although powerful evidence shows Orx1R involvement in the regulation of feeding behavior, more experiments are still required to elucidate the exact mechanism(s) of MJ interplay with Orx1R neurons to modulate feeding behavior in the brains of rats.

CONCLUSION

Overall, the data indicated that central infusion of phytohormone MJ induced an anorexic effect in rats. Moreover, it decreased Orx1R expression in hypothalamic nuclei. However, more studies are needed to determine the exact mechanism(s) of MJ effects on feeding behavior and Orx1R neuron activity in rats.

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Ethics

Ethics Committee Approval: All the experiments were approved by the Animal Experimentation Ethics Committee of Jiroft University of Medical Sciences, Jiroft, Iran (IR.JMU.REC.1400.001).

Informed Consent: Not necessary.

Peer-review: Externally peer reviewed.

Authorship Contributions

Surgical and Medical Practices: M.Z., M.P., Concept: M.A., Design: R.K., Data Collection or Processing: M.P., R.K., A.A., Analysis or Interpretation: A.A., F.S., Literature Search: M.P., F.S., Writing: R.K., M.A.

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

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ORIGINAL ARTICLE

Phenolic Compound Composition of *Sambucus nigra* L. Wild-Growing Plants from Kosovo

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ABSTRACT

Objectives: The aim of this study was to determine the phenolic components in the flowers and leaves of wild-growing *Sambucus nigra* L.

Materials and Methods: Plant materials were collected from eleven localities in Kosovo. Before LC-DAD-ESI-MS^n analysis, an ultrasonic-assisted method with 70% methanol for 30 min extraction was used.

Results: In total, 34 and 37 phenolic compounds were identified in flower and leaf extracts, respectively, with a total content of 61321.82-85961.64 mg/kg dry weight (DW) and 36136.62-93890.37 mg/kg DW. In all of the analyzed extracts, 15 phenolic acids, 20 flavonoids, one lignan, and one coumaroyl iridoid were detected. The major components were flavonoids, especially flavonols (quercetin-3-rutinoside, caffeoyl-kaempferol, and isorhamnetin-3-rutinoside), followed by phenolic acids (dicaffeoylquinic acid isomer, caffeic acid derivative, dicaffeoylquinic acid isomer, and dicaffeoylquinic acid isomer).

Conclusion: In general, the methanolic extracts of flowers have shown higher polyphenolic content than those found in leaves. The multivariate statistical analysis of the phenolic content of the samples resulted in PLS-DA models with appropriate correlation coefficients of 0.903 and 0.921 for flower and leaf extracts, respectively. The models revealed distinctive clustering patterns, and the loading scatter plots depicted the unique phenolic compounds specific to each sample group.

Key words: *Sambucus nigra* L., flower, leaf, phenolic compounds, LC-DAD-ESI-MS^n

INTRODUCTION

Sambucus nigra L. (known as European elder, elderberry, black elder, or elder)[1] is a European species with an oceanic to suboceanic, cool-temperate, and west Mediterranean range.[2] *S. nigra* tolerates poor soil conditions or disturbed soils known as eutrophic soils and grows in sunlight-exposed locations.[1] It can be found in forests, thickets, parks, balks, and in-home gardens.[3]

S. nigra leaves, as its principal biomass, are a valuable source of flavonoids.[4] Historically, the leaves were considered to relieve pain and promote healing when applied as a poultice. The flowers and fruits of *S. nigra* have been used successfully for centuries for medicinal purposes and to prepare tea, wines, and liquors. Traditional folk medicine uses the infusion from dried flowers because of its diuretic effects, which include reducing fever, promoting perspiration, and moderating cough.[5] Elder cultivars are used as beverages and food flavoring.[6] Moreover, both the fruits and the flowers are sources of flavonols, proanthocyanidins, and phenolic acids. Dried flowers of elder (*Sambuci flos*) are most often used in various tea compositions,[7] but they are also used in several cosmetics and medicinal products.[5,8]

The beneficial health-promoting effects of elderberries and elderflowers are well known, including beneficial effects against

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degenerative diseases (cardiovascular and inflammatory diseases), cancer, and diabetes, as well as antioxidant, anti-inflammatory, immunostimulant, chemopreventive,[1,2] and atheroprotective effects.[1] More recent pharmacological studies have shown it to have antibacterial,[8] antiviral, anti-inflammatory,[2] and immunomodulatory activities.[4] Recent experimental evidence also suggests that it is a powerful antioxidant.[1,2,9]

Polyphenols represent a large and diverse group of plant secondary metabolites abundantly found in the plant kingdom.[10,11] Apart from their use as food supplements or as additives in functional foods, natural phenolic compounds have also become increasingly attractive from a technological point of view because of their possible exploitation in materials science.[12]

Elderberry flowers possess a substantial supply of bioactive flavonoids, antioxidants, and phenolic compounds, which have been frequently used in traditional medicine and healing. Different solvents and infusion times in extraction may induce differences in phenolic composition and bioactive features.[9] Several extraction methodologies have been reported for the extraction of phenolic compounds from plant materials, mostly based on the use of organic solvents such as methanol, ethanol, acetone[12], ethyl acetate, and their combinations, often with different proportions of water.[11]

Ultrasonic-assisted extraction is suitable for the extraction of plant bioactive molecules such as polyphenols, providing extracts with higher concentrations of active compounds and enhanced biological activity.[13]

The aim of this work was to determine the phenolic compounds in the methanolic extracts of flowers and leaves of wild-grown *S. nigra* using LC/DAD/ESI-MS^n.

MATERIALS AND METHODS

Plant material: Plant materials were collected at the flowering stage from eleven localities in Kosovo (Table 1) during May-June, 2021. Plant identity was verified by Professor Shkelzim Ukaj and voucher specimens were deposited at the Herbarium at the Department of Pharmacognosy, Faculty of Pharmacy, UBT, Prishtina, Kosovo (PhGNFF/UBT-02). They were air-dried and stored in a paper box until analysis.

Extraction of phenolic compounds: The plant material (flowers and leaves) was collected separately, dried, and homogenized (Ika Laborteknik, Staufen, Germany) for 30 s. The obtained powders were used for phenolic extraction. A total of 0.5 g of powdered plant material was extracted with 20 mL of 70% methanol for 30 min using an ultrasonic bath at room temperature. The supernatant was filtered through a 0.45 µm pore-size polyethersulfone filter before analysis.

LC/DAD/ESI-MS^n analysis: For LC/DAD/ESI-MS^n analysis, an Agilent HPLC 1100 system coupled with an ion trap mass spectrometer was used. A C18 Eclipse SB column (Agilent) with dimensions of 150 mm x 4.6 mm, 5 µm for chromatographic separation of polyphenolic compounds was used. Water-formic acid (0.1%, *v/v*) (A) and acetonitrile (B) were used as a mobile phase starting with 20% B for 5 min and after it was installed to reach 30% B at 10 min (5 min linear), 55% B at 25 min (5 min linear), 65% B at 45 min, and 100% B at from 50 to 65 min. The flow rate was 0.4 mL min^{-1} and the injection volume was 20 µL.

Spectral data from all peaks were accumulated in the 190-600 nm range, and chromatograms were recorded at 280, 330, and 350 nm.

MS and MS^2 spectra were acquired in the negative ionization mode with an electrospray ionization (ESI) system using nitrogen as the nebulizing gas at a pressure and flow of 50 psi and 12 L min^{-1}, respectively. The heated capillary and voltage were maintained at 325 °C and 4 kV, respectively. The full scan covered the mass range at *m/z* 100-1200.

Identification and quantification of polyphenolic compounds

The identification of various classes of polyphenols was based on a comparison of their retention times, UV-Vis spectra, and MS/MS^n fragmentation patterns with those of the available standards and literature data. Quantification was performed using the area under the peaks in HPLC/DAD chromatograms and the corresponding regression curves ($R^2 \geq 0.999$) of authentic standards. Phenolic acid derivatives were quantified as caffeic acid equivalents at 330 nm, whereas flavonoids were quantified as quercetin equivalent at 350 nm.

Statistical analysis

Statistical analysis was performed using Simca 14.1 statistical software (Umetrics, Sartorius Group, Sweden). Principal component analysis (PCA) was employed to elucidate patterns of polyphenolic content that was specific to certain samples (flowers or leaves), and hierarchical cluster analysis (HCA). For that purpose, Ward's method was performed to group the samples based on their PCA scores. A subsequent partial least-square discriminatory analysis (PLS-DA) was conducted to build a correlation model among the sample's group affiliation (dependent - Y- variables) and their quantitative polyphenolic composition (independent - X- variables). Correlation coefficients were used to determine the model goodness of fit, whereas score scatter plots and loading plots were employed to depict the polyphenolic compounds that made an important contribution to sample classification.

RESULTS and DISCUSSION

Phenolic compounds in wild-grown *S. nigra* methanolic extracts were identified by their retention times, UV spectra, deprotonated molecular ions, and corresponding ion fragments using LC/DAD/ESI-MS^n (Table 2).

A total of 34 and 37 individual compounds was identified in the methanolic extracts of flower (SN-FL) and leaf (SN-LE) of *S. nigra,* representing 61321.82 (SN10-FL) - 85961.64 (SN3-FL) mg/kg DW (Table 3) and 36136.62 (SN4-LE) - 93090.37 (SN8-LE) mg/kg DW (Table 4) of the total content, respectively.

Phenolic compounds in the SN-FL and SN-LE samples were classified into three groups, *e.g.* phenolic acids (14 and 15), flavonoids (18 and 20), and lignans (1 and 1), as one compound of the coumaroyl iridoids group (1 and 1), respectively.

Table 1. Collection of data for plant material from eleven different natural populations of *Sambucus nigra* L. from Kosovo

No	Localities	*Sambucus nigra* Voucher specimen		Latitude	Longitude	Altitude (m)
		Flower	Leaf			
1.	Zllakuqan (Klinë)	SN1-FL	SN1-LE	42°39′59″ N	20°32′14″ E	401
2.	Istog	SN2-FL	SN2-LE	42°47′11″ N	20°29′09″ E	519
3.	Vitomiricë	SN3-FL	SN3-LE	42°41′30″ N	20°19′13″ E	525
4.	Pejë	SN4-FL	SN4-LE	42°39′41″ N	20°15′43″ E	565
5.	Skivjan	SN5-FL	SN5-LE	42°26′11″ N	20°22′21″ E	428
6.	Strellc (Deçan)	SN6-FL	SN6-LE	42°32′49″ N	20°17′26″ E	617
7.	Krushë e made (Gjakovë)	SN7-FL	SN7-LE	42°19′13″ N	20°37′58″ E	314
8.	Sopij (Suharekë)	SN8-FL	SN8-LE	42°20′09″ N	20°50′50″ E	443
9.	*Nerodime e epërme* (Ferizaj)	SN9-FL	SN9-LE	42°22′13″ N	21°04′35″ E	638
10	Graçanicë	SN10-FL	SN10-LE	42°36′29″ N	21°10′24″ E	588
11	Podujevë	SN11-FL	SN11-LE	42°53′46″ N	21°13′02″ E	595

Phenolic acids, a subclass of plant phenolics, possess phenol moiety and resonance stabilized structure, which causes H-atom donation and results in antioxidant properties through a radical scavenging mechanism.[14]

Total amount of phenolic acids in the SN-FL and SN-LE samples ranged from 15816.44 to 26985.19 mg/kg DW and 8632.18 to 28883.19 mg/kg DW, respectively. Four peaks with MS fragmentation ions at *m/z* 353 were attributed to the dicaffeoylquinic acid isomer (Table 2).

Dicaffeoylquinic acid isomer (peak no: 24) (2997.09-7675.36 mg/kg DW) and caffeic acid derivative (peak no: 4) (1018.73-7675.36 mg/kg DW) comprised 44.35-73.74% of the total phenolic acid content, followed by dicaffeoylquinic acid isomer (peak no: 23), dicaffeoylquinic acid isomer (peak no: 25), and *p*-coumaroyl-caffeoylquinic acid isomer (peak no: 33) were the dominant phenolic acids in flower (Table 3). In the leaves (Table 4), the major phenolic acids were caffeic acid derivative (peak no: 4) (2965.58-8571.72 mg/kg DW), dicaffeoylquinic acid isomer (peak no: 24) (201.23-4830.37 mg/kg DW) followed by 4-caffeoylquinic acid (peak no: 5), and dicaffeoylquinic acid isomer (peak no: 23).

Flavonoids, a class of polyphenol secondary metabolites, are believed to have various bioactive effects, including antiviral, anti-inflammatory, cardioprotective, antidiabetic, anticancer, and antiaging *etc.*[15] Flavonoids were the dominant group of polyphenols in the flower (Table 3) and leaves (Table 4) of *S. nigra* and represented 57-66.50% and 56.65-84.60% of the total analyzed polyphenolics, respectively. The total content of flavonoids in SN-FL and SN-LE samples ranged from 34997.17 to 57124.80 mg/kg DW and 20474.20 to 78757.38 mg/kg DW, respectively.

Flavonols were the main flavonoids in all samples of flowers and leaves with a total content of 34997.17-57124.80 mg/kg DW and 20474.20-78757.38 mg/kg DW, respectively. The prevailing compounds of flavonols in flowers were quercetin-3-rutinoside (peak no: 19) (6970.56-26685.21 mg/kg DW), caffeoyl-kaempferol (peak no: 6) (5734-12724.98 mg/kg DW), isorhamnetin-3-rutinoside (peak no: 22) (2583.7-11456.17 mg/kg DW), comprising 46-95% of the total flavonols, followed by quercetine malonyl diglucoside (peak no: 10), kaempferol-3-rutinoside (peak no: 21), and quercetin galloyl pentoside (peak no: 27). The component quercetin coumaroyl-rhamno-glucoside (peak no: 8) was found only in sample SN4-FL (3182.32 mg/kg DW). The dominant components of flavonols in leaves were caffeoyl-kaempferol (peak no: 6) (1157.34-27342.46 mg/kg DW), quercetin-3-rutinoside (peak no: 19) (1393.79-11917.56 mg/kg DW), and quercetin coumaroyl-rhamno-glucoside (peak no: 8) (2271.74-8958.14 mg/kg DW), comprising 25-62% of the total flavonols, followed by quercetin malonyl diglucoside (peak no: 10). Qualitatively and quantitatively, flavones and flavanones were the flavonoid representatives with the lowest content.

Lignan coumaroyl glucoside (peak no: 13) is a polyphenol of the non-flavonoid group with contents of 2224.9-4243.42 mg/kg DW and 1387.27-5071.77 mg/kg DW in SN-FL and SN-LE samples, respectively.

p-Coumaroyl dihydromonotropein (peak no: 34) belongs to the group of coumaroyl iridoids and was present in all analyzed samples of flowers (989.70-3276.52 mg/kg DW), whereas it was found only in some analyzed leaf samples (77.91-166.5 mg/kg DW).

Generally, the polyphenol content is higher in methanolic extracts of the flowers than in the leaves, except for the SN8-LE sample, where the polyphenol content is higher (93090.37 mg/kg DW). The SN6 sample was characterized by an approximate polyphenolic content in the flowers and leaves of 65296.79 and 69548.62 mg/kg DW, respectively. In other samples, a difference was observed between the polyphenolic content in the flowers and leaves of *S. nigra.*

Moreover, in agreement with previous studies, flavonoid amounts are greater in flowers than in leaves of *S. nigra.*[18]

Uzlasir et al.[9] analyzed the phenolic compositions of methanol, ethanol, and aqueous extracts of elderberry flowers using LC-

Table 2. Spectral data and retention time of polyphenolic compounds identified in methanol extracts of *Sambucus nigra* L. flowers and leaves

	Components				
Peak no.	Phenolic acids and their derivatives	t_R/min	λ_{max}/nm	[M-H]$^-$	MS^2
1	Quinic acid	3.927	235, 255	191	127, 173
2	3-Caffeoylquinic acid	4.116	288, 328	353	191, 173
3	5-Caffeoylquinic acid	4.557	288, 298 sh, 328	353	191
4	Caffeic acid derivative	5.221	290, 326	709	621, 534, 463, 353, 324
5	4-Caffeoylquinic acid	5.986	218, 300 sh, 326	353	191, 179, 173, 161, 135, 127
7	Caftaric acid	7.069	234, 294, sh, 324	311	179, 135
14	Coumaroylquinic acid	10.03	244, 294, sh, 324	337	191
23	Dicaffeoylquinic acid isomer	17.153	266, 346	515	447, 353, 299, 203, 173
24	Dicaffeoylquinic acid isomer	17.462	246, 298, sh, 328	515	353, 191, 179
25	Dicaffeoylquinic acid isomer	18.002	232, 300, sh, 314	515	471, 353, 299, 203, 173
29	*p*-Coumaroyl-caffeoylquinic acid isomer	20.106	234, 300, sh, 316	499	337, 163
30	*p*-Coumaroyl-caffeoylquinic acid isomer	20.338	234, 300, sh, 316	499	353, 191
31	Dicaffeoylquinic acid isomer	20.619	262, 298, 328	515	353
32	*p*-Coumaroyl-caffeoylquinic acid isomer	21.37	316	499	353, 337, 163
33	*p*-Coumaroyl-caffeoylquinic acid isomer	21.636	318	499	353, 337, 191
36	Caffeic acid derivative	26.817	234, 296, sh, 320	709	671, 353
Flavonols					
6	Caffeoyl-kaempherol	6.235	242, 298, sh, 328	447	439, 401, 285
8	Quercetin coumaroyl-rhamno-glucoside	7.574	256, 354	755	591, 489, 343, 300, 271
9	Quercetin coumaroyl-rhamno-glucoside	7.995	232, 298, sh, 326	755	737, 609, 593, 573, 489, 343, 301, 271
10	Quercetin malonyl diglucoside	8.335	316	771	667, 625, 505, 487, 365, 301
11	Quercetin malonyl diglucoside	8.696	332	771	667, 625, 505, 487, 365, 301
12	Quercetin 3-*O*-diglucoside	8.732	312	623	505, 445, 343, 301, 271
15	Kaempferol coumaroyl-rhamno glucoside	10.964	266, 348	739	593, 575, 473, 327, 285, 255
16	Quercetin caffeoyl pentoside	11.438	246, 296, sh, 328	595	475, 445, 301
17	Isorhamnetin diglucoside	12.871	234, 254, 343	639	607, 459, 315
18	Kaempferol coumaroyl -glucoside	13.219	226, 292, sh, 312	593	521, 359, 329, 285
19	Quercetin-3-rutinoside	14.211	256, 354	609	343, 301, 271
20	Quercetin-3-glucoside	15.887	254, 330	463	301
21	Kaempferol-3-rutinoside	16.17	266, 348	593	357, 327, 285, 229, 211
22	Isorhamnetin-3-rutinoside	16.489	256, 356	623	357, 315, 301, 271, 255
26	Kaempherol-3-malonylglucosede	18.422	266, 346	533	489, 285
27	Quercetin galloyl pentoside	18.909	254, 354	585	541, 459, 315, 301
28	Isorhamnetin octylglucoside	19.233	254, 354	519	477, 357, 315, 271
Flavones					
36	Acetyl-isoorientin	29.34	312	312	327, 291
37	Hydroxy trimethoxy flavonoid	27.484	363	368	291, 271, 229, 211, 171
Flavanones					
38	Naringenin	28.156	288	271	151
Lignans					
13	Lignan coumaroyl glucoside	9.428	228, 312		
Iridoids					
34	*p*-Coumaroyl dihydromonotropein	22.536	236, 292, sh, 320	537	389, 373, 331, 313, 193, 163

sh: Shoulder

Table 3. Phenolic content (mg/kg DW) in the methanolic extract of *Sambucus nigra* L. flower (SN-FL) (peak no: Compound according to Table 2)

Peak no.	SN1-FL	SN2-FL	SN3-FL	SN4-FL	SN5-FL	SN6-FL	SN7-FL	SN8-FL	SN9-FL	SN10-FL	SN11-FL
Phenolic acids and their derivatives											
1	355.58	76.04	95.71	87.46	72.17	60.68	62.56	97.89	72.58	100.99	46.99
2	828.82	412.51	608.42	437.26	550.8	471.46	561.44	462.37	496.71	510.37	553.61
3	387.86	593.52	791.81	257.58	490.16	496.66	496.27	800.25	592.07	701.32	429.53
4	5193.94	5090.66	4846	5252.27	5840.06	6087.05	5977.34	5313.38	5453.59	4018.73	7675.36
5	40.9	122.19	125.64	126.31	110.99	73.01	77.59	109.25	116.18	84.36	114.02
7	328.1	188.28	288.32	291.67	195.78	240.84	306.36	203.54	323.21	128.19	303.15
14	90.99	90.35	93.61	133.27	116.45	85.54	182.12	107.21	176.58	92.36	119.98
23	1817.29	1459.72	2008.99	1471.75	1281.27	1371.71	1485.39	1291.26	1359.09	1275.94	1198.03
24	8965.25	7420.57	12223.06	4197.8	8930.07	6615.19	8458.61	7702.74	9760.46	9462.73	2997.09
25	-	1907.36	1803.57	1504.67	1580	1355.74	1361.32	1518.79	917.84	772.2	1625.98
29	777.6	590.94	621.91	315.59	575.82	467.08	817.33	580.04	598.69	878.16	274.05
30	979.59	779.07	709.34	517.36	777	795.76	1231.32	902.48	857.56	955.84	365.23
31	-	-	-	-	-	-	-	-	-	-	-
32	1442.15	1010.47	1244.48	1431.38	1278.06	1191.25	1436.6	1189.02	1289.73	1064.62	1108.88
33	1178.45	989.7	1244.48	3276.52	1278.06	1191.25	1436.6	1189.02	1289.73	1528.8	-
36	115.01	133.62	279.84	171.61	212.73	512.22	191.14	137.16	145.31	97.9	202.57
Flavonols											
6	8886.34	10182.37	8738.78	5734	8088.94	9385	9387.17	12724.98	8536.82	9731.51	9384.68
8	-	-	-	3182.32	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-
10	1617.89	2630.86	3611.13	1981.46	1462.29	2214.63	1544.1	1593.8	1552.91	1684.56	1912.21
11	1651.32	2659.23	-	1334.75	2077.66	1333.66	1675.55	1358.87	1339.24	1650.73	1310.2
12	-	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-	-
16	310.83	618.72	286.19	473.69	385.74	367.54	529.81	455.2	594.13	476.42	381.38
17	1543.47	1558.02	1706.34	1762.07	1943.74	1610.53	1910.31	1889.47	1657.88	1493.58	-
18	-	276.02	381.87	271.04	105.66	313.13	332.55	296.57	185.86	81.88	74.44
19	12746.03	21829.87	26685.21	11660.99	16427.71	10881.45	12006.64	12411.14	22686.34	6970.56	11374.09
20	1052.34	1085.05	1049.48	1239.46	1124.08	1060.88	1164.5	1271.33	-	-	1075.97
21	2398.14	1767.28	2441.29	1390.08	1378.72	1471.7	1689.34	1556.49	1683.33	1173.2	1272.23
22	8536.84	2583.7	4159.93	8113.13	6611.06	3650.84	9515.6	7870.72	11456.17	7431.44	9416.35
26	1819.36	1083.51	1256.33	1229.4	1274.39	1103.44	1317.37	1259.92	1268.08	1144.56	1125.02
27	2560.25	1154.33	1321.74	1929.12	1500.48	1389.63	3184	2842.77	2487.37	1461.26	2793.77
28	1080.71	1061.51	1194.27	1324.17	1292.18	1421.12	1200.25	1297.17	-	-	1315.88
Flavones											
35	1097.03	931.57	1000.52	956.56	1017.08	1017.51	1091.02	1012.75	1046.32	1062.99	902.87

Table 3. Continued											
37	895.22	764.72	1007.57	1044.04	884.89	1240.76	803.32	809.13	747.66	967.18	1302.3
Flavanones											
38	1.18	4.79	5.6	5.7	6.41	11.86	6.74	4.15	5.42	5.79	19.39
Lignans											
13	2879.48	3419.24	3164.58	2224.91	3377.39	2810.71	3562.25	4010.78	3187.96	4243.42	3070.25
Iridoids											
34	424.32	658.19	865.63	2097.35	530.93	2996.96	922.26	754.42	664.25	70.23	938.01

(-): Not detected

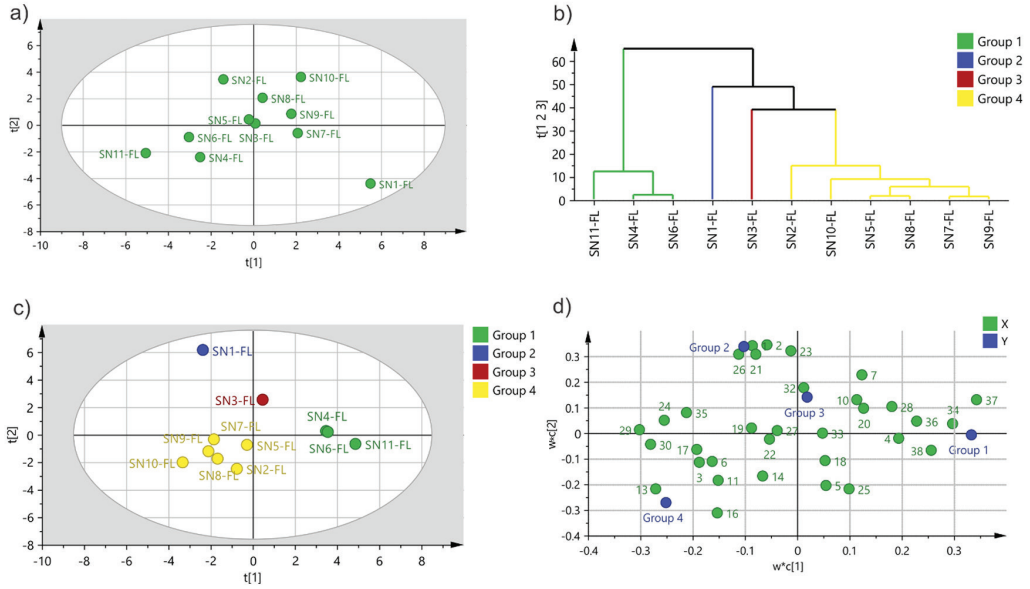

Figure 1. Multivariate analysis of the phenolic content of the flower extracts: a) score scatter plot from the first two components of the PCA model; b) HCA dendrogram with colored group clusters; c) score scatter plot from the first two components of the PLS-DA model colored according to the clustered sample groups; d) loading scatterplot for the first two components of the PLS-DA model

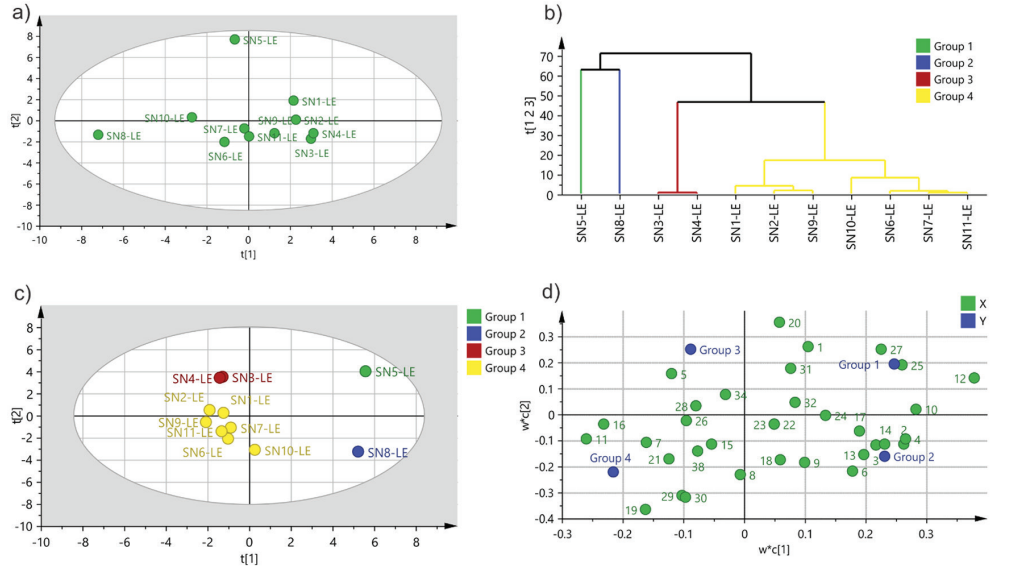

Figure 2. Multivariate analysis of the phenolic content of leaf extracts: a) score scatter plot from the first two components of the PCA model; b) HCA dendrogram with colored group clusters; c) score scatter plot from the first two components of the PLS-DA model colored according to the clustered sample groups; d) loading scatterplot for the first two components of the PLS-DA model

Table 4. Phenolic content (mg/kg DW) in the methanolic extract of *Sambucus nigra* L. leaf (SN-LE) (peak no: Compound according to Table 2)

Peak no.	SN1-LE	SN2-LE	SN3-LE	SN4-LE	SN5-LE	SN6-LE	SN7-LE	SN8-LE	SN9-LE	SN10-LE	SN11-LE
Phenolic acids and their derivatives											
1	44.76	60.84	67.05	65.19	62.33	31.65	61.06	47.05	24.33	51.04	19.45
2	508.77	570.68	516.34	520.83	941.46	980.2	676.83	1272.66	634.89	729.53	768.26
3	512.9	940.11	765.74	681.43	1711.3	2012.96	1291.38	3201.23	138.1	2214.58	1250.49
4	2965.59	3585.7	3309.91	3370.72	7109.55	6857.9	5011.2	8571.72	4984.25	5062.84	4826.13
5	974.03	855.74	3326.92	3353.26	252.65	1071.23	1141.95	943.27	3955.87	1397.89	908.63
7	-	116.81	99.92	40.82	-	70.43	106.46	59.97	77.32	55.22	64.51
14	-	-	-	-	137.74	48.81	-	485.96	-	326.23	57.65
23	2048.53	1074.78	1511.94	1098.61	1262.14	1083.56	1431.71	1550.62	1150.31	1076.47	1267.19
24	2954.96	281.28	342.56	322.54	5858.62	4351.7	1311.58	1711.67	663.17	4830.37	431.7
25	584.91	415.94	517.23	-	4654.29	631.87	372.82	530.55	187.94	695.26	135.62
29	123.05	106.61	-	-	-	128.36	82.08	102.3	60.33	198.62	-
30	118.82	99.95	-	-	-	122.3	87.66	106	63.56	190.54	-
31	1078.71	1035.75	1032.45	-	1145.54	-	-	-	-	-	-
32	1054.89	998.34	-	-	1066.49	-	-	-	-	-	-
33	-	-	-	-	-	-	-	-	-	1036.63	1003.52
36	-	-	-	-	-	-	-	-	-	-	-
Flavonols											
6	3659.09	4799.82	1157.34	1172.72	15375.53	19682.63	13326.51	27342.46	14526.94	15186.14	13465.24
8	4403.38	3314.95	4121.89	3312.19	-	5596.39	5752.36	8958.14	2982.94	7126.36	2271.74
9	1067.2	1520.37	1287.61	968.54	-	1104.19	777.96	3603.82	1176.64	957.84	1638.58
10	1531.06	1759.99	2659.38	2625.23	2653.66	2418.92	1989.14	5112.39	2494.34	2262.43	1874.89
11	1409.15	1542.27	2029.89	1637.58	-	1626.97	1489.62	1352.16	1467.83	1405.38	1532.11
12	1519.1	1279.68	1612.73	1751.45	3072.88	1558.27	1503.39	2991.26	1404.39	1349.35	1454.99
15	2516.14	-	-	-	1742.2	-	1834.4	-	1687.95	1601.79	1516.69
16	137.51	413.22	395.08	397.18	-	538.38	210.97	192.29	375.05	284.6	276.06
17	-	-	-	-	1451.5	-	1220.4	1262.7	-	1265.34	1290.86
18	260.44	446.65	431.45	433.75	121.85	120.89	1140.32	1295.5	615.82	921.33	295.33
19	6369.16	7412.85	4314.14	4337.13	1393.29	9901.72	8975.85	9288.86	7076.68	9231.39	11917.56
20	1043.53	1007.82	1433.5	1425.61	1091.36	1034.22	1021.5	1116.92	1048.71	963.82	1024.2
21	2617.2	1356.44	1361.96	1862.86	1415	1942.63	2527.97	1736.97	1600.06	1629.56	1886.53
22	1474.25	1476.64	1954.64	1346.72	1409.05	1366.45	1604.54	1859.85	1451.37	1773.68	1740.3
26	1086.3	-	1178.34	1065.49	-	1275.99	1011.54	1009.99	1076.47	-	1154.72
27	1237.56	-	1197.61	1226.6	1521.36	-	1168.29	1219.42	-	-	-
28	1053.47	1010.98	1258.45	1265.16	-	1007.53	977.86	1008.49	-	-	1063.67
Flavones											
35	-	-	-	-	991.71	-	-	-	-	-	-
37	-	-	-	-	-	634.11	-	-	-	-	-

Table 4. Continued											
Flavanones											
38	6.15	-	4.11	4.23	4.47	9.13	4.54	5.87	11.25	5.55	14.68
Lignans											
13	1844.59	1382.27	1821.43	1742.06	1513.8	2172.73	1773.02	5071.77	1970.62	2489.88	1993.38
Iridoids											
34	-	-	111.13	111.72	-	166.5	84.48	78.51	-	-	77.91

(-): Not detected

DAD-ESI-MS/MS. Chlorogenic acid, 5-*p*-coumaroylquinic acid, dicaffeoylquinic acid, and quercetin-3-rutinoside were the most abundant phenolic compounds in all the extracts. The results revealed significant differences among aqueous, methanol, and ethanol extracts of elderberry flowers with respect to the detected phenolics.[9] These components were also present and dominant in our samples of methanolic extracts of *S. nigra* flowers and leaves.

The aqueous extract of elderflower obtained at 90 °C was analyzed by GC-MS and HPLC-MS, which allowed the identification of 46 compounds, including quercetin and chlorogenic acid derivatives, representing 86% of the total phenolic compounds identified in the hydrophilic fraction of the aqueous extract. Naringenin (27.2%) was the major compound present in the lipophilic fraction.[8] In our examined samples of methanolic extracts from SN-FL and SN-LE, naringenin was the component with the lowest content. The reason for this difference might derive from the dichloromethane used in the extraction solution, which is selective for the isolation of lipophilic components from plant materials.[8]

Mikulic-Petkovsek et al.[17] reported phenolics in three elderflower extracts (one methanolic extraction and two water extracts prepared as fresh drinks according to local recipes). Hydroxycinnamic acids and flavonol glycosides are the major phenolic constituents in elderflowers.[17] These results are similar to our results for the methanolic extracts of flowers and leaves.

The methanolic extract contained higher levels of all phenolic groups than the aqueous extracts. The outcome of elderflower extracts is reliable on the solution used for extraction and time of extraction.[16]

A literature search also revealed that the efficiency of phenolic extraction depends on the extraction method, solvent type, and drying process used for the plant material.[6] We used an ultrasonic-assisted method for extraction of the plant air dried material and methanol as the solvent.

According to Tundis et al.,[18] the flowers and leaves of *S. nigra* were extracted by maceration using methanol and ethanol as solvents. Selected phenolic acid and flavonoid contents of extracts were analyzed by HPLC-DAD. Overall, the obtained data showed that target compounds exhibited higher content in methanol extracts than in ethanol extracts. However, the extraction yield depends not only on the extraction method but also on the solvent used for the extraction process. Generally, methanol has been found to be more efficient in the extraction of polyphenols with lower molecular weight.[18] Phenolic compounds are more soluble in polar organic solvents because of the presence of a hydroxyl group; therefore, methanol was selected as the extracting solvent.[10]

Elderberry growth phases represent an irreversible process involving a series of biochemical changes that have an extremely important impact on nutritional characteristics. The green buds and flowers had a high quercetin 3-rutinoside.[18] This compound was also dominant in the samples of *S. nigra* flowers and leaves collected during the flowering stage of the plant in the present study.

Total phenolic and flavonoid compounds are considered to be important antioxidant components. They are responsible for deactivating free radicals based on their ability to donate hydrogen atoms to free radicals based on their structural characteristics.[19]

Considering the multitude of phenolic compounds present in the flowers and leaves, a multivariate data analysis was performed to elucidate possible patterns in the phenolic content of the samples and further develop a correlation model. The initial PCA of the polyphenolic content of the flower extracts was performed using three main components that explain 60.6% of the variations in the X matrix (chemical composition of the flowers). The model score scatter plot (Figure 1a) reveals some grouping patterns of the samples that were further analyzed using HCA. The HCA dendrogram (Figure 1b) differentiated four samples groups based on the PCA scores. Group 4 contained 6 samples (SN2-FL, SN5-FL, SN7-FL, SN8-FL, SN9-FL, SN10-FL), Group 1 had 3 samples (SN4-FL, SN6-FL, SN11-FL), while groups 2 and 3 consisted of only one sample (SN1-FL and SN3-FL, respectively.

To analyze the relationship between the sample grouping and the polyphenolic content of the samples, further PLS-DA modeling was applied. Three main components were used to build the model that delivered a high correlation coefficient (R2Y= 0.903) and a distinctive grouping of the samples in the score scatter plot (Figure 1c). The variance important for projection (VIP) values revealed that nearly 20 phenolic components were assigned VIP coefficients larger than 1, indicating that more than half of the analyzed polyphenols were important and contributed to the sample classification. The VIP values are calculated by summing the squares of each loading weight, thus summarizing

the contribution of the variables to explain X and correlate to Y, where VIP values larger than one indicate important X variables (phenolic compounds).[20] The loading scatter plot (Figure 1d) reveals the relationships among the phenolic compounds and their corresponding groups, where the relative distance between each X (phenolic compound) and Y point (sample group) showcases the specificity of the phenolic chemical contents of each group. Considering the aforementioned, it can be observed that the Group 4 samples exhibit specific phenolic content that is richer in 5-caffeoylquinic acid, caffeoyl-kaempferol, quercetin malonyl diglucoside, lignan coumaroyl glucoside, and quercetin caffeoyl pentoside, whereas the Group 1 samples could be differentiated by higher contents of caffeic acid derivatives, *p*-coumaroyl dihydromonotropein, hydroxy trimethoxy flavonoid, and naringenin. The Group 2 sample presented specific phenolic content with high concentrations of quinic acid, 3-caffeoylquinic acid, kaempferol-3-rutinoside, and kaempferol-3-malonylglucoside, and the Group 3 sample was distinctive by its high *p*-coumaroyl-caffeoylquinic acid isomer content.

Statistical Analysis

Statistical analysis of the chemical composition of the leaf extracts was performed as described for the flower samples. The PCA model was built using three main components explaining 63.4% of the variability in the X-matrix, and the score scatter plot (Figure 2a) revealed two outlying samples (SN8-LE and SN5-LE) with distinctive scores in the first and second components, respectively. The subsequent HCA produced four distinctive sample clusters (groups) (Figure 2b), two of which were represented by the aforementioned samples (Group 1 - SN5-LE and Group 2 - SN8-LE), while Group 3 was composed of two samples (SN3-LE and SN4-LE) and Group 4 of seven samples (SN1-LE, SN2-LE, SN6-LE, SN7-LE, SN9-LE, SN10-LE, and SN11-LE).

The PLS-DA modeling of the data using group clustering as a Y-variable and phenolic content as an X-variable produced a model using three main components with R2Y= 0.921. Seventeen phenolic compounds were considered important in the regression model (VIP > 1), while the loading scatter plot (Figure 2d) revealed the distinctive phenolic compounds whose content was specific to each group. Therefore, high contents of dicaffeoylquinic acid isomer and quercetin galloyl pentoside were characteristic of SN5-LE (Group 1), whereas 3-caffeoylquinic acid, 5-caffeoylquinic acid, caffeic acid derivative, caffeoyl-kaempferol, lignan coumaroyl glucoside, coymaroylquinic acid, and isorhamnetin diglucoside were present in high concentrations in SN8-LE (Group 2). On the other hand, Group 3 samples were rich in 4-caffeoylquinic acid, and the phenolic content of the Group 4 samples was distinctive by its high concentrations of caftaric acid, quercetin malonyl diglucoside, quercetin caffeoyl pentoside, quercetin-3-rutinoside, and kaempferol-3-rutinoside.

CONCLUSION

A few dissimilarities in the qualitative composition of phenolic compounds between the methanolic extracts of flowers and leaves of *S. nigra* collected in the flowering stage from eleven different localities in Kosovo were revealed, nevertheless the quantitative differences were more evident. The multivariate statistical analysis revealed distinctive clustering patterns for the flower and leaf extracts, and several statistical indicators depicted the phenolic compounds that were present in higher concentrations and were specific for each group of samples.

Ethics

Ethics Committee Approval: Not necessary.

Informed Consent: Not necessary.

Peer-review: Externally peer reviewed.

Authorship Contributions

Concept: B.Q., V.E., Design: H.K., S.D., Data Collection or Processing: V.Q., Analysis or Interpretation: J.P.S., M.C., Literature Search: B.Q., V.E., N.G., Writing: B.Q., V.E., N.G.

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ORIGINAL ARTICLE

Vitamin D was Superior to Omega-3 as a Simvastatin Adjuvant in Improving Blood Lipids and Atherogenic Index in Type-I Dyslipidemic Rats

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ABSTRACT

Objectives: Adjuvant therapy is often used to optimize the antihyperlipidemic effect of simvastatin. Omega-3 and vitamin D supplementation are recommended as adjuvant therapies to low-intensity statins. This study aimed to compare the effects of vitamin D and omega-3 as adjuvant therapy to simvastatin to improve the lipid profiles and atherogenic index of plasma (AIP) in type-I dyslipidemic rats.

Materials and Methods: Thirty-six male rats were randomized and divided into six groups: healthy control, dyslipidemic rats with no treatment, and dyslipidemic rats treated with either low-dose simvastatin only or omega-3 or vitamin D at low and high doses. Dyslipidemia was induced with high-fat diets for four weeks, followed by treatment for the next two weeks. Blood samples were withdrawn before and after simvastatin treatment. In addition, aspartate transaminase (AST) and alanine transaminase (ALT) levels were analyzed to assess liver function.

Results: Administration of a high-fat diet-induced type 1 dyslipidemia and increased ALT levels ($p < 0.05$). Treatment with low-dose simvastatin did not significantly improve triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDLc) or non-HDLc levels. When combined with a high-dose vitamin D, simvastatin significantly reduced TG and increased HDLc levels ($p < 0.05$), thereby improving AIP levels. This improvement was not observed in rats treated with omega-3 or vitamin D at a lower dose.

Conclusion: We concluded that high-dose vitamin D as an adjuvant to simvastatin therapy was superior to omega-3 in improving TG, HDL, and AIP levels. High-dose vitamin D also improved ALT levels in type-I dyslipidemic rats. This result may be translated in clinics to reduce the risk of coronary syndrome in patients with type-I dyslipidemia.

Key words: Vitamin D, omega-3, atherogenic index, simvastatin, adjuvant therapy

INTRODUCTION

Simvastatin is one of the most widely used antihyperlipidemic drugs. It competitively inhibits the action of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase). The reduction in hepatocellular cholesterol promotes increased lipoprotein [low-density lipoprotein (LDL)] receptors at the surface and thus increases LDL uptake, decreases LDL plasma and other apolipoprotein-B, and lowers triglyceride (TG) levels.[1] Other effects of simvastatin include reducing oxidative stress and inflammation of the vesicle walls, thereby providing protection against atherosclerotic lesions.[2]

The use of simvastatin is associated with cheaper and more affordable treatment for most patients, but it is still inferior in decreasing LDL and increasing high-density lipoprotein (HDL) compared with other statin agents.[3] Various studies show that up to 65-75% of the incidence of atherosclerosis cannot be prevented by lowering LDL-cholesterol with statin therapy.[4] One of the reasons is that statins are often not titrated to their optimal dose in practice, mainly due to concerns about their side effects, including myopathy and impaired liver function. Another reason is that a small proportion of patients have refractory hypercholesterolemia and cannot reach targets

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even, when treated with high doses of statins.[5] Therefore, combination therapy of statins with other lipid-lowering agents has become one of the most commonly used strategies in the clinic.[6]

The combination of a statin with polyunsaturated fat (PUFA) omega-3, based on the guidelines for the management of dyslipidemia, is a recommended option for the treatment of dyslipidemia, especially hypertriglyceridemia.[2] Fish oil, which is rich in omega-3 fatty acids, docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA), is one form of safe and well-tolerated adjuvant therapy.[7] Growing evidence both from epidemiological data and case-control studies have indicated that routine consumption of fatty fish as well as long-chain n-3 PUFAs may lower the risk of cardiovascular death.[8]

However, the effect of omega-3 PUFAs on cardiovascular mortality, based on various studies, has been inconsistent. Studies in Japan reported that EPA therapy was associated with a 19% reduction in cardiovascular events and that combination therapy of EPA plus a statin would be more cost-effective than statin monotherapy in primary and secondary prevention.[9,10] One meta-analysis found that omega-3 supplementation for 4.4 years did not significantly reduce the incidence of coronary heart disease or other major vascular events.[11] Based on these inconsistent data, another candidate must be considered for adjuvant therapy to statin treatment for patients with dyslipidemia.

Vitamin D (known as calciferol) has long been associated with bone growth and strength.[12] However, substantial evidence suggests that vitamin D deficiency is also associated with an increased risk of cardiovascular disease.[13] The mechanisms by which vitamin D may improve cardiovascular disease are still unclear, but several hypotheses have been proposed, including direct effects on the myocardium, downregulation of the renin-angiotensin-aldosterone system (anti hypertensive effect), and improved glycemic control.[14-16]

Various data regarding the relationship between vitamin D and blood lipid levels remain inconsistent. Another study evaluating the association of vitamin D deficiency with the risk of dyslipidemia and heart disease found serum 25(OH)D levels to be inversely related to TG and LDLc levels and positively associated with total cholesterol (TC).[17] A meta-analysis found that vitamin D supplementation at various doses (from 300 IU *per* day to a 200,000-IU single dose) had an effect on LDLc levels but no significant effect on TC, HDL or TG levels.[18]

In many studies, vitamin D deficiency has been strongly associated with liver disease. The role of vitamin D in the liver has been greatly understood since the development of the vitamin D receptor.[19] Vitamin D correlates inversely with nonalcoholic fatty liver disease in a dose-dependent manner.[20] One study with 6,800 patients found that lower serum levels of vitamin D in patients correlated with an elevation of alanine transaminase (ALT) levels.[21]

Accordingly, this study aimed to compare the effect of vitamin D and omega-3 as adjuvant therapy to simvastatin to improve the lipid profiles and atherogenic index of plasma (AIP) in dyslipidemic rats. In addition, serum liver enzyme levels were measured to assess liver function following a high-fat diet and therapy. The results of this study may be important to determine, whether vitamin D and its recommended dose can be used as adjuvant therapy to simvastatin to improve the lipid profile and reduce the risk of atherosclerotic disease and liver dysfunction in dyslipidemic patients or not.

MATERIALS AND METHODS

Drugs and chemicals

Drugs of choice

Simvastatin was obtained from 10 mg simvastatin tablets (Kimia Farma Co., Indonesia). Omega-3 was obtained as soft capsules containing 180 mg of EPA and 120 mg of DHA (Blackmores Co., Australia). Vitamin D was obtained as tablets containing 400 IU cholecalciferol (Novapharin Co., Indonesia). All drugs were purchased from local pharmacies. The dose of simvastatin was 2 mg/kg body weight (*b.w.*) of rats, which is equivalent to a low-intensity simvastatin dose of 20 mg/day in humans. The dose of omega-3 was 206 mg/kg of rat *b.w.*, which is equivalent to 2,000 mg/day in humans. Vitamin D was administered in two different doses: 62 IU/kg rats' *b.w.*, equivalent to 600 IU/day in humans; and 620 IU/kg rats' *b.w.*, equivalent to the dose recommended for vitamin D insufficiency and deficiency treatment of 6,000 IU/day in humans.

Chemicals

Serum TC, TG, HDL levels, aspartate transaminase (AST), and ALT levels were determined using reagent kits (Human Diagnostic Worldwide, Germany).

Animals

Thirty-six male Wistar rats (150-200 g) were obtained from a certified animal house (UD, Wistar Bantul, Yogyakarta). The rats were then transported and cared for in the Laboratory of Pharmacology and Toxicology, Faculty of Pharmacy, Hasanuddin University. They were housed under a controlled room temperature of 25 °C and humidity with 12 h light and dark cycles. The animals were fed standard pellets and water *ad libitum* during the adaptation period. All animal protocols complied with the Institutional Standard of Animal Care, and ethical clearance was obtained (409/UN 4.6.4.5.31/PP36/2022).

Experimental design

After a week of acclimatization, the rats were randomized and assigned to one of the six groups (n: 6 *per* group). The healthy control rats were fed standard pellets, whereas the dyslipidemia groups received a high-fat diet containing 5% duck egg yolk and 18% beef lard mixed with standard pellets and fructose in water (15%) for four consecutive weeks to induce dyslipidemia. After four weeks, the dyslipidemia groups were randomly assigned to receive either no treatment, simvastatin treatment only, omega-3 with simvastatin, low-dose vitamin D (62 IU/kg), or high-dose vitamin D (620 IU/kg) with simvastatin. These treatments were administered for 2 weeks along with the high-

fat diet. The rats' body weight was measured daily during the experimental period.

Blood collection and biochemical analysis

Blood samples were collected following the 4-week high-fat diet administration (at the end of week 4) and after all treatments were administered for two weeks (at the end of week 6). All rats were fasted for 12 h and anesthetized with ether before blood sampling. A 3 mL blood sample was collected from the lateral veins using a Vacutainer tube (BD Vacutainers) and then centrifuged at 4,000 rpm for 10 min at room temperature. The clear supernatant serum was then frozen and stored at 20 °C for biochemical analysis.

Lipid profile measurement

TC, TG, and HDLc levels were analyzed using a blood chemical analyzer (Humalyzer 3500, Human Diagnostic Worldwide, Germany). The serum non-HDLc level was calculated using the equation:

Non-HDLc= TC-HDLc [1]

Atherogenic index of plasma

AIP, an indicator of small dense LDLc and a predictor of coronary atherogenicity, was calculated using the following formula:

AIP=Log (TG/HDLc)

AIP can be used to determine the size of atherogenic lipoprotein particles. If AIP increases, the atherogenic lipoprotein particles become smaller, making it easier to move and undergo oxidation, thereby accelerating the process of atherosclerosis.[22]

Serum liver marker function enzyme measurement

AST and ALT levels were analyzed using a blood chemical analyzer (Humalyzer 3500, Human Diagnostic Worldwide, Germany).

Statistical analysis

Data were analyzed using SPSS version 25. The normality of the collected data was tested using the Shapiro-Wilk test and then further analyzed using one-way ANOVA (95% confidence interval), if the data were normally distributed or Kruskal-Wallis analysis, if they were not normally distributed. Statistical significance was defined as a p value of < 0.05.

RESULTS

Effects of simvastatin, omega-3, and vitamin D on body weight

Figure 1 shows the pattern of body weight gained by the rats after exposure to a high-fat diet and different treatments. Regardless of the high-fat diet treatment, the dyslipidemic rats did not show a significant weight gain compared with rats that did not receive a high-fat diet. Hence, treatments with simvastatin only or adjuvant therapies did not cause any significant change in the *b.w.* at any time point.

Anti-dyslipidemia activity of simvastatin, omega-3, and vitamin D

The results of the lipid profile measurements following four weeks of high-fat diet administration are shown in Table 1.

In all dyslipidemia groups, serum TG levels were significantly higher than in the healthy controls ($p < 0.05$), but TC, HDLc, and non-HDLc levels did not significantly increase. This indicates that administration of a high-fat diet and 15% fructose in water for six weeks only induced Frederickson phenotype-1 dyslipidemia.

Serum lipid levels after two weeks of treatment are depicted in Figure 2. Treatment with low-dose simvastatin in dyslipidemic rats only caused an insignificant decrease in TC and TG levels, with mean reductions of 13.6% and 12.4%, respectively. HDLc and non-HDLc levels were also not significantly changed by simvastatin treatment. In the groups treated with omega-3 as an adjuvant to simvastatin, the TC level was slightly increased, and the TG and HDL levels were slightly reduced compared with simvastatin treatment alone. In contrast, the administration of vitamin D, especially at a high dose, led to a significant reduction in TG by 36.4% compared with the placebo group (112.66 ± 19.36 *vs.* 177.20 ± 17.93, $p = 0.019$). Moreover, the combination therapy of simvastatin and high-dose vitamin D significantly increased HDLc levels compared with the administration of simvastatin alone (64.94 ± 10.59 *vs.* 39.13 ± 6.89, $p = 0.016$). This group had higher HDLc levels than the control group (64.94 ± 10.59 *vs.* 43.46 ± 7.01, $p = 0.042$). Interestingly, we found that the HDLc levels of the simvastatin plus omega-3 group were lower than those of all the dyslipidemia groups, including the untreated dyslipidemia group (25.06 ± 2.10 *vs.* 46.71 ± 5.20, $p = 0.040$).

Anti-atherogenic potential of omega-3 and vitamin D as adjuvant therapies

Figure 3 shows the AIP of rats after two weeks of treatment. The normal rats had an average AIP of 0.155 ± 0.082. Meanwhile, the groups that received a high-fat diet (dyslipidemia groups) predominantly had higher AIPs. The highest mean AIP was found in rats receiving simvastatin plus omega-3 therapy (0.702 ± 0.077), which was significantly higher than that of the controls. Unlikely, the AIP value of the rats treated with simvastatin plus a high-dose vitamin D therapy was near that of the normal controls and substantially lower than that of simvastatin alone ($p < 0.05$) or simvastatin plus omega-3 therapy groups ($p < 0.05$).

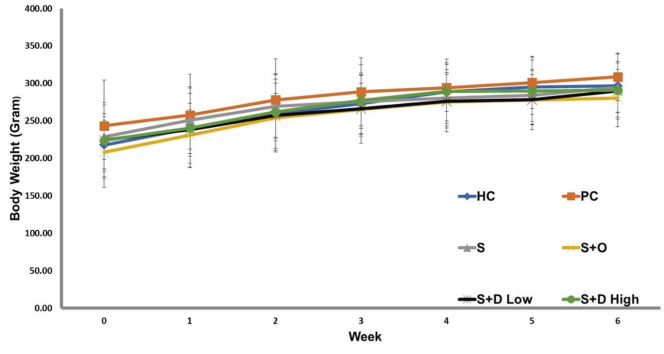

Figure 1. The effect of simvastatin, omega-3, and vitamin D on the bodyweights of rats of different therapeutic groups. Data are presented as mean ± standard deviation

Table 1. Lipid profile levels of rats after consuming a high-fat diet for 4 weeks before therapy administration

Group	TC	TG	HDL	Non-HDL
	(mg/dL)	(mg/dL)	(mg/dL)	(mg/dL)
Healthy control (HC)	102.18 ± 6.63	101.16 ± 7.91	33.73 ± 3.18	74.53 ± 7.59
Placebo control (PC)	103.58 ± 14.67	255.70 ± 42.66*	47.07 ± 3.05	62.77 ± 21.23
Simvastatin (S)	115.60 ± 6.14	192.64 ± 15.96*	52.59 ± 10.07	70.01 ± 11.13
Simvastatin + Omega-3 (S + O)	113.83 ± 13.10	224.22 ± 19.50*	30.48 ± 3.42	92.76 ± 10.23
Simvastatin + MVD (S + D low)	119.58 ± 11.32	197.40 ± 34.87*	47.39 ± 6.78	77.1 ± 9.66
Simvastatin + HVD	100.58 ± 14.95	170.00 ± 36.66	43.86 ± 9.21	73.77 ± 18.84
(S + D high)				
***P* value**	**0.791**	**0.022**	**0.177**	**0.771**

Data are presented as mean ± standard deviation. *$p \leq 0.05$ compared with the healthy control group.
TC: Total cholesterol, TG: Triglyceride

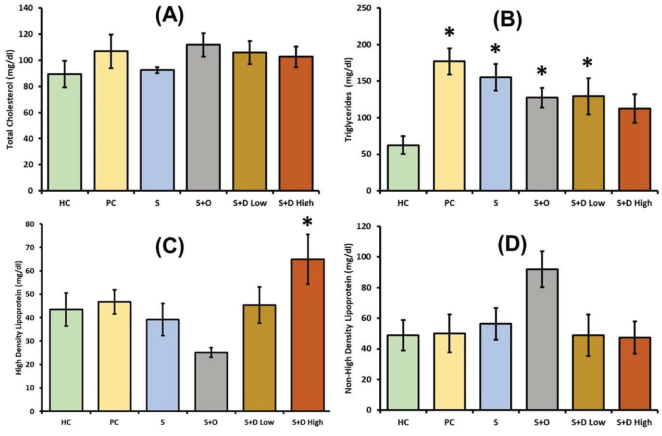

Figure 2. The effect of different treatments on serum lipid levels in rats. Total cholesterol (TC) (A), triglyceride (TG) (B), high-density lipoprotein cholesterol (HDLc) (C), and non-high-density lipoprotein cholesterol (non-HDLc) (D)

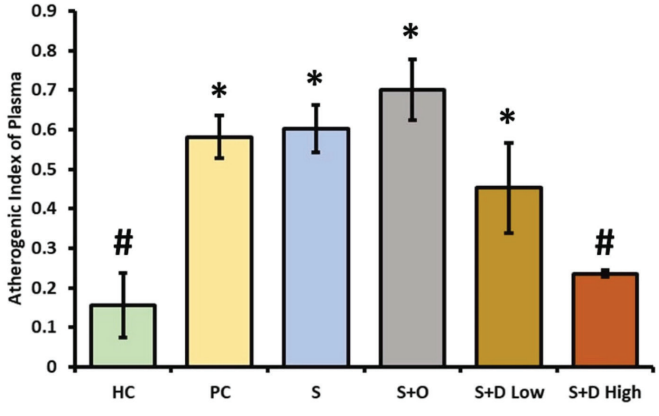

Figure 3. Results of atherogenic index of plasma calculation after administration of therapies for 2 weeks in each group

Liver function test after different treatments

The levels of serum liver enzymes after two weeks of treatment are depicted in Figure 4. In the placebo control group, rats only receiving a high-fat diet without antihyperlipidemic treatment

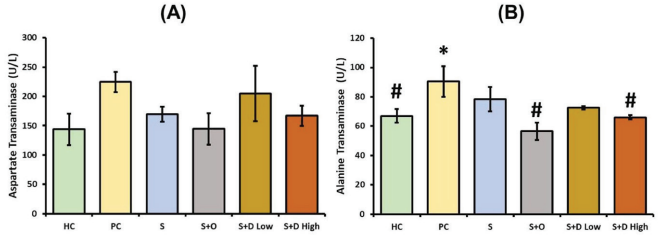

Figure 4. The effect of adjuvant treatments on the levels of serum liver enzymes in rats. Aspartate transaminase (A) and alanine transaminase (B)

experienced an increase in AST and ALT levels, although only the ALT level achieved statistical significance ($p < 0.05$). After the 2-week treatment with simvastatin, AST and ALT of the dyslipidemic rats were not significantly improved with mean reductions of 24.5% and 13.4%, respectively. Although the AST levels were not significantly changed with added adjuvant therapies, the administration of omega-3 or a high-dose vitamin D as an adjuvant to simvastatin led to a significant reduction in ALT by 37.60% and 27%, respectively, compared with the placebo group (56.5 ± 5.86 *vs.* 90.5 ± 10.45, $p = 0.003$ and 66.0 ± 1.39 *vs.* 90.5 ± 10.45, $p = 0.018$). The ALT value of rats treated with omega-3 and high-dose vitamin D as an adjuvant to simvastatin therapy was near that of normal controls.

DISCUSSION

Several studies have shown that consumption of a high-fat diet and fructose can induce lipid abnormalities, obesity, metabolic diseases, and cardiovascular diseases.[23,24] In general, experimental animals fed a high-fat diet and fructose solution experienced Frederickson phenotype-1 dyslipidemia, characterized by TG abnormalities > 99% due to lipoprotein lipase deficiency. Phenotype-1 dyslipidemia can occur due to the intake of high triacylglycerol content and enhanced lipogenesis activity that produces TGs.[12,24]

The addition of omega-3 as an adjuvant to simvastatin therapy had a superior effect on lowering TG levels with a mean value of 127.38 ± 13.36 mg/dL compared with 155.20 ± 18.05 mg/dL

with simvastatin alone. This is in accordance with the 2019 ESC/EAS Guidelines for the management of dyslipidemia, which state that omega-3 fatty acid therapy has the main effect of lowering TG levels, so its use is more recommended in hypertriglyceridemia conditions. Omega-3 can reduce serum TG concentrations by approximately 30%.[2] In the liver, omega-3 suppresses endogenous triacylglycerol production by decreasing levels of sterol receptor element-binding protein-1c (SREBP-1c) and selectively increasing the degradation of apo B-100, further reducing the production of triacylglycerol-rich VLDL. Another mechanism is the upregulation of fatty acid oxidation in the liver and skeletal muscle, thereby reducing the VLDL synthesized material.[25,26]

The group receiving combination therapy with simvastatin and a high-dose vitamin D showed the greatest reduction in TG levels, even resembling the TG levels of the normal group. However, the TC and non-HDLc levels did not significantly improve. These results are consistent with those of Paloma et al.,[27] who found that vitamin D supplementation (4,000 IU/day) could reduce serum TGs without affecting other lipid profile parameters. Another study examining the effect of vitamin D on serum showed that vitamin D could lower TG levels by 30.5% every time the dose was doubled.[28] *In vitro* studies have revealed that incubation with calcitriol ($1,25(OH)_2D_3$) may increase the expression and activity of lipoprotein lipase in cultured adipocytes, resulting in enhanced TG hydrolysis in chylomicrons and VLDL hydrolysis in blood vessels.[15,29]

This study also displayed that the combination therapy of simvastatin and high-dose vitamin D could increase HDL levels in the dyslipidemia group to a level high enough to exceed HDL levels in the normal group. This is in accordance with the research of Elmi et al.,[30] who reported that vitamin D3 levels were positively related to HDL2-C levels. Skaaby et al.[28] showed an increase in HDLc levels every time the dose of vitamin D was doubled. Although the molecular mechanism of the relationship between vitamin D and HDLc has not been fully elucidated, various observational studies have shown the relationship between them. The observational study of Williams et al.[31] of 4,274 children in the UK showed that high 25-OH-D levels were associated with cardioprotective levels of HDLc, apoprotein A1, and adiponectin. The results of this observational study are reinforced by the results of the experimental study by Tavakoli et al.,[32] who provided vitamin D supplementation to children aged 10-14 years and found a substantial rise in serum HDL levels compared with the control group.

In this study, adjuvant therapy with omega-3 or vitamin D did not have a beneficial effect in reducing TC levels compared with simvastatin treatment alone. These results are in accordance with those of a study conducted by Wang et al.,[18] where vitamin D supplementation lowered LDLc levels but had no significant effect on TC. Although the mechanism is not clear, various studies have shown an association between vitamin D deficiency and dyslipidemia. Vitamin D is hypothesized to increase lipolysis by suppressing parathyroid hormone secretion.[28] 25-Hydroxyvitamin D also inhibits sterol regulatory element-binding protein as a major regulator of lipogenesis.[33]

According to the 2019 ESC/EAS Guidelines for the management of dyslipidemia, omega-3 does not reduce TC levels but lowers TG.[2] However, Ibrahim Fouad[34] found that the administration of omega-3 monotherapy (500 mg/kg) in rats with hyperlipidemia induced by a high-fat diet for six weeks had a TC-lowering effect. Further research is required to evaluate the comparative effect of each therapy with simvastatin, vitamin D, and omega-3.

The non-HDLc level was calculated in this study to estimate the total number of atherogenic particles in plasma.[1] It also correlates with apolipoprotein-B levels and is a good predictor of cardiovascular risk.[35] In this study, the combination of simvastatin and adjuvant therapy (either omega-3 or vitamin D) had no significant impact on non-HDLc levels. A confounding factor is that the administration of the high-fat diet and fructose in rats only induced type-I dyslipidemia; therefore, rats did not experience an increased level of TC. Because the non-HDLc level was calculated from the TC level, the non-HDLc levels also remained unchanged after receiving a high-fat diet. Therefore, observing the role of either vitamin D or omega-3 in non-HDLc levels was difficult in this study. However, another study reported a similar result in a clinical setting, where vitamin D supplementation in patients with type 2 diabetes mellitus had no significant effect on non-HDLc levels.[35]

The administration of combination therapy with simvastatin and high doses of vitamin D in this study exhibited a superior effect in reducing AIP in the dyslipidemia group. The AIP of the group receiving combination therapy of simvastatin and high doses of vitamin D almost resembled the AIP of normal rats. This result is consistent with another study by Wang et al.,[17] which reported that serum 25(OH)D concentrations were negatively associated with AIP in men. Reduced TG and increased HDL will result in a smaller AIP calculation, indicating the normal diameter of LDL (atherogenic lipoprotein) and a lower risk of atherosclerosis. Vitamin D can inhibit TG synthesis and secretion by increasing calcium absorption in the intestine. Increased intestinal calcium levels may decrease fatty acid absorption because of the formation of insoluble calcium-fat complexes. In addition, calcium can increase the conversion of cholesterol into bile salts so that cholesterol levels decrease.[17,36] Further study is important to provide a complete measurement of atherogenic index parameters, including the cardiac risk ratio and atherogenic coefficient, as well as confirmation by histopathological examination of the heart and blood vessels.

Study limitations

In this study, we also found that the addition of omega-3 or high-dose vitamin D as an adjuvant to simvastatin therapy had a superior effect on lowering ALT levels compared with simvastatin alone. Vitamin D in high doses could also lower ALT levels with a mean value of 66.0 ± 1.39 µ/L. The hepatoprotective effects of omega-3 and vitamin D were previously studied. Omega-3 PUFA in low doses has a hepatoprotective effect, if administered daily.[37] Omega-3 prevents hepatic damage by improving hepatic function and normalizing lipid profiles in the serum and liver.[38] Similarly, vitamin D deficiency correlates with increased hepatic inflammation by increasing toll-like

receptors.[39] Lorvand Amiri et al.[40] reported that a combination of vitamin D and calcium had a superior effect on lowering serum ALT levels and could improve the stage of non-alcoholic fatty liver disease on ultrasonography. This means that vitamin D benefits on liver function may be related to its effect on lipid profiles. This hepatoprotective effect of vitamin D can improve clinical outcomes in patients with dyslipidemia.

CONCLUSION

Administration of high doses of vitamin D as an adjuvant to simvastatin therapy was more effective than omega 3 in improving TG and HDL levels. Vitamin D (both maintenance and high doses) as an adjuvant to simvastatin therapy was also superior to omega-3 in improving AIP in a rat model of type-1 dyslipidemia. Vitamin D in high doses (6,000 IU/day) as adjuvant therapy to simvastatin may not just alleviate dyslipidemia and decrease the risk of atherosclerosis. Furthermore, the administration of high doses of vitamin D or omega-3 as an adjuvant to simvastatin therapy had a hepatoprotective effect. Further work is needed to clarify the molecular mechanism underlying the cardioprotective, atheroprotective, and hepatoprotective potentials of vitamin D.

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Ethics

Ethics Committee Approval: All animal protocols complied with the Institutional Standard of Animal Care, and ethical clearance was obtained (409/UN 4.6.4.5.31/PP36/2022).

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Authorship Contributions

Surgical and Medical Practices: D.L, A.A., Concept: D.L, Y.Y.D, A.A., Design: D.L, Y.Y.D, A.A., Data Collection or Processing: D.L, Y.Y.D, B.O.M., Analysis or Interpretation: D.L, Y.Y.D, A.A., Literature Search: D.L, B.O.M., Writing: D.L, Y.Y.D.

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ORIGINAL ARTICLE

Head Space Single Drop Micro Extraction Gas Chromatography Flame Ionization Detection (HS-SDME-GC-FID) Method for the Analysis of Common Fatty Acids

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ABSTRACT

Objectives: Post-marketing/surveillance studies show that most of the many vegetable oils that are sold with health-promoting claims or statements with high nutritional values and are beneficial against diseases are off-limits of related monographs/criteria. Defining the oil with a fast, cheap, and efficient analytical method is needed to express fatty acids in any herbal product to authenticate, trace, specify, and classify the content.

Materials and Methods: Here, we define a new simple tool with a headspace single drop microextraction (HS-SDME) method coupled with a gas chromatography-flame ionization detector (GC-FID) for the analysis of common fatty acids (FAs) in oils. Linolenic acid, γ-linolenic acid, and linoleic acid in olive oil, thyme oil, and fish oil were determined. Derivatization was performed with 0.2 mL of 2 mol/L KOH in methanol to transfer the FAs of oils into their methyl esters (FAMEs). Then, FAMEs were extracted using a head space single drop, which is 2.0 µL of sodium dodecyl sulfate:1-butanol (1:3, *v/v*) mixture.

Results: The most suitable extraction condition was that 360 µL of the FAMEs, 2.0 mL vial, 0.07 g NaCl as a salting-out effect, 45 °C extraction temperature, and 35 min extraction time. The precision of the method was below 12%, with accuracy validated by the GC-FID reference method.

Conclusion: The HS-SDME can be used effectively for extracting FAs from oils for improved analysis of other FAs. The method is of direct importance and relevance for the herbal, pharmaceutical, and cosmetics industries.

Key words: Head space, microextraction, gas chromatography flame ionization detection, HD-SDME-GC-FID, fatty acids

INTRODUCTION

Fatty acids are monocarboxylic acids with double carbon numbers, cis structures, unbranched or straight chains, or acyclic structures. Vegetable-derived oils usually contain fatty acids with one or more double bonds in their structure. The melting points of fatty acids vary depending on the length of the chain and the degree of unsaturation. The melting points of short-chain fatty acids are lower than those of long-chain fatty acids. Thus, the melting point decreases with an increase in the number of double bonds.[1,2]

The most common fatty acid in nature is oleic acid (18:1, n-9 or omega-9); however, there is also linoleic acid (18:2, n-6 or omega 6), linolenic acid (18:3, n-3 or omega-3), and arachidonic acid (20:4, n-6 or omega-6). Although animal organisms can synthesize only a single pair of double-bond fatty acids, fatty acids with multiple double bonds (*i.e.* linoleic acid, α-linolenic acid) are also essential and should be taken externally.[3,4] Omega-3 and omega-6 fatty acids, which cannot be synthesized by the body, are converted into longer-chain fatty acids when taken into the body. Linoleic acid is metabolized to arachidonic acid in the

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body, while α-linolenic acid is metabolized to docosahexaenoic acid (22:6, DHA) and eicosapentaenoic acid (20:5, EPA), which play roles in cell membrane formation, blood clotting, wound healing, and inflammation.[3,5]

Oily fish are rich in omega-3 and omega-6, which are the fatty acids required for growth and development. Insufficient digestion of these fatty acids may result in dermatitis in children, which is characterized by a slowdown in brain development, kidney failure, and hematuria. Olive oil, which is a vegetable oil, is quite rich in terms of oleic acid (56-85%), linoleic acid (3.5-20%), and linolenic acid (1.2%).[6] Digestion of linoleic acid-rich food may have prophylactic and therapeutic effects in diseases such as cystic fibrosis, diabetes, and dermatitis.[5] There is a linear relationship between the appropriate consumption of fat (especially conjugated linoleic acid, CLA) and weight loss.[4,7]

The fatty acid content of vegetable oils depends on the plant seed because fatty acids are partially hydrolyzed by lipase enzymes because of bacterial contamination in the presence of high temperature and humidity.[8] Thus, fatty alcohols are formed through ketones, aldehydes, and short-chain fatty acids, which are produced during the contamination of certain fungi such as *Aspergillus niger*.[9] Auto-oxidation of oils may result in free radicals that cause food to decay, loss of taste quality, damage tissues, cause cancer, atherosclerosis, inflammatory events, and accelerate aging. Several factors affecting lipid auto-oxidation are the type and amount of fatty acids in the composition of fat, the presence of oxygen in the environment, metals (Cu and Fe), light, temperature, moisture, and storage conditions.[10]

Fatty acids are widely analyzed by different analytical methods such as high-performance liquid chromatography (HPLC),[11] gas chromatography (GC),[12] and capillaries electrophoresis.[13] Derivatization, which is a chemical process that increases chromatographic selectivity and sensitivity, is required to transform compounds into volatile states to prepare them for GC analysis. Compounds containing polar functional groups such as -COOH, -OH, -NH, and -SH are less volatile for gas chromatographic analysis because they form hydrogen bonds by themselves, thereby decreasing their volatility or interaction with the column. In contrast, gas chromatographic analyses can be performed by making these compounds volatile through derivatization because of the polarization of the compounds. The carboxyl group of fatty acid reacts with alcohols in the presence of the catalyst (acid, or base) to give the ester species, which is more volatile.[14-16]

Lipids are extracted from matrices using non-polar solvents and saponified with base (NaOH or KOH) to produce free fatty acid salts. The fatty acid salts are further derivatized to fatty acid methyl esters by refluxing with methanolic sodium or potassium hydroxide to improve peak symmetry, increase volatility, and decrease sample activity.[6,15] Finally, the FAMEs were extracted with a non-polar solvent (*e.g.*, heptane) for GC analysis.

The preliminary process plays a major role in the separation and enrichment of analytes in a complex matrix environment for precise, accurate, and fast analysis. Most sample preparation methods include purification steps that are time-consuming and expensive because of the chemicals used during the process.[14] In recent years, techniques such as flow injection, liquid-phase extraction, and solid-phase extraction methods have been developed to simplify and reduce the amount of solvents used in purification.[17] Although the liquid-liquid extraction (LLE) method is one of the most preferred classical sample preparation methods, it is still time-consuming and requires a large amount of organic solvents. On the other hand, the solid-phase microextraction method requires less solvent than the LLE method but contains complex extraction steps. Due to these challenges, interest has increased in micro-extraction methods compatible with gas chromatography, capillary electrophoresis, and high-performance liquid chromatography, which can be applied more easily, simplify the extraction steps, provide automation, reduce the use of organic solvents to microliter (μL) levels, and provide better enrichment. Single drop microextraction (SDME) is the most preferred method of liquid phase microextraction techniques because it is cheap, easy to apply, reduces solvent usage, and can be applied in analytical systems in the form of direct pre-concentration. It can be easily applied to GC, inductively coupled plasma mass spectrometry (ICP-MS), capillary electrophoresis (CE), and electrothermal atomic absorption spectrometry.[18] In the SDME method, the extraction solvent droplet (1-10 μL), which does not mix with the gas or liquid sample, hangs on the nozzle. Following extraction, the substances are resuspended from the aqueous sample by passive diffusion and analyzed *via* GC, HPLC or CE.[19]

Volatile and semi-volatile compounds can be determined using headspace SDME (HS-SDME).[20-23] The HS is used for extracting volatile compounds from gaseous and aqueous phases using a stir bar[24] or a single liquid collecting droplet.[25] The HS-SDME method is based on placing the microdrop of the appropriate solvent in the tip of a microsyringe needle into the space at the top of the vial (HS), which contains the sample solution, and the extraction of volatile analytes to the microdrop. This method, which has three phases: aqueous phase, gas phase, and organic drop, is used for the enrichment of volatile organic compounds and allows easy removal of analytes from the complex matrix.[20,26,27]

In our study, we aimed to develop a high-precision analytical method with a simple preliminary process for fast and accurate analysis of linoleic acid, linolenic acid and, γ-linolenic acid in fish oil, olive oil, and thyme oil. For this purpose, an esterification reaction with methanol and an alkaline catalyst was performed to transform the essential fatty acids in the samples into their methyl esters, which were extracted from the matrix using the HS-SDME method and analyzed *via* GC-FID.

MATERIALS AND METHODS

Reagents and chemicals

All chemicals used were of gas chromatographic quality. Linolenic acid, γ-linolenic acid, linoleic acid, potassium hydroxide (KOH), heptane (C_7H_{16}, 99%), and sodium dodecyl

sulfate were purchased from Sigma-Aldrich (Germany). The standards of FAs were stored at -20 ˚C. Methanol, (CH_3OH, containing less than 0.5% water) and 1-butanol were obtained from Merck (Darmstadt, Germany). NaCl, purchased from J.T. Baker, Deventer, Holland, was used to adjust the ionic strength of the solution. Helium, hydrogen, and dried air gases (for gas chromatography) were purchased from Oksan Co., Ltd. (Ankara, Türkiye). Deionized water was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA). The screw-top vial used in single-drop microextraction was 2 mL of amber (Agilent Technologies Inc., US). The three commercial oil samples (olive oil, thyme oil, and fish oil) were purchased in 2018 from a local store in Ankara (Türkiye) and stored in the absence of light at ambient temperature until analysis. All solutions containing FAs were prepared in an ice bath.

Chromatographic conditions

GC-FID analysis was performed on an Agilent Technologies 7890A GC System (Santa Clara) equipped with an autosampler (Agilent 7693A Automatic Liquid Sampler, China), and J&W HP-5 (5%-phenyl)-methylpolysiloxane non-polar capillary column (30 m x 0.32 µm ID x 0.25 µm film thickness, Agilent Technologies, US). The oven temperature program was as follows: hold at 80 ˚C for 2 min, increase from 4 ˚C/min to 210 ˚C, then hold for 5 min, increase from 15 ˚C/min to 300 ˚C, and hold for 5 min. The column carrier gas was high-purity helium (≥ 99.999%) at a constant flow rate of 1 mL/min. The injection and detector temperatures were 270 ˚C and 280 ˚C, respectively. The injection volume was 1 µL with a split ratio of 100:1. Flame gases were hydrogen and dried air gases. Identification of the fatty acid methyl esters of oil samples was performed on the basis of the comparison of their retention times (t_R) with those of pure standards under the same chromatographic conditions. Peak areas were used to evaluate the number of FAMEs as a percentage.

Derivatization of the standards

Linolenic acid, γ-linolenic acid, and linoleic acid (10 mg of each) standards were dissolved in 200 µL of heptane, separately, and incubated in an ice bath. 0.2 mL of 2 mol/L potassium hydroxide in methanol was added to each solution, and caps were closed immediately, mixed with vortexing for approximately 30 s, and waited until the upper phase became clear. The FAME-containing upper phase was analyzed *via* GC-FID for 12 h.[28]

Derivatization of the samples

Each sample (olive oil, thyme oil, and fish oil) was heated in a closed system at 100 ˚C for about 3 h. One gram of oil sample was weighed, 2 mL of heptane was added, shaken, and then 0.2 mL of 2 mol/L KOH in methanol solution was added. The caps were closed and mixed with vortexing for 30 s. The clear upper phase, which contains fatty acid methyl esters, was taken for enrichment with HS single drop microextraction.

The intraday precision of FAMEs was evaluated by GC-FID analysis of the olive oil sample on the same day at four different times (0^{th}, 6^{th}, 12^{th} and 24^{th} hour) and the relative standard deviation (RSD %) was found to be 2.1-18.3% at 12 h and 2.1-25.0% for 24 h. FAMEs were used on the HS-SDME-GC-FID method for 12 h, and all analyses were performed in triplicate.

Headspace single-drop microextraction

After the derivatization of samples, microextraction and injection procedures were performed using a Hamilton gastight syringe (1700 series, SL syringe, 50 µL, needle size 22s ga, Germany). 360 µL of fatty acids methyl esters sample and 0.07 g of NaCl were placed in a 2 mL glass vial (Agilent Technologies Inc., US) equipped with a screw cap and silicone septum. Hamilton gastight syringe containing 2.0 µL of sodium dodecyl sulfate: 1-butanol mixture (1:3, *v/v*) was immersed from the septum up to 0.5 cm of the solution. The temperature was adjusted to 45 ˚C for 35 min. Following microextraction, the solvent was retracted into the gastight syringe, and the syringe's valve was turned off until it was transferred to the heated injection port of the GC-FID.

RESULTS AND DISCUSSION

All microextraction parameters were determined in the derivatized olive oil sample according to the peak area of the linolenic acid methyl ester. The optimization assay used a successive optimization approach, in which one parameter was changed at a time, and the others were kept constant. The most important step for the HS-SDME method is selecting the appropriate extraction solvent. Thus, the boiling point of the solvent and its viscosity are important parameters in the selection criteria for the solvent in the micro-extraction method. Hence, the solvent's boiling point must be low enough to conform to gas chromatographic analysis, but higher than the boiling point of the analytes to prevent evaporation during the extraction process. Moreover, the solvent must have a high viscosity sufficient to adhere to the tip of the syringe needle, as well as a viscosity low enough to allow rapid diffusion of the analyte to the drop that significantly affects the extraction time. Various types of extraction solvents (1-butanol, 1-octanol, acetonitrile, diethyl acetate, toluene, hexane, heptane, dichloromethane: hexane: toluene, sodium dodecyl sulfate: 1-butanol) were attempted to select the best solvent for the microextraction of FAMEs. Sodium dodecyl sulfate:1-butanol mixture (1:3, *v/v*) was the most suitable extraction solvent for HS-SDME. The extraction efficiency of the analyte depends on the microdrop volume; however, as the drop volume increases, the drop on the needle of the syringe becomes unstable. Therefore, 2 µL of extraction solvent was used in further steps of the experiment.

Optimization of other variables in the HS-SDME was the quantity of the salting-out reagent (NaCl), the micro-extraction time, and temperature. The total vial volume was tested with 10 mL and 2 mL of vial, where 2 mL of vial was selected without stirring in further studies.

In conventional extraction methods, the addition of salt to the aqueous sample solution is an accepted approach to increase the extraction efficiency and ensure that the polar analytes in the organic phase remain intact. As the ionic strength of the medium increases with the addition of electrolytes to the

samples prepared in the aqueous medium, the water solubility of the polar analytes and the organic compounds decreases. Thus, the rate of analyte passing to the organic phase and the extraction efficiency of the method increases. The reduction of the solubility of analytes in water occurs when the ionic salt molecules (salting out effect) in the aqueous medium surround the hydration layer. In addition, salt molecules may interact with polar molecules electrostatically, leading to reduced mass transfer of analytes. Depending on the solubility of analytes, the amount of extraction may increase with the salting out effect at high salt concentrations or the extraction of analytes may decrease due to the attraction forces between charged particles, electrolytes, and analytes dispersed in the solution. Although adding salt to the sample solution generally increases the extraction efficiency, the presence of salt in high concentrations may change the physical properties of the extraction film and decrease the diffusion rate of the analyte in the organic phase.[29,30] In the present study, the extraction efficiency of FAs from the aqueous phase to a single drop was determined using NaCl as the inorganic salt. The effect of increasing the ionic strength of the solution was evaluated by adding NaCl with the highest peak area of 0.07 g NaCl.

The extraction time was tested between 15 and 45 min with the optimum extraction time being 35 min (Figure 1). The extraction temperature was determined between 25 and 45 °C , and the maximum peak area was obtained at 45 °C (Figure 2).

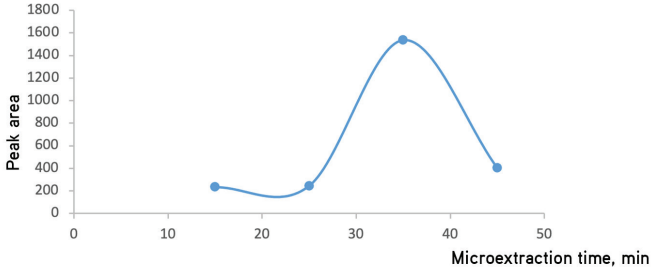

Figure 1. Effect of time on the extraction efficiency for HS-SDME-GC-FID method (360 µL sample, 2 µL microdrop of sodium dodecyl sulfate: 1-butanol (1:3, *v/v*), extraction temperature 45 °C, 0.07 g NaCl)

HS-SDME-GC-FID: Headspace single-drop microextraction- gas chromatography-flame ionization detector

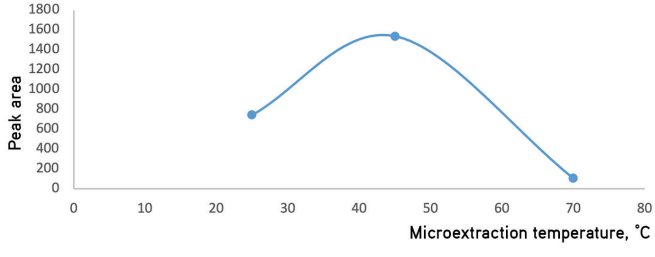

Figure 2. Effect of temperature on the extraction efficiency for HS-SDME-GC-FID method (360 µL sample, 2 µL microdrop of sodium dodecyl sulfate: 1-butanol (1:3, *v/v*), extraction time 35 min, 0.07 g NaCl)

HS-SDME-GC-FID: Headspace single-drop microextraction-gas chromatography-flame ionization detector

Further microextraction studies were conducted by 360 µL FAMEs, 0.07 g NaCl in 2.0 mL vials, and applying microextractions for 35 min at 45 °C. The applied extraction conditions and obtained highest efficiency values are summarized in Table 1.

The GC-FID chromatograms of the linolenic acid, γ-linolenic acid, and linoleic acid methyl ester standards are presented in Figure 3. The retention times (t_R) of linolenic acid methyl ester, γ-linolenic acid methyl ester, and linoleic acid methyl ester were found to be 35.82, 35.04, and 34.32 min, respectively.

Identification of the fatty acid methyl esters in oil samples was performed on the basis of the comparison of their retention times (t_R) with those of pure standards. The peak areas were used to evaluate the number of FAMEs as a percentage under the optimum HS-GC-FID chromatographic conditions. Chromatograms of thyme oil, olive oil, and fish oil fatty acid methyl esters with GC-FID and HS-SDME-GS-FID at optimum conditions are shown in Figures 4-6, respectively.

The accuracy was determined by comparing the HS-SDME-GC-FID quantitative results with the GC-FID reference[6,31] and methods (Table 2). Olive oil, thyme oil, and fish oil fatty acid methyl esters were calculated on the basis of the total fatty acid methyl ester amounts according to the normalization method received from the GC-FID and HS-SDME-GS-FID analysis results. The results were found to be compatible with the European Pharmacopoeia criteria.[6] Statistical analysis was performed at 95% confidence limit (p = 0.05). HS-SDME-GC-FID and GC-FID reference method values were compared using Student's t-test at 95% confidence limit. "t" values were calculated from equation 1,[32,33] and s values were calculated from equation 2.[32,33] Because the calculated "t" values were smaller than t_{table}, they are statistically acceptable and the proposed method is significant for accurate use in further studies.

$$t = [(|x_{ort} - y_{ort}|)/s\ ] \times \sqrt{[(m \times n) / (m+n)]}$$

Equation 1

$$s = \sqrt{[(\sum x_i^2 - (\sum x_i)^2/m + \sum y_i^2 - (\sum y_i^2)/n) / (m+n-2)]}$$

Equation 2

s: standard deviation of two series

x_{mean}: Mean value of Serie 1 (HS-SDME-GC-FID results)

y_{mean}: Mean value of Serie 2 (GC-FID results)

m: Number of data points of Serie 1

n: Number of data points of Serie 2

The precision of the HS-SDME-GC-FID method was determined

Table 1. Factors and experimental condition applied for optimization of the HS-SDME

Factors	Experimental conditions			
NaCl (g)	0.02	0.04	0.07*	
Extraction time, min	15	25	35*	45
Extraction temperature, °C	25	45*	70	
Sodium dodecyl sulfate:1-butanol (*v/v*)	1:0	1:1	1:2	1:3*

*Optimum value

Table 2. Percentage of FAMEs obtained with GC-FID (n: 3) and HS-SDME-GC-FID (n: 3). % (*w/w*) and *t*-value (p = 0.05)

Sample	γ-Linolenic acid methyl esther (mean % ± SD*)	Linolenic acid methyl esther (mean % ± SD*)	Linoleic acid methyl esther (mean % ± SD*)
Fish oil (HS-SDME-GC-FID)	1.0 ± 0.2	2.9 ± 0.4	1.2 ± 0.3
Fish oil (GC-FID)	1.5 ± 0.1	2.8 ± 0.2	1.0 ± 0.2
$t_{calculated} < t_{table}$	1.78 < 2.78	0.61 < 2.78	1.1 < 2.78
Reference value of fish oil (EU 2011)		0.5-3.0	0.5-3.0
Thyme oil (HS-SDME-GC-FID)	23.7 ± 1.3	1.0 ± 0.3	2.5 ± 0.1
Thyme oil (GC-FID)	25.4 ± 1.5	1.4 ± 0.2	2.7 ± 0.2
$t_{calculated} < t_{table}$	2.55 < 2.78	1.00 < 2.78	2.08 < 2.78
Reference value of thyme oil (EU 2014)		max. 1.2	3.5-20.0
Olive oil (HS-SDME-GC-FID)	5.2 ± 0.5	1.7 ± 0.2	2.2 ± 0.1
Olive oil (GC-FID)	5.9 ± 0.2	1.9 ± 0.1	2.1 ± 0.2
$t_{calculated} < t_{table}$	1.21 < 2.78	0.51 < 2.78	0.78 < 2.78
Reference value of olive oil (EU 2011)		Max 1.2	3.5-20

*Standard deviation
HS-SDME-GC-FID: Headspace single-drop microextraction-gas chromatography-flame ionization detector

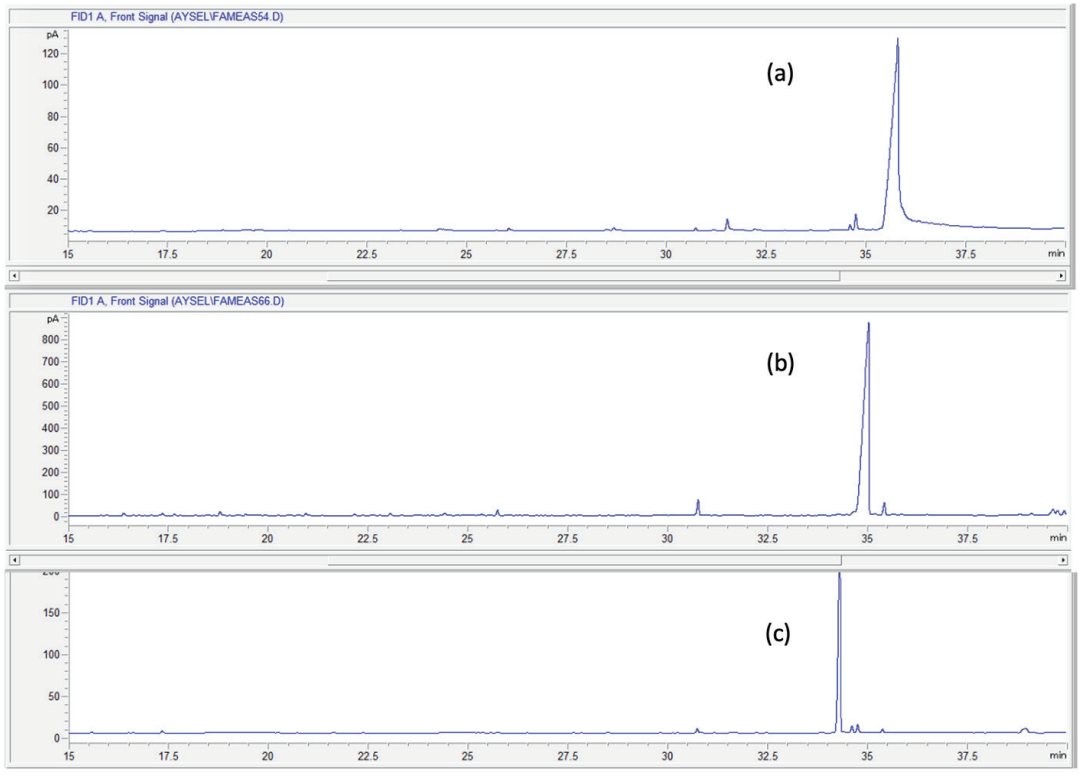

Figure 3. GC-FID chromatogram of (a) linolenic acid methyl esther (t_R: 35.82 min), (b) γ-linolenic acid methyl esther (t_R: 35.04 min), (c) linoleic acid methyl esther (t_R: 34.32 min)
GC-FID: Gas chromatography-flame ionization detector

as RSD % (n: 5) as 11.7% for linoleic acid methyl ester, 12.4% for γ-linolenic acid methyl ester, and 11.2% for linolenic acid methyl ester.

The extraction efficiency of the method was calculated to be 41.5 ± 3.7% according to the GC-FID and HS-SD-GC-FID peak areas.

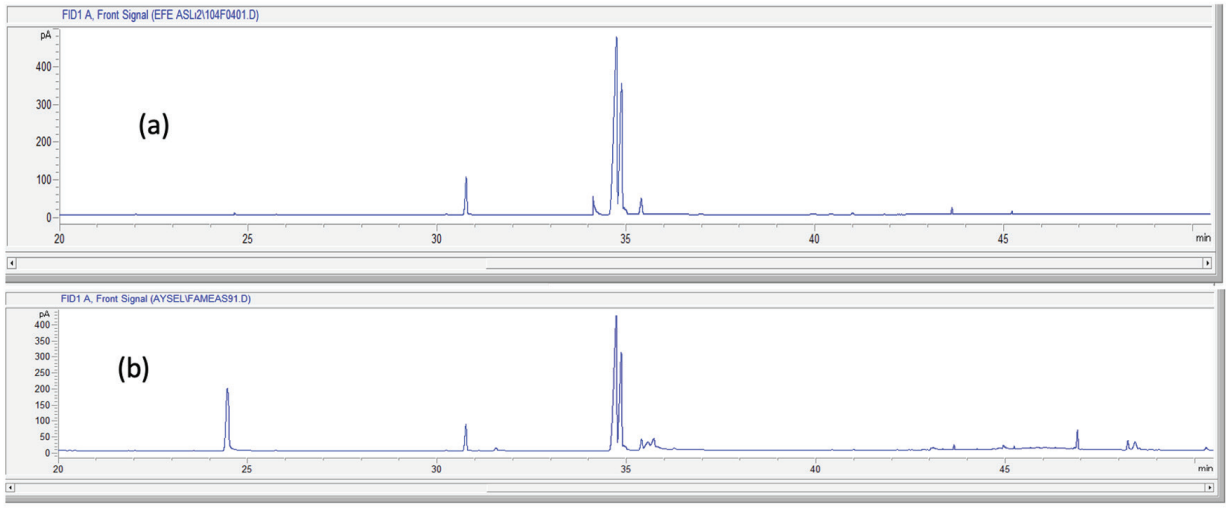

Figure 4. Chromatogram of thyme oil methyl esters obtained with a) GC-FID b) HS-SDME-GC-FID.
HS-SDME-GC-FID: Headspace single-drop microextraction- gas chromatography-flame ionization detector

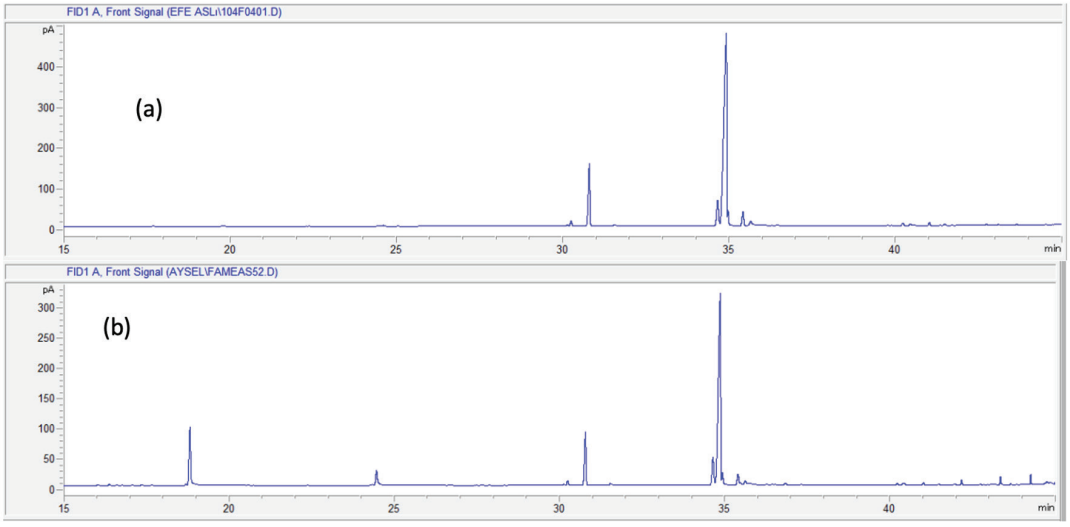

Figure 5. Chromatogram of olive oil methyl esters obtained with a) GC-FID b) HS-SDME-GC-FID.
HS-SDME-GC-FID: Headspace single-drop microextraction- gas chromatography-flame ionization detector

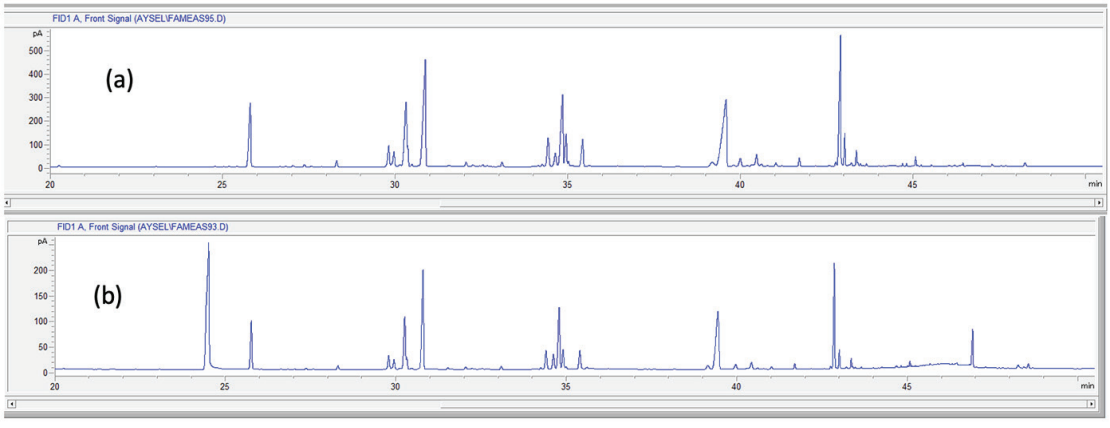

Figure 6. Chromatogram of fish oil fatty acid methyl esters obtained with a) GC-FID b) HS-SDME-GC-FID.
HS-SDME-GC-FID: HS-SDME-GC-FID: Headspace single-drop microextraction- gas chromatography-flame ionization detector

CONCLUSION

HS-SDME-GC-FID is a rapid, reproducible, and accurate method for the analysis of FAMEs (γ-linolenic acid, linolenic acid, and linoleic acid methyl esters). This method reduces the amount of extraction solvent (green chemistry) and thereby the cost. High precision (below 12.4%), simple sample preparation, enrichment of the analyte, and removal of the matrix from the analyte may allow the use of this proposed method for routine analysis in industry and research laboratories.

It is also possible to determine fatty acids in samples using an external calibration method using the standards of γ-linolenic acid, linolenic acid, and linoleic acid methyl esters. However, because of the cost of standard fatty acids, studies are often designed on percentage quantities. However, fatty acid methyl esters can also be easily analyzed using a GC mass spectrometry (GC-MS), its existing library without using costly standards, although libraries must contain accurate all *m/z* spectra of oil, which type of library may cost high as standards' itself. Here, the method developed was analyzed using carbon-sensitive FID detector due to the long carbon chain structures of fatty acids. The method we developed with easy sample preparation and repeatable results could also be used successfully in the analysis of different types of oils and fatty acids using standards containing methyl esters of all fatty acids. Because it is defined using a fast, cheap, and efficient analytical method, the new technique can also be used to express fatty acids in any food or product, to authenticate, trace, specify, and classify the content, whatever or wherever such technique is needed.

Ethics

Ethics Committee Approval: There is no requirement for ethical approval.

Peer-review: Externally peer reviewed.

Authorship Contributions

Concept: A.E., O.K.U., A.B., Design: A.E., O.K.U., A.B., Data Collection or Processing: A.E., O.K.U., A.B., Analysis or Interpretation: A.E., O.K.U., A.B., Literature Search: A.E., O.K.U., A.B., Writing: A.E., O.K.U., A.B.

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

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SHORT COMMUNICATION

Spectrophotometric Quantification of Atomoxetine Hydrochloride Based on Nucleophilic Substitution Reaction with 1,2-Naphthoquinone-4-Sulfonic Acid Sodium Salt (NQS)

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ABSTRACT

Objectives: A simple, sensitive, selective, and cost-effective colorimetric method has been established for the quantitative estimation of atomoxetine hydrochloride in bulk and formulation.

Materials and Methods: It was established based on the visible reaction between atomoxetine hydrochloride and 1,2-naphthoquinone-4-sulfonic acid sodium salt in a basic medium (potassium hydroxide). The resulting orange colored chromogen exhibited an absorption maximum at 474 nm.

Results: Based on the optimization studies, distilled water as the solvent, 1% *w/v* potassium hydroxide (2 mL), and 0.3% *w/v* 1,2-naphthoquinone-4-sulfonic acid sodium salt (2 mL) were used in the method. The developed method was validated *per* the International Council for Harmonization (ICH) guidelines. The linearity was found at a concentration of 10-50 µg/mL. The method showed a good correlation between the concentration of atomoxetine hydrochloride and its absorbance. The correlation coefficient (r^2) of 0.999 evidenced the same. The limits of detection and quantification were 0.20 and 0.606 µg/mL, respectively, for atomoxetine hydrochloride. The accuracy and precision of the method were also evaluated and the results obtained were within the acceptance criteria (relative standard deviation % < 2.00). The percentage assay of atomoxetine hydrochloride proved to be 101.52, which is in accordance with its label claim.

Conclusion: The developed method is non-complex and can be effectively employed in the analytical practices of atomoxetine hydrochloride in pharmaceutical dosage forms.

Key words: Colorimetry, atomoxetine hydrochloride, accuracy, precision, linearity

INTRODUCTION

Atomoxetine hydrochloride is chemically known as (*R*)-*N*-methyl-3-phenyl-3-(*o*-tolyloxy) propylamine hydrochloride (Figure 1).[1] As a norepinephrine reuptake inhibitor, it is used in the management of attention/deficit hyperactivity disorder. Atomoxetine hydrochloride is the subject of a monograph published in the Indian Pharmacopoeia. Its titration with acetous perchloric acid in the presence of acetous mercuric acetate and further potentiometric determination at the end-point were described in Indian Pharmacopiea.[2]

Several methods have been presented in the literature for the determination of atomoxetine hydrochloride. A few of them include visible spectrometric methods involving oxidative

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coupling with sodium per-iodate and folic reagent,[3] nucleophilic substitution with 1,2-naphthoquinone-4-sulfonic acid sodium salt (NQS) in alkaline medium[4] and condensation with vanillin/*para*-dimethylamino benzaldehyde,[5] ultraviolet spectrophotometric methods using solvents, such as double distilled water,[6] acetonitrile,[7] 0.05 N hydrochloric acid,[8] spectrofluorimetric methods based on the emergence of binary complex with eosin Y in Teorell-Stenhagen buffer[9] and reaction with 4-chloro-7-nitro-2,1,3-benzoxadiazole in chloroform.[4] Furthermore, reverse-phase high-performance liquid chromatographic methods with combinations of stationary and mobile phases,[10-15] ultra-performance liquid chromatographic method,[16] and liquid chromatography-tandem mass spectroscopic method[17] have also been mentioned in the literature.

A literature survey revealed that even though sophisticated instrument techniques are available for atomoxetine hydrochloride (reverse phase high-performance liquid chromatography, ultra-performance liquid chromatography, and liquid chromatography-tandem mass spectroscopy), their use is limited as they require costly instruments, solvents/reagents, skilled operators, and tedious extraction protocols. It was identified that the colorimetric method reported used borate buffer-chloroform-NQS in alkaline medium and the color was developed with the aid of heat (70 °C) after 40 min of addition of the reagents.[4] NQS is one of the extensively used chromogenic reagents for the determination of primary or secondary amine-containing drugs.[18] Colorimetry is persistently competitive with chromatographic techniques due to its simplicity, sensitivity and selectivity, cost-effectiveness, fair accuracy, precision, and easy access in most quality control laboratories for pharmaceutical analysis.[19]

Keeping these facts in mind, the present work was attempted by dissolving the analyte in the most economical and easily available solvent, *i.e.*, distilled water, and using NQS as the reagent. Furthermore, the reaction was performed at room temperature. The details of the methods and materials adopted and the results obtained are discussed in the following chapters.

MATERIALS AND METHODS

Materials and instrumentation

Atomoxetine hydrochloride standard (Hetero Drugs Pvt. Ltd., India) and marketed tablets (Axepta-10, contains 10 mg of atomoxetine hydrochloride *per* tablet) from a local drugstore were used as received. We used analytical grade chemicals and reagents for establishing the analytical method. Colorimetric measurements were performed using a double-beam ultraviolet-visible spectrophotometer (Shimadzu 1800, Japan). The standard statistical functions were computed using the options available in MS-EXCEL.

Reagents and standard solutions

NQS reagent (0.3% w/v)

An accurately weighed NQS (0.30 g) was transferred into a 100 mL volumetric flask and dissolved in an appropriate volume of distilled water.

Potassium hydroxide solution (1% w/v)

In a 100 mL volumetric flask, accurately measured potassium hydroxide (1.00 g) was transferred, and the volume was made with distilled water.

Standard stock solution of atomoxetine hydrochloride

Atomoxetine hydrochloride (1,000 µg/mL) was produced by solubilizing a precisely weighed substance (10.00 mg) in distilled water contained in a volumetric flask (10 mL). Transferred 1 mL into another 10 mL volumetric flask from the above stock, and the volume was finally made with distilled water to acquire 100 µg/mL as the end concentration.

Analysis of atomoxetine hydrochloride

Aliquots of the standard drug solution of atomoxetine hydrochloride (100 µg/mL) ranging from 1 to 5 mL were transferred to a group of 10 mL volumetric flasks. They were shaken vigorously after adding potassium hydroxide solution (1% *w/v*, 2 mL) and NQS reagent (0.3% *w/v*, 2 mL). The volume was adjusted with water to prepare a batch of analytical solutions comprising 10-50 µg/mL of atomoxetine hydrochloride. The absorbance of the resulting colored complex was recorded after 20 min at λ_{max} 474 nm against the corresponding reagent blank. Beer-Lambert's plot was used to compute the amount of atomoxetine hydrochloride.

Analytical method optimization

The optimum concentration of potassium hydroxide and NQS reagent, reaction time, and mole ratio were studied during method development, and the details are provided in the results and discussions.

Analytical method validation

Validation of the method was attempted by checking linearity, accuracy, precision, sensitivity, and robustness by adopting the procedures stated in the International Council for Harmonization (ICH) guidelines.[20]

Linearity

Aliquots of stock solution (100 µg/mL) of atomoxetine hydrochloride were conveyed to a set of volumetric flasks to obtain final concentrations in the span of 10-50 µg/mL. Potassium hydroxide solution (1% *w/v*, 2 mL) and NQS reagent (0.3% *w/v*, 2 mL) were added to the above analyte solution, and the volume was made using distilled water. Using an appropriate blank, the absorbance of colored chromogen was recorded after 20 min at λ_{max} 474 nm. The calibration curve was developed by plotting the absorbances against the drug concentrations.

Accuracy

Standard solutions of atomoxetine hydrochloride were added at 80, 100, and 120% levels to pre-quantified sample solutions of atomoxetine hydrochloride (20 µg/mL). Each sample was tripled at the individual level. The atomoxetine hydrochloride quantity was estimated using acquired absorbance values in the regression equation.

Precision

The intra-day and inter-day precisions of the proposed colorimetric technique were established by estimating the responses in six replicates of atomoxetine hydrochloride (30 µg/mL) on the corresponding day and three distinct days in a week, respectively. The outcomes are described in terms of percentage relative standard deviation (% RSD).

Sensitivity and robustness

The lowest detectable amount [limit of detection (LOD)] and the lowest quantifiable amount [limit of quantitation (LOQ)] in the method were determined using samples containing very low concentrations of atomoxetine hydrochloride, as stated in the ICH guidelines. The LOD was calculated as 3.3 multiplied by the ratio of standard deviation and slope. Similarly, the LOQ was calculated by 10 multiplied by the ratio of the standard deviation and slope. Sandell's sensitivity of atomoxetine hydrochloride was computed from the quotient of molecular weight and molar absorptivity. Furthermore, the method was also tested with minor modifications in the concentration of NQS and potassium hydroxide to ensure robustness and the resulting responses were recorded.

Assay of atomoxetine hydrochloride in the marketed tablets

The marketed tablets (Axepta-10 containing 10.00 mg atomoxetine hydrochloride) were precisely measured and ground to a fine powder. The powder analogous to 10.00 mg analyte was dispersed in distilled water (10 mL) to produce 1,000 µg/mL and then sonicated for 5 min. The resulting mixture was then shaken vigorously and filtered using Whatman's filter paper (no: 41). Clear filtrate (1 mL) was shifted into a 10 mL volumetric flask and diluted with distilled water up to the mark. From this 100 µg/mL solution, a 3 mL portion was moved to another 10 mL volumetric flask, 2 mL of 1% *w/v* potassium hydroxide and 2 mL of 0.3% *w/v* NQS were added. Final fabrication was performed with distilled water. This solution was used to estimate atomoxetine hydrochloride after 20 min. The absorbance was recorded at 474 nm using an appropriate reagent blank. The amount of atomoxetine hydrochloride was computed by incorporating responses into the regression equation, with correction for dilution, and the results were statistically validated.

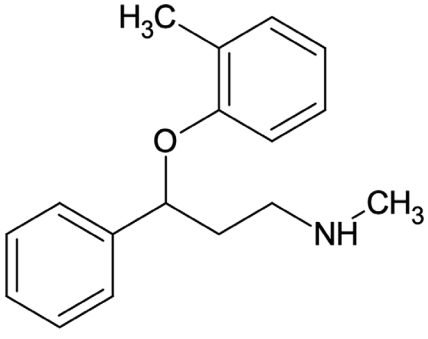

Figure 1. Structure of atomoxetine hydrochloride

RESULTS AND DISCUSSION

Development and optimization of the analytical method

This colorimetric method was developed on the basis of the reaction between NQS and atomoxetine under basic conditions at ambient temperature. The reaction yielded orange-colored chromogen, which displayed a maximum absorbance at 474 nm after 20 min of addition of the reagent (Figure 2). The probable reaction of NQS with atomoxetine hydrochloride in the basic medium is depicted in Figure 3.

Selection of the solvent

The ultraviolet (UV) absorbance of atomoxetine hydrochloride was determined by dissolving the analyte in different solvents such as distilled water, methanol, ethanol, and acetonitrile (Supplementary Table 1). Distilled water was selected as the solvent based on its high absorbance, low cost, and ease of availability.

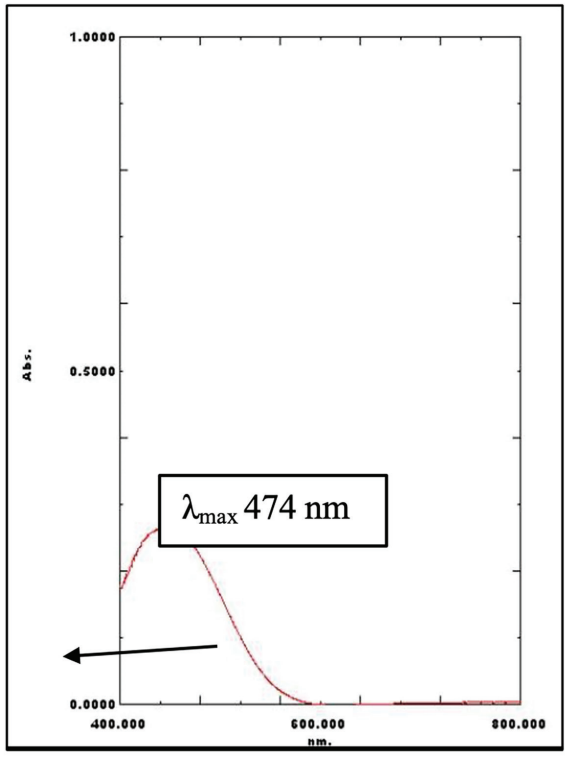

Figure 2. 2 UV absorption spectrum of atomoxetine hydrochloride at λ_{max} 474 nm in distilled water (10 µg/mL)

UV: Ultraviolet

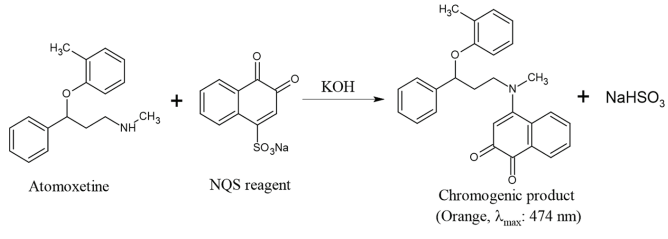

Figure 3. Probable reaction between atomoxetine hydrochloride and NQS

NQS: 1,2-Naphthoquinone-4-sulfonic acid sodium salt

Table 1. Accuracy data of the analytical method

% Level	Formulation (µg/mL)	Conc. of std. spiked (µg/mL)	Amount found (AM ± SD, n: 3)	Bias	% Recovery	% RSD*
80	20	16	35.283 ± 0.301	0.174	98.00	0.85
100	20	20	40.451 ± 0.313	0.181	101.13	0.77
120	20	24	43.524 ± 0.675	0.389	98.92	1.55

AM: Arithmetic mean, SD: Standard deviation, Bias: Standard deviation/square root of sample size, RSD: Relative standard deviation
*Acceptance criteria: % RSD should not exceed 2.00

Optimization of the concentration of potassium hydroxide and NQS

Atomoxetine hydrochloride solution (100 µg/mL, 1 mL) was shifted to a volumetric flask (10 mL), mixed with various concentrations of potassium hydroxide (2 mL, 0.5, 1.0, 1.5, and 2.0%, *w/v*), NQS reagent (0.3 and 0.5%, *w/v*) and finally diluted with distilled water. Each analyte solution was produced by changing only one variable (either concentration of potassium hydroxide or NQS) at a time, and the response of the resulting solutions was recorded at 474 nm after 20 min (Figures 4 and 5). The results indicated that 0.3% (*w/v*) concentration of NQS and 1% (*w/v*) concentration of potassium hydroxide showed constant and highest absorbance among all the combinations. Therefore, the same were selected for further investigation.

Selection of the wavelength

The analyte solution (10 µg/mL in distilled water) along with the above mentioned reagents was scanned in the visible range (400-800 nm), and the spectrum obtained is shown in Figure 2. From the spectrum, λ_{max} was found to be 474 nm for atomoxetine hydrochloride. The absorbance of the analyte solutions at the same wavelength was recorded throughout this investigation.

Optimization of the reaction time

Color development was monitored at discrete time intervals (5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 min) to optimize the reaction time. Maximum absorbance was noted after 20 min (Figure 6). Stable color development was noticed up to 6 h under optimized conditions.

Stoichiometry of the reaction

The continuous variation method was used to investigate the stoichiometry of the reaction. Samples were prepared with equimolar amounts of atomoxetine hydrochloride (3.43 x 10^{-5} M) and NQS, while other reaction conditions were the same as mentioned earlier. The drug and reagent (NQS) were assorted to produce different mole ratios (0.2:0.8, 0.4:0.6, 0.5:0.5, 0.6:0.4, and 0.8:0.2, respectively). The stoichiometric relationship between the two variables is shown in Figure 7 and a mole ratio of 0.5:0.5 gave the highest absorbance value.

Analytical method validation

The response of atomoxetine hydrochloride at 474 nm was deliberated in 10-50 µg/mL concentration range. The data for the calibration curve and regression analysis of the calibration curve are shown in Figure 8. The study resulted in a correlation coefficient (r^2) value of 0.999. From the results, it was observed that with an increase in the concentration of atomoxetine hydrochloride, the absorbance also increased linearly.

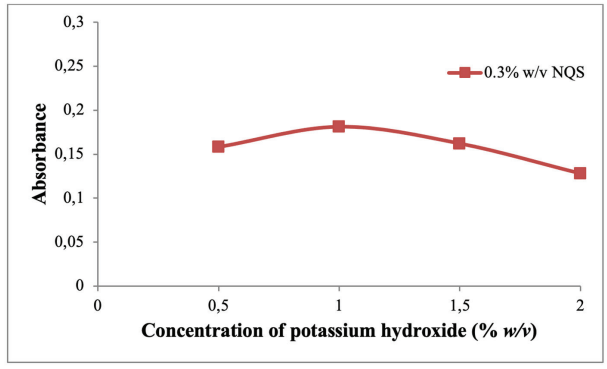

Figure 4. Effect of the concentration of potassium hydroxide and 0.3% (*w/v*) NQS in X-axis scale as- 0, 0.5, 1, 1.5 and 2, Y-axis scale as 0, 0.05, 0.1, 0.15, 0.2, 0.25, and 0.3

NQS: 1,2-Naphthoquinone-4-sulfonic acid sodium salt

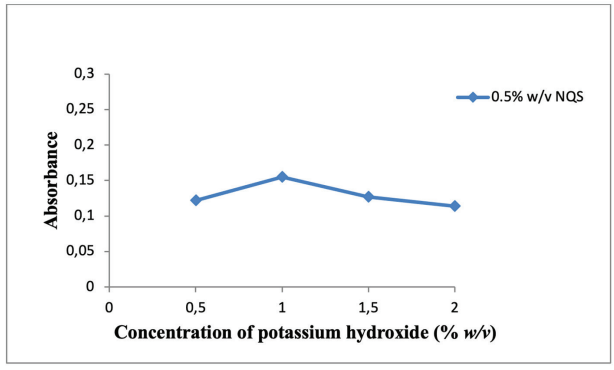

Figure 5. Effect of the concentration of potassium hydroxide and 0.3% (*w/v*) NQS, X-axis scale as - 0, 0.5, 1, 1.5, and 2, Y-axis scale as - 0, 0.05, 0.1, 0.15, 0.2, 0.25, and 0.3

NQS: 1,2-Naphthoquinone-4-sulfonic acid sodium salt

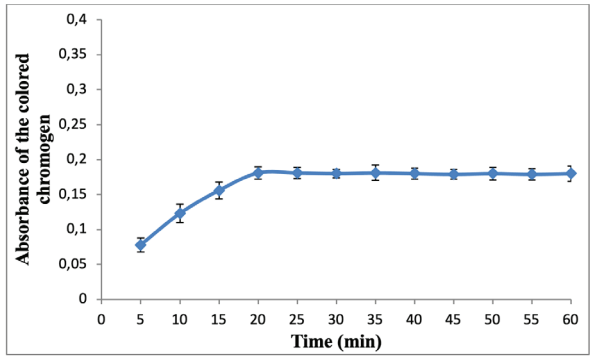

Figure 6. Effect of time on the stability of the colored complex at 474 nm

Y-axis scale as 0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, and 0.4

The exactness of the method was confirmed by spiking the standard analyte in 80, 100, and 120% concentrations to the fixed concentration of atomoxetine hydrochloride in the formulation (20 µg/mL). The details are presented in Table 1. They were found to be significant under specification limits, with percent recovery of 98.00-101.13% and the percentage RSD was found to be < 2.00 for the drug. The percentage recovery of atomoxetine hydrochloride for the three levels was found to be satisfactory.

Atomoxetine hydrochloride at a concentration of 30 µg/mL was used to determine the repeatability (intra-day precision) and intermediate precision of the method. The data obtained in the precision studies are provided in Table 2. The precision of the method was confirmed by observing a % RSD value less than 2.00 in both studies.

The sensitivity of the methodology was resolute in terms of LOD and LOQ, which were calculated as 0.2 and 0.606 µg/mL, respectively. The Sandell's sensitivity of the method was determined and calculated as 0.0553 µg/cm^2. The influence of minor dissimilarities in the concentrations of potassium hydroxide and NQS (1.0 ± 0.1 and 0.3 ± 0.1%, *w/v*, respectively) was studied to ensure the robustness of the method. The results revealed that these dissimilarities did not affect the absorbance of the formed colored complex to a large extent.

Assay of atomoxetine hydrochloride in the marketed tablets

The assay of commercial tablets was performed to determine the reliability of the proposed method. The results obtained were compared against the corresponding labeled claim. The amount of atomoxetine hydrochloride was determined as 10.152 ± 0.049 mg and the % assay was computed as 101.52%. The results denoted that the assay results agreed with the respective labeled claim and that there was no interference of excipients from the formulation at the determined wavelength (Table 3).

The details of the current and literature colorimetric and UV spectrophotometric methods developed for atomoxetine hydrochloride are provided in Table 4. The colorimetric method described by Ulu[4] used borate buffer and chloroform as solvents, and the color was developed at 70 °C after 40 min. In the current method, distilled water was used as the solvent and NQS in potassium hydroxide was used as the chromogenic reagent. The color developed at room temperature after 20 min. Thus, the temperature and time required for the analysis of atomoxetine hydrochloride were found to be minimal in this investigation. Lower LOD and LOQ values established in the current colorimetric method compared with the literature methods further evidence the high sensitivity of the method (Table 4).

CONCLUSION

This nucleophilic addition-based spectrophotometric method developed for the quantification of atomoxetine hydrochloride using NQS in potassium hydroxide was found to be a simple,

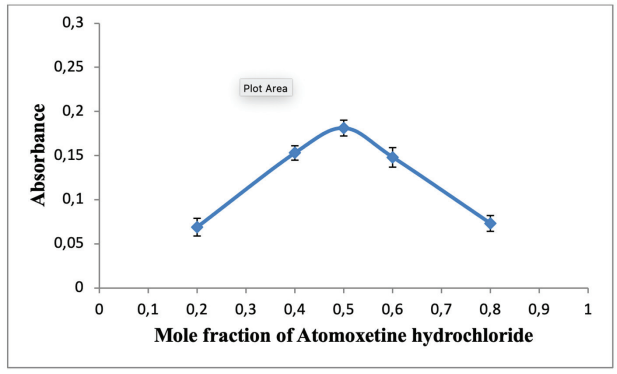

Figure 7. Job's continuous variation plot for the analytical method, X-axis scale as 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1, Y-axis scale as - 0, 0.05, 0.1, 0.15, 0.2, 0.25, and 0.3

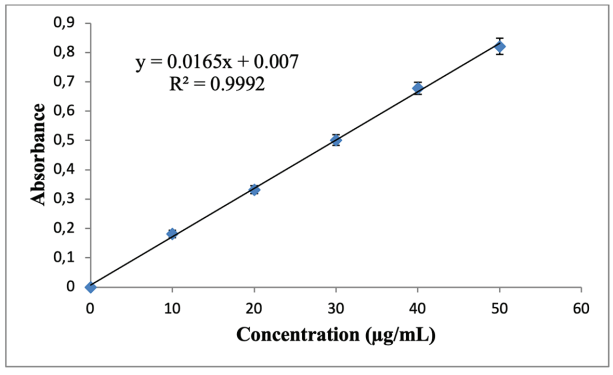

Figure 8. Calibration plot of atomoxetine hydrochloride (10-50 µg/mL) in distilled water, Y-axis scale as - 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 0.9

Table 2. Precision data of the analytical method

Statistical variables	Concentration estimated (µg/mL)[b]	
	Intra-day precision	Inter-day precision
AM ± SD	30.514 ± 0.548	30.646 ± 0.359
% RSD[a]	1.79	1.17

AM: Arithmetic mean, SD: Standard deviation, [a]Acceptance criteria: % RSD should not exceed 2.00, [b]Concentration taken: 30 µg/mL, RSD: Relative standard deviation

Table 3. Assay data of atomoxetine hydrochloride in tablets using the developed method

Formulation	Label claim (mg)	Amount found (mg) (AM ± SD, n: 3)	Bias	% Assay	% RSD*
AXEPTA-10	10	10.152 ± 0.049	0.028	101.52	0.48

AM: Arithmetic mean, SD: Standard deviation, Bias: Standard deviation/square root of sample size.
*Acceptance criteria: % RSD should not exceed 2.00

Table 4. Method conditions in the current and literature methods

S. no.	Method conditions	Validation parameters	References
1	Solvent: Borate buffer and chloroform Reagent: NQS in an alkaline medium, Temperature: 70 °C, Time for development of colour: 40 min, λ_{max}: 449 nm	Rectilinear: 5 - 40 µg/mL LOD: 0.02 µg/mL LOQ: 0.06 µg/mL	4
2	Method A: Solvent: Methanol Reagent: Vanillin in sulphuric acid λ_{max}: 560 nm	Linearity: 1 - 5 µg/mL	5
	Method B: Solvent: Methanol Reagent: *para*-Dimethyl amino benzaldehyde in sulphuric acid λ_{max}: 600 nm	Linearity: 10 - 50 µg/mL	
3	Solvent: Double distilled water, λ_{max}: 270 nm	Linearity: 20 - 180 µg/mL LOD: 4.04 µg/mL LOQ: 12.25 µg/mL	6
4	Solvent: Acetonitrile λ_{max}: 271 nm	Linearity: 20 - 140 µg/mL LOD: 3.5 µg/mL LOQ: 10.62 µg/mL	7
5	Solvent: 0.05 N Hydrochloric acid λ_{max}: 225 nm	Linearity: 5 - 40 µg/mL LOD: 0.154 µg/mL LOQ: 0.467 µg/mL	8
6	Solvent: Distilled water, Reagent: NQS in potassium hydroxide, Temperature: Room temperature, Time for development of colour: 20 min λ_{max}: 474 nm	Linearity: 10 - 50 µg/mL LOD: 0.20 µg/mL LOQ: 0.606 µg/mL	Current method

expeditious, and extraction-free strategy. The use of universal solvents, such as distilled water, makes it a non-pollutant methodology (as no organic solvent was used). Furthermore, a shorter reaction time and analysis at room temperature are more promising features of this method. The proposed analytical method was passed through validation parameters as *per* ICH specification. The % assay was congruent as stated on the label. In addition, mediation of the formulation excipients was found to be nil in the estimation. With these benefits, the suggested methodology can be adopted in routine quality control testing of atomoxetine hydrochloride in its pharmaceutical dosage forms.

Peer-review: Externally and internally peer reviewed.

Authorship Contributions

Surgical and Medical Practices: P.Y., Concept: S.N., D.P., Design: S.N., Data Collection or Processing: P.Y., S.N., Analysis or Interpretation: S.N., P.Y., Literature Search: P.Y., S.C., Writing: S.N., S.C.

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

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Supplementary Table 1. Data for the selection of solvent

S. no.	Solvent	Absorbance* (AM ± SD)
1	Distilled water	0.181 ± 0.001
2	Methanol	0.117 ± 0.003
3	Ethanol	0.148 ± 0.006
4	Acetonitrile	0.073 ± 0.004

AM: Arithmetic mean, SD: Standard deviation, *Measured using 10 µg/mL analyte solution

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