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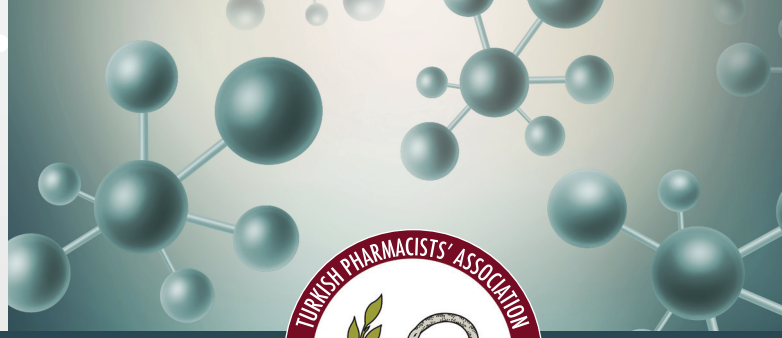

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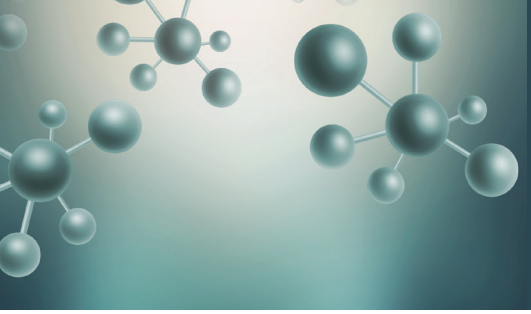

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Original Articles

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ORIGINAL ARTICLE

Gemini Curcumin Suppresses Gastric Cancer AGS Cell Proliferation Through Modulation of *lncRNA CCAT2* and *c-Myc* Genes

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ABSTRACT

Objectives: Gemini surfactant nanocurcumin (Gemini-Cur) is a novel formulation of Curcumin (Cur) with dramatic suppressive effects on cancer cells. Here, we investigated the cancer effects of Gemini-Cur in a human gastric adenocarcinoma cell-line (AGS) through the evaluation of the expression of long non-coding RNAs colon cancer-associated transcript-2 (*CCAT2*) and its downstream *c-Myc* as known oncogenic modulators of tumorigenesis.

Materials and Methods: The AGS cells were treated with Gemini-Cur and pure Cur in a time- and dose-dependent manner. The toxicity of Gemini-Cur was studied using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and scratch tests. Furthermore, real-time polymerase chain reaction and Western blotting techniques were employed to evaluate the expression of genes.

Results: Gemini-Cur significantly affected the viability of AGS cells in a dose- and time-dependent manner with inhibitory concentration 50 values of 59.32, 40.88, and 19.63 μM during 24, 48, and 72 h, respectively. Our findings showed that Gemini-Cur effectively decreased the expression levels of *lnc-CCAT2* and *c-Myc* genes. Western blotting analysis also confirmed the down-regulation of *c-Myc* in treated samples compared to controls.

Conclusion: Gemini-Cur attenuates the proliferation of AGS cells partly through modulation of the *lncCCAT2*-related pathway.

Key words: AGS cells, gastric cancer, gemini curcumin, metastasis, *lnc-CCAT2* and *c-Myc*

INTRODUCTION

The term cancer refers to a complex disease that has the main characteristic is unregulation of cell growth, aggression and spreading from the original place to the other organs of the body.[1] Gastric cancer is the fourth most common tumor malignancy worldwide and is the second cause of mortality.[2] However, the widespread of gastric cancer is higher in developing countries.[3] Colon cancer-associated transcript-2 (*CCAT2*) gene is a member of the long non-coding RNAs (*lncRNAs*), which are notably overexpressed in microsatellite-stable colorectal cancer and promote oncogenesis, metastasis, and chromosomal instability.[4,5] In the area with high expression, the number of point mutations and centromeric displacements increases dramatically.[6] The expression of this gene enhances the number of fragile chromosomes in the body,[7] on the other hand, it causes fragile X and Huntington's disease.[8] A comprehensive literature survey revealed that the *CCAT2* gene is a causative agent in infections related to fragile chromosomes.[4] The gene accession number (nr_109834.1) has a total of one exon, which is categorized in the *lncRNAs* classification.[9] Various studies also confirmed that the *CCAT2* gene is up-regulated in various cancers including ovarian, colon, gastric, liver and lung.[10,11] Guo et al.[12] realized that *CCAT2* directly enhanced the *c-Myc* expression in glioma cells. On the other hand, micro-RNA-33b prevents osteosarcoma cell invasion, proliferation and migration by targeting the *c-Myc* expression,[13] so that the

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understanding of the dynamic *lncRNAs-MYC* network is found to be more complicated.[14] On this occasion, Yan et al.[15] concluded that *CCAT2* overexpression significantly increased the LATS2 and *c-Myc* expression in osteosarcoma cells. Therefore, finding the new bioactive resources or modifying them to suppress the expression of *lnc-CCAT2* and its downstream *c-Myc* genes are of particular interest for researchers as tumor suppressor candidates.

Curcumin (Cur) is a diarylheptanoid natural product isolated from the rhizomes of *Curcuma longa* L. (Zingiberaceae).[16] Extensive studies show that Cur can effectively modulate many cancer symptoms, including anti-invasive behavior, uncontrolled cell proliferation, cancer-associated inflammation, cell death, angiogenesis, and metastasis.[17] Particularly, Cur can inhibit epithelial-to-mesenchymal transition/metastasis *via* various pathways and mechanisms in human tumors.[18] However, its weak solubility in water, metabolism and rapid excretion from the body are the main obstacles that limit the use of Cur as an anti-cancer therapeutic compound.[19] Different methods have been developed to increase the effectiveness of Cur, one of which is the employment of gemini surfactant nanoparticles (Gemini-Cur).[20] This special structure provides the advantages such as low critical micelle concentration, high solubility, and low cost for these nanoparticles, which have been considered drug carriers.[21] Therefore, Cur coating with Gemini-Cur may increase the cellular absorption of Cur and increase its anti-cancer effects. In this study, we aimed to evaluate the anticancer properties of Gemini-Cur on gastric cancer AGS cells *via* the expression of *lncRNA CCAT2* and its downstream *c-Myc*, a well-known oncogenic transcription factor linked to tumorigenesis in most types of cancers.

MATERIALS AND METHODS

Chemicals and reagents

Gemini-Cur was a kind gift from Dr. Farhood Najati. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder and RPMI-1640 medium (R5886) were purchased from GIBCO Co. (USA). Dimethyl sulfoxide (DMSO, 99.9%) and Hoechst were purchased from Merck (Germany). Fetal bovine serum (FBS), phosphate-buffered saline (PBS 1X), and trypsin (0.25% EDTA solution) were obtained from Gibco (Taiwan).

Gemini curcumin preparation

Gemini-Cur was prepared by single-step nano-precipitation method described in our previous works.[22] Briefly, 6 mg Cur and 100 mg of ethoxyl-poly urethane gemini surfactants (both of them as a gift by Dr. Farhood Najafi, Institute for Color, Science and Technology, Tehran, Iran) were dissolved in 3 mL methanol. After the evaporation of methanol in a rotary evaporator at room temperature for at least 6 h, Gemini-Cur was lyophilized and stored at 4˚C until use. Characterization of Gemini-Cur was studied according to our recent works.[21,23]

Cell culture

The human gastric cancer cell line AGS was acquired from the National Cell Bank of Iran (Pasteur Institute of Tehran, Iran). These cells were cultured in the Roswell Park Memorial Institute (RPMI 1640) medium supplemented with 10% FBS and 0.5% penicillin/streptomycin and kept in a humidified cell culture incubator containing 5% CO_2 at 37˚C.

Cell viability assay

Cytotoxicity of free Cur and Gemini-Cur on AGS cells was evaluated using the MTT assay. In brief, 1×10^4 cells were seeded in 200 µL of media in a 96 well plate for 24 h. Having sufficient density, AGS cells were treated with various concentrations ranging from 0 to 100 µM of Cur in free and nanoforms in 5% FBS-RPMI medium and incubated for 24, 48, and 72 h. At the end of incubation time, 20 µL of MTT solution (5 mg/mL) was added to each well and then incubated for 3 h. Finally, MTT containing media was replaced with 100 µL DMSO and furthermore incubated for 30 min. The absorbance was at 570 nm. The inhibitory effect on AGS cells was measured using the following formula:

$$I\% = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100$$

Where A_{blank} is the absorbance of the control reaction and A_{sample} is the absorbance of the test compound. By using the obtained inhibition percentages, cytotoxicity was expressed as inhibitory concentration 50 (IC_{50}) (the concentration causing 50% inhibition).[24]

Scratch test

The cell migration ability was examined using a scratch assay. After scratching by the yellow tip head in the middle of the monolayer, AGS cells were seeded in 6 well plates and then treated with 40.88 µM Gemini-Cur. The plates containing cells were photographed at the cleft site at zero times, immediately after scratching, and after 24, 32, and 48 h, using an Invert microscope under a 10X magnification lens.[25]

RNA extraction and real-time polymerase chain reaction (PCR)

Total RNA from cultured cells was extracted using BRIzol reagent (Fara gene, Iran) following the phenol guanidinium thiocyanate method and its manufacturer's protocol. The quality and quantity of RNAs were checked using agarose gel electrophoresis and a Picodrop spectrophometer (Termo Fischer, USA). DNase I treatment was employed to eliminate any DNA contamination and then, complementary DNA (cDNA) synthesis was accomplished by cDNA synthesis kit (Thermo Scientific, USA). The reaction was applied with SYBR Green master mix (AMPLICON, Iran) and appropriate primers. The primers were planned using Oligo7 software (Table 1). In this study, the total volume for the real-time PCR (RT-PCR) reaction reached 10 µL consisted of 5 µL of SYBR green, 1 µL of forward and reverse primers, 1 µL of cDNA template, and 3 µL of ddH_2O. Step one TM real-time PCR system quantitative PCR was performed to evaluate the expression of *lnc-CCAT2* and *c-Myc* genes. All tests were done at least twice with the accordance situation: 95˚C for 10 min, 95˚C for 15 s and 60˚C for 30 s. The

melting curve was specified as 60°C to 95°C. Relative gene expression was computed as 2^{-ddcT}.[26]

Western blotting assay

Having lysed the treated AGS cells, total protein from established cells was extracted and stored in the freezer -20°C. Based on the difference in light absorption at 630 nm wavelength in the device, the protein concentration was determined by the Bradford protein assay. Then, prepared protein samples must be concentrated before pouring into the well and mixed with the sample buffer. The electrophoresis was performed on sodium dodecylsulphate plate gel. After Western blotting or immunostaining, in which *c-Myc* proteins were detected by specific antibodies, the samples were electrically transferred from the gel to the surface of the polyvinylidene fluoride paper. The paper was mixed and diluted with the primary *β*-actin antibody (sc-47778, 1:300) and incubated. In the secondary antibody incubation phase, the paper was quenched with anti rabbit (1:1000) for all primary antibodies at room temperature. This diagnosis was used by the advanced ECL reagent kit, which includes skim milk and reagents A and B used in this study.

Statistical analysis

The difference between groups was analyzed by the student's *t*-test. Results were presented as the mean value ± standard deviation. Statistical significance was considered $p<0.05$. For the cytotoxicity test, IC_{50} was calculated using the program Curve Expert 1.3 (Cure Expert statistical software).

RESULTS

Cell viability

Cytotoxicity of free Cur and Gemini-Cur on AGS cells was analyzed by MTT assay (Figure 1). Gemini-Cur treatment for 24, 48, and 72 h demonstrated cytotoxicity on AGS cells in a time- and dose-dependent manner. However, we did not detect any significant toxicity of free Cur at similar doses. Viability of AGS cells was minimized and achieved almost 80% after treatment with 20 μM ($p= 0.0004$) of Gemini-Cur for 48 h. Interestingly, incubation of AGS cells with 20 μM of Gemini-Cur for 72 h, meaningfully decreased the cell viability and reached around 50%. Hence, IC_{50} values were calculated to be 59.32, 40.88, and 19.63 μM during 24, 48, and 72 h, respectively.

Table 1. Sequences of primers used in real-time polymerase chain reaction

Genes	Sequences (5'→3')	T_m (°C)
CCAT2	F: 5'-*CTACCAGCAGCACCATTTCAG*-3'	59.2°C
	R: 5'-*CACCAGATACACCCCAGAGAG*-3'	
c-Myc	F: 5'-*CTCGGTTTCTCTGCTCTCCTC*-3'	59.8°C
	R: 5'-*TTCCTCATCTTCTTGTTCCTCC*-3'	
β-actin	5'-*AGAGCTACGAGCTGCCTGAC*-3'	57°C
	5'-*AGCACTGTGTTGGCGTACAG*-3'	

Cell scratch test

The assessment of cell metastasis and width of the scratch were measured under an inverted microscope under a 10X lens at 0, 24, 32, and 48 h. It showed that scratches of the treated cells were wider than those of the control group at the same time (Figure 2A). This indicated that non-treated AGS cells invaded the scratched parts in a time-dependent manner. However, cells treated with 40.88 μM showed fewer cells in scratched spaces ($p<0.0001$). Considering all factors, our data show that Gemini-Cur inhibits the invasion of gastric cancer AGS cells (Figure 2B).

Lnc-CCAT2 and c-Myc expression studies

Gemini-Cur affects the expression of *lnc-CCAT2* and its subgene *c-Myc* at both the gene and protein levels. RT-PCR data showed that *CCAT2* is significantly down-regulated in treated cells rather than *c-Myc* ($p<0.01$, Figure 3). Western blotting also confirmed that *c-Myc* expression is decreased in treated cells compared with controls ($p<0.01$, Figure 4).

DISCUSSION

Gastric cancer is the second leading cause of cancer-related death.[27,28] *CCAT2* expression is significantly elevated in gastric cancer tissues compared to with adjacent non-tumoral gastric specimens.[29] The level of *CCAT2* expression is also positively correlated with the lymph node involvement and distance metastasis[30] and serves as an independent predictive factor

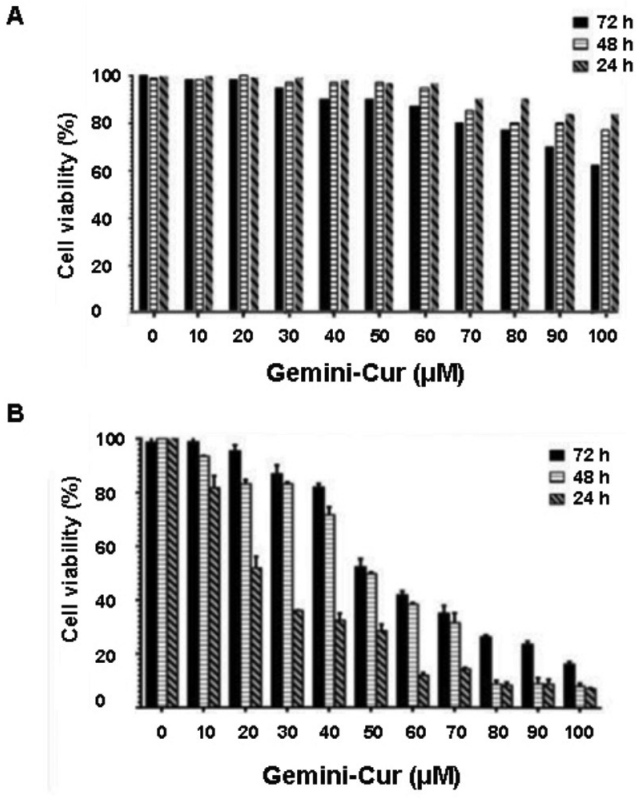

Figure 1. Cellular viability of AGS cells treated with free curcumin (A) and Gemini-Cur (B) at three-time intervals of 24, 48, and 72 h

Gemini-Cur: Gemini surfactant nanocurcumin

for shorter overall survival in gastric cancer patients.[31] These findings indicate that up-regulation of *CCAT2* is correlated with gastric cancer development and metastasis and might function as a potential prognostic biomarker for stratifying gastric cancer patients with different clinical outcomes.

It is worth mentioning that previous research indicated that pure Cur shows a significant inhibitory effect on AGS cell lines in a dose- and time-dependent manner.[32,33] Meanwhile, our results of MTT assay showed that Gemini-Cur reduces viability of the same cell lines in a dose- and time-dependent manner. These

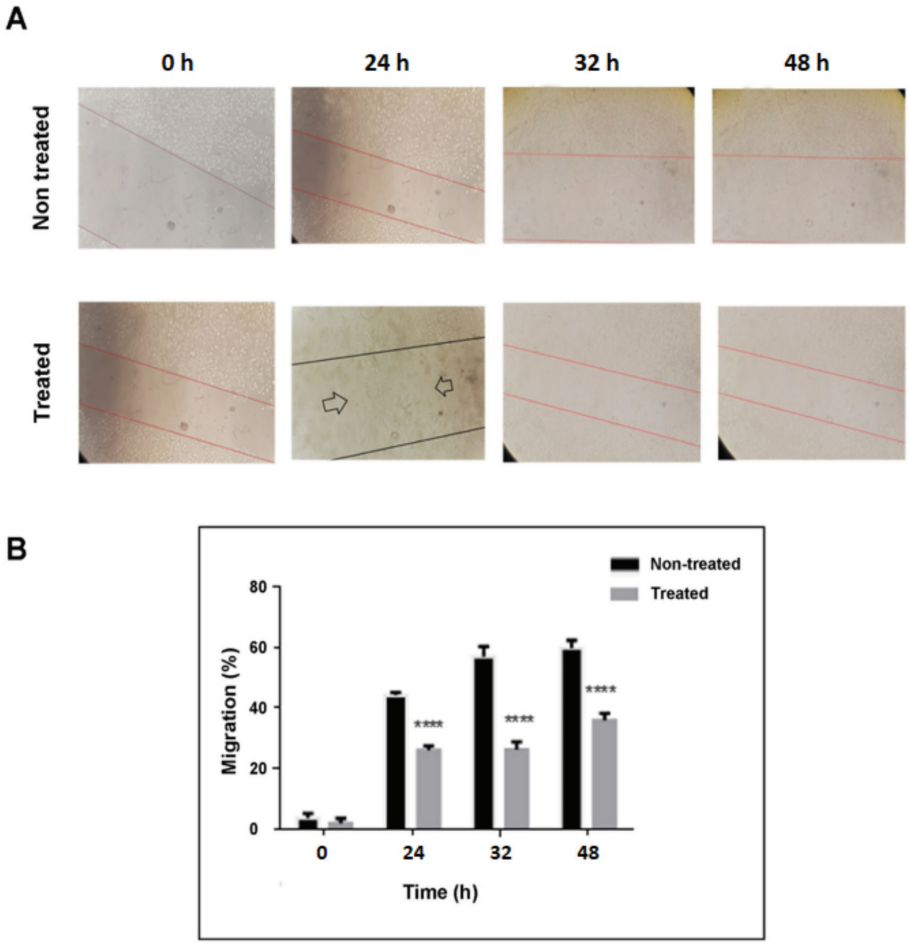

Figure 2. A) Evaluation of the effect of Gemini-Cur on the migration of treated and non-treated AGS cells using the scratch test. B) Percentage of migrated cells. Data analysis indicated a significant reduction in the number of cells in scratched spaces in treated samples compared to control

****: $p<0.0001$, Gemini-Cur: Gemini surfactant nanocurcumin

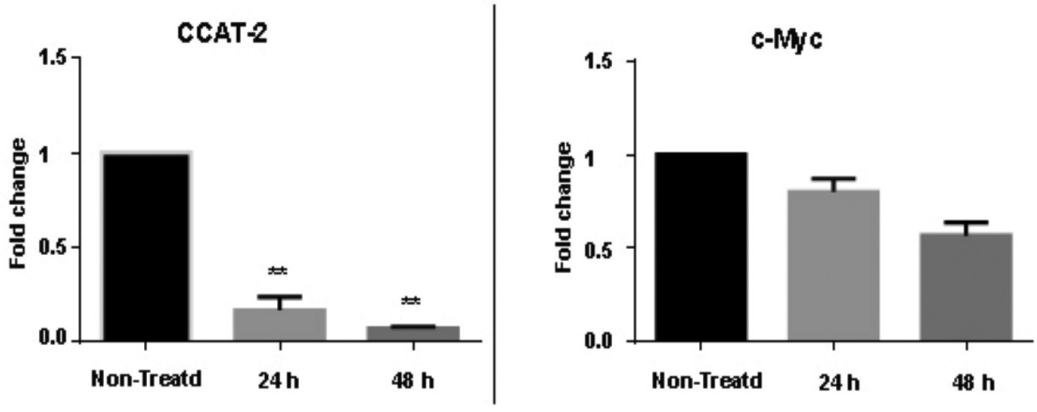

Figure 3. Evaluation of the expression of *CCAT2* and *c-Myc* in treated AGS cells compared with non-treated cells (control) in a time-dependent manner

**: $p<0.001$, *CCAT2*: Colon cancer-associated transcript-2

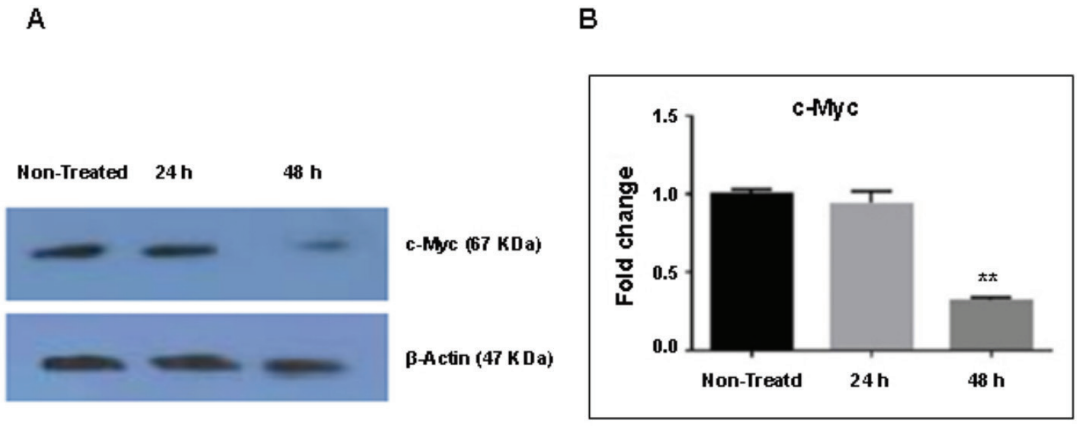

Figure 4. Western blotting data of *c-Myc* protein expression compared with non-treated cells (control). As protein bands (A) and diagram (B) show, *c-Myc* is down-regulated in treated cells after 48 hours

**: $p<0.001$

outcomes are in accordance with that of Karimpour et al.[23] and Zibaei et al.[21] studies regarding the enhancement of toxicity of Cur in the form of Gemini-Cur nanoparticles on breast and colon cancer cells.

Considering the capacity of *CCAT2* in the migration and invasion of AGS cells, which was an imperative process for metastasis, the results showed that Gemini-Cur at a concentration of 40.88 µM not only had no cytotoxic impact on normal cells but also ceased the migration of AGS cells. It effectively reduces the expression of *CCAT2* gene and its downstream *c-Myc* as well (Figure 2). Kim et al.[34] examined the expression of *CCAT2* gene in pancreatic cancer patients and healthy individuals in the American community by RT-PCR. The authors concluded that *CCAT2* expression was increased compared to control. Moreover, the *CCAT2* gene plays an important role in pancreatic cancer.[34-36] Also, Wang et al.[37,38] studies in hepatocellular carcinoma demonstrated the oncogenic role of this gene and concluded that it played a major role in cell proliferation and cancer cell migration. All the findings of this experiment were in agreement with the previous data.

In a study on the expression of *CCAT2* gene in gastric cancer and its effect on invasion and metastasis, Wang et al.[39] concluded that *CCAT2* expression increased in gastric cancer, which was directly related to invasion and metastasis. Given that cell migration is an essential process for metastasis, our results manifest that the Gemini-Cur at a concentration of 40.88 µM significantly suppresses the metastasis. It is worth mentioning that the present findings confirm that naturally occurring metabolites such as Cur interrupt the metastasis by affecting multiple pathways.[40] Therefore, the plant preparation and natural drug combinations can act as an effective therapeutic approach for tumor suppressors without having toxic side effects on healthy and common tissues.[41-43] Of note, there has been no significant reaction of treated cells at 32 and 48 h, in other words, the drug will act only dose-dependently. Xin et al.[29] examined the levels of *CCAT2* gene expression by real-time PCR in normal and ovarian cancer tissue and cells. The authors concluded this gene was more expressed in cancer tissues than normal specimens, and the higher *CCAT2* expression, the shorter the cells survived. In this study, the expression of *CCAT2* gene in the AGS gastric cancer cell line was evaluated and a significant correlation was observed with $p<0.001$. These findings indicate not only an important role of the *CCAT2* gene in gastric cancer but a direct relationship between the *CCAT2* gene expression and cell metastasis.

Study limitations

Future cancer studies especially pathway and whole genome studies, are warranted to validate the findings of this study by using clinically relevant animal models for the diverse therapeutic uses of Gemini-Cur. Besides, a comprehensive analysis of the biocompatibility and toxicity of Gemini-Cur should be conducted using appropriate cell lines *in vitro* and *in vivo*.

CONCLUSION

In this study, gastric cancer cell line AGS was treated with a nanoform of Cur (Gemini-Cur). The results of our study were in line with the former studies conducted on other cancer cell lines. To the best of our knowledge, a significant effect of Gemini-Cur on the modulation of *CCAT2* gene expression and its underlying *c-Myc* was observed, which is also associated with preventing metastasis. Scratch tests revealed a decreasing effect of metastasis in Gemini-Cur treated AGS cells indicating an anti-cancer potency of Gemini-Cur in appropriate concentration and time. This phenomenon can reduce both cell proliferation and cell migration in the AGS gastric cancer cell line. Finally, yet importantly, the further deep investigation of Gemini-Cur and its various applications are strongly recommended to strengthen the claims of being Cur-related nanoparticles as anti-cancer agents.

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Ethics

Ethics Committee Approval: The study was approved by the graduate studies committee at the University of Tabriz (3/11242/100).

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: N.J., E.B., S.H.G., Design: N.J., E.B., Data Collection or Processing: N.J., H.S., Analysis or Interpretation: N.J., B.M.H., E.B., Literature Search: N.J., H.S., B.M.H., Writing: N.J., S.H.G., E.B.

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

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ORIGINAL ARTICLE

Protective Effect of *Apis dorsata* Honey on Chronic Monosodium Glutamate-Induced Testicular Toxicity in *Mus musculus* Mice

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ABSTRACT

Objectives: This study proves the protective effect of *Apis dorsata* honey against chronic monosodium glutamate (MSG)-induced testicular toxicity on the Leydig cell necrosis count and malondialdehyde (MDA) serum level in *Mus musculus* mice.

Materials and Methods: In this study, 25 male mice were used and grouped into two large groups: The control group consisting of negative control (C-) and positive control (C+). C+ group was fed with 4 mg/g body weight (gBW) of MSG followed by distilled water. The treatment group consisted treatment 1, treatment 2, and treatment 3 groups with *A. dorsata* honey dosage 53.82 mg/20 g, 107.64 mg/20 g, 161.46 mg/20 g *per os* (*p.o.*), respectively, followed by MSG 4 mg/g BW of MSG *p.o.* For the difference analysis between the group used the one-way ANOVA test and Duncan test.

Results: The result of this study showed that there was a significant difference between the treatment group and control group ($p<0.05$) in the Leydig cell necrosis count and MDA levels. The highest Leydig cell necrosis count and MDA level were found in C+ with values 13.20 ± 2.05 cell and 37.08 ± 9.17 µmol/L compared to C-, while in the treatment group, T3 showed the lowest Leydig cell necrosis value and MDA level 4.64 ± 0.55 cell and 14.22 ± 2.01 µmol/L compared to the C+ group.

Conclusion: It can be concluded that *A. dorsata* honey could reduce the Leydig cell necrosis number and MDA level of mice (*Mus musculus*) exposed to MSG.

Key words: Reproductive health, *Apis dorsata* honey, MSG, necrosis, Leydig cells, MDA

INTRODUCTION

The development of human lifestyles in the era of globalization has led to significant changes in the needs and means of fulfilling nutrition. The fast lifestyle causes people to choose fast food as a fast and cheap alternative. Fast food is an option because of savory taste due to additive added to enhance taste, the most common additive is monosodium glutamate (MSG).[1] MSG consumption has increased every year in Indonesia from 1.53 g/*capita*/day in 1998 to 9.62 g/*capita*/day in 2011.[2] This excessive consumption behavior could damage the reproductive system due to the production of excess free radicals subsequently infertility.[3]

MSG can cause infertility due to the activation of several glutamatergic receptors such as metabotropic glutamic receptor (mGluR), ionotropic GluR, and *N*-methyl D-aspartate receptor (NMDAR). Activation of these receptors will initiate phospholipase C (PLC) signaling due to activation of G protein and increase intracellular calcium from cells.[4] Increased calcium levels will increase the production of reactive oxygen species (ROS) in the synapses of hypothalamic neurons and cause ablation. However, the ablation will disrupt the hypothalamic signalling axis - anterior pituitary - testes and interfere with the production of reproductive hormones such as follicle stimulating hormone (FSH) and interstitial cell stimulating hormone (ICSH).[3]

Leydig cell damage is also caused by excessive production of ROS in the tubules and causes cells to be in a state of oxidative stress, which is characterized by increasing

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levels of malondialdehyde (MDA) as a waste product of lipid peroxidation reactions and decreasing glutathione (GSH). The damage caused by ROS can be prevented with exogenous antioxidants because they have the ability to donor the hydrogen ions and neutralize ROS.[5] *Apis dorsata* forest honey is multiflora honey that is produced from multiple flowers and nectar. It has a more diverse bioactive antioxidant content than *Apis mellifera* honey, which is only harvested from one flower.[6] Based on the explanations above, this study proves the protective effect of *A. dorsata* honey against chronic MSG-induced testicular toxicity with the parameter of the Leydig cell necrosis count and MDA serum level in *Mus musculus* mice.

MATERIALS AND METHODS

This research is an experimental laboratory study using a completely randomized design of 25 male mice (*Mus musculus*) divided into five treatment groups using preventive doses and five replications. Mice were obtained from the Center for Veterinary Farma (PUSVETMA). The mice were then acclimatized for 7 days to minimize stress and were then given a standard feed of Hi-Pro-Vite Medicated 593 feed.

Mice were grouped into two large groups: the control group consisting of negative control (C-) and positive control (C+) and the treatment group consisting of treatment 1 (T1), treatment 2 (T2), and treatment 3 (T3). The C- was only given a placebo (aqua dest), the C+ were induced with 4 mg/g body weight (gBW) MSG and given *aqua dest* post 1 hour. The treatment group including T1, T2, and T3 was given with *A. dorsata* forest honey with dosages 53.82 mg/20 gBW, 107.64 mg/20 gBW, and 161.45 mg/20 gBW *p.o.*, respectively, and post 1 h, they were induced with MSG 4 mg/gBW *p.o.* The dosage is based on research conducted by for *A. dorsata* forest honey and for MSG doses.[7,8] All the treatments were carried out for 52 days.

At the end of the treatment, the mice were euthanized using atlanto-occipital cervical dislocation, then the tests were prepared and put in 10% formalin solution for histopathological examination with hematoxylin and eosin staining and intra-cardiac blood collection for MDA level measurement.

Histopathological was examined using a Nikon Eclipse microscope with 400x magnification to observe the number of necrotized Leydig cells. Leydig cell necrosis was counted in five visual fields and then averaged. MDA examination was carried out using serum samples and using the ELISA colorimetric method, whose levels were given units of µmol/L.

Statistical analysis

For the difference analysis between groups used the one-way ANOVA test and Duncan tests, and the data obtained were analyzed statistically by SPSS 20.00 version. To understand which groups are significant to each other, the superscripts (a, b, c, d) show the different values and different superscripts show significant differences between the groups.

RESULTS AND DISCUSSION

The average number of necrotic Leydig cells was observed on histopathological preparations using the Nikon Eclipse E-100 and calculated using a raster image application with a magnification of 400x in five fields of view. MDA levels were measured using a colorimetric method using a spectrophotometer with an absorbance of 450 nm, which was then compared with a standard curve. Generally, the results showed that there was a significant difference ($p<0.05$) between the control group and treatment groups in the Leydig cell necrosis count and MDA serum level.

In the Leydig cell necrosis count, there were significant differences between the control group and the treatment group as shown in Table 1 and in the Figure 1 showing necrotic Leydig cells marked with pyknotic. In the control group, the highest necrosis cell count was found in C+ with 13.20 ± 2.05 cells, this value is significantly different with T1, T2, T3, and C- (as shown with different superscript), meanwhile, the lowest necrosis cell count was found in C- with 2.56 ± 0.51 cells and significantly different with C+, T1, T2, and T3. In the treatment group consisting of T1, T2, and T3, the T3 group with the highest dose of *A. dorsata* forest honey had the lowest necrosis cell count of 4.64 ± 0.55 cells and is significantly different compared to another treatment group (T1, and T2) and control group (C- and C+). These results indicated that along with an increasing dose of *A. dorsata* honey given in MSG-induced testicular toxicity, there was a decrease in Leydig cell necrosis count even though T3 is still significantly different with the lowest value in C-.

MDA serum-level results are shown in Table 2. There were significant differences between the groups. In the control group, C+ was significant with C- and all treatment groups (T1, T2, and T3) but C- was only significant with C+, and T1 and not significantly different with T2 and T3. The C+ had the highest value (37.08 ± 9.17) compared to all groups and the lowest MDA value was found in C- (11.87 ± 3.81). In the treatment group consisting of T1, T2, and T3, the T3 group with the highest dose of *A. dorsata* forest honey had the lowest MDA serum level 14.22 ± 2.01, although it was not significantly different with

Table 1. The average number of necrotic Leydig cells in each group

Group	Leydig cell necrosis number (mean ± SD)
C-	2.56^{e} ± 0.51
C+	13.20^{a} ± 2.05
T1	9.84^{b} ± 0.74
T2	8.12^{c} ± 1.08
T3	4.64^{d} ± 0.55

$^{a, b, c, d}$Means within the same column with differing superscripts are significantly different ($p<0.05$). C-: Control (distilled water). C+: MSG *p.o.* 4 mg/gBW + distilled water. T1: *Apis dorsata p.o.* (53.82 mg/20 g) + MSG *p.o.* 4 mg/gBW. T2: *Apis dorsata* honey *p.o.* (107.64 mg/20 g) + MSG *p.o.* 4 mg/gBW. T3: *Apis dorsata p.o.* honey (161.46g/20 g) + MSG *p.o.* 4 mg/gBW. All treatments were carried out for 52 days. C+: Positive control, C-: Negative control, SD: Standard deviation, T: Treatment, MSG: Monosodium glutamate, gBW: Gram body weight, *p.o.*: *Per o*s

T2 17.65 ± 5.72 and compared to C- in the control group. The results also showed that the treatment group values, including T1, T2, and T3, are significantly different from C+ in the control group. These results indicated that the MDA value of each treatment group decreased with the dose of *A. dorsata* honey in the treatment group (T1, T2, and T3) and was statistically significant compared with C+ even though the lowest value of MDA was in the C- group.

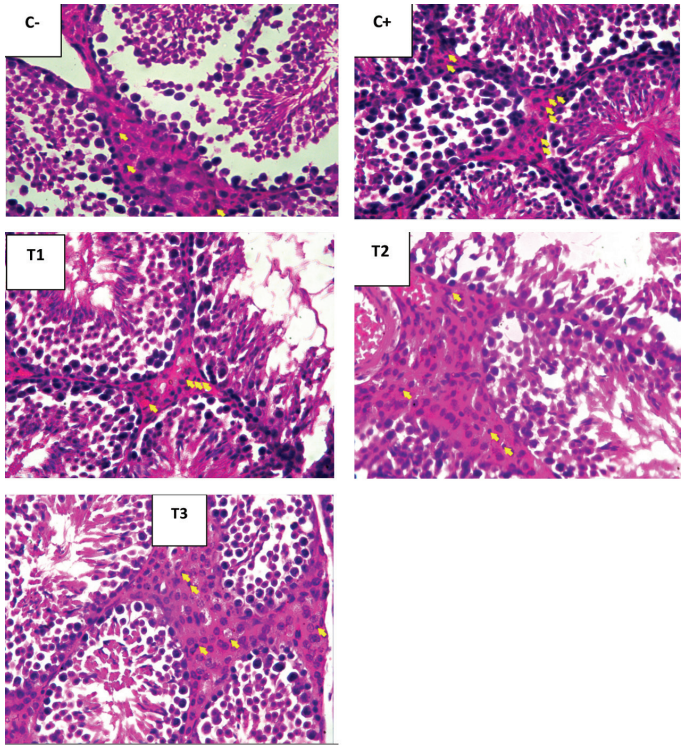

Figure 1. The testicular histopathology (HE) of mice (*Mus musculus*) given *Apis dorsata* forest honey as a preventive dose with a magnification of 400x, yellow arrows showed necrotic Leydig cells marked with pyknotic. C-: Control (distilled water). C+: MSG *p.o.* 4 mg/gBW + distilled water. T1: *Apis dorsata p.o.* (53.82 mg/20 g) + MSG *p.o.* 4 mg/gBW. T2: *Apis dorsata* honey *p.o.* (107.64 mg/20 g) + MSG *p.o.* 4 mg/gBW. T3: *Apis dorsata p.o.* honey (161.46 g/20 g) + MSG *p.o.* 4 mg/gBW. All treatments were carried out for 52 days. HE: Hematoxylin and eosin, C+: Positive control, C-: Negative control, T: Treatment, gBW: Gram body weight, *p.o.*: *Per os*

Table 2. MDA levels in serum

Group	MDA level (µmol/L) (mean ± SD)
C-	11.87^{c} ± 3.81
C+	37.08^{a} ± 9.17
T1	23.87^{b} ± 11.88
T2	17.65^{bc} ± 5.72
T3	14.22^{bc} ± 2.01

$^{a, b, c}$Means within the same column with differing superscripts are significantly different ($p<0.05$). C-: Control (distilled water). C+: MSG *p.o.* 4 mg/gBW + distilled water. T1: *Apis dorsata p.o.* (53.82 mg/20 g) + MSG *p.o.* 4 mg/gBW. T2: *Apis dorsata* honey *p.o.* (107.64 mg/20g) + MSG *p.o.* 4 mg/gBW. T3: *Apis dorsata p.o.* honey (161.46 g/20 g) + MSG *p.o.* 4 mg/gBW. All treatments were carried out for 52 days. MDA: Malondialdehyde, C+: Positive control, C-: Negative control, SD: Standard deviation, T: Treatment, gBW: Gram body weight, *p.o.*: *Per os*

Chronic consumption of MSG will increase L-glutamate levels in blood vessels, which will activate the mGluR then will increase the binding activity of D-aspartate with NMDAR.[8] Normally, in the steroidogenesis process, NMDAR is activated *via* the mitogen-activated protein kinases (MAPK) and cyclic adenosine monophosphate signaling pathways to activate the steroidogenic acute regulatory (STAR) protein complex, which actively converts cholesterol into testosterone through biosynthesis of testosterone.[9]

Chronic high L-glutamate levels in the blood will increase the influx of Ca^{2+} in the hypothalamic nerve synapses and will cause nerve cell death due to excessive excitation known as excitotoxicity.[4] This condition will cause ablation of the hypothalamic neuron cells and affect the hypothalamus-pituitary-testis axis and affect the production of ICSH directly.[3] This is evidenced by a study conducted by[10] that there was a significant decrease in ICSH levels along with the increase in the dose of MSG induction.

The disruption of the endocrine axis will cause a hypostimulation state in Leydig cells.[3] However, excessive NMDAR stimulation facilitates excessive intracellular Ca^{2+} secretion and stimulates the activation of ROS-forming enzymes such as xanthine oxidase, lipoxygenase, and NADPH oxidase. Excessive production of ROS will result in a state, where endogenous antioxidants such as GSH and superoxide dismutase are unable to keep up the production of ROS, known as oxidative stress.[11] The excessive activation will disrupt MAPK signaling pathway; so that, it will interfere with the STAR-mediated steroidogenesis process.[12]

ROS will bind to polyunsaturated fatty acid (PUFA) and initiate a lipid peroxidation event, where a chain reaction occurs which results in a radical lipid. Oxidized lipid cell membranes will produce MDA and 4-hydroxinonenal (4-NHE), which are toxic to tissues, especially reproductive tissue.[11] Increased levels of MDA were positively correlated with cell necrosis and tissue damage.[13] This statement was proved by administering MSG 4 mg/gBW in the C+, which increased the number of necrotic Leydig cells (13.20 ± 2.05) and an increase in MDA levels (37.08 ± 9.17 µmol/L) compared to the C- and the treatment groups (T1, T2, and T3).

In the treatment group, there was a decrease in the number of necrotic Leydig cells sequentially along with an increase in the preventive dose of *A. dorsata* forest honey. In the T3 group, the minimum number of necrotic Leydig cells was 4.64 ± 0.55 cells and significantly different compared to C+ 13.20 ± 2.05 cells ($p<0.05$). In the MDA level analysis using the colorimetric method, the T3 group showed the lowest MDA level of 14.22 µmol/L and was significantly different compared with the C+ group 37.08 ± 9.17 µmol/L ($p<0.05$) and not significantly different ($p>0.05$) with C- 11.87 ± 3.81 µmol/L. These results are closely related to the potential of *A. dorsata* forest honey as an antioxidant and testicular protector potential.

The content of *A. dorsata* forest honey consists of flavonoids, phenolic components, enzymatic antioxidants such as (glucose oxidase, catalase), carotenoids, amino acids, and vitamin C

(ascorbic acid).[6] Phenolic analysis of *A. dorsata* forest honey by[14] showed the highest yield of 352.73 gallic acid equivalent compared to *A. mellifera* honey at 186.70 gallic acid equivalent and *Apis cerana* at 206.33 gallic acid equivalent. *A. dorsata* forest honey also has antioxidant potential measured using DPPH radical scavenging method leading to IC_{50} of 5453.57 ppm.[15] This high antioxidant potential can overcome the formation of ROS caused by MSG.

The phenolic compounds present in *A. dorsata* forest honey play an important role in inactivation of ROS produced by excessive NMDAR activation. Anthraquinone compounds reduce ROS such as singlet oxygen, hydroxyl radical, and superoxide, make these radicals inactive and unable to bind to PUFAs thus preventing auto-oxidation.[16] The content of vitamin C in *A. dorsata* forest honey also acts as a chain-breaking antioxidant that protects PUFAs. The content of flavonoids also plays a role in chelating transition metals such as Fe (II), Fe (III), and Cu (II) that play a role in the formation of ROS.[17] In this study, giving forest honey as a preventive dose was proved to reduce the number of necrotic Leydig cells and reduce MDA levels.

However, forest honey also plays a role in preventing hypothalamic ablation caused by excitotoxicity and reducing oxidative stress that occurs in the brain due to excessive excitatory postsynaptic stimulation of neurons. Repair in the hypothalamus-pituitary-testicular axis directly normalizes ICSH production from the anterior pituitary and normalizes the function of steroidogenesis.[3] Through this mechanism, giving *A. dorsata* forest honey a preventive dose can prevent oxidative stress caused by chronic MSG consumption by reducing the number of necrotic Leydig cells and decreasing MDA levels.

CONCLUSION

This study concludes that giving *A. dorsata* forest honey as a preventive dose can reduce the Leydig cells necrotic counts and MDA levels of in mice that are chronically exposed to MSG.

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Ethics

Ethics Committee Approval: This research received ethical clearance number: 1. KE.075.08.2020 released by the Animal Care and Use Committee, Faculty of Veterinary Medicine Universitas Airlangga.

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: E.M.L., A.T.A., W.W., V.F.H., Design: E.M.L., A.T.A., W.W., V.F.H., Data Collection or Processing: E.M.L., A.T.A., W.W., V.F.H., Analysis or Interpretation: E.M.L., A.T.A., W.W., V.F.H., Literature Search: E.M.L., A.T.A., W.W., V.F.H., Writing: E.M.L., A.T.A., W.W., V.F.H.

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ORIGINAL ARTICLE

Methylphenidate Fast Dissolving Films: Development, Optimization Using Simplex Centroid Design and *In Vitro* Characterization

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ABSTRACT

Objectives: The focus of this study was to design and optimize methylphenidate hydrochloride mouth dissolving film (MDF) that can be beneficial in an acute condition of attention deficit hyperactivity disorder (ADHD) and narcolepsy.

Materials and Methods: Solvent casting method was used for the preparation of this film. Optimization of the effect of independent variables such as the number of polymers and active pharmaceutical ingredients [hydroxypropyl methyl cellulose (HPMC) E5, HPMC E15, and maltodextrin], % of drug release, disintegration time, and tensile strength of the film done using simplex centroid design. Complex formation of the film was tested using fourier-transform infrared spectroscopy and differential scanning calorimetry study. The multiple regression analysis was obtained from equations of the results that adequately describe influence of the independent variables on the selected responses. Polynomial regression analysis, contour plots, and 3-D surface plots were used to relate dependent and independent variables.

Results: Experimental results indicated that different polymer amounts had complex effects on % drug release from the film, disintegration time as well as the tensile strength of the film. The observed responses were in near alignment with expected values calculated from the developed regression equations as shown by percentage relative error. Final formulation showed more than 95% drug release within 2 min and was shown to disintegrate within a minute that had good tensile strength.

Conclusion: These findings suggest that MDF containing methylphenidate hydrochloride is likely to become a choice of methylphenidate hydrochloride preparations for treatment in ADHD and narcolepsy conditions.

Key words: HPMC, maltodextrin, mouth dissolving film, ADHD, simplex centroid design

INTRODUCTION

Oral drug administration has been most convenient and commonly recognized routes of delivery of most medicinal agents since the dawn of time. Oral drug formulations are solid and liquid preparations that are taken orally, chewed or swallowed, and travel into the gastrointestinal tract for post buccal absorption.[1] Nowadays, the most common solid oral dosage types used today are tablets and capsules, which include traditional tablets, controlled-release tablets, along with hard and soft gelatin capsules.[2,3]

One of the major problems correlated with use of these oral dosage forms is the time required for onset of action, which is at least half an hour in case of conventional dosage forms and even more in the controlled and sustained release dosage forms. Dysphagia (difficulty in swallowing) is a chronic problem in people of all ages, but it is more prevalent in the elderly and pediatric patients due to physiological differences. Uncooperative, mentally ill, and patients suffering from fatigue, vomiting, motion sickness, allergic attack or coughing are some of the other groups who have issues. This issue affects 35-50% of the population according to reports.[4,5]

These concerns created mouth-dissolving films (MDF), a new kind of solid oral dosage medium. These delivery mechanism degrades or disintegrates quickly in mouth, requiring of water

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to facilitate swallowing. Such technologies make it easier for those with swallowing problems as well as the public to take their drugs. Upon ingestion, saliva serves to rapidly disperse/dissolve the MDF. The saliva containing dissolved medicament is absorbed from mouth, pharynx, and esophagus. Because of the above-mentioned advantages, bioavailability of drugs is significantly increased than those observed from conventional dosage forms such as tablets and capsules.[2,3]

Methylphenidate hydrochloride is a psychostimulant drug. The drug is useful in the condition of attention deficit hyperactivity disorder (ADHD), a condition that requires immediate medication. By blocking dopamine delivery or carrier proteins, this drug prevents dopamine uptake in central adrenergic neurons. It also induces a heightened sympathomimetic activity in central nervous system by operating on brain stem arousal system and cerebral cortex. Methylphenidate hydrochloride is a biopharmaceutics classification system class-I (high permeability and solubility) drug and its bioavailability is only 11-52% due to its hepatic metabolism. Therefore, main objective of this work was to provide immediate release of the psychostimulant drug methylphenidate HCl for immediate action in ADHD condition in order to improve patient compliance and to avoid hepatic first-pass metabolism of the drug.[4,5]

Therefore, the current study was conducted to develop MDFs of methylphenidate hydrochloride to provide quicker onset of action in the condition of ADHD.[4]

MATERIALS AND METHODS

Methylphenidate hydrochloride was given as a gift sample from Ipca Laboratories Ltd., Mumbai, India. Different hydroxypropyl methyl cellulose (HPMC) grades were gifted from Colorcon Asia Pvt. Ltd. Goa, India. Maltodextrin was purchased from Himedia Laboratories Pvt. Ltd, Mumbai, India.

Calibration curve of methylphenidate HCl

Preparation of standard stock solutions

Methylphenidate HCl (100 mg) was weighed accurately into a 100 mL volumetric flask and dissolved with phosphate buffer pH 6.8. The volume was made up to 100 mL with the same solution to get a concentration of 1000 µg/mL (1 mg/mL).[6]

Scanning of drugs

Ultraviolet (UV) spectrum was taken of the stock solution between wavelengths of 200-400 nm. It gave a peak at 257.2 nm and the same was selected as λ_{max}. The absorption maxima of methylphenidate hydrochloride in a pH buffer of 6.8 are shown in Figure 1.[7]

Preparation of calibration curve

The stock solution was diluted with a pH buffer of 6.8 to get a concentration range of 100 to 1000 µg/mL. Absorbance of these solutions was measured against a blank at 257.2 nm using a UV visible spectrophotometer (Shimadzu Corporation, Japan) and the absorbance values are summarized in Table 1. The calibration curve, which was plotted against absorbance *versus* drug concentrations, is given in Figure 2.[8,9]

Preparation of mouth dissolving film of methylphenidate HCl

Calculation of dose of methylphenidate HCl

Methylphenidate is an effective drug against ADHD treatment with a good safety profile; evidence shows that dose optimization can improve the safety and effectiveness of treatment. Dose optimization is used widely in general medicine and psychiatry to achieve optimum therapeutic impact, thus minimizing the likelihood of adverse effects. Dose optimization is typical with virtually all psychotropic drugs and may be critical, particularly in therapeutic dose-response relationships with high individual heterogeneity, such as the use of stimulants to manage ADHD. Genetic diversity, patient's weight, age, sex, drug-induced resistance, and associations with other drugs or medical conditions are all considerations that can affect the need for dosage optimization.[10]

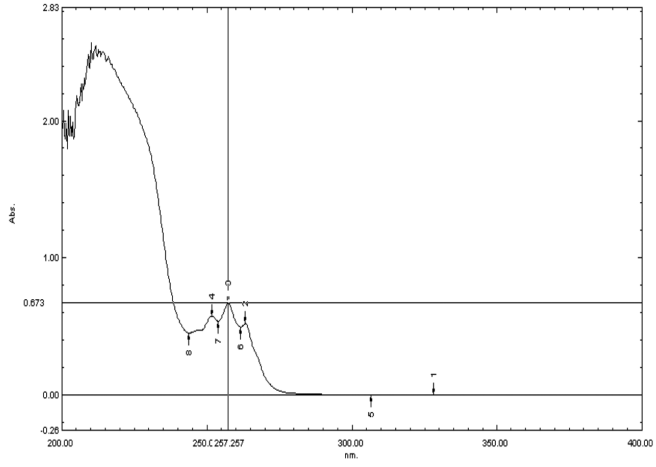

Figure 1. Absorption maxima of methylphenidate HCl in pH 6.8 phosphate buffer

Table 1. Calibration data of drug in pH 6.8 phosphate buffer at 257.2 nm

Concentration (µg/mL)	Absorbance			Mean absorbance*
	I	II	III	
0	0	0	0	0
100	0.056	0.084	0.068	0.0693 ± 0.014
200	0.119	0.140	0.131	0.13 ± 0.013
300	0.186	0.214	0.205	0.2017 ± 0.014.
400	0.251	0.289	0.271	0.2703 ± 0.019
500	0.327	0.369	0.349	0.3483 ± 0.021
600	0.402	0.443	0.414	0.4197 ± 0.021
700	0.456	0.485	0.471	0.4707 ± 0.014
800	0.544	0.559	0.552	0.5517 ± 0.017
900	0.602	0.649	0.623	0.6247 ± 0.023
1000	0.664	0.682	0.673	0.673 ± 0.014

*Results are shown in mean ± SD (n= 3), SD: Standard deviation

Dosage to be used in the film was measured using the equation below.[11]

Drug input= $C_{ss} \times K_e \times V_d$

= 133 µg L-1 × 0.3465 hr-1 × 2.7 L

= 6872.399 µg hr-1=6.87 mg

Here, C_{ss}= 133 µg L^{-1}

V_d= 2.7 L

K_e= 0.3465, where C_{ss} is the concentration at a steady state.

K_e= Elimination rate constant

V_d= Volume of distribution

Dose of methylphenidate HCl is 7.17 mg. Therefore, 7.17 mg dose of methylphenidate HCl was required in a film containing 4 cm^2 area. Total area of 9.4 cm diameter petri dish was 69.43 cm^2. So, the amount of drug present in 69.43 cm^2 of petri dish was 124.42 mg for all formulations. Therefore, the amount of methylphenidate HCl in each film (4 cm^2) was 7.17 mg.[12,13]

Preparation of film by solvent casting method

Various methods have been used for film preparation. Among the methods, the solvent casting method is the widely used method to get a good and smooth film. MDF of methylphenidate HCl was made by the solvent casting method. The aqueous solution was prepared by dissolving the chosen polymers in 25 mL purified water and allowed to rest for 1 hour to eliminate any trapped air bubbles. Then, the active pharmaceutical ingredients and plasticizer were dissolved in this polymeric solution. After that, the mixture solution was poured into a silicone petri dish and dried in a 50˚C oven for 24 hours. The film was then gently withdrawn from the petri dish and examined for flaws. The samples were wrapped in butter paper and aluminum foil and stored in a desiccator until further analysis.[14-17]

Preformulation study

Melting point

Melting point of methylphenidate HCl was measured by digital melting point apparatus. The drug sample was filled in a capillary tube and stored using a mercury thermometer in an aluminum block of the apparatus. The block was heated by two elements clamped to the sides in the apparatus and the sample tube was viewed through the magnifying lens by adjusting a dark or bright background. Temperature was recorded at which the sample started to melt and the point, at which it was completely melted.[18,19]

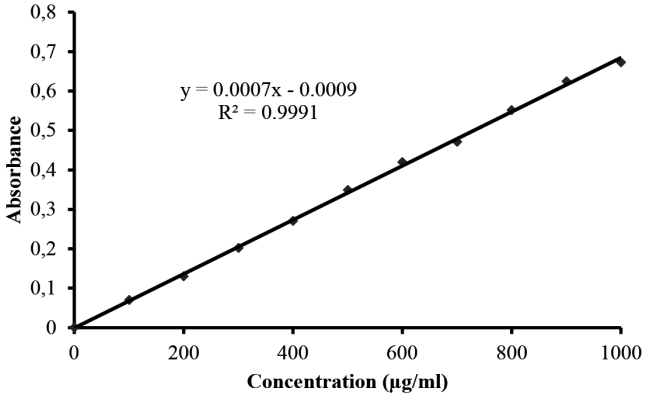

Figure 2. Standard curve of methylphenidate HCl in phosphate buffer (pH 6.8)

Partition coefficient

Methylphenidate is soluble in alcohol, ethyl acetate, and ether. Hence, ether is chosen for determination of partition coefficient. For this purpose, ether and water were saturated with each other for the period of 24 h in a 500 mL volumetric flask. In a 100 mL volumetric flask, 10% (w/v) of the drug was transferred to mixture of the above-saturated solution and stirred for 24 hours at room temperature on a rotary shaker. After 24 hours of equilibrium, the system was centrifuged for 15 minutes at 3000 rpm for 15 minutes. Concentration of methylphenidate HCl in ether and water was analyzed by a UV-visible spectrophotometer at 257.2 nm after appropriate dilution with methanol. Partition coefficient was determined using the equation below. The experiment was replicated thrice.[19]

$$\text{Partition coefficients} = \frac{\text{Concentration of drug in ether}}{\text{Concentration of drug in water}}$$

Optimization of mouth dissolving film components

The placebo films were made using polymers like maltodextrin, HPMC E3, HPMC E5, and HPMC E15 by solvent-casting method. Polymers were selected from the abovementioned placebo film by an appearance *via* visual inspection and disintegration time. An identical approach was used to optimize plasticizers (glycerin, propylene glycol) using the previously optimized concentration of respective components. The plasticizer was optimized based on film tensile strength, folding endurance, and disintegration time.[20,21]

Statistical analysis

Statistical analysis has been performed using simplex centroid design.

Simplex centroid design

The use of simplex centroid experimental designs in pharmaceutical research is well known. They are especially useful in formulation optimization procedures, where the overall number of ingredients being considered must remain constant. In the films, the total amount of polymer, if changed, can lead to a large extent change in the mechanical properties of the film, so, simplex centroid is the appropriate design to be applied to the film formulation. The values of dependent and independent variables can be used to develop a polynomial first-order linear interactive model.

$Y= B_1X_1 + B_2X_2 + B_3X_3 + B_{12}X_1X_2 + B_{23}X_2X_3 + B_{13}X_1X_3 + B_{123}X_1X_2X_3$

where Y is the response parameter and Bi are the projected coefficients for factor X_i. The main effects (X_1, X_2, and X_3) represent average results of changing one factor from its low to high value at a time. The interaction terms (X_1X_2, X_2X_3, X_1X_3,

$X_1X_2X_3$) show how the response changes when two or more factors are changed simultaneously (Tables 2, 3).[22-24]

Other common ingredients used for each formulation

Other ingredients used include propylene glycol, 0.5 mL, as a plasticizer, and brilliant blue as color. Glycerin was used to the lubrication the petri dish to facilitate smoother peeling of the film.

Evaluation parameters for prepared films

Scanning of methylphenidate HCl in UV spectrophotometer

Scanning of methylphenidate HCl has been performed.[25] A UV spectrum was run between the wavelengths 200-400 nm and is described in Figure 1.

Calibration curve of methylphenidate HCl

Methylphenidate HCl (100 mg) was weighed accurately into a 100 mL volumetric flask and dissolved with phosphate buffer pH 6.8. The volume was made up to 100 mL with the same solution to get a concentration of 1000 µg/mL. From this, solutions of concentrations ranging from 100 µg/mL to 1000 µg/mL were prepared and their absorbance was measured at 257.2 nm wavelength in a UV spectrophotometer.[25,26]

Thickness measurement

A screw gauge was used to measure the thickness of the MDF (2 × 2 cm^2). Each film's thickness was measured in three locations and the standard deviation (SD) was estimated.[27]

Drug content uniformity

A 4 cm^2 MDF was cut into small pieces and placed in a graduated glass-stoppered flask with 10 mL of 6.8 pH phosphate buffer. The flask was kept for 24 hrs. The solution from the flask was filtered through Whatman filter paper and the amount of drug present was determined by UV spectrophotometric method at 257.2 nm wavelength.[28]

Table 2. Independent variables and their respective levels

Independent variables	0	0.33	0.5	1
HPMC E5 (X1)	217	250	267	317
HPMC E15 (X2)	150	183	200	250
Maltodextrin (X3)	300	333	350	400

HPMC: Hydroxypropyl methyl cellulose

Weight variation

Three films of size (2 × 2 cm^2) from every batch of MDF were weighed on an electronic balance (Citizen CY 220C, Mumbai, India) and the average weight with SD was calculated.[29,30]

Tensile strength

Tensile strength was used to precisely calculate the mechanical properties of polymeric MDF. Using a handcrafted tensile strength instrument, the tensile strength of the MDF was measured. MDF was then applied to the assembly and the weights needed to split was measured. The following formula was used to measure tensile strength (formula 1).[31,32]

$$\text{T.S.= Break force/A} \quad (1)$$

where A= Cross-sectional area of the film

Percentage elongation

After calculating tensile strength of the film, percentage elongation was determined using the formula below (formula 2).[32]

$$\text{Percentage elongation} = \frac{(L_F - L_O)}{L_O} X\ 100 \quad (2)$$

Here, L_F = final length, L_O= initial length

Moisture content (%)

This measure was also used to determine the film's credibility in dry weather. A film with a surface area of 4 cm^2 was cut-out, weighed, and placed in a desiccator containing fused anhydrous calcium chloride. The films were removed and re-weighed after 24 hours. Formula 3 was used to calculate the percentage moisture content of the film.[33,34]

$$\%\ \text{Moisture content} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100 \quad (3)$$

% Moisture uptake

The formulation was exposed to an atmosphere of 84% RH at 28°C for three days using a saturated solution of NaCl. After three days the films were removed, weighed and the percentage moisture absorbed was calculated. Calculated the

Table 3. Simplex centroid design

Formulations*	Coded values			Actual values (mg)		
	X_1	X_2	X_3	X_1	X_2	X_3
F1	1	0	0	317	150	300
F2	0	1	0	217	250	300
F3	0	0	1	217	150	400
F4	0.5	0.5	0	267	200	300
F5	0.5	0	0.5	267	150	350
F6	0	0.5	0.5	217	200	350
F7	0.33	0.33	0.33	250	183	333

average percentage moisture absorption of each film using the following formula 4.[34]

$$\% \text{ Moisture uptake} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100 \quad (4)$$

In vitro disintegration time

The test was carried out using a slightly modified version of the procedure described by Mishra and Amin[20]. A glass petri dish containing 10 mL of distilled water was used to hold the film size needed for dosage distribution (2 × 2 cm). Time that took to break the film was recorded as the *in vitro* disintegration time.[20,35]

Solubility study

The solubility of methylphenidate hydrochloride was determined in different types of solvent like water, methanol, ethanol, 0.1 N HCl, chloroform, ethyl acetate, acetone, and pH 6.8 phosphate buffer at room temperature. Saturated solutions were prepared by adding excess drug into the solvents to form a suspension and continued stirring for 24 h in the presence of drug particles. The saturated suspensions were filtered (using 0.2 µm PTFE filters) to remove drug particles and the clear solutions were diluted to measure the drug concentration (Table 4).

In vitro dissolution study

The test was performed with a slight modification using the same method as mentioned by Dinge and Nagarsenker[38] A film of 4 cm^2 was placed in a glass petri dish and 25 mL of dissolution medium (phosphate-buffered saline pH 6.8) was added. A stirring speed of 100 rpm was selected for the dissolution of the batches. An aliquot of 2.5 mL was withdrawn and replaced with equal volumes of pH buffer 6.8 at regular intervals of 1, 2, 3, 4, 5, 7.5, and 10 minutes to maintain sink condition. The collected samples were filtered through the Whatman filter and using a UV-visible spectrophotometer, the concentration of dissolved methylphenidate HCl was measured at the required wavelength.[36-38]

Folding endurance

Folding endurance was observed and determined by repeated folding of strip at the same place until strip broke due to folding. The number of times the film was folded without breaking was determined as the folding endurance value.[39,40]

Table 4. Solubility data of methylphenidate HCl

Solvent	Solubility (mg/mL)
Water	>100
Methanol	>100
Ethanol	>25
0.1 N HCl	>100
Chloroform	>100
Ethyl acetate	0.08
Acetone	0.9
Phosphate buffer pH 6.8	>100

Stability study

Stability testing's goal was to show how the consistency of a drug ingredient or drug product changes over time, when exposed to a range of environmental factors including temperature, humidity, and light, allowing for recommended storage conditions, retest times, and shelf-life. International Conference on Harmonization (ICH) specifies the length of study and storage conditions.[41-43]

Method

The sample was wrapped in aluminum foil and subjected to stability studies as *per* the ICH guidelines. After that, they were held in a stability chamber at 40°C/75°F for 3 months and tested for their physical appearance, drug quality, *in vitro* disintegration duration, and drug release at 1 month intervals with the findings being released.[41,43,44]

Release kinetics and mechanisms

Data obtained from dissolution studies were fitted to various kinetic equations. The kinetic models used were zero order (cumulative percentage of drug unreleased *vs.* time in min), the first order (log cumulative percentage of drug remaining *vs.* time), Hixon-Crowell model (M01/3-M1/3 *vs.* time in min), Higuchi's model (cumulative percentage of drug released *vs.* square root of time), and Korsmeyer-Peppas model (log cumulative percentage of drug released *vs.* log time) equation. These data are used to find R^2 value.

RESULTS AND DISCUSSION

λ_{max} of the drug was determined by scanning 1000 µg/mL concentration solution prepared with pH 6.8 buffer in range 200-400 nm using a double beam UV-visible spectrophotometer. λ_{max} was found to be 257.257 nm (Figure 1). Therefore, further studies were conducted in a wavelenght of 257.2 nm.

Fourier-transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC) studies

An FTIR spectrophotometer was used to conduct the compatibility tests. A KBr disc was used to investigate IR spectrum of a pure substance and a physical combination of drug and polymer.[45,46] In different samples, the distinctive peaks of methylphenidate hydrochloride were obtained at different wavenumbers (Figure 3, Table 5)

The spectra for all formulations are shown below.

In the above spectrum, the characteristic (principal) peaks of methylphenidate hydrochloride are presented as follows.

FTIR spectra of methylphenidate hydrochloride+ HPMC E5 (Figure 4) exhibited peaks at 711 cm^{-1} (monosubstituted benzene), 1593 cm^{-1} in the presence of (aromatic stretch), 2411-2681 cm^{-1} (secondary amine salt), 1756 cm^{-1} (C=O stretch), 1182-1201 cm^{-1} (C-O stretch). Here, all the principal peaks are exhibited in the range. FTIR spectra of methylphenidate hydrochloride+ HPMC E15 (Figure 5) exhibited peaks at 699 cm^{-1} (monosubstituted benzene), 1592 cm^{-1} presence of (aromatic stretch), 2411-2588 cm^{-1} (secondary amine salt), 1745 cm^{-1} (C=O stretch), 1110-1210 cm^{-1} (C-O stretch). Here, all the principal peaks

are exhibited in the range. FTIR spectra of methylphenidate hydrochloride+ maltodextrin (Figure 6) exhibited peaks at 701-721 cm^{-1} (monosubstituted benzene), 1592 cm^{-1} presence of (aromatic stretch), 2419-2633 cm^{-1} (secondary amine salt), 1734 cm^{-1} (C=O stretch), 1115-1145 cm^{-1} (C-O stretch). Here, all the principal peaks are exhibited in the range. FTIR spectra of MDF formulation (Figure 7) exhibited peaks at 713 cm^{-1} (monosubstituted benzene), 1595 cm^{-1} presence of (aromatic stretch), 2398-2511 cm^{-1} (secondary amine salt), 1731 cm^{-1} (C=O stretch), 1141-1190 cm^{-1} (C-O stretch). Here, all the principal peaks exhibit in the range.

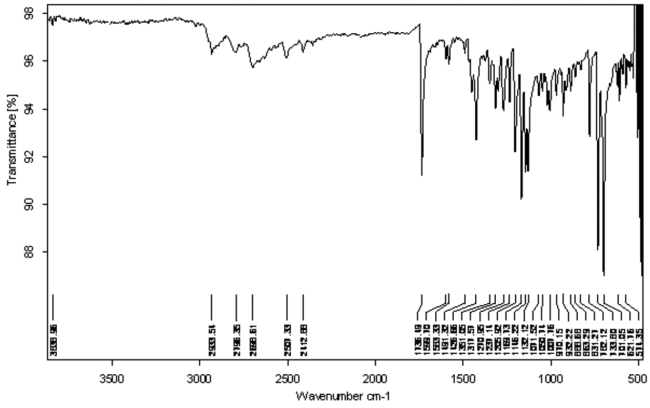

Figure 3. FTIR spectrum of pure methylphenidate hydrochloride

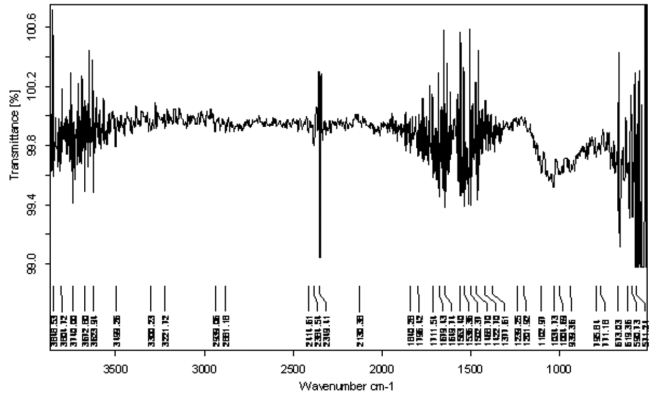

Figure 4. FTIR spectrum of methylphenidate HCl + HPMC E5

Table 5. FTIR characteristic (principal) spectral details

Pure methylphenidate hydrochloride	Stretching
701, 733	Monosubstituted benzene
1599	Aromatic stretch
2412-2698	Secondary amine salt
1736	C=O stretch
1146-1169	C-O stretch

FTIR: Fourier-transform infrared spectroscopy

In the spectrum of the drug-polymer mixture, all the peaks are present and in the formulation. This indicates that there is no interaction between the drug and the formulation components.

DSC

DSC thermogram of methylphenidate hydrochloride showed an endothermic peak at 229.41°C corresponding to its melting

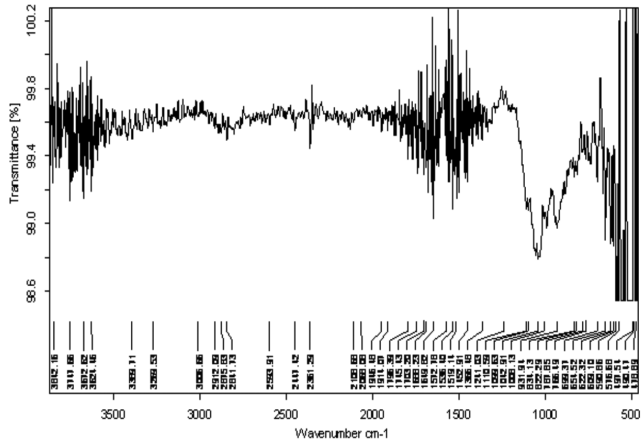

Figure 5. FTIR spectrum of methylphenidate HCl + HPMC E15

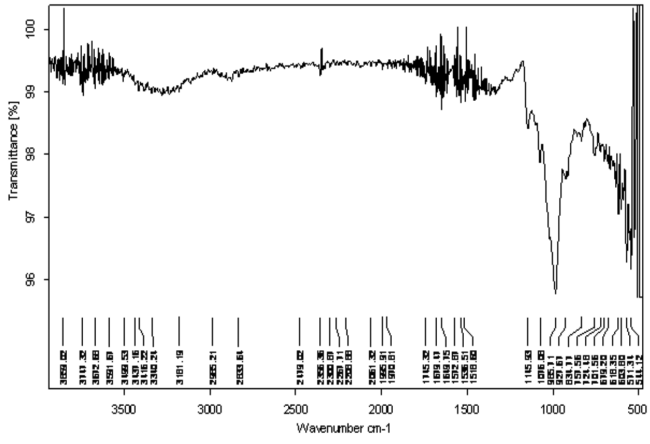

Figure 6. FTIR spectrum of methylphenidate HCl + maltodextrin

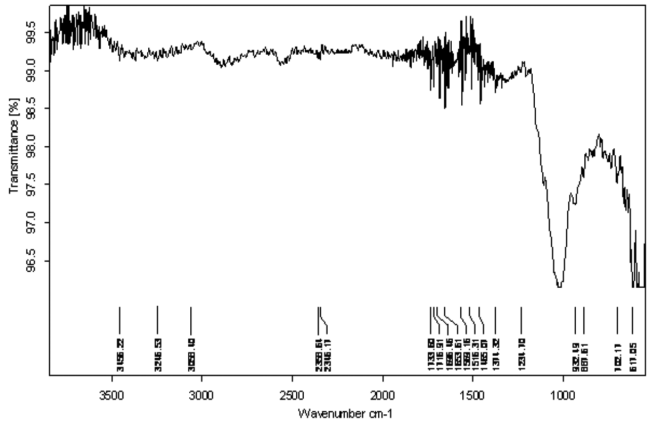

Figure 7. FTIR of mouth dissolving film formulation

point.[38] DSC thermograms of the drug with other excipients do not display a profound shift in peaks (229.41°C), which indicates compatibility. DSC thermograms of the individual drug and final formulation are shown in Figure 8 and 9.[47]

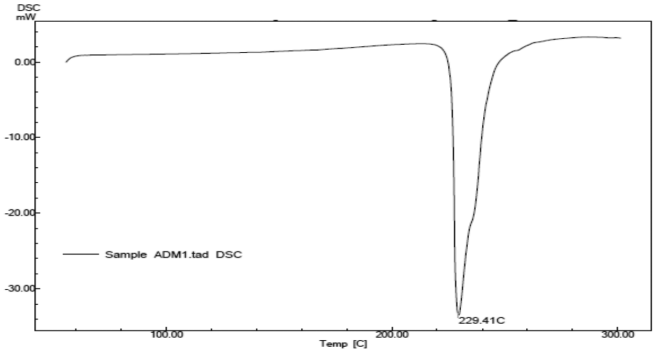

Figure 8. DSC of pure drug

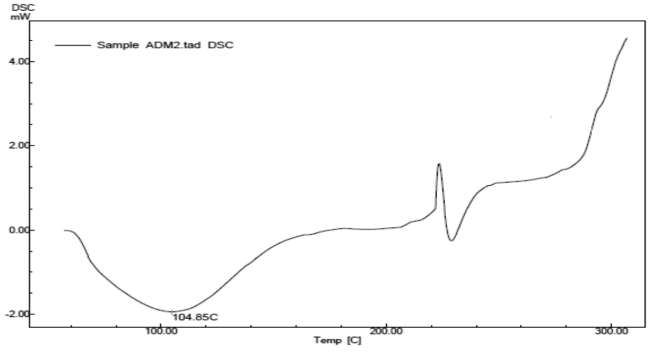

Figure 9. DSC of formulation

Preliminary studies on the selection of polymers

A preliminary research was conducted to identify appropriate polymers and a suitable plasticizer capable of manufacturing films with favorable mechanical properties and disintegration times.[48] The solvent casting process was used to make the casting solution. The composition of various batches, number of polymers used, and their appearance and disintegration time are given in Table 6.

Optimization of polymer

Placebo films were prepared using maltodextrin, HPMC E3, HPMC E5, and HPMC E15 as film-forming agents in various amounts. The placebo films prepared using maltodextrin as a film former in various amounts of 750, 1000, 1250, and 1500 mg were not having acceptable physical characteristics. The lowest amount of maltodextrin (PB1), when cast in the plastic petri dish having an area of 70 cm^2, was insufficient for making the film. In other batches of maltodextrin (PB2 to PB4), amounts were sufficient for making the film, which was sticky. Thus, maltodextrin alone was not selected as the film-forming polymer.

HPMC is a hydrophilic polymer that is suitable for the MDF. Various grades of HPMC could make films that were very transparent and had excellent mechanical properties. Placebo films of different grades of HPMC E3, HPMC E5, and HPMC E15 were prepared to verify their film-forming capacity and suitability for MDF. From all HPMC batches, PB7 for HPMC E3, PB9 for HPMC E5, and PB11 for HPMC E15 were easily removed from the petri dish and had good acceptable physical characteristics and low disintegration time in accordance with other batches (Table 6).

Films prepared from single polymers (PB7, PB9, PB11) gave good results for disintegration time, but other properties were not so good, so, combinations of different grades of HPMC were

Table 6. Characteristics of placebo film prepared using different polymers

Batch	Polymer	Amount (mg)	Remarks	Disintegration time* (sec)
PB1	Maltodextrine	750	Insufficient	-
PB2		1000	Sticky	-
PB3		1250	Sticky	-
PB4		1500	Very sticky	-
PB5	HPMC E3	500	Insufficient	-
PB6		750	Good	32 ± 1.732
PB7		1000	Very good	44.67 ± 1.527
PB8	HPMC E5	500	Average	38.67 ± 2.081
PB9		750	Very good	42.67 ± 0.577
PB10		1000	Good	51.67 ± 2.081
PB11	HPMC E15	500	Very good	36.67 ± 1.527
PB12		750	Good	56.33 ± 1.527
PB13		1000	Average	66 ± 2.645

*Results are shown in mean ± SD (n= 3), SD: Standard deviation, HPMC: Hydroxypropyl methyl cellulose

taken, which exerted better results in terms of disintegration time, folding endurance, and tensile strength.

A combination of different grades of HPMC and maltodextrin was tried and as a result, films having a much smoother texture were obtained. The combination yielded smoother films with less disintegration time, and finally, among the preliminary batches, PB22 was shown to give the best results (Table 7). Therefore, a combination of HPMC E5, HPMC E15, and maltodextrin was selected as the film-forming combination for the current work.[49,50]

Optimization of plasticizer

The films were prepared using propylene glycol and glycerol as plasticizers in different amounts ranging from 0.25 to 1.25 mL (Table 8). The results indicated that, with the least amount of plasticizer, films were very brittle and with the highest amount of plasticizer, films could not be dried properly and peeling off the problem was observed. Amongst the prepared films, PB24, PB25, PB30, and PB31 were good but their disintegration time was much higher than PB29 because of more amount of plasticizer. Based on folding endurance, tensile strength, and disintegration time, 0.5 mL of propylene glycol was selected as the optimum amount of plasticizer.[50,51]

Statistical analysis

Simplex centroid design is a type of mixture design that is often used to modify formulation variables with the simple prerequisite of knowing how independent variables interact. Preliminary investigations of the process parameters revealed that factors such the amount of HPMC E5 (X_1), amount of HPMC E15 (X_2), and amount of maltodextrin (X_3) showed a significant influence on the amount of drug dissolved in 2 min (CPR Q_2; R_1), disintegration time (R_2) and tensile strength (R_3) of the drug-loaded fast dissolving film. As a result, it was used in further research. All three chosen dependent variables (X_1, X_2, and X_3) showed large variance in disintegration time, volume of drug released in 2 minutes, and tensile strength for all 7 batches (Table 9). The data showed that X_1, X_2, and X_3 had a major effect on those responses (R_1, R_2, and R_3). Since considering the magnitude of coefficients and statistical signals, polynomial equations can be used to determine, whether the response is positive or negative. The statistical analysis (ANOVA) results for the design batches are shown below.[46,52]

Table 7. Optimization of mixture of polymers

PB14	E3 + E5	500 + 375	Good	56.33 ± 0.577
PB15	E3 + E15	500 + 250	Good	57.33 ± 1.527
PB16	E5 + E15	375 + 250	Good	50.33 ± 1.154
PB17	E3 + maltodextrin	500 + 500	Good and smooth	43.67 ± 1.154
PB18	E5 + maltodextrin	375 + 500		41.33 ± 0.577
PB19	E15 + maltodextrin	250 + 500		35.33 ± 0.577
PB20	E3 + E5 + maltodextrin	333 + 250 + 333	Very good and smooth	42.67 ± 2.081
PB21	E3 + E15 + maltodextrin	333 + 166 + 333		39.33 ± 1.527
PB22	E5+E15 + maltodextrin	250 + 166 + 333		34.67 ± 1.154

*Results are shown in mean ± SD (n= 3), SD: Standard deviation

Table 8. Characteristics of placebo films prepared using different plasticizer

Batch#	Plasticizer	Amount (mL)	Folding endurance	Disintegration time* (sec)	Tensile strength* (n/cm^2)
PB23	Glycerin	0.25	142	Brittle	-
PB24		0.5	156	66.33 ± 2.081	3.11 ± 0.061
PB25		0.75	-	74.66 ± 4.167	3.18 ± 0.017
PB26		1	-	Peel off problem	-
PB27		1.25	-	Peel off problem	-
PB28	Propylene glycol	0.25	-	Brittle	-
PB29		0.5	148	46 ± 1.73	2.42 ± 0.023
PB30		0.75	152	59.66 ± 3.055	2.74 ± 0.068
PB31		1	156	64.33 ± 2.516	2.96 ± 0.066
PB32		1.25	-	Peel off problem	-

#,$Each formulation contains HPMC E5, HPMC E15 and maltodextrin (250 + 166 + 333), *Results are shown in mean ± SD (n= 3). SD: Standard deviation, HPMC: Hydroxypropyl methyl cellulose

Response 1: CPR Q_2 (R_1)

The magnitude of coefficients and mathematical signs can be used to determine whether the polynomial equations express positive or negative information. Statistical analysis was carried out in Design-Expert software (7.1.5), which suggested that a special cubic model (SCM) was followed for drug release % in 2 minutes with a *p* value of 0.0385. This indicated that the model was highly significant.

Polynomial equation

R_1 (CPR Q_2)= +104.21*A + 86.83*B + 94.30*C - 9.16*A*B + 8.62*A*C + 23.53*B*C + 55.72*A*B*C

To determine contribution of each component and their interaction, an ANOVA for SCM was carried out.

The statistical analysis (ANOVA) results (Table 10), contour plot, and 3D surface plot for cumulative percentage release (CPR), Q_2 (Figure 10) presents a strong effect of three factors (amounts of HPMC E5, HPMC E15, and maltodextrin). A polynomial equation of Q_2 indicates that three polymer amounts have a positive effect on the Q_2. *In vitro* dissolution of the films increased with the increase in amount of the polymer. It was noted that, when the amounts of polymer were selected within the limits of the design, *in vitro* dissolution rate increased to a greater extent with the amount of HPMC E5 and increased to a lesser extent in the case of maltodextrin followed by HPMC E15. As *per* the equation, better release can be achieved with the combination of the three polymers, rather than combining any two of them.[53]

Response 2: Disintegration time (R_2)

Statistical analysis was carried out in Design-Expert software (7.1.5), which recommended that a SCM was followed for release at $T2_{min}$ with a *p* value of 0.0385. This indicated that the model was highly significant.[53]

Polynomial equation

R_2 (disintegration time)= +38.50*A + 78.00*B + 35.00*C - 25.00*A*B + 37.00*A*C + 26.00*B*C -235.50*A*B*C

To find the contribution of each component and their interaction, an ANOVA for SCM was carried out.

ANOVA results (Table 11), contour plot, and 3D surface plot for the disintegration time (Figure 11) indicates the strong effect of the three factors (amounts of HPMC E5, HPMC E15, and maltodextrin). A polynomial equation of disintegration time indicates that the three polymers amounts have a positive effect on the disintegration time. *In vitro* disintegration time of the films was observed to increase as the volume of polymer was increased. It was noticed that, when the amounts of polymer

Table 9. Design summary

Formulation code	R_1	R_2	R_3
	$Q_{2\ min}$*	Disintegration time (sec)*	Tensile strength (n/cm^2)*
F1	104.44 ± 2.91	38 ± 0.57	2.7 ± 0.02
F2	97.08 ± 2.89	78 ± 1.15	3.43 ± 0.06
F3	99.80 ± 0.80	35 ± 2.01	2.39 ± 0.03
F4	98.12 ± 1.62	52 ± 2.64	3.1 ± 0.07
F5	101.41 ± 1.89	46 ± 1.73	2.52 ± 0.01
F6	98.86 ± 3.18	63 ± 2.31	2.94 ± 0.04
F7	99.73 ± 1.78	46 ± 2.64	2.84 ± 0.02
F1 (R)	103.94 ± 0.27	39 ± 1.52	2.72 ± 0.02

*Results are shown in mean ± SD (n= 3), SD: Standard deviation, R_1: Response 1, R_2: Response 2, R_3: Response 3

Table 10. ANOVA for special cubic model (% release at 2 min)

Source	Sum of squares	DF	Mean square	F value	Prob > F
Model	253.82	6	42.30	395.44	0.0385
Linear mixture	210.05	2	105.02	981.76	0.0226
AB	3.81	1	3.81	35.62	0.1057
AC	3.38	1	3.38	31.60	0.1121
BC	23.06	1	23.06	215.56	0.0433
ABC	2.62	1	2.62	24.52	0.1269
Pure error	0.11	1	0.11	-	-
Cor total	253.92	7	-	-	-

DF: Degree of freedom

were selected within the limits of the design, *in vitro* dissolution rate decreased the most, when more amounts of maltodextrin were used in the formulation, which increased gradually with HPMC E5 followed by HPMC E15. As *per* the equation, a shorter disintegration time can be achieved with the combination of the three polymers, rather than the single polymer or with the combination of any two of them.

Response 3: Tensile strength (R_3)

Statistical analysis was carried out in Design-Expert software (7.1.5), which suggested that SCM was followed for release at $T2_{min}$ with a *p* value of 0.0385. It revealed that the model was highly significant.

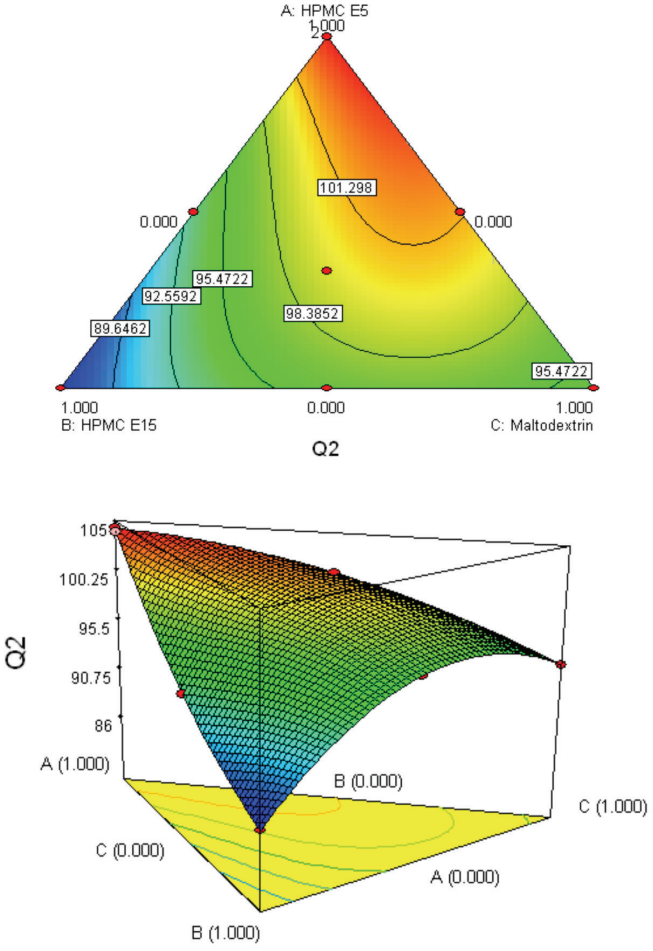

Figure 10. Contour plot and 3D surface plot of CPR Q_2 (%) against amounts of HPMC E5, HPMC E15, and maltodextrin

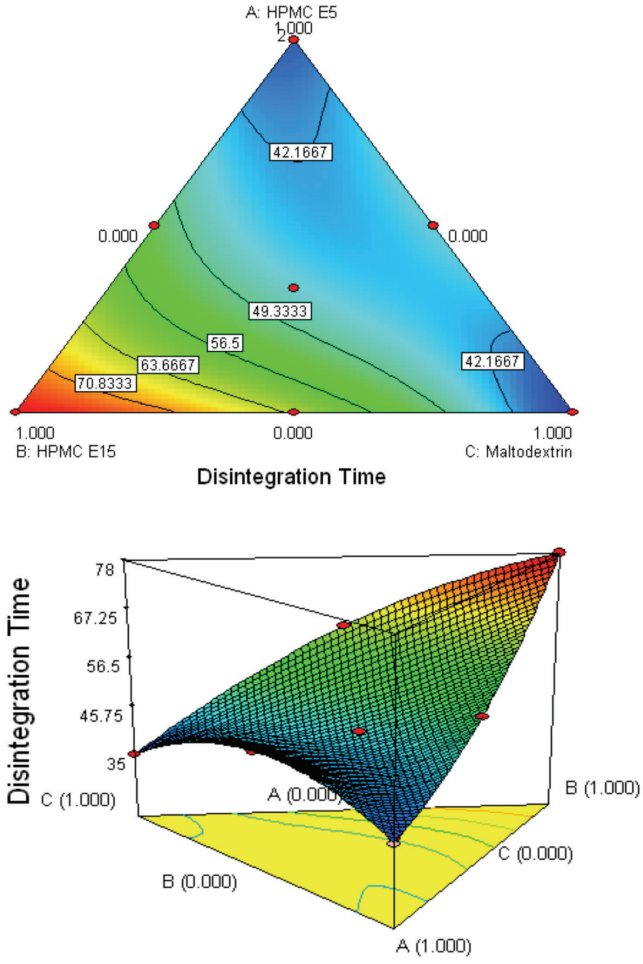

Figure 11. Contour plot and 3D surface plot of disintegration time (seconds) against amounts of HPMC E5, HPMC, E15, and maltodextrin

Table 11. ANOVA for special cubic model (disintegration time)

Source	Sum of squares	DF	Mean square	F value	Prob > F
Model	1477.38	6	246.23	492.46	0.0345
Linear mixture	1320.95	2	660.48	1320.95	0.0195
AB	28.41	1	28.41	56.82	0.0840
AC	62.23	1	62.23	124.45	0.0569
BC	28.17	1	28.17	56.33	0.0843
ABC	46.86	1	46.86	93.72	0.0655
Pure error	0.50	1	0.50	-	-
Core total	1477.88	7	-	-	-

DF: Degree of freedom

Polynomial equation

R_3 (tensile strength)= +2.71*A + 3.43*B + 2.39*C + 0.15*A*B - 0.11*A*C + 0.12*B*C - 0.45*A*B*C

To determine impact of each component and their interaction, ANOVA for SCM was carried out. The ANOVA results (Table 12),

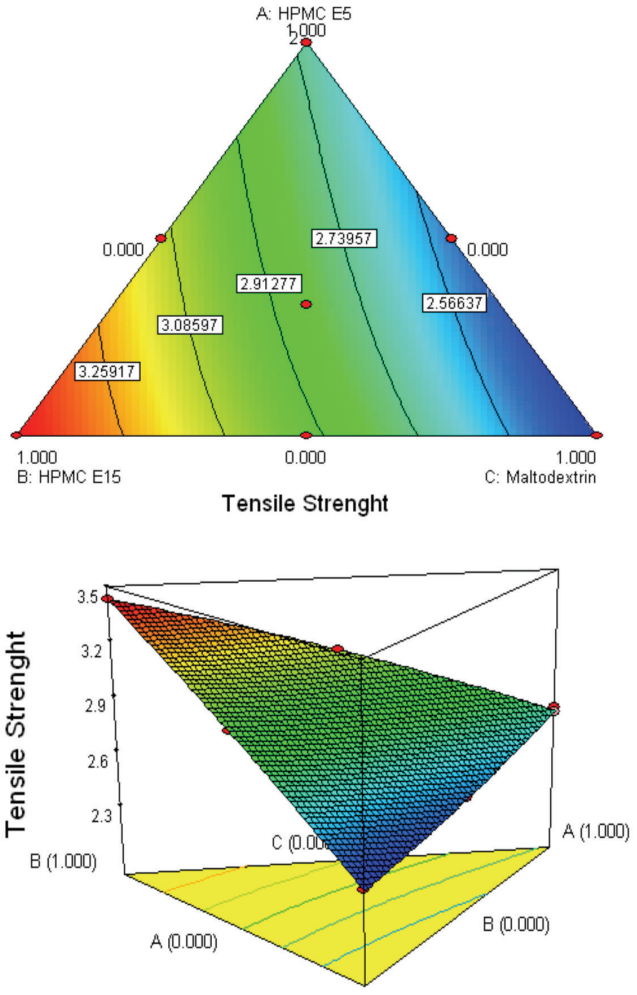

Figure 12. Contour plot and 3D surface plot of tensile strength (n/cm^2) against amounts of HPMC E5, HPMC, E15 and maltodextrin

3D surface plot, and contour plot for the tensile strength (Figure 12) indicated the strong effect of the three factors (amounts of HPMC E5, HPMC E15, and maltodextrin). A polynomial equation of tensile strength indicates that all the all the three-polymer amount have a positive effect on the tensile strength. It was observed that when the amounts of polymer were selected within the limits of the design, tensile strength was increased when more amounts of HPMC E15 were used in the formulation and it increased to a lesser extent in HPMC E5 followed by maltodextrin. As *per* the equation, values of tensile strength were decreased with the combination of all three polymers.[53,54]

Evaluation parameters for film formulation

Weight variation tests

Table 13 summarizes weight difference % for all formulations. They were under the pharmacopeial limits of 7.5%, so both of the films passed weight variation test. It was found to be in the range of 37 ± 2.081 to 81.67 ± 2.081 mg. Films having more amount of maltodextrin exhibited higher weight, whereas films having HPMC E5 were lighter in weight. Weight of the films was uniform.[55]

Thickness

The formulated films were observed to have thicknesses ranging from 0.103 ± 0.015 to 0.207 ± 0.02 mm. Table 13 lists the mean values. In both formulations, the values are almost identical. Films containing maltodextrin resulted in increased thickness, which was required for comfortable handling of the film.[56]

Folding endurance

The films' folding endurance was measured by folding a small strip of film at the same location before it separated and the average folding endurance of all films is shown in Table 13. All the batches have folding endurance of 101 ± 2.645 to 177.67 ± 3.51. The folding endurance increases as concentration of the polymer increases.[57,58]

Drug content

Drug content and uniformity tests were carried out to ensure that the drug was distributed uniformly and accurately. The content uniformity of all nine formulations was determined, where the

Table 12. ANOVA for special cubic model (tensile strength)

Source	Sum of squares	DF	Mean square	F value	Prob > F
Model	0.77	6	0.13	450.86	0.0360
Linear mixture	0.76	2	0.38	1348.51	0.0193
AB	9.924E-004	1	9.924E-004	3.51	0.3122
AC	5.767E-004	1	5.767E-004	2.04	0.3889
BC	5.709E-004	1	5.709E-004	2.02	0.3905
ABC	1.690E-004	1	1.690E-004	0.60	0.5811
Pure error	2.828E-004	1	2.828E-004	-	-
Cor total	0.77	7	-	-	-

DF: Degree of freedom

results are listed in Table 13. A spectrophotometer was used to examine three trials for each formulation. Mean values of all the formulations and SDs were calculated. The findings showed that both formulations had the same drug material. In *in vitro* release trials, the total % of drug released from each film was calculated using the mean quality of the drug contained in the film. Ranges of drug content in the formulations were 95.218% to 98.00%.[58]

In vitro dissolution study

In vitro release studies of methylphenidate hydrochloride films were performed in phosphate buffer (pH 6.8). Cumulative drug release was calculated based on the drug content of methylphenidate hydrochloride. Rapid drug dissolution was observed in F1, F5, which released 104.44% and 101.41%, respectively, at the end of 2 min. Comparatively, slow drug dissolution was observed in F6, F7 with the release of 96.45% and 99.73%, respectively. At end of 2 min, remaining formulations had slower drug release than the above-mentioned formulations. As the concentration of polymer HPMC E15 increased, the time for drug release was found to be increasing. This might be due to the higher viscosity of the polymer, which results in the formation of a strong matrix layer decreasing mobility of drug particles in swollen matrices, which leads to a delay in drug release.[36]

Table 14 shows the data of dissolution of prepared design batches. Figure 13 shows the graph of CPR *versus* time in minutes. The data indicated the data up to 2 min only, so that, we can easily compare the dissolution and % of drug release within our desired time limit. From Figure 13, we may conclude that in the first minute, drug release for every batch is almost the same, but for the consecutive minutes, number of drug release changes. So, we may say that polymers having a lower viscosity release the drug quickly than the polymers with higher viscosity. Thus, in order to get a quicker release, lower viscosity-grade polymers are desirable.[47]

Optimized batch analysis by statistical analysis

The optimized formulation was chosen based on criteria, a higher amount of drug release in 2 minutes, shortest disintegration time, and a medium value of tensile strength. Overlay plot was drawn to obtain an optimized batch using Design Expert (7.1.5) (Figure 14).

An optimized batch of the film was prepared experimentally using the same procedure/the results of stated parameters were compared with the computed values from regression equations. When the experimental and theoretical values were compared, error % was found to be less than 8% for the responses (Table 15).

Stability studies

A stability study was conducted according to the ICH guidelines for a short time. The developed formulations were tested for stability at 40°C and 75% relative humidity for 6 months and were evaluated for tensile strength, disintegration time, and *in vitro* drug release at 1, 3, and 6 month intervals. Effects of the formulations were deemed within acceptable limits as seen in Table 16. The measurable parameters showed no major differences. So, the formulation was found to be stable.[47]

Release kinetics and mechanisms

Data of *in vitro* release were fit into different equations and kinetic models to explain release kinetics of methylphenidate from these films. Release kinetics of methylphenidate followed zero order from the films (Table 17). A better fit (highest R^2 values) was observed in the case of Higuchi's model than Hixon-Crowel model except film I. Hence, mechanism of drug release

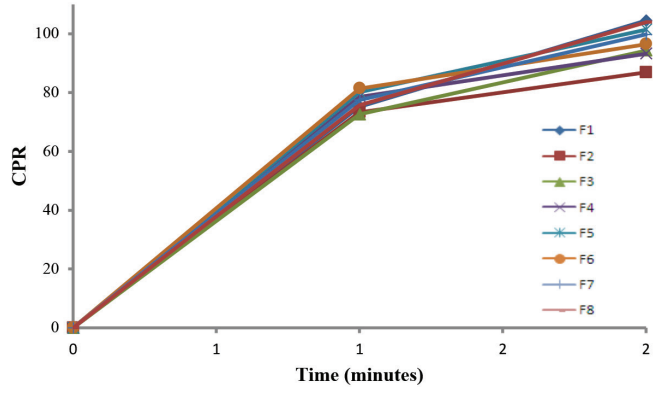

Figure 13. *In vitro* release of methylphenidate hydrochloride in phosphate buffer (pH 6.8) from the film formulation

Table 13. Evaluation parameters of experimental design batches

Batches	Weight variation ± SD* (mg)	Thickness ± SD* (mm)	Folding endurance ± SD*	Drug content ± SD* (%)
F1	37.33 ± 2.081	0.117 ± 0.011	108 ± 3.51	95.21 ± 0.52
F2	72.66 ± 1.527	0.167 ± 0.005	101 ± 2.645	95.41 ± 0.63
F3	81.67 ± 2.081	0.207 ± 0.02	116 ± 3.05	96.41 ± 0.46
F4	54.33 ± 1.527	0.137 ± 0.011	103 ± 2.0	98.00 ± 0.87
F5	80.33 ± 2.081	0.17 ± 0.02	117.67 ± 4.15	95.41 ± 0.56
F6	76.33 ± 2.301	0.103 ± 0.015	109 ± 5.03	97.40 ± 0.58
F7	62.66 ± 1.527	0.133 ± 0.011	115 ± 5.291	96.01 ± 0.48
F1 (R)	37.66 ± 2.31	0.17 ± 0.10	108 ± 3.60	95.41 ± 0.52

*All results are shown in mean ± SD (n= 3), SD: Standard deviation

from the remaining films followed is diffusion controlled and drug release from film I followed dissolution controlled (Table 18).

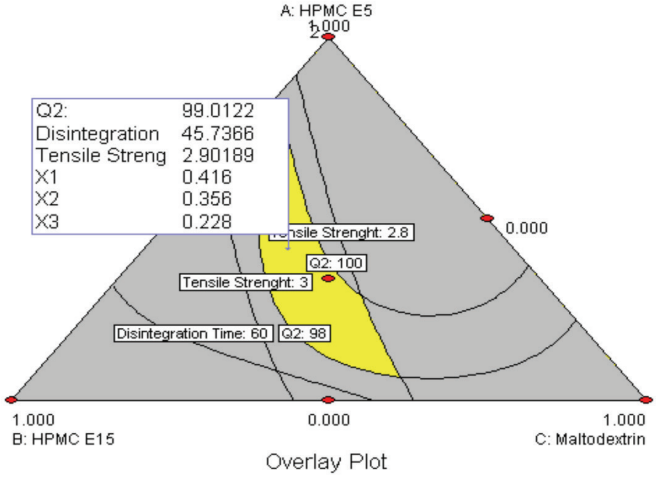

Figure 14. Overlay plot

Application of the Hixon-Crowell cube root law, the equation (M01/3-M1/3)= kt, provides information about the release mechanism, namely the dissolution rate limited. Application of Higuchi's equation (M= K $t_{1/2}$) provides information about the release mechanism, namely the diffusion rate limited. Korsmeyer-Peppas model indicates that the release mechanism is not well-known or that more than one type of release phenomenon could be involved. The "n" value could be used to characterize different release mechanisms (Table 19).

R^2 values are higher for Higuchi's model compared to Hixon-Crowell for the films except film I. Hence, drug release from film I followed a dissolution rate-controlled mechanism and drug release from the remaining films followed a diffusion rate-controlled mechanism.

According to the Korsmeyer-Peppas model, a value of slope between 0.5 and 1 indicates an anomalous behavior (non-Fickian). So, it indicates that the release mechanism from the films follows non-Fickian diffusion (anomalous behaviour). However, film I follows case II transport.

Table 14. Cumulative% of drug release from film formulations

Time (min)	0	1	2	3	4
F1	0.0 ± 0.0	75.19 ± 2.30	104.44 ± 2.91	-	-
F2	0.0 ± 0.0	73.34 ± 1.04	86.83 ± 1.00	89.64 ± 3.40	97.08 ± 2.89
F3	0.0 ± 0.0	72.62 ± 3.88	94.30 ± 2.04	99.80 ± 0.80	-
F4	0.0 ± 0.0	78.60 ± 2.98	93.23 ± 2.02	98.12 ± 1.62	-
F5	0.0 ± 0.0	80.12 ± 2.27	101.41 ± 1.89	-	-
F6	0.0 ± 0.0	81.40 ± 2.53	96.45 ± 2.81	98.86 ± 3.18	-
F7	0.0 ± 0.0	77.46 ± 1.42	99.73 ± 1.78	-	-
F1 (R)	0.0 ± 0.0	75.74 ± 0.378	103.94 ± 0.27	-	-

*All results are shown in mean ± SD (n= 3), SD: Standard deviation

Table 15. Evaluation of optimized batch

Responses	Predicted value	Experimental value*	Relative error (%)
$Q_{2\ min}$	99.01	98.45 ± 0.99	-0.56
Disintegration time (sec)	45.73	49 ± 3	7.15
Tensile strength (n/mm^2)	2.90	2.98 ± 0.14	2.75

*All results are shown in mean ± SD (n= 3), SD: Standard deviation

Table 16. Results of accelerated stability studies

Evaluation parameters	Time period for sampling*			
	Initial	After 1 month	After 3 months	After 6 months
CPR at 2 min (%)	98.45 ± 0.99	98.06 ± 5.44	98.15 ± 4.78	98.42 ± 2.35
Disintegration time (sec)	49 ± 3	47 ± 1	48 ± 0.57	49 ± 0.57
Tensile strength (n/cm^2)	2.98 ± 0.14	2.95 ± 0.081	3.01 ± 0.07	2.99 ± 0.14

*All results are shown in mean ± SD (n= 3), SD: Standard deviation

CPR: Cumulative percentage release

Table 17. Comparison of orders of *in vitro* release from all the patches

Batches	*In vitro* release in phosphate buffer pH 6.8 Regression equations	
	Zero order	First order
I	y= -1.6731x + 90.129 R^2= 0.9799	Log y= -0.0227x + 2.1477 R^2= 0.8944
II	y= -1.1987x + 86.842 R^2= 0.9817	Log y= -0.0247x + 2.2969 R^2= 0.6074
III	y= -0.8962x + 96.53 R^2= 0.9944	Log y= -0.014x + 2.2549 R^2= 0.6323
IV	y= -1.0745x + 93.923 R^2= 0.9933	Log y= -0.0166x + 2.223 R^2= 0.6606
V	y= -1.356x + 91.964 R^2= 0.9921	Log y= -0.0236x + 2.2586 R^2= 0.6991
VI	y= -0.7912x + 86.63 R^2= 0.9944	Log y= -0.0146x + 2.1439 R^2= 0.6421
VII	y= -1.0745x + 93.923 R^2= 0.9947	Log y= -0.0214x + 2.2547 R^2= 0.6666

Table 18. Comparison of regression equations of *in vitro* release from all the patches

Batch	*In vitro* release of drug in phosphate buffer pH 6.8		
	Hixon-Crowell model	Higuchi's model	Korsmeyer-Peppas model
I	y= 0.0159x - 0.0399 R^2 = 0.9762	y= 13.552x - 11.116 R^2= 0.9744	y= 1.0295x + 0.255 R^2= 0.9464
II	y= 0.014x - 0.0571 R^2= 0.8862	y= 11.717x - 8.1596 R^2= 0.9733	y= 0.9141 + 0.3521 R^2= 0.9074
III	y= 0.0092x - 0.0988 R^2= 0.8606	y= 10.24x - 18.435 R^2= 0.9239	y= 0.8815x + 0.2008 R^2= 0.9688
IV	y= 0.0111x - 0.0775 R^2= 0.8668	y= 11.012x - 14.728 R^2= 0.9397	y= 0.9136x - 0.2446 R^2= 0.9561
V	y= 0.0149x - 0.0777 R^2= 0.9094	y= 12.606x - 13.274 R^2= 0.9624	y= 0.979x + 0.2519 R^2= 0.9524
VI	y= 0.0261x - 0.0411 R^2= 0.9662	y= 12.255x - 12.111 R^2= 0.9777	y= 0.9812x + 0.522 R^2= 0.9644
VII	y= 0.012x - 0.0617 R^2= 0.9288	y= 11.177x - 9.634 R^2= 0.9755	y= 0.9144 + 0.5312 R^2= 0.9047

Table 19. Slope of Korsmeyer-Peppas equation and proposed release mechanisms

Slope (n)	Mechanism
<0.5	Fickian diffusion (Higuchi matrix)
0.5< n <1	Non-Fickian diffusion
1	Case II transport

CONCLUSION

The prepared film of methylphenidate hydrochloride obtained using the solvent casting method showed the desired % drug release, disintegration time, and tensile strength. The prepared film had a very smooth surface because of maltodextrin and without any interactions between the drug and polymer. The optimization of the film was done by simplex centroid design. The multiple regression analysis of the results led to equations that adequately describe the influence of the selected variables on the responses under study. Formulations with a % drug release of more than 95% within 2 minutes were found in a specific region containing more amounts of HPMC E5 resulting in quicker drug release. Formulations with *in vitro* disintegration time <60 sec were found in a specific region containing high levels of HPMC E5 and maltodextrin and low levels of HPMC E15. The desired level of tensile strength was achieved, when the optimum amount of HPMC E15 was present in the film. High drug release % of the film in simulated saliva (pH buffer 6.8) indicated that it could be helpful for treating acute ADHD and narcolepsy, where quick bioavailability of the drug is desired.

Therefore, all designed batches were prepared and their evaluations were carried out which showing acceptable results. Based on the results, we may conclude that aim of the current work was successfully fulfilled.

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Ethics

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

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: B.B., Design: B.B., Data Collection or Processing: B.B., A.M., A.D., Analysis or Interpretation: B.B., A.M., A.D., Literature Search: A.M., A.D., Writing: B.B.

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

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ORIGINAL ARTICLE

Development and Validation of Chromatographic and Spectrophotometric Methods for the Quantitation of Rufinamide in Pharmaceutical Preparations

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ABSTRACT

Objectives: Two optimized and validated high performance liquid chromatography (HPLC) and spectrophotometric methods are proposed. The developed methods were quantified with high sensitivity, accuracy, and precision at low concentrations to determine rufinamide (RUF) in active pharmaceutical ingredients (API) and pharmaceutical preparations.

Materials and Methods: HPLC method was developed using a base deactivated silica Hypersil C_{18} column and a combination of methanol: acetonitrile: water (15: 10: 75, v/v/v) as the mobile phase and detected at 210 nm. A reaction of RUF with sodium nitrite and hydrochloric acid occurred, absorbed maximally at 385 nm was extended to develop a ultraviolet (UV)-visible spectrophotometric method to determine RUF in API and pharmaceutical preparations.

Results: Different analytical validation parameters, including specificity, linearity, accuracy, precision, the limit of detection, quantification, ruggedness, and robustness, were determined as *per* International Conference on Harmonization guidelines. The linearity range of RUF was 0.15-3.5 and 10-100 µg/mL for HPLC and spectrophotometric methods, respectively.

Conclusion: The proposed investigations were valuable for drug monitoring and regular analysis of RUF in quality control and research laboratories. Moreover, the accuracy and precision obtained with the UV-visible spectrophotometer implied that it could be a cheap, easy, and alternative method, while HPLC could be sensitive to determine RUF at low concentration levels.

Key words: Rufinamide, validation, quality control laboratories, HPLC, UV-visible spectrophotometry

INTRODUCTION

Rufinamide (RUF) is a third-generation antiepileptic drug used to treat a neurological disorder characterized by seizure symptoms linked to Lennox-Gastaut syndrome (LGS). LGS is rare and one of the most severe forms of epilepsy among children between typically 3 to 5 years and adults. Therefore, the treatmeant of LGS is highly important, particularly in patients with childhood epilepsy. However, treatment success is limited by this condition.[1,2] RUF is a triazole derivative classified as an orphan drug, chemically known as 1-[(2,6-difluorophenyl) methyl]-1H-1,2,3-triazole-4 carboxamide (mol. formula: $C_{10}H_8F_2N_4O$, MW: 238.2 g/mol) developed in 2004 and has been authorized by the US Food and Drug Administration (US FDA) in 2008 for managing seizures associated with LGS[3-8] in children (4 years and above) and adults. RUF is believed to increase the refractory period of voltage-dependent sodium channels, reducing the possibility of fire in neurons.[9] The carboxamide group of RUF is extensively metabolized *via* carboxylesterase-mediated hydrolysis in a pharmacologically inactive carboxylic acid derivative and finally excreted in the urine. It has been recommended to monitor the absorption of this drug (slow and dose-dependent), as its peculiar and probable interaction with

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co-administered antiepileptic agents leads to pharmacokinetic variability. Therefore, regular therapeutic drug monitoring must adjust optimal dosage according to the patient's individual needs with epileptic seizures.[10] Chromatographic methods with different detection techniques, such as high performance liquid chromatography/ultraviolet (HPLC/UV) and liquid chromatography-tandem mass spectrometry[11-25] (LC-MS) have been developed. Recently, stability-indicating reversed phase-HPLC and first derivative ratio assays were designed to determine RUF in the presence of an alkaline degradation product in dosage forms.[26] A validated high-performance thin-layer chromatographic (TLC) assay in bulk drug and its formulations were also developed.[27] Only a few low sensitive spectrophotometric methods in pharmaceutical dosage forms, human and animal biological fluids,[28-30] and extraction-based spectrophotometric methods have been developed to determine RUF.[31,32]

Most reported methods have several drawbacks and are not stability-indicating. Hence, there is a need to develop sensitive, validated, and simple analytical methodologies such as HPLC and UV-visible spectrophotometry, which are widely employed in pharmaceutical quality control laboratories to quantify drug substances, and to estimate accurate and precise drug content in pharmaceutical preparations. HPLC method is characterized by sensitivity, repeatability, specificity, and spectrophotometric techniques, considered inexpensive, simple, fast, and direct.

This research aimed to develop two well-optimized and validated analytical methods (HPLC and spectrophotometry) with high sensitivity, accuracy, and precision with a good linearity range for RUF determination in pure and pharmaceutical preparations.

MATERIALS AND METHODS

Products and reagents

Sodium nitrite ($NaNO_2$), hydrochloric acid (HCl), methanol (CH_3OH), dimethylformamide [$(CH_3)_2NCH$], and acetonitrile (CH_3CN) were purchased from Sigma Aldrich through a local vendor. All the reagents are analytical grade and can be utilized without additional purification. Banzel 200 and 400 mg of the pharmaceutical product belonged to Eisai Co. Ltd.

Instrumentation and analytical conditions

Shimadzu, LC-2010 CHT HPLC was used to separate, which consists of a pump (LC-20AD), autosampler (SIL-20AC), column oven (CTO-20AC), and photodiode array detector (SPD-20A). LC solution software was used to integrate the chromatograms. The column used to separate the analytes was base-deactivated silica (BDS) Hypersil C_{18} (250 mm × 4.6 mm, 5 µm). The column temperature was maintained at 30°C with a mobile phase comprised methanol: acetonitrile: dimethylformamide (7:5:8, v/v/v) with a fixed flow rate (1 mL/min). An injection volume of 10 µL was chosen and detected at 210 nm. All the spectral runs were performed using Jenway (UV-vis 6300) and cecil (CE-7400) spectrophotometers with 10 mm path length at a wavelength of 385 nm.

Extraction of RUF from the dosage forms

Five RUF tablets (200 mg/tablet) were ground into powder, shifted into a 1000 mL beaker and dissolved in dimethylformamide and distilled water (1: 10). Stationary phase used in column chromatography was silica gel. Mobile phase consisted of methanol: water: glacial acetic acid (6.3: 1.3: 0.5 v/v/v), separated and dried as a solid RUF.

Preparation of standard solutions

For HPLC method, RUF stock solution (50 µg/mL) was prepared in a 100 mL volumetric flask by transferring the correct amount of the drug in dimethylformamide (DMF). Then, sonicated the mixture was for 15 min, and finally, the volume was completed with DMF. This solution was further diluted as *per* the requirement of the analysis.

A stock of RUF (1 mg/mL) was prepared for the spectrophotometric method in DMF. The HCl (0.50 M) and $NaNO_2$ (0.10 M) were diluted and prepared with distilled water, and further dilutions were continued as necessary.

Optimization of variables

Trial of current HPLC procedure was performed using several columns such as ODS Hypersil C_{18} (250 mm × 4.6 mm, 5 µm), ODS Hypersil C_{18} (150 mm × 4.6 mm, 5 µm), ODS Hypersil C_8 (250 mm × 4.6 mm, 5 µm), ODS Hypersil C_8 (150 mm × 4.6 mm, 5 µm), BDS Hypersil C_{18} (250 mm × 4.6 mm, 5 µm), BDS Hypersil C_{18} (150 mm × 4.6 mm, 5 µm), BDS Hypersil C_8 (250 mm × 4.6 mm, 5 µm), and BDS Hypersil C_8 (150 mm × 4.6 mm, 5 µm). The best separation was achieved with BDS Hypersil C_8 (250 mm × 4.6 mm, 5 µm). Different solvents with ratio, as the mobile phase, were studied and the highest separation occurs with methanol: acetonitrile: dimethylformamide (7: 5: 8, v/v/v) at a controlled oven temperature 30°C with detection at 210 nm.

Effect of volume of 0.50 M HCl concentration was studied using spectrophotometry by keeping a constant concentration of RUF (100 µg/mL) and 1 mL $NaNO_2$ (0.10 M) with a varied concentration of HCl (0.1-1.1 mL) in a final volume of 10 mL solution. Similarly, influence of 0.10 M $NaNO_2$ solution concentration was also studied by keeping the constant concentrations of RUF (100 µg/mL) and the optimized concentration of 0.50 M HCl (0.9 mL) and varying the concentration of $NaNO_2$ (0.1-2.4 mL) in a final volume of 10 mL solution. Figure 1 shows an increase in the absorbance of 0.5 M HCl concentration up to 0.7 mL and the influence of 0.10 M $NaNO_2$ solution concentration on the absorbance up to 1.8 mL. Therefore, concentrations of 0.9 mL of 0.50 M HCl and 2.1 mL of 0.1 M $NaNO_2$ were used throughout the experiment. The figure also includes an error bar with the respective standard deviations for optimizing HCl and $NaNO_2$.

Analytical method validation

The optimized spectrophotometric method was validated by evaluating the linearity, accuracy, precision, the limit of detection (LOD), the limit of quantitation (LOQ), specificity, standard addition, ruggedness, and robustness following the International Conference on Harmonization (ICH) guideline Q2 (R1).[33]

Linearity

Aliquots of 0.1-1.0 mL from 100 µg/mL RUF were pipetted into a series of 10 mL standard volumetric flasks. To each flask, 0.9 mL of 0.50 M HCl was added, followed by 2.1 mL of 0.10 M $NaNO_2$. The volume was completed with double distilled water. The contents of each flask were mixed well and heated at 100˚C. The increase in absorbance was recorded immediately at 385 nm.

Into a sequence of ten volumetric flasks with a 50 mL capacity, different RUF (50 µg/mL) volumes were transferred to prepare in the range of 0.15-3.5 µg/mL. 10 µL of each one was injected in 5 replicates and average peak area was recorded to evaluate the developed method's linearity range.

LOD and LOQ

Both methods (spectrophotometric and HPLC) sensitivities were established with the LOD and the LOQ. The LOD and LOQ values were computed with the help of a calibration curve, following the equations given below:

$LOD = 3.3 \times S_0/m$, and $LOQ = 10 \times S_0/m$,

where S_0= standard deviation of the y-intercept of a regression line:

m= Slope of the calibration curve

Accuracy and precision

HPLC and spectrophotometric method's accuracy precision were assessed. It determines the drug concentration at three different concentration levels (low, medium and high) within one day (intraday) and 5 consecutive days (interday). The standard deviation (SD) and percentage relative SD (RSD%) were determined. The standard addition method was continued to obtain percentage recoveries.

Robustness

For assessing method robustness, a slight variation was considered with the current experimental parameters. The analysis was presented at the deliberately varied experimental conditions using two wavelengths (± 2 nm) and a mobile phase composition ratio. SD and RSD% were calculated.

Ruggedness

Small changes in the environment conducted experiments, and an instrument model means little variation with operating conditions than the standard proposed analysis method.

Statistical analysis

Detailed statistical data analyses are presented in Table 1 for the proposed methods. The results proved an outstanding correlation between peak area and each drug's concentration within the specified range.

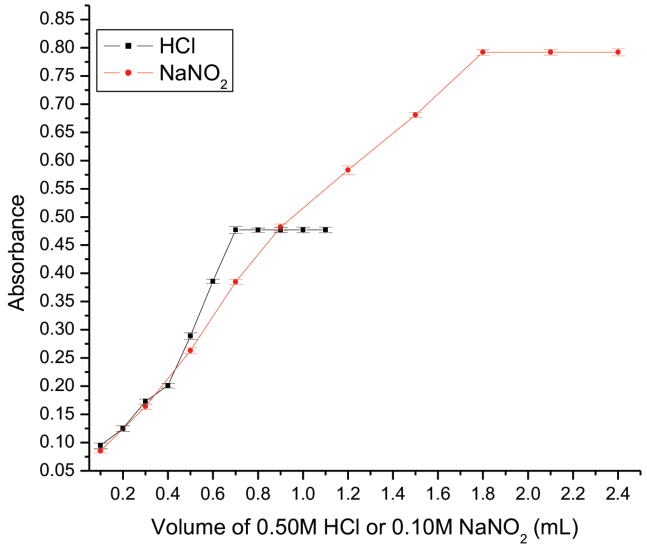

Figure 1. Effect of concentration and error bars with standard deviations of HCl/ $NaNO_2$

RESULTS AND DISCUSSION

RUF, an US FDA-approved drug, is a triazole derivative structurally unrelated to other marketed antiepileptic drugs. It is highly susceptible to acidic and alkaline hydrolysis. Simultaneously, it remained stable under oxidative, thermal, and photolytic stress conditions.[34] Literature reported that RUF could extensively metabolize after the hydrolysis of the carboxamide group of the drug *via* a primary biotransformation pathway (carboxylesterases) into an inactive acid derivative that is eliminated mainly in the urine.[35,36] Based on the above facts, a reaction of RUF with $NaNO_2$ and HCl performed at 100˚C undergoes hydrolysis of the carboxamide group of the drug and is expected to convert into a yellow-coloured acid derivative that absorbs maximally at 385 nm. A scheme was proposed based on a literature survey (Figure 2).

Under optimized chromatographic conditions, RUF was separated with a higher number of theoretical plates, good resolution, and peak shape. There was no interference from other components with a retention time of 4.65 min (Figure 3).

The specificity/system suitability test runs to ensure the current procedures connect all the requirements to start the analysis. Generally, it determines the presence of common excipients available with the pharmaceutical dosage form to know the

Table 1. Summary of linearity data for spectrophotometry and HPLC methods

Parameter	UV-visible spectrophotometry	HPLC
Beer's law range (µg/mL)	10-100	0.15-3.5
Regression equation	y= 0.0078x - 0.0059	y= 2332.2x + 970.72
S_0	0.009697	42.82088
M (slope)	0.007863	2332.155
Regression coefficient (r^2)	0.9984	0.9998
LOD (µg/mL)	4.07	0.061
LOQ (µg/mL)	12.33	0.184

LOD: Limit of detection, LOQ: Limit of quantitation

methods' ability to separate without interference. RSD% was calculated for both practices and found to be less than 2%.

Under optimized experimental conditions described, Beer's law obeyed the concentration ranges of 10-100 µg/mL for spectrophotometric method. The linear regression analysis used the least square method to assess the slope, intercept, and regression coefficient. High values of the regression coefficient and the small values of the regression equation intercept proved the calibration curve's linearity. The detection and quantification limit values reveal the proposed methods' high sensitivity. The HPLC procedure was rectilinear within 0.15-3.5 µg/mL.

LOD and LOQ are the smallest concentrations that provide a noticeable response and possibly be quantified. Consequently, signal to noise ratio was computed. Then, the current methods calculated the LOD and LOQ values of 0.061, 4.07, and 0.184, 12.33 µg/mL, respectively. The replicated analysis (n= 5) of RUF corresponding to 1, 2, and 3, as well as 20, 60, and 100 µg/mL of the proposed HPLC and UV-visible spectrophotometric methods were performed, determining its intraday and interday

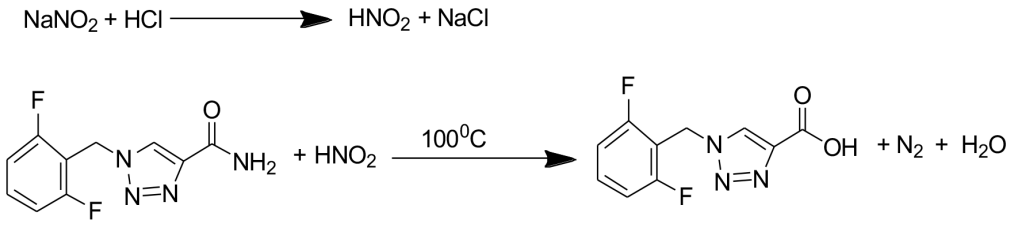

Figure 2. Proposed reaction scheme

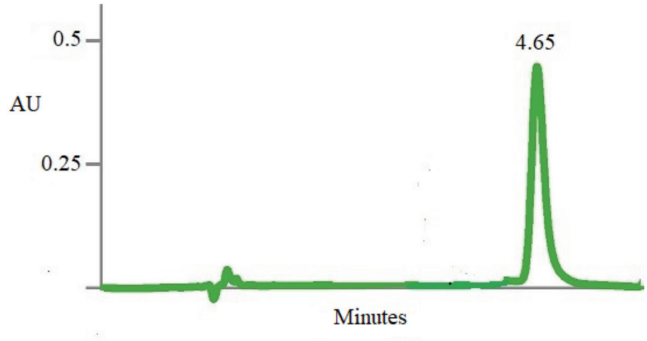

Figure 3. RUF chromatogram with a retention time of 4.65 min

Table 2. Determination of RUF in pharmaceutical formulations for precision

The proposed methods		Amount (µg/mL)		Recovery%	RSD%[a]	SAE[b]	CL[c]
		Taken	Found ± SD[a]				
UV-visible spectrophotometry	Intraday	20	19.67 ± 0.112	98.35	0.569	0.050	0.139
		60	59.92 ± 0.154	99.87	0.257	0.069	0.191
		100	99.81 ± 0.101	99.81	0.101	0.045	0.125
	Interday	20	19.63 ± 0.125	98.15	0.637	0.056	0.155
		60	59.13 ± 0.177	98.55	0.299	0.079	0.219
		100	99.56 ± 0.131	99.56	0.132	0.059	0.163
HPLC	Intraday	1	0.989 ± 0.005	98.90	0.506	0.002	0.006
		2	1.987 ± 0.006	99.35	0.302	0.003	0.008
		3	2.963 ± 0.009	98.77	0.304	0.004	0.011
	Interday	1	0.992 ± 0.008	99.20	0.807	0.004	0.010
		2	1.976 ± 0.011	98.80	0.557	0.005	0.014
		3	2.983 ± 0.014	99.43	0.469	0.006	0.017

Mean for 5 independent analyses. [a]SD: Standard deviation, RSD: Relative standard deviation, [b]SAE: Standard analytical error, [c]CL: Confidence limit at 95% confidence level and 4 degrees of freedom (t= 2.776)

Table 3. Standard addition method to determine accuracy of the proposed methods

The proposed methods		Amount (µg/mL)			% Recovery	% RSD[a]	SAE[b]	CL[c]
		Taken	Added	Found ± SD[a]				
UV-visible spectrophotometry	Intraday	20	20	39.75 ± 0.145	99.38	0.365	0.065	0.180
		20	40	59.92 ± 0.123	99.87	0.205	0.055	0.153
		20	60	78.99 ± 0.132	98.74	0.167	0.059	0.164
	Interday	20	20	39.88 ± 0.167	99.70	0.419	0.075	0.207
		20	40	59.64 ± 0.153	99.40	0.257	0.068	0.190
		20	60	79.11 ± 0.148	98.87	0.187	0.066	0.184
HPLC	Intraday	0.8	0.8	1.58 ± 0.003	98.75	0.190	0.001	0.004
		0.8	1.6	2.39 ± 0.004	99.58	0.167	0.002	0.005
		0.8	2.4	3.17 ± 0.007	99.06	0.221	0.003	0.009
	Interday	0.8	0.8	1.57 ± 0.006	98.13	0.382	0.003	0.007
		0.8	1.6	2.38 ± 0.009	99.17	0.378	0.004	0.011
		0.8	2.4	3.19 ± 0.005	99.69	0.157	0.002	0.006

Mean for 5 independent analyses. [a]SD: Standard deviation, RSD: Relative standard deviation, [b]SAE: Standard analytical error, [c]CL: Confidence limit at 95% confidence level and 4 degrees of freedom (t= 2.776)

precision. For spectrophotometric and HPLC methods, the % RSD was 0.101-0.637% and 0.302-0.807, respectively (Table 2). The accuracy parameter was determined with help of the standard addition method. Due to that, 50, 100, and 150% were spiked with the original drug components and determined its % recovery. The computed value was 98-100% for both methods (Table 3).

The method's robustness relative to each functioning parameter was studied and verified. The impacts of variation with wavelength (± 2) and mobile phase composition (± 2%) were analyzed to determine the method's robustness. Recovery% and RSD were 99.15-99.56 and 0.123-0.612% for both methods.

Ruggedness studies were conducted with a different model of instrument. As *per* ICH guidelines, recovery% ± RSD resulted within 98-102 and ± 2%.[33] All results were reproducible and indicated that the proposed methods are robust enough to determine the RUF in pharmaceuticals.

CONCLUSION

HPLC and UV-visible spectrophotometric methods were appropriate to quantify RUF in pure and pharmaceutical preparations. Therefore, precise and selective HPLC and spectrophotometric methods were developed to estimate RUF in pharmaceutical preparations. Although HPLC is a modern and sophisticated technique, it is expensive and time-consuming. A narrow range of RUF concentrations (0.15-3.5 µg/mL) could be estimated using HPLC. However, the UV-visible spectrophotometric method is easy, inexpensive and performed almost in all quality control and research laboratories. It can also determine various RUF concentrations (10-100 µg/mL). The chromatographic method presented sensitive and reliable results with good recoveries. In contrast, the spectrophotometric method offers a simple, accurate, precise, and time-saving method. It could be recommended as an equivalent alternative method. These two methods could be successfully applied to quantify RUF in research laboratories, hospitals, and quality control laboratories.

Ethics

Ethics Committee Approval: The ethics committee approval is not required for the proposed research. We have not used any human beings or animal matrix.

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: H.R., S.M.H., Design: S.M.H., Data Collection or Processing: H.R., Analysis, or Interpretation: H.R., S.M.H., Literature Search: H.R., Writing: H.R., S.M.H.

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

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ORIGINAL ARTICLE

Assessment of Commercially Safflower Oils (Carthami Oleum Raffinatum) in Terms of European Pharmacopoeia Criteria and Their Weight Control Potentials

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ABSTRACT

Objectives: Safflower oils, which are sold commercially, are in demand with food, cosmetics, and health-promoting claims. In this study, safflower oil samples belonging to 11 different brands were evaluated in terms of European Pharmacopoeia Criteria 7.0. Additionally, *in vitro* weight control potential of all samples was investigated.

Materials and Methods: Samples to be analyzed were purchased from pharmacies, herbal, online, and cosmetics stores. Acid and peroxide values of 11 safflower samples and analysis of their fatty acids by gas chromatography-mass spectrometry (GC-MS) were carried out according to the "Carthami oleum raffinatum" monograph registered in the European Pharmacopeia 7.0. To test the effects of all samples on weight control, their inhibitory effects on carbohydrate-digesting enzymes (α-glucosidase and α-amylase) were evaluated using spectrophotometric methods.

Results: Out of 11 oil samples, only two of them had acid and peroxide values below the reference value. According to GC analysis, safflower oil samples are rich in monounsaturated fatty acids (oleic acid) and polyunsaturated fatty acids (linoleic acid) (67.10-99.53%) of total fatty acids in its content are oleic, linoleic, palmitic, and stearic acids. Saturated fatty acids were 0.58 to 12.18% of the total fatty acid methyl esters in oils. When evaluated in terms of the inhibition of α-amylase and α-glucosidase enzymes that hydrolyze carbohydrates, the results showed that safflower oil samples had no inhibitory activity on these enzymes.

Conclusion: It has been determined in this report that many safflower oil samples on the market do not meet the quality criteria recommended in European Pharmacopoeia 7.0. It was observed that safflower oil did not show any inhibitory effect on these two enzymes, which is considered a rational approach for weight control.

Key words: Fatty acids, gas chromatography-mass spectrometry, quality control, safflower oil

INTRODUCTION

Due to oil, protein, carbohydrate, mineral, and vitamins they contain, some plant seeds have a critical place in human and animal nutrition and biodiesel production. Most of the oils needed in human nutrition are obtained from vegetable oils. 92% of the world's oil production is obtained from vegetable sources and 8% from animal sources.[1] Seeds are an important source of various vegetable oils. Fatty acid content and biological activity of seeds are the factors that determine the use of a vegetable oil in nutrition, pharmaceutical or industrial areas.[1,2] Vegetable oil production in the world is mainly met with palm oil, soybean, rapeseed, olive, safflower, corn, sunflower, peanut, sesame, castor oil, poppy, flax, hemp, and jojoba. Most of the vegetable oil production in Türkiye is based mainly on sunflower; sunflowers constitute 69% of vegetable oil

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production, 32% of total oil consumption. Therefore, safflower, whose production is low, may be a hope of closing vegetable oil gap in the market. 80% of world safflower production is carried out by Kazakhstan (24.6%), Russia (19.2%), Mexico (16.1%), India (11.9%), and Türkiye (8.2%).[1]

Carthamus tinctorious L. is a bushy, herbaceous plant from Asteraceae family and grown in arid or semi-arid regions or where moderately salty water is used. Safflower oil (Carthami oleum raffinatum) is a fixed oil obtained by squeezing or extracting the seeds of *C. tinctorius* and then refining.[3-5]

Except using these oils for dietary purposes, many vegetable oils are sold with health-promoting claims or statements that they are beneficial against diseases, while safflower oils are one of them, as they are sold in "natural", "organic products", "local products" shops, cosmetics store chains, and pharmacies. Safflower oil, which is marketed for health benefits and cosmetics, must meet European Pharmacopeia criteria. Currently, these safflower oils are marketed as fixed oils with the producer's own marketing and quality criteria, while a pharmacopeia's core mission is to protect public health by creating and making available public standards to help ensure the quality of products while the user or procurer can make an independent judgment regarding quality, thus safeguarding the health of the public.

In European Pharmacopoeia 7.0, "safflower oil, refined; Carthami oleum raffinatum" is registered under the name "refined safflower oil", which is defined as oil obtained from the seeds of *C. tinctorius* (type I) or hybrid *C. tinctorius* seeds (type II) from extraction and/or extraction followed by refining.[6] Type II oil is rich in oleic acid and contains antioxidant.[7] Fatty acid content is one of the main factors that determine the use of a vegetable oil in nutrition, pharmaceutical or industrial areas.[2]

Safflower seeds contain high levels of polyunsaturated fatty acids and are used for dietetic, medical, and industrial purposes.[8,9] 96-99% of total fatty acids in its content are oleic, linoleic, palmitic, and stearic acids, 9.7-10.8% of saturated fatty acids.[2] Fatty acid composition in safflower seeds usually consists of 71 to 75% linoleic acid (C 18:2), 16 to 20% oleic acid (C18:1), 6 to 8% palmitic acid (C16:0), and 2 to 3% stearic acid (C18:0).[2,10-12]

There is much news about the use of safflower oil in weight control in the media and on the internet. Pharmacists also state that especially women often demand safflower oil for this purpose. Literature surveys have shown us that there is no scientific study on the effect of this oil on weight control. Therefore, in this study, we tested the effects of safflower oil on enzymes that digest carbohydrates, which is a rational approach to weight control. On the other hand, quality control evaluations of 11 safflower oil samples obtained from pharmacies, akhtars, and cosmetics shops were made in terms of the criteria in the relevant monograph in the European Pharmacopoeia 7.0. For this purpose, acid and peroxide values of the oil samples by volumetric method and fatty acid analysis were performed using gas chromatography-mass spectrometry (GC-MS) technique.

MATERIALS AND METHODS

Chemicals and instruments

All chemicals used were of analytical reagent grade. Heptane (99%), potassium hydroxide (KOH), methylene chloride (CH_2Cl_2) for GC-MS Supra Solv®, sodium chloride (NaCl), sodium sulfate (Na_2SO_4), Supelco 37-component fatty acid methyl ester (FAME) mix (FAME37, C4-24), chloroform ($CHCl_3$), glacial acetic acid, acetone, phosphomolybdic acid, ether, phenolphthalein, 3,5-dinitrosalicylic acid, sodium potassium tartrate were purchased from Sigma Aldrich Co. and Riedel-de Haën (Seelze, Germany). Methanol (MeOH) containing not more than 0.5% (w/w) water, starch, potassium iodide (KI), sodium thiosulfate, and peroxide-free ether were purchased from Merck, Germany. Oksan Co., Ltd. (Ankara, Türkiye) provided helium, hydrogen, and dried air gas for GC with 99.99% purity. Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

GC (7890A GC System, Agilent Technologies Inc, US), a capillary column Rt-2560 (100 m, 0.25 mm ID, 0.2 µm) (Restek Corporation Bellefonte, US), vial insert, 250 µL, glass with polymer feet, vial, screw top, 2 mL, amber and cap, screw, blue, polytetrafluoroethylene/red silicone septa (Agilent Technologies Inc., US), and ELISA (Versamax Tunable Microplate Reader) were used in GC analysis and enzyme activity studies, respectively.

Safflower oil samples

Eleven different brands of safflower oils were purchased from pharmacies, akhtars, online, and cosmetics stores in Ankara/Türkiye in 2019. The samples were stored at 4˚C until used in studies.

Acid value (V_A)

About 10 g of the oil (m) was dissolved in 50 mL of 96% MeOH and ether mixture (1:1, v/v). 0.1 M KOH was used as a titrant in the presence of phenolphthalein indicator until the pink remained stable for at least 15 s (n mL of 0.1 M KOH). Acid values of samples were calculated from the equation of V_A= (5.610 x n)/m and compared with value of maximum of 2.0 in a 5.0 g oil sample.[13]

Peroxide value (V_P)

About 5 g oil (m) was placed in a 250 mL conical flask fitted with a ground-glass stopper. 30 mL of a mixture of $CHCl_3$ and glacial acetic acid (2:3, v/v) was added. After the oil dissolved, 0.5 mL of saturated KI solution was added and shaken exactly 1 min, then 30 mL water was added. It was titrated with 0.01 M sodium thiosulfate until yellow color was almost discharged. 5 mL of starch solution was added and continued the titration, until the color was discharged (n_1 mL of 0.01 M sodium thiosulfate). We carried out a blank test under the same conditions (n_2 mL of 0.01 M sodium thiosulfate). Volume of 0.01 M sodium thiosulfate used in the blank titration did not exceed 0.1 mL. Peroxide value was calculated from the equation of V_P= 10 (n_1 - n_2)/m, and compared value of max 15.0 in 5 g oil.[14,15]

Fatty acid analysis by GC-MS

Standard mixture of 37 fatty acids methyl esters (Supelco™ 37 Component FAME Mix, FAME37, C4-24) used for the GC analyses. FAME37 (100 mg) was stored at -20˚C and all standard solutions were prepared in an ice bath. To prepare 400 mg/mL FAME 37 standard solution, 250 µL of CH_2Cl_2 solution was added to 100 mg of FAME37 standard, and vortexed. 75 µL of this solution was taken into a GC vial and 925 µL CH_2Cl_2 was added, and then vortexed by closing the mouth. FAME37 standard solution (100 µL; 30 mg/mL) was placed in a 250 µL polymer-footed glass tube placed in 2 mL amber colored vial and sealed, then analyzed by GC-MS under the following chromatographic conditions (Table 1).

FAME were prepared by *trans*-esterification of the oils with MeOH, using KOH as a catalyst before GC analysis. 2 mL of safflower oil sample was placed in a flat bottom, approximately 50 mm diameter and 30 mm long container and dried in the oven at 100-105˚C. It was allowed to cool in a desiccator with silica gel. 1 g of the oil was weighed into a 25 mL round-bottomed flask with a ground-glass neck fitted with a reflux condenser and a gas port into the flask. Anhydrous MeOH (10 mL) and 0.2 mL of 60 g/L KOH in MeOH were added. Then, flux condenser was attached, passed nitrogen through the mixture at a flow rate of about 50 mL/min, mixed, and heated to boiling. When the solution was clear (usually after about 10 min), it was continued heating for a further 5 min, and cooled the flask and transferred the contents were to a separating funnel. The flask was rinsed with 5 mL heptane, transferred the rinsing to the separating funnel, and stacked. NaCl solution (10 mL of a 200 g/L) was added and stacked vigorously. It was allowed to separate two phases and transferred the upper organic layer to a vial containing anhydrous Na_2SO_4, allowed to stand, then filtered, and FAME compositions were determined by GC-MS under the chromatographic conditions (Table 1).[16] Results are expressed as % (w/w) with respect to all fatty acids detected.

Table 1. GC-MS conditions

GC conditions	
Component	**Condition**
Device	Agilent Technologies 7890A GC system, Agilent Technologies Inc, Santa Clara, MS detector
Column	Restek-2560, bissyano propyl polysiloxane (100-m x 0.25 µm ID x 0.20 µm)
Oven temperature	100˚C (hold 4 min), increased to 24˚C by 3˚C min (hold 15 min)
Injection temperature	225˚C
Detector temperature	250˚C
Carrier gas, flow rate	He, 1.0 mL/min
Injection volume	2 µL
Split ratio	100:1
MS condition	
Component	**Condition**
Device	Agilent Technologies 5977E MS
Mode	Scan
Solvent Delay	11.5 min
Mass range	20-300
Step size	0.1 *m/z*
Scan speed	3.125 (u/s)
Frequency	9.0 scan/sec
Cycle time	110.96 ms

Enzyme inhibition methods

α-Amylase enzyme inhibition

The effects of safflower oil samples in α-amylase enzyme were determined by modifying the method of Ali et al.[17] α-Amylase enzyme (EC 3.2.1.1, type I-A, Sigma) was prepared in water at a concentration of 4 U/mL. Potato starch solution (0.5%) prepared in 20 mM phosphate buffer was used as the substrate.

Safflower oil samples were prepared in 100% MeOH at concentrations of 1, 0.5, and 0.25 mg/mL. The samples were treated with α-amylase enzyme (4 U/mL) at 37˚C. They were incubated at 37˚C and starch solution was added. Further, 50 mL of DNS color solution (96 mM 3,5-dinitrosalicylic acid, 5.31 M sodium potassium tartrate in 2 M NaOH) was added and kept at 85˚C for 15 min. Later, the mixtures were diluted with water and the tubes were allowed to cool to complete the reaction. The absorbance of the mixture was measured at 540 nm with an ELISA (Versamax Tunable Microplate Reader) plate reader. Acarbose was used as the reference substance. The change in absorbance resulting from the amount of maltose formed was calculated using equation 1.

The amount of maltose formation was measured using the maltose standard calibration curve (between 0.0 and 0.1%, w/v) maltose concentration *versus* net absorbance value, y= 0.4428x + 0.0264, r^2= 0.9926. Percentages of inhibition were calculated using equation 1.

[A (control solution) / A (oil sample)] = [A (oil sample) / A (blank)] (equation 1)

A: Absorbance

α-Glucosidase enzyme inhibition

Inhibitory effects of the safflower oil samples on α-glucosidase were determined using the method of Lam et al.[18] The α-glucosidase (type IV) obtained from *Bacillus stearothermophilus* was prepared in 0.5 M phosphate buffer (pH 6.5).

Safflower oil samples were prepared by dissolving in 100% MeOH at concentrations of 1, 0.5, and 0.25 mg/mL. The solutions were incubated with α-glucosidase in a 96 well microplate reader at 37˚C. Subsequently, substrate solution (10 µL, 20 mM *p*-nitrophenyl-α-D-glucopyranoside) was added and the reaction was allowed to occur at 37˚C. At the end of the

period, color intensity was measured with an ELISA (Versamax Tunable Microplate Reader, USA) plate reader at a wavelength of 405 nm was used as the reference substance. Percentages of inhibition were calculated using equation 2.

Inhibition (%)= [1 - (A (oil sample) / A (control)] × 100 (equation 2)

A: Absorbance

Statistical analysis

While evaluating the test results, standard deviations (SD) were calculated in MS Excel program on the Windows XP operating system. All the results were given for at least as triplicate and values were expressed as mean ± SD.

RESULTS

Acid and peroxide values of the oil samples

Acid and peroxide values of the safflower oil samples are given in Table 2. According to European Pharmacopoeia 7.0, acid value of the safflower oils should be at most 0.5 and the peroxide value should be 10. Except for n_1 and n_5 oils, acid value of 9 other oils is above the reference value of 0.5. On the other hand, only 2 (n_1 and n_4) out of 11 oil samples have the peroxide value below the reference value.

Fatty acid composition of the oil samples

The pharmacopeia mentions two types of safflower oil. The oil obtained from the seeds of *C. tinctorius* from expression and/or extraction is called type I and the oil obtained from the seeds of the hybrids of this plant is called type II fixed oil. Since it is not stated on the packages of the purchased oil samples whether the plant from which the oil is obtained is hybrid or not, the results of the fatty acid analysis were evaluated according to the results of type I oil (Table 3).

Quantitative analysis of fatty acids contained in the samples was carried out over the peak areas by comparing the retention times of standard fatty acids and the results are given in Table 4. According to this, safflower oil samples are rich in monounsaturated (oleic acid) and polyunsaturated (linoleic acid) fatty acids (67.10-99.53%) of total fatty acids in its content are oleic, linoleic, palmitic, and stearic acids. Saturated fatty acids are 0.58 to 12.18 % of the total FAMEs in oils. The fatty acid composition in safflower oils consisted of 47.04 to 92.59% linoleic acid (C18:2), 4.09 to 40.34% oleic acid (C18:1), 0.54 to 12.01% palmitic acid (C16:0), and 0.05 to 1.35% stearic acid (C18:0). Considering all these results and the values in the monograph, it was seen that none of the oil samples were suitable for the pharmacopeia.

Table 2. Acid and peroxide values of the oil samples

Samples	n_1	n_2	n_3	n_4	n_5	n_6	n_7	n_8	n_9	n_{10}	n_{11}
V_A[a] ± SD[c]	0.2 ± 0.1	1.4 ± 0.1	1.3 ± 0.2	1.2 ± 0.0	0.4 ± 0.0	0.7 ± 0.1	0.8 ± 0.2	1.3 ± 0.1	1.9 ± 0.1	0.8 ± 0.1	1.1 ± 0.1
V_P[b] ± SD[c]	7.5 ± 1.6	28.6 ± 0.2	14.6 ± 0.3	8.2 ± 0.4	12.8 ± 2.1	14.8 ± 1.5	157.7 ± 6.1	25.4 ± 3.4	18.4 ± 1.9	87.8 ± 0.0	30.4 ± 1.5

[a]: Acid value, [b]: Peroxide value, [c]SD: Standard deviation, n= 3

Table 3. Fatty acid composition of safflower oil (type I) according to European Pharmacopeia monograph 7.0[14]

FAMEs	% (w/w)
C16:0	4-10
C18:0	1-5
Σsaturated FAMEs (C16:0 and C18:0)	5-15
C18:1	8-21
C18:2	68-83
Σunsaturated FAMEs (C18:1 and C18:2)	76-105

Table 4. Fatty acid compositions of the oil samples by using GC-MS

FAMEs	Samples (% w/w)										
	n_1	n_2	n_3	n_4	n_5	n_6	n_7	n_8	n_9	n_{10}	n_{11}
C16:0	3.27	4.08	12.01	5.32	3.57	6.16	0.54	2.24	2.21	2.43	2.73
C18:0	0.05	0.43	0.17	1.35	1.28	0.52	0.04	0.23	0.09	0.46	0.58
Σsaturated FAMEs (C16: 0 and C18:0)	3.32	4.51	12.18	6.67	4.85	6.68	0.58	2.47	2.30	2.89	3.31
C18:1	4.09	6.32	32.06	40.34	24.51	23.17	9.71	9.61	6.25	12.29	9.75
C18:2	92.59	87.73	47.04	49.69	68.20	69.52	89.64	89.92	91.45	84.81	86.90
Σunsaturated FAMEs (C18:1 and C18:2)	96.68	94.05	79.10	90.03	92.71	92.69	99.35	99.53	97.70	67.10	96.65

α-Amylase and α-glucosidase enzyme inhibitory activities of the oil samples

All oil samples were tested at concentrations of 0.25, 0.5, and 1.0 mg/mL to evaluate the inhibitory effects on both enzymes (Table 5). The results showed that safflower oil samples had no activity on α-glucosidase, which is an enzyme that digest carbohydrates. A feeble α-glucosidase inhibitory effect was detected only in sample number 11. The inhibition values of this sample were calculated as 6.70 ± 2.55% at a concentration of 0.25 mg/mL and 4.58 ± 1.03% at a concentration of 1 mg/mL. Inhibition values of acarbose used as the reference was determined as 98.19 ± 0.53% (0.25 mg/mL), 99.53 ± 0.04% (0.5 mg/mL), and 99.57 ± 0.04% (1 mg/mL).

Generally, it was found that the oil samples did not show a significant inhibitory effect on α-amylase. Only sample number 1 showed a moderate inhibitory activity (52.13 ± 2.87%) at a concentration of 1 mg/ml. At the same concentration, acarbose displayed 100% inhibitory activity.

DISCUSSION

Acid value is used as a shelf-life monitoring parameter in the quality control of oils. The high free fatty acidity is one of the rancidity indicators of any oil and an increase in the oxidation potential of the oil and decreases stability. Except for oil samples 1 and 5, all samples were found to be rancid and oxidized. Peroxide value is a measure of the amount of active oxygen in oils, and the amount of peroxide in 1 kg oil in milliequivalent grams of oxygen. Oxygen causes smaller molecule fatty acids. Oils can deteriorate due to various factors (storage conditions, metal ions, temperature, light, *etc.*). The peroxide value shows oxidation state of the oil. It also indicates whether the deodorization process has been done effectively.[17,18] Again, it was determined that all samples were highly oxidized except for oil samples 1 and 4.

Palmitic, stearic, oleic, and linoleic acids were detected in all oil samples. It was determined that oleic acid (8.0-21.0%) and linoleic acid (68.0-83.0%), which were reported as the main fatty acids in the European Pharmacopoeia 7.0, were not among the desired amounts in the samples. The contradictory situation we detected in the samples for these two fatty acids led us to predict that oleic acid is oxidized into linoleic acid.

There are many *in vitro* and *in vivo* methods to evaluate the effects of pure compounds or plant extracts on weight control. In this study, the effects of safflower oils, which have been in high demand from pharmacies for weight control recently, on α-amylase and α-glucosidase that help carbohydrate digestion were evaluated and the oil samples did not show any inhibitory effect. However, the fact that none of the oil samples met the quality standards may have caused unpredictable interactions in activities of the enzymes.

Previously, Takahashi and Miyazawa[19] studied the effects of MeOH extracts of safflower seeds on α-glucosidase enzyme. As a result, compounds with stronger inhibitory effects than acarbose (*N-p*-coumaroyl serotonin and *N*-feruloyl serotonin) were isolated from the extract.[19] However, it is clear that the secondary metabolite contents of seed oils will be different from seed MeOH extracts. In a study examining the activities of 10 fatty acids on enzymes known to be associated with diabetes, Su et al.[20] showed that oleic and linoleic acids have potent glucosidase enzyme inhibitory activity. In short, the fact that the oils analyzed in this study were highly oxidized and the fatty acid content was not between the desired values should be a factor to be considered in determining the effectiveness.[20]

Safflower oils are among the commonly used oils because they have many activities (antioxidant, antiulcer, cardioprotective, antinociceptive, anti-inflammatory, hepatoprotective, anti-

Table 5. α-Amylase inhibition results of the oil samples

Samples	Inhibition % ± SD		
	0.25 mg/mL	0.5 mg/mL	1 mg/mL
n_1	-	5.37 ± 2.87	52.13 ± 2.87
n_2	13.81 ± 3.57	-	7.73 ± 0.86
n_3	-	15.53 ± 3.26	6.32 ± 1.89
n_4	13.96 ± 3.50	3.42 ±1.05	13.81 ± 1.06
n_5	8.59 ± 3.50	4.91 ± 1.77	-
n_6	-	11.52 ± 1.07	-
n_7	8.11 ± 2.86	5.38 ± 1.18	21.08 ± 7.68
n_8	16.09 ± 3.73	17.23 ± 1.05	24.45 ± 5.06
n_9	28.37 ± 4.90	20.93 ± 2.40	24.66 ± 2.81
n_{10}	40.38 ± 3.25	18.15 ± 4.14	16.98 ± 2.98
n_{11}	-	7.54 ± 1.83	4.01 ± 5.50
Acarbose	92.70 ± 5.80	98.45 ± 6.66	100.00 ± 0.04

-: No inhibition, SD: Standard deviation, n= 3

cancer, antidiabetic, and weight control activities[21-26] as well as their use in food and cosmetics, while most of these health benefits are associated with the oil content and the fatty acid composition of the respected oils. These oils, which contain significant number of oxygen radicals, will cause serious health problems after chronic exposure, especially considering that they are used daily by people who buy them for weight control. Meanwhile, evaluating the effectiveness of safflower oils on weight control with other methods can be considered another research topic. In conclusion, the high oxidation rates and significant variability in fatty acid content (especially for unsaturated fatty acids) detected in 11 purchased samples indicated that more attention should be paid to the quality control of these oils, which points out to fact that they would be beneficial in terms of the health benefits expected from safflower oils.

CONCLUSION

Our literature survey has shown that quality control analysis of the safflower oils in Turkish market and their effectiveness on two enzymes (α-amylase and α-glucosidase) was performed in this study for the first time. Within this study, we conducted in 11 safflower oil samples we bought from the market; it was concluded that these oil samples did not meet the quality criteria required by European Pharmacopoeia for safflower oil. Interestingly, it was observed that the amount of oleic acid was very low, while the amount of linoleic acid was high. This result suggested that oleic acid in oils was oxidized to linoleic acid due to the production methods and storage conditions. The results of enzyme inhibitory activities of the safflower oil samples showed that these oils could not have any effect on weight control by inhibiting the enzymes that provide carbohydrate digestion.

Safflower, whose market cap and production are low, may be the hope of closing the vegetable oil gap for the present/future of the growing world's population demand. In addition to increasing oil production, the end product also meets the expectations in terms of quality. Likewise, if the oil is used or at least marketed with health claims (with or without pharmaceutical usage), to establish the necessary quality criteria and show no harm to the user, the safflower oil (if for human use) needs to be encouraged to be European Pharmacopoeia compliance.

Ethics

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

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: D.D.O., Design: D.D.O., Data Collection or Processing: D.D.O., D.S., S.P., B.Ö., A.B., O.K.U., Analysis or Interpretation: D.D.O., D.S., S.P., B.Ö., A.B., O.K.U., Literature Search: D.D.O., O.K.U., Writing: D.D.O., O.K.U.

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ORIGINAL ARTICLE

The Safety of Herbal Medicines (Phytovigilance) from Community Pharmacists' Perspective: A Cross-Sectional Study

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ABSTRACT

Objectives: The "safe if natural" perception of herbal products may have several undesirable side effects. It is important to raise awareness in public order to change this perception and ensure safer use of herbal products. The role of pharmacists is to supply herbal medicines safely and to provide accurate information to the patients about herbal products. The aim of this study was to analyze the perspective, knowledge, attitude, and behavior of community pharmacists about phytovigilance.

Materials and Methods: A cross-sectional analysis was performed using community pharmacists (n= 879) using face-to-face surveys between April-June 2019 in Istanbul. For statistical analysis, student's *t*-test and one-way ANOVA were used to compare the mean values of the groups.

Results: It was determined that 58.1% of the pharmacists heard phytovigilance for the first time and 93.5% of them had not reported any safety-related herbal medicine reported so far. Among the reasons for not reporting, well-known adverse reactions were found to be 24.2% and the difficulty of reporting was found to be 21.8%. 84.6% of pharmacists have never received training related to phytovigilance. It was found that pharmacists with a working experience of more than 20 years primarily selected herbal products provided in their pharmacy based on the manufacturing company primarily, whereas others selected based on the efficacy of the products.

Conclusion: The results of this study have revealed the necessity to increase training on the safety of herbal medicines to cover all stakeholders and to give due importance to phytovigilance. The phytovigilance systems established in some countries for public health should be expanded to other countries. There is a need for a more user-friendly reporting system to increase adverse reaction reporting by pharmacists and other healthcare professionals. In the future, plan to perform studies to raise awareness in the public and promote reporting with digital technologies.

Key words: Adverse drug reactions, community pharmacists, herbal medicines, herbal products, phytovigilance, safety of herbal medicines

INTRODUCTION

The use of plants for therapeutic purposes is as ancient as human history. There is a public perception that plants and herbal products in widespread use are safe because of their natural origin and traditional use.[1] On the other hand, studies and regulatory guidelines have shown that this perception is inaccurate.[2-7] Likewise, the lack of adverse reaction reports does not mean that these herbal products have no adverse effects. Some plants may also be poisonous. For example; the ancient Greek philosopher Socrates was sentenced to death with *Conium maculatum L.,* which is a highly toxic plant. Additionally, factors including improper use, high dose, drug interactions, drug addiction, ineffectiveness may also harm the patient. The most important differences in herbal products compared to conventional drugs are their unknown active metabolites, complex structures, difficulties in standardization and stability, quality of raw material, quality in manufacturing, use of different plants or plant parts, errors in botanical identification, insufficient clinical and ethnopharmacovigilance studies, poor packaging and labeling, lack of regulations, uncontrolled distribution channels, and lack of knowledge.[8-14] Herbal products contain complex structures. Their qualitative and quantitative composition varies depending on their geographical origin, genotype, which part of the plant is used, time of harvest, storage conditions, extraction and other related process.[12-14]

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Hepatotoxicity, nephrotoxicity, and carcinogenic effects are particularly remarkable in the toxicity of herbal medicines and herbal supplements.[12,15-17] Herbal cosmetics can cause undesirable conditions on the skin, such as irritation, phototoxicity, immediate-type of allergy *etc.*[15] However, it is difficult to access data regarding the incidence and causality assessment of adverse effects caused by the herbal products.[7] One of the main reasons for this is that a phytovigilance system is not available in many countries.

Phytovigilance system established in Italy has drawn attention. Research on the adverse effects of herbal food supplements used in weight control has revealed that safety-related risks are associated with product quality and self-medication. Spontaneous reporting is considered the only way to monitor these products.[16] Similarly, another retrospective analysis on the Italian Phytovigilance System Data examined herbal products used in children and determined that herbal products containing more than two active substances and those that are used along with conventional drugs have potential safety risks in children.[17]

In many studies conducted on herbal slimming products sold on the internet, there is adulteration. In a study, in which content analyzes were conducted by nine different herbal slimming products sold over the internet, it was seen that three of them contained sibutramine, three caffeine, three caffeine + temazepam, and the amount of sibutramine in each capsule was over 10 mg. These chemical substances were in high doses. They also contained trace toxic metals.[18]

It is seen that the other distribution channels, except the pharmacies, misinform public about the products, especially those that are sold over the internet. The fact that herbal products are easily accessible and that patients are directed to these products with incomplete and incorrect information increases the need for monitoring.[19]

Due to the increased safety concerns, many researchers recommend integrating herbal products into the existing pharmacovigilance system and using a single reporting form.[20] Similarly, a guideline on the monitoring of herbal medicines in the pharmacovigilance system was published by the World Health Organization (WHO) in 2004.[21] As emphasized by The International Pharmaceutical Federation, pharmacists need to play an active role in ensuring patient safety.[22] Considering that pharmacists provide consultancy and supply herbal products to the patient, the approach of pharmacists to such herbal products needs to be examined. Therefore, this study aims to understand the perspective, knowledge, attitude, and behavior of pharmacists, who are easily accessible as primary healthcare providers, about herbal products.

MATERIALS AND METHODS

Study design and population

A survey was conducted among community pharmacists between April 2019 and June 2019 in Istanbul. Face-to-face surveys were determined based on the number of pharmacies in Istanbul districts randomly. If a pharmacist was not available, the survey was conducted with the nearest pharmacy to make up a sufficient number in the same district. Each subgroup was weighted according to its representative share of the main population. In this context, the sample size of this study was calculated to be 879 community pharmacists at a 95% +/- 3 confidence level and cross-sectional analysis was performed.

Questionnaire design

The questionnaire was developed based on existing literatures.[23-32] The survey consisted of 30 questions and three sections: Pharmacists' socio-demographic and professional characteristics; pharmacists' knowledge and opinions about herbal medicines; pharmacists' behaviors and attitudes toward herbal medicines. The questions of third section consisted of a 5 point Likert-type scale ranging from "strongly disagree" to "strongly agree".

After obtaining ethical approval, a pilot study was conducted on 20 pharmacists, who were excluded from the main study. Thus, the final version of the questionnaire was comprised.

Statistical analysis

Parametric tests were used without the normality test according to the Central Limit Theorem.[33] In statistical analysis, student's *t*-test was used to compare the mean values of two groups. The one-way ANOVA test was used to compare the mean values of more than two groups. The significant difference found with ANOVA was confirmed with Tukey as the *post-hoc* test. Chi-square test statistics were used to evaluate the relationship between categorical variables. The mean and standard deviation, the minimum and maximum values of the variables were used when analyzing the continuous data in the scales; whereas, frequency and percentage values were used when analyzing categorical variables. E-picos New York software and the MedCalc statistics package program were used to evaluate the data. Statistical significance was defined as $p<0.05$.

RESULTS

The demographic data of the participants are listed in Table 1.

Our findings showed that the distribution of male and female was almost similar to 46.8% and 53.2%, respectively. Many pharmacists obtained bachelor's degree (86.3%). Since the highest rates in the years of practice were more than 21 years (25.1%) and 6-10 years (23.9%), the sample comprised both old and young pharmacists. Their average age was 40.4 ± 11.9. In terms of the location of pharmacy, most of the pharmacy were on a street (49.1%) and near a hospital (23.3%).

As seen in Table 2, it was determined that most of the pharmacists heard the concept of phytovigilance for the first time (58.1%). Others mostly had familiarity with this concept in undergraduate education (20.8%). The knowledge of the concept of phytovigilance differed by pharmacists' age ($p<0.001$). Similarly, there was a statistically significant difference between the working experience and the knowledge of the concept of phytovigilance ($p<0.001$). It has been determined that those who were young and had less working experience

gained information about the safety of herbal medicines during undergraduate education, while others learned phytovigilance from vocational training with the increase in working experience and age. According to these results, we can make an assessment that the importance given to phytovigilance and safety of herbal medicines has been increased over time and the education curriculum has been revised accordingly. However, the higher rate of those unaware of phytovigilance reveals that there is a need for additional training in herbal medicines.

On the other hand, it has been observed that 84.6% of pharmacists have never received training related to phytovigilance (n= 744). It was determined that 54.8% of those, who received training have received it during his/her undergraduate education.

93.5% (n= 822) of the participants have not completed any safety reporting for herbal medicines so far. The answer to the question of "In which case do you report adverse reactions?" was that "if the effect is serious" at a rate of 41.4% (n= 345). This is followed by the answers of "if the effect is unusual" (32.3%, n= 269), "if the effect is due to a new product" (25.9%, n= 216) and "if the adverse effect is certain" (25.2%, n= 210). However, it is impossible for a pharmacist to understand by himself/herself that the adverse reaction certain. This can only be decided by the national pharmacovigilance center through the analysis and evaluation of the reports. Achieving the minimum reportability criteria would be sufficient for the pharmacist to make a report.

Regarding the question of "where they should report the adverse effects", the answer of the majority of the pharmacists was Turkish Pharmacovigilance Center (TÜFAM), established within Turkish Medicines and Medical Devices Agency (TİTCK) (47.7%, n= 387).

However, it was observed that more than half of the participants did not know exactly how to obtain the suspected adverse drug reaction (ADR) reporting form (52.4% no, 14.6% partially).

A significant difference was observed between the working experience of the pharmacist and the knowledge of how to obtain the reporting form (p<0.006). According to the results, pharmacists with a working experience of 15 years or more did not know how to obtain the reporting form.

Regarding the barriers to reporting, pharmacists stated primarily as the well-known adverse reaction at a rate of 24.2% and the difficulty of reporting at a rate of 21.8%. This was followed by "Do not know reporting rules" (16.1%), "ADR forms not easily available" (12.7%), "Not enough information for reporting" (10.6%), "The patient's privacy" (5.1%), "Unsure if it is an ADR" (4.7%), "Time constraints" (2.7%), "The doctors' responsibility" (0.8%) "Fear of making mistakes" (0.7%), and "Not receiving feedback after reporting" (0.6%). These results again emphasize insufficient knowledge and the difficulty of the process (Figure 1).

When the pharmacists were asked to give an example of herbal medicine with potential adverse effects, 43.6% of them did not know any example, whereas 7.4% answered as digoxin and 6.6% answered as *Ginkgo biloba*.

A significant difference was determined between age and priorities in herbal product selection (p<0.05). Patient/consumer preference and price promotions were the primary reasons for product selection for young pharmacists, while the manufacturing company and efficacy of herbal product became a priority in selection of herbal products as the age increases.

Table 1. Socio-demographic characteristics of the pharmacists

Socio-demographics	The mean ± SD	Min - max
Age	40.4 ± 11.9	23-82
	Frequency (n)	**%**
Gender		
Male	411	46.8
Female	468	53.2
Educational level		
Bachelor	753	86.3
Master	105	12
Doctorate	15	1.7
Working experience		
1-5 years	190	21.7
6-10 years	209	23.9
11-15 years	142	16.2
16-20 years	115	13.1
21+ years	220	25.1
Location of community pharmacy		
Neighborhood pharmacy	116	13.2
Pharmacy on a street	432	49.1
Pharmacy near the hospital	205	23.3
Pharmacy near a family health center	123	14
Pharmacy in a mall	3	0.3

SD: Standard deviation, Min: Minimum, max: Maximum

Table 2. Correlation between pharmacists' age and the familiarity with the concept of phytovigilance

	n	%	Mean ± SD	p
Now, I learned in this survey	504	58.1	39.925 ± 12.08	
During undergraduate education	180	20.8	37.139 ± 9.48	
In continuous education programs	114	13.1	45.018 ± 11.67	
At the congresses	10	1.2	42.1 ± 15.358	**<0.001**
In scientific articles	44	5.1	44.886 ± 14.728	
From manufacturers	12	1.4	40.167 ± 11.907	
Other	3	0.3	44.667 ± 18.009	

SD: Standard deviation

Table 3. Correlation between pharmacists' years of practice and the priority of pharmacists in herbal product selection

Working experience (years)	1-5 n (%)	6-10 n (%)	11-15 n (%)	16-20 n (%)	20< n (%)	p
Product quality	49 (26.9)	70 (34)	47 (33.8)	30 (26.8)	47 (22.5)	
Manufacturing company	53 (29.1)	50 (24.3)	38 (27.3)	37 (33)	80 (38.3)	
Efficacy of the product	54 (29.7)	72 (35)	48 (34.5)	42 (37.5)	73 (34.9)	<0.002
Prices and promotions	15 (8.2)	9 (4.4)	6 (4.3)	2 (1.8)	6 (2.9)	
Patient/consumer preferences	11 (6)	4 (1.9)	-	1 (0.9)	3 (1.4)	

Similarly, a significant difference was also determined by working experience (p<0.002). It was found that pharmacists with a working experience of more than 20 years primarily selected herbal products provided in their pharmacy based on the manufacturing company primarily, whereas others selected herbal products based on efficacy of herbal product (Table 3).

To the question of "Why the safety of herbal medicines is important", 23% of the pharmacists responded as unknown adverse reactions (n= 202), 17% as the complex structure of plants (n= 149), 10.5% as poor knowledge (n= 92) and 10.1% as insufficient clinical trials (n= 89) (Table 4).

Table 4. Importance of the safety of herbal medicines

	n	%
The complex structure of the plants	149	17
Difficult standardization	74	8.4
Poor impurity	67	7.6
Unknown adverse reactions	202	23
Insufficient clinical trials	89	10.1
Low production quality	55	6.3
Adulteration/counterfeiting	81	9.2
Different distribution channels	70	8
Poor knowledge	92	10.5

A significant difference was determined between the importance of the safety of herbal medicines and the working experience of pharmacists (p<0.05). It has been observed that pharmacists with more than 25 years of working experience considered the unknown adverse reactions, whereas pharmacists who have less than 5 years of working experience remarkably considered difficult standardization of herbal products as important. This result could be related to the revised education curriculum over time (Table 5).

It is a remarkable result that 37.1% of the participants agreed to the statement that "I do not sell herbal products in my pharmacy". There was a significant difference between this statement and the location of the pharmacy (p<0.001). While neighborhood pharmacies strongly disagreed, hospital pharmacies agreed with this statement. We can assume that hospital pharmacies do not supply herbal medicines because they mostly provide prescription drugs and their patient profiles are different as compared to neighborhood pharmacies. Similarly, there was a statistically significant difference between this statement and the knowledge of the concept of phytovigilance (p<0.001).

Another remarkable result was that 47.4% of pharmacists agreed to the statement that "Herbal medicines have fewer side effects than synthetic drugs because they are natural", which is also a common understanding by public. Similarly, there was a significant difference between pharmacy location and this statement (p<0.009). It is observed that neighborhood pharmacies strongly agreed with this statement and were

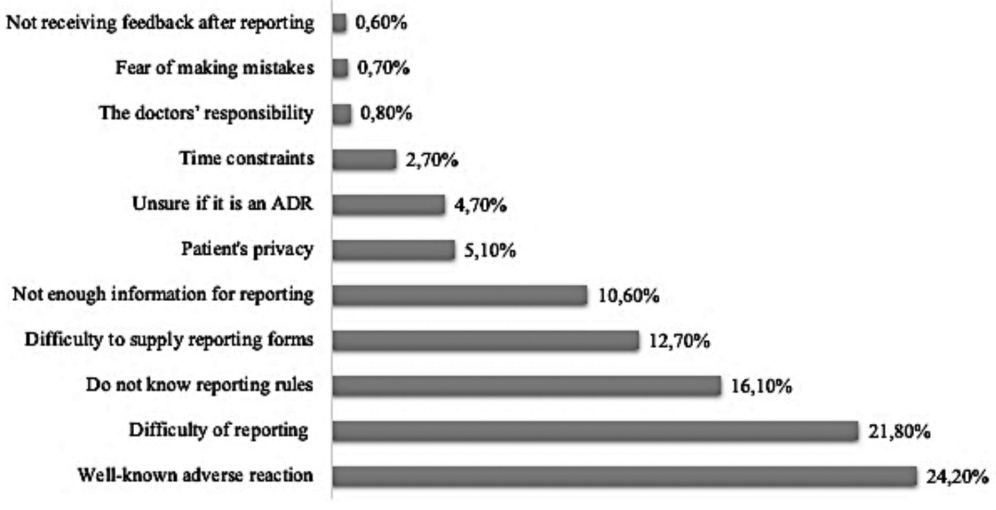

Figure 1. Barriers to the reporting of adverse reactions

more confident in herbal medicines compared with synthetic drugs.

Many pharmacists (74.8%) agreed that herbal medicines should only be sold in pharmacies. These results show that there may be pharmacists who supply herbal medicines only if they are sold only in pharmacies. Similarly, a significant difference was found with gender (p<0.006). It was determined that women were undecided, while male participants agreed with this statement. These data could be related to women's need for more detailed information.

Whereas 78.7% of pharmacists agreed to the statement that "Herbal medicines are used to maintain health and prevent diseases", more than two-thirds of pharmacists agreed to the statement that "Herbal medicines are used to treat diseases". There was a significant difference between gender and the statement that "Herbal medicines are used to treat diseases" (p<0.004). Accordingly, it was observed that male participants disagreed with this statement, while the female participants agreed.

71.4% of participants agreed that the therapeutic ineffectiveness of herbal medicines should be reported. 41.2% of pharmacists agreed to the statement that "Herbal medicines have adverse effects limited to hepatotoxicity and nephrotoxicity". In Türkiye, pharmacists can report only hepatotoxicity and nephrotoxicity-related adverse reactions to herbal medicines.

"The stability and expiration date should be questioned for herbal medicines" (75.5%) and "I inform my patients about the points that should be considered in the use of herbal medicines" (73.4%) statements were largely confirmed by community pharmacists.

A significant difference was found between gender and the statement that "I inform my patients about the points to be considered in the use of herbal medicines" (p<0.03). Male participants disagreed with this statement, while the female ones agreed. According to these results, it can be concluded that male participants give more importance to synthetic drugs than herbal medicines.

The pharmacists also stated that they can participate in awareness-raising campaigns for the safe use of herbal medicines as resolution advisory (58.1%). This result shows that pharmacists are eager to participate in such studies.

To encourage adverse reaction reporting, establishing phytovigilance contact points in hospitals was suggested at a rate of 20.9%, increasing informative campaigns at a rate of 18.6% and organizing more training for pharmacists at a rate of 17.9%. This was followed by increasing the priority of phytovigilance by the Ministry of Health (TİTCK, TÜFAM) (17.7%), increasing the role of pharmacists in improved phytovigilance system (16.1%), and the development of mobile applications that facilitate reporting (8.8%) (Figure 2).

DISCUSSION

Interest in herbal products has increased worldwide. The availability of herbal products from various sources raises safety problems.[8-14,18-19] At this point, there is a greater need for pharmacies and pharmacist counseling. In our study, it is found that pharmacists are eager to participate in informative activities, although they have a lack of knowledge and education.

Table 5. Distribution of the participants according to the Likert scale scores

	Strongly disagree, n (%)	Disagree n (%)	Neither agree nor disagree n (%)	Agree n (%)	Strongly agree n (%)
It is almost impossible to determine whether herbal medicine is responsible for a particular adverse reaction	34 (3.9)	158 (18)	320 (36.4)	290 (33)	77 (8.8)
I do not sell herbal products in my pharmacy	185 (21)	187 (21.3)	181 (20.6)	276 (31.4)	50 (5.7)
Herbal medicines are used to maintain health and prevent diseases	11 (1.3)	46 (5.2)	130 (14.8)	458 (52.1)	234 (26.6)
Herbal medicines are used to treat diseases	21 (2.4)	69 (7.9)	185 (21.1)	457 (52.2)	144 (16.4)
If the herbal medicine is ineffective, I report	12 (1.4)	44 (5)	195 (22.2)	461 (52.4)	167 (19)
Herbal medicines have fewer side effects than synthetic drugs because they are natural	100 (11.4)	174 (19.8)	188 (21.4)	320 (36.4)	97 (11)
The stability and expiration date should be questioned for herbal medicines	14 (1.6)	49 (5.6)	152 (17.3)	401 (45.6)	263 (29.9)
Herbal medicines have adverse effects limited to hepatotoxicity and nephrotoxicity	102 (11.6)	166 (18.9)	249 (28.3)	260 (29.6)	102 (11.6)
Herbal products should be sold only in pharmacies	12 (1.4)	40 (4.6)	169 (19.2)	352 (40)	306 (34.8)
I want to participate in awareness-raising campaigns for the safe use of herbal medicines	29 (3.3)	75 (8.5)	264 (30)	403 (45.8)	108 (12.3)
I inform my patients about the points that should be considered in the use of herbal medicines	13 (1.5)	59 (6.7)	162 (18.4)	428 (48.7)	217 (24.7)

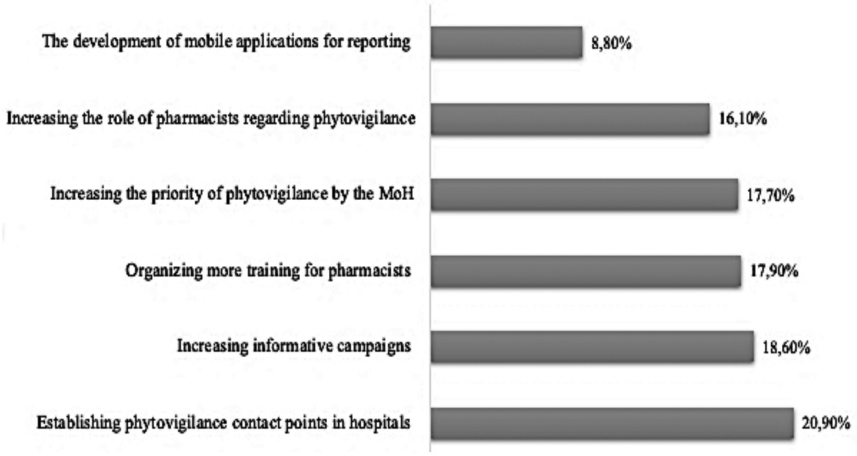

Figure 2. Suggestions for encouraging adverse reaction reporting

It was found that 58.1% pharmacists never heard of phytovigilance in our study. In contrary, in a study by Pellegrino et al.[34], it was found that 90% family pediatricians, who are other healthcare professionals, were able to answer correctly on the definition of phytovigilance in Italy.

Whereas in our study 13.1% of pharmacists stated that they learned the term of phytovigilance during continuous education programs and 42.3% of pharmacists sold herbal medicines, Chang et al.[35] showed that 45.1% of the participants had previous continuing education on herbal medications, and 73.6% sold herbal medicines.

In this study, it was found that more than half of the participants did not know exactly how to obtain the suspected ADR reporting form for herbal drugs (52.4% no, 14.6% partially). Similarly, Toklu and Uysal[36] showed that 87.6% of pharmacists admitted that they do not know how and where to obtain ADR reporting forms for medications in Türkiye.

When comparing the literature covering plants with adverse reactions, the most reported plants in VigiSearch are *Hypericum perforatum, Citrus x paradisi*, *Ginkgo biloba*, *Cannabis sativa*, and *Digitalis purpurea*.[20] The use of these herbal products without professional advice from a pharmacist or a physician increases the risk.[20] Similarly, *Ginkgo biloba* with its potential adverse effects was also stated by pharmacists, who participated in our study.

On the other hand, it has been determined that some pharmacists (37.1%) have negative perceptions of herbal products. It is thought that those perceptions will be changed in time by using improving the quality, efficacy and safety of herbal products and increasing related training of pharmacists.

Similar to the pharmacovigilance system, harmonization of the phytovigilance system is critical among countries. Manufacturers, healthcare professionals, and patients should be informed by providing the necessary training. At this point, pharmacists may provide guidance to the public. As recommended by WHO, it will be crucial to integrate phytovigilance into the pharmacy education curriculum and Good Pharmacy Practices (GPP).[3] The results of our study support this recommendation.

CONCLUSION

The phytovigilance systems established in some countries should be expanded to others. There is a need for a more user-friendly reporting system to increase adverse reaction reporting by pharmacists and other healthcare professionals.

It should be taken into account that pharmacy is a health center and a business center and pharmacists should have sufficient knowledge about the product (P), which is the main component of the marketing mix required for the sustainability of the business. This not only ensures the sustainability of the business but also contributes to public health through the safe use of herbal products. The pharmacist needs to guide the community at this point, however, it will be critical for the pharmacist to update knowledge about the herbal products first and focus on patient health with a holistic approach.

Not to ignore the patient's safety, authorities should establish a phytovigilance system that identifies the possible risks and monitors adverse reactions of herbal products.

Ethics

Ethics Committee Approval: Ethical approval was provided by Biruni University Ethical Committee (CSS ref: 2019-27-43).

Informed Consent: Informed consent was obtained from all individual participants included in the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

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

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

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ORIGINAL ARTICLE

A Folk Medicine: *Passiflora incarnata* L. Phytochemical Profile with Antioxidant Potency

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ABSTRACT

Objectives: *Passiflora incarnata* L., commonly called folk medicine declaredly used for an enormous range of therapeutic purposes, one such is antioxidant potency. The study prioritized to determine the phytochemical analysis of total phenolics, flavonoids, alkaloids, and tannins contents as well as the antioxidant properties through 1,1-diphenyl-2-picrylhydrazyl (DPPH) quenching assay, 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) cation decolorization test, superoxide, and hydrogen peroxide radical scavenging assays of ethanol extract of *P. incarnata* leaves.

Materials and Methods: The organoleptic characteristics such as color, odor, appearance, taste, and other characters such as drying range and fiber contents were analyzed as preliminary data. Analytical parameters like total phenolic content, total tannins, total alkaloid content, and total flavonoid with multiple antiradical scavenging activity (DPPH, ABTS, superoxide and H_2O_2 scavenging assays) with IC_{50} (µg/mL) in terms of inhibition percentage with various concentrations of the ethanolic extract studied.

Results: *P. incarnata* possessed a high radical scavenging activity with a phenolic content of 2.48 mg gallic acid equivalent/g of extract in leaves, whereas the total flavonoid content was 2.1, respectively.

Conclusion: High antioxidant activity was noticed in *P. incarnata* extract, in which might be of higher levels of flavonoids and phenols. Findings in the studies revealed that *P. incarnata* is a veritable source for antioxidant drug bioprospecting in scientific research and pharmaceutical industries.

Key words: *Passiflora incarnata*, phytochemical profile, antioxidant activity, DPPH, ABTS, radical scavenging activity

INTRODUCTION

Singlet oxygen is formed in the biological system in aerobic organisms for normal cell functions, if exceed, the reactive oxygen species (ROS) level in the living system causes oxidative stress and leads to oxidative damage. These ROS intermediate threaten various biomolecules including proteins, enzymes, lipids, and DNA[1,2] and also cause physiological errors like blocks in arteries, strokes, cancer, and nervous disorders, which highly possess to increase in the study of compounds that protect against ROS and can prevent diseases.[3,4] As a result, high priority to be enlightened to the purpose of antioxidants, particularly organic-based to protect from damage due to free radicals.[5] Antioxidants, compounds that can delay or prevent the oxidation of biomolecules by blocking engagement of oxidative progress that can avoid or restore the damage by ROS.[6] However, synthetic antioxidants, *e.g.* propylgallate, butylated hydroxyanisole, butylated hydroxytoluene, and tertiary butyl-hydroquinone, are recognized as good oxidative costs, yet, they have limitations because of their carcinogenic effects on the lungs and liver parts.[7] So, recently, several excessive efforts have been outworthed to safeguard potent organic tagged antioxidants from ethnomedicinal sources.

Natural antioxidants, principally from plants with that sort of phenolic compounds, vitamin C, and carotenoids. Ethnomedicinal-based compounds have multiplex biological effects, including antioxidant potentials with its phytoconstituents including phenolics and flavonoids.[8] Nutritional components from plants are the dynamic cause of various classes of polyphenolic components and some flavonoids.[9] Some phytomedicines are traditional, among them, *Passiflora* sp. has been reported as folk medicine. *Passiflora* genus, Passifloraceae, includes about 520

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species,[10] which are spotted mostly in tropical and subtropical regions of the world. *Passiflora incarnata* L., *P. alata* Curtis, *P. mucrinata* Lam., and *P. edulis* Sims reveal their potential biological activity by its various phytometabolites such as phenolic substances, alkaloids, and flavonoid contents, and it is known for its sedative properties as well as in the food and pharmaceutical industry.[11] In spite of extensive research on plant products, the efficacy of plant sources as novel drugs is still meagerly documented. Only a least fraction has been studied phytochemically and therefore, the fractions recommended for biological or pharmacological screening are even smaller. Hence, an attempt made to evaluate the leaves of *P. incarnata* to reveal its antioxidant potential.

MATERIALS AND METHODS

Collection of plant material

P. incarnata was collected from Keezanatham, Ariyalur (Dt.), Tamil Nadu, India (Figure 1). Identification of plant material was validated by Prof. Jegadeesan Head, Department of Environmental and Herbal Sciences, Tamil University, Thanjavur). Furthermore, it was confirmed with Herbarium sheets available in the Rabinat Herbarium, St. Joseph's College, Thiruchirappalli, Tamil Nadu, India. The specimen was kept in the Herbarium of Arignar Anna Government Arts College, Department of Botany (AAGAC/BOT-07). The fresh and fully-grown plant leaves were selected. The collected plant leaves were cleaned to remove mud and other adhering weed plants. Fresh leave samples were desiccated at the room temperature and then shade-dried for 2-3 days and powdered mechanically, sieved using 80 meshes, and refrigerated.[12]

Preliminary phytochemical studies

Organoleptic characters

Color, odor, appearance, taste and other characters like drying range and fiber contents of the grounded sample were determined. The sample (2 g) was allowed to dry in a tarred dish and with the temperature of 100-105°C, it was then allowed to cool and weigh again.

Figure 1. *Passiflora incarnata* L.
Photo courtesy: P. Subramaniam

Analytical parameters

Total ash

5 g sample was exposed *in silica* crucible, which is ignited prior, allowed to cool and weighed. It was allowed to incinerate with slow progression of heat, up to 450°C, allow to cool and weigh again. The percentage of total ash was calculated using the reference range and repeated again until, a constant weight was noted.

Acid-insoluble ash

Total ash was allowed to boil with 25 mL (10%) of diluted HCl for about 5 min and filter it, then ignite it to obtain acid insoluble ash.

Water-soluble ash

To check the water-soluble ash, a portion of the total ash was allowed to boil in 25 mL of H_2O for 5 min and wash the filtered debris was with hot water. The water-soluble ash was calculated.

Water soluble ash= Total ash value - Water-insoluble ash

Sulfated ash

A portion of the sample was ignited with 1 mL of H_2SO_4. It was then cooled, and percentage of sulfated ash was calculated.

Extractive values

The dried sample (5 g) was impregnated with moderately hot petroleum ether overnight. The extract was concentrated and weighed.[13]

Preparation of the extract

The shade-dried leaves were pulverized to get a coarse powder.[14] The ground plant material (1 kg) was soaked separately in ethanol for 48 hrs. The aqueous extraction was gained through filtration method. The extract was then subjected to dryness in an evaporator under controlled pressure and temperature (40-50°C).

Chemicals

2,2-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 1, Folin-Ciocalteu's phenol reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, Folin-Danis reagent, bromocresol green solution (BCG), dimethyl sulfoxide (DMSO), potassium persulfate, methanol, and sodium carbonate were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Estimation of total phenolic content

The extract was allowed to incubate for 5 minutes prior with 0.3 mL of Folin-Ciocalteu's reagent. Na_2CO_3 (7%, 10 mL) solution was mixed and incubated for 2 h absorbance was measured at 740 nm. Quantification was conducted using a gallic acid (GA) as the standard. The results are expressed as milligrams *per* gram dry weight.[15]

Estimation of tannins

Folin-Danis reagent (0.5 mL) was added to each tube containing different concentrations of the sample and kept for 3 minutes. Further, 2 mL of 20% Na_2CO_3 solution was added and gently vortex. The test tubes were kept in boiling

for 1 min and cooled down. The absorbance was measured at 650 nm.[16]

Estimation of total alkaloid content

A portion of extract residue was dissolved in 2N HCL for 20 minutes and then filtered. 1 mL solution was transferred to a separatory funnel and washed with 10 mL chloroform (thrice). The hydrogen ion range of the solution was adjusted to neutral. BCG with phosphate buffer (5 mL) was mixed with the mixture. The extract with chloroform was mixed by continuous shaking, then the extract was collected in a 10 mL flask, and diluted again with chloroform. The precipitate was collected and dried at 105°C to constant weight and weighed.

Estimation of total flavonoid content

The sample (10 g) was impregnated with 60 mL methanol and allowed to stand overnight. The residue was filtered and washed twice with 20 mL methanol. Filtrate was washed and concentrated to 10 mL. The concentrated solution was added drop wise continuously shaken into 100 mL ether. Mix vigorously for 10 minutes and allowed to stand for 10 minutes to settle. Filtrate was evaporated to dry and calculated.

In vitro antioxidant activity

DPPH radical scavenging activity

The plant extract at different concentrations was diluted with DMSO to get a sample solution. The sample (5 µL) was seeded in a 96-well plate followed by 195 µL DPPH working solution to each well. After 20 min reaction, the absorbance was measured at 515 nm. The free radical activity of the extract was determined by comparing its absorbance with blank.[17] The scavenging ability by DPPH radical was expressed as a percentage of inhibition and was calculated.

DPPH scavenging activity (%)= $(A_0 - A_1)/ A_0 \times 100$

where A_0: Absorbance of the control, and A_1: Absorbance of the sample.

ABTS radical scavenging activity

ABTS radical was formed with the addition of 5 mL of ABTS stock solution and 2.45 mM $K_2S_2O_8$ solutions respectively, and stored in the dark at room temperature for 16 hs. Before use, this solution was diluted and the absorbance was notably at 0.700 ± 0.020 at 734 nm and maintained at 30°C. The extract at various concentrations was diluted with DMSO, which counts for the sample solution. 5 µL of sample solution was mixed with 195 µL ABTS + solution, and incubated at room temperature for 6 min and the absorbance was recorded at 734 nm.[18] ABTS scavenging activity was expressed as IC_{50} (µg/mL) and the inhibition percentage was calculated.

ABTS scavenging activity= $(A_0 - A_1) / A_0 \times 100$

Hydrogen peroxide scavenging activity

Hydrogen peroxide was prepared with phosphate buffer (pH 7.4). Different concentrations of sample (200 µL) were mixed with 0.6 mL of H_2O_2 solution. A test tube containing 200 µL of phosphate buffer was processed as discussed above act as a control. Different concentrations of ascorbic acid were used as the reference compound. Absorbance of H_2O_2 was determined against a blank.[19]

Superoxide scavenging activity

The sample (200 µL) of different concentrations was taken in test tubes. Superoxide radicals were produced by equal addition of 1 mL of Tris-HCl buffer (16 mM, pH-8), nitro blue tetrazolium (50 µM), nicotinamide adenine dinucleotide (78 µM) solution and phenazine methosulphate (10 µM) respectively. The mixtures were then incubated at 25°C for 5 min and measured the absorbance (560 nm).[20]

Statistical analysis

The experimental results were expressed as ± standard deviation. Data were analyzed with ANOVA and determined by Duncan's Multiple Range test using Graph Pad Prism software version 5.0 (San Diego, USA).

RESULTS

The organoleptic characteristics and analytical parameters of the leaf powder of *P. incarnata* were studied (Table 1). The ratio of active chemical components in unpolished drugs are mainly based on air-dried. Therefore, ranging the dryness of plant materials should be evaluated, particularly for the materials that imbibe moist easily. The residue remaining after incineration of the plant material is the ash content, which simply represents some inorganic salts and occurs naturally in unprocessed crude drug materials.

Phytochemical analysis

The total phenolic content in examined leaf extract was 2.8 mg GA equivalent/g and showed flavonoids (2.1 mg/g) but in merger range compared with phenolic contents (Table 2). Total tannin content of *P. incarnata* leaf extracts show 1.9 mg/g and alkaloids 0.031 mg/g. The high antioxidant activity was observed in *P. incarnata* extract, which evidenced the high level of flavonoids and phenolic presence in the plant (Table 3).

Table 1. Organoleptic characteristics of *Passiflora incarnata* leaves

Appearance	Coarse powder
Color	Green
Odor	No characteristic
Taste	Slightly bitter
% Loss on drying	7.12
Crude fiber	9.4

Table 2. Analytical parameters

Parameters	Value in w/w
Total ash	9.23
Acid-insoluble ash	10.3
Water-soluble ash	6.50
Sulfated ash	18.20

In vitro antioxidant activity

Inhibition% of DPPH by the extract at different concentrations (1, 2, 4, and 8 µg/mL) was observed to be 17.43 ± 0.31, 30.12 ± 0.29, 51.69 ± 0.43, and 80.91 ± 0.37, respectively, whereas the percentage inhibition of ascorbic acid was found to be 22.13 ± 0.28, 39.87 ± 0.33, 57.28 ± 0.25, and 82.55 ± 0.41. IC_{50} values for DPPH scavenging activity of the leaf extract of *P. incarnata* and ascorbic acid were 4.30 µg/mL and 3.69 µg/mL, respectively (Figure 2, Table 4). The extract exhibited good ABTS radical scavenging activity and inhibition% (with various concentration 1, 2, 4, 8 µg/mL) was found to be 14.65 ± 0.17, 32.16 ± 0.24, 49.87 ± 0.30, and 75.23 ± 0.33, respectively, which were comparable to that of ascorbic acid 19.32 ± 0.21, 40.28 ± 0.27, 60.71 ± 0.31, and 77.56 ± 0.29. IC_{50} values for scavenging ability on ABTS radical of the extract of *P. incarnata*, while IC_{50} values by ascorbic acid were 4.60 µg/mL and 3.82 µg/mL, respectively (Figure 3, Table 5).

P. incarnata leaf extracts exhibited a strong scavenging effect on hydrogen peroxide were recorded as 12.43 ± 0.87, 28.57 ± 0.67, 41.32 ± 0.72, and 62.84 ± 0.60 respectively (Figure 4, Table 6). Contrast, 18.21 ± 0.52, 39.52 ± 0.41, 50.43 ± 0.39, and 66.16 ± 0.47 was noted in standard ascorbic acid. IC_{50} values for H_2O_2 scavenging activity for ethanol extract of leaves of *P. incarnata* and ascorbic acid were 5.79 and 4.80 µg/mL. Superoxide radical scavenged by the plant extract at different concentrations were observed as 20.15 ± 0.45, 29.42 ± 0.38, 48.88 ± 0.35, and 74.37 ± 0.51 for the leaf extract, while the reference was found to be 25.53 ± 0.29, 37.19 ± 0.33, 55.26 ± 0.34, and 77.55 ± 0.39 respectively. IC_{50} value of ascorbic acid was 3.90 µg/mL, while the leaf extract's was 4.63 µg/mL (Figure 5, Table 7). Values are the average of triplicate and represented as mean ± standard deviation.

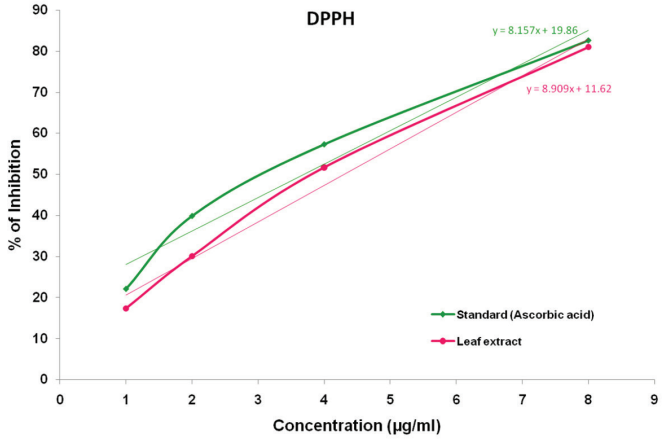

Figure 2. DPPH radical scavenging activity

Table 3. Polyphenol content of the ethanolic leaf extract of *Passiflora incarnata*

Total phytochemicals	Leaf extract
Total phenolic content	2.48
Total tannins content	1.9
Total alkaloid content	0.031
Total flavonoid content	2.1

DISCUSSION

Uptrended studies have suggested the prevalence with increased levels of plant-derived compounds with low optional remedy rates of many diseases. These results suggest a connection between the protecting role of plant compounds in

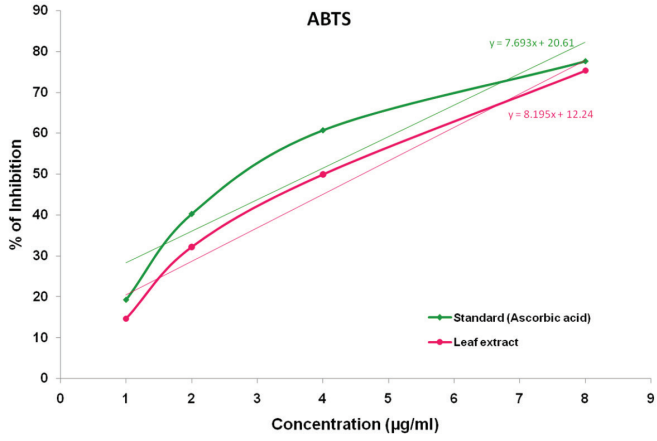

Figure 3. ABTS radical scavenging activity

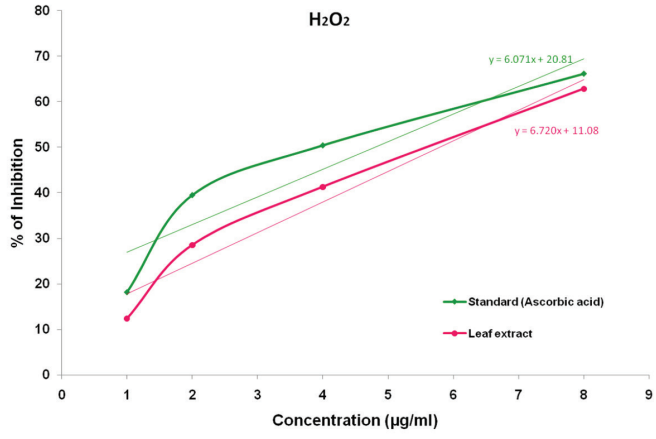

Figure 4. H_2O_2 scavenging activity

Table 4. DPPH

Group	Concentration (µg/mL)	% of inhibition	IC_{50} value
Ethanol extract of leaves of *Passiflora incarnata*	1	17.43 ± 0.31	4.30 µg/mL
	2	30.12 ± 0.29	
	4	51.69 ± 0.43	
	8	80.91 ± 0.37	
Ascorbic acid	1	22.13 ± 0.28	3.69 µg/mL
	2	39.87 ± 0.33	
	4	57.28 ± 0.25	
	8	82.55 ± 0.41	

increasing the average life span of human health. Plant derived compounds have an enormous range of polyphenols, which play a prime role in minimizing the balance between free radicals and antioxidant potential. Substantially, it is needed to optimize these flavonoids to be acknowledged for their efficient action.[21]

Accumulated evidence has suggested that the ethanolic extracts of *P. incarnata* showed a prominent and potent *in vitro* antioxidant activity with high flavonoid contents (2.1 µg/mL). Flavonoids - a phenolic substance, labeled for its phytoconstituents of *P. incarnata*[22] that can steadily repair the unpaired electrons located in its aromatic ring and can minimize the cause of free radical development. There are scientific reports showing that the number of phenolic compounds is directly proportional to antioxidant activity.[23] *In vitro* study has also evidenced a potential activity in aqueous and ethanolic extracts of *P. incarnata*.[24] A group of scientists from Italy studied the methanol extracts from five species of *Passiflora* obtained by zygotic embryo culture showed DPPH and ABTS radical scavenging activity.[25] Among the flavonoid constituents, in *P. incarnata*, vicenin, isovitexin, and orentin have major roles in the *in vitro* antiradical scavenging activity.[26] A number of flavonoid glycosides - isochaftoside, schaftoside, isoorientin, orientin, vitexin, and isovitexin are considered standard markers to identify different *Passiflora* species,[27] such as *P. edulis, P. incanata, P. tripartita*. Isoorientin, major flavonoid credentials contributing in poly(2-methoxyethyl acrylate) fractions can be the most accountable for the antioxidant activity. Moreover, *in vivo* approaches, Wistar rats were gavaged with vitexin and isovitexin, noticing the increasing antioxidant capacity.[28] Most pharmacological studies have demonstrated effects on central nervous system, *e.g.* anxiolytic, sedative action, and anticonvulsant properties. About 294 volatile compounds have been identified in several passion fruit extracts.[29] From the results of this study, it is concluded that the ethanol extract of *P. incarnata* with its remarkable phytochemical profile can ensure the promising antioxidant potential.

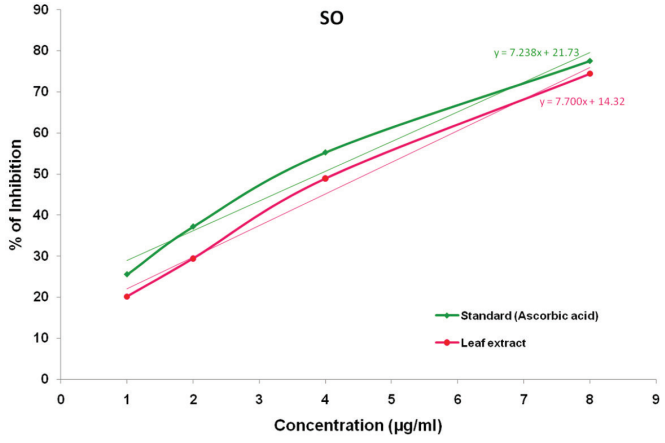

Figure 5. Superoxide scavenging activity

Table 5. ABTS

Group	Concentration (µg/mL)	% of inhibition	IC_{50} value
Ethanol extract of leaves of *Passiflora incarnata*	1	14.65 ± 0.17	4.60 µg/mL
	2	32.16 ± 0.24	
	4	49.87 ± 0.30	
	8	75.23 ± 0.33	
Ascorbic acid	1	19.32 ± 0.21	3.82 µg/mL
	2	40.28 ± 0.27	
	4	60.71 ± 0.31	
	8	77.56 ± 0.29	

CONCLUSION

In green chemistry, the influence of basic phytochemical extraction methods was studied in the *P. incarnata* leaves in order to screen or to obtain its high phenolic compounds. This study investigated the leave extract act as an antioxidant agent in *in vitro* studies. The results revealed that the flavonoid contents present in *P. incarnata* (2.1 µg/mL) can be an optional to decrease or eradicate the ROS in active levels. Recently, the use of therapeutic ethnomedicinal products has been reliable with adverse effects. *P. incarnata* is a nutraceutical enriched with bioactive compounds, which are evident to possess it as

Table 6. H_2O_2

Group	Concentration (µg/mL)	% of inhibition	IC_{50} value
Ethanol extract of leaves of *Passiflora incarnata*	1	12.43 ± 0.87	5.79 µg/mL
	2	28.57 ± 0.67	
	4	41.32 ± 0.72	
	8	62.84 ± 0.60	
Ascorbic acid	1	18.21 ± 0.52	4.80 µg/mL
	2	39.52 ± 0.41	
	4	50.43 ± 0.39	
	8	66.16 ± 0.47	

Table 7. SO

Group	Concentration (µg/mL)	% of inhibition	IC_{50} value
Ethanol extract of leaves of *Passiflora incarnata*	1	20.15 ± 0.45	4.63 µg/mL
	2	29.42 ± 0.38	
	4	48.88 ± 0.35	
	8	74.37 ± 0.51	
Ascorbic acid	1	25.53 ± 0.29	3.90 µg/mL
	2	37.19 ± 0.33	
	4	55.26 ± 0.34	
	8	77.55 ± 0.39	

folk medicine for many years and possibly subsidizes to the prevention and cure of many disorders. Further investigations are required to determine the potential use of *P. incarnata* leaves in the pharmaceutical fields could be considered.

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Ethics

Ethics Committee Approval: Not applicable.

Informed Consent: Not applicable.

Authorship Contributions

Concept: S.P., W.E.G., Design: N.B.M., Data Collection or Processing: H.S.R.M., W.E.G., Analysis or Interpretation: S.P., N.B.M., Literature Search: H.S.R.M., N.B.M., Writing: H.S.R.M., N.B.M.

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

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ORIGINAL ARTICLE

Analysis of Drug-Related Impurties by HPLC in Ciprofloxacin Hydrochloride Raw Material

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ABSTRACT

Objectives: In this study, we report the quality control results of drug-related impurity analysis of seven raw materials of ciprofloxacin hydrochloride marketed in Algeria.

Materials and Methods: According to the European Pharmacopoeia (Eur. Ph.), high-performance liquid chromatography (HPLC) was used to analyze (B, C, D and E) impurities, while thin layer chromatography (TLC) used to control impurity A.

Results: HPLC analysis showed that the C1, C2, C3, C4, and C6 samples have individual contents of specified impurities (B, C, D, E), unspecified and the total of all present impurities conform to norms. The C5 sample contains a very high content (0.579%) of impurity C, which is a photodegradation product and the impurities total (0.625%) exceeding limit, while C7 sample has a slightly higher content (0.118%) of unspecified impurity. The control solution of impurity A was not migrated in all developed TLC plates, so the system is not compliant, for this reason, an HPLC analysis protocol was developed.

Conclusion: The results showed that impurity A content conformed in all samples except for the C6 sample, which has equal content to the limit. Therefore, we recommend revising the detecting technique of impurity A by TLC in the Eur. Ph. or replacing it with a more sensitive technique such as HPLC.

Key words: Drug-related impurities, specified, HPLC, TLC, ciprofloxacin hydrochloride

INTRODUCTION

The identification and quantification of impurities in raw materials is critical to ensure effective and safe treatment. So, impurity control is a key component and a big challenge in the pharmaceutical industry.[1,2] Impurities relate to starting materials, by-products, breakdown products or polymorphs. They can appear at active pharmaceutical ingredient (APIs) production level as well as during or after the formulation process. Their concentrations may change upon storage of the product.[2,3]

Chemical determination of related impurities in APIs is important because a long exposure at low concentrations, can have undesirable side effects or toxicity and/or may interfere with the drug's activity.[3,4] There are no toxicity studies for the majority of impurities, so impurity analysis is a critical step in quality control.[5,6] Therefore, specific requirements for impurities are set by the regulatory authorities.[6,7]

Ciprofloxacin hydrochloride (CPF HCl) (Figure 1) is a synthetic antibiotic that is part of the list of essential drugs established by the World Health Organization (WHO), manufactured by several generic laboratories in Algeria, their high rate of prescription by clinicians thanks to their numerous indications in the different infections (gynecological, urinary, digestive, and respiratory, *etc.*). CPF HCl has several associated impurities, which are well described and defined in European Pharmacopoeia (Eur. Ph.) 8th edition. The specified impurities are A, B, C, D, and E, which are individually cited and limited by a specific acceptance criterion, while the impurity F is not specified that is present but limited by an overall acceptance criterion.[8] According to Eur. Ph., impurities B, C, D, and E are searched by high-performance

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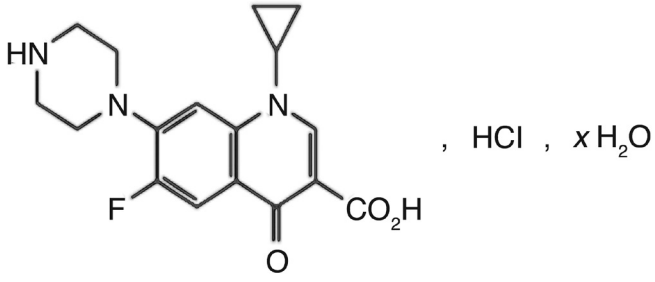


Figure 1. Chemical structure of CPF HCl[8]

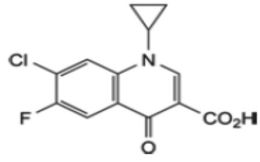
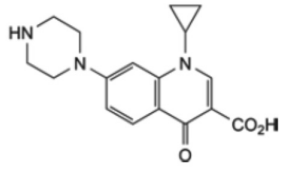
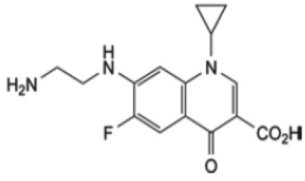
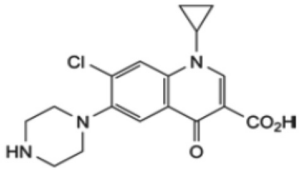
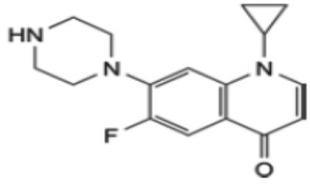
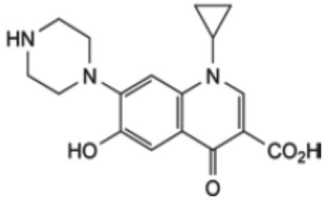
liquid chromatography (HPLC), while impurity A by thin layer chromatography (TLC) (Table 1).

In this paper, we analyzed and evaluated the drug-related impurities of seven samples of CPF HCl APIs marketed in Algeria using HPLC.

MATERIALS AND METHODS

Seven samples of CPF HCl were collected from pharmaceutical producers located in Algeria.[9] They are labeled as follows: C1, C2, C3, C4, C5, C6, and C7 (Table 2).

Table 1. Related substances of CPF-HCl[7,8]

Origin	Impurity	Structure	Analysis method
Synthesis by-product	**Impurity A** (specified) Fluoroquinolonic acid: 7-chloro-1-cyclopropyl-6-fluoro-4-oxo-1, 4-dihydroquinoline-3-carboxylic acid		TLC
Synthesis by-product	**Impurity B** (specified) Defluorinated derivative: 1-Cyclopropyl-4-oxo-7- (piperazin-1-yl) -1, 4-dihydroquinoline-3-carboxylic acid		HPLC
Photodegradation product	**Impurity C** (specified) Ethylenediamine derivative: 7 - [(2-aminoethyl) amino] -1-cyclopropyl-6-fluoro-4-oxo-1, 4-dihydroquinoline-3-carboxylic acid		HPLC
Synthesis by-product	**Impurity D** (specified) 7-Chloro-1-cyclopropyl-4-oxo-6- (piperazin-1-yl) -1, 4-dihydroquinoline-3-carboxylic acid		HPLC
Degradation products resulting from decarboxylation	**Impurity E** (specified) Dicarboxylic derivative: 1-cyclopropyl-6-fluoro-7- (piperazin-1-yl) quinolin-4 (1H)-one		HPLC
Hydroxylation product	**Impurity F** (unspecified) 1-Cyclopropyl-6-hydroxy-4-oxo-7- (piperazin-1-yl) -1, 4-dihydroquinoline-3-carboxylic acid		HPLC

Research and quantification of impurities B, C, D, and E by HPLC

Standards, reagents, and apparatus

The standard impurities "CPF HCl for identification of SCR peaks (containing impurties B, C, D, and E)" were purchased from Eur. Ph. (Strasbourg, France). Acetonitrile (HPLC grade), triethylamine and phosphoric acid were produced by Sigma-Aldrich.[8]

An HPLC-ultraviolet (UV) device (Thermo Scientific Dionex UltiMate 3000 Rapid Separation LC systems) equipped with an automatic injector and UV detector.

Analysis protocol

Mobile phase: Thirteen volumes of acetonitrile were mixed with 87 volumes of phosphoric acid at 2.45 g/L.

Test solution: 25 mg of CPF HCl of each sample was dissolved in 50 mL of mobile phase.

Control solution (c): 1 mL of the test solution was diluted in 500 mL of mobile phase.

Control solution (b): 2.5 mg of CPF HCl for identification of SCR peaks is dissolved in 5 mL of mobile phase.

Chromatographic conditions: Temperature: 40°C; flow: 1.5 mL/min; injection volume: 50 µL of control solution (b) and (c); detection: 278 nm; column C18: 5 µm, 250 × 4.6 mm.[8]

Research on impurity A by TLC

Standards, reagents, and apparatus

The standard "impurity A of CPF SCR" was purchased from Eur. Ph. acetonitrile (HPLC grade), ammonia, dichloromethane, and methanol were obtained from Sigma-Aldrich. Silica gel plate F254 for TLC, chromatography tank, and UV lamp at 254 nm were from CAMAG.

Procedure

Test solution: 50 mg of CPF HCl was dissolved in 5 mL water.

Control solution: Impurity A standard (10 mg) is dissolved in a mixture (0.1 mL of diluted ammonia and 90 mL water), completed to 100 mL with water, and 2 mL is diluted in 10 mL of water.

Mobile phase: Acetonitrile, concentrated ammonia, methanol, and methylene chloride (10:20:40:40 V/ V/ V/ V); the deposit volume: 5 µL.

Development: At the bottom of the chromatography tank, a container of 50 mL concentrated ammonia was deposited. The vessel was closed, and the plate was exposed to ammonia vapors for 15 min. The plate was developed on ¾; drying in air and exanimated under UV at 254 nm.

Limits

The sample is compliant, if the impurity A spot is not more intense than main spot of the control solution (0.2%).[10]

Research and quantification of impurity A by HPLC

Analysis protocol

Mobile phase: Acetonitrile (50 volumes) were mixed with 50 volumes of phosphoric acid at 2.45 g/L.

Standard stock solution: Standard (5 mg) is dissolved in 50 mL of mobile phase.

To determine the maximum absorption of impurity A, the standard solution was scanned in UV over a range of 200 to 400 nm.

The establishment of the calibration curve: Five dilutions were prepared from the standard stock solution (0.1 mg/mL) (Table 3).

Test solution: CPF HCl (50 mg) was dissolved in 5 mL of water.

Chromatographic conditions: Temperature: 25.9°C, flow: 1.5 mL/min, injection volume: 20 µL, detection at 260 nm, column C18: 5 µm, 150 × 4.6 mm.

Table 2. Collection of CPF HCl raw material from local producers

Sample	Local producer	Batch number	Expiration date	Manufacturer/supplier
C1	Lab C1	A004801	04/2017	Unknown
C2	Lab C2	CIC 0074	01/2017	Baselux (Spain)
C3	Lab C3	CICA 4066	12/2019	Chemo (Swiss)
C4	Lab C4	10271610	07/2018	Dr. Reddy's Laboratories (India)
C5	Lab C5	120801	08/2016	Pharmaceutical Co. Ltd. (China)
C6	Lab C6	KOFA0062	03/2017	Dr. Reddy's Laboratories (India)
C7	Lab C7	0251103F	07/2019	Unknown

Table 3. Dilution range of the calibration curve

	1st Dilution	2nd Dilution	3rd Dilution	4th Dilution	5th Dilution
Stock solution (mL)	0.5	1	1.5	2	2.5
Solvent (mL)	9.5	9	8.5	8	7.5
Diluted solution (%)	0.05	0.10	0.15	0.20	0.25

System compliance: Linearity of the calibration curve with a correlation coefficient greater than 0.990. The symmetry factor of the impurity (A) peak must be between 0.8 and 1.5.

Identification of impurity A: Its retention time.

Results expression

The impurity A content of each sample is expressed by extrapolating its area on the calibration curve: y = a X + b

y: Impurity (A) area, X: Impurity (A) concentration (%)

Calculus formula of impurity A content

$$\textbf{Impurity A content}\ (\%) = \frac{(\text{Peak Area} - b)}{a} \times \frac{50}{\text{Wieght (mg)}}$$

In this study, there was no statistical data analysis.

RESULTS AND DISCUSSION

Research and quantification of impurities B, C, D, and E by HPLC

System compliance

Figures 2 and 3 showed the obtained chromatogram of control solution (b) and typical chromatogram.

These two chromatograms were superimposable and comparable, which enabled us to identify the CPF HCl main peak and impurity E, B, C, and D peaks corresponding.

Retention time (RT) of CPF HCl is 8.962, a value close to that required by Eur Ph that must be at about 9 min. The RT obtained for each impurity (E, B, C, and D) is respectively (3.547 min, 5.977 min, 6.650 min and 11.855 min). All these values are close to those given in the standard chromatogram or calculated from RRT (RT_impurity E: 3.58 min, RT_impurity B: 5.377 min, RT_impurity C: 6.273 min, RT_impurity D: 10.754 min).

The resolution between peaks of impurity B and C is 3, value complies with the required standard (at least 1.3). The symmetry factor of the CPF HCl peak is 1.4, conforming to Eur. Ph. standard (between 0.8 and 1.5). The symmetry factor of peaks belonging to impurities E, B, C, and D (1.16, 1.31, 1.30, and 1.17). All these values were conformed. Therefore, the system compliance is validated.

Sample analysis

Obtained chromatograms of the sample analysis were presented in Figures 4-6, and 7. Table 4 presents individual contents of (B, C, D, E, unspecified) impurity, and the impurities total.

According to the Eur. Ph. standards, the individual content of impurities B, C, and D must be less than or equal to 0.2%, impurity E, less than or equal to 0.3% and unspecified impurity less than or equal to 0.1%. Any other impurity with individual content less than or equal to 0.05% (exclusion limit) shall not be taken into consideration. The impurities total content shall not exceed 0.5%.

Samples C1, C2, C3, C4, and C6 have individual content of specified impurities B, C, D, and E or unspecified, and the impurities total in the required standards.

C5 sample contains very high content (0.579%) of impurity C compared to the limit, and a total (0.625%) exceeding the norm. This explains that the sample has degraded in impurity C, which is a photodegradation product despite having been well preserved. This result is consistent since the sample was analyzed in date close to its expiry date (August 2016) or it degraded during handling.

C7 sample has individual content of unspecified impurity (known structure such as impurity for unknown structure) equal to 0.118%, slightly higher than the general acceptance criterion and a total in the norm.

Research on impurity A by TLC

Figure 8 shows the TLC plates revelation under UV lamp. The first plate revealed 4 main spots corresponding to test solutions of C1, C2, C3, and C4 samples and no spot of the control solution appeared. The second plate revealed three main spots corresponding to the test solution of C5, C6, and C7 samples and no spot of the control solution appeared. Because of the absence of control migration, a third plate was prepared in

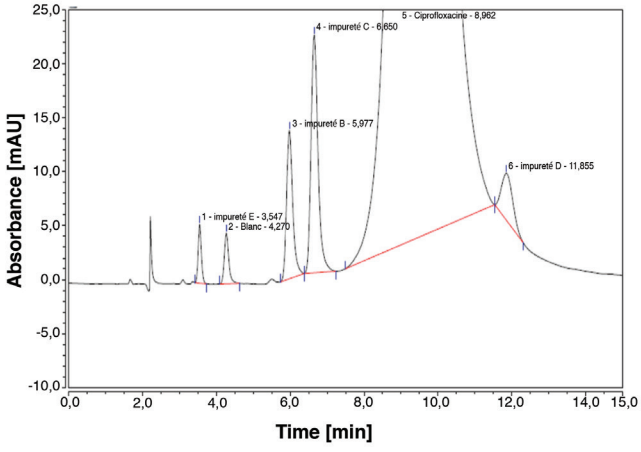


Figure 2. Chromatogram of control solution

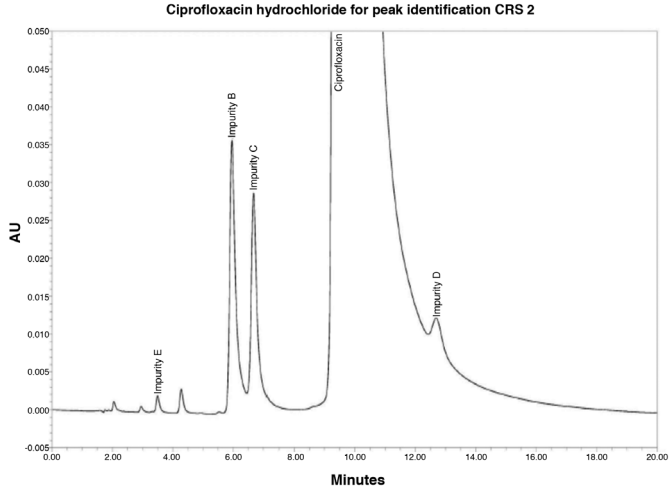


Figure 3. Typical chromatogram

which the stock control solution (0.1 mg/mL) was deposited but still has not been migrated.

TLC was re-tested several times, while using

- New reagents to prepare the mobile phase (plate 3);
- New plates silica gel F254 for TLC (plate 3);
- Second control solution prepared from the first vial (plate 4);
- The third control solution was prepared from a second vial of impurity standard (plate 5).

The control was not migrated in all developed TLC plates, so the system is not compliant. For this reason, an HPLC analysis protocol for impurity A was developed.

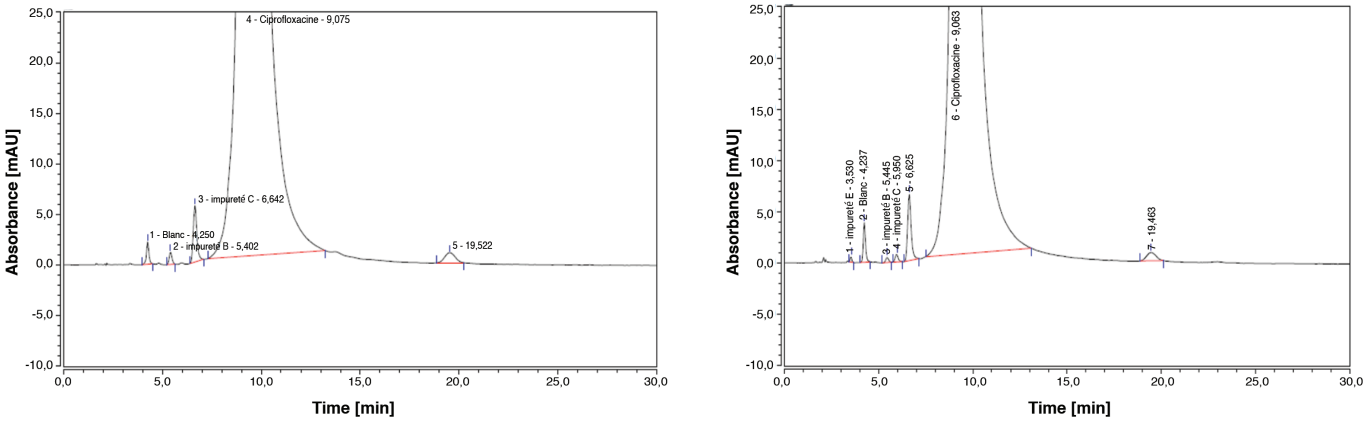


Figure 4. Chromatograms of samples C1 and C2

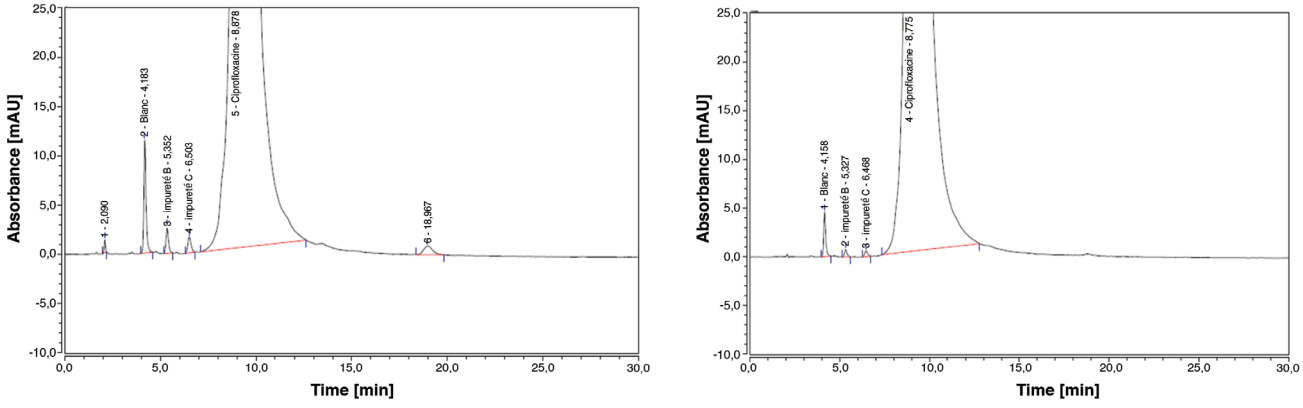


Figure 5. Chromatograms of C3 and C4 samples

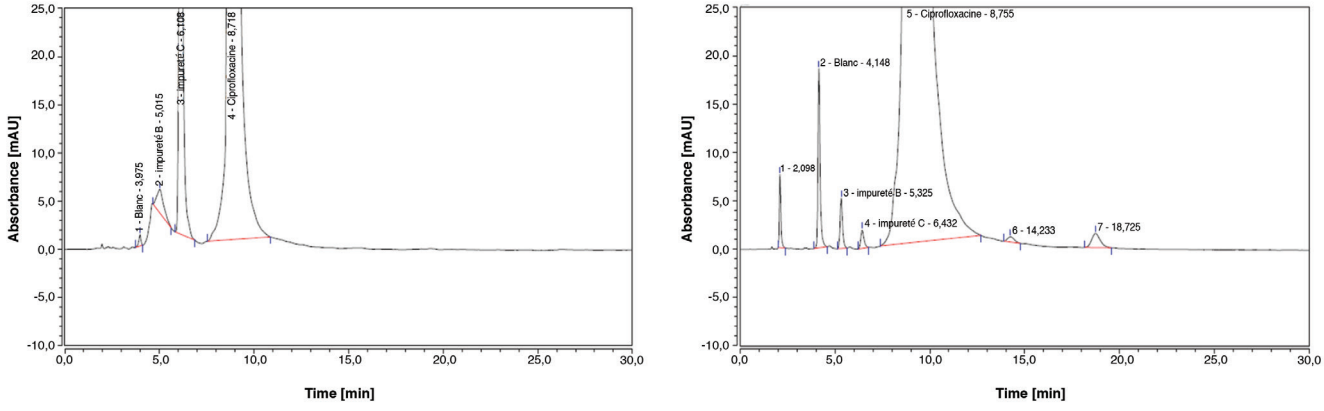


Figure 6. Chromatograms of samples C5 and C6

Research and quantification of impurity A by HPLC

Maximum absorption of the standard solution

Figure 9 shows the absorption spectrum of impurity A in UV.

System compliance

The chromatograms obtained with various standard solutions of the calibration range are shown in Figures 10 and 11.

The maximum absorption of impurity A is 260 nm and its RT is 3.208 min. The symmetry factor of impurity A peak was 1.40, conforming to the norm. The correlation coefficient of the calibration curve is 0.997, which shows that the curve linearity is validated. Therefore, the system is compliant.

Sample analysis

Figures 12 and 13 show the obtained chromatograms of all sample analysis. Table 5 shows the individual contents of impurity A.

According to Eur. Ph., the individual content of impurity A must be less than 0.2%.

Impurity A was not detected in the C2 sample, while C1, C3, C4, C5, and C7 had a content conform but C6 had content equal to the limit.

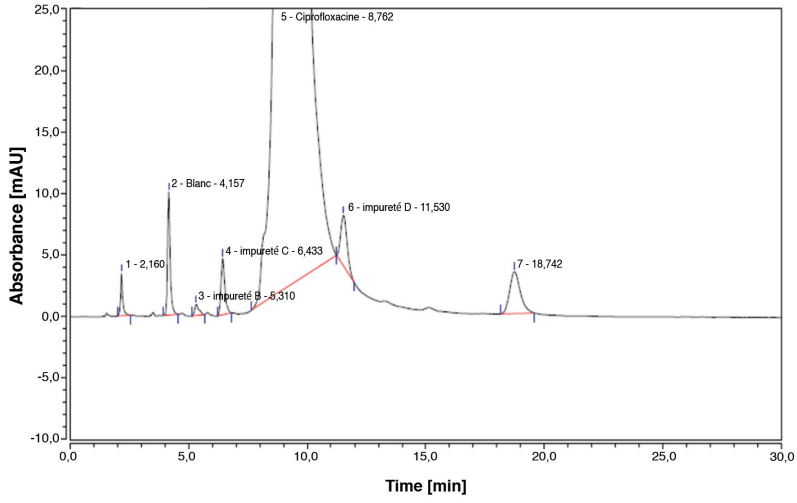


Figure 7. Chromatogram of sample C7

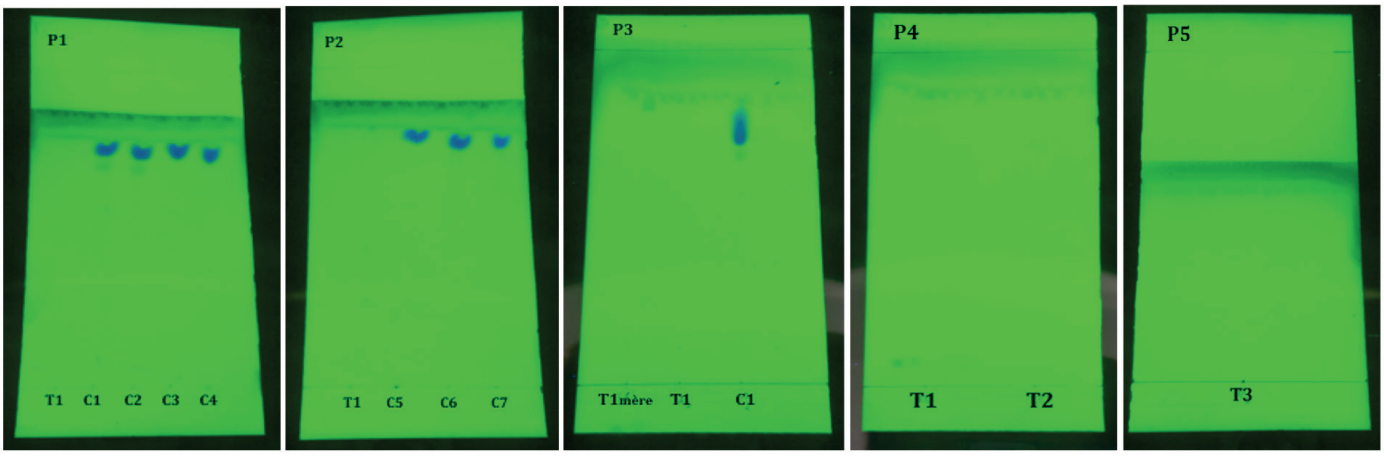


Figure 8. TLC plates revealated under UV lamp

T1: 1st control solution (0.02 mg/mL) prepared from the first vial

T1 stock: 1st stock control solution (0.1 mg/mL) prepared from the first vial

T2: 2nd control solution (0.02 mg/mL) prepared from the first vial

T3: 3rd control solution (0.02 mg/mL) prepared from a second standard vial

Table 4. Individual content of (B, C, D, E, and unspecified) impurity and the impurities total

Ciprofloxacin hydrochloride sample	Impurity	Impurity area (mAU-min)	Control (c) area (mAU-min)	Control (c) weight (mg)	Dilution factor control (c)	Control concenration (µg/mL)	Theoric concenration of control (c) (µg/mL)	Theoric concenration of control (c) (%)	Correction factor	Real concenration control (c) (%)	Individual content of impurity (%)	Impurities total (%)	Norms
C1	Imp B	0.202	3.375	50.5	0.00002	1.01	1	0.2	0.7	0.202	0.008	0.087	Imp B ≤0.2 Imp C ≤0.2 Imp D ≤0.2 Imp E ≤0.3 Imp unspf ≤0.1 Imp total ≤0.5 Exculsion limit: 0.05
	Imp C	1.189	3.375	50.5	0.00002	1.01	1	0.2	0.6	0.202	0.043		
	Imp D	ND	3.375	50.5	0.00002	1.01	1	0.2	1.4	0.202	ND		
	Imp E	ND	3.375	50.5	0.00002	1.01	1	0.2	6.7	0.202	ND		
	Imp unspf 1	0.606	3.375	50.5	0.00002	1.01	1	0.2	1	0.202	0.036		
C2	Imp B	0.086	3.514	50.7	0.00002	1.014	1	0.2	0.7	0.203	0.003	0.136	
	Imp C	0.137	3.514	50.7	0.00002	1.014	1	0.2	0.6	0.203	0.005		
	Imp D	ND	3.514	50.7	0.00002	1.014	1	0.2	1.4	0.203	ND		
	Imp E	0.052	3.514	50.7	0.00002	1.014	1	0.2	6.7	0.203	0.020		
	Imp unspf 1	1.404	3.514	50.7	0.00002	1.014	1	0.2	1	0.203	0.081		
	Imp unspf 2	0.465	3.514	50.7	0.00002	1.014	1	0.2	1	0.203	0.027		
C3	Imp B	0.411	3.190	50.2	0.00002	1.004	1	0.2	0.7	0.201	0.018	0.070	
	Imp C	0.424	3.190	50.2	0.00002	1.004	1	0.2	0.6	0.201	0.016		
	Imp D	ND	3.190	50.2	0.00002	1.004	1	0.2	1.4	0.201	ND		
	Imp E	ND	3.190	50.2	0.00002	1.004	1	0.2	6.7	0.201	ND		
	Imp unspf 1	0.093	3.190	50.2	0.00002	1.004	1	0.2	1	0.201	0.006		
	Imp unspf 2	0.473	3.190	50.2	0.00002	1.004	1	0.2	1	0.201	0.030		
C4	Imp B	0.126	3.559	50.2	0.00002	1.004	1	0.2	0.7	0.201	0.005	0.010	
	Imp C	0.153	3.559	50.2	0.00002	1.004	1	0.2	0.6	0.201	0.005		
	Imp D	ND	3.559	50.2	0.00002	1.004	1	0.2	1.4	0.201	ND		
	Imp E	ND	3.559	50.2	0.00002	1.004	1	0.2	6.7	0.201	ND		
C5	Imp B	1.038	3.190	50.2	0.00002	1.004	1	0.2	0.7	0.201	0.046	0.625	
	Imp C	**15.342**	3.190	50.2	0.00002	1.004	1	0.2	0.6	0.201	**0.579**		
	Imp D	ND	3.190	50.2	0.00002	1.004	1	0.2	1.4	0.201	ND		
	Imp E	ND	3.190	50.2	0.00002	1.004	1	0.2	6.7	0.201	ND		

Table 4. Continued

Ciprofloxacin hydrochloride sample	Impurity	Impurity area (mAU-min)	Control (c) area (mAU-min)	Control (c) weight (mg)	Dilution factor control (c)	Control concenration (µg/mL)	Theoric concenration of control (c) (µg/mL)	Theoric concenration of control (c) (%)	Correction factor	Real concenration control (c) (%)	Individual content of impurity (%)	Impurities total (%)	Norms
C6	Imp B	0.775	3.284	50.0	0.00002	1	1	0.2	0.7	0.200	0.033	0.147	Imp B ≤0.2 Imp C ≤0.2 Imp D ≤0.2 Imp E ≤0.3 Imp unspf ≤0.1 Imp total ≤0.5 Exculsion limit: 0.05
	Imp C	0.366	3.284	50.0	0.00002	1	1	0.2	0.6	0.200	0.013		
	Imp D	ND	3.284	50.0	0.00002	1	1	0.2	1.4	0.200	ND		
	Imp E	ND	3.284	50.0	0.00002	1	1	0.2	6.7	0.200	ND		
	Imp unspf 1	0.664	3.284	50.0	0.00002	1	1	0.2	1	0.200	0.040		
	Imp unspf 2	0.221	3.284	50.0	0.00002	1	1	0.2	1	0.200	0.013		
	Imp unspf 3	0.763	3.284	50.0	0.00002	1	1	0.2	1	0.200	0.046		
C7	Imp B	0.189	3.071	50.5	0.00002	1.01	1	0.2	0.7	0.202	0.009	0.302	
	Imp C	0.861	3.071	50.5	0.00002	1.01	1	0.2	0.6	0.202	0.034		
	Imp D	1.287	3.071	50.5	0.00002	1.01	1	0.2	1.4	0.202	0.119		
	Imp E	ND	3.071	50.5	0.00002	1.01	1	0.2	6.7	0.202	ND		
	Imp unspf 1	0.349	3.071	50.5	0.00002	1.01	1	0.2	1	0.202	0.023		
	Imp unspf 2	1.790	3.071	50.5	0.00002	1.01	1	0.2	1	0.202	0.118		

Imp: Impurity, Unspf: Unspecified, ND: Not detected

Table 5. Individual content of impurity A

Ciprofloxacin hydrochloride sample	Injection number	Weight (mg)	Peak area	a	b	Impurity content (%)	Average (%)	Norm (%)
C1	1 2	50.15 50.15	2.412 2.440	119.4887 119.4887	0.84257 0.84257	0.01 0.01	0.01	Impurity A <0.2
C2	1 2	50.20 50.20	ND ND	119.4887 119.4887	0.84257 0.84257	ND ND	ND	
C3	1 2	50.00 50.00	20.457 20.471	119.4887 119.4887	0.84257 0.84257	0.16 0.16	0.16	
C4	1 2	50.10 50.10	1.536 1.529	119.4887 119.4887	0.84257 0.84257	0.01 0.01	0.01	
C5	1 2	50.25 50.25	5.496 5.460	119.4887 119.4887	0.84257 0.84257	0.04 0.04	0.04	
C6	1 2	50.20 50.20	24.431 24.516	119.4887 119.4887	0.84257 0.84257	0.20 0.20	0.20	
C7	1 2	50.20 50.20	18.704 18.772	119.4887 119.4887	0.84257 0.84257	0.15 0.15	0.15	

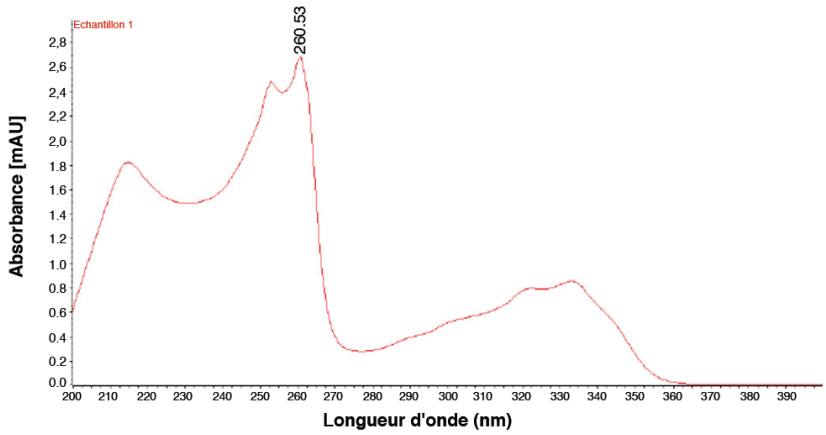


Figure 9. Absorption spectrum of impurity A in UV

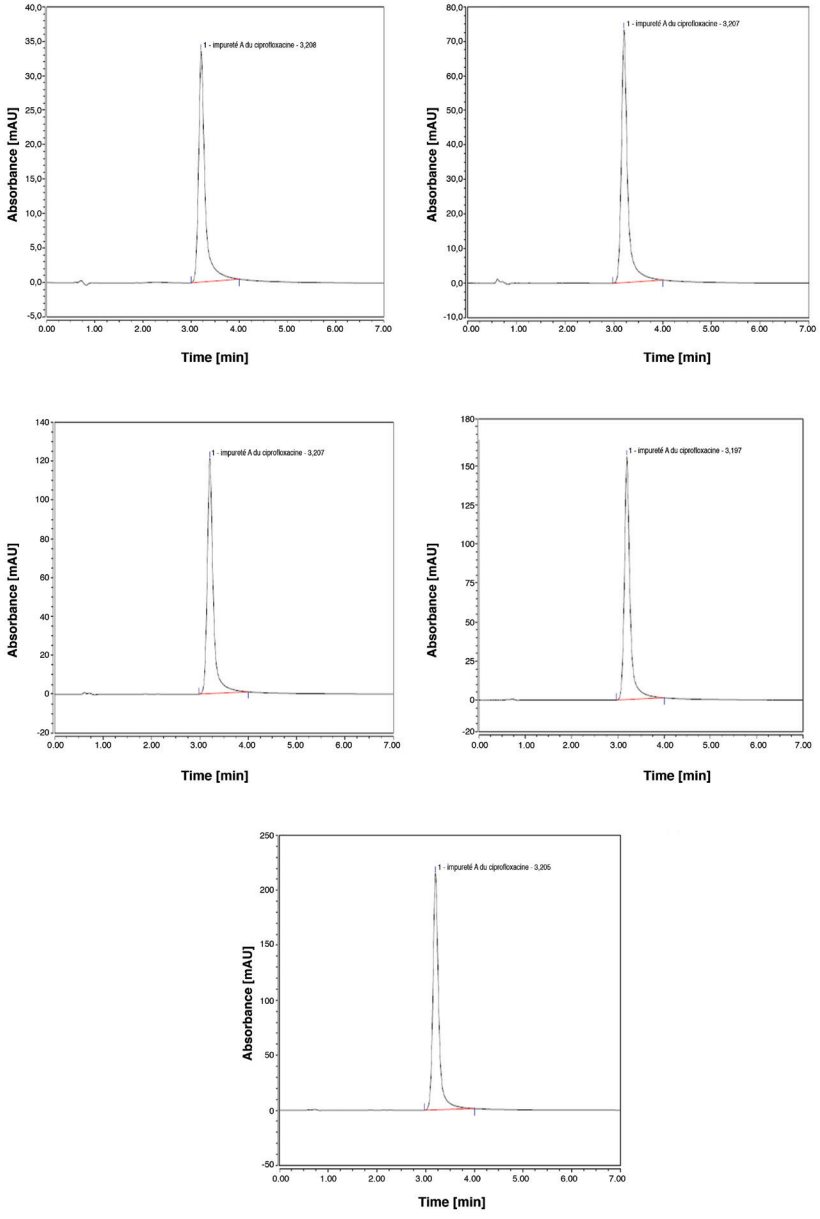


Figure 10. Chromatograms of standard solution at 0.05%, 0.1%, 0.15%, 0.2%, and 0.25%, respectively

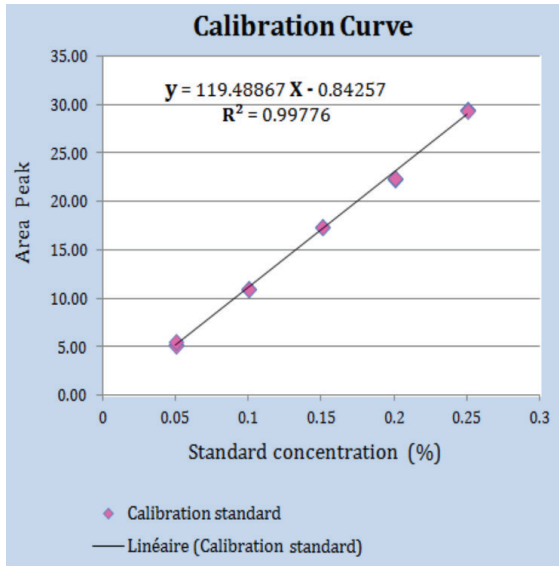


Figure 11. Calibration curve of standard solution

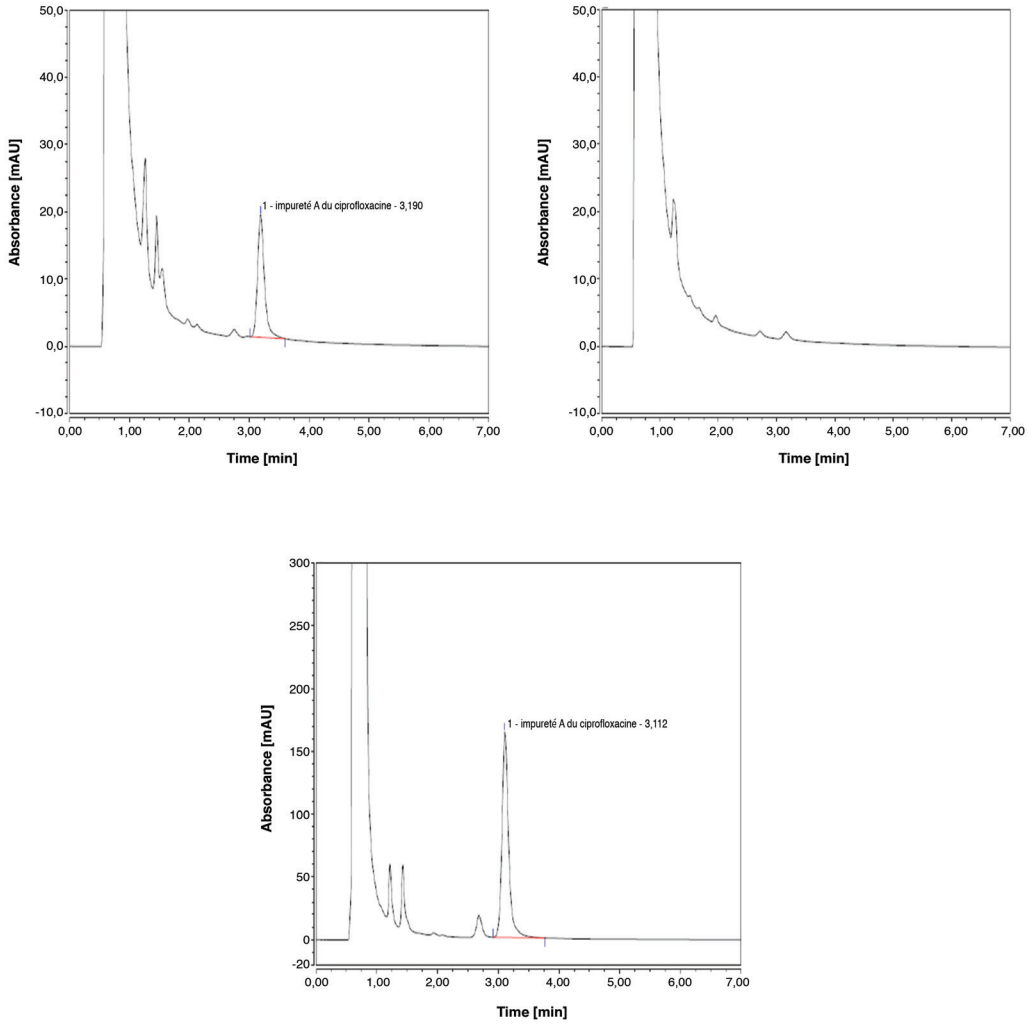


Figure 12. Chromatogram of C1 samples, C2 and C3

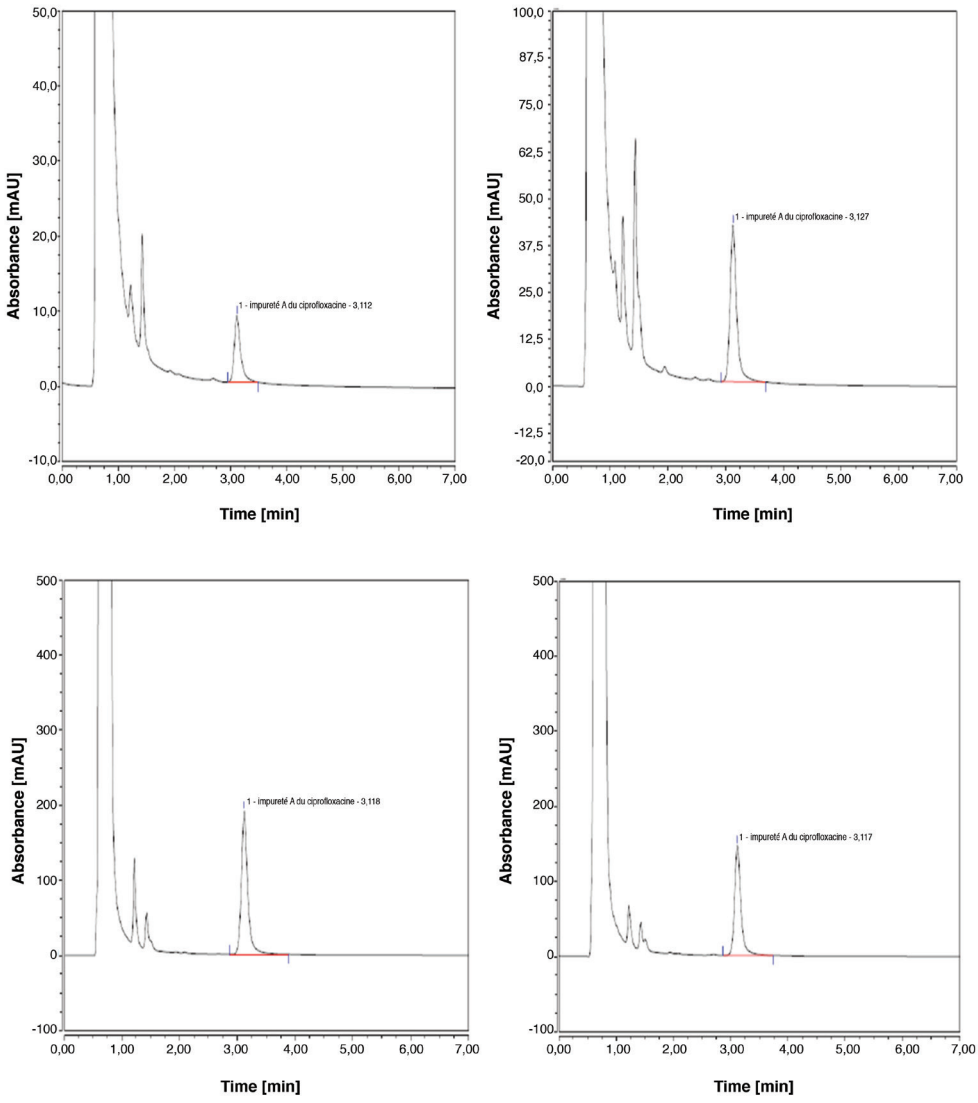


Figure 13. Chromatograms of C4, C5, C6, and C7

CONCLUSION

The specified and unspecified impurities (A, B, C, D, and E) was precisely determined in seven samples of CPF_HCl by HPLC. The C1, C2, C3, C4, and C6 samples have individual contents of specified impurities (B, C, D, and E), unspecified and the total of all present impurities conforms to norms. The C5 sample contains very high content of impurity C, which is a photodegradation product and the impurities total exceeding limit, while sample C7 has a slightly higher content of unspecified impurity. Impurity A content is conformed in all samples except for the C6 sample, which has equal content to the limit. According to the detecting technique of impurity A by TLC in the Eur. Ph., the control solution was not migrated, so we recommend revising this method or replacing it with a more sensitive technique such as HPLC.

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Ethics

Ethics Committee Approval: Not applicable.

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: D.M., H.T., Design: D.M., Data Collection or Processing: D.M., N.H., K.F.E.H., Analysis or Interpretation: D.M., K.F.E.H., N.H., N.H.Z., Literature Search: D.M., Writing: D.M.

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ORIGINAL ARTICLE

Potentially Inappropriate Medication Use in Older Adults with Chronic Kidney Disease

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ABSTRACT

Objectives: This study aimed to identify the prevalence of potentially inappropriate medication use (PIMU) in adults above the age of 65 with chronic kidney disease (CKD) according to the American Geriatric Society Beers Criteria (Beers), Screening Tool of Older People's Potentially Inappropriate Prescriptions Criteria (STOPP) and medication appropriateness index (MAI) 30 criteria and to compare them to justify their use in this specific patient group.

Materials and Methods: This was a retrospective and descriptive study conducted between October 1st, 2019 and March 18th, 2020 at Ibni Sina Hospital, Nephrology Department, Faculty of Medicine, Ankara University.

Results: Among 269 patients discharged from the hospital during the study period, 100 of them were eligible for the study. The mean age was 73.3 ± 6.9 years and 51.9% of them were male. The prevalence of 35 PIMU was 91%, 42%, and 70% according to the Beers, STOPP, and MAI criteria, respectively. There was a statistically significant difference in terms of prevalence among 3 criteria (p<0.001). Beer detected more PIMU (11.3% *vs.* 6.4%) and had higher sensitivity among older adults with CKD (0.97 *vs.* 0.56) compared to the STOPP criteria. Most patients had at least one drug-drug interaction (DDIs) in their discharge prescription (93%) and DDI was one of the main contributors of PIMU. Proton pump inhibitors were the most common medication associated with PIMU in all 3 criteria.

Conclusion: The prevalence of PIMU was high among older adults with CKD at discharge according to these criteria. To improve the prescriptions after hospital discharge, it is considered appropriate to use Beers criteria under guidance of a clinical pharmacist.

Key words: Potentially inappropriate medication use, older adults, chronic kidney disease

INTRODUCTION

Number of elderly people has been increasing gradually in recent decades. It is estimated to reach 1.5 billion by 2050 in both developing and developed countries.[1] In Türkiye, the average life expectancy is projected to be 82.5 and 89.1 years in 2050 and 2100, respectively.[2] As life expectancy increases, number of older adults in the population increases, thus causing a high number of people with many comorbidities. Due to comorbidities, older adults take many medications that make them prone to potentially inappropriate medication use (PIMU). PIMU can cause undesirable consequences, such as adverse drug reactions, hospital admissions/readmissions, increased treatment costs, morbidity, and mortality.[3,4]

Chronic kidney disease (CKD) is one of the most common comorbidities seen in older adults.[5] This is mainly due to the traditional risk factors for CKD, including cardiovascular disease, hypertension, and diabetes.[5] The prevalence of CKD in older adults in Türkiye is 5% (age ≥60 years), while it is 4.1% (age 65-74) in Switzerland, 25.4% (age 65-74) in northeast Germany and 39.4% (age >60 years) in the United States of America (USA).[5-7] A systematic review, which included 10 studies from USA, 8 studies from Europe, and 8 studies from Asia and Australia, found that the prevalence of CKD among older adults ranged from 23.4% to 35.8% (age ≥64 years).[8] Physiological changes caused by aging and decreased kidney function in older adults with CKD affect pharmacokinetic

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and pharmacodynamic properties of medicines, leading to various problems in absorption, metabolism, distribution, and elimination stages.[9,10] These problems can alter the effectiveness of medications or increase the frequency of side effects or toxicities. Therefore, PIMU is often observed in older adults with CKD and is estimated to be 62-67% in the hospital and ambulatory care settings.[4,9,10]

Several screening tools have been developed to improve medication use among older adults. These tools for elderly patients are classified as explicit implicit. Explicit tools are usually developed from published reviews, expert opinions, and consensus reports. These tools are mostly drug-specific and/or disease-specific and can be applied with little or no clinical judgment.[11] The commonly used tools in practice are as follows: The American Geriatric Society Beers criteria (Beers criteria), Screening Tool of Older People's Potentially Inappropriate Prescriptions Criteria (STOPP).[12,13] Implicit tools are judgement-based, patient-specific, and consider the patient's entire medication regimen. Implicit criteria are based on the pharmacist's and/or geriatrics' knowledge, experience, and attitude.[11] Medication appropriateness index (MAI) is an implicit screening tool.[14] These tools provide useful information about what can be potentially inappropriate, when prescribed for older adults.[15,16] They are also widely and easily implemented in many healthcare settings.[15,16]

Discharge from the hospital can put patients at a high risk, when they are prescribed new medications or do not receive any counseling about the appropriate use of medications. Pharmacists can provide medication review services at discharge to identify PIMU for patients, particularly older adults with CKD taking many medicines. This service is crucial for them because they are known to require dose adjustments based on the glomerular filtration rate (GFR), polypharmacy, comorbidities, and age-related physiological changes. There are limited data on the frequency of PIMU among older adults with CKD. Therefore, the primary aim of this study was to describe PIMU among older adults with CKD by using the Beers, STOPP, and MAI criteria. The secondary aim was to compare these criteria in terms of their identifiability, sensitivity, and specificity for PIMU among patients with CKD by examining discharge prescriptions.

MATERIALS AND METHODS

Study design and setting

This descriptive cross-sectional study was conducted between October 1st, 2019 and March 18th, 2020 at Ibni Sina Hospital, Nephrology Department, Faculty of Medicine, Ankara University. Ibni Sina Hospital is a 1,000-bed, government-run tertiary university hospital in Türkiye. The nephrology ward accepts patients mainly from Ankara, but a considerable number of patients are admitted to the ward, as it is one of the largest university hospitals in Türkiye. This ward has 34-bed and patients are followed up by 6 physicians and 7 nurses, however, there is no clinical pharmacist.

Ethics approval

The study was approved by the Ethics Committee for Human Research of the Ankara University Faculty of Medicine (date: September 12, 2019; no: İ3-70-19).

Data collection

Patients who were discharged from the nephrology ward during the study period were screened using their electronic discharge notes. Patients, who were 65 years old or older, discharged from the nephrology service and diagnosed with CKD, were considered eligible (the classification of kidney function was based on the Kidney Disease Improving Global Outcomes-KDIGO guidelines in this study). Patients discharged due to transfer to another hospital or service were excluded from the study.

A data collection form was used to obtain patients' admission diagnosis, length of stay, age, sex, and list of medications during discharge. All information was retrospectively gathered from the electronic medical records of all eligible patients. Detailed information regarding the patients' admission diagnoses and prescription medications was also collected. Prescription records included names, therapeutic classes, doses, dosage forms, and dosage regimens of the prescribed medications.

Evaluating potentially inappropriate medication use

To identify PIMU at discharge, 3 criteria were used: Beers,[12] STOPP,[16] and MAI.[14]

Beers criterion was developed by American Geriatric Society.[12] The recent Beers criteria published in 2019 include the following 5 categories for PIMU:[12]

1. PIMU: In older adults,
2. PIMU due to drug-disease/syndrome interactions that exacerbate the disease/syndrome,
3. PIM to be used with caution,
4. Potentially clinically important drug-drug interactions (DDIs) that should be avoided,
5. Medication that should be avoided or have reduced dosage with varying levels of kidney function.

STOPP criteria were developed by O'Mahony et al.[16] It consists of a section related to the indication of medications that might be prescribed without an evidence-based clinical indication, prescribed beyond the recommended duration, although the treatment duration is well-defined or duplicated.[16] Other sections consist of criteria for each medication group such as cardiovascular system medications and gastrointestinal system medications.[16]

MAI includes 10 parameters such as indication (1), effectiveness (2), dosage (3), correct directions (4), practical directions (5), DDIs (6), drug-disease interactions (7), duplications (8), durations (9), and expenses (10).[14] The scoring of MAI uses a different process from the mentioned tools. This tool requires the user to answer 10 questions regarding a particular medication to determine its appropriateness for a patient. All "yes" responses have a score of zero, while "no" responses have values ranging from 1 to 3 depending on their importance in assessing the appropriateness

of a particular drug. The maximum score of 18 is interpreted as a maximum inappropriateness.[14] In patients' discharge records, it was not stated whether the correct and practical instructions were given to them.[14] All equivalent medications are likely to have the same price in Türkiye.[17] Therefore, these 3 parameters of MAI were not scored.

Lexicomp® drug interaction checker was used to identify DDIs.[18] DDIs were classified as categories A, B, C, D, and X according to Lexicomp®.[18] DDIs belonging to categories D and X were assumed to be clinically important interactions present in the MAI, whereas DDIs belonging to categories A, B, and C were assumed to be minor interactions present in the MAI.

PIMU is defined by the pharmacist as occurring when a medication was categorized as inappropriately used according to Beers, STOPP or MAI.

Comparison between Beers, STOPP and MAI criteria

The tools were compared based on the frequency of detected PIMU among the study population. Sensitivity and specificity were calculated according to the optimal cut-off value. MAI criteria were selected as a reference since their reliability and validity were tested in previous studies.[14,15,19]

Statistical analysis

Categorical variables were described with percentages, and continuous variables were described with the mean ± standard deviation (SD). The chi-square test was used, and a p value <0.05 was considered statistically significant. The degree of agreement was determined using the Kappa statistic. A receiver operating characteristic (ROC) curve was used to estimate the areas under the ROC curves. Data were analyzed using SPSS version 21.0 (IBM SPSS Statistics for Windows, Version 21.0; IBM Corp., Armonk, NY, USA). Microsoft Excel for Windows version 2016 was used to calculate PIMs, prevalence, and medication usage rates.

RESULTS

Demographic and clinical characteristics of the patients

During the study period, 269 patients were discharged from the nephrology ward. Among these, patients were excluded, if they were younger than 65 years (n= 154), died before discharge (n= 3), discharged without any prescription (n= 8) or were transferred to another hospital/ward (n= 4) (Figure 1).

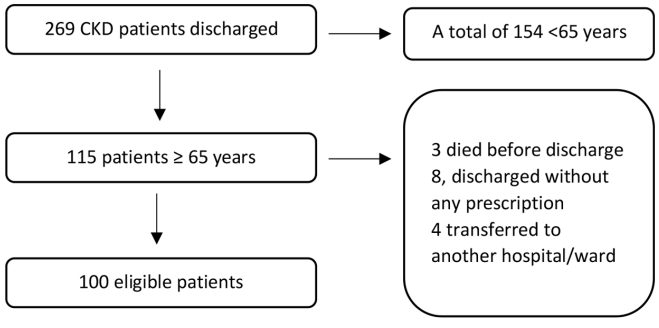

Figure 1. Selection of the patients

In total, 100 patients (mean ± SD age, 73.3 ± 6.9 years; 51.0% male) were included in the study. The most common comorbidities of the patients were hypertension (83.0%), diabetes mellitus (57.0%), and coronary artery diseases (31.0%). Patients' duration of hospital stay (mean ± SD) was 10.7 ± 7.4. The number of comorbidities and medications (mean ± SD) between the patients were 3.6 ± 1.3 and 9.4 ± 3.2, respectively. The percentages of patients based on CKD stages 1, 2, 3, 4, and 5 were 3.0%, 10%, 33%, 38%, and 18%, respectively. Patients' hemoglobin levels (mean ± SD) were low (10.7 ± 1.9 g/dL), while serum uric acid (7.6 ± 0.2 mg/dL) and parathormone levels were high (166.3 ± 141.2 pg/mL) according to KDIGO guidelines (Table 1).

A total of 928 medications were prescribed. The most commonly prescribed medication classes were for cardiovascular system (35.2%), alimentary tract and metabolism (22.3%), and blood and blood-forming organs (16.7%). The most commonly used medications in this study were pantoprazole/esomeprazole/lansoprazole (65%), atorvastatin/rosuvastatin/pravastatin/pitavastatin (58%), and aspirin (49%). Among these patients, 30% were prescribed at least one oral antidiabetic agent (20% linagliptin, 9% metformin, 3% sitagliptin, 2% vildagliptin, and 1% empagliflozin).

PIMU according to the Beers criteria

Most participants were prescribed at least one PIM according to Beers criteria [91.0%, 95% confidence interval (CI): 85.0-96.0]. Among these, 31 patients (31.0%, 95% CI :22.0-40.0) received only one PIM, 40 (40.0%; 95% CI: 30.0-50.0) received two PIMs, 19 (19.0%, 95% CI: 12.0-27.0) received three PIMs, and one (6.3%; 95% CI: 0.0-3.0) received four PIMs.

Overall, 11.3% of the medications were potentially inappropriate (n= 105 out of 928) according to Beers criteria. The most common PIM classes were proton pump inhibitors (PPIs) (65%), diuretics (50%), antiplatelets/anticoagulants (31%), and alpha-1 adrenergic blockers (30%). The most common reasons for PIM were a high risk of side effects (71.4%), long duration (71.4%), and risks of the medicine outweighing the benefits (34.1%) (Table 2).

A total of 13 DDIs were identified on the basis of Beers criteria. The most common DDI was between doxazosin and furosemide (92%). However, these common DDIs were not present in the Lexicomp® drug interaction checker.

PIMU according to STOPP criteria

According to the STOPP criteria, 42 patients (42.0%; 95% CI: 33.0-52.0) were prescribed at least one PIM. Among these patients, 34 (34.0%; 95% CI: 26.0-43.0) received only one PIM, 7 (7.0%; 95% CI: 2.0-12.0) received two PIMs, and one (1.0%; 95% CI: 0.0-4.0) received three PIMs.

Overall, 6.3% of the medications were potentially inappropriate (n= 58/928) according to STOPP criteria. The most common PIM classes were PPIs (10%), psychotropic drugs (9%), and antiplatelets/anticoagulants (8%). The most common reasons for PIM were medication use without indication (78.6%), and risks of the medicine outweighing the benefits (19.0%) (Table 2).

PIMU according to MAI criteria

The mean ± SD MAI score *per* drug was 8.7 ± 1.2, while the mean ± SD MAI score *per* patient was 80.4 ± 28.9. Based on MAI, 70 patients (70.0%; 95% CI: 61.0-78.0) used at least one PIM.

Table 1. Demographic and clinical characteristics of the patients (n= 100)

Characteristics	Values
Male, n (%)	51 (51.0)
Age (years), mean ± SD	73.3 ± 6.9
Age (years), n (%)	
≥80	18 (18.0)
Number of comorbidities, mean ± SD	3.6 ± 1.3
Number of comorbidities, n (%)	
≥5	26 (26.0)
Hypertension	83 (83.0)
Diabetes mellitus	57 (57.0)
Coronary artery disease	31 (31.0)
Duration of hospital stay, mean ± SD	10.7 ± 7.4
Number of medications, mean ± SD	9.4 ± 3.2
Number of medications, n (%)	
≥5	92 (92.0)
Common medications at discharge, n (%)	
Pantoprazole/esomeprazole/lansoprazole	65 (65.0)
Atorvastatin/rosuvastatin/pravastatin/pitavastatin	58 (58.0)
Aspirin	49 (49.0)
CKD stages, n (%)	
Stage 1	3 (3.0)
Stage 2	10 (10.0)
Stage 3	33 (33.0)
Stage 4	38 (38.0)
Stage 5	18 (18.0)
Laboratory findings, mean ± SD	
Calcium (mg/dL)	8.8 ± 0.9
Phosphorus (mg/dL)	4.1 ± 1.4
Magnesium (mg/dL)	2.0 ± 0.6
Uric acid (mg/dL)	7.6 ± 0.2
Albumin (g/dL)	4.3 ± 0.7
LDL-cholesterol (mg/dL)	110.5 ± 41.5
Parathormone (pg/mL)	166.3 ± 141.2
Folic acid (ng/mL)	9.3 ± 4.7
Hb (g/dL)	10.7 ± 1.9

CKD: Chronic kidney disease, Hb: Hemoglobin, LDL: Low-density lipoprotein, SD: Standard deviation

More than a quarter of medications were rated inappropriate based on 6 criteria of MAI (25.9%). Most medications were rated inappropriate in 1-4 criteria of the MAI (92.5%). Among the medications that met at least one of MAI criteria, 51.5% were due to DDIs.

The most common PIM classes were PPIs (22%), steroids (20%), insulins (18%), oral antidiabetic drugs (14%), and antiplatelets/anticoagulants (11%). The most common reasons for PIM were DDIs (68.6%), medication use without indication (47.1%) and the need for dose adjustment for kidney function (21.4%) (Table 2).

According to the Lexicomp® drug interaction checker, most patients had at least one DDI in their discharge prescription (93%). Nearly half of the them had at least one DDI belonging to categories D or X (43%). A total of 752 DDIs were identified. The percentages of DDIs in categories A, B, C, D, and X were 0.8%, 10.5%, 78.9%, 7.9%, and 1.9%, respectively (Figure 2). Among these, the most common DDI was between aspirin and furosemide (2.8%), which belonged to category C. Information on the most common DDIs in category X and D is shown in Table 3.

Comparison of Beers, STOPP and MAI criteria

There was a statistically significant difference between the prevalence of PIMU according to Beers (91.0%), STOPP (42.0%), and MAI (70.0%) criteria (p<0.001). PIMU was more likely to be present in patients with polypharmacy (medications ≥5) according to Beers criteria (p= 0.023) (Table 2). Patients with PIMU according to MAI criteria had a longer hospital stay (p= 0.001) (Table 2). Among the patients, 39% had at least one PIM met all 3 criteria.

The most common medication group associated with potentially inappropriate use was PPIs based on all 3 criteria (65.0% *vs.* 10.0% *vs.* 22.0%). The most common reasons for PIMU varied between the criteria (Table 2).

The ROC results showed that beer had higher sensitivity than STOPP (0.97 *vs.* 0.56) and that STOPP had higher specificity than beer (0.21 *vs.* 0.46). The measures of agreement (Kappa index) were 0.26 between Beers and MAI (p<0.001) and 0.36 between STOPP and MAI (p<0.001) (Table 4). These results indicated moderate agreement between the criteria.

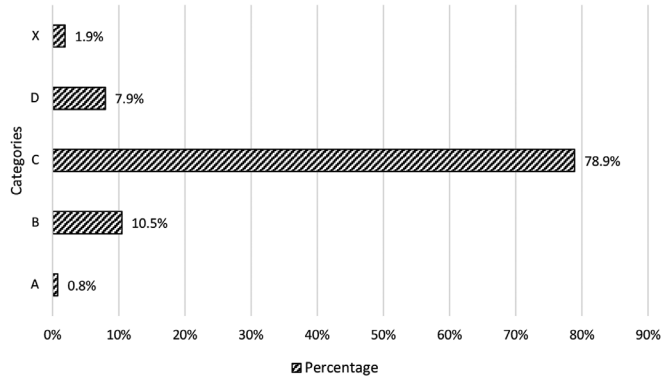

Figure 2. Percentage of drug-drug interaction categories based on Lexicomp®

Table 2. Comparison of Beers, STOPP, and MAI criteria

	Beers criteria			STOPP criteria			MAI criteria		
	Patients with PIMU, n= 91	Patients without PIMU, n= 9	*p* value	Patients with PIMU, n= 42	Patients without PIMU, n= 58	*p* value	Patients with PIMU, n= 70	Patients without PIMU, n= 30	*p* value
Gender			0.525			0.565			0.930
Male	45 (49.5)	6 (66.7)		20 (47.6)	31 (53.4)		35 (50.0)	16 (53.3)	
Female	46 (50.5)	3 (33.6)		22 (52.4)	27 (46.6)		35 (50.0)	14 (46.7)	
Number of medications			0.023			0.134			0.050
<5	5 (5.5)	3 (33.3)		1 (2.4)	7 (12.1)		3 (4.3)	5 (16.7)	
≥5	86 (94.5)	6 (66.7)		41 (97.6)	51 (87.9)		67 (95.7)	25 (83.3)	
Duration of hospital stay (mean ± SD)	10.9 ± 7.6	8.0 ± 3.9	0.255	11.7 ± 7.8	9.9 ± 7.0	0.224	11.9 ± 8.0	7.8 ± 4.5	0.001
CKD stage			1.000			0.519			0.497
Stage 1-2 (eGFR ≥60 mL/min/1.73 m^2)	10 (11.0)	1 (11.1)		6 (14.3)	5 (8.6)		9 (12.9)	2 (6.7)	
Stage 3-5 (eGFR <60 mL/min/1.73 m^2)	81 (89.0)	8 (88.9)		36 (85.7)	53 (91.4)		61 (87.1)	28 (93.3)	
Number of comorbidities			1.000			0.149			0.201
0-4	66 (72.5)	7 (77.8)		27 (64.3)	46 (79.3)		48 (68.6)	25 (83.3)	
≥5	25 (27.5)	2 (22.2)		15 (35.7)	12 (20.7)		22 (31.4)	5 (16.7)	
Common reasons for PIMU									
The risks of medicine outweigh the benefits	31 (34.1)	N/A		8 (19.0)	N/A		0 (0.0)	0 (0.0)	
High risk of side effects	65 (71.4)	N/A		0 (0.0)	N/A		0 (0.0)	0 (0.0)	
Long duration	65 (71.4)	N/A		2 (4.8)	N/A		0 (0.0)	0 (0.0)	
Need dose adjustment for kidney function	12 (13.2)	N/A		0 (0.0)	N/A		15	15 (21.4)	
Drug-drug interactions	13 (14.3)	N/A		N/A	N/A		43	48 (68.6)	
Medication use without Indications	N/A	N/A		33 (78.6)	N/A		33	33 (47.1)	
Use of duplicated medications	N/A	N/A		2 (4.8)	N/A		2	2 (2.9)	
Common medicines associated with PIMU									
Proton pomp inhibitors	65 (71.4)	N/A		10 (23.8)	N/A		22 (31.4)	N/A	
Diuretics	50 (54.9)	N/A		0 (0.0)	N/A		0 (0.0)	N/A	
Antiplatelets/anticoagulants	31 (34.1)	N/A		8 (19.0)	N/A		11 (15.7)	N/A	
Alpha-1 blockers	30 (32.9)	N/A		1 (2.4)	N/A		8 (11.4)	N/A	
Insulins	2 (2.2)	N/A		0 (0.0)	N/A		18 (25.7)	N/A	
Psychotropic drugs	10 (10.9)	N/A		9 (21.4)	N/A		7 (10.0)	N/A	
Oral antidiabetic drugs	0 (0.0)	N/A		1 (2.4)	N/A		14 (20.0)	N/A	
Steroids	0 (0.0)	N/A		0 (0.0)	N/A		20 (28.6)	N/A	

CKD: Chronic kidney disease, eGFR: Estimated glomerular filtration rate, N/A: Unavailable, PIMU: Potentially inappropriate medication use, STOPP: Screening Tool of Older People's Potentially Inappropriate Prescriptions Criteria, MAI: Medication appropriateness index, SD: Standard deviation

DISCUSSION

The results of this cross-sectional descriptive study showed that there was a high prevalence of PIMU among older adults with CKD, with the most common medication associated with PIMU being PPIs. To the best of our knowledge, this is the first study to describe the PIMU among elderly adults in Türkiye. This study included the discharge prescriptions of older patients with CKD.

In this study, the prevalence of PIMU based on Beers version 2019, STOPP, and MAI criteria was 91.0%, 42.0%, and 70.0%, respectively. The prevalence of PIMU was 48.0% and 83.3% according to Beers version 2015 and MAI, in a study including the same patient group in Australia.[20] In another study from USA, the prevalence of PIMU was 59.2% and 33.0% according to Beers version 2015 and STOPP, respectively, among the patients' last prescriptions in a nephrology ward.[21] The prevalence of aged-based PIMU was 32.7% according to both Beers version 2015 and STOPP criteria among the subcohort of patients with CKD.[4] In Lebanon, the prevalence of PIMU was 34.1% according to Beers version 2019 among patients with CKD.[22] The prevalence of PIMU was 32.0% according to STOPP criteria among patients with CKD in France.[23] Compared with these studies specific to CKD patients, our patients had a high prevalence of PIMU at discharge. The high prevalence in this study might have resulted from the lack of a clinical pharmacist to review medications at discharge. It was suggested that the most significant reduction in PIMU could be seen, when the physicians received immediate and concurrent feedback from a clinical pharmacist.[24]

In this study, the most common medications associated with PIMU therapy were PPIs, diuretics, antiplatelets/anticoagulants, alpha-1 blockers, insulins, psychotropics, and oral antidiabetic drugs. Similarly, PPIs,[25] benzodiazepines,[20] antiplatelets/anticoagulants, psychotropics,[21] antiplatelets/anticoagulants,[22] metformin and diuretics[4] were commonly observed as medications associated with PIMU in older adults with CKD. Moreover, most patients had used 5 or more medications in past studies.[4,20-22] Older adults with CKD often have a high drug burden and are at risk of polypharmacy-associated adverse outcomes.[24] Identification of PIMU is critical in this patient group, especially at hospital discharge, where patients may no longer be under the control of healthcare professionals. Incorporating pharmacists into discharge medication reviews to identify PIMU may improve medication use.[26-29] Additionally, collaboration and good communication between nephrologists, nurses, and pharmacists are required to review the appropriateness of medication prescription.[25] Interventions to prevent PIMU in older adults with CKD should be implemented in all healthcare settings.

According to Beers criteria, use of PPIs for more than 8 weeks was not recommended due to the risk of *Clostridium difficile* infections, osteoporosis, and bone fracture.[12] The most common medicine associated with PIMU in all 3 criteria was PPIs in our study. PPIs, statins, and oral antidiabetic agents are commonly prescribed without any indication for older adults with CKD.[24] Therefore, long-term use of PPIs could also be placed under the category of "any drug prescribed without an evidence-based clinical indication" in STOPP and "no indication" in MAI criteria. The risks and benefits of PPI use should be considered during deprescribing interventions in older adults with CKD.[24] However, specific guidance for deprescribing in this patient group does not exist.[24] There is a need for future studies to assess how PPIs can be deprescribed and what the potential clinical outcomes are after discontinuation. Medication reviews, education of health professionals and the use of decision support systems were among the strategies suggested to control the use of PPIs.[28]

Table 3. The most common drug-drug interactions according to category D or X in Lexicomp®

Drug-drug interactions	Category of DDIs	Number of DDIs
Insulin glargine-linagliptin	D	8
Methylprednisolone-sodium bicarbonate	D	6
Insulin aspart-linagliptin	D	6
Calcium carbonate-methylprednisolone	D	3
Atorvastatin-fusidic acid	X	2
Insulin lispro-linagliptin	D	2
Calcium carbonate-levofloxacin	D	2
Calcium carbonate-levothyroxine	D	2
Cefuroxime-sodium bicarbonate	D	2

DDIs: Drug-drug interactions

Table 4. Sensitivity, specificity and a measure of agreement between the criteria

Variable	Beers criteria	STOPP criteria	MAI criteria
Prevalence of PIMU (95% CI)	91.0 (85.0-96.0)	42.0 (32.0-52.0)	70.0 (61.0-78.0)
AUC (95% CI, *p* value)	0.60 (0.47-0.73, $p>0.05$)	0.73 (0.63-0.83, $p<0.001$)	Reference
Sensitivity	0.97	0.56	Reference
Specificity	0.21	0.46	Reference
Kappa index (*p* value)	0.26 ($p<0.001$)	0.36 ($p<0.001$)	Reference

AUC: Area under the curve, CI: Confidence interval, PIMU: Potentially inappropriate medication use, STOPP: Screening Tool of Older People's Potentially Inappropriate Prescriptions Criteria, MAI: Medication appropriateness index

KDIGO guidelines contain a strong recommendation about statin use in all patients with CKD above the age of 50.[30] High levels of low-density lipoprotein (LDL) cholesterol are a risk factor for cardiovascular disease among adults with CKD.[31,32] The key therapy to lower LDL cholesterol levels includes statins.[31] The risk of atherosclerotic events and mortality can be lowered as much as 25% with statin therapy in adults with CKD.[32] More than half of our patients were prescribed statins (58%) at discharge. This might has been because not all nephrologists in the ward were likely to use KDIGO guidelines or because the guidelines and recommendations differed or due to lack of data from large randomized controlled trials on the side effects of statins in older adults with CKD.[32] These patients are also vulnerable to statin-related myopathy.[24] There are still mixed findings regarding the benefits of statins for adults 75 years or older or frail patients with many comorbidities such as CKD.[24]

Metformin is the first-line treatment in diabetes guidelines.[33] Due to its low cost, low hypoglycemia risk, and potential cardiovascular benefits, metformin is prioritized over other antidiabetic drugs.[33] Initial guidelines suggested not to using metformin if a patient's estimated GFR (eGFR) is less than 60 mL/minute/1.73 m^2.[33] However, recent KDIGO guidelines published in 2020 recommend the use of metformin if the patient's eGFR is more than 30 mL/minute/1.73 m^2.[30] Over the years, the risk of lactic acidosis has diminished with evidence that metformin did not pose a high risk in patients.[33] Therefore, more relaxed rules are now followed for the metformin use based on eGFR. In our study population, a few patients were prescribed metformin, whereas the majority used linagliptin. The possible reasons for preference for dipeptidyl peptidase-4 inhibitors are their availability for use in all stages of CKD, once-daily dosing, low risk of hypoglycemia in patients with CKD and potential cardiovascular and renal benefits.[24]

Beers and STOPP criteria include suggestions for renal dose adjustment, while MAI criteria only include a suggestion for the appropriate dose and are not specific to the renal dose. However, suggestions based on the renal dose are only for a limited number of medications. Beers and STOPP criteria are known as explicit measures that are for universal use in all patients,[20] so they may not cover all case scenarios with medications. MAI is an implicit measure that is more patient-specific but requires a detailed search for patient data and databases.[20] Therefore, although it was time consuming, a detailed search to identify each medication's renal dose was employed in this study using other medication databases. This highlights the need for specific guidelines for older adults with CKD to improve practice.

Most patients had at least one DDI in their discharge prescription (93%). Nearly half of the them had DDIs in either category X or D that required avoiding the combination or modifying the regimen (46%). Most DDIs were in the moderate severity category and required monitoring drug therapy (78.9%). Although a lower number of DDIs was determined according to Beers compared to MAI (13 *vs*. 752), the recommendations from Beers were from clinical observations and thus more likely to be associated with clinically relevant adverse events among older adults.[34] A high number of DDIs were identified by the MAI because the drug interaction checker database was used for the evaluation. Similarly, DDIs were found to be common among older adults with CKD at discharge in Australia.[20] CKD was independently associated with DDIs in older adults.[34] This high number of older adults affected by PIMU showed that there is a need for guidance regarding the appropriate concomitant use of medications by older adults with CKD. Pharmacists have enough skill and knowledge to determine DDIs and make suggestions regarding optimal medication use for this vulnerable group of patients. Our nephrology ward could have benefited from the presence of a clinical pharmacist, who routinely checks for DDIs during discharge and seeks to prevent PIMU. Moreover, educational interventions specific to DDIs are needed to improve existing practices.

In this study, Beers criterion had higher sensitivity (0.97 *vs*. 0.56) and detected more PIMU (11.3% *vs*. 6.4%) than STOPP criteria. The measures of agreement were moderate between 2 sets of criteria. In contrast to our study, a local study conducted among older adults in Türkiye found STOPP criteria were more successful than Beers version 2012 in detecting PIMU.[35] Compared to Beers version 2015, STOPP criteria had the highest sensitivity and measure of agreement among older adults to detect PIMU in a study from Kuwait.[15] The differences might have arisen because the most updated 2019 version of Beers was likely to detect more PIMU among the older adults with CKD since it had renal dose adjustment recommendations from version 2015. Another reason might be the differences in patient characteristics in the studies. This study included only older adults with CKD. The number of older adults with CKD was higher than that of older adults with any comorbidities. Therefore, Beers version 2019 appears more sensitive and able to detect more PIMU in older adults with CKD.

Study limitations

There were several limitations to the present study. Firstly, the study included only the prescription records of the patients. Data on use of dietary supplements without prescription at the time of discharge could not be collected. However, it is routine practice to write down the names of dietary supplements such as vitamins in prescriptions in nephrology wards. Second, the findings could not be generalized to all older adults with CKD due to the limited number of study participants and the study was conducted in a single ward. Finally, the clinical outcomes of PIMU were unknown as this was a retrospective study.

CONCLUSION

In conclusion, the high prevalence of PIMU is a major concern among older adults with CKD. DDIs are the main contributor to PIMU. To detect PIMU, use of Beers criteria seemed appropriate, although there is a great need for more specific guidance. Well-designed coordination between healthcare professionals and especially involving a clinical pharmacist to review the medication prescribed at discharge can help improve appropriate medication use among older adults with CKD.

Ethics

Ethics Committee Approval: The study was approved by the Ethics Committee for Human Research of the Ankara University Faculty of Medicine (date: September 12, 2019; no: İ3-70-19).

Informed Consent: Since it was a retrospective study, informed consent were not obtainable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: A.P., A.S., Design: A.P., A.S., Ş.E., Data Collection or Processing: A.P., A.S., Ş.E., Analysis or Interpretation: A.P., A.S., Ş.E., Literature Search: A.P., A.S., Writing: A.P., A.S., Ş.E., Ş.Er., A.T.Ö.

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ORIGINAL ARTICLE

Cytotoxic Effects of Britannin on Acute and Chronic Myeloid Leukemia Cells Through Inducing p21-Mediated Apoptotic Cell Death

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ABSTRACT

Objectives: Following the success of natural compounds for treating solid tumors, interest in applying such agents for treating hematologic malignancies has been fired up more strikingly. Thus far, anti-leukemic effects of several compounds have been examined in different leukemia cell lines, especially in acute lymphoblastic leukemia. The agent that has recently attracted tremendous attention is Britannin, which is derived from *Inula aucheriana* DC., a plant that grows in Iran (Azerbaijan) and Türkiye. In this study, we evaluated the effects of this compound in myeloid leukemia for the first time.

Materials and Methods: We treated chronic myeloid leukemia (CML)-derived K562 and acute myeloid leukemia (AML)-derived U937 cells with different concentrations of britannin. We used several assays, including trypan blue, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, bromodeoxyuridine/5-bromo-2'-deoxyuridine, flow cytometry, and quantitative real-time polymerase chain reaction analysis, to study anti-leukemic effects of the compound.

Results: Our results show that while britannin remarkably reduced the survival of both cell lines in a concentrations-dependent manner, it had cytotoxic effects neither on mouse fibroblast-derived L929 cells nor on normal peripheral mononuclear cells. Moreover, among the tested cell lines, the viability of CML-derived K562 cells was inhibited at higher concentrations of the compound compared with AML-derived U937 cells. We found that britannin induced apoptotic cell death in both cell lines by altering the expression of anti- and pro-apoptotic genes. Britannin also hampered proliferative capacity of the cells in a p21/p27-dependent manner.

Conclusion: Overall, we suggest that based on the lack of toxicity on the normal cells and valuable anti-leukemic activities, britannin could be a promising agent in the treatment strategies of both CML and AML. However, further investigations must more precisely study this compound's mechanism of action and evaluate its safety profile.

Key words: Acute myeloid leukemia, chronic myeloid leukemia, britannin, cytotoxic effects, apoptosis

INTRODUCTION

For a long time, herbal medicines have catered to the basic needs humans to treat various diseases such as cancer.[1] The success of vinca alkaloids, a group of drugs isolated from the periwinkle plant; *Catharanthus roseus* (L.) G. Don, for treating hematologic malignancies -indeed- has begun a new era in terms of cancer treatment strategies.[2] Thus far, many compounds provided from natural sources have found their way into the treatment of different human cancers.[3] Curcumin,[4] trabectedin,[5] and resveratrol[6] are all-natural compounds that recently enjoyed unprecedented success for treating human cancers. Ergolid is another natural compound isolated from *Inula oculus-christi* L., which showed anti-leukemic effects in acute lymphoblastic leukemia (ALL)-derived cell lines. Also, *Artemisia annua* L.

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extract has been reported to not only to induce caspase-3-dependent apoptosis in ALL cells, but also to increase the sensitivity of the leukemic cells to chemotherapeutic drugs.[7] *Bryonia aspera* Steven ex Ledeb. extract has also been reported to have anti-proliferative and pro-apoptotic effects in ALL-derived Nalm-6 and REH cells.[8] During the burgeoning success of these reports and the astonishing roles of natural compounds in treating hematologic malignancies, there is a consensus that natural compounds could probably change the paradigm of treatment in human leukemia. Recently, promising results have been published about the cytotoxic effects of britannin, a sesquiterpene lactone (SL), which is derived from *Inula aucheriana* DC., a plant that grows in Iran (Azerbaijan) and Türkiye.[9-11]

It has been declared that apart from the anti-inflammatory effects, there is the presence of α-methylene-γ-lactone in sesquiterpene lactones (SLs) that endows their ability to induce cell death in cancer cells.[12] For example, in breast cancer, Hamzeloo-Moghadam et al.[13] have reported that low concentrations of britannin reduced the viability of MCF-7 cells through the suppression of cyclin D1 and CDK4 protein. In another study, this compound exerted cytotoxic effects in hepatocarcinoma, breast cancer, and lung cancer[9] Cui et al.[14] also suggested that britannin induced apoptotic cell death in liver cancer cells. In the most recent study, Mohammadlou et al.[15,16] reported anti-leukemic effects of britannin in ALL cells. The authors suggested that this compound induced G1-cell cycle arrest and increased intracellular levels of reactive oxygen species (ROS) in ALL cell lines. Although many studies have been focused on anticancer effects of britannin, as far as we are aware, no study has evaluated the effects of this compound in myeloid leukemia cells and our study examined anti-leukemic effect of britannin in chronic myeloid leukemia (CML)-derived K562 and acute myeloid leukemia (AML)-derived U937 cells for the first time.

MATERIALS AND METHODS

Britannin isolation

I. aucheriana was collected and its scientific name was confirmed at the Herbarium of Traditional Medicine and Materia Medica Research Center (TMRC), Shahid Beheshti University of Medical Science (voucher number: TMRC 3173). To obtain britannin, 600 g of *I. aucheriana* dried powder was soaked with *n*-hexane (plant: solvent ratio, 1:10) for three days. After filtration, the same process was repeated with chloroform with the residue of the plant. Seven g of the dried chloroform extract was set to vacuum liquid chromatography (silica gel 40-63 µm; mobile phase: ethyl acetate and methanol); 400 mg of the fraction eluted with EtOAc-MeOH (2: 1) was further processed by solid-phase extraction (2.5 × 7.5 cm silica gel 40-63 µm; mobile phase: dichloromethane, ethyl acetate, and methanol) to afford britannin (70 mg).

Cell culture

To evaluate the effect of the britannin on AML and CML derived cell lines, we cultured U937 and K562 cells in RPMI1640 medium containing 10% fetal bovine serum, 50 mg/mL streptomycin, and 30 mg/mL penicillin in a humidified incubator, respectively. We treated the cells with different concentrations of the compound (0-10 µM) for 24 and 48 h. We also cultured L929 cells, a non-cancerous cell line derived from mouse fibroblasts, and peripheral mononuclear cells (PBMCs) as a control to determine the safety of the compound.

Trypan blue assay

After incubating U937 and K562 cells with different concentrations of britannin (0, 3, 5, and 10 µM) for 24 and 48 h, we stained the drug-treated cells with trypan blue dye. After remaining for 2-3 min at room temperature, the number of viable cells was counted manually using a light microscope.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

MTT assay was used to evaluate whether britannin could reduce metabolic activity of the leukemic cells. Drug-treated leukemic cells were exposed to 100 µL MTT solution (0.5 mg/mL) and were incubated for 4 h. After centrifugation and discarding the medium, 100 µL dimethyl sulfoxide was added to each well and, then, absorbance of each sample was measured at 570 nm by an ELISA reader.

Annexin-V/PI staining assay

Annexin V-FITC staining was used to assess the effect of britannin on the induction of apoptosis in leukemic cells. Both cell lines were treated with the desired concentrations of the compound, and after 48 h, 5 µL of annexin V-FITC dye was added to each sample. Leukemic cells were incubated in the dark for 15 min and the induction of apoptosis was evaluated using BD FACS Calibur (BD biosciences, SanJose, CA, USA). The Flowjo 7.6 software was used for data analysis.

Gene expression analysis

To study the alterations in gene expression, the first RNA was extracted from drug-treated leukemic cells using Trizol reagent. After evaluating the quality of the extracted RNAs by Nanodrop, cDNA was synthesized by TAKARA kit (Japan). Real-time polymerase chain reaction (RT-PCR) was performed using SYBR green PCR. ABL was the housekeeping gene in this study. The primers used to evaluate the expression of genes are shown in Table 1.

Statistical analysis

One-way ANOVA and *post-hoc* Tukey multiple comparisons were used for data analysis. All analyses were performed using SPSS software version 22. A p value <0.05 was considered statistically significant.

RESULTS

Effects of britannin on the viability and the metabolic activity of U937 and K562 cells

To evaluate whether britannin could reduce the population of leukemic cells, two myeloid leukemia cell lines, U937 and K562, were treated with increasing concentrations of the

Table 1. Nucleotide sequences of primers used for RT-PCR

Gene	Accession number	Forward primer (5'-3')	Reverse primer (5'-3')	Size (bp)
ABL	NM-005157	*AGTCTCAGGATGCAGGTGCT*	*TAGGCTGGGGCTTTTTGTAA*	290
p21	NM-000389	*CCTGTCACTGTCTTGTACCCT*	*GCGTTTGGAGTGGTAGAAATCT*	130
p27	NM-004064	*AACGTGCGAGTGTCTAACGG*	*CCCTCTAGGGGTTTGTGATTCT*	139
Bax	NM-138761	*CGAGAGGTCTTTTTCCGAGTG*	*GTGGGCGTCCCAAAGTAGG*	242
PUMA	NM-014417	*GACCTCAACGCACAGTACGAG*	*AGGAGTCCCATGATGAGATTGT*	98
Bad	NM-004322	*CCCAGAGTTTGAGCCGAGTG*	*CCCATCCCTTCGTCGTCCT*	249
Bcl-2	NM-000633	*CGGTGGGGTCATGTGTGTG*	*CGGTTCAGGTACTCAGTCATCC*	90
MCL-1	NM-021960	*AGAAAGCTGCATCGAACCAT*	*CCAGCTCCTACTCCAGCAAC*	183
BCL-xl	NM-138578	*GAGCTGGTGGTTGACTTTCTC*	*TCCATCTCCGATTCAGTCCCT*	119

RT-PCR: Real-time polymerase chain reaction

compound. The results of trypan blue assay showed that, while britannin reduced number of U937 cells in a concentration-dependent manner, anti-leukemic effects of this compound on K562 cells were exerted at a concentration of 10 µM (Figure 1A). Moreover, to confirm anti-leukemic effects of britannin on myeloid leukemia cell lines, we also evaluated effect of the compound on metabolic activity of the cells. MTT assay showed that britannin significantly reduced the metabolic activity of both U937 and K562 cells (Figure 1B). In agreement with the results of the trypan blue exclusion, anti-leukemic effects of britannin were more potent in U937 cells as the concentration of 5 µM of this compound diminished metabolic activity of U937 cells by almost 50%. However, a significant anticancer effect of britannin on K562 cells was observed at a concentration of 10 µM (Figure 1B). We also found that U937 cells with an inhibitory concentration 50 (IC_{50}) value of 3.9 µM were more sensitive to britannin compared to K562 with an IC_{50} value of 8.1 µM (Figure 1A, B). To check whether the compound at the mentioned concentrations had cytotoxic effects on normal cells, non-cancerous L929 cells, and PBMCs were treated with increasing concentrations of the compound (3, 5, and 10 µM). As presented in Figure 1C, britannin had minimal effects on viability of both PBMCs and L929 cells, suggestive of safety of the compound. To better study anti-leukemic effects of britannin on leukemic cell lines, we decided to treat K562 cells with 5, 7, and 9 µM concentrations of the compound and U937 cells with 3, 5, and 7 µM of britannin for further analysis.

Effects of britannin on the proliferation of the leukemic cells

Having established that britannin has anti-leukemic effects on K562 and U937 cells, we evaluated effect of the compound on proliferation of the cells. We treated K562 and U937 cells with different concentrations of the compound (0-9 µM), and then DNA synthesis rate was determined using the bromodeoxyuridine/5-bromo-2'-deoxyuridine (BrdU) assay. Our results showed that britannin could reduce capacity of the leukemic cells to replicate DNA (Figure 2A). In agreement with this finding, we also found that britannin could reduce the expression of both p21 and p27 in both cell lines (Figure 2B).

Britannin induced apoptotic cell death in K562 and U937 cells

To determine whether the treatment of cell lines with britannin could induce apoptotic cell death, we treated both cell lines with different concentrations of the compound (0-9 µM) and then binding of annexin-V combined with PI was analyzed by flow cytometry. As presented in Figures 3A and B, we found a significant elevation in the percentage of annexin-V and annexin-V/PI double-positive cells compared to the control group. In agreement with the MTT and trypan blue assay results, U937 cells were more sensitive to britannin than K562 cells. Moreover, to confirm these results, we also evaluated effect of the compound on expression of both anti- and pro-apoptotic genes. The results of the quantitative RT-PCR analysis indicated that while britannin could remarkably increase the expression levels of pro-apoptotic genes, including *Bax*, *Bad*, and PUMA, it could reduce the expression of anti-apoptotic genes such as *Bcl2, Bcl-xl*, and *MCL-1* (Figure 4).

DISCUSSION

From the first description of human leukemia until now, efforts were made to find a proper treatment for this type of disease. However, the heterogeneity between different sub-types of leukemia, whether they are categorized as chronic leukemia or acute leukemia, did not lead to the development of a single treatment regimen for both disease.[17] For example, while CML takes advantage of tyrosine kinase inhibitors such as imatinib for treatment,[18] AML is mainly treated with vincristine, daunorubicin, and other conventional chemotherapeutic drugs.[19] Even targeted therapies also fail to introduce one specific small-molecule inhibitor to treat both acute and CML.[17] Another challenge of the current treatment of human leukemia is the incidence of severe side effects for the patients, which eventually restrict the clinical dosage of the agents, an event that leads to the disease relapse.[20,21] A stream of efforts for finding new agents with more potent anti-leukemic effects and lower side effects is on the way. Attention has also been attracted to herbal medicines, as many drugs isolated from plants have been shown to be safe and well-tolerated in clinical investigations.[22] In this study, we evaluated the effect of britannin, a compound

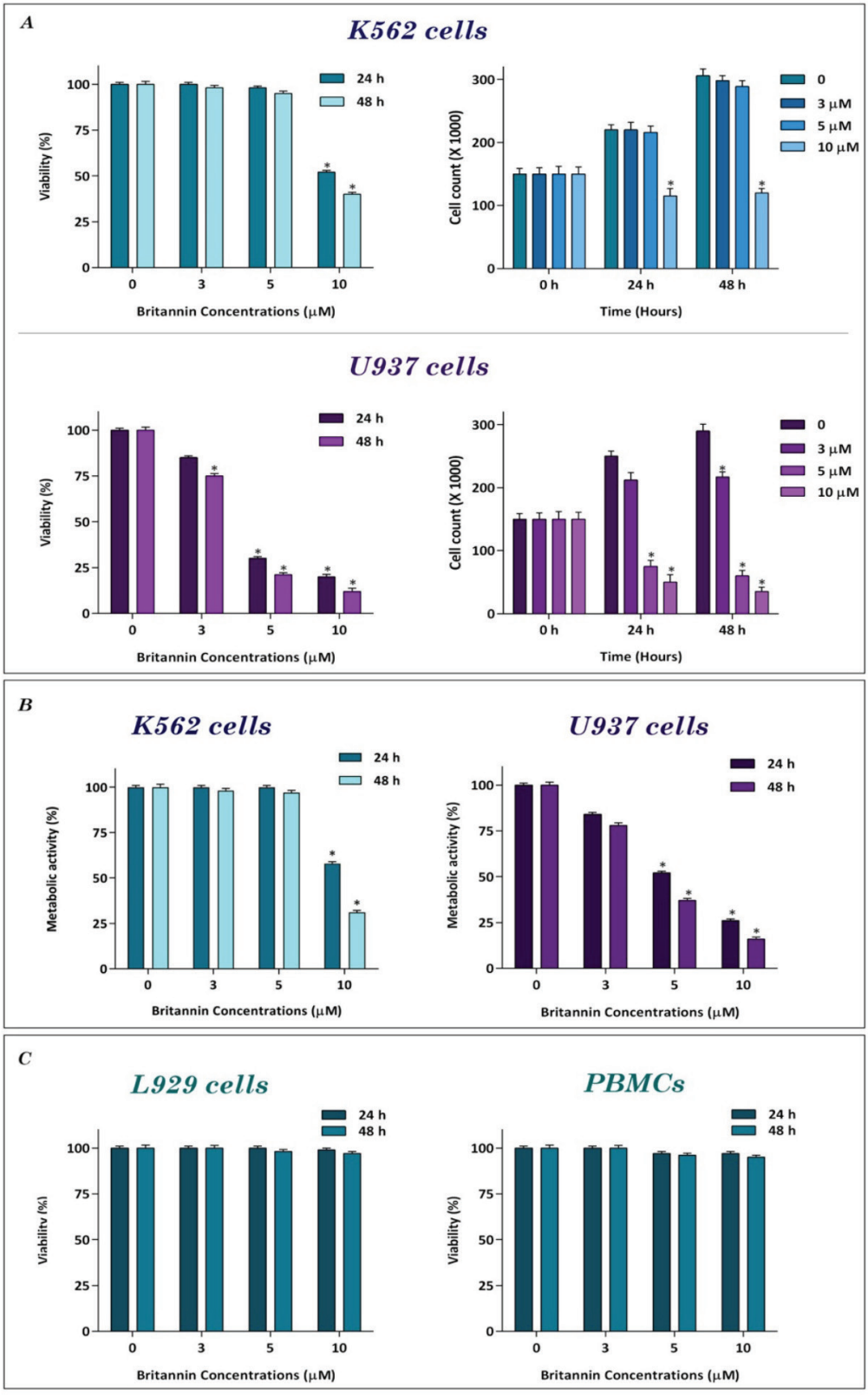

Figure 1. The effect of britannin on the viability and the metabolic activity of K562 and U937 cells. (A) Britannin concentrations of 3, 5, and 10 µM significantly reduced the survival and number of viable cells in both leukemic cell lines. (B) The results of MTT assay revealed that britannin could hamper the metabolic activity of the cells in a concentrations-dependent manner. (C) Britannin at the concentrations of 3, 5, and 10 µM had toxic effects neither on L929 cells or on PBMCs. Values are given as mean ± SD of three independent experiments.

*$p \leq 0.05$ represent significant changes from untreated control, MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, PBMCs: Peripheral mononuclear cells, SD: Standard deviation

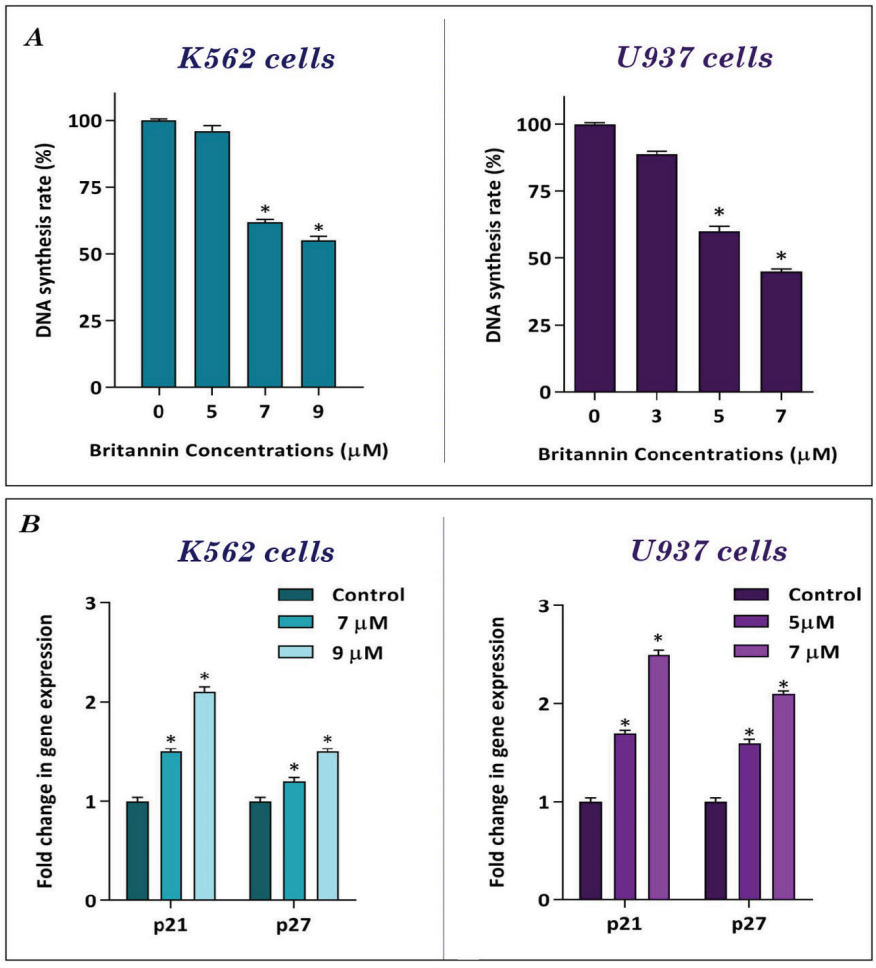

Figure 2. Anti-proliferative effects of britannin on K562 and U937 cells. (A) Treatment of K562 and U937 cells with increasing concentrations of britannin led to a decrease in the DNA synthesis rate of the cells. (B) Britannin significantly increased the expression of p21 and p27 in both cell lines. Values are given as mean ± SD of three independent experiments

*$p \leq 0.05$ represent significant changes from untreated control, SD: Standard deviation

isolated from *I. aucheriana,* on two myeloid leukemia cell lines, *e.g.* K562 and U937, which are derived from CML and patients with AML, respectively.

Interestingly, our results showed that britannin remarkably reduced the number of viable cells, cell growth, and metabolic activity of U937 cells in a concentration-dependent manner. However, for K562 cells, a significant anti-leukemic effect of the compound was obtained at a higher concentration (10 μM). This finding suggested that, compared with U937 cells, CML-derived K562 cells were more resistant to the lower concentrations of britannin. Our results also showed that this single compound at the same concentrations had neither cytotoxic effects on L929 cells nor normal PBMCs.

Note that among the tested cell lines, we found that viability of CML-derived K562 cells was inhibited at higher concentrations of the drug, as compared with AML-derived U937 cells. This difference in cell sensitivity could be due to the excessive activation of PI3K/Akt signaling pathway in K562 cells because of the presence of BCR-ABL.[23] It has been well-established that the PI3K/Akt activity could induce the drug-resistance in CML cells.[24,25] However, britannin reduced survival of K562 cells at concentrations that had no toxic effects on the normal cells.

Having established anti-leukemic activity of britannin on both leukemic cell lines, we also examined the mechanism through, which this compound might induce its effects. Our results showed that britannin reduced the capability of the leukemic cells to replicate DNA. The results of the BrdU assay revealed that in the presence of britannin, there was a significant decrease in the DNA synthesis rate in both K562 and U937 cells, which in turn hampered the ability of the cells to proliferate. Similarly, britannin concentration-dependently reduced the number of viable cells in both cell lines. In agreement with our finding, Zhang et al.[26] also indicated that britannin could reduce proliferation of cancer cells. It has been indicated that britannin interacts with *c-Myc* and thereby hampers the proliferative activity of PDL-1-positive T-cells in the tumor site, an event that in turn prevents the growth and metastasis of cancer cells.[26] Proliferation of leukemic cells could be regulated *via* the alterations in the genes controlling the progression of the cell cycle. Thus

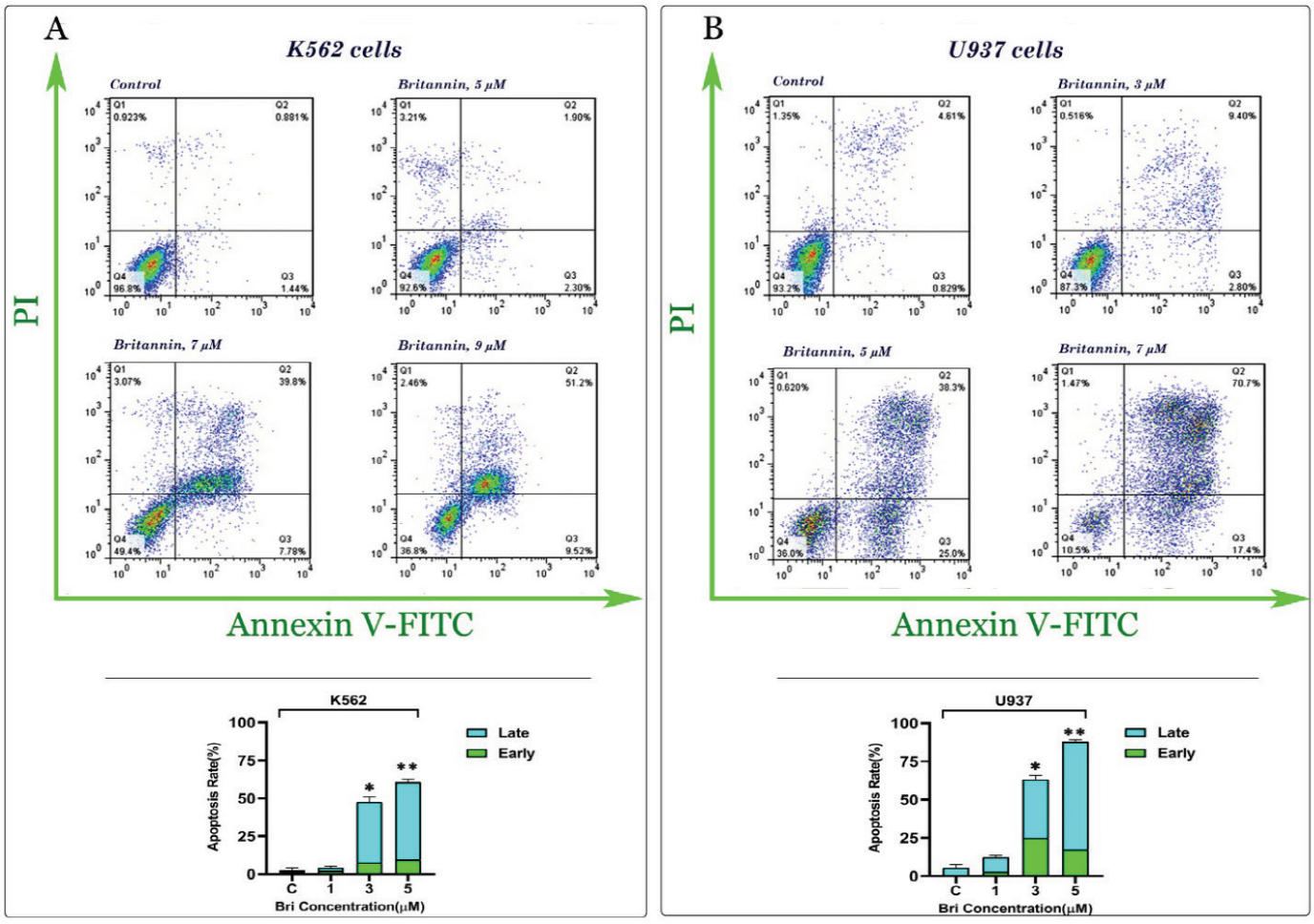

Figure 3. Britannin induces apoptotic cell death in leukemic cells. (A, B) Britannin elevated the percentage of both annexin-V and annexin-V/PI double-positive cells in K562 and U937 cells. Values are given as mean ± SD of three independent experiments

*$p \leq 0.05$ represent significant changes from untreated control, SD: Standard deviation

far, several cell-cycle regulatory genes such as p21 and p27 have been identified.[27] Moreover, the results of a previous study indicated that britannin could reduce the expression of cyclin D1 and CDK4, two other cell-cycle regulatory genes in breast cancer cells.[13] In line with these results, we also found that britannin increased expression levels of both p21 and p27 in leukemic cell lines, suggesting that anti-proliferative capacity of britannin in K562 and U937 cells is mediated, at least in part, through altering the expression of the genes controlling the distribution of the cells in the cell cycle. Apart from regulating progression of the cells from G1 phase of the cell cycle to S phase, p21 could also increase expression of pro-apoptotic genes such as *Bax* in the cells.[28] It has also been declared that elevation in the expression of p21 could increase the sensitivity of cancer cells to apoptosis by increasing the expression of p53-related genes.[29] In another study, Hastak et al.[30] reported that ablation of p21 in prostate cancer cells prevented epigallocatechin-3-gallate, a polyphenolic compound of green tea, induced apoptotic cell death. In agreement with the up-regulation of p21, we found that not only britannin increased the expression of p53-dependent pro-apoptotic genes but also diminished the expression of anti-apoptotic genes such as *Bcl2*, *MCL-1*, and *BCL-xl*. Moreover, this compound significantly increased the population of apoptotic cells in both K562 and U937 cells. Cui et al.[14] also showed that this compound hampered viability of liver cancer cells by elevating the intracellular concentrations of reactive oxygen specious, which eventually induced apoptotic cell death. As the most straightforward interpretation of our results, we suggested that probably britannin reduced survival and proliferative capacity of myeloid leukemia cells *via* p21-mediated suppression of DNA synthesis and induction of apoptotic cell death.

CONCLUSION

Taken together, the results of this study indicated that based on minimal toxicity on the normal cells and valuable anti-leukemic activities, britannin could be introduced in the treatment strategies of leukemia, whether CML or AML. However, further investigations must more precisely investigate the mechanism

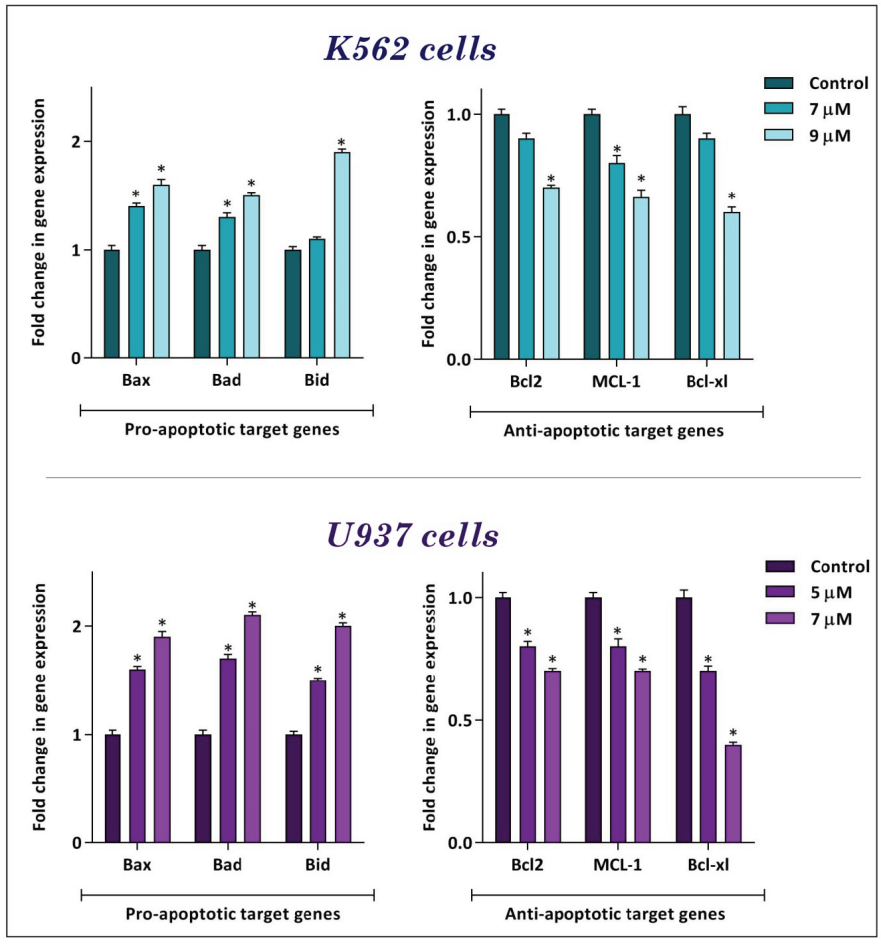

Figure 4. Britannin altered the expression of pro-and anti-apoptotic genes in K562 and U937 cells. The results of the qRT-PCR analysis showed that, while britannin elevated the expression of pro-apoptotic target genes, this compound diminished the expression of anti-apoptotic target genes in both cell lines. Values are given as mean ± SD of three independent experiments

*$p \leq 0.05$ represent significant changes from untreated control, qRT-PCR: Quantitative real-time polymerase chain reaction

of action of the compound and to evaluate its safety profile in a xenograft model.

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Ethics

Ethics Committee Approval: The current study has been approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences and its code of ethics is:1398.614

Informed Consent: The current study has been done on cell line and there was no need having informed consent.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: H.M., M.H.M., M.M., A.G., Design: H.M., M.H.M., M.M., A.G., Data Collection or Processing: H.M., M.H.M., M.M., A.G., Analysis or Interpretation: H.M., M.H.M., M.M., A.G., Literature Search: H.M., M.H.M., M.M., A.G., Writing: H.M., M.H.M., M.M., A.G.

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

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ORIGINAL ARTICLE

Evaluation of Marketed Almond Oils [*Prunus dulcis* (Mill.) D.A. Webb] in Terms of European Pharmacopoeia Criteria

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ABSTRACT

Objectives: Almond oil marketed for health benefits and cosmetic purposes should be in compliance with the European Pharmacopoeia (EP) criteria. Therefore, in this study, 17 almond oil samples sold in pharmacies, herbal shops, online, and cosmetics stores were analyzed in terms of "almond oil" monograph criteria, which have been mentioned in EP 7.0.

Materials and Methods: In this study, 17 almond oil samples sold in pharmacies, herbal, online, and cosmetics stores were analyzed in terms of "almond oil" monograph criteria, which have been mentioned in EP 7.0. Appearance, acidity value, and peroxide value of each sample were determined and the ingredients were identified by thin layer chromatography. Fatty acids were analyzed by gas chromatographic method using flame ionization detector.

Results: It was determined that two of the 17 samples complied with EP 7.0 criteria.

Conclusion: Almond oil, which is currently marketed according to the manufacturer's own marketing and quality criteria, is excluded from the Turkish Food Codex Standards. Our research has shown that most of the products do not comply with the EP standards. For this reason, it should be ensured that almond oil is listed in this codex and urgent arrangements should be made for quality control analysis.

Key words: Fatty acids, gas chromatography-mass spectrometry, *Prunus dulcis*, quality control

INTRODUCTION

Türkiye, with its diversity of plant species, is one of the world's most important gene sources. The almond is one among the important species in this gene source, and is grown or cultivated across widespread in Türkiye. Almond [*Prunus dulcis* (Mill.) D.A. Webb, syn. *P. amygdalus* Batsch, and *P. communis* (L.)] are divided into two varieties,[1] pomologically, sweet almond (*P. dulcis* var. *dulcis*) and bitter almond (*P. dulcis* var. *amara*) (*P. amygdalus* Batsch), where all belongs to the family Rosaceae, along with raspberries, peaches, apples, and pears. About of 20 species have been reported to grow in Iran, while there have been more than 30 wild species in the world.[2]

Almonds, which are typically used as snack foods or found as an ingredient in many products,[3,4] have a considerable economic value with their different usage areas (gastronomy, confectionery *etc.*).[5-9] They are commonly cultivated for their fruits[10] and place number one among the products of tree nuts.[11] Due to their high nutritional content and their promising effects on human health with their high levels of monounsaturated fatty acid and polyunsaturated fatty acid content, consumption of and demand for almonds remains high.[5-7] The almond fixed oil obtained from almond seeds is used as medicine, pharmaceutical, and cosmetic products to treat dry skin disorders such as psoriasis.[12] Many studies on the biological value and chemical properties of nut proteins and oils have been reported.[13] *In vivo* studies have reported that almond seeds and oils possess hepatoprotective, anti-inflammatory, anticancer, and immunestimulant effects. Among tocopherols, phytosterols, and many other health-promoting micronutrients,[14] almond with

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its high content of mono-unsaturated fatty acids, can reduce gastric carbohydrate absorption rate and increase insulin sensitivity,[15] while also helpful for constipation and restless bowel syndrome.[16-18] The importance of almond fruit with rich oleic, linoleic, and linolenic acid content has increased due to the positive effects on cholesterol and cardiovascular disease in human,[12] while the observed blood cholesterol-lowering effects of nuts were far better than what was predicted according to their dietary fatty acid profiles.[19,20]

The almond oil market was valued at $1118 million in 2016 and is expected to reach up to $2680 million by 2023, while the growth of almond oil market is driven by the rise in production of aromatherapy products, increase in preference of customers toward cosmetic products containing natural ingredients, rapid urbanization, and growth in applications of almond oil in pharmaceutical industry.[21]

Differences in the major and minor components of the medicinal oils significantly affect their nutritional, health-promoting activities, and their organoleptic properties. Therefore, in this study, 17 almond oil samples sold in pharmacies, herbal shops, online, and cosmetics stores were analyzed in terms of the criteria specified in the "almond oil" monograph in European Pharmacopoeia (EP) 7.0.[22]

MATERIALS AND METHODS

Materials

Seventeen different brands of almond oils were purchased from pharmacies, herbal, online, and cosmetics stores in Ankara, Türkiye between 2017 and 2018. All chemicals used were of analytical reagent grade. Oksan Co., Ltd. (Ankara, Türkiye) provided helium, hydrogen, and dried air gases for gas chromatography (GC) with 99.99% purity. Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

GC (7890A GC System, Agilent Technologies Inc, US), a capillary column Rt-2560 (100 m, 0.25 mm ID, 0.2 µm) (Restek Corporation Bellefonte, US), vial insert, 250 µL, glass with polymer feet, vial, screw top, 2 mL, amber and cap, screw, blue, PTFE/red silicone septa (Agilent Technologies Inc., US) were used.

Methods

Seventeen brands of almond oils were analyzed according to the criteria of EP 7.0[22] (appearance, identification, acid value, peroxide value, and composition of fatty acids) and the results were compared with the pharmacopeia quality of reference standard almond oil from *P. dulcis* (63445-250 mL, Sigma Aldrich, Lot BCBV9057).

Statistical analysis

All the results are given for at least in triplicate and the values are given as mean ± standard deviation. Calculations made in the statistical analysis were made using Microsoft Excel software.

RESULTS

Appearance

All oil samples were placed into glass droppers and their colors were compared with the pharmacopeia quality of reference almond oil (Table 1).[22]

Identification

Thin layer chromatography (TLC) was used to identify the almond oils.[23] C-18 silica TLC plate (Supelco 10 x 10 cm, 0.2 mm) was used as the stationary phase. An approximately 20 mg of oil sample, and a reference solution of almond oil complying with "Almond Oil European Pharmacopoeia" standards were dissolved in 3 mL of CH_2Cl_2 (for GC MSSupraSolv®). TLC plate was first eluted with ether (Sigma Aldrich, Germany) mobile phase up to 0.5 cm. Then, the plate was then removed from the tank and immersed in the other tank containing CH_2Cl_2: glacial acetic acid (Sigma Aldrich, Germany): Acetone (Sigma Aldrich, Germany) (2:4:5, by volume) mobile phase, and eluted 8 cm. The plate removed from the tank and dried in air, and then 100 g L^{-1} solution of phosphomolybdic acid (Sigma Aldrich, Germany) in alcohol as revelator was sprayed. TLC plate was heated at 120°C for 3 min. Then, retention times and stain of the commercial almond oils were compared with the retention times and stains of reference almond oil from *P. dulcis* (Figure 1). A thin layer chromatogram of the samples and reference almond oil (Figure 1) was also compared with the reference chromatogram in EP 8.0 (Figure 2).[23]

Acid (I_A) and peroxide (I_P) values

Acid value is expressed as milligrams of KOH (Sigma Aldrich, Germany) required to neutralize the free acids in 1 g of the oil. Therefore, about 10 g of each oil sample was dissolved in 50 mL of 96% methanol (Merck, Germany) and peroxide-free ether (Merck, Germany, Germany) mixture (1:1, by volume), then titrated with 0.1 M KOH in the presence of phenolphthalein (Sigma Aldrich, Germany) indicator until the pink remained stable for at least 15 s acid values of samples were compared with the value of maximum of 2.0 in a 5.0 g oil sample (Table 1).[22,24]

The peroxide value is expressed as milliequivalent of active oxygen, the quantity of peroxide contained in 1000 g of the substance. So, about 5 grams of oil was placed in a 250 mL conical flask fitted with a ground-glass stopper. 30 mL of a mixture of chloroform (Merck, Germany) and glacial acetic acid (2:3, by volume) was added. After the oil dissolved, 0.5 mL of saturated potassium iodide (Merck, Germany) solution was added and shaken for exactly 1 min, then 30 mL water was added. It was titrated with 0.01 M sodium thiosulfate (Sigma Aldrich, Germany) until the yellow was almost discharged. 5 mL of starch solution was added and continued the titration, until the color was discharged. It was carried out with a blank test under the same conditions. Peroxide values were compared the value of maximum 15.0 in 5 g oil (Table 1).[22,24]

Preparation of fatty acid methyl esters (FAMEs) standard

All standard solutions were prepared in an ice bath and stored at -20°C. 0.1 g of FAME37, C4-24 (Sigma Aldrich, Germany)

reference standard was dissolved in 250 µL of CH_2Cl_2 (400 mg mL^{-1} FAME37, C4-24), and then, 75 µL of 400 mg mL^{-1} FAME37, C4-24 was diluted to 1.0 mL with CH_2Cl_2 (30 mg mL^{-1} FAME37).

Preparation of FAMEs in almond oils

Each oil was dried at 100-105°C. The oils (1.0 g for each) were weighed into a 25 mL round-bottomed flask with a ground-glass neck fitted with a reflux condenser and a gas port into the flask. Anhydrous methanol (10 mL) and 60 g L^{-1} KOH (0.2 mL) in methanol were added. The reflux condenser was attached, passed nitrogen through the mixture at a rate of about 50 mL min^{-1} shacked, and heated to boiling. When the solution was clear, it was continued heating for a further 5 min and cooled the flask and transferred the contents to a separating funnel. The flask was rinsed with 5 mL of anhydrous chromatographic quality of heptane (99%, Sigma Aldrich, Germany), then, the rinsing was transferred to a separating funnel and stacked. 10 mL of a 200 g L^{-1} NaCl (Sigma Aldrich, Germany) solution was added and stacked vigorously. It was allowed to form two separate phases and transferred the upper organic layer to a vial containing anhydrous Na_2SO_4 (Sigma Aldrich, Germany), allowed to stand, then filtered.

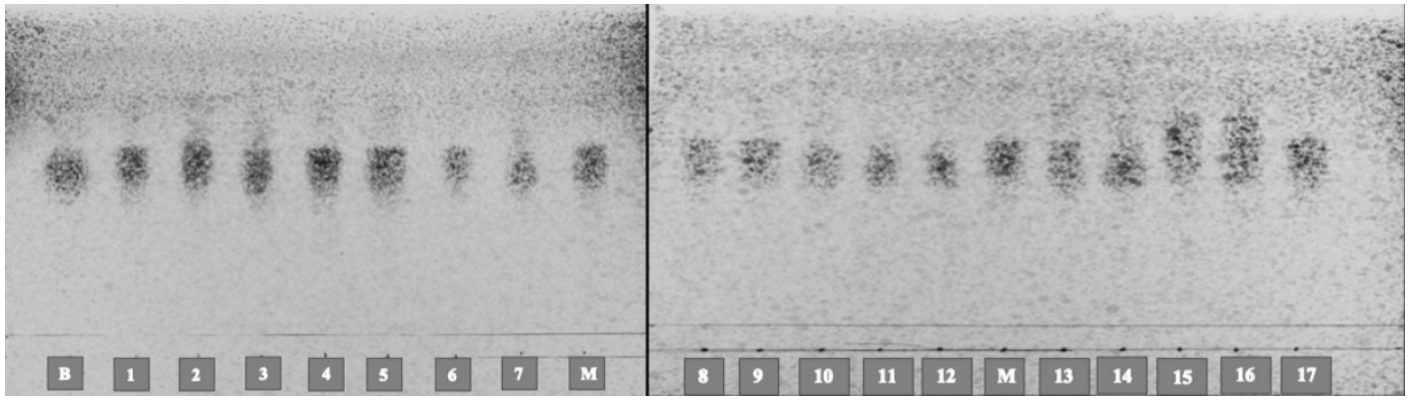

Figure 1. Thin layer chromatography of almond oil samples (s1 to s17), corn oil (M), and reference almond oil obtained from *Prunus dulcis* (B)

Table 1. Appearance, acid value and, peroxide value of almond oils

Sample no	Appearance	I_A^a ± SD[b] mg KOH g^{-1} oil	I_P^c ± SD[b] mL g^{-1}
s1	Clear liquid	0.34 ± 0.04	10.61 ± 0.86
s2	Pale yellow	0.48 ± 0.01	17.30 ± 0.87
s3	Pale yellow	3.30 ± 0.01	4.85 ± 0.99
s4	Clear liquid	0.36 ± 0.04	10.94 ± 1.60
s5	Clear liquid	0,80 ± 0,03	9.40 ± 0.82
s6	Clear liquid	0.32 ± 0.001	8.16 ± 0.77
s7	Dark yellow	9.18 ± 0.09	3.06 ± 0.77
s8	Clear liquid	0.36 ± 0.01	3.76 ± 0.14
s9	Pale green	1.22 ± 0.02	5.40 ± 0.18
s10	Yellowish green	8.65 ± 0.32	4.46 ± 0.32
s11	Clear liquid	0.18 ± 0.01	7.14 ± 0.07
s12	Pale yellow	1.24 ± 0.06	7.68 ± 0.28
s13	Clear liquid	0.35 ± 0.04	9.52 ± 3.30
s14	Dark yellow	6.04 ± 0.16	6.10 ± 0.28
s15	Pale yellow	0.32 ± 0.02	2.43 ± 0.10
s16	Clear liquid	0.35 ± 0.01	19.59 ± 0.41
s17	Dark yellow	0.38 ± 0.03	8.98 ± 0.09
Almond oil (pharmacopoeia quality)	Pale yellow	0.44 ± 0.02	3.46 ± 0.29

[a]Reference acid value is maximum 2.0, determined on 5.0 g, [b] SD: Standard deviation, n: 3, [c]Reference peroxide value is maximum 15.0

Analysis of FAMEs with GC- flame ionization detector (FID)

FAME37, C4-24 standard, and fatty acids in almond oil were analyzed by GC equipped with an auto sampler model Agilent 7693, and FID.[25] A capillary column Rt-2560 (100 m, 0.25 mm ID, 0.2 µm), vial insert, 250 µL, glass with polymer feet, vial, screw top, 2 mL, amber and cap, screw, blue, and PTFE/ red silicone septa were used as the column and sample vial, respectively. GC oven was programmed to 100°C, held for 4 min, and then increased by 3°C min ramp to 240°C, held for 10 min. The injector and FID detector temperatures were 225°C and 250°C, respectively. Injection volume was 2 µL with a split ratio 200:1. Helium was used as the carrier gas at 1.2 mL min^{-1}, 20 cm s^{-1} at 175°C. FAMEs in the oil samples were identified from the chromatogram by comparing their retention times with standard FAME37, C4-24, and the number of FAMEs in the oil samples was expressed as a percentage by weight of all FAMEs from the total detected fatty acids (Figures 3, 4). Peak area was used for quantitative analysis of FAMEs, where their content in almond oil samples were listed in Tables 2a and b.

DISCUSSION

EP 7.0 states that the appearance of almond oil should be clear or pale yellow. The appearance, acid values, and peroxide values of almond oil samples are given in Table 1. In the evaluations, it was observed that the samples coded s7, s9, s10, s14, and s17 do not have this appearance and their colors are pale green or dark yellow (Table 1).

Calculation of the acid value in fixed oils is an important quality criterion. It also gives an idea of whether the oil has exceeded its shelf life. The increase in the amount of free fatty acids is an indication that there will be a decrease in the stability of the oil

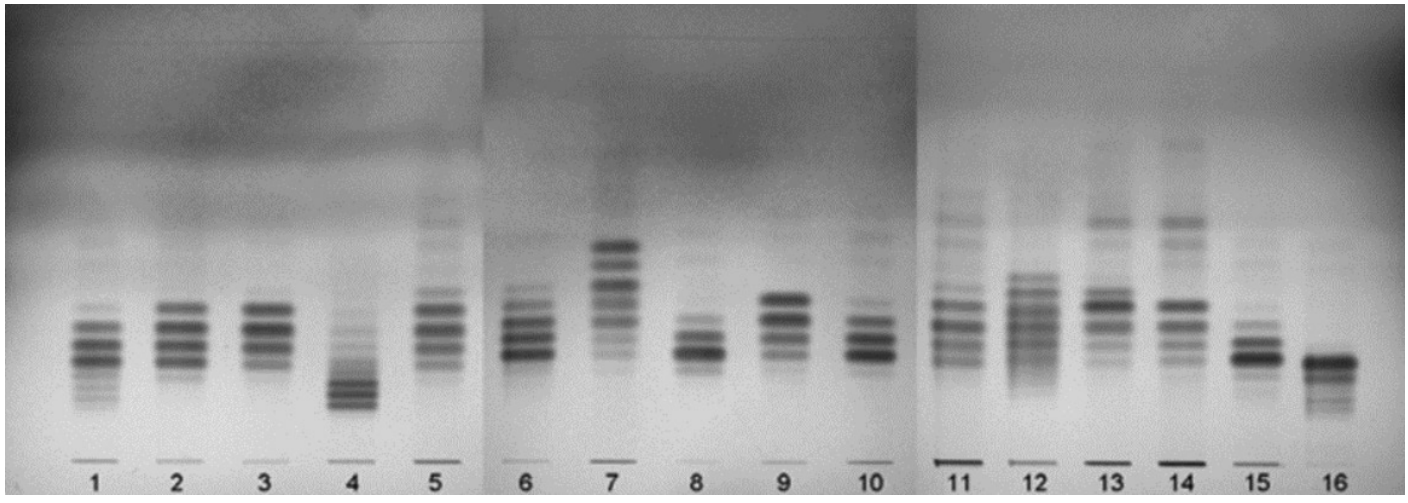

Figure 2. Thin layer chromatograms for the identification of fatty acids in European Pharmacopoeia 8.0[23] [1: Arachis oil, 2: Sesame oil, 3: Corn oil, 4: Rapeseed oil, 5: Soya-bean oil, 6: Rapeseed oil (erucic acid-free) 7: Linseed oil, 8: Olive oil, 9: Sunflower oil 10: Almond oil, 11: Wheat-germ oil, 12: Borage oil, 13: Evening primrose oil, 14: Safflower oil (type I), 15: Safflower oil (type II), 16: Hydrogenated arachis oil]

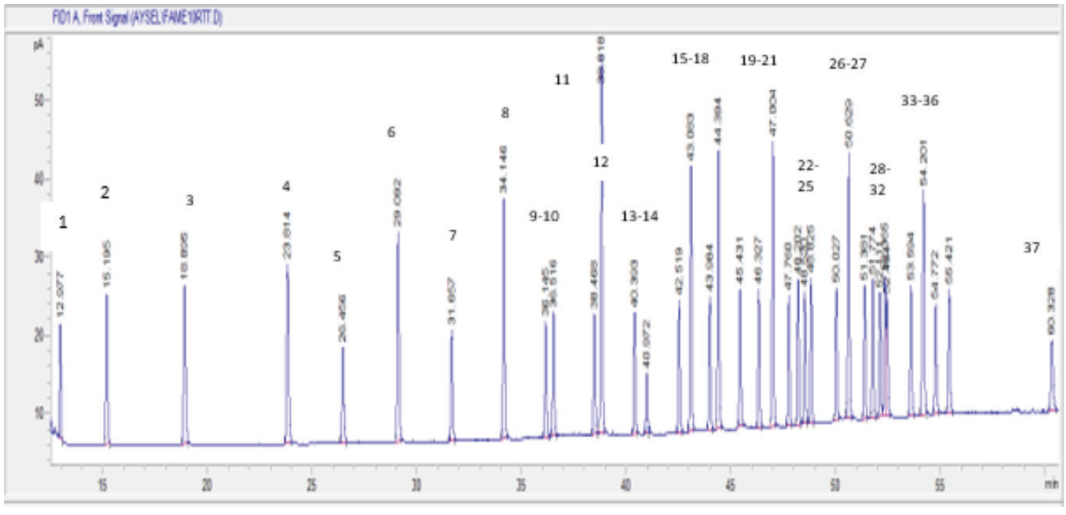

Figure 3. GC-FID chromatogram of FAME37 mix, C4-C24 reference standard material (1- C4:0, 2- C6:0, 3- C8:0, 4- C10:0, 5- C11:0, 6- C12:0, 7- C13:0, 8- C14:0, 9- C14:1 (*cis*-9), 10- C15:0, 11- C15:1 (*cis*-10), 12- C16:0, 13- C16:1 (*cis*-9), 14- C17:0, 15- C17:1 (*cis*-10), 16- C18:0, 17- C18:1 (*trans*-9), 18- C18:1 (*cis*-9), 19- C18:2 (*trans*-9,12), 20- C18:2 (*cis*-9,12), 21- C20:0, 22- C18:3 (*cis*-6,9,12), 23- C20:1 (*cis*-11), 24- C18:3 (*cis*-9,12,15), 25- C21:0, 26- C20:2 (*cis*-11,14), 27- C22:0, 28- C20:3 (*cis*-8,11,14), 29- C22:1 (*cis*-13), 30- C20:3 (*cis*-11,14,17), 31- C20:4 (*cis*-5,8,11,14), 32- C23:0, 33- C22:2 (*cis*-13,16), 34- C24:0, 35- C20:5 (*cis*-5,8,11,14,17), 36- C24:1 (*cis*-15), 37- C22:6 (*cis*-4,7,10,13,16,19)

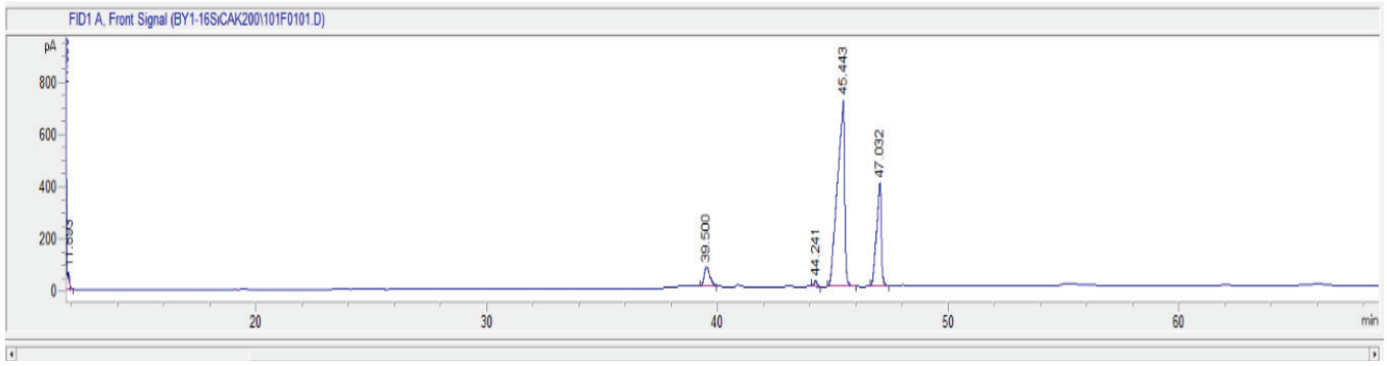

Figure 4. GC-FID chromatogram of reference almond oil (from *Prunus dulcis*)

Table 2a. Content of FAMEs in almond oil samples 1-9

FAME	Content, percent in almond oil ± SD[a]								
	s1	s2	s3	s4	s5	s6	s7	s8	s9
C14:0	0.1 ± 0.0	0.1 ± 0.0	-	-	-	-	0.1 ± 0.0	-	-
C16:0	6.2 ± 0.5	10.2 ± 0.3[b]	4.9 ± 0.3	5.7 ± 0.3	5.5 ± 0.3	5.7 ± 0.3	6.5 ± 0.3	5.5 ± 0.3	5.7 ± 0.3
C16:1	-	-	0.3 ±0.0	-	-	-	0.4 ± 0.1	0.2 ± 0.0	-
C18:0	3.4 ± 0.1[b]	4.6 ± 0.1[b]	1.2 ± 0.2	3.8 ± 0.1[b]	3.9 ± 0.1[b]	3.6 ± 0.1[b]	1.2 ± 0.2	3.5 ± 0.2[b]	2.8 ± 0.2
C18:1	29.3 ± 0.4[b]	25.9 ± 0.5[b]	66.1 ± 0.6	30.5 ± 0.6[b]	29.9 ± 0.5[b]	30.4 ± 0.5[b]	64.2 ± 1.0	33.8 ± 0.3[b]	36.4 ± 0.5[b]
C18:2	57.8 ± 1.3[b]	51.4 ± 0.3[b]	20.6 ± 0.1	57.6 ± 0.6[b]	58.3 ± 0.6[b]	57.9 ± 0.6	24.9 ± 1.5	54.3 ± 1.2[b]	52.9 ± 1.2[b]
C20:0	0.3 ± 0.0[b]	0.6 ± 0.1[b]	0.4 ± 0.0[b]	0.3 ± 0.0[b]	0.3 ± 0.0[b]	0.3 ± 0.0[b]	-	0.3 ± 0.0[b]	-
C18:3	0.3 ± 0.0	0.6 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
C20:1	-	5.2 ± 0.3[b]	5.5 ± 0.3[b]	0.1 ± 0.0	-	-	-	-	-
C18:3	0.3 ± 0.0	0.1 ± 0.0	-	-	0.1 ± 0.0	0.1 ± 0.0	-	-	-
C20:2	0.9 ± 0.2	0.6 ± 0.1	0.3 ± 0.0	0.9 ±0.1	0.9 ± 0.1	0.9 ± 0.1	-	0.9 ± 0.1	0.8 ± 0.1
C24:0	-	-	-	0.4 ± 0.0	-	-	-	-	-
C20:5	0.4 ± 0.2	-	-	-	0.4 ± 0.0	0.4 ± 0.0	-	0.5 ± 0.0	-

[a]SD: Standard deviation, n= 3, [b]Out of limit, FAME: Fatty acid methyl ester

Table 2b. The content of FAMEs in almond oils samples 10-17 and reference almond oil

FAME	Content, % in almond oil ± SD[a]								
	s10	s11	s12	s13	s14	s15	s16	s17	Reference almond oil
C16:0	5.8 ± 0.0	5.7 ± 0.2	5.5± 0.2	3.9 ± 0.1[b]	6.3 ± 0.3	6.9 ± 0.3	5.7 ± 0.2	5.7 ± 0.1	5.5 ± 0.2
C16:1	-	0.4 ± 0.1	0.4 ± 0.1	-	0.4 ± 0.1	-	0.4 ±0.1	0.2 ± 0.0	-
C18:0	1.2 ± 0.0	0.8 ± 0.2	0.5 ± 0.2	2.5 ± 0.5	1.0 ± 0.2	4.3 ± 0.4[b]	3.9 ± 0.2[b]	1.9 ± 0.3	0.9 ± 0.1
C18:1	69.8 ± 1.5	67.6 ± 0.5	68.8 ± 0.9	67.8 ± 1.3	65.6 ± 0.6	23.2 ± 0.6[b]	31.3 ± 0.5[b]	16.7 ± 0.7[b]	68.3 ± 0.1
C18:2	23.2 ± 0.3	24.5 ± 0.7	23.3 ± 0.6	22.7 ± 0.9	25.3 ± 0.6	28.5 ± 0.2	20.2 ± 0.2	74.0 ± 1.6[b]	24.7 ± 0.4
C20:0	-	-	-	0.3 ± 0.0[b]	-	3.7 ± 0.9[b]	4.2 ± 0.3[b]	0.5 ± 0.0[b]	-
C18:3	-	-	-	0.3 ± 0.0	0.3 ± 0.0	2.7 ± 0.0	2.8 ± 0.0	0.3 ± 0.1	-
C20:1	-	-	-	-	-	31.3 ± 0.1[b]	32.9 ± 0.4[b]	-	-
C20:2	-	-	-	1.6 ± 0.1	-	-	-	0.6 ± 0.0	-
C22:0	-	-	-	-	-	0.2 ± 0.1	0.2 ± 0.1	-	-
C24:0	-	-	-	0.9 ± 0.0	-	-	-	-	-

[a]SD: Standard deviation, n= 3, [b]Out of limit, FAME: Fatty acid methyl ester

against oxidation. This situation is defined as the oil becoming bitter. According to EP 7.0, it has been reported that acid value in the almond oil should be "maximum 2.0 in 5 g oil". When evaluated in terms of this criterion; acid values of the samples coded s3, s7, s10, and s14 (I_A= 3.30 to 9.18) were found to be well above the criteria reported (Table 1).

Peroxide content of the oil samples indicates that the oil has started to oxidize. Peroxidation process occurs due to high temperature and light exposure. Contact with metal surfaces can also cause the oil to oxidize faster. Additionally, oxygen breaks down unsaturated fatty acids, resulting in smaller aldehyde molecules such as malondialdehyde. The lower peroxide values indicate the longer shelf life of the oil. A high peroxide value usually indicates poor processing and poor oil quality. According to EP 7.0, it has been reported that the peroxide values in almond oil should be "maximum 15.0 in 5 g oil". When the results were investigated, the samples, s2 (I_P= 17.30) and s17 (I_P= 19.59) were found to have high peroxide values compared to the maximum values (Table 1).

The fatty acids in commercial almond oils were identified by comparing their TLC profiles with that of the reference almond oil from *P. dulcis* under the same conditions (Figure 1). Results of the fatty oils reported in the EP 8.0 (Figure 2)[23] were also used for identification of fatty acids of the samples. TLCs obtained from samples s5, s6, s8, s9, s11, s12, and s13 were similar to the reference almond oil chromatogram and the corresponding pharmacopeia chromatogram as shown in Figure 2. Nevertheless, the general profile of samples s1, s2, s3, s4, s7, s10, s14, s15, and s16 coded expressions are not suitable.

Quality of the oil is related to its contents, types, and number of fatty acids. In the almond oil monograph in EP 7.0, defined as "*Amygdalae oleum raffinatum*" and its fatty oil was obtained from the ripe seeds of *P. dulcis* var. *dulcis* or *P. dulcis* var. *amara* or a mixture of both varieties by cold expression. Then, it is refined. A suitable antioxidants may be added. Another almond oil registered in the EP 7.0 is "almond oil, virgin, "*Amygdalae oleum virginale*," and its fatty oils were obtained from cold expression from the ripe seeds of *P. dulcis* var. *dulcis* or *P. dulcis* var. *amara,* or a mixture of both varieties.

According to EP 7.0, fatty acid compositions of refined almond oil and virgin almond oil are given as; palmitic acid (4.0-9.0%), palmitoleic acid (0.8% max), margaric acid (not more than 0.2%), stearic acid (3.0% max), oleic acid (62-86%), linoleic acid (20-30%), linolenic acid (0.4% max), arachidic acid (not more than 0.2%), eicosenoic acid (0.3% max), behenic acid (not more than 0.2%), and erucic acid (0.1% max).[22] Supelco FAME37 mix, C4-24 was used as a standard reference material to detect the fatty acids in almond oil with GC-FID.

Fatty acids are defined as the organic compounds formed by a hydrocarbonated chain and a carboxylic acid group, which are normally bound with glycerol-forming acylglycerides (mono-, di- or triglycerides).[26] While α-linolenic acid and linoleic acids are the essential fatty acids, which the human body cannot produce, the unsaturated fatty acids in almond oil (50-81% oleic and 6-37% linoleic acid) become more desirable for the physicochemical and nutritional properties and its significant role in human diet.[27-31] Their fatty acid profiles depend on the oil's variety and origin, which affects their stability against rancidity during transport, storage, and directly affecting their products and influencing their price.[32] The oil content and composition of almond seed and the fatty acids are usually referred to as the quality characteristic of almond conditions,[32] while it depends on the genotype, climatic conditions, agriculture, and harvest. The amounts of oleic acid, oleic/linoleic acid ratio and tocopherol concentration are used as quality indicators, while the oleic/linoleic acid ratio is significant in determining the quality of the kernel due to its preventive effect on lipid oxidation.[32] When fatty acid contents of the almond oil samples analyzed are evaluated according to the EP 8.0,[25] it is seen that oleic acid (C18:1) amounts of samples with s1, s2, s4, s5, s6, s8, and s9 codes are lower than the reference value and linoleic acid (C18:2) amounts are higher than the reference value (Table 3). This difference may also be due to production of gums, as well as possibility of oxidation of oleic acid ($C_{18}H_{34}O_2$) to linoleic

Table 3. Comparison of FAMEs in almond oils according to the EP 7.0

FAMEs	s1	s2	s3	s4	s5	s6	s7	s8	s9	s10	s11	s12	s13	s14	s15	s16	s17
C16:0	R	↑	R	R	R	R	R	R	R	R	R	R	↓	R	R	R	R
C16:1	-	-	R	-	-	-	R	R	-	-	R	R	-	R	-	R	R
C17:0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C18:0	↑	↑	R	↑	↑	↑	R	↑	R	R	R	R	R	R	↑	↑	R
C18:1	↓	↓	R	↓	↓	↓	R	↓	↓	R	R	R	R	↓	↓	↓	R
C18:2	↑	↑	R	↑	↑	↑	R	↑	↑	R	R	R	R	R	R	R	↑
C18:3	R	R	-	-	R	R	-	-	-	-	-	-	-	-	-	-	-
C20:0	↑	↑	↑	↑	↑	↑	-	↑	-	-	-	-	↑	-	↑	↑	↑
C20:1	-	↑	↑	R	-	-	-	-	-	-	-	-	-	-	↑	↑	-
C22:0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↑	↑	-
C22:1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

R: Between reference range, ↑: More than reference value, ↓: Less than reference value, -: Not found, FAME: Fatty acid methyl ester, EP: European Pharmacopoeia

acid ($C_{18}H_{32}O_2$) depending on the production methods (refined, infiltration, cold press *etc.*), storage conditions (heat, light, *etc.*), antioxidant addition and amount. However, the samples with s7, s10, s11, and s12 codes were found to meet the criteria required by the EP 7.0.[22] When all the results are evaluated; among the 17 almond oil samples sold in the pharmacy, only 2 (s11 and s12) of them were found to meet the EP 7.0[22] criteria for quality (Table 4).

CONCLUSION

Many vegetable oils are sold with health-promoting claims or statements that they are beneficial against diseases, while almond oils are one of them, as they are sold in "natural", "organic products", "local products" shops, cosmetics store chains, and pharmacies. Almond oil, which is marketed for health benefits and cosmetics, must meet the EP criteria. Currently, these almond oils are marketed as fixed oils with the producer's own marketing and quality criteria, while our study shows that most of the products are off-limits of pharmacopeia. A pharmacopeia's core mission is to protect public health by creating and making available public standards to help ensure the quality of products, while the user or procurer can make an independent judgment regarding quality, thus safeguarding the health of the public. To establish the necessary quality criteria and show no harm to the user, the almond oil (if for human use) needs to be encouraged to be pharmacopeia compliance. Additionally, currently, almond oil is excluded from the Turkish Food Codex Standards. For this reason, it should be ensured that almond oil is listed in this codex and urgent arrangements should be made for quality control analysis.

Table 4. Comparison with criteria according to EP 7.0

Sample no	Appearance	TLC	FAMEs	I_A	I_P
s1	+	-	-	+	+
s2	+	-	-	+	-
s3	+	-	-	-	+
s4	+	-	-	+	+
s5	+	+	-	+	+
s6	+	+	-	+	+
s7	-	-	+	-	+
s8	+	+	-	+	+
s9	-	+	-	+	+
s10	-	-	+	-	+
s11	+	+	+	+	+
s12	+	+	+	+	+
s13	+	+	-	+	+
s14	-	-	-	-	+
s15	+	-	-	+	+
s16	+	-	-	+	-
s17	-	+	-	+	+

+: Appropriate -: Not appropriate

Ethics

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

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: D.D.O., Design: D.D.O., Data Collection or Processing: D.D.O., O.K.U., A.B., S.P., B.N.D.T., Analysis or Interpretation: D.D.O., O.K.U., A.B., S.P., B.N.D.T., Literature Search: D.D.O., O.K.U., B.N.D.T., Writing: D.D.O., O.K.U.

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

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ORIGINAL ARTICLE

In Vitro Activity of Some Medicinal Plants on Blood Coagulation

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ABSTRACT

Objectives: The concern for finding natural and curative agents without adverse side effects has prompted the interest in discovering hemostatic agents from plants. Therefore, *in vitro* activity of *Aizoon hispanicum* L. (Aizoaceae), *Centaurea hyalolepis* Boiss. (Asteraceae), *Heliotropium maris-mortui* Zohary. (Boraginaceae), *Parietaria judaica* L. (Urticaceae), *Polygonum arenarium* Waldst. & Kit. (Polygonaceae), and *Verbascum sinuatum* L. (Scrophulariaceae) on blood coagulation was estimated by two common tests, which are the prothrombin time test (PT) and the activated partial thromboplastin time test (aPTT).

Materials and Methods: The extracted powders from the plants under this study were adjusted to be 50 mg/mL. Then, *in vitro* effect of these extracts on the platelet poor plasma samples was measured by an automated coagulation analyzer using PT and aPTT tests.

Results: Based on the obtained results, all plant extracts affected the coagulation cascade by rising either PT or aPTT or both, except for *V. sinuatum* extract, which reduced only aPTT value. Moreover, the recorded PT values showed that *A. hispanicum*, *H. maris-mortui*, and *P. arenarium* significantly prolonged the PT ($p<0.05$). Additionally, the results clearly showed that *V. sinuatum* acted as a coagulant agent based on aPTT values, while all other plants, in contrast, acted as strong anticoagulants. Among the plant species under study, *A. hispanicum, H. maris-mortui*, and *P. arenarium* extracts prolonged both PT and aPTT significantly ($p<0.05$). This could be referred to their additional effect on the common pathway. However, *C. hyalolepis*, *P. judaica*, and *V. sinuatum* showed no significant effect on PT values ($p>0.05$).

Conclusion: The positive recorded data from this research could serve as identification of new hemostatic remedies that could be used for the commercial economic purposes and for managing several cardiovascular diseases.

Key words: Coagulation cascade, medicinal plants, prothrombin time, activated partial thromboplastin time

INTRODUCTION

Medicinal plants have been and still are used significantly in healthcare by different populations throughout the world.[1] The natural compounds extracted from medicinal plants have been used as conventional or complementary remedies for both treatable and untreatable diseases.[2] However, these natural products are considered as a valuable source of several compounds that exhibit various biological activities, which in turn be useful for developing alternative therapies.[3] For example, herbal medicines prepared from garlic *Allium sativum* L. are supposed to inhibit platelet activation.[3] Also, other herbs such as *Salix alba* L. are ethnomedicinally used as an anti-inflammatory agent. Later on, the extracted salicylic acid from the second plant species was transformed into a powerful anti-platelet drug acetylsalicylic acid that also called aspirin.[4] For that reason, the current research worked on six different plants, which are *Aizoon hispanicum* L., *Centaurea hyalolepis* Boiss., *Heliotropium maris-mortui* Zohary., *Parietaria judaica* L., *Polygonum arenarium* Waldst. & Kit., and *Verbascum sinuatum* L. These plant species were selected for this research according to their medical histories in various fields. However, to date, there are no previous studies on the effect of these plant species on blood coagulation, which is closely related to thromboembolic diseases.[5] The coagulation cascade is a complicated system of two pathways, which are the extrinsic (tissue factor) and intrinsic (contact factor) pathways. At the

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end of each of the two pathways, a solid hemostatic clot was formed from fluid blood. This clot must maintain hemostasis by repairing the damage vascular vessel and diminishing the blood flow through it. Thus, any malfunction in the coagulation cascade can be harmful to the human body.[6]

In this aspect, researchers have offered anticoagulant and antithrombotic drugs such as heparin, aspirin and warfarin that have been widely used nowadays as therapies for thromboembolic diseases.[7] Also, researchers found that plants can be a useful source of new anticoagulant drugs due to their promising biological activities. Based on all previously mentioned information, the idea of this study emerged to assess *in vitro* activity of six plant species that are growing wild in Palestine in the coagulation cascade. The anticoagulant or coagulant properties of their leaf aqueous extracts were evaluated by measuring their effect on the prothrombin time (PT) and the activated partial thromboplastin time (aPTT).

MATERIALS AND METHODS

Plant collection and extract preparation

Six wild plant species [*A. hispanicum* (Jericho), *C. hyalolepis* (Jericho), *H. maris-mortui* (Tulkarm), *P. judaica* (Jericho), *P. arenarium* (Tulkarm), and *V. sinuatum* (Nablus)] were collected from the West Bank, Palestine, and identified by Ghadeer Omar, An-Najah National University, Palestine. Representative plant samples were deposited at An-Najah National University herbarium with specific voucher numbers (1940, 1950, 1960, 1954, 1965, and 1970 respectively). After collection, the plant leaves for coagulation study were washed, dried, crushed, and stored in a dry place at room temperature until the day of extract preparation. For aqueous extraction, 5 g from each crushed plant material was soaked in 100 mL sterile and warm distilled water with a continuous rotary shaking at 25°C. After 72 h, all plant mixtures were macerated using a probe sonicator (3 seconds sonication and 5 seconds rest) for 15 min at 25°C. Then, the macerated extracts were subjected to centrifugation at 4500 rpm for 10 min and the gained supernatants were dried by lyophilization. The lyophilized powder from each plant species was solubilized in sterile distilled water to obtain 50 mg/mL concentration.

Blood collection and platelet poor plasma (PPP) preparation

The study was approved by the Ethics Committee (IRB) of the Faculty of Health Sciences at An-Najah National University (ref: SC 12/1/20). Blood samples were collected from eight volunteers, who were healthy, not under any medication, and not smokers. Each collected blood sample was transferred to a citrated centrifugation tube. Then, the citrated tubes were centrifuged at 1500 rpm for 15 min to obtain PPP.[8] All (PPP) samples were analyzed in duplicate by PT and aPTT tests within 2 h from the time of blood collection. The clotting time for two tests was automatically recorded by Coa DATA 4004 coagulation analyzer (LAberBioMedical Technologies, Germany), using standard reagent kits from Human diagnostics (Germany).

In vitro prothrombin time assay

In a single cuvette, 50 µL of (PPP) was incubated for 5 min with 50 µL of each plant aqueous extract at 37°C. Then, the clotting time was directly recorded after the addition of 100 µL PT reagent (Hemostat thromboplastin-SI, Human, Germany).[9]

In vitro activated partial thromboplastin assay

In a single cuvette, 50 µL of (PPP) was incubated for 2 min with 50 µL from each plant aqueous extract at 37°C. After that, 50 µL aPTT reagent (Human, Germany) was added. Following that, the sample was further incubated for 3 min at 37°C. Then, the aPTT clotting time was directly recorded after the addition of 100 µL calcium chloride solution (Human, Germany).[9]

Statistical analysis

Statistical analysis of the PT and aPTT results was performed using a statistical package SPSS by applying mean values using one-way ANOVA with *post-hoc* test. The purpose of this analysis was to determine whether there was any significant difference between the different plant aqueous extracts being studied and the controls. The obtained results were considered significant, when p value was less than 0.05.

RESULTS

This study aimed to evaluate the effect of different plant leaves aqueous extracts on the coagulation cascade by using PT and aPTT tests. PT measures the time required to produce fibrin after factor VII activation. However, aPTT measures the time required to create fibrin starting from intrinsic pathway initiation. According to the used kits and reagents in the running experiment, the normal range value for PT is between 10 and 15 sec and for aPTT is between 22 and 32 sec.[10] Blood samples of the participants were considered to be a representative sample for the running study with no individual variations among them (p= 0.000). Based on the obtained results, all plant extracts influenced the coagulation cascade by increasing either PT or aPTT or both except for *V. sinuatum* extract, which reduced only the aPTT value (Table 1).

Moreover, the recorded PT values showed that *A. hispanicum*, *H. maris-mortui* and *P. arenarium* significantly prolonged PT with respect to the control (p<0.05) at the concentration under study (Figure 1). Additionally, the results clearly showed that *V. sinuatum* acted as a coagulant agent based on aPTT values. While all other plant species, in contrast, acted as strong anticoagulants. Thus, they could prevent blood coagulation (Figure 2). Among the plant species being studied, *A. hispanicum, H. maris-mortui,* and *P. arenarium* extracts prolonged both PT and aPTT significantly (p<0.05). This could be referred to their additional effect on the common pathway. However, *C. hyalolepis*, *P. judaica*, and *V. sinuatum* had no significant effect on PT values (p>0.05).

DISCUSSION

The blood coagulation cascade is a physiological phenomenon that comprises intrinsic, extrinsic, and common pathways. Briefly, the activation of the intrinsic pathway occurs because

of trauma and contact between kininogen, prekallikrein, and factor XII with underlining collagen on endothelium.[11] Otherwise, activation of the extrinsic pathway occurs upon tissue injury, which causes the release of tissue factor into the bloodstream.[12] After activation, both the intrinsic and extrinsic pathways end with a common pathway, whose activation starts after the generation of factor Xa from one of the previous two pathways. This factor cleaves prothrombin into thrombin. Later on, thrombin converts fibrinogen to fibrin that in turn causes clot formation.[13] In fact, blood coagulation is a highly regulated pathway and the imbalance due to genetic and environmental factors could alter the normal coagulation system that leads to the formation of unusual clots in the blood vessels. This pathological phenomenon called thrombosis, which is considered one of a cardiovascular and cerebrovascular risk.[14]

Table 1. Prothrombin time and activated partial thromboplastin time values of the study plant extracts on eight participants' platelet-poor plasma samples

Plant species	PT (sec)	*p* value*	aPTT (sec)	*p* value*
Aizoon hispanicum	420	0.000	420	0.000
Centaurea hyalolepis	31.35	0.428	48.27	0.000
Heliotropium maris-mortui	325.45	0.000	420	0.000
Parietaria judaica	43.02	0.128	414.07	0.000
Polygonum arenarium	70.65	0.001	420	0.000
Verbascum sinuatum	27.19	0.806	16.63	0.018

**p* value <0.05 was significant among the different study plant species relative to the control (blood sample without plant extract). PT: Prothrombin time, aPTT: Activated partial thromboplastin time

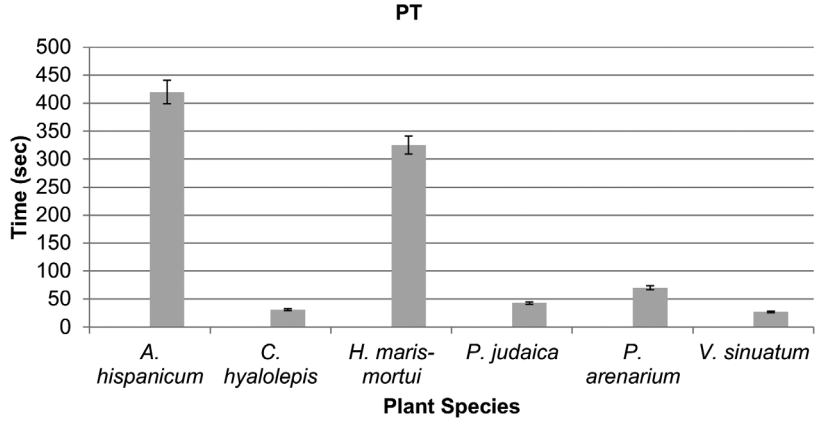

Figure 1. The effect of six plant species aqueous extracts on the prothrombin time for eight participants platelet-poor plasma samples
PT: Prothrombin time

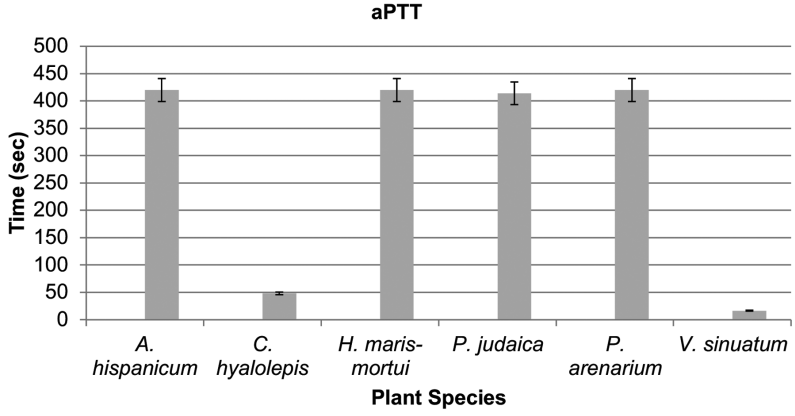

Figure 2. The effect of six plant species aqueous extracts on the partial thromboplastin time for eight participants's platelet-poor plasma samples
aPTT: Activated partial thromboplastin time

The present study evaluated the effect of six different types of the leaf aqueous extracts, which belong to *A. hispanicum, C. hyalolepis, H. maris-mortui, P. judaica, P. arenarium*, and *V. sinuatum*, on the coagulation cascade. Since, clotting can be initiated and proceed according to two different cascading pathways, which are the intrinsic (contact factor) or the extrinsic (tissue factor), the impact of all aqueous extracts under study on blood coagulation was estimated by PT and aPTT tests. Consequently, compared to the standard values of PT and aPTT, it is noticeable that some of the plant extracts examined in this study had a coagulant or anticoagulant effect on the tested blood samples. This investigation considering the effects of previous plant species on the blood coagulation was not previously studied, which in turn provides the novelty of this research.

As noted in the results, all examined plant extracts prolonged aPTT values except *V. sinuatum*, which in contrast, demonstrated a pronounced decreasing effect on the aPTT at the studied concentration. The noticed anticoagulation effect of these plant species may be related to inhibition of the contact factors of intrinsic pathway,[11] whereas extrinsic pathway tissue clotting factors may be inhibited by *A. hispanicum, H. maris-mortui,* and *P. arenarium* aqueous extracts as they prolong PT values.[7] Remarkable anticoagulation activity was seen in *A. hispanicum, H. maris-mortui* and *P. arenarium* as they prolonged both PT and aPTT. This bioactivity indicated that those plant species acted on the common pathway in addition to their impact on the extrinsic and intrinsic pathways. Thus, the common pathway factors; X, V, II, and I, could be the target for their inhibition.[11] Regarding the obtained results, coagulation activity reduction may be mediated through inhibition or diminishion of activity of several factors including tissue factors, thrombin, and other clotting factors.[15,16] Accordingly, it is recommended to evaluate the mechanism of action of these plant extracts on the coagulation cascade.

Actually, plants have unlimited ability to synthesize aromatic substances, including phenolics and polyphenols like simple phenols, phenolic acids, quinine, flavones, flavonoids, flavonols, tannins, coumarins, terpenoids, essential oils, alkaloids, lectins, and polypeptides.[17] In this respect, several experiments relying on the chemical analysis of secondary metabolite content of plants in general and the plant species being studied in particular were carried out. Particularly, in some wild Aizoaceae species, phytochemical analysis indicated that they are rich in secondary compounds such as phenolic compounds, flavonoids, tannins, and alkaloids.[18] Also, chemical studies on the genus *Centaurea* revealed the presence of many compounds belonging mainly to the groups of sesquiterpene lactones, flavonoids, coumarins, indole alkaloids, and lignans.[19] A previous experiment showed that *C. depressa* flower extract had anticoagulant activity by extending both PT and aPTT times.[20] These results match the obtained one in the current research concerning different species of *C. hyalolepis.* Moreover, many bioactive components, especially pyrrolizidine alkaloids, quinones, terpenoids, flavonoids, and other phenolic compounds were reported to be present in *Heliotropium* genus.[21] The results from the former research regarding other species *H. indicum* explain why leaves of these plant species have been traditionally used as a remedy for thrombosis.[22] Hence, the ethanol, petroleum ether, carbon tetrachloride, and chloroform extracts of *H. indicum* leaves showed clot lysis activity.[23] The same anticoagulant activity was observed for *C. hyalolepis* aqueous extract. Furthermore, phytochemical evaluation of *P. judaica* indicated phenolic and flavonoid compounds in this species.[24] Recent research illustrated that the ethanol extract from *P. judaica* exhibited a prolonged effect on aPTT and no significant effect on PT, which coincide with the obtained results in the current study regarding the aqueous extract.[9] Similarly, the main chemical ingredients of the genus *Polygonum* are flavonoids, quinones, phenyl propanoids, and terpenoids.[25] However, the results obtained in this study agreed with anticoagulant activity of other species from the same genus, that is *P. cuspidatum*. It was noticed that *P. cuspidatum* ethanol extract-prolonged coagulation time *via* aPTT aPTT. Moreover, *P. cuspidatum* ethanol extract also exhibited effective fibrinolytic and antiplatelet activity.[26] Subsequently, presence of these phytochemicals in the plant species being studied may elucidate their influence on the coagulation cascade. For example, the effect of some phenolic compounds on various stages of blood coagulation and fibrinolytic mechanism was examined. Here, phenol has a complicated action on blood coagulation as it accelerated thrombin-fibrinogen interaction. This in turn retards clot retraction, enhancement of streptokinase activity on plasminogen, and inhibition of plasmin.[27] Additionally, PT and aPPT times were reported to be affected by flavonoids and tannins.[28] The results presented in a previous study elucidated that flavonoids might be potential structural bases for the design of new naturally, safely, and orally bioavailable direct FXa inhibitors.[29] Besides, tannins demonstrated an efficient anticoagulant activity due to their significant inhibitory effect on thrombin.[30] Likewise, terpenoids manifested a tendency to extend both PT and aPPT times that explain their inhibitory effect on platelet aggregation.[31] Moreover, quinone is a strong anticoagulant, whose action is mediated through its ability to inhibit vitamin K-dependent carboxylase that controls blood clotting.[32] From the previous literature, alkaloids are plentifully available in medicinal plants and most of them possess antiplatelet activity. From a perspective, plant alkaloids are mechanically very versatile and interfere with various mediators of clot formation.[33] Moreover, coumarin derivatives are an important class of phytochemicals that display both *in vitro* and *in vivo* anticoagulant activity *via* PT and aPTT assays with no significant toxicity.[34] Furthermore, plant carbohydrates like pectins and hemicelluloses exhibit antithrombin and thrombolytic activity.[35] Similar to that, plant lectins increased both PT and aPTT times.[36] Additionally, some plant polypeptides, such as cysteine proteases reduced clotting time because of their fibrinolytic activity.[37]

However, *Verbascum* species contain biologically active compounds, such as iridoid glycosides, saponins, flavonoids, phenylethanoids, and neolignanglycosides.[38] Iridoids glycosides showed no effect on both PT and aPTT tests as provided by a previous experiment.[39] This may explain the activity of this

plant in the conducted research, as it demonstrated no effect on PT and significant low inhibitory effect on aPTT. The same observation was also recorded by previous research considering *V. fruticulosum*.[40] But, contrary to the coagulant activity of *V. sinuatum*, other *Verbascum* species have been shown to exert anticoagulant effect. Likewise, the aqueous and alcoholic extracts from the vegetative and generative organs of *V. thapsus* and *V. densiflorum* increased the blood clotting time.[41]

CONCLUSION

The positive recorded data from this research could serve as identification of new hemostatic remedies that could be used for the commercial economic purposes and for managing several cardiovascular diseases. Though, it is not conclusive as *in vivo* studies are yet to be investigated. However, from the recorded results, it is apparent that these plants should be cautiously consumed with anticoagulant drugs (*e.g.* heparin) and stops their consumption before surgery. Furthermore, phytochemical and pharmacological elaborated experiments are required to purify and to characterize possible active constituents of the examined plant species in parallel to their cytotoxicity evaluation.

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Ethics

Ethics Committee Approval: The study was approved by the Ethics Committee (IRB) of the Faculty of Health Sciences at An-Najah National University (ref: SC 12/1/20).

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: L.A., Design: L.A., G.O., Data Collection or Processing: L.A., A.B., D.A., Analysis or Interpretation: L.A., D.A., R.S., T.Q., Literature Search: L.A., I.S., R.S., G.O., Writing: L.A., D.A., I.S., T.Q., R.S.

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ORIGINAL ARTICLE

Analgesic Effects of Vilazodone, Indatraline, and Talsupram in a Rat Model of Neuropathic Pain

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ABSTRACT

Objectives: Drugs that inhibit the reuptake of serotonin, norepinephrine, and/or dopamine are widely used for treating depressive disorders and have emerged as effective drugs for neuropathic pain. They have no substantial anti-nociceptive effects but are considered, with gabapentin/pregabalin, first-line drugs for neuropathic pain.

Materials and Methods: In this study, three different antidepressant agents were used in different doses to investigate their anti-hyperalgesic effects in rat models of neuropathic pain using hot plate and tail flick methods. They have different mechanisms of action; vilazodone hydrochloride is a selective serotonin inhibitor and a 5-HT_{1A} partial agonist; talsupram hydrochloride is a selective noradrenaline inhibitor, and it has a high affinity for noradrenaline transporter (NET), whereas indatraline hydrochloride is a triple reuptake inhibitor that inhibits transporters for 5-HT (SERT), dopamine (DAT), and NET.

Results: All the drugs used in the experiment were found to have an anti-hyperalgesic effect in both tests compared to the sham group. When anti-hyperalgesic effects of the three agents were compared to each other, it was found that talsupram hydrochloride was significantly more effective than the two other drugs in hot plate test. However, there was no statistically significant difference in the tail flick test. Indatraline hydrochloride was more effective than vilazodone hydrochloride at the same doses in the tail flick test.

Conclusion: Our data suggest that three drugs are effective analgesics in rat models of neuropathic pain and inhibition of noradrenaline reuptake represents the cornerstone of analgesic mechanisms of effective antidepressants.

Key words: Neuropathic pain, antidepressant, vilazodone, talsupram, indatraline, hot plate, tail flick, anti-hyperalgesic, sciatic nerve ligation

INTRODUCTION

The International Association for the Study of Pain defined neuropathic pain as "pain caused by a lesion or disease of the somatosensory nervous system".[1] According to a report published in 2011, one-third of Americans experience chronic pain. This exceeds total amount of cardiovascular diseases, diabetes, and cancer cases.[2] The prevalence rate of chronic pain in Europe is about 25-30%.[3] Almost one-fifth of people affected by chronic pain have neuropathic pain.[4,5] These high prevalence rates of chronic pain, especially neuropathic pain, are due to the lack of effective drugs. While nociceptive pain can be managed with analgesic drugs such as opioids and non-steroidal anti-inflammatory drugs, the medications used to manage neuropathic pain have a mild effect and in a small percentage of patients. This is mainly because they are unable to target the exact underlying mechanisms; this is why syndromes like fibromyalgia, whose pathophysiological mechanisms are not unclear, have lower treatment success rates.[6] The existing medications for neuropathic pain are non-specific and often inadequately effective.[7] Other medications such as opioids, on the other hand, have serious side effects. Therefore, there is a persistent need for improved and more specific therapeutic strategies. Before clinicians can prescribe precise medications for neuropathic pain patients, main targets in the pathway must be understood.

The pharmacological treatment of neuropathic pain is complicated and there is no effective treatment for many patients. While a general consensus indicates as to which drugs should be used as first-line medications, controversy over second- and third-line drugs continues, particularly regarding weak and strong opioids. Although opioids are effective in the management of neuropathic pain, they are

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not prescribed as first-line drugs because of their adverse reactions and concerns about abuse and addiction.[8]

Antidepressants have been proven to have analgesic effects in chronic pain even though they were not initially designed to be used as analgesic drugs. Antidepressants have practically no antinociceptive effects, but are considered with pregabalin and gabapentin first choice drugs for neuropathic pain[7,9-11] and fibromyalgia.[12]

There is no full understanding of how antidepressants are effective in pain management. An early concept of analgesic mechanisms of antidepressants for neuropathic pain was that these drugs could potentiate effectiveness of the descending noradrenergic and serotonergic inhibitory pathways that extend from the brain stem to the dorsal horn of the spinal cord. This is done by inhibiting the reuptake of serotonin and noradrenaline released into the spinal synapses between the first-order neurons (nociceptors) and the second-order neurons (spinothalamic neurons). The synaptic transmission between these neurons can be inhibited by the neurotransmitters released from the inhibitory descending fibers, such as noradrenaline, which binds α-2 adrenergic receptors. They can also induce spinal interneurons to release inhibitory materials like GABA and endogenous opioids, such as serotonin at its metabotropic receptors or noradrenaline at α-1 adrenergic receptors.[13]

Our study aimed to explore the anti-hyperalgesic effect of three different antidepressant drugs at different doses in rat models of neuropathic pain using hot plate and tail flick methods, and to compare analgesic activity of these drugs. Anti-hyperalgesic effects of these agents have not been studied extensively before.

MATERIALS AND METHODS

Animals

Experiments were performed on adult male Wistar albino rats weighing 200-225 g. The animals were kept at 22 ± 1°C, four in each cage, and maintained with a light-dark cycle of 12: 12 h and free access to water and food. Sivas Cumhuriyet University Animal Ethics Committee approved all experiment protocols (approval no: 65202830-050.04.04-284).

Drugs

5-[4-[4-(5-Cyano-1*H*-indol-3-yl) butyl]-1-piperazinyl]-2-benzofurancarboxamide hydrochloride (vilazodone hydrochloride) (BLDpharm), 1,3-dihydro-*N*,3,3-trimethyl-1-phenylbenzo[*c*]thiophene-1-propanamine hydrochloride (talsupram hydrochloride) and (1*R*,3*S*)-*rel*-3-(3,4-dichlorophenyl)-2,3-dihydro-*N*-methyl-1H-inden-1-amine hydrochloride (indatraline hydrochloride) (Tocris Bioscience) were diluted in dimethyl sulfoxide. Solutions were freshly prepared on the days of experimentation. Intraperitoneal (*I.P.*) vilazodone hydrochloride (5-HT1A partial agonist and SSRI 2.5, 5, and 10 mg/kg), talsupram hydrochloride (selective inhibitor of noradrenalin transporters 2.5, 5, and 10 mg/kg), and indatraline hydrochloride (5-HT noradrenalin and dopamine reuptake inhibitor 2.5, 5, and 10 mg/kg) were applied before the analgesia tests.

The experimental protocol and the analgesia tests

All experiments were carried out blindly between 10.00 and 16.00 h in normal light and temperature (22 ± 1°C) in a quiet room. The rats were allowed to adapt to the laboratory for at least 2 h before the test and their tails were marked to differentiate the treatment groups. The rats were randomized into 10 groups [3 groups for each drug (1 group for each dose) and 1 group as a sham]. Each experimental group had six rats. The same person performed all neuropathic operations and analgesia tests to minimize experimental variability.

Surgical intervention

The neuropathic pain model was produced by partial sciatic nerve ligation. Surgical interventions were performed at Sivas Cumhuriyet University Medical Faculty Experimental Animals Laboratory. Anesthesia was performed using intramuscular ketamine (90 mg/kg) and xylazine (3 mg/kg). Under aseptic conditions, a 1 cm incision was applied to biceps femoris and the sciatic nerve was reached in the middle thigh level of the right leg. Then, the sciatic nerve was freed of adherent tissues with careful blunt dissection and the dorsal one-third to half of the nerve was tightly ligated with 4.0 chromic catgut. The incision was closed with 4.0 silk. In sham group of rats, the same intervention was applied but without nerve ligation. After surgery, the rats were returned to their cages and kept for 21 days under the abovementioned same conditions.[14,15]

Analgesia tests

To evaluate thermal pain standard tail flick test (May TF 0703 Tail beat unit, Commat) and hot plate test (May AHP 0603 Analgesic HP, Commat) devices were used. In the tail flick test, an intensive light beam was aimed at the animal's tail and a timer begins. When the animal flicks its tail, the timer is stopped and recorded time (latency) represents the pain threshold. Tail-flick latencies were measured before the administration of the vehicle or investigational drugs to obtain a baseline and 15, 30, 60, 90 and 120 min after the *I.P.* administration. The maximum response time was set to 15 seconds (cut-off latency) to avoid tissue damage. Rats that did not show a response within 15 seconds were excluded. The hyperalgesic responses in this test reflect the mechanisms of pain in the central nervous system.[16-18]

A hot plate device was used to evaluate thermal pain. In this test, the rats were placed on a hot plate with the temperature set at 53 ± 0.5°C for a maximum time of 30 s to prevent injury. Response time was recorded (when the animals licked their fore and hind paws or jumped) before and 15, 30, 60, 90, and 120 min after *I.P.* administration of the vehicle or test drugs. The hyperalgesic reactions in this test reflect the mechanisms of pain in both the central and peripheral nervous systems.[16,18]

Statistical analysis

In all groups for each rat, antinociceptive effects of the drugs were measured as tail flick and hot plate latencies and

transformed into a percentage maximum possible effect (% MPE). MPE was obtained using the formula: [MPE= (post-drug latency-pre-drug latency) / (cut-off latency-pre-drug latency) × 100]. Pre-drug and post-drug *N* values were the same in each group. The data were analyzed using one and two-way analysis of variance (ANOVA) and repeated measures ANOVA followed by a Tukey *post-hoc* test (SPSS 14.0 for Windows) for multiple comparisons between groups. All data are presented as a mean ± standard error of the mean. The significance level was determined as $p<0.05$.

RESULTS

Determination of neuropathic pain formation by sciatic nerve ligation

The occurrence of neuropathic pain in the rats was detected using the paired student's *t*-test. The post-surgery basal latencies of the rats were considerably lower than the pre-surgery basal latencies in both hot plate and tail flick tests ($p<0.05$) (Figures 1 and 2).

Effects of vilazodone hydrochloride on neuropathic pain

Vilazodone hydrochloride was applied at three doses: 2.5, 5, and 10 mg/kg. In both tail flick and hot plate tests, the responses were measured before the drug was administered *I.P.* and after the administration at 15, 30, 60, 90, and 120 min. Maximum % MPE was observed in 90 mins after administration of these three doses. One-way ANOVA test was applied to compare the different doses with the sham group and with each other. In both tail flick and hot plate tests, doses at 5 and 10 mg/kg were found to be effective against neuropathic pain compared with the sham group. Dose at 10 mg/kg was effective from 30 to 120 mins in both tests with a statistically significant difference compared with 2.5 mg/kg dose at 90 min in the hot plate test and in 90, 120 min in the tail flick test. Dose at 5 mg/kg was effective from 30 to 90 min in both tests. Dose at 2.5 mg/kg was not statistically different from the sham group in the hotplate test at all minute points, while it was noticed to be effective only in the tail flick test at 60 and 90 min (Figure 3).

Effects of talsupram hydrochloride on neuropathic pain

Talsupram hydrochloride was applied intraperitoneally at three doses: 2.5, 5, and 10 mg/kg. The maximum percentage MPE was observed 60 min after the drug was administered for all three doses. One-way ANOVA test was used. The first dose of 2.5 mg/kg was effective from 30 to 120 mins in the hot plate test and from 30 to 90 min in the tail flick test. The other two doses at 5 and 10 mg/kg were effective at all

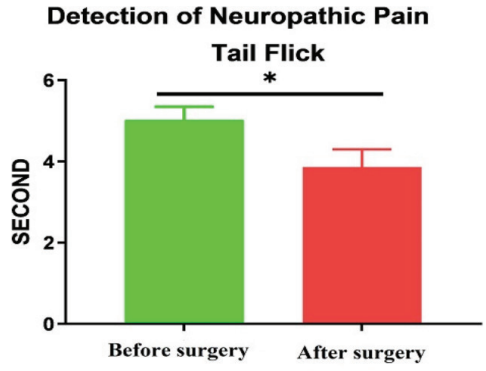

Figure 1. Tail flick basal latencies of rats before and after surgery (*$p<0.05$ paired student's *t*-test)

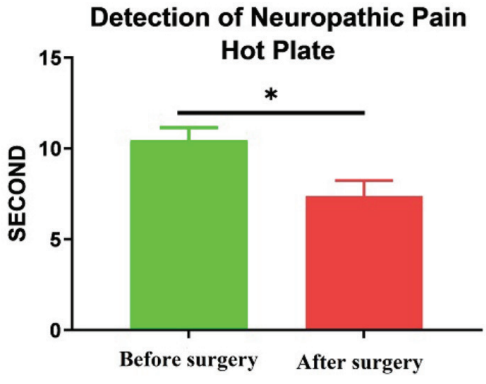

Figure 2. Hot plate basal latencies of rats before and after surgery (*$p<0.05$ paired student's *t*-test)

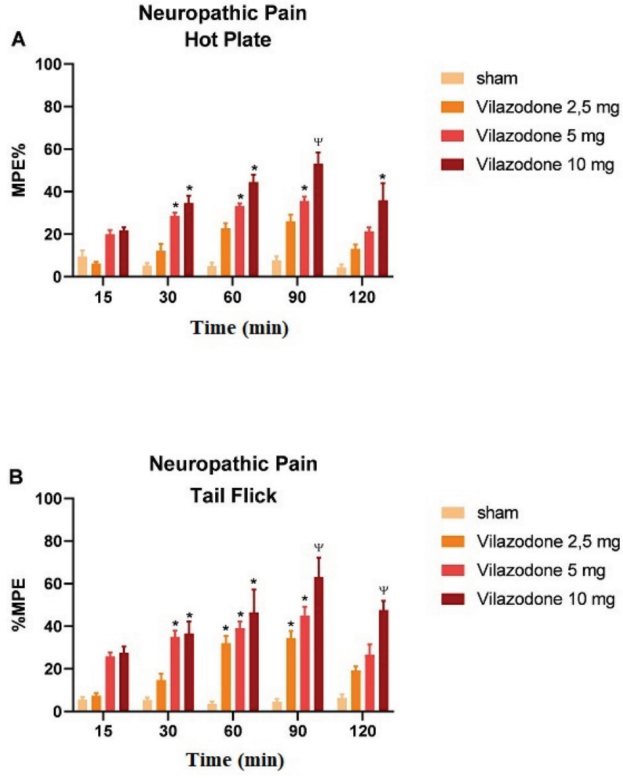

Figure 3. The effect of vilazodone hydrochloride intraperitoneal administration on the neuropathic pain model in the hot plate test (A) and tail flick test (B). This was expressed as a percentage of the maximal possible effect (MPE). Each point represents the mean ± SEM of % MPE for 6 rats

*$p<0.05$ when the groups were compared to the sham group, Ψ: $p<0.05$ when the groups were compared to the sham and 2.5 mg/kg dose groups, SEM: Standard error of the mean

minute points in the hot plate test, while their effectiveness in the tail flick test was noticed from 15 to 90 min compared to dose of 2.5 mg/kg, a statistically significant difference was noticed only with dose at 10 mg/kg at 60 min in the hot plate test (Figure 4).

Effects of indatraline hydrochloride on neuropathic pain

Indatraline hydrochloride was administered intraperitoneally at 3 doses; 2.5, 5, and 10 mg/kg. Maximum MPE% was observed at 60 min after the drug was administered for all three doses. One-way ANOVA test was applied. The drug was effective at all three doses in both tests. After the first dose 2.5 mg/kg was administered, the anti-hyperalgesic effect was statistically significant in 60 min in the hot plate test and from 30 to 90 min flick test. The second dose at 5 mg/kg was effective at 30 and 60 mins in the hot plate test and from 15 to 90 min in the tail flick test. The anti-hyperalgesic effect for the third dose of 10 mg/kg from 30 to 90 min was statistically significant compared to the sham group, and at 60 min the first 2.5 mg/kg dose in the hot plate test. While in the tail flick test, anti-hyperalgesic effect of 10 mg/kg dose was clear from 15 to 90 min compared to the sham group (Figure 5).

Comparison of the anti-hyperalgesic effects of vilazodone hydrochloride, talsupram hydrochloride, and indatraline hydrochloride on neuropathic pain

We used two-way variance analysis followed by Tukey HSD test in this comparison. In hot plate test, MPE% values obtained from doses of 2.5, 5, 10 mg/kg of talsupram hydrochloride dose were significantly higher than values obtained from the same doses of vilazodone hydrochloride and indatraline hydrochloride. Even at lower doses, talsupram was more effective than the other two drugs. MPE% of talsupram at 2.5 mg/kg was › vilazodone 5 mg/kg and indatraline 10 mg/kg, while at 5 mg/kg, MPE% was › vilazodone 10 mg/kg (p<0.05). Whereas in the tail flick test, MPE% values obtained from the different doses of talsupram hydrochloride were not statistically different from those of the same doses of vilazodone hydrochloride and indatraline hydrochloride (p>0.05), except for MPE% of 2.5 mg/kg dose of indatraline hydrochloride, which was higher than that of the same dose of talsupram hydrochloride (p<0.05). There was a statistically significant difference between the MPE% values obtained from the same doses of vilazodone hydrochloride and indatraline hydrochloride in favor of the latter in the tail flick test (p<0.05), whereas no statistically significant difference

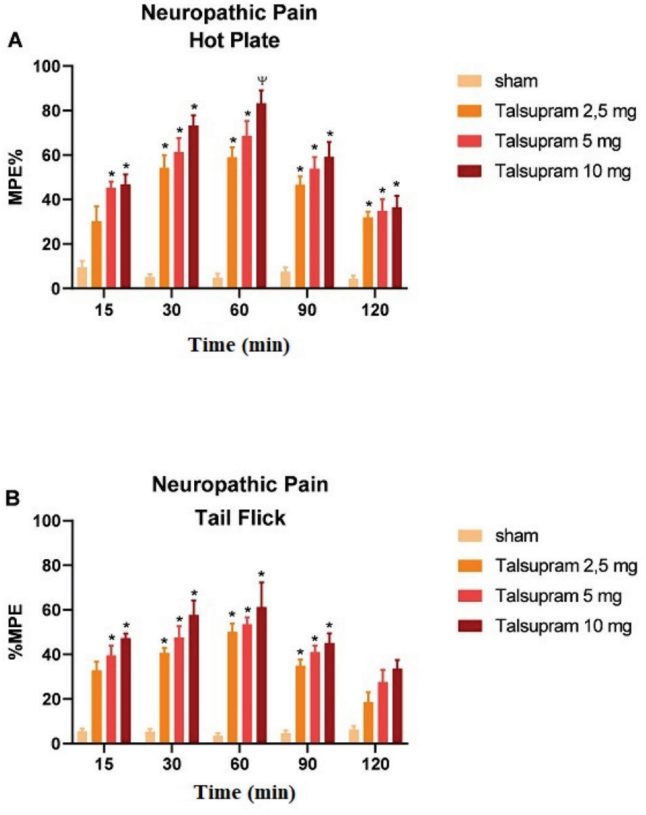

Figure 4. The effect of talsupram hydrochloride intraperitoneal administration on the neuropathic pain model in the hot plate test (A) and tail flick test (B). This was expressed as a percentage of the maximal possible effect (MPE). Each point represents the mean ± SEM of % MPE for 6 rats

*p<0.05 when the groups were compared to the sham group, Ψ: p<0.05 when the groups were compared to the sham and 2.5 mg/kg dose groups, SEM: Standard error of the mean

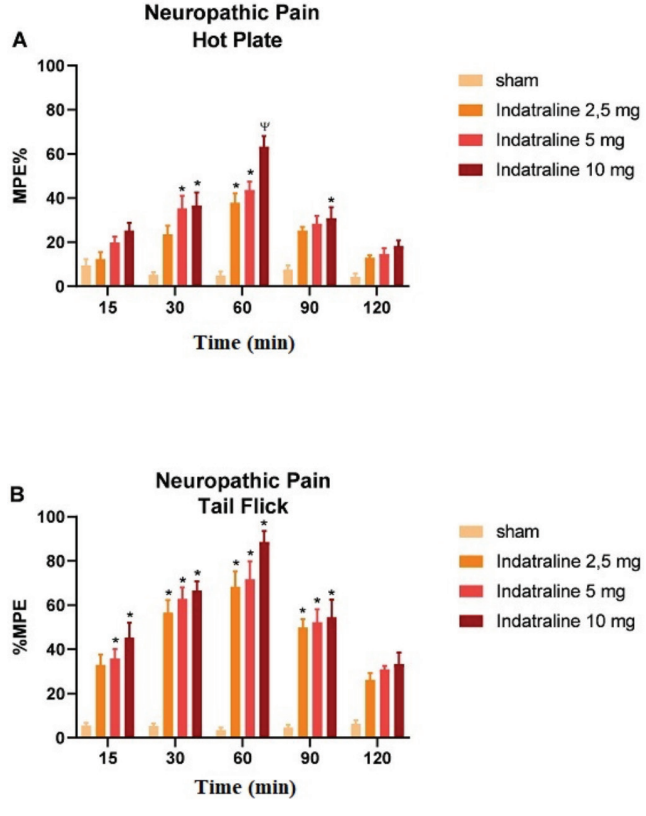

Figure 5. The effect of indatraline hydrochloride intraperitoneal administration on the neuropathic pain model in the hot plate test (A) and tail flick test (B). This was expressed as a percentage of the maximal possible effect (MPE). Each point represents the mean ± SEM of % MPE for 6 rats

*p<0.05 when the groups were compared to the sham group, Ψ: p<0.05 when the groups were compared to the sham and 2.5 mg/kg dose groups, SEM: Standard error of the mean

was found between them in the hot plate test (p>0.05) (Figure 6).

DISCUSSION

Neuropathic pain, a pain syndrome caused by a lesion or disease of the somatosensory system, is a main public health issue and becoming a global burden.[1,19,20] An epidemiological study indicated that the prevalence rate of neuropathic pain is in the range of 6.9% to 10% and increases year after year.[21,22] Patients with neuropathic pain report significantly lower levels of health-related quality of life.[23]

The high rate of comorbidity between pain and depression[24] has led to the wide use of antidepressants in chronic pain treatment. Tricyclic antidepressants (TCA), particularly desipramine, amitriptyline, nortriptyline, and imipramine, are the most effective antidepressants in neuropathic pain management. TCAs have effects on various targets. This lack of selectivity is related to their efficacy. For instance, amitriptyline has a local anesthetic effect by blocking voltage-gated sodium channels.[25] TCAs are effective in many neuropathic conditions. However, these multiple actions of TCAs also contribute to many adverse effects that limit their use, in particular their anticholinergic effects that increase the risk of cardiotoxicity, orthostatic hypotension, mouth dryness, constipation, and urinary retention. To avoid these issues, serotonin-norepinephrine reuptake inhibitors (SNRI), particularly duloxetine, have been suggested in the management of neuropathic pain. SNRIs, *e.g.* duloxetine, have shown consistent efficacy in several neuropathic syndromes, including painful polyneuropathy, post-herpetic neuralgia, low back pain, and painful diabetic neuropathy.[7] Opioids are recommended to be used as second- and third-line treatments because of their adverse effects. Tramadol and FDA-approved tapentadol[26] are used in second-line treatment, while the strong opioids, oxycodone, and morphine[27] are used in the third-line treatment. Therefore, there is still a need for more effective drugs with less serious adverse effects for neuropathic pain. In this study, we investigated anti-hyperalgesic effects of three different antidepressant drugs at different doses in rat models of neuropathic pain using the hot plate method. These drugs have different mechanisms of action; vilazodone hydrochloride is a selective serotonin inhibitor, while talsupram hydrochloride is a selective noradrenaline inhibitor, and it has a high affinity for the noradrenaline transporter, whereas indatraline hydrochloride inhibits transporters for 5-HT, dopamine, and noradrenaline. All the drugs used in the experiment were found to have an anti-hyperalgesic effect compared to the sham group. These results support the evidence for role of noradrenaline, serotonin, and probably dopamine in the analgesic effects of antidepressants on neuropathic pain and corroborate a previous study highlighted that indatraline has analgesic profile in neuropathic mice.[28] Some preclinical studies on animals have indicated the important roles of noradrenaline and serotonin in the processing of pain. Experimental studies have demonstrated that intrathecal administration of serotonin and norepinephrine receptor agonists inhibits pain behavior.[29,30] Other data indicate that serotonin agonists such as fenfluramine trigger the neuronal release of substance P and thus pain behavior.[31] Furthermore, intrathecal administration of serotonin receptor antagonists such as ondansetron inhibits the experimental pain response in rats.[32] 5-HT1A, 5-HT2A/2C, 5-HT3, and 5-HT7 receptors that highly contribute to the transmission of nociceptive messages are expressed in the dorsal horn of spinal cord.[33-36] It seems that serotonin both inhibits and enhances pain sensation by various physiological mechanisms, contrary to norepinephrine, which is essentially inhibitor. A review of studies on SSRIs showed inconsistent efficacy for migraine, diabetic neuropathy, and fibromyalgia; however, some studies of SSRI treatment for mixed-chronic pain are positive.[37]

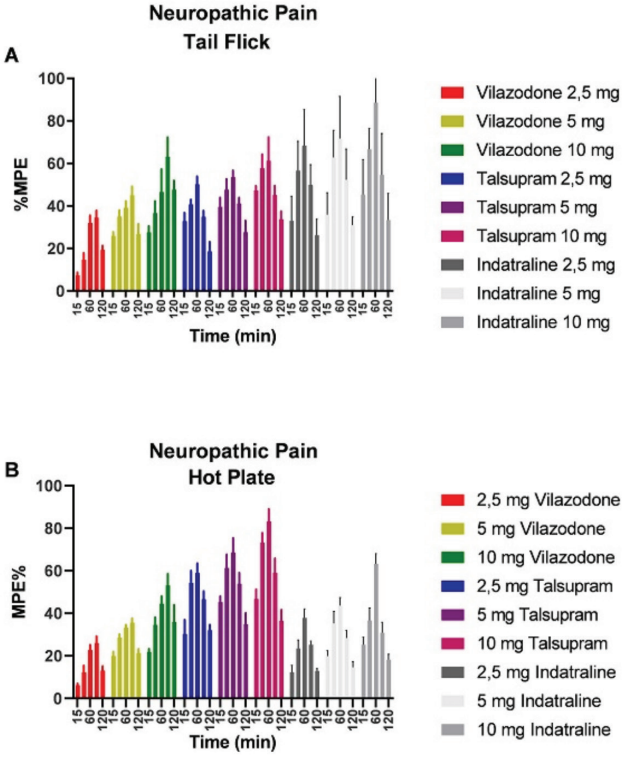

Figure 6. The effect of vilazodone hydrochloride, talsupram hydrochloride and indatraline hydrochloride on the neuropathic pain model (in hot plate and tail flick tests) was expressed as a percentage of the maximal possible effect (MPE). All drugs were administered intraperitoneally. Each point represents the mean of % MPE for 6 rats.

When anti-hyperalgesic effects of three agents were compared to each other, it was found that talsupram hydrochloride was significantly more effective than viazodone hydrochloride and indatraline hydrochloride in the hot plate test. This could be related to the high affinity of talsupram hydrochloride for norepinephrine transporters and the more important role of noradrenaline in the anti-hyperalgesic activity compared to serotonin and dopamine. However, there was no statistically significant difference in the tail flick test.

While the response in the tail flick test is a spinal reflex rather than an indication of pain behavior involving higher brain

centers,[38] the response in hot plate test is considered to integrate supraspinal pathways.[39] Therefore, comparative results suggest that analgesic effect of talsupram hydrochloride is more effective than that of vilazodone hydrochloride and indatraline hydrochloride at the supraspinal level. MPE% values obtained from indatraline hydrochloride were more than values obtained from vilazodone hydrochloride at the same doses in the tail flick test. This could be due to a greater anti-hyperalgesic effect of the inhibition of the reuptake of noradrenaline, serotonin, and dopamine compared to the inhibition of reuptake of serotonin alone. Although vilazodone hydrochloride is less effective against neuropathic pain than the other drugs, its relatively benign sexual side effect profile may be worth considering because in addition to serotonin reuptake inhibition, it acts as 5-HT_{1A} partial agonist.

CONCLUSION

In conclusion, our data suggest that the three drugs used in this study are effective analgesics in rat models of neuropathic pain. Inhibition of noradrenaline reuptake represents the cornerstone of analgesic mechanisms of effective antidepressants. Although SSRIs have a more tolerable side effect profile and the SSRI used in our experiment, *i.e.* vilazodone hydrochloride, was effective in a rat model of neuropathic pain, the evidence to support the use of SSRIs in the clinical management of chronic pain is still not convincing.[40]

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Ethics

Ethics Committee Approval: All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. Animal experimental procedures were approved by the Animal Ethical Committee at Sivas Cumhuriyet University (Sivas, Türkiye) (approval no: 65202830-050.04.04-284).

Informed Consent: Not human subject research.

Authorship Contributions

Surgical and Medical Practices: L.H., Concept: B.S., Design: B.S., Data Collection or Processing: L.H., Analysis or Interpretation: Z.J., Literature Search: L.H., Writing: L.H.

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

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REVIEW

Non-Oral Drug Delivery in Parkinson's Disease: Current Applications and Future

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ABSTRACT

Parkinson's disease (PD) is a type of movement disorder that affects the ability to perform daily activities. It is considered that 1 million people in the U.S. and more than 10 million people worldwide live with PD. It is a chronic and progressive disease, so symptoms worsen over the time. Patients experience motor symptoms such as tremors, stiffness and slow motion, and non-motor symptoms such as sleep problems, constipation, anxiety, depression and fatigue. Dopaminergic drugs are critical for treating motor symptoms in PD. Levodopa (L-DOPA) is the "gold standard" medication for the control of motor symptoms. Because of the progression of the disease, the effectiveness of oral L-DOPA decreases over time and motor fluctuations such as "delayed ON", "no ON" and unpredictable "ON-OFF" periods appear. These motor fluctuations affect the quality of life of the patient at a high rate and the patient has problems in fulfilling his daily morning routines. Gastrointestinal (GI) problems, as the common non-motor symptom, are the most important cause of motor fluctuations that occur because of inadequate oral treatment with the progression of PD. When oral treatments are not sufficient, non-oral treatments that are not affected by GI problems are required. In this review, the treatment strategies, developed and approved non-oral drug delivery systems in the early and advanced stages of PD are emphasized.

Keywords: Parkinson's disease, oral and non-oral treatment, motor and non-motor fluctuations

INTRODUCTION

Parkinson's disease (PD) is the most common neurodegenerative movement disorder that can affect the ability to perform daily activities.[1] It is considered that 1 million people in the U.S. and more than 10 million people worldwide have PD. PD is usually diagnosed in people over the age of 55. Although it is rare, it can also be seen in the young population between the ages of 21-45. The disease is called late-onset, when diagnosed in older people, and young-onset, when diagnosed in the young population.[2]

PD is a chronic and progressive disease. Motor and non-motor symptoms are seen in these patients. However, it is characterized by motor symptoms associated with movement. These symptoms are rhythmic shaking tremors, stiffness or rigidity of the muscles and slowness of the movement (bradykinesia). Movements are controlled by neurons in the brain and messages are transmitted to each other and to the rest of the body by chemicals called neurotransmitters. Dopamine, a neurotransmitter that control movement, is produced in *substantia nigra* area of the brain. In PD, 70-80% of dopamine-producing cells disrupt by stages and are lost, which called neurodegeneration. The damage to neurons causes low levels of dopamine in the part of the brain that controls balance and movement. When neurons do not pass on brain messages properly, their movements have not been controlled smoothly and the motor symptoms of PD appear. In addition to motor symptoms, non-motor symptoms related to PD can occur in patients. Non-motor symptoms are sleep problems, constipation, depression, anxiety, and fatigue. For many of these non-motor symptoms, definitive clinicopathological correlations are still not fully understood.[3] Dopaminergic drugs are critical for treating motor symptoms in PD. Levodopa (L-DOPA) is known as the "gold standard" for the control of motor symptoms in PD. Because of the progression of PD, effectiveness of oral L-DOPA decreases over time.[4] It has been reported that in 5-10 years, patients treated L-DOPA will develop motor fluctuations and dyskinesias in 70-80%.[5] The fluctuations in motor functions

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are due to ON responses (good antiparkinsonian effect) and OFF responses (the symptoms are not efficiently controlled) seen just before the next dose of L-DOPA. In the ON period, patients can fully move and function independently, and the patient is unable to function such as move, talk, smile as easily during the OFF period. These motor fluctuations can occur diversely. These are foreseeable end-of-dose "wearing OFF" phenomena, peripheral problems such as "delayed ON" or "no ON", and unpredictable "ON-OFF" periods. The delayed effect of oral medications causes an early morning OFF period.[6,7] This condition affects quality of life of the patients at a high rate and the patient has problems in fulfilling his daily morning routines. The results of an international multicenter study of EUROPAR, a partner of the European Parkinson's Disease Association, show that the incidence of OFF period is 60% even in patients undergoing optimized PD therapy.[8] L-DOPA dose is usually increased to manage these problems. However, increasing L-DOPA dose can cause involuntary movements or painful dyskinesia. Gastrointestinal (GI) problems, as a common non-motor symptom, are the most important cause of motor fluctuations that occur because of inadequate oral treatment with the progression of PD. Dysphagia, gastric dysfunction, colonic dysmotility, small-intestine motility, and delayed gastric emptying (GE) can be considered GI problems. When oral therapies are not insufficient, alternative drug delivery systems that are not affected by GI problems are necessary, which are known as non-oral treatments. Guidelines published in 2017 at the National Institute for Health and Care Excellence mention that non-oral treatments will be safe, important and effective for PD treatment.[9,10] In this review, importance of non-oral therapy in PD treatment is emphasized. It also includes available non-oral drug delivery systems and current studies of non-oral formulations.

METHODS

We used the websites of the American Parkinson Disease Association and the European Parkinson Disease Association for this review. Additionally, references for this review have been identified through PubMed, ScienceDirect and Google Academic using the terms "Parkinson's disease", "Parkinson's disease treatment strategy" and "Non-oral treatment of Parkinson's disease". We primarily selected articles published between 2000 and 2020. Only publications in English were evaluated. We evaluated more than 200 citations, of which 81 are included in this review.

Current oral treatment options for Parkinson's disease

There is no definite cure for PD, but the medicines used in treatments can provide important symptomatic control of the motor symptoms. Current pharmaceutical strategies for the control of symptoms are L-DOPA, catechol-*O*-methyl transferase (COMT) inhibitors, dopamine agonists, monoamine oxidase B (MAO-B) inhibitors, anticholinergic, and amantadine medications.[10]

L-DOPA is a medicine used since 1970 to treat PD and is still most effective for symptomatic treatment. It is effective in the early stages of PD but remains effective as PD progresses without intolerance developing over time. L-DOPA is routinely used along with a DOPA-decarboxylase inhibitor to reduce some treatment complications, prolong half-life, and increase L-DOPA availability to the brain.[11] However, after long-term use of L-DOPA oral formulations, problems such as motor and non-motor fluctuations and L-DOPA-induced dyskinesia can be observed because of pharmacokinetic properties of L-DOPA. Patients do not experience any fluctuations in motor or non-motor symptoms during the first years of L-DOPA use. Patients begin to aware of these fluctuations after 2-5 years of L-DOPA use. In this way, as the disease progresses, patients must make frequent adjustments to the dosage regimen and they should use L-DOPA more frequently due to the shortened effect time and reduced effect.[12] Increasing the dose and frequency of L-DOPA to control of motor symptoms may provide some improvement, but involuntary movements and painful dyskinesia may occur due to the high plasma concentration of L-DOPA. Dyskinesia can cause the problems in walking and balance; therefore, patients may have difficulties in social life. Additionally, in the later stages of PD, patients become completely dependent on care, and those caring for their care has a heavy social responsibility, both socially and economically.[13,14]

Since motor fluctuations greatly affect the course of the disease, clinicians occasionally have difficulty managing the disease. After 5 years of L-DOPA treatment, approximately 50% of patients experience wearing off, and this rate rises to about 80% after 10 years.[15] Clinicians should choose the appropriate PD medicines to manage symptoms effectively and improve the patient's quality of life. The most important reason for fluctuations in the use of oral PD medications are GI dysfunctions such as slow GE, irregular jejunal absorption, and competition with dietary amino acids in the areas of absorption.[16,17]

COMT inhibitors are drugs that inhibit the enzyme COMT that acts on dopamine breakdown and extend the duration of L-DOPA activity. Doctors use them along with L-DOPA to treat the motor symptoms of PD.[18] Because they prolong L-DOPA duration of action by increasing half-life and delivery to the brain. In some patients, COMT inhibitors provide control of motor symptoms by reducing off-time compared with standard L-DOPA/DOPA decarboxylase inhibitor combinations.[19] Tablet formulations of COMT inhibitors are available on the market. Although they are able to improve motor function in some patients, they are not prescribed alone because they offer a limited effect on PD symptoms. Entacapone and tolcapone, approved COMT inhibitors, are reversible COMT inhibitors approved for treating PD. A third COMT inhibitor, opicapone, is available in Europe but has not yet been approved by the Food and Drug Administration (FDA). Each of these COMT inhibitors has problems in terms of pharmacokinetics, pharmacodynamics, clinical efficacy or safety. Additionally, their elimination half-lifes are approximately 2-3 hours.[20]

The most common adverse effects associated with the addition of COMT inhibitors to carbidopa/L-DOPA treatment are strengthening the dopaminergic effects of drugs, such as nausea, dyskinesia, orthostatic hypotension, sleep disorders, hallucinations, and vomiting. L-DOPA dose adjustment must avoid these events. Dark yellow or orange urine discoloration is related to the colour of the COMT inhibitors and their metabolites. Entacapone from COMT inhibitors is preferred as the first-line treatment in patients with PD. Because tolcapone causes hepatotoxicity. The descriptions of acute, fatal fulminant hepatitis and potentially fatal neurological reactions in association with tolcapone led to the suspension of its marketing authorization in Europe and Canada. In many other countries, use of the drug is restricted to patients, who are not responding to other therapies. If tolcapone is used in PD treatment, proper monitoring of liver function, and liver enzymes is required during the first six to eight months of the treatment.[21,22]

MAO-B inhibitors have been used for treating PD as both early monotherapy and combined therapy in patients with the more advanced disease.[23] Selegiline and rasagiline are selective MAO-B inhibitors approved for PD treatment.[24] Both selegiline and rasagiline were originally developed as antidepressants. However, low and medium doses of selegiline required to provide an irreversible MAO-B inhibition have not had antidepressant activity. The most important differences between these two active substances are their metabolism, their interaction with cytochrome (CYP) P450 enzymes and their molecular biological/genetic level properties.[25] Amphetamine metabolites occur because of the metabolism of selegiline with CYP enzymes. These metabolites can occur after oral use and can cause sleep problems in patients.[24]

The oral bioavailability of selegiline is about 10%. This low bioavailability has led to the development of different non-oral drug delivery systems such as transdermal, buccal, and nasal.[26,27] Another MAO-B inhibitor is rasagiline and because of the metabolism of rasagiline, unwanted metabolites such as amphetamine-like metabolites do not form.[18] Studies have shown that amphetamine-like metabolites occur only in the plasma of patients with PD during the use of selegiline, and not during chronic rasagiline therapy.[28,29] Additionally, rasagiline administered orally is rapidly absorbed from GI tract and reaches the highest plasma concentrations within an hour. Rasagiline's oral bioavailability is about 36% due to its high hepatic first-pass metabolism.[30]

"Cheese reaction", which is a serious side effect, occurs especially when non-selective MAO inhibitors are administered with certain foods such as cheese and drugs such as decongestants. Because of this reaction, hypertensive crisis, palpitations, tachycardia, blurred vision, arrhythmias, and other sympathomimetic problems can be seen. The "cheese reaction" occurs particularly, when older MAO inhibitors are administered with biogenic amine-like substances such as decongestants or high dietary tyramine (more than 500 mg *per* day). Although there are clinical pharmacology and safety data showing that rasagiline and selegiline are selective MAO-B inhibitors, concerns remain regarding interactions with tyramine and the potential for hypertensive crisis. Despite being rare, cases of the "cheese reaction" have been informed during treatment with selegiline. It has been stated that normal dietary tyramine for both selegiline and rasagiline does not cause clinically meaningful interactions, but taking more than 150 mg tyramine *per* day may increase the risk.[25] In the study of Goren et al.[29], rasagiline at the recommended therapeutic dose of 1 mg/day provides a selective MAO-B inhibition. Simultaneously, it has also been noted that, when rasagiline is used at doses >2 mg/day, its selectivity for MAO-B decreases and tyramine sensitivity increases.[29]

Dopamine agonists demonstrate antiparkinsonian effects by directly acting on dopamine receptors and mimicking the endogenous neurotransmitter. Oral L-DOPA/DOPA decarboxylase inhibitor application is inevitably necessary with the advance of PD. In the long term, chronic administration of oral L-DOPA formulations in a fixed combination with inhibitors of the main metabolizing L-DOPA enzymes results in the onset of so-called motor complications. When the disease progresses, the duration of L-DOPA response shortens in addition to the short plasma L-DOPA half-life.[31] L-DOPA converts to dopamine in both the center nervous system and the periphery. To increase the bioavailability of L-DOPA and decrease its side effects, it is often administered along with peripheral decarboxylase inhibitors (such as carbidopa and benserazide). Dopamine decarboxylase inhibitors prevent the conversion of L-DOPA to dopamine in the periphery, allowing for more L-DOPA to cross the brain-blood barrier.[32]

Compared to L-DOPA, dopamine receptor agonists do not require the enzymatic conversion to an active metabolite, and do not have potentially toxic metabolites. However, they do not compete with other substances for their active transport across the blood and blood-brain barrier, and are not depended on the functional capacity of nigrostriatal neurons.[18] Dopamine agonists are classified as ergot or non-ergot types, and these active agents have essential differences associated with receptor affinities. Bromocriptine and cabergoline, as ergot derivatives, are dopamine agonists and they are not commonly used for treating PD. Ropinirole and pramipexole rotigotine are non-ergot-derived dopamine agonists, which are approved for PD therapy.[33] Apomorphine is the most potent dopamine agonist, but it effectively stimulates both D1 and D2 receptors like dopamine. However, due to some limitations, apomorphine cannot be used as an oral drug.[34]

Anticholinergic agents recently used to treat PD are benztropine and trihexyphenidyl. Since these drugs non-selectively block cholinergic receptors in the body, some side effects are seen. There are some hesitations about the use of these drugs for this reason. When selective cholinergic receptor antagonists were tried, significant benefits could not be obtained in PD treatment. Anticholinergics can alleviate dystonia and tremors caused by wearing off. However, it has no significant effect on other PD symptoms.[35]

Dysfunctions of gastrointestinal system in patients with PD

Dysphagia

Chewing and swallowing functions require regularly contracting and relaxing of many muscles. Therefore, it is inevitable that dysphagia is common in patients with PD. Dyspgahia is a problem that reduces quality of life and obstructs intake of the medication and increases the risk of aspiration, which is the cause of death of most patients in PD. PD-related dysphagia is not fully understood. Nevertheless, dopaminergic and non-dopaminergic mechanisms are effective in the development of dysphagia in PD.[36]

Lately, results of the studies disclosed that the dysphagia prevalence based on subjective conclusions, in patients with PD is 35% and rises to 82% by taking objective measures of swallowing dysfunction into account.[37] Aydogdu et al.[35] evaluated the dysphagia prevalence with the Videofluoroscopic Swallowing Study (VFSS) using the guidelines of the United Kingdom Parkinson's Disease Brain Bank. In this study, VFSS evaluation was performed on 23 patients with PD and 16 of the total sample were diagnosed with dysphagia.[35] Some clinical predictors should be considered when evaluating a patient with PD for the presence of dysphagia. For example; in patients with PD, weight loss without any reason or a body mass index below 20 is highly indicative of dysphagia. It is stated that 20% of patients develop malnutrition during PD. Another predictor of dysphagia and aspiration pneumonia is sialorrhea or drooling.[37,38]

Drooling

Drooling has many negative effects on quality of life, such as social embarrassment, decreased oral hygiene, bad breath, increased oral bacteria, difficulty speaking and eating, and increased risk of aspiration pneumonia.[39] There are no standard description and criterion for the diagnosis of drooling in patients with PD. For this reason, the prevalence forecast varies. Leibner et al.[37] conducted a questionnaire study on the drooling problem with 58 patients with PD and 51 healthy volunteers. In the end of the study, when patients with PD and control groups were compared, the rate of drooling was 59% and 14%, respectively.[37] Müller et al.[38] managed a study to examine the emergence and severity of autonomic and sensory symptoms in 207 newly diagnosed, untreated patients with PD and 175 healthy volunteers. The most obvious difference was observed in drooling, which was present in 42% of patients with PD but just 6% of the control group.

Gastric emptying

Disrupted GE (gastroparesis) is a common problem in patients with PD. In gastroparesis, patients experience symptoms such as abdominal discomfort or postprandial bloating, nausea, early satiety and weight loss.[9] It is thought that the cause of delayed and motor fluctuations in PD is delayed GE.[39]

Tanaka et al.[40] conducted a study with three groups. These groups were 20 patients with PD with newly diagnosed, untreated; treated with L-DOPA for a long time, advanced-stage 40 patients with PD; 20 healthy volunteers. The half-emptyting time ($T_{1/2}$) of healthy volunteers, newly diagnosed untreated and long-treated patients were found to be 86 min, 122 min, and 125 min, respectively. Goetze et al.[41] conducted a study with 36 patients with PD (divided into two as mild and advance) and 22 healthy volunteers. Because of this study, 97% of patients with PD had delayed GE. $T_{1/2}$ was found to be significantly longer in patients with PD compared in the control group. (169 *vs*. 107 min). Delayed GE was associated with degree of the disease. GE was found 149 and 196 min for patients with mild and advanced PD, respectively.[41] Unger et al.[42] subjected 20 healthy volunteers, 21 drug-naive and early-stage patients with PD and 18 patients with PD treated with dopaminergic medicines to 13C octanoate breath test to determine the duration of GE. Because of the study, it was observed that GE test (1/2) differs significantly between the groups. GE test (1/2) was found in control, drug-naive, early-stage patients with PD and treated patients with PD, 123.3 min ± 16.6, 166.6 min ± 32.4, and 203.6 min ± 46.8, respectively.[42] Most of these studies reported significantly increased the GE test in the PD group compared with the controls.

Small intestinal bacterial overgrowth (SIBO)

SIBO and changing gut microbiota raise doubts about the effectiveness of oral drug therapy in PD.[41] Recent studies showed that incidence of SIBO is high in PD. Additionally, GI symptoms and worsening of motor functions in PD have been reported to be related to SIBO.[43] Fasano et al.[44] showed that patients with PD and SIBO have more serious motor fluctuations (off time, delayed on-time, and non-on-time) than those without SIBO.

Colonic dysmotility

One of the most important GI problems observed in PD is decreased bowel movement. However, many PD drugs, such as anticholinergics and dopamine agonists, have been shown to cause constipation.[39]

In the study by Cheon et al.[45], the rate of constipation in patients with PD is 65.8%. In a survey study, the rates of difficulty in defecation in patients with PD and control group were reported as 59% and 20.9%, respectively. In the same study, the rate of laxative prescribing was reported as 29.9% and 9.5%, respectively.

Non-oral treatment necessity in PD treatment

Although orally administered L-DOPA is considered as "gold standard" drug for the control of motor symptoms in PD, the duration of benefit is seen to decreases in use long-term at an oral dose of L-DOPA.[4] Patients begin experiencing fluctuations in motor function in the later stages of PD. Due to the late effects of oral medications, the early morning OFF-periods are the most challenging situation in PD. This problem can complicate the patient's daily morning routines and seriously affect their quality of life.[7] As the disease progresses, the most important reason for oral treatment failure and motor fluctuations are the above-mentioned GI problems. Dysfunctions in the GI system occur at all levels of PD and this cause motor fluctuations in the advanced stages of PD, which make management of the disease difficult.[46] Especially, dysphagia may induce silent

aspiration and delayed GE. Problems such as delayed "on" and non "on" responses may arise due to gastroparesis in PD's oral dopaminergic treatment.[7,16] GI problems, such as gastroparesis, which occur in 70-100% of patients, can decrease the effectiveness of oral medications by delaying their absorption and delivery into the bloodstream.[16] Delayed ON and even dose failure, which causes motor fluctuations, may occur because of inadequate levels of medication plasma levels.[47,48] Besides, it has been stated in recent studies that the pathological process of PD can be managed and even started by the intestinal microbiota through the intestinal-brain axis.[49,50] Additionally, studies have shown that bacterial metabolites that may affect the enteric nervous system differ between patients with PD and healthy control groups.[51] At the same time, it has been indicated that previous studies have that some PD drugs may change the microbiota content. The increasing recognition of multilevel GI dysfunction in patients with PD has contributed to the development of non-oral methods for treating PD's motor and non-motor symptoms.[46]

Current studies on non-oral formulations

The liquid intranasal rotigotine is formulated of a pharmaceutically satisfactory acid addition salt of rotigotine and α-cyclodextrin. α-Cyclodextrin is used to predominantly stabilize rotigotine hydrochloride used. A formulation for intranasal use of rotigotine has been developed for therapy in PD and restless leg syndrome. The formulation underwent two phase 2 studies to assess efficacy, safety, and tolerability in a randomized, double-blind, placebo-controlled, and proof-of-concept manner. However, the results of these studies did not show improvement in secondary outcome measures such as a change in Unified Parkinson's Disease Rating Scale III (UPDRS III) administration administration and "OFF" reversals. Development of the drug was discontinued.[52]

Priano et al.[53] completed a pilot study on a new preparation of apomorphine, which was included in microemulsion and administration *via* the transdermal route (APO-MTD). Twenty-one patients were treated and the results obtained showed that APO-MTD delivered an average of 5.1 h of therapeutic plasma levels, improved the UPDRS III scores, and reduced the overall length of "OFF" periods. However, as promising as this treatment may seem, because of the time taken of 1 h to reach therapeutic concentrations, APO-MTD may not be the "ideal" treatment for the rapid relief of the "OFF" periods suffered by patients with PD.

The sublingual formulation of the D2-D3 agonist piribedil, S90049, was designed to abort "OFF" episodes in PD. A phase 2, double-blind, randomized, and placebo-controlled study showed superiority of S90049 in UPDRS III post-application in advanced-stage patients with PD. Additionally, the switch from "OFF" to "ON" was significantly greater in patients using S90049 inhalation than in placebo. Despite these results, no further activity has been reported since 2010.[54]

Sintov et al.[55] have suggested that transdermal L-DOPA administration can be effective to provide continuous dopaminergic stimulation. Considering that L-DOPA is insoluble in most solvents and has limited permeability through the skin, a modern self-assembling nanomicellar system with 2% L-DOPA and 1% carbidopa has been developed. Because of *in vitro* tests and *in vivo* studies in rabbits, it has been observed that transdermal permeability and systemic absorption of L-DOPA from the skin increased significantly through this formulation developed.

Non-oral formulations are required because of high liver metabolism and poor oral bioavailability of selegiline. Accordingly, the buccal film formulation with the poly(lactide-co-glycolide) (PLGA) nanospheres of the selegiline was developed. By evaluation of *in vitro* and *in vivo* studies, buccal films prepared with selegiline-loaded nanospheres have been observed to show great properties such as good physical properties, sufficient bioadhesion, and controlled drug release. Besides, thanks to the formulation prepared, it was seen that a higher amount of selegiline could be administered through the buccal mucosa. With this study, it is supported that buccal administration of the selegiline is an advantageous and promising approach that can overcome the problems limiting the successful delivery of this drug.[56]

Mishra et al.[24] developed a nanolipid carrier (NLC) formulation with selegiline hydrochloride to be administered nasally, considering that the nasal route is a convenient way to target the drug directly to the brain. NLC formulation loaded with selegiline hydrochloride showed 93 ± 5.25% entrapment efficiency and 51.96% loading capacity. It has been shown that with the optimized NLC formulation, 70% release can be achieved within 10 h, and then the drug release continues for up to 22 h (97%). The drug was found to improve behavioral parameters in rotenone-induced rats.

Ravi et al.[57] have developed a nasal thermosensitive gel formulation to provide effective treatment of PD by considering the low oral bioavailability of rasagiline mesylate. Because of pharmacokinetic studies in rabbits, *in situ* gels were found to provide a significant increase in the bioavailability of rasagiline mesylate.

Çelik et al.[58] have developed buccal mucoadhesive tablets to increase the low bioavailability of piribedil and provide a controlled release treatment for PD. Generally, buccal tablets prepared with hydroxypropyl methylcellulose can provide the necessary controlled release and physical properties. Because of the study, it was concluded that buccal mucoadhesive tablets provide various advantages such as controlled-release compared to traditional oral dosage forms. It is thought that side effects can be reduced because of the high bioavailability with lower doses to provide the desired effect.

The use of drugs targeted to the brain continuously and safely in PD is critical in the treating of this disease. In a study, surface-modified biodegradable PEG-PLGA nanoparticles were prepared with lactoferrin (Lf) to target rotigotine intranasally to the brain for PD treatment. When all the results of the study were examined, Lf nanoparticles were shown to be a suitable carrier for targeting rotigotine to the brain intranasally in PD treatment.[59]

Developed non-oral PD formulations

Levodopa/carbidopa intestinal gel (Duopa™) (LCIG)

Although L-DOPA is the gold standard in PD treatment, because of its short plasma half-life, oral L-DOPA treatment cannot effectively stimulate receptors. Motor fluctuations are seen due to insufficient plasma level.[60] LCIG formulation has been developed to be used to provide a continuous effect by keeping the plasma level of L-DOPA constant.[61] With the help of this pump, small doses of L-DOPA/carbidopa are administered into the small intestine at regular intervals, by passing the stomach. LCIG allows safe titration of L-DOPA to high doses, even more than 2000 mg/day and leads to more stable L-DOPA plasma concentrations.[61] Through this formulation, irregular absorption of L-DOPA caused by prolonged GE time in patients with PD is prevented.[61] In a study, when evaluating the effectiveness of the LCIG formulation against L-DOPA-carbidopa tablets, it was reported that LCIG significantly reduced "OFF" times and increased "ON" time without troubling dyskinesias. Because of the study, percentages of the patients, who were reported as "better" for dyskinesia, tremor, and gait disturbance called motor symptoms, were 80%, 55%, 65%, and 85%, respectively. Percentages reported for non-motor symptoms, pain, sleep disorders, depression, and incontinence were 50%, 50%, 42.5%, and 32.5%, respectively. Studies have shown that LCIG formulation is a promising alternative for advanced patients with PD with motor complications.[62]

Intrajejunal TriGel infusion (LECIG)

TriGel is a novel formulation obtained by adding entacapone to LCIG. Entacapone reduces conversion of L-DOPA to 3-O-methyldopa by blocking the second-largest pathway of L-DOPA. Thus, the plasma concentration of L-DOPA increases.[63] In a clinical study, LCIG and LECIG treatments were compared. Because of this study, dose-adjusted L-DOPA exposure was found to be significantly higher in the LECIG formulation compared to LCIG. It was observed that 3 patients had a 20% increase in systemic exposure to L-DOPA and a 40% or higher increase in six patients, and 2 patients could not achieve the target systemic exposure.[64] It is thought that the combination of opicapone, a newly developed COMT inhibitor, and LCIG can provide a similar effect.[65]

Inhaled levodopa powder (Inbrija®)

L-DOPA inhalation powder (Inbrija®) is a dry powder formulation administered orally with an inhaler, enabling rapid drug absorption in the pulmonary system. It is manufactured by Acorda Therapeutics and has been approved by FDA to treat the symptoms of Parkinson's patients during "OFF" periods.[66] Each capsule contains 42 mg spray-dried L-DOPA powder, 1,2-dipalmitoyl-snglycero-3-phosphocholine, and sodium chloride. The dry powder particles (5-10 μm diameter) are homogeneous, low in density, and highly porous for aerosolizability and lung deposition. Inbrija® was developed to achieve a rapid effect by providing a consistent and rapid increase in the concentration of the drug in the bloodstream. Pulmonary administration provides rapid absorption of L-DOPA due to its large surface area and low metabolic activity, so delayed "in" period or dose failures can be avoided.[66] Because of a study to determine the pharmacokinetics and tolerability of the formulation, T_{max} was 15 min in patients who are administered inhaler L-DOPA powder, but after oral administration, T_{max} ranged from 20 min to 90 min. However, no changes in lung function parameters were observed in patients and no patient complained of cough or shortness of breath.[67,68]

Rotigotine patch

The transdermal patch formulation of rotigotine (Neupro®), a dopamine agonist, has been developed for use alone in the early stages of PD or in addition to L-DOPA in the advanced stage of the disease. Rotigotin transdermal patch has been approved in EU, China, and Japan as a combination therapy with monotherapy and L-DOPA for early PD treatment. With the developed transdermal patch formulation, stable rotigotin plasma levels could be achieved for 1-2 days with a single daily administration.[69] A double-blind, placebo-controlled, and randomized study demonstrated that rotigotin patch can well manage both motor function and sleep problems in patients with PD with motor dysfunction, when waking up in the morning.[70] Additionally, other important effects of rotigotine patch on non-motor symptoms include pain, mood, and anhedonia associated with dopamine fluctuations.[71] Compared with rotigotine patch and other conventional oral dopamine agonists, impulse control disorder was reported to be less common with the use of rotigotine patch. It has been reported that the most common side effects after the application of the rotigotin patch are skin reactions in the application area and some neuropsychiatric problems.[71,72]

Subcutaneous rotigotine-polyoxazoline

We provided continuous dopaminergic stimulation by preparing a subcutaneous formulation of rotigotine with polyoxazolines.[73] *In vivo* studies using rat models with 6-hydroxydopamine lesions have shown that rotigotine-polyoxazoline slow-release conjugate relieves motor symptoms by repeated dosing and provides a long rotigotin half-life. With these promising results, a slow-release conjugate of rotigotine has FDA confirmation to enter phase 1 study (NCT02579473) with anew patients with PD.[73] Olanow et al.[74] evaluated the safety, tolerability, and pharmacokinetics of polymer-conjugated rotigotine in patients with PD with a multicentre open-label, multiple incremental, and dose-spaced cohort studies. Because of this study, it has been observed that, when the polymer-conjugated rotigotine is subcutaneously administered once a week, relatively constant plasma rotigotine levels can be achieved, which are safe and well-tolerated.

Subcutaneous apomorphine

Subcutaneous apomorphine has been developed to manage unpredictable and predictable "OFF" periods, in patients with PD well. The subcutaneous apomorphine has been developed in two different formulations. These are intermittent injection of apomorphine and a continuous infusion of apomorphine with a removable infusion pump without surgery. It is specified that it

is a very suitable formulation to prevent delayed or failed "ON" situations caused by GE and L-DOPA absorption problems and to alleviate early dystonia or akinesia quickly and safely. It has been reported that a consistent antiparkinsonian response with subcutaneous apomorphine was obtained and no significant circadian changes were observed during this response. The effect of subcutaneous apomorphine injection on "ON" time was evaluated by a multicentre and open-label phase IV study in patients with PD with morning akinesia. In this study, firstly, the normal morning dose of oral L-DOPA was applied to the patients and "ON" times were recorded. Then, "ON" times of the patients were recorded again for a week using apomorphine injection instead of oral L-DOPA. Because of the study, it was observed that apomorphine injection shortened 37 min the patients' become "ON" status by compared to oral L-DOPA. With several open-label clinical trials, apomorphine infusion significantly reduced OFF time by up to 85% compared with baseline and increase ON time by an average of 5.5 h daily in patients with PD.[75,76]

Inhaled apomorphine (VR040)

In PD, it has been observed that "OFF" periods can be managed with subcutaneous apomorphine, but some patients may experience difficulty in application because it requires the injection. For this reason, it is thought that inhaled apomorphine may be useful. To determine optimal efficacy, safety, and tolerability for inhaled apomorphine in patients with PD, randomized, double-blind, active, and placebo parallel-group, and increased dose titration studies were conducted in 16 centers in 3 countries. Because of this study, the meantime to "ON" in 33 patients in the OFF period was found to be 8.1 min for inhaled apomorphine and 13.1 min for placebo. Additionally, the proportion of those who became "open" within 40 min in patients who received inhaled apomorphine and placebo (except "partially open") was found to be 60.0% and 26.7%, respectively. In the double-blind phase 2 study, tolerability, safety, and effectiveness of VR040 were evaluated. It was reported that development of a UPDRS III in 47 patients was 26.8 points for inhaled apomorphine and 14.9 for placebo.[77,78]

Sublingual apomorphine (APL-130277)

APL-130277 is a film strip in clinical development that is investigated for treating OFF periods. It consists of a thin bilayered film designed to improve apomorphine delivery, while optimizing tissue compatibility and film disintegration. The first layer consists of apomorphine and is designed to provide stability, rapid drug diffusion and enhanced bioavailability. The second layer is a buffer layer that is designed to increase drug permeability and neutralize acid formation following drug absorption. As a result, it is designed as a "turning ON" medication to acutely manage OFF episodes by rapidly delivering apomorphine from the oral cavity without any mucosal irritation. Hauser et al.[79] conducted a phase 2, open-label, proof-of-concept study to assess tolerability, safety, and efficacy, and to determine the effective doses.

Buccal selegiline (Zydis™ ZELAPAR)

Non-oral alternative formulations have been explored because of the low oral bioavailability of selegiline, high rate of first-pass effects, and conversion to undesired metabolites in the liver. One of these factors is the tablet formulation prepared for application to buccal mucosa the Zydis™ technology.[80] Because of pharmacokinetic studies, Zydis™ selegiline can inhibit MAO-B at one-eighth of the traditional oral dose and reduce amphetamine metabolites by 80-90%. Because of phase 4 studies, it was seen that ZELAPAR was preferred by patients because it was well tolerated and provides ease of use.[25,81] Waters et al.[81] evaluated the safety and efficacy of zydis selegiline in patients with PD with motor fluctuations during L-DOPA therapy with a short-term clinical study. Because of the study, it was seen that an orodispersible tablet of selegiline as an additional treatment to L-DOPA in patients with PD with motor fluctuation problems was effective and safe.

CONCLUSION

Current therapy options for PD remain focused on the symptomatic improvement of motor features related predominantly to the loss of dopaminergic neurons in *substantia nigra*, but do not address the root cause of the disease. Improvements in trial design must evaluate candidate drugs more appropriately, perhaps with the introduction of validated clinical markers. Physicians need practical guidance both to help patients make a judgment on what drug to use and when to initiate it. This remains very much an individual decision and will need to take account of many factors, including the patient's age and co-morbidity and the physician's own interpretation of the data available and the information presented here. Oral dopaminergic treatments were mainly focused on the management of PD symptoms. However, it has been thought and investigated that GI problems in patients with PD can significantly affect the effectiveness of oral treatments. As a result, it has been observed that GI problems such as dysphagia, delayed GE, SIBO, and changes in colon motility complicate oral treatment in PD and cause delayed "in" or early morning "OFF" fluctuations in patients. For this reason, non-oral drug delivery systems have been studied to manage PD symptoms effectively.

We seek to bring further clarity to the non-oral treatment options for patients at different stages of PD. The therapies included in this review have all been shown to result in significant improvements of both motor and non-motor symptoms, but each therapy also has many characteristic advantages and drawbacks that need to be matched with the patient's symptomatology.

The costs related to all non-oral drug delivery systems are significant, and further cost reductions are required to increase access to these therapies. Moreover, there is a need for further development of the non-oral continuous drug delivery techniques-both to increase their ease of use and to reduce the relatively frequent device-related adverse effects. In addition to changing the existing drug administration systems,

new methods of administration are required by examining the current studies.

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Authorship Contributions

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REVIEW

Peroxisome Proliferator-Activated Receptors as Superior Targets for Treating Diabetic Disease, Design Strategies - Review Article

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[Ψ]We commemorate late Prof. Dr. Tijen Önkol with mercy and respect on this occasion. IEO, BK, SAE (The Editorial Board)

ABSTRACT

Thiazolidinedione (TZD), a class of drugs that are mainly used to control type 2 diabetes mellitus (T2DM), acts fundamentally as a ligand of peroxisome proliferator-activated receptors (PPARs). Besides activating pathways responsible for glycemic control by enhancing insulin sensitivity and lipid homeostasis, activating PPARs leads to exciting other pathways related to bone formation, inflammation, and cell proliferation. Unfortunately, this diverse effect of activating several pathways may show in some studies adverse health outcomes as osteological, hepatic, cardiovascular, and carcinogenic effects. Thus, a silver demand is present to find and develop new active and potent antiglycemic drugs for treating T2DM. To achieve this goal, the structure of TZD for research is considered a leading structure domain. This review will guide future research in the design of novel TZD derivatives by highlighting the general modifications conducted on the structure component of TZD scaffold affecting their potency, binding efficacy, and selectivity for the control of T2DM.

Key words: Peroxisome proliferator-activated receptors, thiazolidinediones, structure-activity relationship, drug design, antidiabetic activity

INTRODUCTION

This group of metabolic diseases presents because of a defect in insulin action, insulin secretion, or both that finally leads to chronic hyperglycemia is called *diabetes mellitus* (DM). This proves the importance of insulin as an anabolic hormone in controlling lipid, carbohydrate, and protein metabolic abnormalities. These metabolic abnormalities are due to the low level of insulin secretion that leads to inadequate response or/and resistance of target tissues to insulin. The insulin resistance occurs at the level of insulin receptors, signal transduction system, and/or effector enzymes or genes of skeletal muscles, mainly targeted tissue, adipose tissue, and to a lesser extent, liver.[1,2] The duration and type of diabetes are directly related to the severity of symptoms.[3] To distinguish between DM symptoms and various hypoglycemic cases recorded, it has been assorted into different types. In 1997, DM was classified by the American Diabetes Association (ADA) into four types: type 1, type 2, other types, and gestational DM.[1] Until now, this classification adopted by ADA still the most accepted one.

Type 1 DM (T1DM), which is due to the destruction of pancreatic β-cells. It is also known as the autoimmune T1DM due to the presence of autoantibodies, considered as a hallmark of type 1, that work against pancreatic β-cells, although, its role in the disease pathogenesis is unclear. Those autoantibodies are formed against glutamic acid decarboxylases, insulin, islet cell, transporter protein (ZnT8A), and protein tyrosine phosphatase. Before the onset of the disease by months or years, these pancreatic autoantibodies could be present and thus detected in the serum of type 1 patients with DM. Destruction of pancreatic β-cells is mainly through the humoral (B-cell) response and insulitis (T-cell mediated inflammatory response). This type shows general symptoms that often develop suddenly, such as lack of energy, polyuria, polydipsia, extreme tiredness, blurred vision, enuresis, slow-healing wounds, and sudden weight loss. Regarding adolescents and children, diabetic ketoacidosis and severe dehydration are developed. T1DM accounts 5-10% of diabetes cases, in general, and 80-90% of diabetes in adolescents and children. It is important to notice

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that 78,900 new diabetic cases are recorded *per* year by the International Diabetes Federation (IDF).[4] Regarding the adults and adolescences above 14 years of age, type 1 shows a high prevalence, for example, in 2010, total account of type 1 in the United States (USA) was estimated to be 3 million[5] and, in 2019 was accounted to be 1.93 *per* 1000.[6]

However, type 2 DM (T2DM), characterized by a high blood glucose level due to insulin resistance besides a relatively low level of insulin, shows a higher global prevalence based on a 2019 report published by the IDF compared to type 1. The report mentioned that the global prevalence of T2DM in adults (20-79 years old) was 8.3% (382 million people), mainly intensify within ages of 40-59. Men show a higher prevalence than women, with 14 million more (184 million women *versus* 198 million men). Additionally, during pregnancy, 21 million women are diagnosed with the diabetic disease. These accounts by 2035, are predicted to exceed 10.1% global prevalence with 592 million cases.[6-9] The IDF Diabetes Atlas (2019), showed the current and expected prevalence of diabetes in adults (20-79 years) for different regions worldwide (Figure 1). As shown, the highest accounts in 2019 were reported to the western Pacific region with 163 million and that is expected to increase to 212 million by 2045. Additionally, high concerns are directed to the Middle East besides of Africa and North Africa Regions that are expected to report an increase in the total diabetic cases by more than 100%. 80% of the total cases were counted for the low- and middle-income countries, "where the epidemic is gathering pace at alarming rates".[10]

For patients with DM, for insulin deficiency or resistance, many manifestations have been recognized besides of hyperglycemia, these include essential hypertension, nephropathy, obesity, dyslipidemia, non-alcoholic fatty liver disease, accumulation of lipoprotein, premature adrenarche, ovarian hyper and organism, systemic inflammation, polydipsia, polyphagia besides the aforementioned symptoms for type 1 like weight loss and blurred vision. If the diabetic patient is not controlled, stupor and coma are recognized, and finally, lead to death due to ketoacidosis. Severe dehydration of ketoacidosis, recognized in some pediatric type 2 patients with diabetes, who are not recognized as obese generally lead to misclassification to T1DM.[11-13]

Because 90% of patients with DM around the world counts to T2DM; so considered as the predominant form, a group of drugs, such as glitazones [thiazolidinediones (TZD)], sulfonylureas, glinides, dipeptidyl peptidase IV inhibitors, gliflozins and biguanides, are developed and used to control T2DM. Unfortunately, all the used drugs present uncontrolled side effects like obesity and hypoglycemia.[14] One of the most important classes of drugs used to treat T2DM is the TZD class, which acts as an antiglycemic agent by binding avidly to the gamma type of a nuclear receptor called peroxisome proliferator-activated receptor gamma (PPAR-γ).

TZD class of drugs is an orally administered hypoglycemic agent used to treat T2DM alone or along with other orally or injectable hypoglycemic agents. This class includes a group of drugs that, in the late 1990s that is the time of their introduction, were dispensed as a first and a second line for treating T2DM like pioglitazone and rosiglitazone (Figure 2).[15] Of glycemic control, TZDs displayed better outcomes and physiological effects than the other approved second line agents like sulfonylurea and, in some cases, other 1st line hypoglycemic agents like metformin. As a result, presenting another

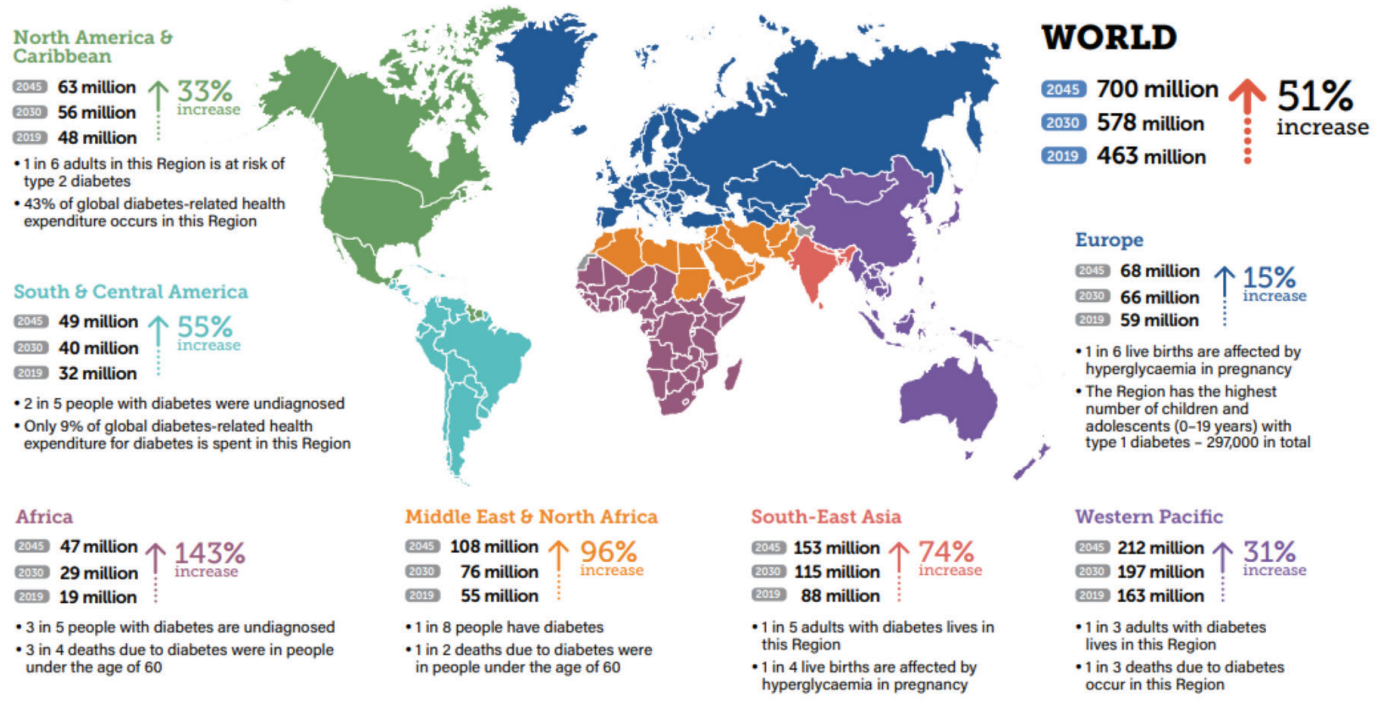


Figure 1. Prevalence of diabetes in adults (20-79 years) in various regions[10]

beneficial effect of TZDs, besides controlling hyperglycemia by enhancing insulin sensitivity, like anti-inflammatory effects, its use as antiglycemic agents was praised.[16]

As PPAR receptors exist in different subtypes that differ in structure, effect, and region of distribution, TZD class of drugs mainly activates the gamma subtypes that are exclusively present in adipose and epithelial tissues including urothelium.[17] However, the gamma subtype also presents in pancreatic β-cells, liver, immune cells, and bone tissues besides of other subtypes (α and β subtypes). Because of activation of PPAR-γ receptors, numerous genes responsible for controlling glucose and lipid metabolism as well as genes that regulate thrombotic function, vascular function, and the inflammatory response are expressed in a different pattern, as up-regulating or down-regulating pattern, and finally leads to both vascular and metabolic effects. Besides, to promote the metabolism of free fatty acids (FFA), TZDs increase the synthesis of triglyceride and non-oxidative glucose disposal, enhance lipid metabolism [raise the level of buoyant and large low-density lipoprotein (LDL) particles and high-density lipoprotein (HDL) level] and lowering the blood pressure besides of improving other common abnormalities related to T2DM like rheological abnormalities and vascular reactivity.[18,19]

Unfortunately, TZDs are accompanied by a set of side effects such as (a) edema and weight gain, which is considered the most common one mainly demonstrated in patients with

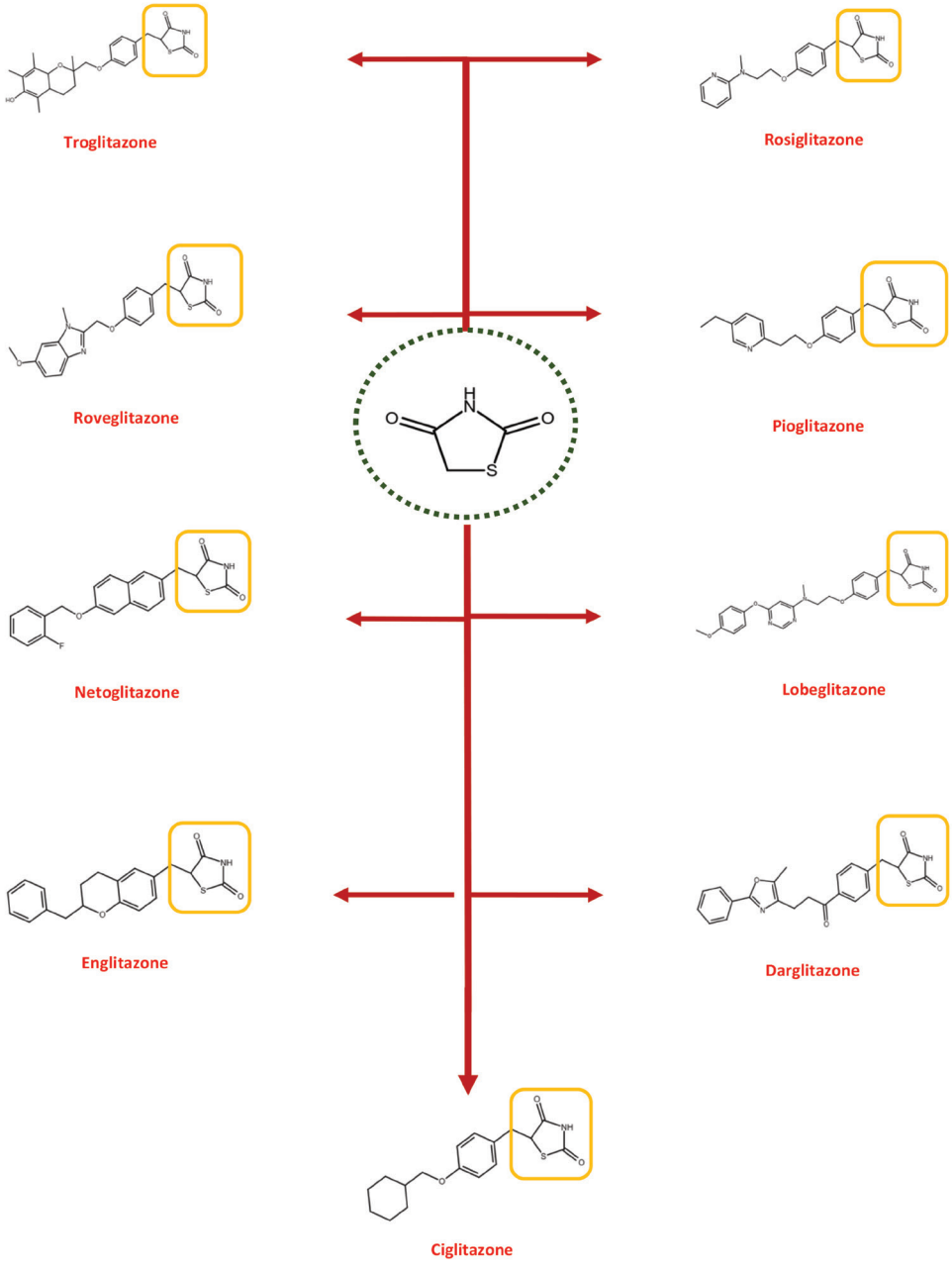


Figure 2. Current approved anti-diabetic drugs that follow thiazolidinediones class[15]

diabetes, who follow TZDs monotherapy or dual therapy along with other oral hypoglycemic agents or with insulin.[18] (b) Hepatotoxic effects, including liver failure, were reported for the troglitazone drug (in January 1997, the Food and Drug Administration in USA approved it as the first marketed drug of TZDs class) and sometimes led to demonstrate deaths. (c) Cardiovascular adverse effects like congestive heart failure (CHF) were also reported in the Framingham Heart Study in patients with diabetes administered TZDs. Also, it is well proved that cardiovascular disease is a prevalent complication of T2DM, and administering TZDs led to elevate the risk of CHF in women patients with diabetes to 5 fold and in men patients with diabetes to 2.5 fold, this presents more highlights in the black side of TZDs.[15] (d) Osteological adverse effects: some records reported that patients with diabetes with T2DM were recognized with an increase in bone mineral density and bone weight. Finally, TZDs were recorded with (e) carcinogenic effects. It has demonstrated that the risk of cancer is accreting to T2DM patients. Many types of cancer have been associated with T2DM, such as liver, gastric, endometrial, pancreatic, renal, ovarian, breast, colon, and bladder cancers. Here, several studies proved the increased rate of cancer mortality rate.[20]

Given above, to diminish the financial burden of T2DM patients and enhance the living equality, many attempts have been conducted by the medicinal chemists to find new antidiabetic agents with better activity and fewer side effects by modifying the existing drugs or discovering new natural leads. To achieve this goal, based on the approved beneficial outcomes of PPAR agonists agents like TZDs on management glucose and lipid metabolism, there is an indispensable need to understand the binding interaction between PPARs and their agonist agents as well as the chemical structure of PPAR. Additionally, to design new ligands with better specificity and binding affinity for PPAR-γ or dual as PPAR-α/γ agonists, more advanced techniques could be used such quantitative structure-activity relationship (QSAR) and pharmacophore modeling and docking to get a more in-depth knowledge about the TZDs besides of other agonist agents, especially regarding stereochemistry, binding groups, and topology is necessary.

Owing to the meaning of these pharmacological and chemical standpoints, this review will summarize the PPAR-α, β, and γ functions and chemical structures. As well, the existing chemical structures of TZDs will be discussed with respect to their agonist effects on PPAR-γ or PPAR-α/γ besides outlining the adverse outcomes specified in the literature. Additionally, we going to delineate the pivotal TZD structures to lead the future attempts to develop the next generation PPAR-γ or PPAR-α/γ agonists as new glitter antidiabetic agents.

Most plant and animal cells that undergo various metabolic functions like cholesterol metabolism, H_2O_2-based respiration, and β-oxidation of FAs generally contain subcellular organelles called PPARs proteins, firstly recognized in 1990, belong to the nuclear hormone receptor superfamily that includes 48 members.[21] From the structure perspective, there is a similarity between the thyroid or steroid hormone receptors and PPARs. As well, the similarity is extended by their responses to small lipophilic ligands. As PPARs exist is three subtypes, each one responsible for mediating the physiological action of huge diverse FAs and fatty acids-derived molecules.

PPAR isoforms

PPARs belong to the nuclear receptor superfamily, which is considered a wide diverse superfamily that constitutes much members such as transcription factors, such as PPARs, glucocorticoid receptors, vitamin D, estrogen, retinoic acid, thyroid in addition to several other protein factors associated with xenobiotic metabolism. As heterodimers, PPARs with retinoid X receptor (RXR) act on DNA response elements. The lipid-derived elements are considered to be the innate activating ligands of PPARs. Three subtypes of PPARs (α, β, and γ) present a fundamental role in energy metabolism; however, the activity spectrum and specificity of these subtypes are different. Regarding PPAR-γ, exists mainly in vascular smooth muscle and endothelial cells and works predominantly in regulating energy storage. PPAR-α presents mainly in the liver but, in the bone, heart, and muscle tissues expressed to a lesser extent. The last subtype (PPAR-β) plays a major role in regulating energy expenditure and displays a ubiquitous expression in the whole body.[22]

Mechanism of action of PPARs

Various genes are transrexpressed and transactivated as a because of binding peroxisome proliferator response elements (PPREs), which exist at the promoter of a target gene that consists of direct repeats of AGGTCA, with a heterodimer complex composed of PPARs and RXR. In the case of no ligands are present, the gene transcription is blocked because of the co-repressor complex role associated with thesis heterodimers. Balakumar et al.,[23] 2007 mentioned that some agonist ligands act on PPARs. RXR, like PPAR, presents as three subtypes: RXR-α, β, and γ. 9-*cis*-retinoic acid acts as an endogenous agonist to all distracted isoforms.[24] With respect to these isoforms within the RXR-PPAR complex, no appointed actions have yet been confirmed. Whilst, antidiabetic action could be obtained through activating the heterodimers complex with retinoids (synthetic RXR agonist), this action is comparable to PPAR agonist effect recognized in mouse models of T2DM. The heterodimerization of RXR with PPARs is facilitated by the ligand-binding domain (LBD) and eventual RXR-PPAR complex, with recruitment of co-factors, subsequently binds to PPRE as briefly shown in Figure 3.[25]

Binding of DNA with RXR/PPAR complex is blocked in the absence of agonists/ligands by a group of co-repressor structures such as G-protein pathway suppressor 2, histone deacetylases, and nuclear receptor co-repressors, high-affinity complexes are formed between the inert heterodimers of RXR-PPAR and these co-repressors. To initiate the transcription process, various transcriptional co-activators/co-factors such as steroid receptor coactivator-1, CREB binding protein, histone acetyltransferase p300, and PPAR coactivator-1 are recruited.[26]

PPAR structure

Figure 4 represents the structure of PPARs as one dimension shape. Many advanced techniques have been incorporated to study the PPAR structure extensively like solvent mapping techniques, molecular modeling, and X-ray crystallography with respect to main structural domains including LBD, co-activator binding site (Co-FBD), and DNA binding domain/region (DBD). This finally led provides insight into the binding mode. PPAR-γ is comprehensively studded in-depth, other than other subtypes, including interactions and structure. Additionally, it is examined in different cases such as the presence or absence of the ligands, co-activator peptides in a DNA bound or unbound state, and as heterodimers with RXR-α or as homodimers. It was proved that *N*-terminal (5′), involved in the phosphorylation of PPAR, contains DBD but the C-terminal (3′) contains the LBD as demonstrated in the 3-D structure of PPARs.[27] 5′-AF-1 domain associated with a region that is independent of A/B domain that is responsible for the phosphorylation of PPAR.

In the promoter region of target gene, peroxisome proliferation response element (PPRE) binds to DNA. The co-FBD/D domain especially binds to co-factors. The binding of specific small molecules to LBD or E/F domain leads to sequential gene expression because of receptor activation. The E/F domain contains a ligand-dependent region called the AF-2 region, which facilitates gene transcription process.[28]

Structure of DNA binding domain of PPARs

A/B domain of PPARs plays a substantial role in protein phosphorylation, works as a functional activator of the transcriptional process, or interacts directly with other regulatory proteins or receptor domains. Additionally, this region does not possess a significant binding side due to the absence of conserved amino acid sequence or residues of high hydrophobic character due to its high mobility. PPAR-γ ligands were recorded with no action on the region of A/B. In the highly conserved and central DNA binding domain, two binding sites of zinc are recognized. As also, architectural elements are present with the ability for sequence-specific binding to DNA.[29,30] Beyond short carboxy terminal extensions (CTE), DBD structure of nuclear receptors was not visualized. Examining the RTX/PPAR-γ complex in depth has demonstrated that the polarity in PPAR-γ is mainly based on CTE. The structure of DBD showed a close vicinity to LBD structure after investigation the structures of PPAR-γ in-depth.

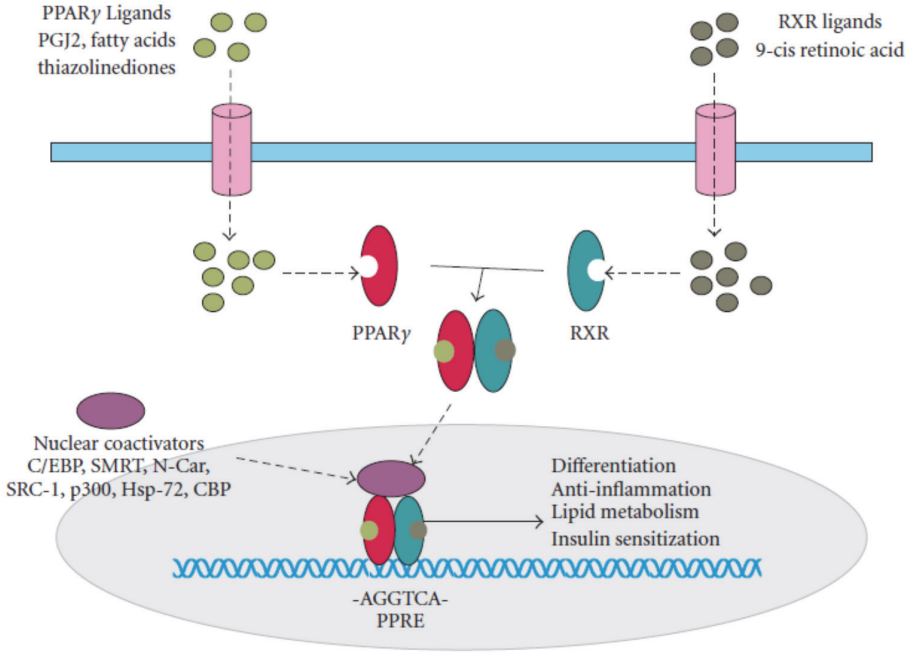


Figure 3. Current mode of action for PPAR-γ. PPAR-γ and RXR form a heterodimer, which is activated by the respective ligands. The activated PPAR-γ/RXR heterodimer will be translocated into nucleus and regulates downstream target genes in concert with nuclear receptor coactivators[25]

PPAR: Peroxisome proliferator-activated receptor, RXR: Retinoid X receptor

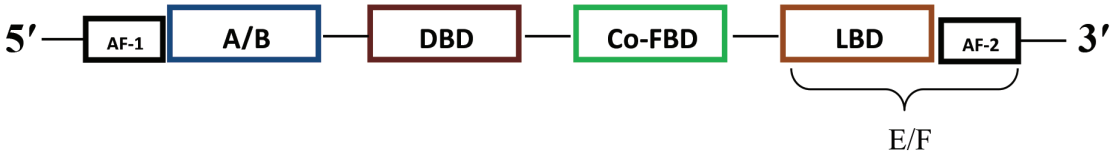


Figure 4. One-dimensional structure of the different binding domains of PPARs[28]

DBD: DNA binding domain/region, Co-FBD: Co-activator binding, LBD: Ligand-binding domain, PPARs: Peroxisome proliferator-activated receptors

Structure of ligand binding domain of PPARs

Various endogenous ligands like FAs including their metabolites considered agonist ligands PPARs isoforms (α, β, and γ).[31] Examining the LBD structure of these three isoforms using the X-ray crystallographic technique showed similar structures. As shown in Figure 5A, the LBD of PPARs structurally consists of thirteen α-helices, H1-H12, and H2' helices, besides small four-stranded β-sheets, S1-S4, folded into a single region/domain. LBD with respect to the secondary structure shows a sandwich-like structure of three layers of antiparallel α-helices. The three long helices (H3, H7, and H10/H11) make up the two outer layers of the sandwich. The helices that made up the middle layer (H4, H5, H8, and H9) are absent from the bottom half of the domain while building the top half. Thus, for ligand binding function, this middle layer exists a large binding cavity (~1400A˚) that displays a Y shape with three arms. This distinct structure of the binding site plays a major role in facilitating the interaction/binding with diverse structure ligands like presenting various conformations with different functional groups or ligands with single/branched chains.[32-34]

In and out Y shape cavity, ten meaningful binding sites (P1- P4, E1, E2, C1, C2, B, and F) have been reported and characterized using solvent mapping techniques on the RXR/PPAR-γ heterodimers, as shown in Figure 5B. Only the four binding sites (P1-P4) exist inside the Y-shape binding cavity of the LBD, while, the other six binding sites are present outside the region. The binding sites E1 and E2 present in the entrance region unlike B and F binding sites, which present in the surface of the Y-shape binding region. Regarding the C1 and C2 binding sites, they are present in the co-activator binding region.

With respect to P1 and P2 binding sites, they constitute a significant hydrophobic pocket that functions as an excellent binding target for all agonists that interact with H12. Examples that could interact with the residues in this binding region are the polar nucleus and carboxyl head group of TZDs and ragaglitazar (a partial agonist ligand), respectively. Compared to P1 and P2 binding sites that are located at arm-I, the P3 and P4 binding sites were present at arm-II and make up a larger hydrophobic pocket. The E2 binding site, the main ligand entrance site, also exhibits a hydrophobic behavior that located at arm-III. The actions including interaction with co-activators and dimerization with RXR are related to the other binding sites located outside the Y-shape cavity.[34]

Structural variations between LBD of PPAR-α and γ

PPAR-α isoform, as well as PPAR-γ isoform, constitute 34 amino acid residues with respect to the Y-shaped binding region of LBD. A high percentage of similarity, around 80%, regarding the size of the binding cavity and the amino acid constituent has been recognized in both isoforms. The ligand specificity of PPARs is a result of the minor differences in the topology, arm-I presents two minor differences in amino acid residues. As the binding sites present in this arm interact mainly with the polar head groups of ligands, these differences in amino acid residues (even these changes are minor) lead to a significant influence on the ligand specificity. The amino acid residues, His323 and Phe363 located at the arm-I of PPAR-γ, are replaced by Tyr314 and Ile354 for PPAR-α, respectively. With respect to arm-II, the Gly284 amino acid residue of PPAR-γ is replaced by a bulky lipophilic Cys275 residue in PPAR-α. The ligand entrance site, located at arm-III, presents a remarkable difference represented by the replacement of Arg288 residue of PPAR-γ by Tr279 in PPAR-α.[27]

SAR studies on TZDs

Medicinal chemistry has an integral mission in relating the molecular structure to its pharmacological effect. To improve

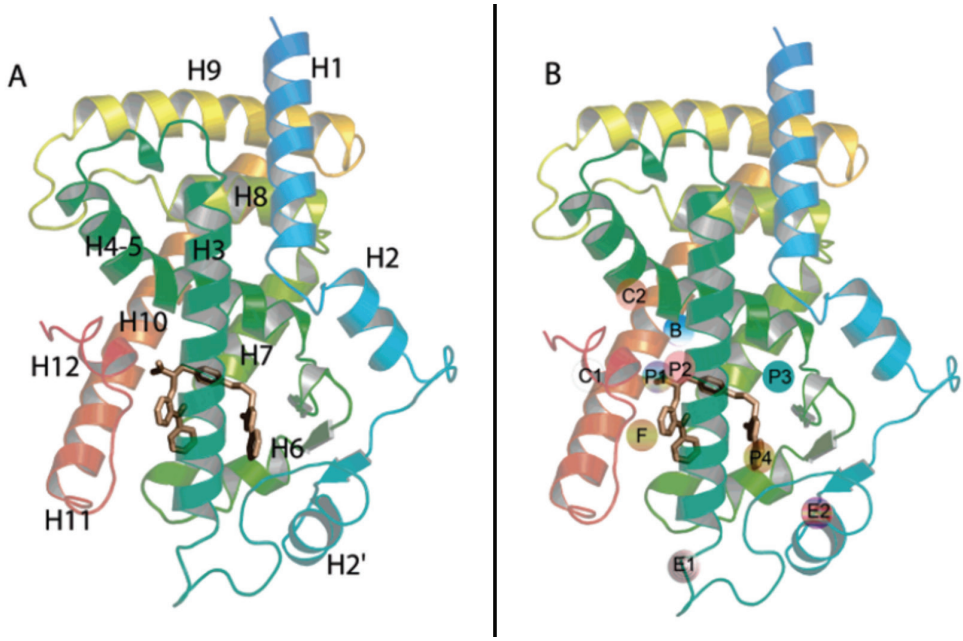


Figure 5. Structure of the PPAR-γ LBD. (A) Polypeptide backbone is shown as a cartoon, indicating the 12 R helices that comprise the domain[34]

PPARs: Peroxisome proliferator-activated receptors, LBD: Ligand-binding domain

the molecular pharmacodynamic and pharmacokinetic properties of a leading drug, including decreasing adverse effects, improving potency and selectivity and enhancing bioavailability, assessing the mode and nature of binding as well as figuring out the pharmacophores is indispensable. For pharmacophore identification, trying to screen, recognize, and synthesize prototype analogs is a common method to get reliable results. However, this approach is time-consuming and is considered an old approach. Currently, various sophisticated advanced computer programs are employed extensively in the drug modification, design, and discovery application.

As a result, to develop new synthetic PPAR-γ agonists, TZDs have been suggested to be stereotype molecules for developing. With respect to the literature, as shown in Figure 6, a simplified topology of a typical synthetic TZD is identified. As shown, the topology structure exhibits a "U" shaped geometry. The head is a TZD functional group, which exhibits polar and acidic character. Linker-1 (L1) should not exceed three carbon atoms in length whilst, linker-2 (L2) could be extended up to four carbon/hetero atoms. The Ar symbol represents a central heteroaromatic/aromatic ring. The tail position should be represented by a bulky lipophilic group like heteroaromatic/aromatic rings.[35]

Significance of TZD structure and its binding interactions

L1 is directly connected to the position 5 of a ring nucleus, thiazolidine-2,4-dione ring, in TZDs (glitazones). With respect to a series of glitazones hypoglycemic agents, *i.e.* rosiglitazone (1) and pioglitazone (2) (Figure 7) are taken as the lead molecules.[36] To control T2DM, an interesting area of research in structural modifications has been directed to these two antidiabetic agents to develop and design new drugs with better profiles. PPAR-γ, in general, considered as a specific, agonist, and strong target for TZD derivatives *via* two styles: The Van der Waals and hydrophobic interactions are employed to bind with lipophilic ligands and, H-bonds are employed to bind with polar acidic ligands.[36] Thus, TZDs have a lipophilic acid behavior, of which the lipophilic binding sites in arm-II and arm-III interact with the lipophilic tail residues of TZDs, while the hydrophilic binding site (P1) existing in arm-I interacts with the acidic head group of TZDs. The aliphatic linkers play a significant role in achieving an efficient binding by acting as spacers; thus the functional groups of TZDs (lipophilic tail and head group) could be positioned properly with the corresponding binding sites of the receptor. Further hydrophobic interaction is obtained from the central phenyl ring. The binding interactions of LBD of PPAR-γ with rosiglitazone antidiabetic drug are sorted out in Figure 8. As shown, rosiglitazone forms a U-shaped conformation through interaction with helix-12.[37,38]

The residues associated with forming H-bond with the acidic head group of TZDs are the Tyr473 of helix-12, His449 of helix-11, and His323 of helix-4. Additionally, through acting as both H-bond donors and acceptors, the oxygen and nitrogen atoms of the nucleus ring could form H-bond with Ser289 (located at helix-3). The β strand besides α-helix 3, 5, 6, and 7, located at arm-II and arm-III of LBD, mainly participate in interacting with the hydrophobic tail moiety of rosiglitazone, which accounts for the potency and efficiency of the binding profile. Helix-3 shows additional hydrophobic interaction with the central phenyl ring.[39]

The ligand binding and co-activators, not the only factors that control the transcriptional activity of PPAR-γ, the state phosphorylation plays a major role as well. For example, many studies have reported that the phosphorylation of Ser273 residue of PPAR-γ led to obesity development. In the case of the rosiglitazone diabetic drug, this phosphorylation is suppressed.[40]

Several research attempts have been conducted to find new TZD derivatives with better therapeutic profiles by improving insulin sensitivity, anti-inflammatory, and anti-cancer effects, as well as decreasing their adverse effects.[41,42] The thiazolidine-2,4-dione ring (nucleus ring of TZDs) has never been subjected to modification, indicating the significance of this moiety. However, a range of heteroaryl, aryl or lipophilic chain

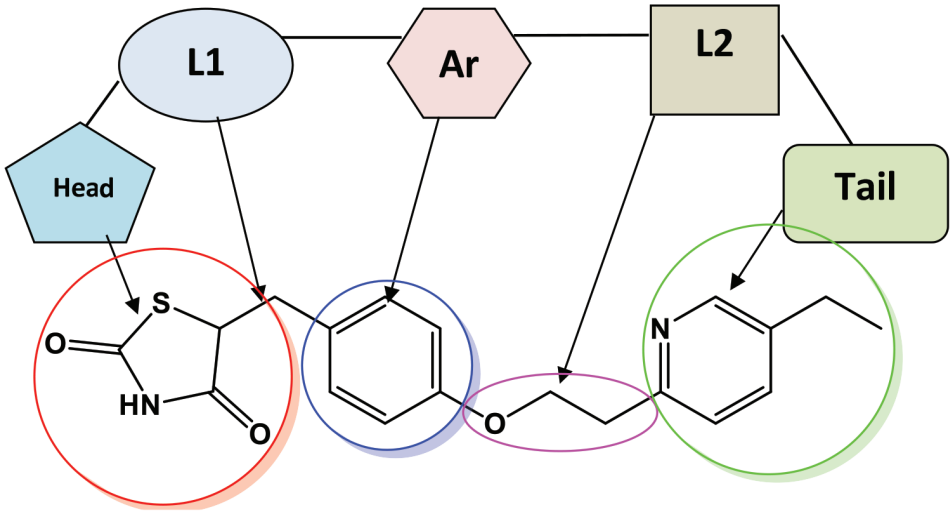


Figure 6. Simplified topology of a typical synthetic thiazolidinedione (pioglitazone)[35]

substituents could be linked to the nucleus ring to develop new antidiabetic agents with better pharmacodynamic and pharmacokinetic profiles.[43]

Modifications on L1

The linker methylene carbon atom located between the central phenyl ring and the nucleus ring of TZDs is displayed in rosiglitazone and pioglitazone (Figure 7). Besides beneficial effects of rosiglitazone by enhancing insulin sensitivity, it displayed a protective effect on the myocardium. The previous studies demonstrated that to retain antidiabetic activity of glitazones, a maximum three carbon atom length of the straight alkyl chain is essential. Branching in the structure is shown if the L1 exceeds the length of the three carbon atoms. The unsaturated bulky alkyl linker in compound (3), Figure 7, provides additional hydrophobic interaction, which leads to improved molecular binding efficiency.[44] Thus, the length and nature of the alkyl linker should be retained, so the space created presents an excellent fitting between functional groups of ligands and the binding cavities of the LBD. The polar residues located at the binding site corresponding to L1 do not exhibit any electrostatic interaction. Hence, the linkers are

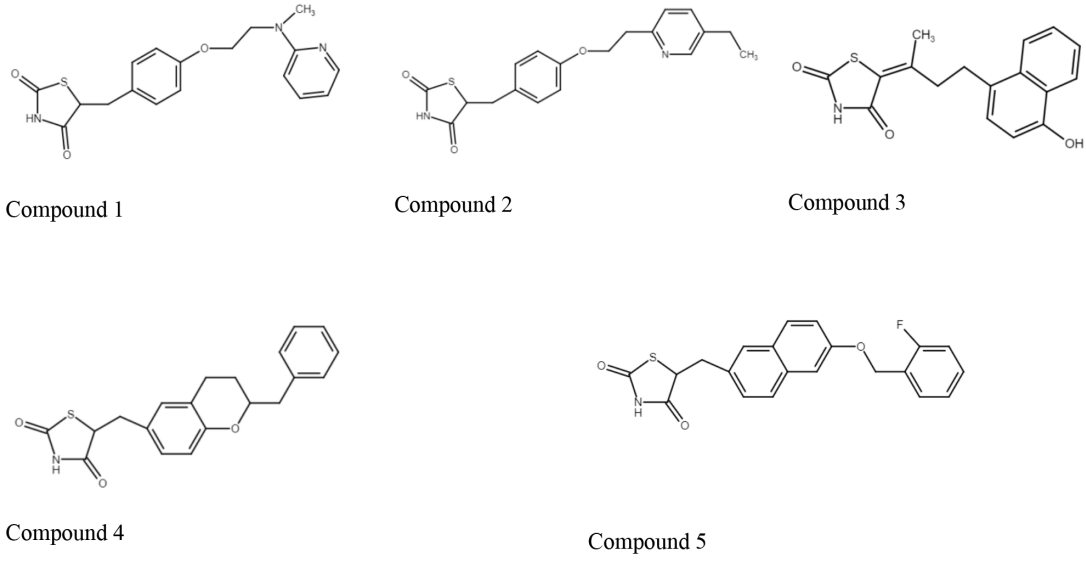


Figure 7. SAR of some TZDs[36,44-46]

SAR: Structure-activity relationship, TZDs: Thiazolidinediones

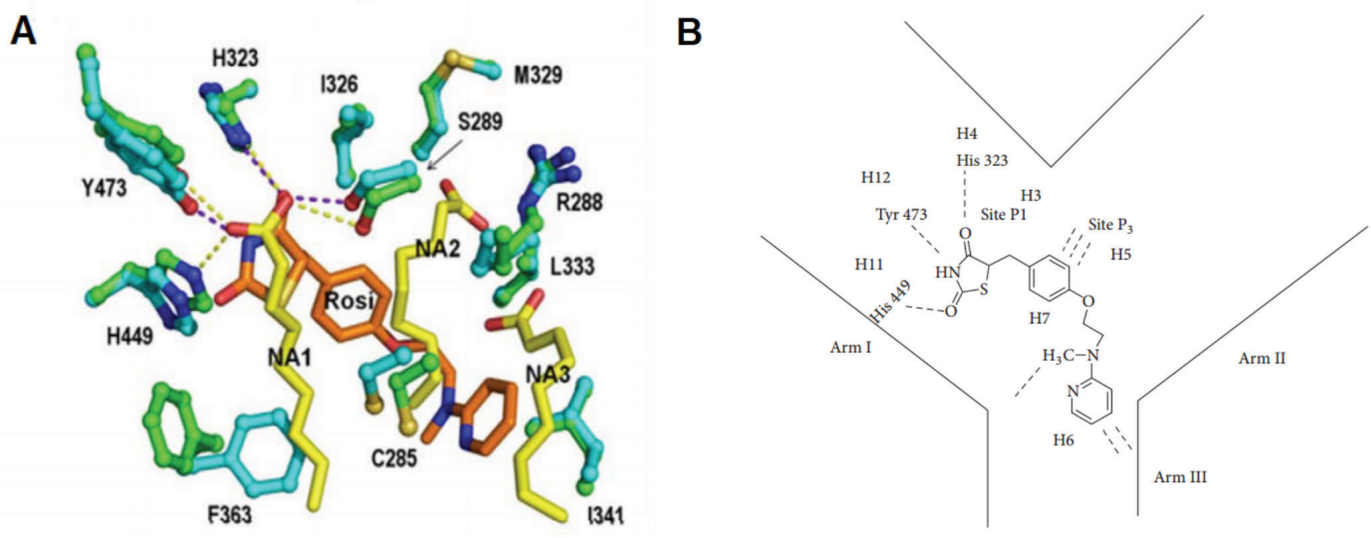


Figure 8. (A) Rosiglitazone binding mode with PPAR-γ (PPAR-γ blue, ligand transparent pink) in the presence of NA (nonanoic acid) (PPAR-γ green, ligand yellow). (B) Binding interactions of rosiglitazone with PPAR-γ revealing the three binding arms within the ligand binding domain[35,38]

PPAR: Peroxisome proliferator-activated receptor

usually represented as aliphatic/hydrocarbon chains without an electronegative atom.

Modifications on central phenyl ring

The aromatic group center plays a major role in the overall ligand activity by introducing hydrophobic interactions with LBD of PPAR-γ, especially with helix 3. With respect to all classical series of glitazones, except englitazone (4) and netoglitazone (5), shown in Figure 7, the aromatic group center is represented by a phenyl ring. With respect to the englitazone ligand (acts as a strong agonist for PPAR-γ), the central aromatic ring is represented by benzdihydropyran whilst, naphthyl moiety is used to occupies the central position for netoglitazone (agonist ligand for PPAR-α and γ). This dual-action of netoglitazone may play a role in minimizing the ligand effect on body weight, to controlling obesity, because of creating partial adipogenesis.[45,46] Thus, it is considered a potential candidate for T2DM patients, who are accompanied by high cardiovascular risk and obesity.[47] This indicates that the binding mode of glitazones is mainly influenced by modifying the central ring structure, so the ability to modify agonist properties, including potency and selectivity as well as minimize adverse effects.

Modifications on L2

L2 exists between the hydrophobic tail and the central aromatic ring. With respect to rosiglitazone (1) as displayed in Figure 7, L2 is represented by ethylene with two terminal heteroatoms, oxygen, and nitrogen that has a methyl branch. As shown, among the glitazones, it follows the SAR guide by displaying the maximum number of atoms (four carbon/hetero atoms length). Whilst, L2 in pioglitazone is represented by an ethylene group connected to just one terminal oxygen atom. The "U" geometry of the binding conformation displayed in Figure 8 for rosiglitazone indicates the vital role of L2. This linker should provide sufficient flexibility to achieve this ideal "U" shape binding conformation. Thus, the binding efficacy would be mainly influenced by chain length besides the heteroatoms that exist in the chain.

A group of conventional glitazones with diverse linkers is shown in Figure 9. This change leads to different ligands with different binding modes and outcomes. A very short L2 is introduced for ciglitazone (6) that is considered a prototype for glitazones. Compared to rosiglitazone, ciglitazone presents oxymethylene linker (-O-CH_2-) and due to its high toxicity, it has been withdrawn from the market. Similar to ciglitazone, L2 is also represented by the oxymethylene linker in troglitazone (7) and rivoglitazone (8). Their high adverse effects led to their use them.[48]

The troglitazone structure was modified into various analogs and their activities were assessed against glucose and triglyceride plasma levels. With maintaining the L2 link (-O-CH_2-) in troglitazone, L1, and the central aromatic ring were modified to an unsaturated branched linker and to a naphthyl spacer, respectively. As a result, analog 9 (Figure 9) was designed and synthesized. This analog displayed a hypoglycemic effect by decreasing glucose level, but the triglyceride level did not change.[49]

Modifying L2 and the hydrophobic tail to an amido-methylene group and trifluoromethyl phenyl ring, respectively, led to get compound 10, called KRP-297. Unfortunately, this drug displayed a carcinogenic effect and was withdrawn from

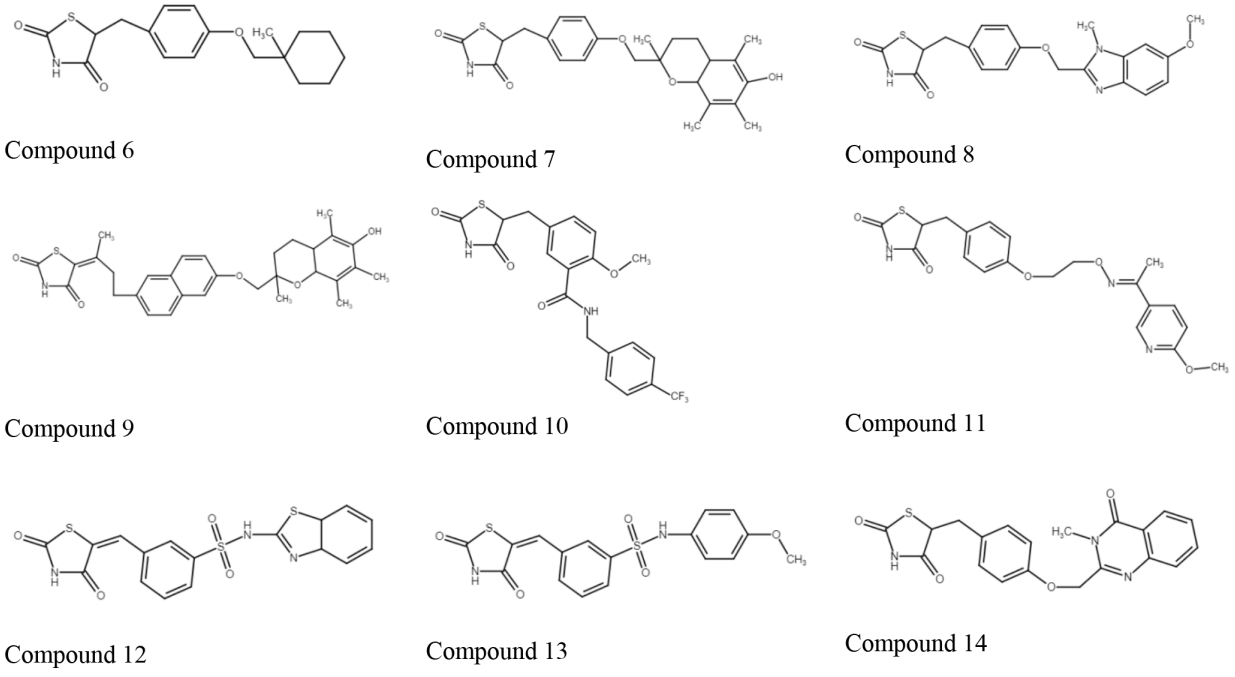


Figure 9. Compound 6, 7 and 8 with short linker, L2. Compound 9 represents troglitazone analogue. Compounds 10, 11, 12 and 13 are TZDs with modifications on L2. Compound 14 is balaglitazone[48-52,58]

TZDs: Thiazolidinediones, L2: Linker-2

therapeutic use in spite of acting as a dual agonist ligand for PPAR-α/γ.[50] Extending L2 to constitute six-carbon/hetero-atoms chain led to produce compound 11. This compound revealed good antihyperglycemic, antihyperlipidemic, and anti-obesity properties. The oxime function group exists in the L2 linker, through boosting the hydrophobic interactions with the receptor, plays an important role in binding.[51] Attempts to insert unusual moieties like sulfonyl groups into L2 linker led to finding new antidiabetic agents related to glitazones. A moderate antidiabetic agent (compound 12) was produced by introducing a benzothiazole group as a high lipophilic tail and a sulfonyl moiety to L2. This led to finding a series of glitazone derivatives bearing sulfonyl L2 that could be administered orally like compound 13. The sulfonyl moiety as the weakest acceptor is suggested to form short H-bonds with the polar residues at the entrance of LBD.[52]

Modifications on lipophilic tail

Regarding the ligand-receptor interaction, in general, extending the hydrophobic area that takes part in the reaction with the receptor moieties leads to enhanced binding efficiency and hence, improves binding affinity.[53] However, the binding affinity here is highly influenced by the desolvation phenomenon presented by the lipophilic moieties of both the ligand and receptor, during the binding process.[44,54] Thus, to decrease the desolvation effect and hence enhance the binding energy, the lipophilic binding cavities of the receptor should be matched properly with lipophilic binding groups of the ligand. Moreover, the hydration status of the receptor as an apo-form (unbound state) should be verified. These two hints are considered the key factors in docking the ligands with the receptors.[55,56] Because of proving that filling 55% of the targeted protein volume represents the optimal binding state, the size of the lipophilic moieties is critical.[57] The binding sites P3 and P4 exist in the LBD of PPAR-γ exhibit large lipophilic cavities.

It is interesting to mention that the strong agonist antidiabetic agent rosiglitazone (1) makes a lipophilic interaction only with the P3 binding site. However, the lipophilic tail group is represented by a large size pyridine ring, its molecular structure is considered small and this large ring is unable to reach the P4 binding site. In contrast, as shown in Figure 9, the lipophilic tail group regarding balaglitazone (14) is represented by a bulky moiety of benzopyrimidinone that makes a contact with both binding sites P3 and P4. This action at both lipophilic sites has a good correlation with it is partial agonist action on PPAR-γ. Representing the hydrophobic tail moiety with a fused heterocyclic or polynuclear aromatic ring would provide additional lipophilic interaction and extend the volume occupied at the binding cavity and hence, the mechanism of action, binding efficacy would be changed. Balaglitazone, developed by Dr. Reddy's labs, completed a phase III trial in Denmark, Finland and Sweden. However of exhibiting superior action compared to pioglitazone such as more potent, minor cardiac arrest, minor risk of fluid retention, and no adverse effects on bone, the clinical studies discontinued due to not demonstrating the competitive potential effect compared to similar products already marketed for T2DM.[58]

Thus, due to the beneficial therapeutic actions associated with the absence of usual adverse effects, the PPAR-γ partial agonists currently attract a lot of interest. Studies have reported that the binding interactions finally change the transcriptional outcomes because of inducing specific conformational variations. To enhance the therapeutic profiles of antidiabetic drugs like pioglitazone and rosiglitazone by diminishing their side effects but while retaining the antidiabetic and antihyperlipidemic action, the co-regulators are considered to be key factors that could be slightly modulated to fine-turn the final pharmacological action. Subjecting the present glitazones drugs for structural modifications could be a reliable strategy to achieve specific modulation of the transcriptional activity.[59,60]

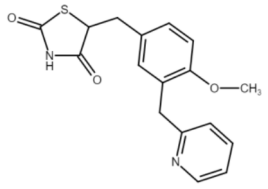
Various ring structures diverse in hydrophobicity and size could be used to identify the topology structure of glitazones. With respect to the lipophilic tail and the topology structure, the TZD analogs are classified into two main types: Conventional and non-conventional TZD. The conventional class fits into the topology of synthetic PPAR-γ and, based on the lipophilic tail, it further sub-classified into (a) pyridyl and pyrimidyl analogs that present large size rings as hydrophobic tail groups. (b) Naphthyl, styryl, diphenyloxy, and pyridyl-pyrrolidinyl analogs, the hydrophobic tails here are represented by bulky groups. And (c) miscellaneous like indolyl, pthalazinyl, quinazolinyl, quinoxalinyl, and benzpyryl (chroman) analogs. Regarding the non-conventional TZDs, that do not fit into the topology of synthetic PPAR-γ, are also sub-classified into the following: TZDs without characteristic lipophilic tail and TZDs without characteristic linker subclasses.

Conventional TZDs

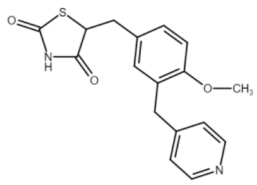
Pyridyl and pyrimidyl analog

Figure 10 shows two pyridyl analogs (compound 15 and 16) and one pyrimidyl analog (compound 17). At micromolar concentrations, the agonistic activity of the pyridyl TZD analogs was reported with enhanced potency. With respect to pyridyl TZDs, L1 and L2 are represented by simple methylene groups attached to a central phenyl ring. Despite the high structural similarity, compound 15 revealed a higher agonistic activity than compound 16. These results could be related to the stereochemistry of the pyridine ring, located at a more favorable position in compound 15 leads to better fitting in the lipophilic cavity.[61,62]

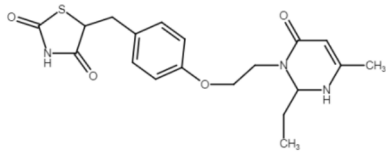
However, compound 17, a pyrimidyl TZD analog, displayed a superior clinical profile compared to the antidiabetic drugs; pioglitazone and rosiglitazone. It exhibited higher transcriptional and agonistic activity for PPAR-γ, lesser side effects, and better oral absorption.[63] It suggested that the bulky lipophilic tail for compound 17, pyrimidine ring-substituted alkyl groups plays a significant role in these superior effects. This bulky tail is expected to obey 55% rule of the volume occupied, thus presenting a better affinity.



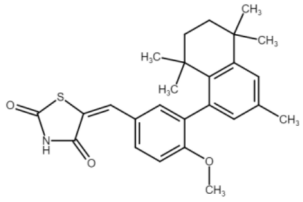
Compound 15



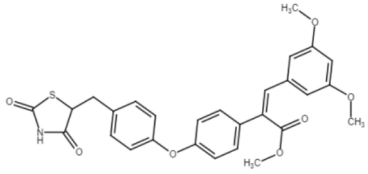
Compound 16



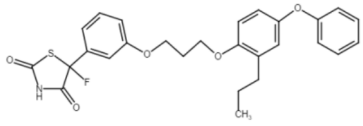
Compound 17



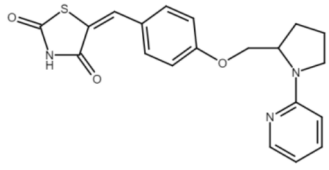
Compound 18



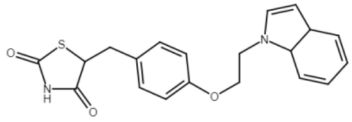
Compound 19



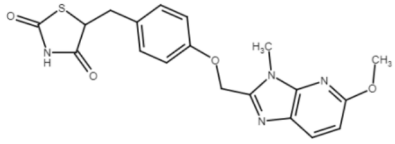
Compound 20



Compound 21



Compound 22



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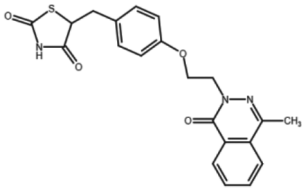

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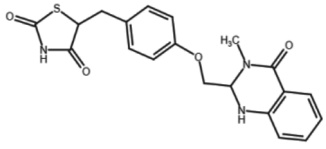

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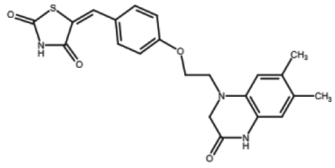
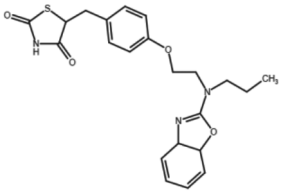
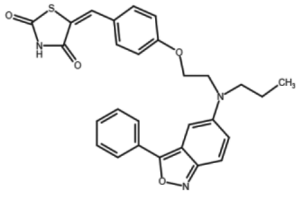


Compound 26



Compound 27



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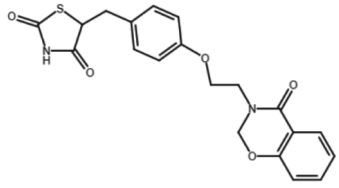
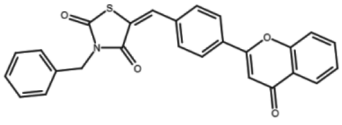
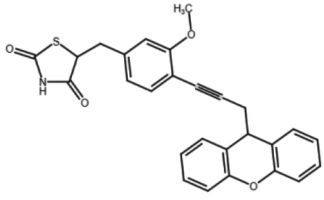


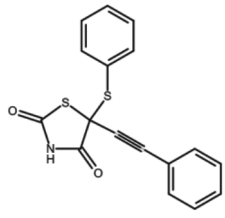
Compound 29



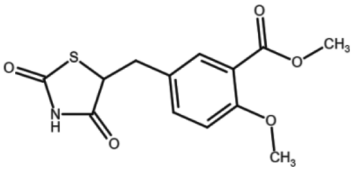
Compound 30



Compound 31



Compound 33



Compound 34

Figure 10. Examples of conventional (compound 15-31) and un-conventional (compound 33 and 34) thiazolidinediones[61-77]

Naphthyl, styryl, diphenyloxy, and pyridyl-pyrrolidinyl analogs

These analogs are characterized by the presence of bulky hydrophobic tails, some examples are shown in Figure 10. Compound 18, a tetrahydronaphthalene analog reported with a moderate hypoglycemic effect, consists of a bulky naphthyl lipophilic tail connected directly to a central phenyl ring, L1 is an unsaturated linker.[64] Because of the absence of L2 and the short length of L1, the ability of this compound to interact properly with the binding sites P3 and P4 is very minimal. Compound 19, a styryl analog, exhibited comparable PPAR-γ agonistic action associated with a better anti-diabetic effect.[65] A considerable effect on insulin sensitivity besides exhibiting dual agonistic action on PPAR-α/γ was reported for compound 20, which contains a bulky diphenyloxy lipophilic tail and an extended L2 chain of five atoms.[66] The hydrophobic tail moiety and L1 linker in compound 21 are represented by a pyrrolidine ring directly attached to the pyridine ring and an unsaturated linker, respectively. This compound displayed a better antidiabetic and antihyperlipidemic effect compared to troglitazone drug.[67]

Miscellaneous

Figure 10 displays the structures of compounds 22 and 23 representing indole and imidazopyridyl derivatives, respectively. Compound 22 exhibited an excellent profile with better insulin sensitivity if compared to the rosiglitazone drug. Also, it exhibited a superior antihyperlipidemic effect compared with troglitazone and rosiglitazone drugs by improving the HDL cholesterol levels.[68] The imidazopyridyl derivative (23) also revealed promising outcomes as a hypoglycemic agent but with less adverse effects often associated with rosiglitazone, like cardiovascular diseases.[69] The indole and imidazopyridyl rings behaved as the best lipophilic moieties to represent the lipophilic tail fraction to minimize the side effects besides improving the agonist activity for PPAR-γ. This proposes that the lipophilic indole and imidazopyridyl residues can interact and fill the lipophilic binding cavities properly; thus the transcriptional activity is specially modulated that usually leads ultimately to reducing adverse effects.

Compounds 24, 25, and 26, shown in Figure 10, represent the pthalazinyl, quinazolinyl, and quinoxalinyl analogs, respectively. Compound 26 displayed a higher PPAR-γ agonistic effect compared to compound 24 (pthalazinyl derivative). Additionally, it is approved that the presence of activating groups like methyl at positions 6 and 7 on quinoxalinyl ring, compared to analogs containing deactivating groups like the phenyl ring, showed excellent hypoglycemic and antihyperlipidemic activity. Regarding quinoxalinyl analogs, a significant antihyperlipidemic effect has been reported with the analogs containing shorter L2 lengths.

To achieve an ideal PPAR-γ agonistic activity, the length of L2 should be represented by a maximum of three atoms. Due to the steric effect, as suggested, the pthalazinyl and quinazolinyl analogs revealed less PPAR-γ agonistic activity compared to quinoxalinyl analogs. This steric effect increases improper contact within the lipophilic binding cavity.[70,71]

Assessing the hypoglycemic and antihyperlipidemic effects of molecule 27, a benzoxazolyl analog, and molecule 28 proved some activities (Figure 10). Representing the L2, in both derivatives, with *n*-propyl substituent led to getting the extreme effect.[72,73] Regarding compound 29, a benzoxazinyl analog, was reported with a dual PPAR-α/γ agonistic activity, indicating that, in designing, the ring expansion strategy could be favorable to target both receptors, PPAR-α and γ (Figure 10).

Analyzing these three series analogs from the structural viewpoint demonstrated that, the molecules reported with an optimum agonistic activity hold short linkers of two to three atoms. This optimal activity turns to a full PPAR-γ agonist if these short linkers are extended to 4 atoms length. TZD analogs with a bulky lipophilic tail like benzoxazine moiety connected to ideal L2 (three atoms) and L1 lengths would result in a potential dual agonistic activity besides lacking adverse effects.[74]

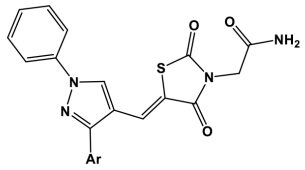
Compound 30, a benzpyryl derivative, exhibited a mild activity on elevated lipid and glucose levels. As shown in Figure 10, compound 18 lacks L2 and has a substituent on the nitrogen N-3 of the TZD ring.[75] Also, Figure 10 shows the chemical structure of compound 31 in which, the lipophilic tail is represented by a bulky dibenzpyryl ring whilst, L2 is an unsaturated chain of three atoms. This molecule exhibited an agonistic activity for all receptor isoforms, PPAR-α, -β, and -γ.[76]

Non-conventional TZDs

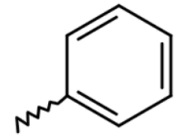
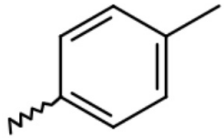
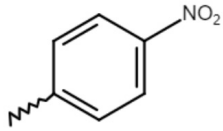
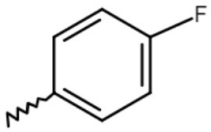
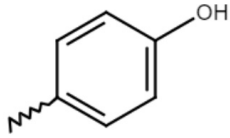
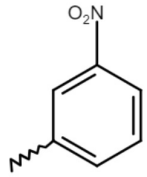
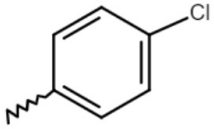
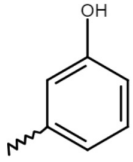
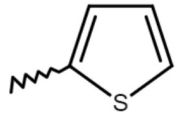
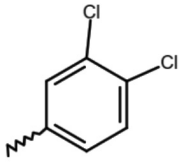
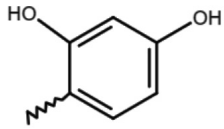
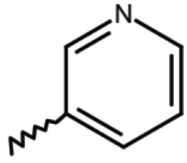
This class of TZDs represents, as mentioned before, the analogs which do not follow the classical topology of synthetic PPAR-γ. A series of non-conventional TZD-based amide analogs were designed and synthesized besides monitoring their blood glucose-lowering action and adverse effects. As shown in Figure 10, this group lacks L2, the lipophilic tail group is represented by a simple phenyl ring, and the L1 moiety is an unsaturated link of one carbon atom. A moiety of pyrazole rings connected directly to an aromatic ring applied as the central phenyl ring in which, the nature of this aromatic ring is the only difference within these analogs.

Based on the recorded data in Table 1, when the aromatic ring is a phenyl ring (32a) or 4-fluorophenyl (32b), a moderate hypoglycemic effect is obtained. Replacing the fluorine with a chlorine atom led to compound 32c that displayed the most promising results compared to other analogs (highest hypoglycemic effect). The results obtained using molecular docking of compound 32c against PPAR-γ showed excellent H-bond interactions with ILE-281 and SER-342 amino acids, with LYS-367 *via* halogen interaction and with ARG-288 *via* π-π interaction and these findings could explain the superior action of 32c. Also, this molecule elevated the gene expression of PPAR-γ by 2.1 fold accompanied by transactivation effects equals to 53.65%, which is comparable to pioglitazone and rosiglitazone with 62.21% and 86.4% transactivation, respectively. Furthermore, the molecule did not display any noticeable hepatotoxic effect or significant change in body weight. Furthermore, compounds 32i and 32l, similar to 32a and 32b, displayed moderate effects whilst, 32n and 32m

Table 1. Amide-based thiazolidinedione analogues[15]



32 a-q

Compound	Ar	Compound	Ar	Compound	Ar
	Phenyl		4-Methylphenyl		4-Nitrophenyl
32a		32g		32m	
	4-Fluorophenyl		4-Hydroxyphenyl		3-Nitrophenyl
32b		32h		32n	
	4-Clorophenyl		3-Hydroxyphenyl		Thiphene-2-yl
32c		32i		32o	
	3,4-Diclorophenyl		3,4-Dihydroxyphenyl		Pyridine-3-yl
32d		32j		32p	
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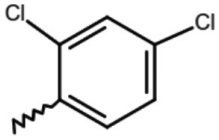
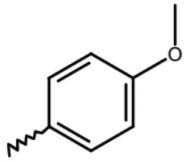
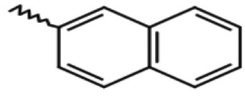
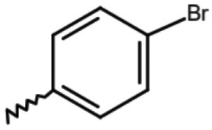
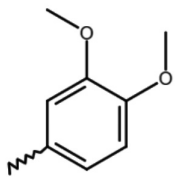
32e		32k		32q	
	4-Bromophenyl		3,4-Dimethoxyphenyl		
32f		32l			

showed significant blood glucose-lowering effects compared to reference drugs.

Evaluating hepatic protectivity of the most advantageous compounds 32c, 32e, and 32m, showed an excellent hepatoprotective effect by returning alkaline phosphatase, alanine transaminase, and aspartate transaminase enzyme levels to normal ranges. Compound 32n displayed excellent hypoglycemic action but unfortunately, reported with mild liver. The other analogs did not display any favorable hypoglycemic effect.[15]

Additionally, Figure 10 displays two non-conventional analogs that contain neither specific bulky hydrophobic tails nor characteristic linkers. Compound 33 elicited a moderate hypoglycemic effect, whilst antihyperglycemic activity was reported for compound 34.[35,77]

Hybrid TZD analogs

Based on the fact that: If two agents present independent pharmacological activity is linked covalently, a synergistic activity could be achieved. This is the concept of pharmacophore merging or hybridization. Very diverse compounds could be used to this strategy, including polypeptides, amino acids, small organic and inorganic molecules, natural ligands, and nucleic acids. Through the rational approach, using compound libraries or computational techniques in drug design, the molecules to be subjected are identified.[78] Figure 11 shows two examples of hybrid TZD analogs. Because of hybridizing a classical TZD structure contains L2, central phenyl ring, and L1 with a potent antioxidant α-lipoic acid to represent the hydrophobic tail (compound 35). This hybrid analog showed an outstanding pharmacological profile exemplified by presenting a potent PPAR-γ agonistic activity accompanied by an extreme reduction in triglyceride levels.[79] Compound 36 represents a novel hybrid analog of TZD, in which, a phenylalanine amino acid is substituted on the ring nitrogen of TZD. The polar residues existing at the head of phenylalanine create additional hydrogen bond interactions, which participate mainly in enhancing its binding efficiency. This analog was reported with a synergistic activity at nanomolar levels.[80]

The hybrid molecules, in application in T2DM, were also effective to control the skin inflammatory conditions, vascular restenosis, and obesity. Additionally, anti-inflammatory and anti-malignant activities have been reported for several other hybrid agents. Thus, for significant antidiabetic effects along with minimum cardiovascular complications, hybrid molecules are currently considered a novel class of PPAR-γ agonists.[81]

FFA Receptor 1 (FFAR1) as emerging target for TZDs

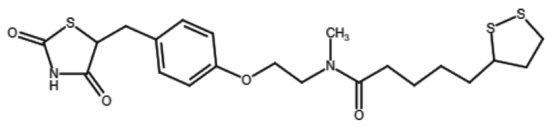
Several favorable research attempts have recently been focused on treating DM by regulating insulin secretion using FFA. It has been recently proved that the medium to long-chain FAs induce insulin release by stimulating FFAR1, also termed as G-protein coupled receptor 40. This stimulation will ultimately improve glucose-dependent secretion of insulin from pancreatic β-cells by affecting the protein kinase-C pathway besides other several signaling pathways.[82] A short to medium carbon chain length presents ten carbon atoms minimum, contains a potential free carbonyl group and unsaturated or saturated carbon is considered the proposed pharmacophore to stimulate these targets.[83]

Lately, FFAR1 has attracted attention because it has been proved to be stimulated positively by TZD hypoglycemic agents, however, the mechanism of activation.[84] Hence targeting the FFAR1 to treat T2DM currently presents a vast scope for developing TZDs as agonists/antagonists ligands. Compounds 37 and 38, shown in Figure 12, represent new TZD analogs that were reported with a dual FFAR1 and PPAR-γ agonistic activity at micromolar levels. This led to a boost in both insulin sensitivity and release. As shown, these analogs follow the conventional topology of the synthetic agonists in which, the biphenyl and benzimidazole bulky groups are used to represent the lipophilic tail of compounds 36 and 37, respectively.[85]

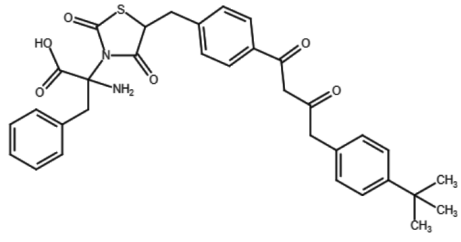
The activation of FFAR1 by the TZD analog rosiglitazone, as demonstrated by a study, led to improve PPAR-γ efficiency by regulating signal transduction.[86] Hence, this regulating effect of these dual agonistic ligands is expected to play a vital role in glucose homeostasis. This new-found concept regarding the advantageous effects of dual agonistic agents has unveiled reliable novel strategies to dominate the T2DM disease.

CONCLUSION

Studying the SAR of TZDs led to discover plentiful diverse derivatives, which mean that TZDs could tolerate the presence of diverse hydrophobic tails, central aromatic rings, and linkers that finally resulted in modifying their pharmacodynamic properties by enhancing selectivity and potency along with decreasing adverse effects. As well, some

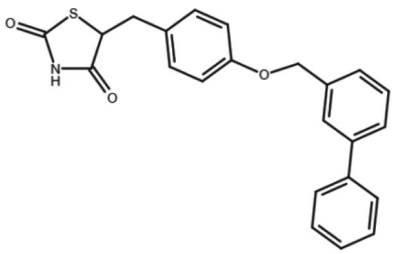


Compound 35

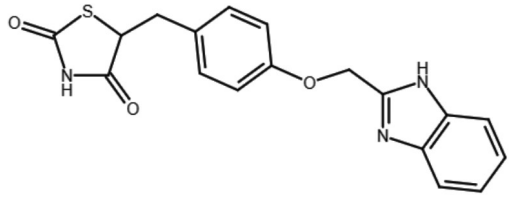


Compound 36

Figure 11. Hybrid compounds of thiazolidinediones[79,80]



Compound 37



Compound 38

Figure 12. Thiazolidinedione dual PPAR-γ and FFAR1 agonists[85]
PPAR: Peroxisome proliferator-activated receptors, FFAR: FFA receptor 1

modifications led to improve their pharmacokinetic properties such as upgrading oral bioavailability. Often, TZDs present a bulky structure that is considered substantial to create proper contact with the large lipophilic and polar cavities of the LBD of the target. Based on the previous studies, the main headlines that could summarize the SAR of TZDs as promising ligands to treat T2DM are the methylene group (one carbon atom) is suggested as the best choice to represent L1 moiety to connect TZD acidic ring with the central phenyl ring. Any advantageous effects had not been achieved in the case of the insertion of a heteroatom in this linker. Regarding the central phenyl ring, it is commonly occupied by a simple phenyl ring. A dual agonistic effect could be obtained if the phenyl ring is expanded or replaced by a naphthyl ring. The insertion of electron-withdrawing groups (Cl, F, NO_2) into central aromatic moieties was more profound in regulating glucose level toward the normal range and maximizing gene expression compared with moieties accommodating electron-withdrawing groups (CH_3, OH, OCH_3). Substituting the para position with electron releasing groups showed a more potent hypoglycemic effect than the meta position. Increasing the number of electron releasing substituents led to better performance. With respect to L2, the carbon chain between the central phenyl ring and the lipophilic tail should be at least two atoms in length. To output optimum agonistic activity, L2 chain should be represented by a maximum four atom chains that accommodates at least one heteroatom. With regard to the lipophilic tail group, the size plays a significant role in determining the efficacy of binding. In conclusion, TZDs with large lipophilic tail and short/lengthy connectors act as potent PPAR-γ agonists. Whilst, a dual PPAR-α/γ or PPAR-γ /FFAR1 agonistic activity could be achieved using a bulky hydrophobic tail and short linkers. These agonist ligands exhibit different binding modes.

Because of the structural requirements to achieve a dual PPAR-α/γ agonistic effects are still not completely identified, TZD analogs exhibiting dual activity is still rare. Further in-depth studies regarding the structure and action mechanisms of dual agonists of PPAR/FFAR1 or PPAR are required. This could be accomplished through applying advanced molecular modeling techniques.

Ethics

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: T.Ö., Design: M.T.Q., Data Collection or Processing: M.T.Q., I.A., Analysis or Interpretation: M.T.Q., I.A., T.Ö., Literature Search: M.T.Q., I.A., T.Ö., Writing: M.T.Q., I.A.

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

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