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RESEARCH ARTICLE

Synthesis of 2-Substitutedbenzimidazolium Tetrachloroplatinate(II) Compounds and Their Cytotoxic Activities on Different Cell Lines

Mahmut GÖZELLE*°, Aysun KILIÇ SÜLOĞLU**

Synthesis of 2-Substitutedbenzimidazolium Tetrachloroplatinate(II) Compounds and Their Cytotoxic Activities on Different Cell Lines

SUMMARY

The aim of the study was the synthesis of novel platinum compounds having benzimidazole ligands and screening for their in vitro cytotoxic activity on human cervical carcinoma HeLa, human lung carcinoma A549, and human lung epithelial Beas-2B cell lines. 2-Substituted benzimidazole ligands were synthesized by using appropriate aldehydes and o-phenylenediamine. Subsequently, 2-substituted benzimidazole ligands and potassium tetrachloroplatinate(II) (K_2PtCl_4) were used to synthesize 2-isopropylbenzimidazole tetrachloroplatinate(II) (K1) and 2-(1-methylpropyl)benzimidazole tetrachloroplatinate(II) monohydrate (K2). HRMS, IR, elemental analysis, ^{1}H-NMR, and melting point were used to characterize the synthesized compounds. Cytotoxic activities against HeLa, A549, and Beas-2B cells after 48 h and 72 h incubation of the platinum compounds were investigated via MTT assay. Cisplatin and carboplatin were used as reference drugs. The cytotoxic activity results showed that K2 platinum compound displayed 53.42%±2.21 (at 160 µM) on HeLa, 88.16%±0.22 (at 160 µM) on A549 and 92.09%±0.57 (at 160 µM) on Beas-2B after 48 h incubation, K2 displayed 27.42%±2.03 (at 160 µM) on HeLa, 93.95%±0.53 (at 160 µM) on A549 and 91.99±0.22 (at 160 µM) on Beas-2B after 72 h incubation. Both of the platinum compounds have higher cell inhibitory effects than reference drug carboplatin after 48 h incubation for tested cells.

Key Words: *Benzimidazole, platinum complexes, cytotoxic activity, HeLa, A549, Beas-2B.*

2-Sübstitüebenzimidazolyum Tetrakloroplatinat(II) Bileşiklerinin Sentezi ve Çeşitli Hücre Hatlarındaki Sitotoksik Etkileri

ÖZ

Bu çalışmanın amacı, benzimidazol ligandı taşıyan yeni platin bileşiklerinin sentezi ve bu bileşiklerin insan servikal karsinoma HeLa, insan akciğer karsinoma A549 ve insan sağlıklı akciğer epitel Beas-2B hücre hatları üzerindeki in vitro sitotoksik etkilerinin araştırılmasıdır. 2-Sübstitüe benzimidazol ligandları uygun aldehit türevleri ve o-fenilendiamin kullanılarak sentezlenmiştir. Ardından, 2-sübstitüe benzimidazol ligandları ve potasyum tetrakloroplatinat(II) (K_2PtCl_4) kullanılarak 2-izopropilbenzimidazol tetrakloroplatinat(II) (K1) and 2-(1-metilpropil)benzimidazol tetrakloroplatinat(II) monohidrate (K2) sentezlenmiştir. Sentezlenen bileşikler HRMS, IR, elementel analiz, ^{1}H-NMR ve erime noktası kullanılarak karakterize edilmiştir. Sentezlenen platin bileşiklerinin HeLa, A549 ve Beas-2B hücre hatlarına karşı MTT testi kullanılarak sitotoksik etkileri araştırılmıştır. Referans ilaç olarak sisplatin ve karboplatin kullanılmıştır. Sitotoksik aktivite çalışmalarında, bileşik K2'nin 160 µM konsantrasyonda 48 saatlik inkübasyonları sonrasında inhibisyon değerleri HeLa hucrelerinde %53.42±2.21, A549 hucrelerinde %88.16±0.22 ve Beas-2B hucrelerinde %92.09±0.57 bulunmuştur. Her iki platin bileşiğinin de test edilen hücrelere karşı 48 saat inkübasyon sonucunda referans ilaç olan karboplatine göre daha etkili olduğu görülmüştür.

Anahtar Kelimeler: *Benzimidazol, platin kompleksleri, sitotoksik aktivite, HeLa, A549, Beas-2B.*

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INTRODUCTION

According to World Health Organization's (WHO) report, an estimated 9.6 million people died of cancer in 2018, and cancer is the second leading cause of death worldwide (WHO, 2019). Lung, colorectal, prostate, liver, and stomach cancer are the most common types of cancer in men, although the most common types of cancers in women are breast, cervical, lung, colorectal, and thyroid cancer. Cervical cancer is the fourth most common cancer among women worldwide, accounting for 7.5 percent of all cancer deaths in women in 2018, with a reported 570.000 new cases (WHO, 2019; WHO, 2020).

After Rosenberg et al. discovered the antiproliferative effect of cisplatin (cis-diamminedichloroplatinum(II)) serendipitously (Rosenberg et al., 1965; Rosenberg et al., 1969), platinum compounds as anticancer agents received attention. Cisplatin, carboplatin, and oxaliplatin (Figure 1.), which have been marketed worldwide, have become the most effective medicines for the treatment of cancers. Platinum compounds, especially cisplatin, are used for neoadjuvant chemotherapy in cervical cancer (Biersack, 2017). DNA is the primary target of anticancer activity for platinum compounds and *in vitro* studies have shown that the N7 position of guanine is more preferable to attack over the other bases in DNA (Ghosh, 2019). Although cisplatin has a great success in the treatment of several tumor diseases, it has several side effects and restrictive effects, including ototoxicity, nephrotoxicity, myelosuppression, and neurotoxicity (Dasari & Bernard Tchounwou, 2014; Daugaard & Abildgaard, 1989; Pérez et al., 1999). Thus, therapeutic use of cisplatin is limited by especially both tumor resistance and toxicological considerations (Jamieson & Lippard, 1999). Owing to the need for platinum compounds, which have less toxicity, good water solubility, fewer side effects, and a broader spectrum of activity, several metal compounds have been synthesized and investigated for their biological effects since the discovery of cisplatin.

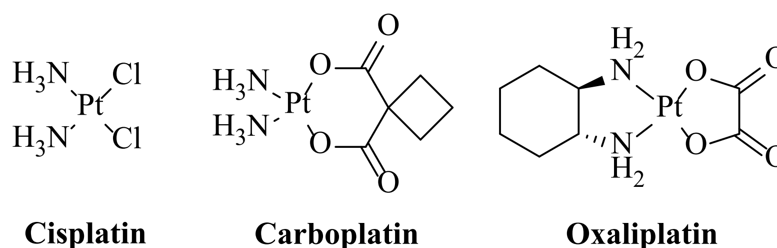

Figure 1. Structures of platinum drugs approved for clinical use worldwide.

Benzimidazole nucleus is one of the important pharmacophores in medicinal chemistry, and benzimidazole compounds have anticancer, antihistaminic, antihypertension, antibacterial, antiviral, and antifungal properties (Gaba & Mohan, 2016; Narasimhan et al., 2012). The presence of benzimidazole core can help to improve solubility. Moreover, the benzimidazole core can bind a wide range of macromolecules in biological systems due to its ability to act as a proton donor or acceptor (Beltran-Hortelano et al., 2020). Platinum compounds containing N-donor ligands such as benzimidazole show better biological efficiency with less toxicity (Al-Khathami et al., 2019; Facchetti & Rimoldi, 2019). According to literature data, benzimidazoles with substituents at the C2 position have activity a variety of cancer cell types (Gozelle et al., 2019; Nashaat et al., 2020; Doğan et al., 2021). Several platinum compounds bearing substituted benzimidazole ligands were reported previously (Zeyrek et al., 2017; Eren et al., 2018; Eren et al., 2019; Gozelle et al., 2019; Niknam et al., 2019; Özçelik et al., 2019).

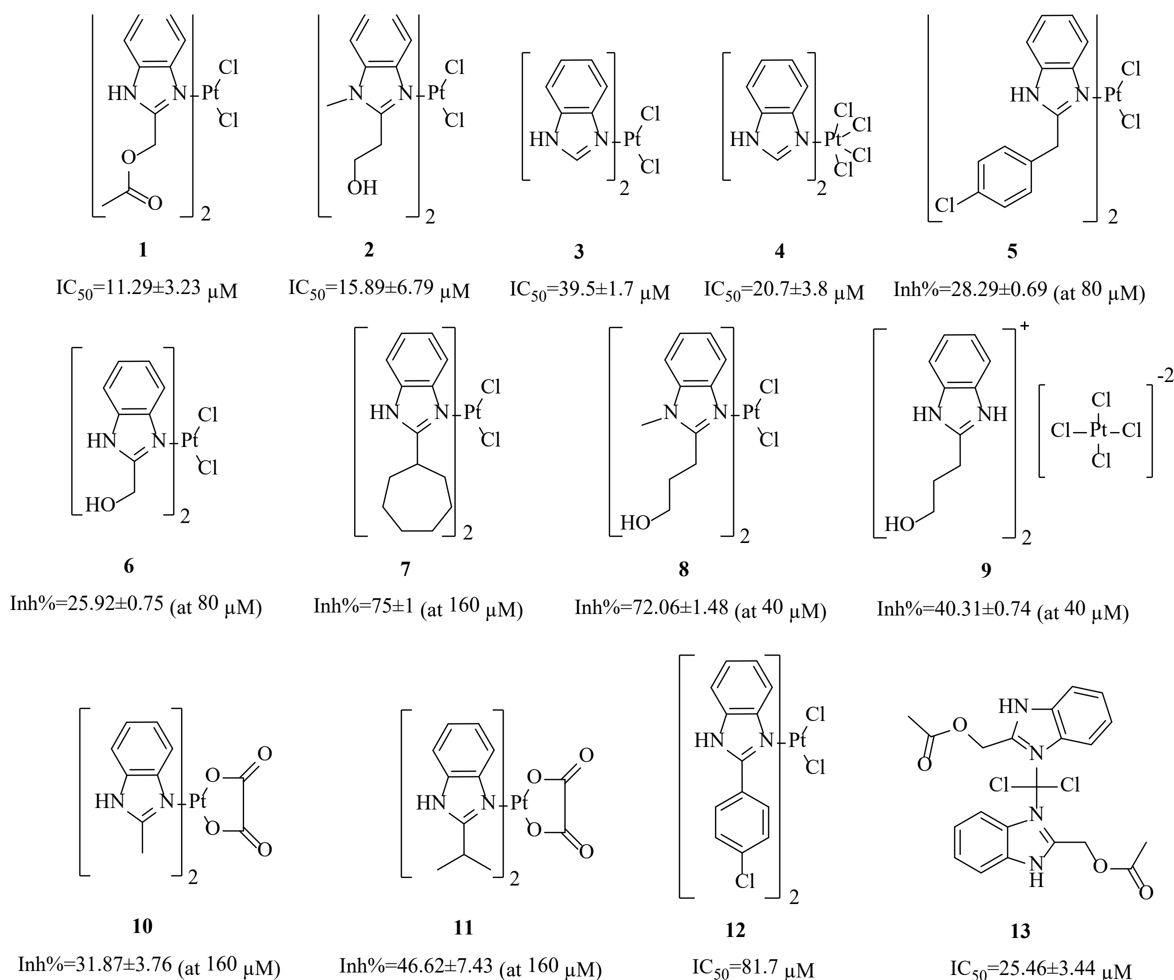

Figure 2. Platinum compounds bearing benzimidazole ligands against HeLa cell line.

In previous studies, several platinum compounds bearing substituted benzimidazole ligands against human cervical carcinoma HeLa cell line have been described, including *cis*-[dichloro-bis(2-acetoxymethylbenzimidazole)platinum(II)] (**1**), *cis*-[dichloro-bis(1-methyl-2-(2'-hydroxyethyl)benzimidazole)platinum(II)] (**2**) (Gümüş et al., 2009), *cis*-[dichloro-bis(benzimidazole)platinum(II)] (**3**), [tetrachloro-bis(benzimidazole)platinum(IV)] (**4**) (Utku et al., 2010), *cis*-[dichloro-bis(2-(4-chlorobenzyl)benzimidazole)platinum(II)] (**5**) (Özçelik et al., 2012), *cis*-[dichloro-bis(2-hydroxymethylbenzimidazole)platinum(II)] (**6**) (Utku et al., 2014), *cis*-[dichloro-bis(2-cycloheptylbenzimidazole)platinum(II)] (**7**) (Özçelik et al., 2015), *cis*-[dichloro-bis(1-methyl-2-(3' - hydroxypropyl) benzimidazole) platinum(II)] (**8**), 2 - (3' - hydroxypropyl) benzimidazolium tetrachloroplatinate (II) (**9**) (Eren et al., 2018), oxalate - bis (2-methylbenzimidazole) platinum(II) (**10**), oxalate-bis (2 - isopropylbenzimidazole)platinum(II) (**11**) (Gozelle et al., 2019), *cis*-[dichloro-bis (2 - (4 - chlorophenyl) benzimidazole) platinum(II)] (**12**) (Özçelik et al., 2019), *trans*-[dichloro-bis(2-acetoxymethylbenzimidazole)platinum(II)] (**13**) (Eren et al., 2019) (Figure 2.).

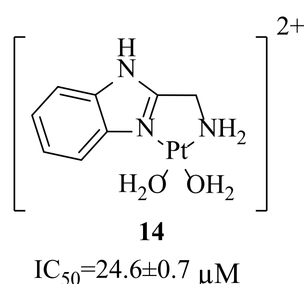

Figure 3. Platinum compound bearing 2-aminomethylbenzimidazole against A549 cell line.

Cytotoxic activity of 2 - Aminomethylbenzimidazol containing diaqua platinum(II) compound (**14**) (Figure 3.) was investigated against human lung carcinoma A549 cell line (Mitra et al., 2016).

In this study, we synthesized two novel platinum compounds with 2-isopropylbenzimidazole and 2-(1-methylpropyl)benzimidazole and then investigated their cytotoxic activities on HeLa, A549, and human lung epithelial Beas-2B cells.

MATERIAL AND METHODS

Chemistry

All chemical compounds and solvents were reagent grade and purchased locally from Merck and Sigma-Aldrich. All chemicals and solvents were used without additional purification. Pre-coated aluminum thin layer chromatography (TLC) was used to monitor the reactions. Dragendorff reagent, iodine vapor, and ultraviolet light were used for TLC visualization methods. The molecular weight of the carrier ligands was assessed by electrospray ionization mass spectrometry (ESI+) in-house on a Waters LCT Premier XE Q-TOF Mass Spectrometer, also coupled to an AQUITY Ultra Performance Liquid Chromatography system (Waters Corporation, Milford, MA, USA). The purity of the final compounds was determined to be >97% by UPLC via an ultraviolet light detector. FTIR spectra were measured on a Perkin Elmer Spectrum 400 FTIR/FTNIR spectrometer (Perkin Elmer, Inc., Waltham, MA, USA) and were reported in cm^{-1} units. Elemental analyses were performed with a LECO-932 CHNS analyzer (LECO Corporation, St. Joseph, MI, USA), and proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Varian Mercury 400 MHz Fourier-Transform (FT)-NMR spectrometer (Agilent Technologies, Palo Alto, CA, USA) by using TMS as the internal standard (Ankara University, Faculty of Pharmacy). The relative integrals of peak areas agreed with those expected for the assigned structures. All chemical shift values were expressed in ppm (δ). Melting points were recorded via an SMP-II Digital Melting Point Apparatus (Schorpp Geraetetechnik, Überlingen, Germany) and are uncorrected.

*2-Isopropylbenzimidazole (**L1**).* Synthesis and detailed structural analyses of ligand **L1** were carried out previously reported (Gozelle et al., 2019).

*R,S-2-(1-methylpropyl)benzimidazole (**L2**).* Synthesis and detailed structural analyses of ligand **L2** were carried out previously reported (Gozelle et al., 2019).

*2-Isopropylbenzimidazolium Tetrachloroplatinate(II) (**K1**).* A solution of K_2PtCl_4 (300.0 mg, 0.72 mmol) in 0.5 N HCl was added to a stirred solution of **L1** (200.0 mg, 1.25 mmol) in 0.5 N HCl dropwise over 30 min at rt. The reaction mixture was stirred at 50 °C for 11 days and protected from light. The resulting precipitate was filtered off and washed with cold water, cold ethanol, cold acetone, and diethyl ether several times before being dried in vacuo.

Infrared (IR) [(Attenuated total reflection (ATR)]: ν (cm^{-1}) 3101, 3063, 2969. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 1.47 (d, *J*=6.8 Hz, 12H), 3.44-3.53 (m, 2H), 7.55 (dd, *J*=6.0 and 3.2 Hz, 4H), 7.96 (dd, *J*=6.4 and 3.2 Hz, 4H). Anal. calcd for $C_{20}H_{26}N_4Cl_4Pt$: C 36.43; H 3.97; N 8.50; found C 36.19; H 4.18; N 8.57. mp >360 °C. Yield 20%. High-resolution mass spectrometry (HRMS) (m/z) calculated for **K1** [M+H] 161.1079, found 161.1077.

*2-(1-Methylpropyl)benzimidazolium Tetrachloroplatinate(II) Monohydrate (**K2**).* A solution of K_2PtCl_4 (300.0 mg, 0.72 mmol) in 0.5 N HCl was added to a stirred solution of **L2** (218.0 mg, 1.25 mmol) in 0.5 N HCl dropwise over 30 min at rt. The reaction mixture was stirred at 50 °C for 6 days and protected from light. The resulting precipitate was filtered off and washed with cold water, cold ethanol, cold acetone, and diethyl ether several times before being dried in vacuo.

IR (ATR): ν (cm^{-1}) 3098, 3034, 2950. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 0.88 (t, *J*= 7.4 Hz, 6H), 1.45

(d, *J*= 6.8 Hz, 6H), 1.77-1.91 (m, 4H), 3.24-3.36 (m, 2H), 7.55 (dd, *J*= 6.0 and 3.2 Hz, 4H), 7.80 (dd, *J*= 6.0 and 3.2 Hz, 4H). Anal. calcd for $C_{22}H_{30}N_4Cl_4Pt.H_2O$: C, 37.46; H, 4.57; N, 7.94; found C, 37.09; H, 4.70; N, 7.95. mp >360 °C. Yield 17%. HRMS (m/z) calculated for **K2** [M+H] 175.1235, found 175.1234.

Cell Culture

HeLa cell line was obtained from Foot and Mouth Disease Institute (Ankara, Turkey). A549 and Beas-2B cell lines were purchased from American Type Culture Collection. Cells were seeded in 25 cm^2 flasks using either Dulbecco's modified Eagle's medium (HyClone Laboratories, Inc., Logan, UT) or RPMI 1640 Medium (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Cegrogen Biotech GmbH, Germany) and 1% penicillin-streptomycin mixture and then grown for 24 h at 37°C in 5% CO_2 in a humidified incubator.

Cytotoxic Activity

Cisplatin and carboplatin were used as reference drugs. *In vitro* cytotoxic effects of these compounds were performed according to MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. $1x10^5$ cells/well were seeded in 96-well plates for MTT assay. HeLa cells were incubated for 24 h at 37°C and 5% CO_2 in a humidified incubator. Cells were exposed to drugs for 24 h, then the medium was replaced with 5 mg/mL MTT and incubated for 4 h in 5% CO_2 incubator. After 48 h and 72 h incubation, the formazan crystals were dissolved in DMSO/ammonia and the optical density at 570 nm was measured with a microplate reader (BioTek Instruments Inc., Winooski, VT). For each compound, three independent experiments were performed. Results were expressed as the mean percentage of cell growth in the drug treatment group/control group. The mean of the control group was assumed as 100% survival.

Statistical Analysis

Statistical analysis was carried out using SPSS version 20 software for Windows (SPSS Inc., Chicago, IL, USA). Data were expressed as mean±standard deviation (SD), and statistical significance was assigned at the $p \leq 0.001$ and $p \leq 0.005$ levels. Results were analyzed using one-way ANOVA, Tukey's post-hoc test.

RESULTS AND DISCUSSION

The present paper investigates the *in vitro* cytotoxic effects of 2-isopropylbenzimidazole tetrachloroplatinate(II) (**K1**) and 2-(1-methylpropyl)benzimidazole tetrachloroplatinate (II) monohydrate (**K2**).

The first phase in the synthesis step to get the platinum compounds was the synthesis of the benzimidazole ligands (**L1** and **L2**). Ligand **L1** and **L2** were synthesized according to the procedure described previously (Gozelle et al., 2019), as shown in Figure 4. The synthesized yields of benzimidazole ligands were 68% and 70%, respectively. The platinum compounds **K1** and **K2** were synthesized, as shown in Figure 5. The synthesized yield of platinum compounds was 20% and 17%, respectively.

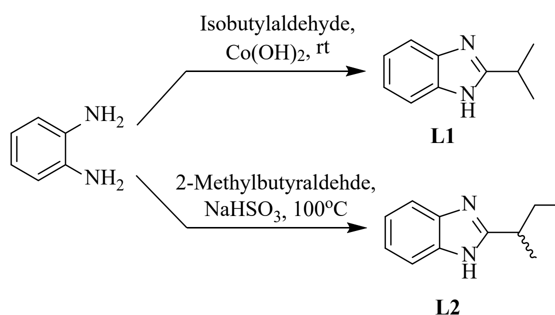

Figure 4. Synthesis of 2-substituted benzimidazole ligands.

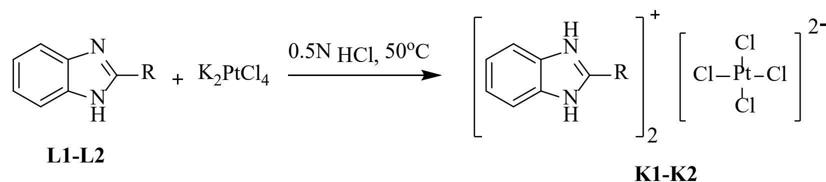

Figure 5. Synthesis of platinum compounds.

HRMS, IR, elemental analysis, ^{1}H-NMR, and melting point were used to characterize the synthesized compounds. X-ray structure of K1 was reported in our previous paper (Zeyrek et al. 2017). All characterized information proposed a 1:2 (platinum:ligand) stoichiometry for the platinum compounds. HRMS analysis of the platinum compounds **K1** and **K2** showed only m/z values of the benzimidazole ligands because **K1** and **K2** are salts that consist of a square planar tetrachloroplatinate(II) anion with hydrogen bonds to 2-substituted benzimidazoles.

Due to the platinum compounds' insolubility in the other NMR solvents, the ^{1}H-NMR spectra of the compounds were acquired by using dimethyl sulfoxide-d_6 (DMSO-d_6). It was observed that the peaks of the platinum compounds shifted towards the paramagnetic.

The cytotoxic activity of the synthesized compounds, cisplatin, and carboplatin was screened against HeLa, A549, and Beas-2B cells. Table 1. shows the inhibition values of compounds and reference drugs after 48h incubation. The inhibition percentage of cell viability on HeLa of **K1** and **K2** platinum compounds were determined as 51.43±0.80 (at 160 µM) and 53.42±2.21 (at 160 µM), respectively. The inhibition percentage of cell viability on A549 of **K1** and **K2** platinum compounds were determined as 54.96±0.47 (at 160 µM) and 88.16±0.22 (at 160 µM), respectively. Compound **K2** was determined more cytotoxic against A549 than carboplatin.

Table 1. Cytotoxic activity values expressed as inhibition % ± SD of compounds, cisplatin, and carboplatin after 48h incubation.

Comp.	µM	HeLa	A549	Beas-2B
L1	20	1.62±1.62	n.i.	n.i.
	40	2.19±1.72	18.75±5.42[a]	25.09±4.21[a]
	80	3.82±2.32	19.88±3.42[a]	27.23±3.81[a]
	160	4.93±2.67[b]	24.96±2.80[a]	29.13±4.18[a]
L2	20	n.i.	n.i.	n.i.
	40	0.65±0.28	6.68±5.48	16.20±3.75[a]
	80	1.57±0.57	16.02±2.30[a]	23.33±1.50[a]
	160	2.78±1.78[b]	19.97±6.35[a]	23.93±1.63[a]
K1	10	4.73±2.73	n.i.	4.65±1.72[a]
	20	5.44±1.50	n.i.	14.93±5.52[a]
	40	5.42±4.45	0.35±0.60	81.57±1.30[a]
	80	11.51±1.77[a]	10.01±1.59[a]	91.85±0.72[a]
	160	51.43±0.80[a]	54.96±0.47	92.11±0.39[a]
K2	10	3.67±1.81	n.i.	n.i.
	20	4.85±2.46	6.31±3.67[b]	3.14±0.63[a]
	40	4.89±2.97	17.30±3.43[a]	89.80±1.29[a]
	80	14.38±5.93[a]	37.98±0.87[a]	90.16±0.58[a]
	160	53.42±2.21[a]	88.16±0.22[a]	92.09±0.57[a]
Carboplatin	10	1.54±0.22[a]	10.13±8.53	17.03±3.74[a]
	20	2.03±0.20[a]	22.30±0.27[a]	20.43±4.18[a]
	40	4.07±0.22[a]	30.61±1.47[a]	33.97±0.32[a]
	80	8.33±0.70[a]	35.55±0.92[a]	47.28±0.26[a]
	160	14.07±0.2[a]	41.32±3.45[a]	51.87±0.85[a]
Cisplatin	5	16.1±0.52[a]	37.73±6.89[a]	39.65±5.37[a]
	10	18.81±1.10[a]	43.89±9.66[a]	62.18±3.59[a]
	20	51.33±0.58[a]	45.70±6.69[a]	84.61±0.22[a]
	40	53.68±1.15[a]	67.58±17.32[a]	89.76±0.25[a]

The results (mean±SD) of three independent experiments. a: statistically significant from the control group ($p \leq 0.01$), b: statistically significant from the control group ($p \leq 0.05$), n.i.: no inhibition.

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The cytotoxic activity of compounds and reference drugs after 72h incubation was shown in Table 2.

The cytotoxic activity results showed that **K1** displayed 29.46±5.50 (at 160 µM) on HeLa, 53.47±1.2 at 160 µM) on A549. The inhibition percentage of cell viability of **K2** was 53.47±1.20 (at 160 µM) on HeLa and 93.95±0.53 (at 160 µM) on A549. Compound **K2** was more cytotoxic against human lung carcinoma cells than carboplatin.

Table 2. Cytotoxic activity values expressed as inhibition % ± SD of compounds, cisplatin, and carboplatin after 72h incubation.

Comp.	µM	HeLa	A549	Beas-2B
L1	20	4.98±0.42[a]	n.i.	n.i.
	40	3.55±0.13[b]	16.23±2.14[a]	19.32±2.44[a]
	80	2.88±0.92[b]	16.51±2.08[a]	24.82±3.77[a]
	160	3.00±2.32[b]	19.59±0.29[a]	26.23±2.49[a]
L2	20	n.i.	n.i.	n.i.
	40	4.16±3.31	14.65±1.18[a]	n.i.
	80	4.64±3.79	15.30±1.45[a]	n.i.
	160	15.52±4.7[a]	20.34±2.32[a]	n.i.
K1	10	13.54±6.79[a]	1.87±1.68	15.45±2.60[a]
	20	10.54±1.04[b]	5.39±2.34	19.36±3.25[a]
	40	15.83±0.35[a]	9.17±3.88	87.71±1.73[a]
	80	13.67±4.66[a]	26.07±3.62[a]	95.06±0.16[a]
	160	29.46±5.50[a]	53.47±1.20[a]	95.15±0.13[a]
K2	10	n.i.	1.95±0.59	n.i.
	20	n.i.	6.97±0.87[a]	n.i.
	40	n.i.	14.34±1.49[a]	90.41±0.24[a]
	80	0.86±1.06	36.57±1.17[a]	91.38±0.57[a]
	160	27.42±2.03[a]	93.95±0.53[a]	91.99±0.22[a]
Carboplatin	10	1.08±0.53	19.63±1.73[a]	22.03±8.41[a]
	20	1.30±0.50[b]	25.61±0.17[a]	33.96±7.79[a]
	40	18.79±0.61[a]	40.49±0.95[a]	36.24±6.63[a]
	80	26.4±0.80[a]	46.68±1.22[a]	62.17±2.85[a]
	160	48.17±0.50[a]	46.73±0.10[a]	86.05±4.21[a]
Cisplatin	5	21.67±0.70[a]	42.88±2.20[a]	60.99±9.51[a]
	10	30.00±1.78[a]	48.00±0.03[a]	82.61±3.44[a]
	20	63.99±0.44[a]	58.74±1.36[a]	91.67±2.11[a]
	40	67.67±1.72[a]	87.96±1.78[a]	93.44±1.63[a]

The results (mean±SD) of three independent experiments. a: statistically significant from the control group ($p \leq 0.01$), b: statistically significant from the control group ($p \leq 0.05$), n.i.: no inhibition.

Figure 6. and Figure 7. show the inhibition percentage of compounds and reference drugs. The benzimidazole ligands (**L1** and **L2**) show no significant inhibition activity on all cell lines after 48h and 72h incubation. The cytotoxic activity of **K1** and **K2** against human cervical carcinoma after 48h incubation is higher than 72h incubation. **K1** and **K2** have no significant inhibition for HeLa cell line after 72h incubation. The cytotoxic activity of **K2** against human lung carcinoma after 72h incubation is higher than 48h incubation.

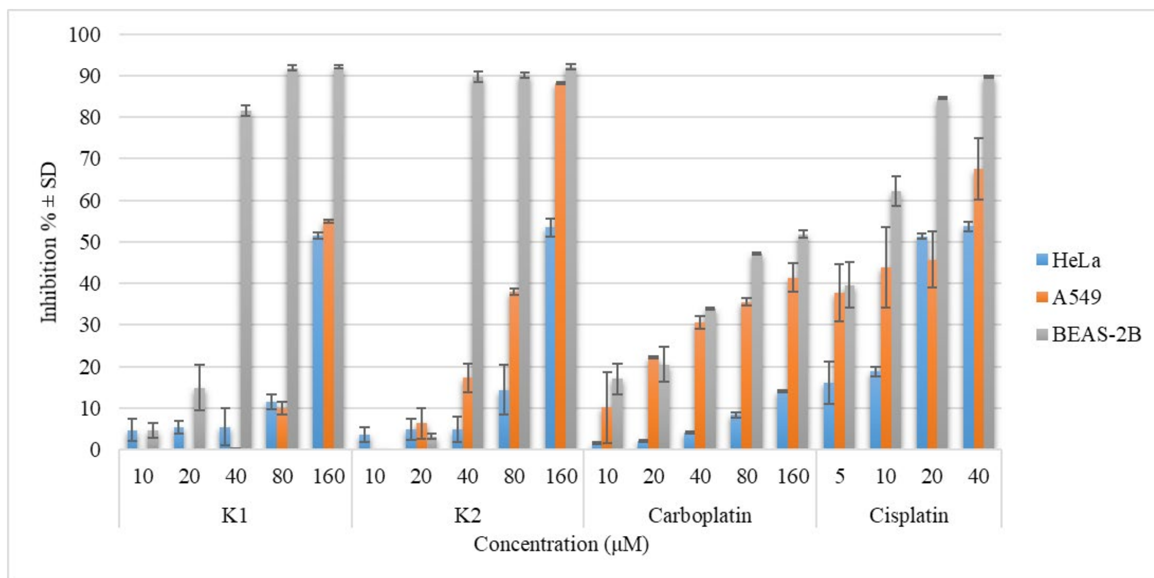

Figure 6. Inhibition percentage of cell viability on HeLa, A549, and Beas-2B cell lines treated with different concentrations of the compounds and positive controls after 48h incubation.

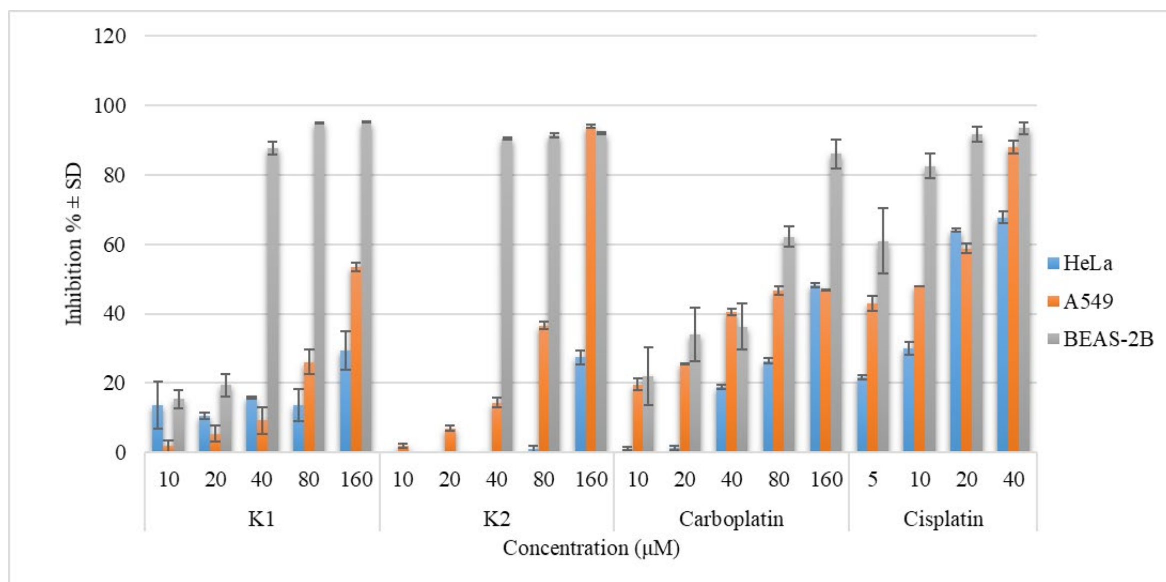

Figure 7. Inhibition percentage of cell viability on HeLa, A549, and Beas-2B cell lines treated with different concentrations of the compounds and positive controls after 72h incubation.

As shown in Table 1, the compounds being tested are arranged in an order to decrease cytotoxic effect cisplatin>**K1**≈**K2**>carboplatin on HeLa cells for 48h incubation. **K1** and **K2** have a moderate cytotoxic effect against HeLa cells for 48h incubation. The compounds being tested are arranged in an order to decrease cytotoxic effect cisplatin>**K2**>**K1**>carboplatin on A549 cells for 48h incubation. Compounds **K1** and **K2** showed similar selectivity to cisplatin against HeLa. Moreover, **K2** showed more selectivity than the other compounds against A549.

As shown in Table 2., the compounds being tested are arranged in an order to decrease cytotoxic effect

cisplatin >carboplatin>**K1**≈**K2** on human cervical carcinoma HeLa cells for 72h incubation. The compounds being tested are arranged in an order to decrease cytotoxic effect cisplatin>**K2**>**K1**>carboplatin on human lung carcinoma A549 cells for 48h incubation. Compounds **K1** and **K2** showed no selectivity against HeLa.

CONCLUSION

The main aim of this paper was to investigate the cytotoxic activities on human cervical carcinoma HeLa cells after 48h and 72h incubation of the platinum compounds. With this purpose, 2-Isopropylbenzimidazole tetrachloroplatinate(II) (**K1**) and 2-(1-methylpropyl)benzimidazole tetrachloroplatinate (II) monohydrate (**K2**) were synthesized, and elemental analysis, IR, and ^{1}H-NMR were used to characterize these compounds. Besides, the effects of these compounds on HeLa, A549, and Beas-2B cells were examined. The primary data obtained in this study lead us to conclude that the platinum compounds (**K1** and **K2**) showed higher inhibition values than carboplatin against HeLa and A549 cells after 48h incubation. Moreover, it is observed that the inhibition values of **K1** and **K2** against HeLa at 160 μM were similar to the inhibition value of cisplatin at 40 μM. The inhibition values of **K2** against A549 at 160 μM were similar to the inhibition value of cisplatin at 40 μM. Despite the fact that **K2** showed more selective than the other compounds against A549. This work highlights the cytotoxic potential on human cervical carcinoma and human lung carcinoma of platinum compounds derived from benzimidazole ligands. Those results must be taken into consideration for further studies.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTION STATEMENT

Design, synthesis, and analysis of compounds, writing the manuscript and statistical analysis: M.G., Cytotoxic activity: A.K.S., critical review: M.G., A.K.S. All authors gave final approval for publication.

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RESEARCH ARTICLE

ABC, VED, and ABC-VED Matrix Analyses for Inventory Management in Community Pharmacies: A Case Study

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ABC, VED, and ABC-VED Matrix Analyses for Inventory Management in Community Pharmacies: A Case Study

SUMMARY

Inventory control methods should be used effectively in community pharmacies to determine and obtain the needs for pharmaceuticals and non-pharmaceutical products at appropriate times and with proper procedures. An effective stock control provides positive outputs in the community pharmacy's economy, the quality of the service to be provided, and the pharmacy's image. In this context, within the scope of this study, it aims to evaluate ABC, VED, and ABC-VED matrix analyses in terms of community pharmacies.
In the study, ABC, VED, and ABC-VED matrix analyzes were applied using the inventory data of a community pharmacy serving in the city center of Van for the 2019-2020 financial year. For this purpose, firstly, annual consumption and expenditure data for each pharmacy item specified for the 2019-2020 financial year were collected. The data were then transferred to an MS Excel spreadsheet, and statistical analysis was performed using MS Excel statistical functions.
As a result of ABC-VED analysis, it was determined that the drugs in the first category were of great importance for effective stock control, the drugs in the second category were of medium importance, and the drugs in the third category were of low importance. They are because the pharmacy from which the research data is taken is close to the family health center and the socioeconomic structure of the pharmacy's environment. This categorization is thought to be appropriate.

Key Words: *ABC analysis, ABC-VED matrix, Pharmacy, Inventory control, VED analysis*

Serbest Eczanelerde Stok Yönetimi için ABC, VED ve ABC-VED Matris Analizleri: Bir Olgu Çalışması

ÖZ

Toplum eczanelerinde ilaç ve ilaç dışı ürünlere olan gereksinimlerin uygun zamanlarda ve uygun yöntemler ile tespit edilip elde edilebilmesi için stok kontrol yöntemlerinin etkin bir şekilde kullanılması gerekmektedir. Etkin bir stok kontrolü gerek serbest eczane ekonomileri açısından gerekse sunulacak hizmetin kalitesi ve eczane imajı açısından olumlu çıktılar sağlamaktadır. Bu bağlamda bu çalışma kapsamında ABC, VED ve ABC-VED matrisi analizlerinin serbest eczaneler açısından değerlendirilmesi amaçlanmıştır.
Çalışmada Van ili merkezin hizmet sunmakta olan bir serbest eczanenin 2019-2020 mali yılı için stok verisi kullanılarak ABC, VED ve ABC-VED matrisi analizlerinin uygulaması yapılmıştır. Bu doğrultuda, öncelikle 2019-2020 mali yılı için belirtilen eczanenin her bir kalemine ait yıllık tüketim ve harcama verileri toplanmıştır. Veriler daha sonra bir MS Excel elektronik tablosuna aktarılmış ve MS Excel istatiksel fonksiyonları kullanılarak istatiksel analiz gerçekleştirilmiştir.
ABC-VED analizi sonucunda birinci kategoride yer alan ilaçların etkili bir stok kontrolü için büyük öneme, ikinci kategoride yer alan ilaçların orta derecede öneme ve üçüncü kategoride yer alan ilaçların düşük derecede öneme sahip olduğu saptanmıştır. Araştırma verilerinin alındığı eczanenin aile sağlık merkezi yakınında olması ve eczanenin bulunduğu çevrenin sosyoekonomik yapısı göz önüne alındığında elde edilen bu kategorizasyonun uygun olduğu düşünülmektedir.

Anahtar Kelimeler: *ABC analizi, ABC-VED matrisi, Eczane, Stok kontrolü, VED analizi*

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INTRODUCTION

Regardless of the size of the business, its field of activity, and the type of material used or needed, each company must keep different types and amounts of inventory to continue its activities. The amount of inventory held is a critical component that impacts the economies of organizations. While the amount of inventory is less than needed, it increases the cost of being out of stock or drug shortages. At the same time, its excess can reduce capital power and increase other expenses (Abromowitz, 1984; Tengilimoğlu and Yiğit, 2017). For this reason, it is essential to manage inventory correctly. When choosing the inventory control method, organizations should evaluate the business size, production and service type, financial possibilities, machinery, and equipment status, building and warehouse capacity, information flow system, communication, registration, and personnel (Abromowitz, 1984; Tengilimoğlu & Yiğit, 2017; Fri et al., 2020).

Inventory management is one of the most important activities that affect community pharmacies' economic development. It is also essential to maintain uninterrupted healthcare services throughout the pharmaceutical supply chain (Arslan et al., 2021). It is possible to follow up questions such as when and how much to order, what level the inventory amount should be, and the expiration date of a large number of various drugs and materials in pharmacies with inventory control. Performing inventory management with appropriate methods can minimize the adverse effects on pharmacy economies against unexpected situations such as price changes, seasonal demand fluctuations, also taking advantage of bulk discounts (Yüksel & Duman, 2017; Yilmaz, 2018; Fri et al., 2020; Fahriati et al., 2021). When the literature is examined, it is seen that the methods that provide the segmentation of the inventory items are more appropriate to ensure the effective management of the pharmaceutical supplies in the healthcare sector. Mani et al. (2014), Pirankar et al. (2014), Singh et al. (2015), Gupta et al. (2019), Dora et al. (2020), Bialas et al. (2020) revealed that to reduce inventory costs in hospital pharmacies and to ensure pharmaceutical services without interruptions, inventories can be categorized according to their relative importance and can be followed more optimally. Inventory management techniques classifying items according to their relative importance are frequently used in the healthcare sector. Always, better and control (ABC), vital, essential, and desirable (VED), and ABC-VED matrix analyses are the most preferred among these methods. Nigah et al. (2010), Anand et al. (2013), Böker and Çetin (2020), Çil Koçyiğit and Doğan Çulha(2020), Gizaw and Jemal (2021) stated that the most suitable methods for inventory control in hospitals/health centers are ABC method, VED method, and ABC-VED matrix methods. Besides, the need for routine implementation of these methods is emphasized in these studies. Ceylan and Bulkan (2020) revealed that the classification of inventory items is also vital in community pharmacy. It is stated that these methods enable material resources and human resources to be used more efficiently by ensuring that the essential inventory items are checked more frequently.

This case study shows the applicability of these inventory item segmentation methods in community pharmacies and increases inventory management performance. In this context, the inventory data obtained from a community pharmacy was analyzed, and suggestions were presented in the study. It is thought that the information presented in this study will be a guide for inventory control, especially for community pharmacists and pharmacy faculty students.

MATERIAL AND METHODS

Within the scope of this study, ABC, VED, and ABC-VED matrix analyzes, which draw attention to inventory control methods, were applied in a community pharmacy serving in the city center of Van. The pharmacy is located near a Family Health Center (FHC). There are five family physicians have been operating in FHC. Also, there is no more pharmacy near the FHC.

The study collected annual consumption and expenditure data for each 1540 pharmacy items determined for the 2019-2020 financial year. Then transferred, the data to an Microsoft (MS) Excel spreadsheet, and statistical analysis was performed separately for each pharmacy item using MS Excel statistical functions.

Analysis of inventory items was carried out in three stages. After ABC analysis and VED analysis, respectively, the ABC-VED matrix was created. In ABC and VED analyzes, items were grouped by the authors and the pharmacist of the responsible manager of the concerning pharmacy.

While performing the ABC analysis, the annual expenditure of inventory items was firstly arranged in descending order. Then cumulative costs, the cumulative percentage of expenditure, and the cumulative percentage of the number of items were calculated. Then items with a cumulative percentage between 0-79.99 are assigned to group A, products between 80-94.99 are assigned to group B, and products between 95-100 are assigned to group C.

While performing VED analysis, 1540 inventory items were examined separatelyparallel to the literature and some other factors such as prescription rates of FHCs, consumer profile of the pharmacy, etc. Inventory classification was made by considering the studies using VED analysis for pharmacies in the literature (Ceylan & Bulkan, 2017; Fahriati et al., 2021), the general prescribing rates of FHCs in Turkey, and the situation regarding the relevant pharmacy. Yavuz et al. (2020) put forth nearly 28% of all prescription requests from FHCs around Turkey are for drugs prescribed for chronic illnesses, containing hypertension, dyspepsia, mood disorders, diabetes mellitus, etc. According to Güner et al. (2020), the general prescription rate of antibiotics was almost 29% in Turkey in 2017, and FHCs' rate of antibiotic prescribing in Istanbul was nearly 26%. Additionally, the Health Statistics Yearbook 2019, prepared by the Turkish Ministry of Health, states that the rate of antibiotics prescribed by family physicians in Van is around 24%. The rate of analgesics is about 40%. In addition, the rate of prescriptions, including injectable drugs, is around 7%.

In the light of the information presented above, the allergy drugs, some antidiabetics and antibiotics, antiasthmatics, antihypertensives, and some others, and medicines and medical materials must be kept in the pharmacy according to the Turkish Medicines and Medical Devices Agency were evaluated under V (Vital) group. Medicines in many pharmacological groups, such as analgesics, antibiotics, antifungals, and antiemetics, which are of moderate importance for the patient, are in the E (Essential) group. Lastly, items and medicines that are not vital for the patient, such as cosmetics, supplements, and pruritus products, are handled under the D (Desirable) group.

In the last stage of the study, inventory items classified according to ABC and VED analyses in the ABC-VED matrix were divided into three categories. The inventory items that make up the first category are items in the AV, AE, AD, BV, and CV groups. The items in this category are the group of both vital and expensive drugs in terms of cost. The inventory items that make up the second category are the BE, CE, and BD groups. The drugs in this category are of medium importance in vitality and medium significance in cost. Finally, non-vital items that make up the third category are the items in the CD group. These are at the pharmacist's initiative according to the pharmacy's characteristics. They are not expensive in terms of cost.

RESULTS AND DISCUSSION

Inventory control is essential for community pharmacies, like many businesses. Several methods can be used in community pharmacies for inventory control. When the literature is examined, it is seen that the ABC-VED matrix method is one of the most suitable methods for pharmacies among them (Ceylan and Bulkan, 2017; Fahriati et al., 2021). Previous studies have evaluated ABC, VED, and ABC-VED inventory

control methods specifically for hospital pharmacies (Bialas et al., 2020; Dora et al., 2020; Singh et al., 2015). However, the number of studies dealing with community pharmacies is quite limited. To fill this gap, in the study, the expenditure of 1540 items, amounting to 365361,5121 Turkish Liras (TL), belonging to the year 2019-2020 of X Pharmacy, which provides service in the city center of Van (one of the biggest cities in the east part of Turkey), was analyzed using ABC, VED, and ABC-VED matrix.

Firstly, the number and percentage distribution of inventory items in ABC analysis, annual expenditure amount, and annual expenditure percentage are given in Table 1. The number of items in group A is 342, the number of items in group B is 491, and group C is 707. The annual expenditure amounts of group A are 292135.43 TL, the amount of items forming group B is 54944.77 TL, and the amount of items in group C is 18281,2321 TL. Annual expenditure percentages are 79.96% for A group items, 15.04% for B group items, and 5.004% for C group items.

Table 1. Number of Items and Expenditure Amounts According to ABC Analysis

ABC	Number of items	% of items	Total annual expenditure (TL)	% annual expenditure
A	342	22.21	292135.43	79.96
B	491	31.88	54944.77	15.04
C	707	45.91	18281.2321	5.004
TOTAL	1540	100.00	365361.4321	100

According to Table 1, 22.21% of 1540 items are in group A, 31.38% in group B and 45.91% in group C. Group A has the most negligible share in terms of expenditure amount. These values are also similar to Yüksel and Duman (2017), who evaluated the drug sales data of a community pharmacy in Turkey.

The categorization is not very significant when only depending on costs. In this regard, the critical value of inventory items should also be considered to improve inventory management. Therefore, table 2 includes the inventory item information depending on the VED analysis result, which analyzes all items critically. Annual expenditure percentages are calculated as 3.64% for V group items, 90.23% for E group items, and 6.13% for D group items.

Table 2. Number of Items and Expenditure Amounts According to VED Analysis

VED	Number of items	% of items	Total annual expenditure (TL)	% annual expenditure
V	57	3.70	13306.88	3.64
E	1302	84.55	329666.7433	90.23
D	181	11.75	22387.80876	6.13
TOTAL	1540	100.00	365361.4321	100.00

The analysis findings made with the VED method were evaluated together with the total amount of items; 3.70% of the 1540 items are in the V group, 84.55% in the E group, and 11.75% in the D group. It can be said that these results show differences from the current literature in which approximately 50 percent of the products were evaluated under group E (Pund et al., 2016; Devnani et al., Gupta et al., 2007). It is thought that this difference in classification is due to the fact that the existing studies were conducted specifically for hospital pharmacies.However, according to Güner et al. (2020) and the Health Statistics Yearbook 2019, especially antibiotics are seen as essential drugs for FHCs and pharmacies around them. Considering that the prescriptions received in the relevant pharmacy mainly include antibiotics, analgesics, anti-

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allergics, and cold and gastrointestinal system diseases drugs which are categorized in the essential group, it is seen that the classification is appropriate.

After ABC and VED analyses were done separately, the ABC-VED matrix was created by cross-tabulation Table 1 and Table2. Table 3, including the subgroups that make up the ABC-VED matrix, shows the number of products in these groups and the annual expenditure amounts.

Table 3. Number of Products and Expenditure Amounts According to the ABC-VED Matrix Analysis

	ABC-VED Matrix	**A**	**B**	**C**
V	Category	**AV**	**BV**	**CV**
V	Number of Items	19	11	27
V	Annual Expenditure	11646.6	1015.98	644.16
E	Category	**AE**	**BE**	**CE**
E	Number of Items	309	428	565
E	Annual Expenditure	265858.98	48721.61	15091.67334
D	Category	**AD**	**BD**	**CD**
D	Number of Items	14	52	115
D	Annual Expenditure	14629.94	5207.17	2545.39876

After creating these groups, they were classified under three categories of their vital and material values (Table 4).

Table 4. Number of Category I-II-III Drugs and Annual Expenditure

CATEGORY	**COMBINED CATEGORY**	**Number of items**	**% of items**	**Total annual expenditure (TL)**	**% annual expenditure**
I	**AV. AE. AD. BV. CV**	**380**	**24.68**	**293795.66**	**80.41**
II	**BE. CE. BD**	**1045**	**67.86**	**69020.45334**	**18.89**
III	**CD**	**115**	**7.47**	**2545.39876**	**0.70**
TOTAL		**1540**	**100.00**	**365361.5121**	**100.00**

According to Table 4, 24.68% (380 items) are in Category I (AV, AE, AD, BV, CV). Considering the annual expenditure, this group constitutes the highest part with 80.41% (293795.66 TL). Category II (BE, CE, BD) is 67.86% (1045 products), and more than 50% of the annual items are in this group. The annual expenditure amount of the items in this group constitutes 18.89% (69020,45335 TL) of the total expenditure. The rate of items in Category III (CD) is 7.47% (115 products). The items in this group are at the lowest level, both in number and value. Considering the annual expenditure amount, it constitutes 0.7% (2545,39876 TL) of the expenditures.

When the ABC, VED, and ABC-VED matrix analyseswere evaluated, some differences were seen between studies conducted in different nations (Mani et al., 2014; Singh et al., 2015; Bialas et al., 2020). It should be noted that these differences can be about the nations' health systems, drug pricing and reimbursement systems, drug purchasing policies, the number of beds in the hospital, the status of the hospital, the health service profile provided, and the supply chain (Gizaw & Jemal, 2021; Yılmaz, 2018). From a different point of view, most of the studies in Turkey were carried out in hospital pharmacies, which is also why the results are different from national studies in general. After all, the results are similar to Ceylan and Bulkan's (2017) study conducted in a community pharmacy in Turkey. Therefore, the distributions of these categories in annual expenditure percentages have closer

values. In the study conducted by Ceylan and Bulkan (2017), the annual expenditure percentage of Category I was 75.25%, while in this study, it was 80.41%. In the study, the annual expenditure percentage of Category II is found as 22.18%, which Ceylan and Bulkan (2017) found as 18.89%. Lastly, while the annual expenditure percentage of Category III was 2.57%, it was calculated as 0.70% in this study. It is estimated that this situation resulting from the comparison is affected by factors such as the city where the pharmacy is located, location, patient profile, drug prices in the year of the study, and differences in drug purchase agreements from drug distribution channels of pharmacies.

In the light of the study findings, supplier performance evaluation should be made, especially for category I items. More sensitivity should be shown when estimating demand for items in groups A than B and C. For example, wrong planning and purchasing in group A will increase inventory costs. In this context, the correct planning of the pharmacy's needs and the consideration of equivalent products by making both pharmacological group-based and company-based evaluations during this planning will increase efficiency in inventory control. As Bialas et al. (2020) stated, significant deficiencies in applying such inventory control methods are frequently discussed theoretically in academic studies. In this context, increasing the practical application of such ways is crucial, reducing pharmacy expenses and saving money by effectively managing inventories. Therefore, the awareness and knowledge of pharmacists on this issue should be increased.

CONCLUSION

It is anticipated that inventory management performance in community pharmacies can be improved, and inventory tracking can be facilitated by using inventory control methods that target the segmentation of inventory types following the characteristics and conditions of each pharmacy. Thus, pharmacy and country resources can be used more effectively and efficiently. To increase the knowledge and awareness of pharmacists about the importance of inventory control, these issues should be given more place in both undergraduate pharmacy education and in-service training programs. The correct and regular application of the inventory control methods discussed in this study will not only provide economic benefits to pharmacies. Still, it will also be effective in continuing service-oriented activities to increase efficiency and prevent being out of stock.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

ED: Data curation, Writing, Original draft preparation, Visualization, Investigation;

MA: Conceptualization, Methodology, Software, Writing-Reviewing and Editing, Supervision.

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RESEARCH ARTICLE

Simultaneous Determination of Spiramycin and Metronidazole in Coated Tablets by Derivative and Wavelet Transforms of UV Spectra and Ratio Spectra

Doan Thanh XUAN[*°], Vu Dang HOANG[**°]

Simultaneous Determination of Spiramycin and Metronidazole in Coated Tablets by Derivative and Wavelet Transforms of UV Spectra and Ratio Spectra

SUMMARY

Signal transformation (derivative and wavelet) was applied to UV spectra and ratio spectra to directly quantify spiramycin and metronidazole in binary mixtures. Linear calibration graphs were examined for either drug in the concentration range of 6.25 – 25 mg/L with $R^2 > 0.990$. First derivative-transformed (i.e., using Savitzky-Golay filter) and wavelet-transformed (i.e., using families such as sym6, haar, db5, bior2.4, rbio2.4, meyr with scaling factor = 256) UV spectrophotometric methods were statistically comparable to the reversed phase-HPLC reference method ($p > 0.05$) with regard to accuracy and precision when assaying spiramycin and metronidazole in their coated tablets. These analytical methods used only green solvent, and proved to be time-saving and cost-effective.

Key Words: *UV spectrophotometry, derivative transform, wavelet transform, spiramycin, metronidazole, coated tablets, RP-HPLC*

UV Spektrum ve Oran Spektrumlarının Türev ve Dalgacık Dönüşümleri ile Kaplanmış Tabletlerde Spiramycin ve Metronidazolün Eşzamanlı Belirlenmesi

ÖZ

İkili karışımlarda spiramisin ve metronidazolün doğrudan kantitatif analizi için UV spektrumlarına ve oran spektrumlarına sinyal dönüşümü (türev ve dalgacık) uygulandı. Her iki ilaç için de $R^2 > 0.990$ ile kalibrasyon grafikleri 6.25 – 25 mg/L doğrusal konsantrasyon aralığnda çalışıldı. Birinci türevle dönüştürülmüş (yani, Savitzky-Golay filtresi kullanılarak) ve dalgacıkla dönüştürülmüş UV spektrofotometrik yöntemler yani skala faktörü = 256 olan sym6, haar, db5, bior2.4, rbio2.4, meyr gibi aileleri kullanarak, kaplanmış tabletlerinde spiramisin ve metronidazol test edilirken doğruluk ve kesinlik açısından RP-HPLC referans yöntemiyle ($p > 0.05$) istatistiksel olarak karşılaştırılabilir bulundu. Bu çalışmada analitik yöntemlerde yalnızca yeşil solvent kullanıldı ve yöntemlerin zamandan tasarruf ve düşük maliyetli olduğu kanıtlandı.

Anahtar Kelimeler: *UV spektrofotometrisi, türev dönüşümü, dalgacık dönüşümü, spiramisin, metronidazol, kaplanmış tabletler, RP-HPLC*

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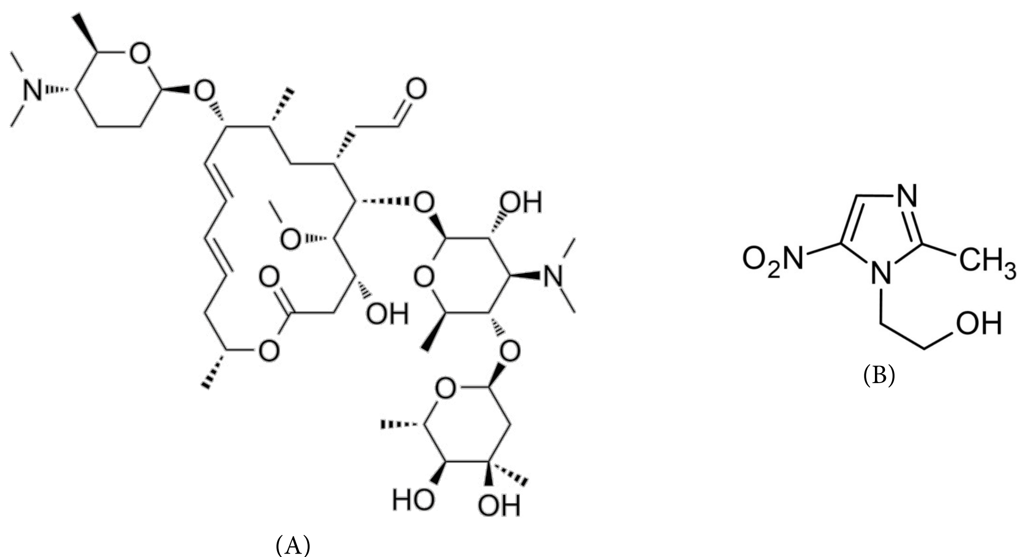
INTRODUCTION

Spiramycin (SPI) is an antiparasitic and antibiotic, first isolated in 1954 from *Streptomyces ambofaciens*. This macrolide is chemically composed of a 16-member lactone ring with the substitution of two amino sugars (mycaminose and forosamine) and one neutral sugar (mycarose) (Figure 1A). It is a mixture of three major components: I (3-OH) being the most important (accounted for at least 85%) together with II (3-*O*-acetyl) and III (3–*O*-propionyl), showing a greater activity against *Treponema* than other macrolides such as erythromycin or oleandomycin (Kwon, 2017). The conversion factor of International Unit (IU) to milligram (mg) for SPI is 10^{-3} (Drugs.com, 2022).

Metronidazole (MET) is a synthetic drug that chemically belonged to the family of nitroimidazole antibiotics. Since its development in 1959 specifically for trichomoniasis treatment, this 5-nitroimidazole medication (Figure 1B) has been still prescribed for anaerobic infections (both parasitic and bacterial) and listed among the 'essential medicines' according to the World Health Organization. This fact is very likely associated with MET's pleiotropic mode of action, separating it from most other antimicrobials i.e., it does target many molecules rather than a few or exactly a single one as it enters the cell and is reduced to its nitro group under low oxygen concentrations (Leitsch, 2017).

As early as the 1980s, the *in vitro* activity of Rodogyl (SPI-MET combined tablet) was reported against putative periodontopathic bacteria (Quee et al., 1983). It was proved that there is synergy between SPI and MET in treating polymicrobial infections (Brook, 1988). The SPI-MET combination (1500000 units/250 mg, three times a day) could mostly eliminate bacterial pathogens in the treatment and follow-up treatment of patients with active periodontitis (except for *Fusobacterium spp*.) (Poulet et al., 2005).



(4R,5S,6R,7R,9R,10R,11E,13E,16R)-10-{[(2R,5S,6R)-5-(dimethylamino)-6-methyltetrahydro-2H-pyran-2-yl]oxy}-9,16-dimethyl-5-methoxy-2-oxo-7-(2-oxoethyl)oxacyclohexadeca-11,13-dien-6-yl 3,6-dideoxy-4-O-(2,6-dideoxy-3-C-methyl-α-L-ribo-hexopyranosyl)-3-(dimethylamino)-α-D-glucopyranoside

2-(2-methyl-5-nitroimidazol-1-yl) ethanol

Figure 1. Chemical structure and IUPAC name of (A) SPI and (B) MET.

In pharmaceutical analysis, the quantification of either drug alone in their bulk, single and combined dosage forms could be done by various chromatographic methods (Akay et al., 2002; Chepkwony et al., 2001; Horie et al., 1988; Tavakoli et al., 2007), NMR spectroscopic methods (Salem & Mossa, 2012; Salem et al., 2006), Visible light (VIS) and Near Infrared (NIR) spectrophotometric methods (Saffaj et al., 2004; Saffaj et al., 2006; Abdel-Kader & Hashem, 2021; Sakira et al., 2021), and electrochemical methods (Li & Xu, 2014). It was also reported that MET could be co-assayed in multicomponent mixtures by using derivative and chemometrics-assisted UV spectrophotometry (Erk & Altun, 2001; Mahrouse & Elkady, 2011; Elkhoudary et al., 2014; Korany et al., 2015; Attia et al., 2016), direct and ratio-subtraction UV spectrophotometry (El-Ghobashy & Abo-Talib, 2010), kinetic spectrophotometric H-point standard addition method (Issa et al., 2013). To the best of our knowledge, there has not been any pharmacopoeial monograph for SPI-MET combined tablets yet. In the literature, the co-assay of SPI and MET in pharmaceutical mixtures was reliably performed by HPTLC and/or RP-HPLC (both isocratic and gradient) (Maher & Youssef, 2009; Elkhoudary et al., 2016). In 2010, Khattab and co-workers used different UV spectrophotometric techniques to determine SPI and MET in bulk and tablets (Khattab et al., 2010). Their study, nonetheless, has some limitations, such as (i) using methanol as the spectrophotometric solvent that is environmentally unfriendly and (ii) impossibility to determine SPI and MET simultaneously by zero-crossing point technique.

This study was undertaken to develop and validate UV spectrophotometric methods for simultaneous determination of SPI and MET in coated tablets by using both signal transform algorithms: derivative and wavelet. It proves to be better than the above-mentioned study by Khattab and co-workers with regard to (i) the use of a much less toxic solvent (ethanol) (Hansen, 2020) and (ii) the outperformance of wavelet transform over derivative transform in resolving UV spectral overlaps of binary mixtures as pointed out by Dinç and co-workers in reviews (Dinç, 2013, Dinç & Yazan, 2018) and experimental studies (e.g., Dinç et al., 2005, Üstündağ, Ö., & Dinç, E., 2021).

MATERIALS AND METHODS

Apparatus and software

A double-beam UV-1800 spectrophotometer (Shimadzu, Japan) equipped with 1-cm pairs of quartz cuvette was employed. Absorption spectra were registered in the range of 200 - 400 nm with a 0.1-nm fixed slit width, slow scanning speed and 0.1-nm sampling interval. Spectral manipulation (i.e., arithmetic, transformation such as smoothing and derivative) was performed using built-in UV Probe software. For wavelet transform, the spectral processing was done using MATLAB R2020a software (MathWorks, Natick, MA, USA).

RP-HPLC analysis was performed using an Agilent 1200 Series Diode-Array-Detector chromatograph (Agilent Technologies, USA). The chromatographic operating conditions were used exactly as proposed by Elkhoudary et al. (Elkhoudary et al., 2016).

Reagents and standard solutions

Chemical reference standards (HPLC purity): SPI (97%) and MET (99.65%) were provided from LGC Standards – UK. All chemicals and solvents were of analytical grade. Stock solutions of SPI and MET (1250 mg/L) were freshly prepared in ethanol. These solutions were appropriately diluted with ethanol to make a set of standard solutions in 25-mL volumetric flasks.

Sample solutions

Four coated tablet formulations (containing SPI 750000 IU + MET 125 mg) were purchased at local retail pharmacies i.e., Rodogyl (Sanofi Aventis, France), Novogyl (Mekophar, Vietnam), Arme-Rogyl (Armephaco, Vietnam), and Zolgyl (Bidiphar, Vietnam). For each formulation, twenty tablets were carefully peeled off the film coating using a very thin blade, then accurately weighed and finely pulverized in a mortar.

An accurate quantity equivalent to one-half of a tablet was dissolved in about 70 mL of ethanol in a 100-mL volumetric flask by 15-min sonication that was filled subsequently to the calibration mark with the same solvent. Further dilution in 25-mL volumetric flasks was required to obtain the test solutions i.e., SPI 12.5 mg/L + MET 6.25 mg/L (for SPI assay) and MET 12.5 mg/L + SPI 25 mg/L (for MET assay). Unless stated otherwise, only Rodogyl's transformed UV spectra and ratio spectra were displayed as "sample" in all Figures to demonstrate the applicability of our UV spectrophotometric methods.

RESULTS AND DISCUSSION

Figure 2 displays the zero-order UV spectra of SPI, MET and their corresponding mixtures at working concentrations, showing that SPI and MET have broad absorption bands peaked at 226.6 and 308.8 nm, respectively. This observation could be attributable to the electronic transition n → π*, provided that the drugs under study are constituted of the chromophores (i.e., 16-member lactone ring and carbonyl group in SPI, and 5-nitroimidazole structure in MET) and auxochromes (i.e., methyl, hydroxyl, and amino groups). In comparison with the data published by Khattab et al (Khattab et al., 2010) (i.e., SPI and MET peaked at ca. 232 and 311 nm in methanol, respectively), the hypsochromic shift for both drugs was noted under our spectrophotometric conditions. It is probably ascribed to the broadening of n → π* electronic transition in ethanol, which is a hydrogen bond donor stronger than methanol.

It is clear to indicate that (i) the obedience of the law of spectral additivity was reasonably justified in the range 200 – 400 nm, and (ii) the assay of SPI in binary mixtures was hindered since MET absorbed UV radiation markedly in the SPI spectral peak region. Although a direct determination of MET in the binary mixture could be done in the range of 280 – 360 nm as suggested by Khattab et al. (Khattab et al., 2010), it is still advisable to apply signal processing techniques (such as derivative and wavelet transforms) for MET quantification. This is because such signal transformation may effectively eliminate baseline drift as well as unwanted spectral absorption of the sample matrix. Thus, in our study, signal transformation was applied to both UV spectra and ratio spectra for the assay of SPI and MET in binary mixtures.

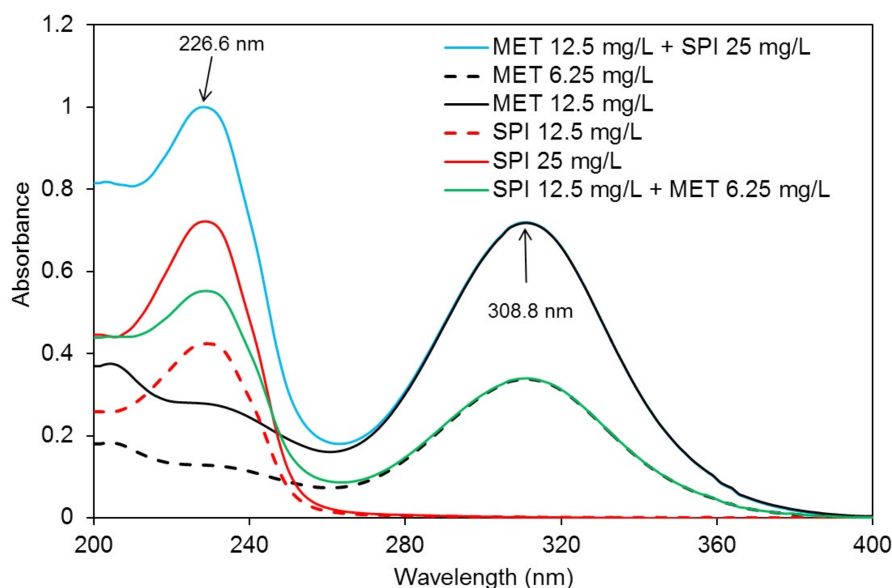


Figure 2. UV absorption spectra of SPI, MET and corresponding SPI-MET mixtures at working concentrations

By using the Savitzky-Golay smoothing and differentiation filter (Savitzky & Golay, 1964), the first-derivative transform is based on the convolution operation exquisitely fitting pieces of 17 data points inclusive of former and later data to a polynomial using least-squares regression. This signal processing was optimally implemented with $\Delta\lambda$ = 4 nm. In Figure 3 A, the first-derivative spectra of SPI and MET exhibited zero-crossing points at 289.8 and 259.8 nm, at which the amplitude of MET and SPI signals was not annulled accordingly. However, the assay of SPI is not feasible herein because a good correlation is not secured for a linear relationship between its derivative response and concentration (Table 1). Figures 3 B and

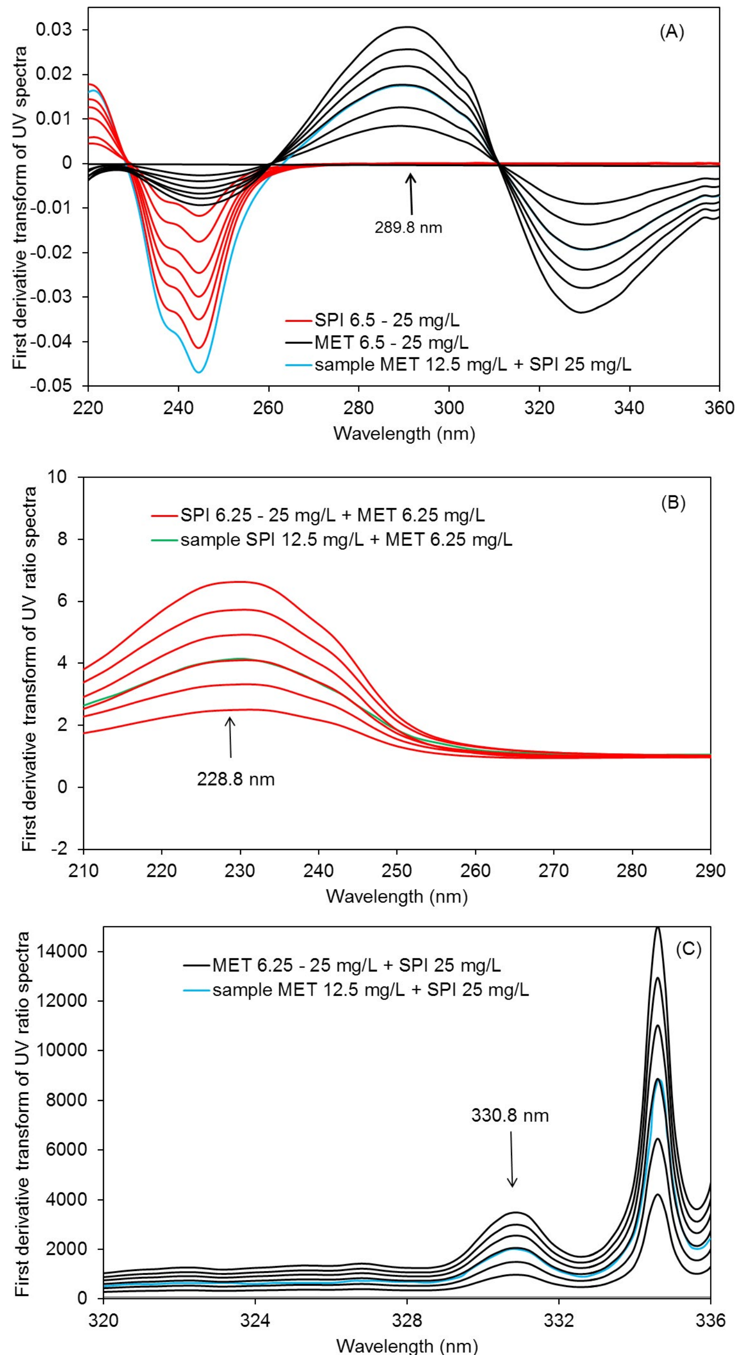
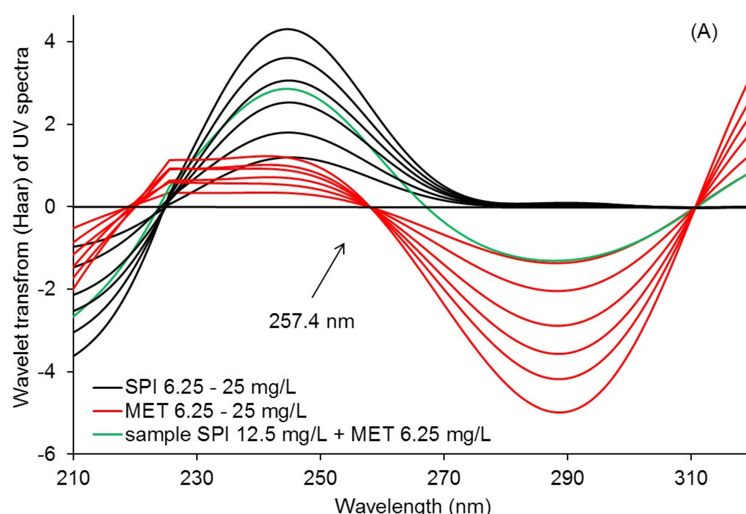


Figure 3: First-order derivatives of UV spectra (A) and UV ratio spectra (B and C)

C present the first-order derivatives of ratio spectra with suitable divisor standard concentrations found to be SPI 25 mg/L (for MET assay) and MET 6.25 mg/L (for SPI assay). The working wavelengths 228.8 and 330.8 nm were subsequently selected to determine SPI and MET, respectively, for their derivative amplitudes were the highest and directly proportional to the concentration ranges under investigation of SPI and MET (6.25 – 25 mg/L).

In the wavelet transform procedure, a signal is decomposed into a set of basic functions (a.k.a. wavelets) by dilating and translating a prototype mother function Ψ(t) (Kaiser, 2011). Spectral data, explicitly speaking, are subjected to mathematical processing for conversion into various coefficients, and each coefficient is then analyzed at a resolution matched to its scale. It means that concerning a wavelet expansion, the representation of spectra could be realized by using coefficients in a linear combination of the wavelet functions. In this study, different wavelet families such as continuous (i.e., Symlets, Coiflets, Mexican hat function, Meyer, Dmeyer, Gaussian, BioSplines, ReverseBio, Morlet) and discrete (Haar, Daubechies) were scrutinized at different dilation parameters (a) to separate overlapping spectral bands of SPI and MET in their binary mixtures. It was shown that the best spectral recovery values were obtained with $a = 256$. Figures 4 and 5 selectively present the wavelet-transformed UV spectra and ratio spectra, respectively. It is essential to indicate that SPI and MET could be simultaneously determined at 287.6 and 300.4 nm, respectively, by using rbio2.4-based transformed UV spectra. Moreover, the wavelet transform-based amplitudes were higher than the corresponding derivative ones, as expected. This is always true, in particular, for the assay of SPI using signal transformation of UV ratio spectra.

The suitability of our spectrophotometric assay was examined by repeating the absorbance measurement of SPI and MET standard solutions at working concentrations (RSD < 1%, n = 6). The developed spectrophotometric methods were validated in conformity with ICH guidelines for some criteria such as linearity, accuracy, within-run precision (repeatability), and ruggedness (intermediate precision) (ICH Harmonised Tripartite Guideline, 2005). Calibration curves were established for SPI and MET at the working wavelengths with $R^2 > 0.990$ for six standard points in the concentration range of 6.25 - 25 mg/L, suggesting a good relation between two variables: concentration – signal amplitude. Based on the statistical analysis of calibration curve data, LOD and LOQ values (expressed in mg/L) for all the UV spectrophotometric methods proposed were also calculated (Table 1).



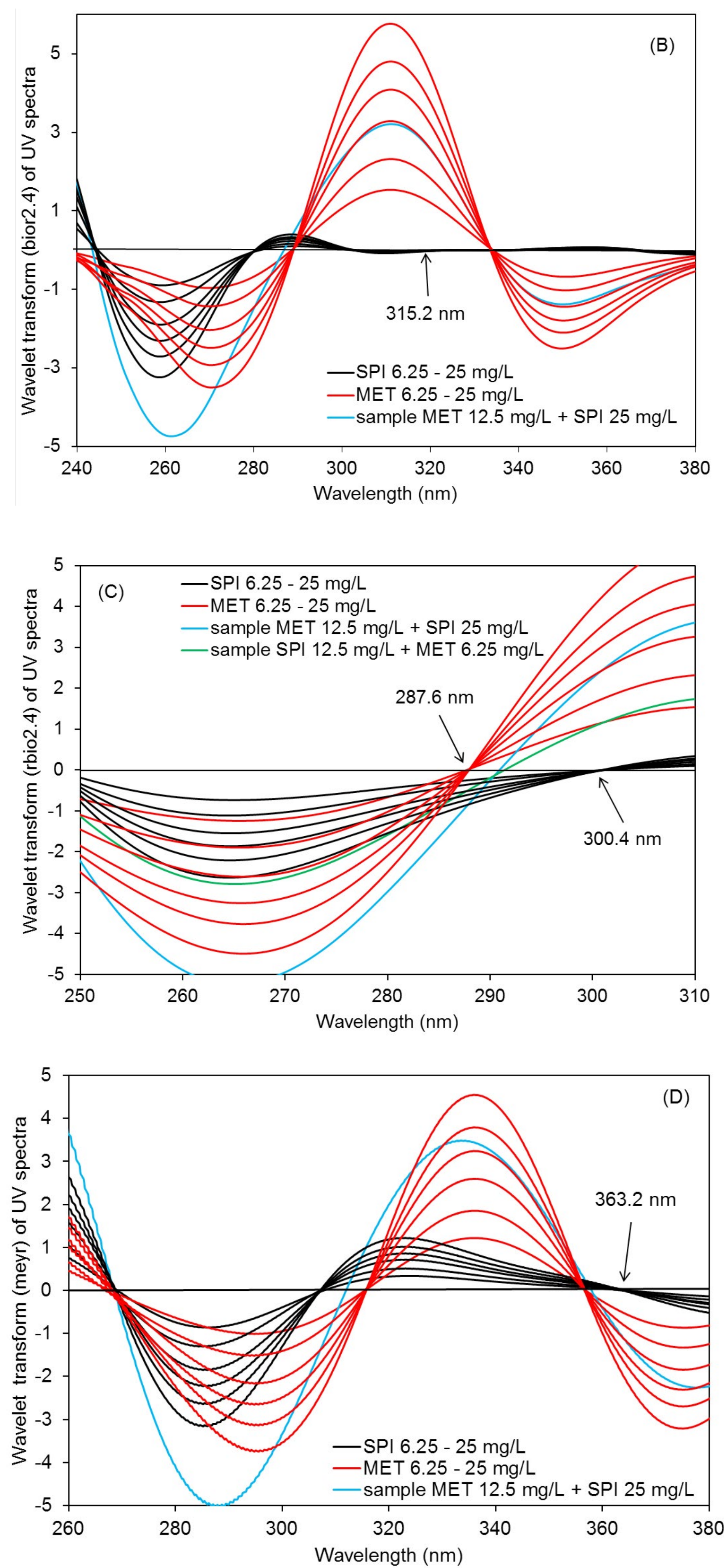
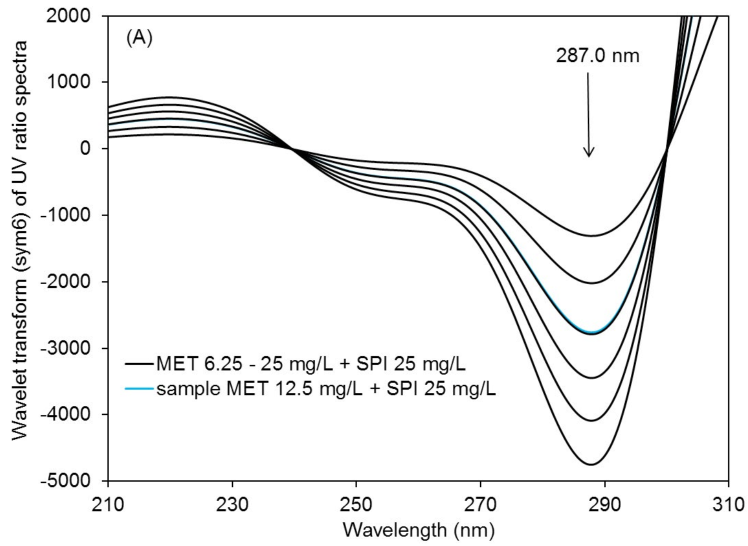
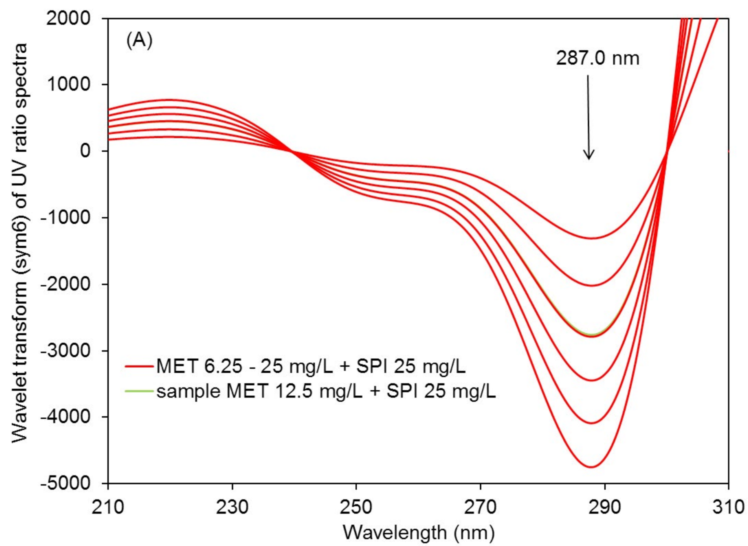
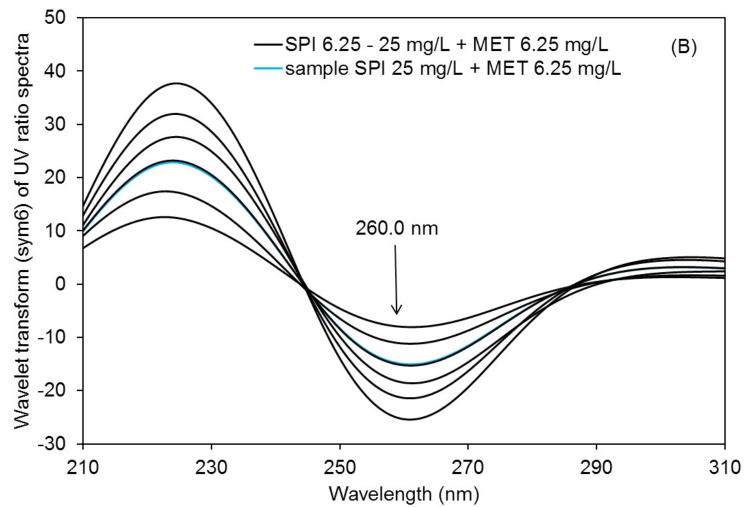
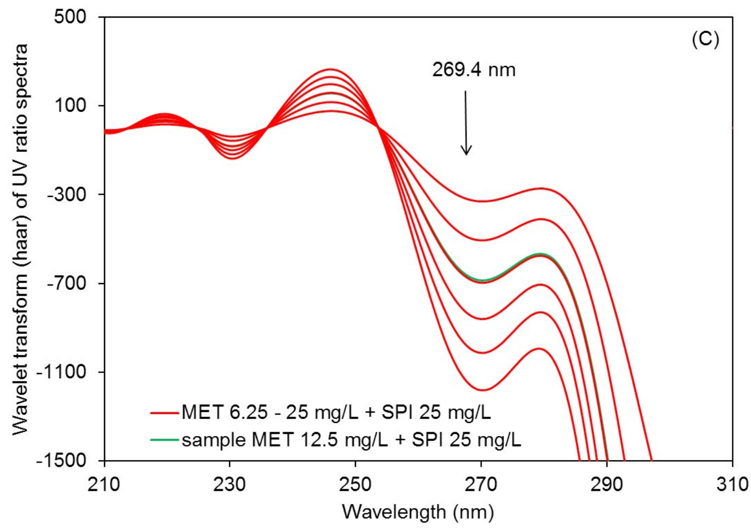


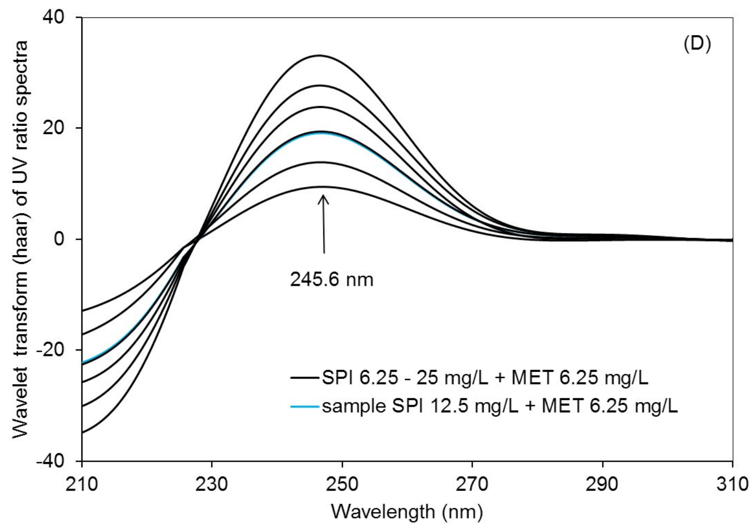
Figure 4. Wavelet transform of UV spectra: (A) haar, (B) bior2.4, (C) rbio2.4, (D) meyr

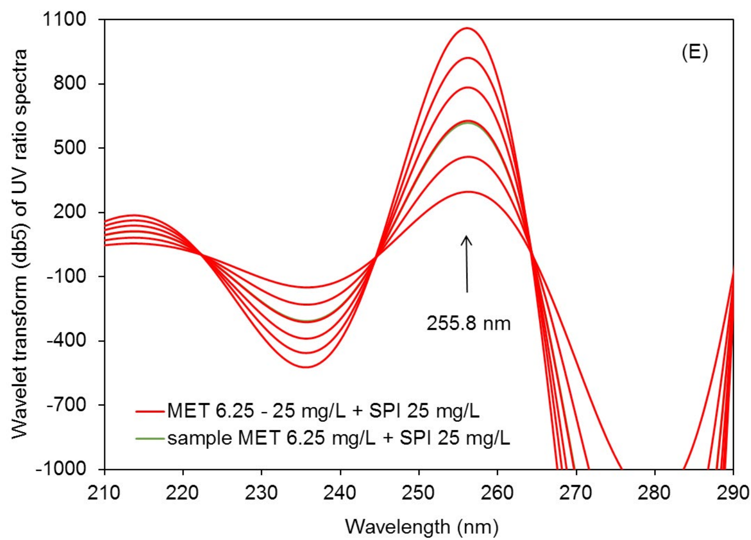

(A)
287.0 nm
MET 6.25 - 25 mg/L + SPI 25 mg/L
sample MET 12.5 mg/L + SPI 25 mg/L
Wavelet transform (sym6) of UV ratio spectra
Wavelength (nm)


(A)
287.0 nm
MET 6.25 - 25 mg/L + SPI 25 mg/L
sample MET 12.5 mg/L + SPI 25 mg/L
Wavelet transform (sym6) of UV ratio spectra
Wavelength (nm)


SPI 6.25 - 25 mg/L + MET 6.25 mg/L
sample SPI 25 mg/L + MET 6.25 mg/L
(B)
260.0 nm
Wavelet transform (sym6) of UV ratio spectra
Wavelength (nm)


(C)
269.4 nm
Wavelet transform (haar) of UV ratio spectra
Wavelength (nm)
MET 6.25 - 25 mg/L + SPI 25 mg/L
sample MET 12.5 mg/L + SPI 25 mg/L


(D)
245.6 nm
Wavelet transform (haar) of UV ratio spectra
Wavelength (nm)
SPI 6.25 - 25 mg/L + MET 6.25 mg/L
sample SPI 12.5 mg/L + MET 6.25 mg/L


(E)
255.8 nm
Wavelet transform (db5) of UV ratio spectra
Wavelength (nm)
MET 6.25 - 25 mg/L + SPI 25 mg/L
sample MET 6.25 mg/L + SPI 25 mg/L

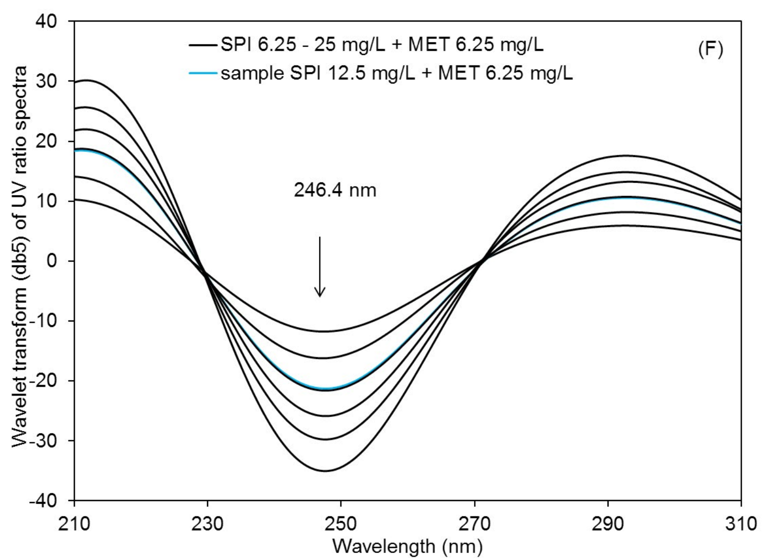


Figure 4. Wavelet transform of UV ratio spectra: (A)-(B) sym6, (C)-(D) haar, (E)-(F) db5

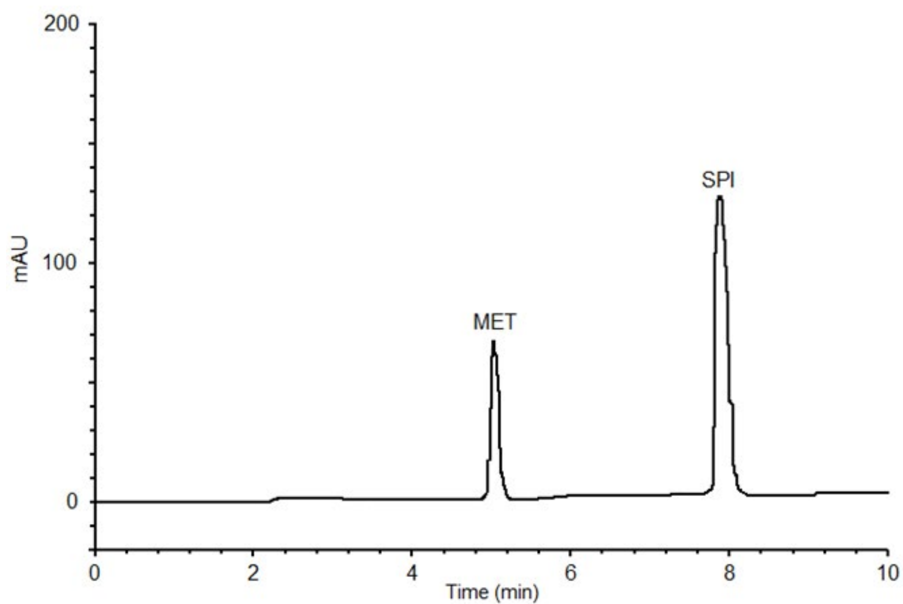


Figure 5. A typical HPLC chromatogram of the mixture of MET 6.25 mg/L + SPI 12.5 mg/L

Table 1. Statistical analysis of 6-point calibration graphs of the proposed spectrophotometric methods (6.25 –25 mg/L)

Method	Compound	Wavelength (nm)	a (×10⁴)	b (×10⁴)	Sa (×10⁴)	Sb (×10⁴)	Sy.x (×10⁴)	R^2	LOD	LOQ
Derivative transform										
UV spectra	MET	289.8	12.68	1.050	0.189	3.09	2.74	0.9991	0.71	2.16
UV ratio spectra	SPI	228.8	2370	923.2	33.2	542	481	0.9992	0.67	2.03
	MET	330.8	1449336	582381	28240	460039	408274	0.9984	0.93	2.82
Wavelet transform										
UV spectra										
haar	SPI	257.4	1047	747.6	18.3	297	264	0.9987	0.83	2.52
bior2.4	MET	315.2	2304	– 474.5	23.2	377	335	0.9995	0.48	1.45
rbio2.4	SPI	287.6	– 353.3	– 240.4	8.68	141	126	0.9975	1.17	3.57
	MET	300.4	1705	84.55	17.1	278	246	0.9995	0.48	1.44
meyr	MET	363.2	– 772.6	309.6	6.05	98.6	87.5	0.9997	0.37	1.13
UV ratio spectra										
sym6	SPI	260.0	– 9969	– 14764	128	2084	1849	0.9993	0.61	1.85
	MET	287.0	– 1984345	– 441943	36668	597338	530142	0.9986	0.88	2.67
haar	SPI	245.6	13567	5720	164	2670	2370	0.9994	0.57	1.74
	MET	269.4	– 489942	– 189578	8623	140473	124667	0.9987	0.84	2.54
db5	SPI	246.4	– 13261	– 31312	142	2320	2058	0.9995	0.51	1.55
	MET	255.8	442718	173415	10983	178923	158790	0.9975	1.18	3.59

Y = aC + b; where C is concentration in mg/L and Y is signal amplitude in arbitrary unit.
a: slope; b: intercept; Sa: SD of the slope; Sb: SD of the intercept; Sy.x: SD of the residuals; R^2: coefficient of determination;
LOD = 3.3×Sy.x/a; LOQ =10×Sy.x/a

Table 2. Assay results for SPI and MET in coated tablets

% of label claim (mean ± SD, = 6)								
Method	Rodogyl		Novogyl		Arme-Rogyl		Zolgyl	
	SPI	MET	SPI	MET	SPI	MET	SPI	MET
RP-HPLC	100.1 ± 1.2	99.5 ± 1.3	100.4 ± 1.0	100.2 ± 1.1	99.9 ± 0.9	99.3 ± 1.2	100.8 ± 1.4	100.9 ± 1.5
Derivative transform								
UV spectra	-	100.2 ± 1.5	-	99.5 ± 1.7	-	99.0 ± 1.8	-	101.4 ± 2.1
UV ratio spectra	99.8 ± 1.5	100.1 ± 1.7	100.8 ± 1.4	99.8 ± 1.8	100.3 ± 1.5	99.3 ± 1.8	100.1 ± 1.8	101.4 ± 1.9
Wavelet transform								
UV spectra								
haar	100.0 ± 1.4	-	101.0 ± 1.6	-	99.9 ± 1.8	-	100.3 ± 1.8	-
bior2.4	-	99.9 ± 1.7	-	99.9 ± 1.6	-	99.6 ± 1.9	-	101.0 ± 2.1
rbio2.4	100.3 ± 1.3	99.6 ± 1.6	100.7 ± 1.8	100.0 ± 1.8	100.1 ± 1.9	99.9 ± 2.0	100.5 ± 2.0	100.9 ± 1.9
meyr	-	100.2 ± 1.8	-	99.7 ± 1.7	-	99.8 ± 1.7	-	101.5 ± 1.9
UV ratio spectra								
sym6	99.7 ± 1.6	100.3 ± 1.6	100.5 ± 1.7	100.5 ± 1.8	100.3 ± 1.9	99.5 ± 2.1	100.5 ± 1.9	101.0 ± 2.2
haar	99.9 ± 1.4	99.9 ± 1.5	100.6 ± 1.8	100.2 ± 1.9	99.8 ± 1.7	99.7 ± 1.9	100.8 ± 1.7	100.7 ± 1.8
db5	100.2 ± 1.6	99.8 ± 1.5	100.8 ± 1.9	99.9 ± 1.8	99.9 ± 1.8	99.9 ± 1.9	100.1 ± 2.1	101.7 ± 2.0

Table 3. Evaluation of one-way ANOVA and Bartlett tests at the significance level 5% for assay results

			One-way ANOVA test		
Source of variation	Compound		Between-groups	Within-groups	Total
Sum of squares	SPI	I	1.680	72.10	73.78
		II	1.491	92.50	93.99
		III	1.526	98.25	99.78
		IV	3.103	116.8	119.8
	MET	I	3.733	112.9	2.976
		II	4.320	130.6	134.9
		III	4.573	150.3	154.8
		IV	5.520	169.9	175.4
Degree of freedom	SPI		6	35	41
	MET		8	45	53
Mean of squares	SPI	I	0.2800	2.060	
		II	0.2486	2.643	
		III	0.2543	2.807	
		IV	0.5171	3.336	
	MET	I	0.4667	2.509	
		II	0.5400	2.902	
		III	0.5717	3.339	
		IV	0.6900	3.776	
Calculated F value	SPI	I	0.1359		
		II	0.0941		
		III	0.0906		
		IV	0.1550		
	MET	I	0.1860		
		II	0.1861		
		III	0.1712		
		IV	0.1828		
Tabulated F value	SPI		2.371		
	MET		2.152		
			Bartlett test		
Degree of freedom	SPI		6		
	MET		8		
Calculated χ^2 value	SPI	I	0.6167		
		II	2.295		
		III	2.997		
		IV	0.8994		
	MET	I	0.6654		
		II	1.630		
		III	1.641		
		IV	0.8768		
Tabulated χ^2 value	SPI		12.592		
	MET		15.507		

I: Rodogyl; II: Novogyl; III: Arme-Rogyl; IV: Zolgyl

The accuracy was accessed using the standard addition technique (i.e., spiking a pre-assayed sample with a known amount of standard substances equal to 20% of the nominal content), revealing that the total recovery of standard addition was 98.5 - 101.4% (data not shown). Table 2 displays the analytical results when applying these UV spectrophotometric methods for the assay of SPI and MET in their coated tablets commercially available, clearly demonstrating the good precision of our methods (RSD < 2%) and the actual content of both drugs i.e., 97.7 ÷ 101.0% for SPI and 99.3 ÷ 101.7% for MET as compared to

the label claim. Assay results obtained by two different analysts on two different days (n = 6 for each day) were found to be insignificant different ($p > 0.05$) with RSD values ≤ 1.9%, meaning that our methods could be considered to be rugged enough. In our study, RP-HPLC proposed by Elkhoudary et al. (Elkhoudary et al., 2016) was used as the reference method. Figure 5 displays a typical HPLC of the binary mixture of MET 6.25 mg/L + SPI 12.5 mg/L, obviously showing that MET and SPI were respectively well resolved at ca. 5.0 and 7.9 minutes with tailing factor < 1.5 and acceptable column efficiency (number of theoretical plates > 2000). Statistical interpretation of both RP-HPLC and UV spectrophotometric data shows that they are comparably precise (Bartlett test: calculated χ^2 values smaller than tabulated ones) and accurate (one-way ANOVA test: calculated F values smaller than tabulated ones) at the significance level of 0.05 (Table 3). It also indicates that the excipients used in the pharmaceutical dosage forms under study did not interfere with the specificity of our spectrophotometric assay.

CONCLUSION

The signal transformation was successfully exploited for UV spectrophotometric assay of SPI and MET in their binary mixtures requiring no separation step for either drug. With regard to the assay of SPI, especially, wavelet transform manifested some noticeable advantages over derivative transform, such as the higher signal amplitude in transformed UV ratio spectra and the possibility to determine SPI and MET using transformed UV spectra simultaneously. In comparison with the work previously reported by Khattab and co-workers (Khattab et al., 2010), our spectrophotometric methods used only ethanol (a less toxic solvent than methanol) and offered the possibility of co-assaying SPI and MET in binary mixtures by zero-crossing point technique (i.e., transforming UV spectra by the mother wavelet rbio2.4). They were also statistically comparable with the RP-HPLC reference method (Elkhoudary et al., 2016) in terms of accuracy and precision when assaying SPI and MET in their combined coated tablets. Taking into consideration that they used only green solvent and proved to be time-saving and cost-effective, these analytical methods are strongly suggested for the routine analysis of SPI-MET coated tablets.

CONFLICT OF INTEREST

There is no conflict of interest among the authors of this manuscript.

AUTHOR CONTRIBUTION STATEMENT

The authors equally contributed to this work.

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RESEARCH ARTICLE

Quercetin Based Standardization of Polyherbal Anti-Gout Remedy and Its Molecular Docking Study Against Anti-Gout and Anti-Inflammatory Protein Targets

Ayisha SHAUKAT*°, Khalid HUSSAIN**

Quercetin Based Standardization of Polyherbal Anti-Gout Remedy and Its Molecular Docking Study Against Anti-Gout and Anti-Inflammatory Protein Targets

SUMMARY

A five-herb-containing traditional homemade medicine is extensively used to treat gout but has not been standardized for quercetin content. Therefore; the current study describes a reversed-phase liquid chromatographic method for quercetin determination in traditional herbal remedy. The elution was carried out using aqueous 2.0% acetic acid, acetonitrile, and tetrahydrofuran (55:40:5, V/V/V) as mobile phase at a flow rate of 0.8 mL/min, and detection was performed using diode array detector operated at 370 nm. The detector's response was linear in the range investigated (2.5-160.0 µg/mL) with R2 = 0.996. The results of recovery (98.26-103.22%, SD<5%), intraday accuracy and precision (94.68-104.08%, RSD<5%), and interday accuracy and precision (92.31-104.92%, RSD<5%) showed that the method was reliable, repeatable and reproducible, hence may be used for determination of quercetin in herbal remedy. The medicine contained 0.2425 mg/g quercetin. The molecular interactions of this marker compound were also studied against anti-gout and anti-inflammatory protein targets. Hence, the developed RP-HPLC method may be applied to standardize anti-gout medicine for quercetin content. Moreover, the molecular interactions help in the understanding of the underlying mechanism of action of this marker compound against gout.

Keywords: *Polyherbal, quercetin, RP-HPLC, standardization*

Anti-gut Poliherbal İlacın Kersetin Tabanlı Standardizasyonu ve Anti-gut ve Anti-inflamatuvar Protein Hedeflerine Karşı Moleküler Yerleştirme Çalışması

ÖZ

Beş bitki içeren geleneksel ilaç, gut tedavisinde yaygın olarak kullanılmaktadır, ancak kersetin içeriği bakımından standardize edilmemiştir. Bu nedenle, mevcut çalışma, geleneksel bitkisel ilaçta kersetin tayini için ters fazlı sıvı kromatografik bir yöntemi açıklamaktadır. Elüsyon, mobil faz olarak sulu %2.0 asetik asit, asetonitril ve tetrahidrofuran (55:40:5, V/V/V) kullanılarak 0.8 mL/dk akış hızında gerçekleştirilmiş ve 370 nm'de çalıştırılan diyot dizi dedektörü kullanılarak tanımlama yapılmıştır. Dedektörün yanıtı, R2 = 0.996 ile araştırılan aralıkta (2.5-160.0 µg/mL) doğrusaldır. Geri kazanım (%98,26-103,22, SD<5), gün içi doğruluk ve kesinlik (%94,68-104,08, RSD<%5) ve günler arası doğruluk ve kesinlik (%92,31-104,92, RSD<%5) sonuçları, yöntemin güvenilir, çoğaltılabilir ve tekrarlanabilir olduğunu, dolayısıyla bitkisel ilaçlarda kersetin tayini için kullanılabileceğini göstermiştir. İlaç 0.2425 mg/g kersetin içermektedir.. Bu işaretleyici bileşiğin moleküler etkileşimleri gut önleyici ve iltihap önleyici protein hedeflerine karşı da incelenmiştir. Bu nedenle, geliştirilen RP-HPLC yöntemi, kersetin içeriği bakımından standardize edilmiş gut önleyici ilaç üretmek için kullanılabilir. Ayrıca, moleküler etkileşimler, bu işaretleyici bileşiğin gut hastalığına karşı altta yatan etki mekanizmasının anlaşılmasına yardımcı olacaktır.

Anahtar Kelimeler: *Poliherbal, kersetin, RP-HPLC, standardizasyon*

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INTRODUCTION

A folklore polyherbal medicine reported in the literature is being extensively used for curing gout (Shaukat et al., 2020). Herbal formulations are inconsistent in terms of chemical constituents due to various genetic, growing, harvesting, and environmental factors. Hence, for a reproducible claim, herbal product needs to be standardized prior to pharmacological evaluation. The markers and characteristics compounds found in the ingredients of the traditional remedy can be used as analytical markers to develop various analytical methods for herbal product standardization (Li et al., 2008).

Quercetin, a flavonoid, found in the ingredients of the remedy, is also reported to have xanthine oxidase inhibitory potential (Zhu et al., 2004; Lin et al., 2015; Nile et al., 2017). Hence, it was selected as an analytical standard to develop HPLC method for standardization of the remedy. The literature review indicated various reversed-phase HPLC methods for the determination of quercetin in extracts, serum, urine, tea, and different matrices using diode array detector, solid-phase extraction and UV detection, electrochemical detection, and coulometric electrode array detection (Careri et al., 2003; Ishii et al., 2003; Fasolo et al., 2007; Goo et al., 2009; Phani et al., 2010; Liu et al., 2011). Most of the methods were expensive, time-consuming and laborious. Hence, there is a need to develop a simple and specific RP-HPLC method for quantification of quercetin which can easily be performed in less equipped laboratories for standardization and routine quality control of the remedy.

Molecular docking is a commonly used structure-based drug design strategy due to its extensive applications in finding molecular interactions and binding energy. Docking uses a scoring function that ranks candidate dockings by searching high-dimensional spaces effectively (Meng et al., 2011; Kalyaanamoorthy and Chen, 2011). Xanthine oxidase (XO) is an essential enzyme catalyzing the hydroxylation of hypoxanthine to xanthine and xanthine to uric acid, which is excreted by kidneys. Excessive production and, or inadequate excretion of uric acid results in hyperuricemia and gout (Borges et al., 2002). Quercetin inhibits xanthine oxidase due to its hydrophobic interaction with the enzyme and binding of the 3-hydroxyl group on benzopyrene ring of quercetin with isoalloxazine ring of FAD domain of the enzyme (Lin et al., 2002; Rasoulzadeh et al., 2009; Nessa et al., 2010). Prostaglandins are generated from arachidonate by the action of cyclo-oxygenase (COX) isoenzymes, and their biosynthesis is blocked by nonsteroidal anti-inflammatory drugs (NSAIDs). Prostaglandins play a vital role in the generation of the inflammatory response, their biosynthesis is significantly increased in inflamed tissue, and they contribute to the development of the cardinal signs of acute inflammation (Ricciotti and Fitzgerald, 2011). Quercetin inhibits COX-2, thus inhibiting the release of inflammatory mediators involved in inflammation, thus, inhibits carrageenan-induced inflammation in experimental rats (Morikawa et al., 2003). The inhibition of carrageenan-induced hind paw edema of rats is well correlated with inhibition of such inflammatory mediators as reported in literature (Shaukat et al., 2021). Phospholipase-A_2 hydrolyze cell membrane phospholipids (ester bonds) to produce arachidonic acid and fatty acid (lysophosphatidylcholine and lysophospholipids), playing an essential role in the production of inflammatory lipid mediators, mainly eicosanoids. Hence, they are considered pro-inflammatory enzymes, and their inhibition is regarded as a desirable therapeutic target (Yedger et al., 2006). Quercetin selectively inhibits phospholipase A_2, thus inhibiting mediators of inflammation (Lindal and Tagesson, 1997).

The present studies are performed to simulate the wet lab results with dry lab studies, hence, a freely available software was used for the blind type of docking to evaluate all possible active sites as well as allosteric sites. Therefore, the present study aimed to quantify quercetin in herbal remedy and to under-

stand its molecular interaction with anti-gout and anti-inflammatory protein targets.

MATERIAL AND METHODS

Chemicals and solvents

The chemicals and solvents used in the current study are acetonitrile, methanol, tetrahydrofuran, quercetin, acetic acid and sodium acetate (Merck, Germany). The double distilled water prepared in-house was used, where required.

Instruments

Double beam UV/Visible spectrophotometer (Model-2550, Shimadzu Scientific Instruments, USA, equipped with Operating system UV Probe 2.21), Fourier Transform Infrared Spectrophotometer (IR Tracer-100, Shimadzu Japan). A liquid chromatography system (Agilent Technologies, 1200 series, Germany) equipped with an isocratic pump (G1310A), auto-sampler (G1329A), thermostatically controlled column oven (G1316A), and diode array detector (G1315B) were used in the current study. Other equipment used included a pH meter (WTW series, Ino lab) and ultrasonicator (Memmert, Germany).

Preparation of anti-gout remedy

The anti-gout remedy was prepared by using method reported in the literature (Shaukat et al., 2020).

Preparation of standard solutions

A stock solution of quercetin (1.0 mg/mL) was prepared in HPLC-grade methanol. Working standard solution having concentration range of 2.5-160 µg/mL was prepared by diluting the standard stock solution with the mobile phase.

Method development

A volume (20 µl) was eluted by the isocratic mobile phase comprising 2.0% acetic acid, acetonitrile, and tetrahydrofuran (55:40:5, *V/V/V*) at 0.8 mL/min flow rate through the C_{18} column (Agilent 5 TC-C_{18} (2) 250×4.6 mm) that was maintained at 35ºC. The detection was carried out using DAD, 370 nm detection wavelength, and 360 nm reference beam. The chromatogram obtained was used to determine system suitability.

System suitability

The system suitability was ensured by determining the number of theoretical plates (N), height equivalent to theoretical plate (HETP), capacity factor (k′), tailing factor, and peak asymmetry.

Method validation

Linearity, Beer's range, limit of detection and quantification

The working standard solution having a concentration range of 2.5-160 µg/mL was analyzed in triplicate. The plot between concentration and peak area was constructed, and linearity was evaluated visually by applying the linear regression equation. The correlation of data points was assessed by the correlation coefficient.

The sensitivity, Limit of detection (LOD) and Limit of quantification (LOQ), were determined using the statistical method. Briefly, five standard solutions of quercetin (5.0-80.0 µg/mL) were analyzed in quintuplicate. The standard calibration curve was constructed to determine the mean slope (S) and standard deviation of intercepts (σ) to determine LOD and LOQ using Equations 1 and 2, respectively.

Recovery

For recovery, the dried herbal extract (20 mg) was spiked with 1 mL of working standard solutions containing quercetin (5.0, 10.0 and 20.0 µg/mL). Un-spiked samples were treated in a similar procedure to prepare respective blanks. The spiked and un-spiked samples were analyzed in triplicate, the peak corresponding to standard was identified, and the peak area was used to determine the amount of quercetin using the calibration curve. The calculated amount was then compared with the spiked amount to assess recovery.

Intra-day and inter-day accuracy and precision

Intra-day and inter-day accuracy and precision were determined using each of the three mixed standard solutions used for recovery studies which were analyzed six times in a single day and once daily for six consecutive days, respectively. Accuracy was determined by quantification of each standard from the respective standard curve, constructed on each day, whereas the RSD of the six readings was taken as precision.

Robustness

The working standard solution of quercetin was analyzed by changing mobile phase pH (± 0.1), detection wavelength (± 2 nm) and column temperature (± 2°C).

Quantification of quercetin in polyherbal anti-gout remedy

The sample for HPLC analysis was prepared by acid hydrolysis of the herbal remedy using the method reported in the literature (Ewais et al., 2016). The sample solution having a concentration of 20 mg/mL was prepared for quantification of quercetin in traditional herbal remedy. The amount of quercetin was calculated using linear regression equation obtained from the standard calibration curve of quercetin.

Molecular docking studies

The 1-Click Docking Mcule and UCSF Chimera 1.12 Software were used for docking and determining the hydrogen-bonding affinity of the ligands against xanthine oxidase, prostaglandin G_1/H_1 synthase, prostaglandin G_2/H_2 synthase and phospholipase A_2.

Quercetin was used as ligand. The data input was in the form of SMILES of the ligands, taken from the PUB-Chem database. The SMILES code of quercetin is given as (C1=CC(=C(C=C1C2=C(C (=O) C3=C(C=C(C=C3O2)O)O)O)O)O), allopurinol is C1=NNC2=C1C(=O)NC=N2 and diclofenac is 1=CC=C(C(=C1)CC(=O)O)NC2=C(C=CC=C2Cl) Cl. The PDB file of each ligand was evaluated using UCSF Chimera 1.12 to find hydrogen bonding and bond lengths.

Interpretation of molecular docking

The least binding energies (kcal/mol) were noted for the ligand-protein interaction. The amino acids involved in interaction and bond angles were noted. The best possible binding pose showing hydrogen bonding of ligand was explored.

RESULTS AND DISCUSSION

In plants, phenolic compounds often occur as glycosides and sugar moieties affect their elution pattern. Moreover, the presence of the type of sugar makes the selection of standard difficult. Therefore, in the present study, the sample of polyherbal anti-gout remedy was subjected to acid hydrolysis to break the glycosidic bond. The aglycone-containing fraction was then extracted by partitioning with ethyl acetate. Ethyl acetate was not miscible with water, so it was removed and the samples were made with the mobile phase. This extraction produced aglycone-rich samples, free from interfering substances. Moreover, it helped to make the method more specific and sensitive.

Quercetin exhibits maximum absorbance at two wavelengths (280 nm due to benzoyl ring and 370 nm due to cinnamyl ring) which can be used to develop UV absorbance-based methods (Duan, 2014). However, it exhibits intrinsic absorption at 370 nm due to functional groups in the flavonoid ring giving a broad absorption peak at this wavelength (Yao et al., 2004; Dmitrienko et al., 2012). Therefore, in the present study, 370 nm wavelength was selected to detect quercetin using diode array detection. In plants, quercetin is also found in the form of glycosides (rutin); hence, the samples of polyherbal anti-gout remedy were prepared like the determination of phenolic and polyphenolic compounds.

The chromatogram of the standard quercetin solution obtained using the optimized chromatographic condition is given in Figure 1. These results showed that the peak of the standard appeared at 5.480 ± 0.1 min. Moreover, the peak was symmetrical/Gaussian which could be used to determine system suitability.

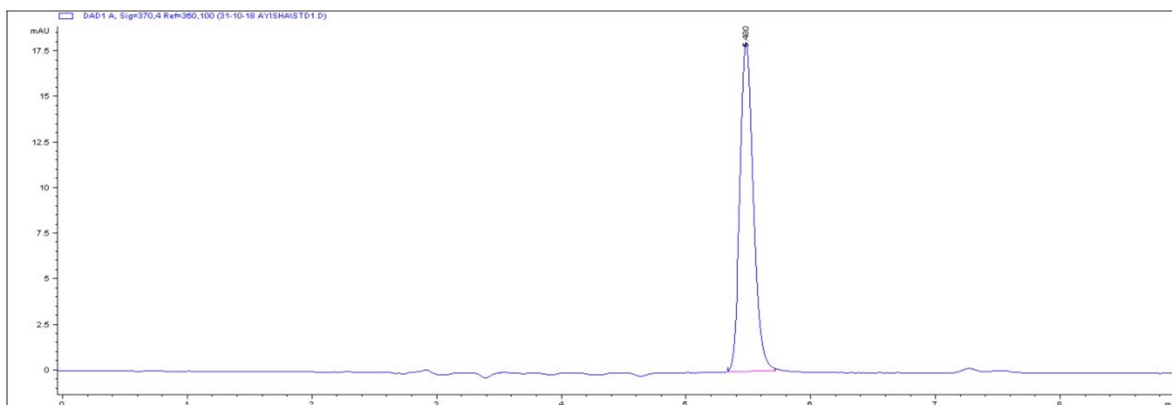

Figure 1. HPLC chromatogram of standard marker (quercetin)

The system suitability parameters are given in Table 1. The results were within the specified limits, which indicated that the chromatographic system and conditions were suitable to be used for quantitative purposes. Hence, the method can be validated for the standardization of anti-gout remedy.

Table 1. System suitability parameters calculated from chromatogram of quercetin.

Parameters	Values	Reference values
Capacity(retention) factor k´	4.48	K ≥ 2
Peak asymmetry/Tailing factor As	1.0	T ≤ 2
Number of theoretical plates N	12012.16	N > 2000
Height equivalent to theoretical plate	20.81	The smaller the value, the higher the efficiency of the column

The plot of concentration versus peak area of quercetin is shown in Figure 2. These results indicated that the method was linear in the concentration range investigated (2.5- 160 µg/mL).

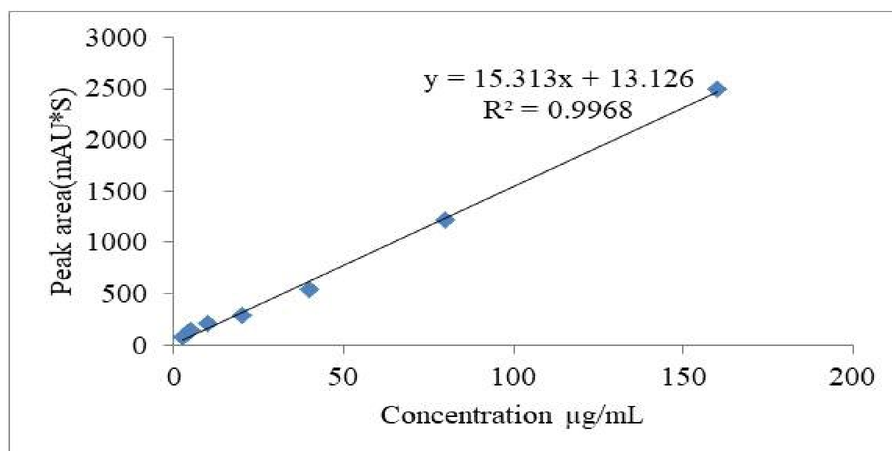

Figure 2. A plot of concentration versus peak area of quercetin.

The sensitivity of the method – LOD and LOQ – was determined statistically using the standard deviation of intercepts and the mean slope (S) of standard calibration curves (n=5), which was found to be 0.36 and 1.10 µg/mL, respectively (Table 2). These results indicate that the method can be used to quantify this marker even at the sub-microgram level.

Table 2. Limit of detection (LOD) and limit of quantification (LOQ) of quercetin by RP-HPLC

Standard curve	Concentration(µg/mL)	Linear regression equation	Slope	Intercept
1	5.0-80.0	Y=14.299x+19.925	14.299	19.925
2	5.0-80.0	Y=13.126x+22.383	13.126	22.383
3	5.0-80.0	Y=13.067x+21.023	13.067	21.023
4	5.0-80.0	Y=12.996x+19.819	12.996	19.819
5	5.0-80.0	Y=12.928x+18.454	12.928	18.454
Mean slope (S) = 13.283				
Standard deviation (SD) = 1.46				
LOD=3.3SD/S = 0.36 µg/mL				
LOQ=10SD/S = 1.10 µg/mL				

The mean recovery of spiked samples with three different concentrations (5.0, 10.0 and 20.0 µg/mL) are given in Table 3. The recovery was found to be ranging from 98.26 to 103.22%, with (SD) less than 5%. These results were within acceptable limits, which showed that the developed method was reliable. The results of intra- and inter-day accuracy and precision are shown in Table 3. The Intra- and Intra-day accuracy for quercetin was found to be (94.68-104.08%; 92.31-104.92%) with the relative standard deviation of less than 5%, which indicated that the developed method was repeatable and reproducible. The chromatograms of the remedy showed that the peak of quercetin was well resolved without the interference from any other component. Hence, the developed method is specific for the determination of quercetin.

Table 3. Recovery, Intraday, inter-day accuracy and precision of the HPLC method for the determination of quercetin (n = 6)

Concentration (µg/mL)	Mean recovery (%)± SD	Intra-day analysis		Inter-day analysis	
		Accuracy %	Precision RSD	Accuracy %	Precision RSD
5.0	102.27±0.057	104.08	1.97	104.92	2.12
10.0	103.22±0.173	97.05	0.52	98.03	1.90
20.0	98.26±0.152	94.68	0.22	92.31	0.46

The developed method was robust since slight variation in mobile phase pH (± 0.1), column temperature (± 2°C) and detection wavelength (± 2 nm) did not affect the chromatographic resolution.

The chromatogram of the polyherbal anti-gout remedy is given in Figure 3. The peak of the standard was identified by comparing retention time, and the peak area was used to quantify the marker in the polyherbal anti-gout remedy. The amount of quercetin in the anti-gout remedy was found to be 0.2425 mg/g.

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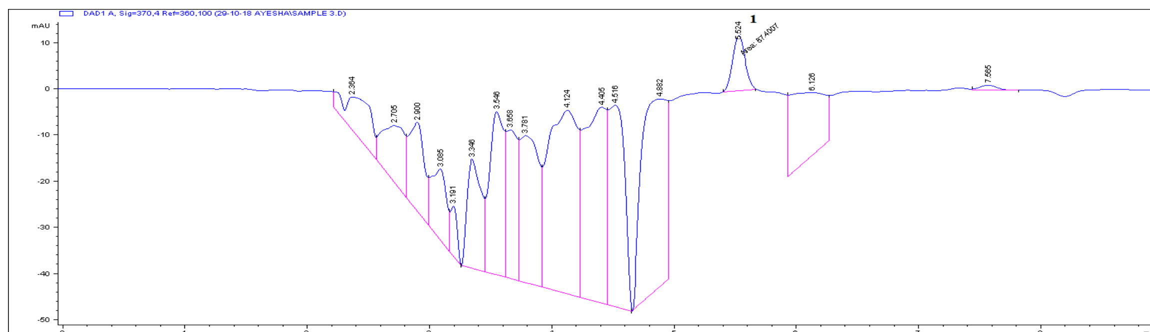

Figure 3. Chromatogram of polyherbal anti-gout remedy; Quercetin (1)

In the present study, we have used quercetin as an analytical marker to standardize a polyherbal anti-gout remedy. The sensitivity of the method was found to be more than the method reported in the literature Sladkovsky et al. (2001), and lesser than some of the studies (Chen and Xiao, 2010; Savic et al., 2013).

Amino acid residues of xanthine oxidase ALA 1054.D, SER 1057.D formed two hydrogen bonds with quercetin and four hydrogen bonds with allopurinol(GLY 772.D, PHE 773.D, GLN 1169.D). The least binding energies of -9.2 and -6.4 were noted for quercetin and allopurinol, respectively (Figure 4 and Figure 5).

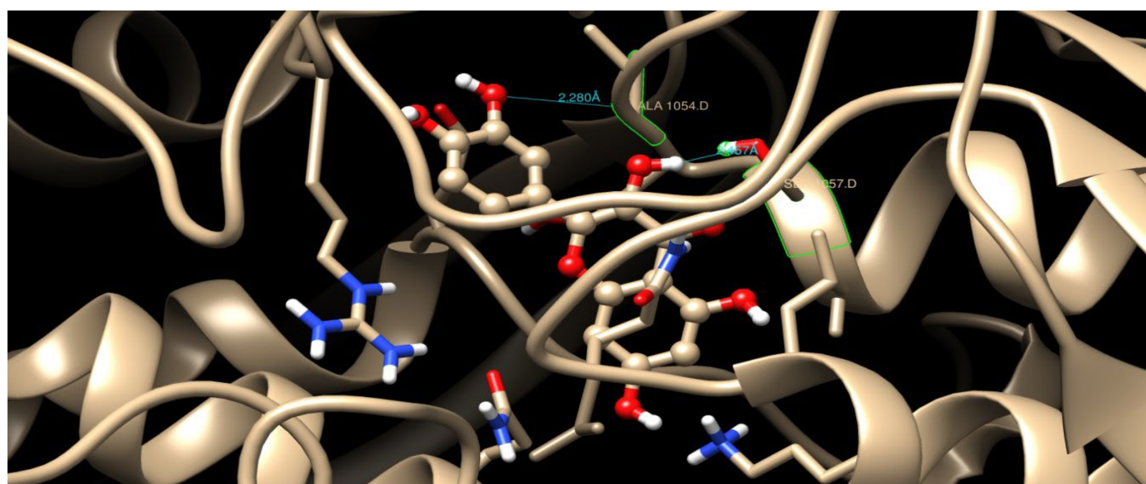

Figure 4. Interaction of quercetin with xanthine oxidase

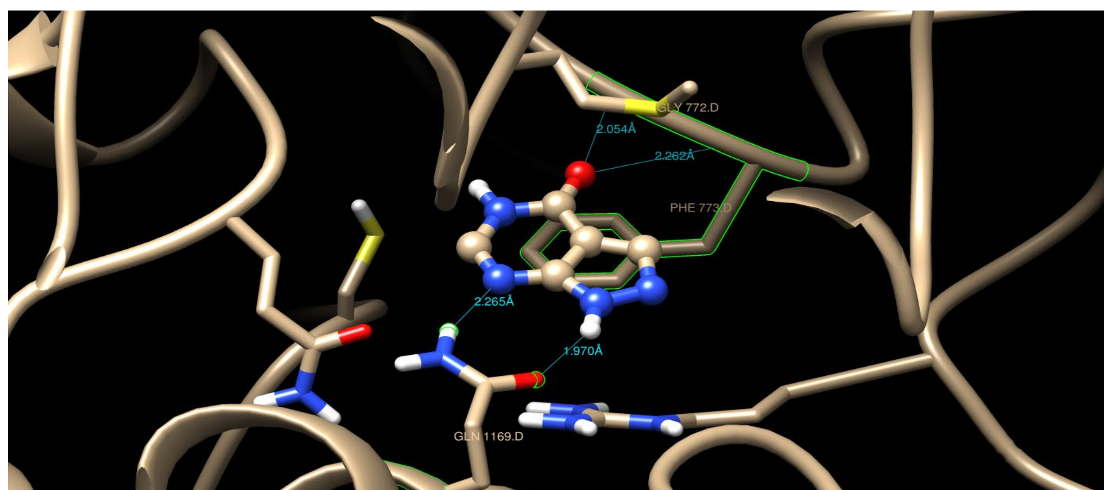

Figure 5. Interaction of Allopurinol with xanthine oxidase

The lower values for the binding energy showed the good binding affinities of ligands with the enzymes. The interaction occurs through the formation of hydrogen bonding between hydroxyl groups of quercetin and allopurinol and catalytic residues of the binding sites, which results in the formation of a conjugated pi-system responsible for stabilizing interaction with the active site.

The docking of prostaglandin G_2/H_2 with quercetin showed that two hydrogen bonds were involved in the binding interaction of SER 499.C and THR 175.C with quercetin (Figure 6). Two hydrogen bonds were involved in the binding interaction of TYR 354.C and SER 499.C with diclofenac (Figure 7). The docking studies of enzyme prostaglandin G_1/H_1 have shown a strong binding interaction via formation of two hydrogen bonds with quercetin and one hydrogen bond with diclofenac (Figure 8 and Figure 9). The docking studies of enzyme phospholipase A_2 have shown a strong binding interaction via formation of two hydrogen bonds, each with quercetin and diclofenac (Figure 10 and Figure 11).

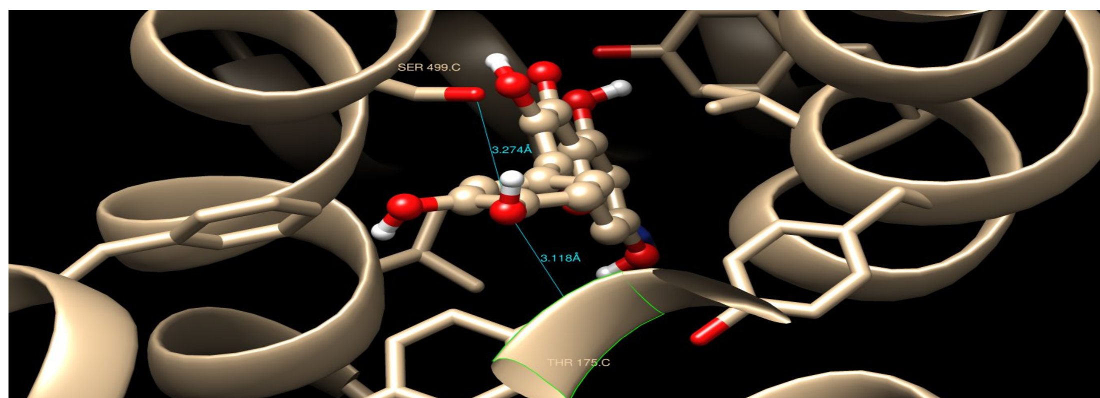

Figure 6. Interaction of quercetin with prostaglandin synthase G_2/H_2

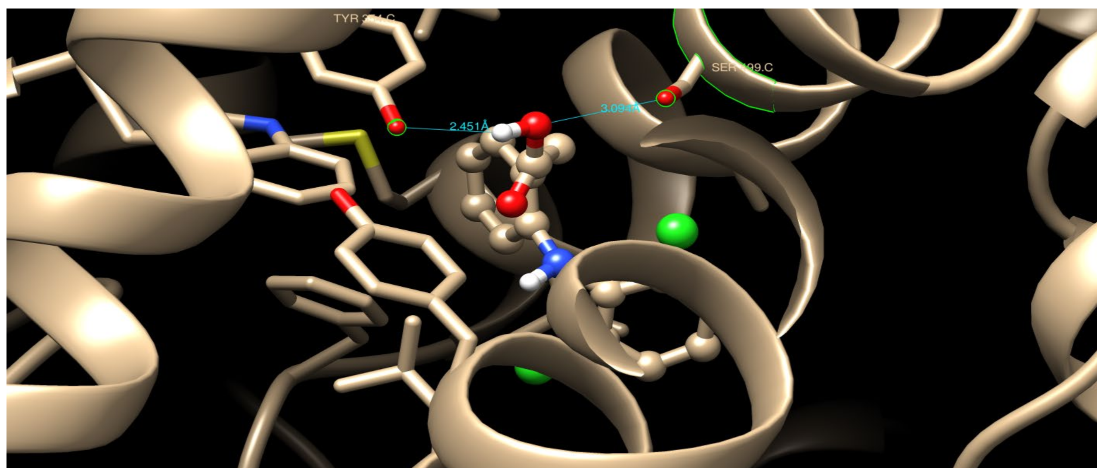

Figure 7. Interaction of diclofenac with prostaglandin G_2/H_2

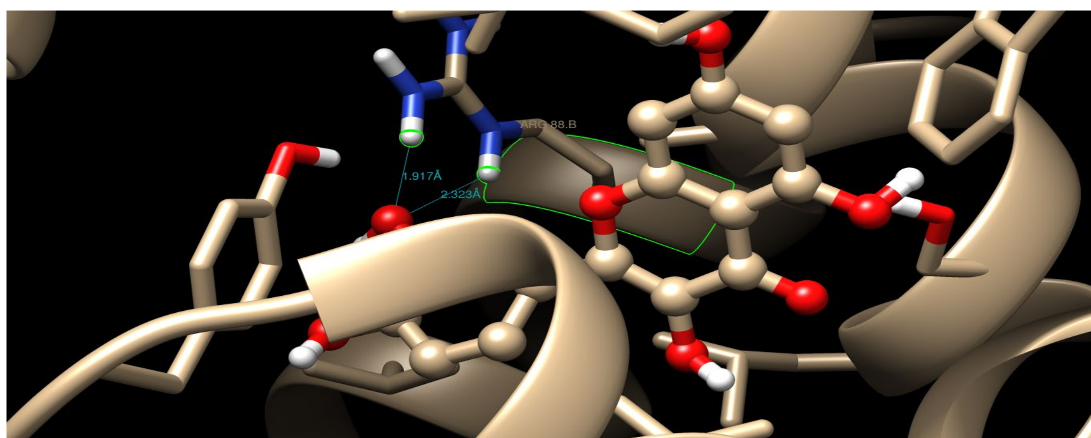

Figure 8. Interaction of quercetin with prostaglandin G_1/H_1 synthase

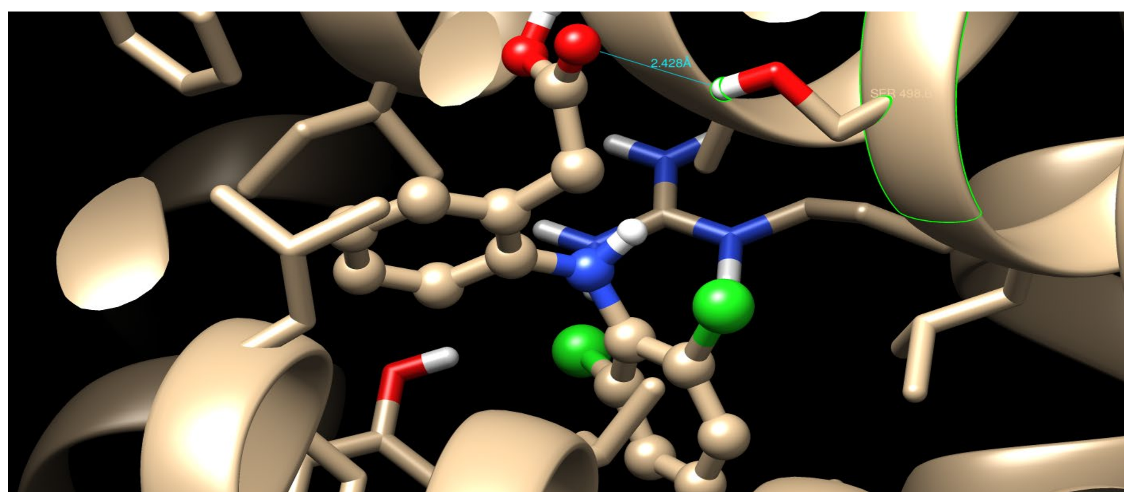

Figure 9. Interaction of diclofenac with prostaglandin G_1/H_1 synthase

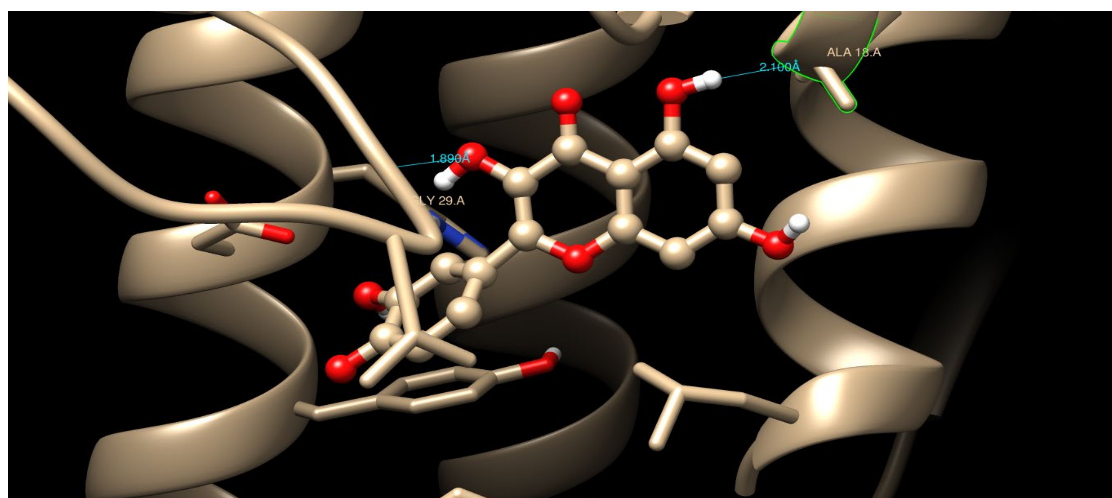

Figure 10. Interaction of quercetin with phospholipase A_2

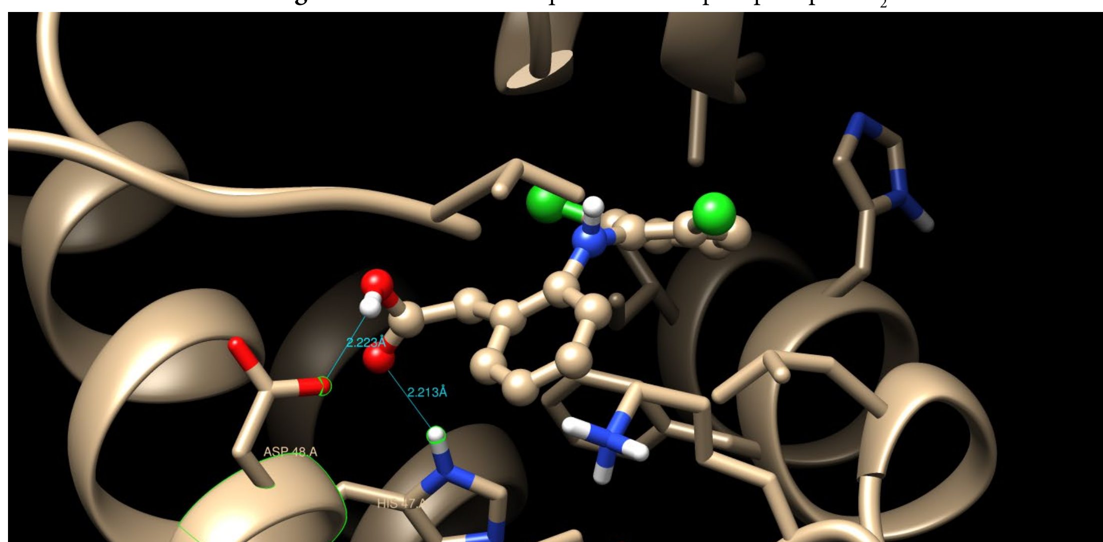

Figure 11. Interaction of diclofenac with phospholipase A_2

The results of ligand-protein binding, binding energy involved in amino acids and bond length of the ligand quercetin and standard antigout compound (allopurinol) and anti-inflammatory compound (diclofenac) with enzymes (xanthine oxidase, prostaglandin G_2/H_2, prostaglandin G_1/H_1 and phospholipase A_2) are given in Table 4.

In mcule software used in the study, few of the enzyme targets (animal origin) show docking sequence with ligand because in mcule, the gene sequence of a particular enzyme in humans is not studied completely, while studied in animals having biochemical and target sequence of animal enzymes showing resemblance to human biochemistry. Hence, to maintain consistency in the study using single software, mcule was used with different organisms enzymes targets available/studied. Moreover, animal enzymes are easily available to further pursue this research for wet-lab screening, sorting and optimizing the SAR. The animal enzymes considered for this study exhibit sufficient homology with human structure. Homology with human enzymes, resolution, availability, cost-effective approach and integrity of structure especially and other complicated errors related to software are considered vital.

Table 4. Molecular interaction of different enzymes with quercetin and standard compounds (Allopurinol and diclofenac)

Enzyme	Ligands	PDB ID	Organism	Amino acid	Binding energy (kcal/mol)	Bond length
Xanthine oxidase	Quercetin	2e1q	Homosapien	ALA 1054.D SER 1057.D	-9.2	2.280 Å 2.457 Å
	Allopurinol (standard inhibitor)	2e1q	Homosapien	GLY 772.D PHE 773.D GLN 1169.D GLN 1169.D	-6.4	2.054 Å 2.262 Å 2.265 Å 1.970 Å
Prostaglandin synthase G_2/H_2	Quercetin	3 mqe	Mus musculus	SER 499.C THR 175.C	-8.2	3.274 Å 3.118 Å
	Diclofenac (standard inhibitor)	3 mqe	Mus musculus	TYR 354.C SER 499.C	-8.0	2.451 Å 3.094 Å
Prostaglandin synthase G_1/H_1	Quercetin	1ht5	Ovis aries	ARG 88.B ARG 88.B	-7.4	1.917 Å 2.323 Å
	Diclofenac (standard inhibitor)	1ht5	Ovis aries	SER 498.B	-7.8	2.428 Å
Phospholipase A_2	Quercetin	Idb4	Homosapiens	GLY 29.A ALA 18.A	-8.0	1.890 Å 2.100 Å
	Diclofenac (standard inhibitor)	Idb4	Homosapiens	ASP 48A HIS 47.A	-7.7	2.223 Å 2.213 Å

The literature review indicated computational tools such as Metapocket and Autodock for finding molecular interaction of phospholipase A_2 with different phytochemicals (Shivashankar et al., 2019). Numerous studies reported that phenolic compounds and flavonoids inhibit eicosanoid biosynthesis, 5-lipoxygenase and cyclooxygenase pathways (Laughton et al., 1991; Ferrandiz and Alcaraz, 1991). Hence, they reduce the release of arachidonic acid and inhibit inflammation (Kim et al., 1998; Rathee et al., 2009).

CONCLUSION

The method developed in the present study was simple, reliable, sensitive, repeatable and reproducible, hence may be used to standardize polyherbal anti-gout remedy. The marker quercetin has shown good binding affinity with amino acid residues of xanthine oxidase, prostaglandin G_2/H_2 synthase, prostaglandin G_1/H_1 synthase and phospholipase A_2. The binding energies of quercetin against anti-inflammatory and anti-gout targets confirm the anti-gout activity of polyherbal medicine.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Development of method, experimentation, content writing and data analysis (SA), Materıals and processing the manuscript (HK, SA), Interpretation of results (HK, SA), Literature Search (HK, SA), Critical Reviews and final approval of manuscript (HK, SA).

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RESEARCH ARTICLE

Leukotriene D4 Levels In Patients With Breast Cancer

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Leukotriene D4 Levels In Patients With Breast Cancer

SUMMARY

Leukotriene D4 (LTD4) is an inflammatory mediator synthesized from LTC4 via gamma-glutamyltransferase (GGT) enzyme in the arachidonic acid pathway and has been reported to induce cell proliferation and survival in cancer. In recent studies, it has been shown that there is an increase in GGT enzyme activity in breast cancer. In recent studies, it has been shown that there is an increase in GGT enzyme activity in breast cancer. The aim of this study is to determine whether there is a change in serum LTD4 levels in patients with breast cancer and to examine the relationship between LTD4 and GGT. For this purpose, serum samples were taken from 43 patients diagnosed with breast cancer and eight healthy controls. Patients were divided into five subgroups, Luminal A, Luminal B, Luminal B-HER2(+), HER2(+), and triple-negative. LTD4 levels were measured by the ELISA method. Mean levels of LTD4 in the patients were significantly higher than in healthy controls (p < 0.05). Based on the molecular subtypes, serum LTD4 levels were found to be considerably higher in the Luminal A, Luminal B, and Triple (-) subgroups than in the controls (p <0.01, p <0.005, and p <0.005, respectively). Higher levels of LTD4 have been observed in post-menopausal patients than in premenopausal patients (p <0,05). A statistically significant positive correlation was observed between GGT activity and LTD4 levels in the whole study group and post-menopausal patients (R=0.349, p=0.014, and R=0.437, p=0.042, respectively). According to the literature, this study is the first to examine LTD4 levels in breast cancer and supports other studies showing the role of leukotrienes in cancer. Because of LTD4's ability to induce proliferation and inhibit apoptosis, increased levels of LTD4 in our study may be associated with cancer development, especially in post-menopausal women.

Key Words: *Leukotriene D4, Gamma-Glutamyltransferase, Breast Cancer, Post-menopausal Status*

Meme Kanseri Hastalarında Lökotrien D4 Düzeyleri

ÖZ

Lökotrien D4 (LTD4) araşidonik asit yolağında gama-glutamiltransferaz (GGT) enzimi aracılığı ile LTC4'ten sentezlenen inflamatuar bir aracıdır ve kanserde hücre çoğalmasını ve sağ kalımını indüklediği bildirilmiştir. Son yıllarda yapılan çalışmalarda, meme kanserinde GGT enzim aktivitesinde artış olduğu gösterilmektedir. Bu çalışmada, meme kanserli hastalarda serum LTD4 düzeylerinde de bir değişiklik olup olmadığını ve LTD4 ile GGT arasındaki ilişkiyi incelemeyi amaçladık. Bu amaçla meme kanserli 43 hasta ve 8 sağlıklı kontrolden serum örnekleri alındı. Hastalar, Luminal A, Luminal B, HER2 (+), Luminal B-HER2 (+) ve üçlü negatif olmak üzere beş alt gruba ayrılmıştır.

Lökotrien D4 seviyeleri ELISA yöntemi ile ölçülmüştür. Hastalardaki ortalama LTD4 seviyeleri sağlıklı kontrollerle karşılaştırıldığında anlamlı derecede yüksek bulunmuştur (p<0.05). Moleküler alt tiplere göre serum LTD4 düzeyleri; Luminal A, Luminal B ve Üçlü (-) alt gruplarında kontrollere göre anlamlı derecede yüksek bulunmuştur (sırasıyla p <0,01, p <0,005 ve p <0,005). Menopoz-sonrası hastalarda menopoz-öncesi hastalara göre daha yüksek LTD4 seviyeleri gözlenmiştir (p <0,05). Tüm çalışma grubunda ve post-menopozal hastalarda GGT aktivitesi ile LTD4 seviyeleri arasında istatistiksel olarak anlamlı bir pozitif korelasyon gözlenmiştir (R=0.349, p=0.014 ve R=0.437, p=0.042, sırasıyla). Bu araştırma literatüre göre, meme kanserinde LTD4 düzeylerini inceleyen ilk çalışmadır ve lökotrienlerin kanserdeki rolünü gösteren diğer çalışmaları desteklemektedir. LTD4'ün hücre proliferasyonunu indükleme ve apoptozu inhibe etme yeteneği nedeniyle, çalışmamızda bulunan artmış LTD4 seviyelerinin, özellikle menopoz sonrası kadınlarda kanser gelişimi ile ilişkili olabileceği düşünülmektedir.

Anahtar Kelimeler: *Lökotrien D4, Gama-Glutamiltransferaz, Meme Kanseri, Post-menopozal Durum*

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INTRODUCTION

Despite many advances made in research in recent years, cancer remains the leading cause of death worldwide. Changes in the tumor microenvironment, including dysregulated immunity, contribute to carcinogenesis and cancer progression. In 1863, Rudolf Ludwig Carl Virchow described the relationship between inflammation and cancer. He supposed that the inflammatory process could provide a suitable environment for tumor development and infiltrated immune cells show the place where cancer lesions start in the inflamed tissue (Korniluk, 2017). Inflammation is responsible for removing dead cells, cell proliferation, and tissue repair, this type of inflammatory response is self-limiting and ceases after the repair is completed. On the other hand, uncontrolled inflammation can become chronic, and cause sustained release of growth factors and reactive oxygen that interact with the DNA of the proliferating epithelium and result in genomic changes which lead to induce tumor initiation and triggers malignant growth in surrounding tissues (Todoric, 2016). Inflammatory cells release pro-inflammatory mediators, including eicosanoids, chemokines, growth factors, and cytokines, which can transform the microenvironment into an abnormal environment (Bellamkonda, 2016). The synthesis of leukotrienes (LTs) is initiated by 5-lipoxygenase-activating protein (FLAP), which presents arachidonic acid to 5-lipoxygenase (5-LOX) enzyme. Although the clinical value of LTs comes from their bronchoconstriction properties during allergic inflammation, they are inflammatory mediators with potent biological activities in the pathogenesis of many diseases. Commonly LTA4 and LTB4 are considered known as LTs. On the other hand, Cysteinyl leukotrienes (CysLTs) mention LTC4, LTD4, and LTE4, and these inflammatory mediators are structurally different from LTA4 and LTB4 (Almutlaq, 2017). CysLTs are mainly synthesized by leukocytes and dendritic cells such as eosinophils, basophils, mast cells and monocytes/macrophages (Peters-Golden, 2007). CysLTs activate their specific receptors ($CysLT_1R$ and $CysLT_2R$) located on the cell membrane. CysLT1R has a high affinity for LTD4 while CysLT2R has a lower affinity (Magnusson, 2011).

The main function of the gamma-glutamyl transferase (GGT) enzyme found in the extracellular membrane is to degrade the extracellular glutathione by transferring the gamma-glutamyl group to acceptor substrates thus providing the cell with intracellular cysteine to re-synthesis glutathione (Verma, 2015). Another essential function of this enzyme, also known as gamma-glutamyl leukotrienase (GGLT), is to catalyze the synthesis of LTD4 by removing the gamma-glutamyl group from LTC4 (Funk, 2001). In recent years, increased GGT enzyme levels in various types of cancer, including breast cancer have been reported (Corti, 2010). This study aimed to examine LTD4 level and its correlation with GGT to estimate the effect of chronic inflammation in breast cancer.

MATERIALS AND METHODS

Forty-three patients with breast cancer who were followed up by Istanbul University, Institute of Oncology, Department of Clinical Oncology, Oncology Surgery Unit were included in this study. Patients have been received and understood all the research-related information before the operation date, and the informed consent was obtained from them. Serum samples were taken from 43 patients before the operation, and eight healthy people admitted to the clinic for breast reduction surgery were also included in this study as controls. Serum samples were taken from healthy women before the operation. Samples were stored at -80°C until use. Baseline characteristics and laboratory results of the patients and controls are summarized in Table 1. Ethics approval for this study was approved by The Clinical Research Ethics Com-

mittee of the Istanbul Faculty of Medicine (Ethics Committee 28.03.2016/106748). Molecular classification of the patient group was performed according to the evaluation criteria of estrogen/progesterone hormone receptor, Ki67, and cerB2 (HER2) (Goldhirsch, 2011).

Accordingly, patients were classified as Luminal A (10 patients with estrogen receptor [ER] positive and, or progesterone receptor [PR] positive, human epidermal growth factor receptor 2 [HER2] negative, low Ki67 expression), Luminal B (8 patients with ER+ and, or PR+, HER2-, Ki67 high), Luminal B/Her2+ (7 patients with ER+ and, or PR+, HER2+, Ki67 high), Her2+ (8 patients with ER-, PR-, HER2+), and triple-negative/basal type (10 patients with ER-, PR-, HER2-).

Table 1. Baseline characteristics and laboratory tests of patients and controls.

Variables	Patients (n=43)	Controls (n=8)
Age (SD)	51.3 (11.9)	39.0 (14.7)
Menopausal Status		
Premenopausal (%)	20 (46.5)	7 (87.5)
Postmenopausal (%)	23 (53.5)	1 (12.5)
Cancer Stage, n (%)*		
I	3 (7.0)	-
II	18 (41.9)	-
III	21 (48.8)	-

*One missing data

Leukotriene D4 Analysis

Serum Leukotriene D4 (LTD4) levels were measured using a commercially available ELISA kit (Oxford Biomedical Research, MI 48371 U.S.A.). In the 96-well microplate, the standards and the samples were analyzed by the kit procedure. Samples were purified using extraction columns before measurement, and then the kit procedure was applied. Measurements were carried out in absorbance (A)
at 400 nm. All data are expressed as mean (standard deviation, SS).

Statistical analysis

The homogeneity of the data was evaluated with the Kolmogorov-Smirnov test. Since the data were not normally distributed, the results were compared using nonparametric tests. Mann-Whitney U test was used to compare differences between patients and healthy controls. Spearman-correlation test was used to examine the relationship between the parameters for the non-normally distributed data. P values of less than 0.05 were regarded as statistically significant. Statistical analyzes were performed using the SPSS 22 Package Program (SPSS Inc, USA).

RESULTS AND DISCUSSION

To determine whether the data from serum LTD4 analysis were distributed normally, a Kolmogorov-Smirnov test was used. According to test results, LTD4 data did not show a normal distribution ($p<0.05$). Mean LTD4 levels in total patients were significantly higher than those in healthy controls ($p<0.005$). According to the molecular subtypes of breast cancer, patients in Luminal A, Luminal B, and Triple (-) groups had significantly higher LTD4 levels than in healthy controls ($p<0.01$, $p<0.005$, and $p<0.005$, respectively) (Table 2). Patients in the Her2(+) group had lower LTD4 levels than those in

other subtypes. However, there was no statistically significant difference between the groups in terms of LTD4 levels in general. Several studies show the direct effect of 5 lipoxygenase derivatives on cellular growth, cell migration, and invasion in cancer cells (Bishayee, 2011).

Here, we demonstrate for the first time the high LTD4 levels in breast cancer patients. It is known that the proinflammatory leukotriene D4 (LTD4) has been observed to increase proliferation, survival, and cell migration (Paruchuri, 2003; 2005). One study showed a significant correlation between high expression of $CysLT_1R$ in tissue from breast cancer patients and histological grade (Magnusson, 2011). Interestingly, other studies showed that the LTD4 receptor $CysLT_1R$ is highly expressed in human colon cancer and negative correles with patient survival (Bellamkonda, 2016; Ohd, 2003). Also, LTD4 has been found to induce migration and proliferation of colon cancer cells (Salim, 2014). In addition to that, LTD4 was reported to have a growth-stimulating effect on human gastric cancer cells (Shimakura, & Boland,1992). In contrast, one study showed that LTB4 and LTD4 inhibit MCF-7 breast cancer cell growth, and a leukotriene receptor antagonist and a 5-LO inhibitor were able to abolish the inhibitory effect of LTB4 and LTD4 on cell growth, suggesting that the leukotriene receptors mediate this effect (Przylipiak, 1998). Increased 5-LO expression and the cysteinyl leukotriene (CysLT) pathway prepare the tumor microenvironment could be by activating different signal pathways and producing inflammation (Tsai, 2021). Findings from studies seem heterogeneous, so more studies are needed to understand the role of the LTD4 in cancer.

Table 2. Levels of serum LTD4 in both patients and healthy controls.

			LTD4 Levels	
		N	**Mean(SD)**	**P**
Controls		8	1.30 (0.39)	--
Total patients		43	3.42 (2.13)	0.002*
Patients in molecular subtypes of breast cancer	Luminal A	10	3.11 (2.18)	0.006*
	Luminal B	8	4.11 (2.56)	0.002*
	Luminal B+Her2	7	3.68 (2.56)	0.104
	Her2(+)	8	2.70 (2.12)	0.114
	Triple(-)	10	3.58 (1.52)	0.003*

* compared with controls

In our study, post-menopausal patients had higher levels of LTD4 than pre-menopausal patients [4.13 (2.35) ng/mL vs 2.61 (1.51) ng/mL, respectively] ($p< 0.05$). Although there is no study examining leukotriene levels in breast cancer, one study has shown the role of inflammation in the pathogenesis and progression of post-menopausal estrogen-dependent breast cancer (Madeddu, 2014). Another study reported that regular use of aspirin, ibuprofen or other NSAIDs may have a significant protective effect against the development of breast cancer in post-menopausal women (Harris, 2003).

The mean GGT activity was found to be significantly higher in total patients than in healthy controls [27.89 (11.21) vs. 18.85 (4.41), respectively] ($p<0.05$).

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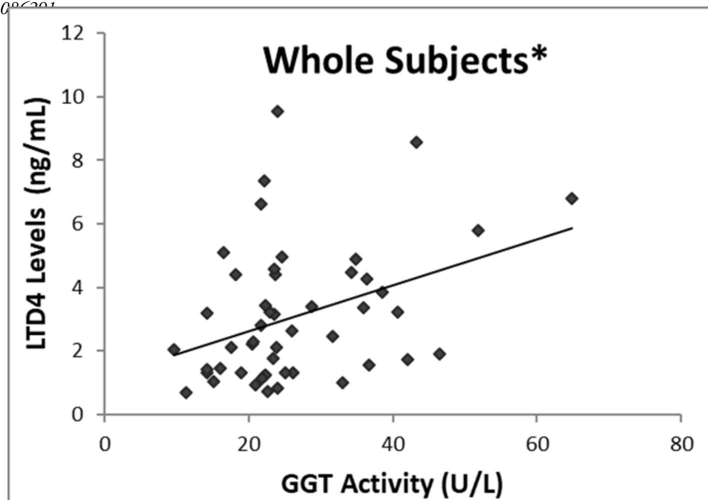
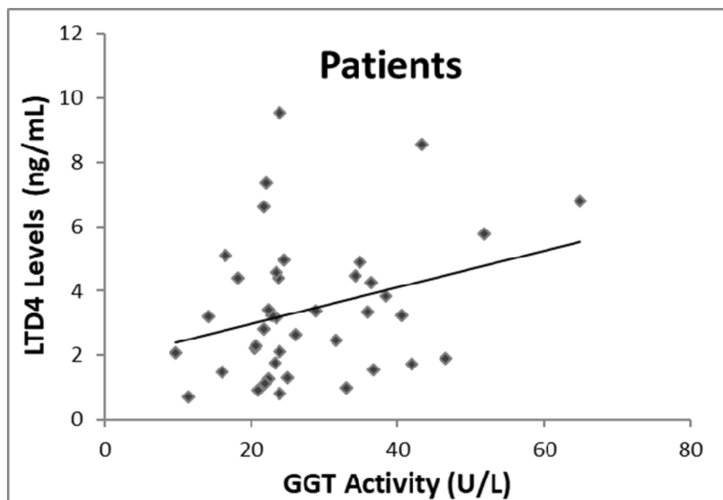
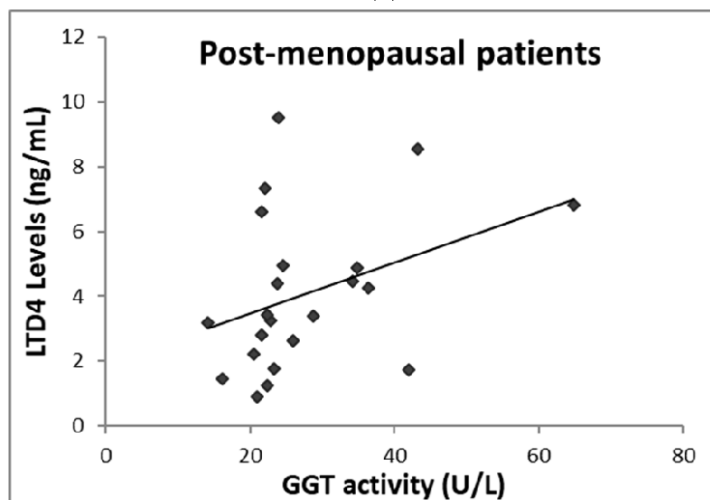


(a)



(b)



(c)

* patients + controls (n=51)

Figure 1. Correlations between LTD4 and GGT in whole subjects (a), only patients (b) and, post-menopausal patients (c).

When the whole study group was examined, a statistically significant positive correlation was observed between GGT activity and LTD4 levels (R=0.349, p=0.014) (Figure 1a). However, a positive but not statistically significant correlation was observed between GGT activity and LTD4 levels in patients (Figure 1b). There was also a positive correlation between these parameters in post-menopausal patients (R=0.437, p=0.042) (Figure 1c). A significant correlation between high GGT activity and LTD4 levels in breast cancer patients shows the role of GGT in producing LTD4. Data from previous studies demonstrated the function of the GGT family could be catalysis LTC4 to LTD4 (Hanigan, 2014). Here we indicate that GGT participates in the inflammatory response in breast cancer patients. Our findings are consistent with the studies that showed the importance of the GGT family in the inflammation process in mice (Shi, 2001), and pulmonary epithelial cancer cells (Lukic, 2016).

CONCLUSION

In conclusion, our study is the first to examine LTD4 levels in different molecular subclasses of breast cancer, higher LTD4 levels were observed in patients with both hormone-dependent and triple (-) breast cancer than controls. The participation of GGT in LTD4 synthesis, and the correlation between GGT and LTD4 indicates the important role of these two parameters in breast cancer. More studies with larger numbers of molecular subgroups may reveal the effect of inflammation in groups.

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CONFLICT OF INTEREST

Authors declare that there is no conflict of interest

AUTHOR CONTRIBUTION STATEMENT

Conception or design of the work (SYA), sample collection and clinical data (HK, SD), experiments and interpretation (SYA, EMS, SR), drafting the article (SYA, SR), critical revision of the article (SYA), final approval of the version to be published (SYA, SR, EMS, HK, SD)

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RESEARCH ARTICLE

The Role of Community Pharmacists in Increasing Patients' Drug Compliance

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The Role of Community Pharmacists in Increasing Patients' Drug Compliance

SUMMARY

In this study, the importance of compliance to treatment for the patient to benefit from the treatment, and the effects of patient compliance were determined. The study is a decisive type of research. The forms with 5-point Likert-type questions created by the researchers were asked to community pharmacists via the Internet. 110 pharmacists from different parts of Turkey participated in the survey. The data obtained in the research were analyzed with the SPSS ver. 25.0 program. The significance level (α) was determined as 0.05 in the analyzes made in the study. The Cronbach-alpha reliability coefficient for the developed scale was found to be 0.847. In this study, it is found that community pharmacists make an effort to increase the drug compliance of patients. There was no significant difference between the age and professional experience of the pharmacist in improving patient compliance. Pharmacists need to work more systematically to improve patients' drug compliance. It is thought that the concept of drug compliance is frequently included in the education curriculum of Pharmacy Schools. Still, the necessary educational content and learning opportunities are not sufficient to increase it.

Keywords: *compliance, drug compliance, community pharmacy, pharmacist*

Hastaların İlaç Uyumunun Arttırılmasında Toplum Eczacılarının Rolü

ÖZ

Bu çalışmada, hastanın tedaviden yarar görmesi için tedaviye uyumunun önemi ve hasta uyumunun nelerden etkilendiği belirlenmiştir. Çalışma, betimleyici tiptedir. Araştırmacılar tarafından oluşturulan 5'li Likert tipi soruların bulunduğu formlar, anket tekniği ile toplum eczacılarına internet ortamında uygulanmıştır. Ankete Türkiye'nin farklı yerlerinden 110 eczacı katılmıştır. Araştırmada elde edilen veriler SPSS ver. 25.0 programı kullanılarak analiz edilmiştir. Araştırmada yapılan analizlerde anlamlılık düzeyi (α) 0,05 olarak belirlenmiştir. Geliştirilen ölçek için Cronbach-alpha güvenirlik katsayısı 0,847 olarak bulunmuştur. Bu çalışmada, toplum eczacılarının hastaların ilaç uyumunu artırmak için çaba sarf ettikleri görülmektedir. Hasta uyumunu arttırmada eczacının yaş ve mesleki deneyimi arasında anlamlı bir fark görülmemiştir. Eczacıların hastaların ilaç uyumunu artırmak için daha sistemli çalışması gerekmektedir. Eczacılık Fakültesi eğitim müfredatında ilaç uyumu kavramının sıklıkla yer aldığı ancak arttırmak için gerekli eğitim içeriği ve öğrenme fırsatlarının yeterli olmadığı düşünülmektedir.

Anahtar Kelimeler: *uyum, ilaç uyumu, toplum eczacılığı, eczacı*

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INTRODUCTION

The patient's compliance with medication is following the instructions regarding the treatment and taking an active role in this process. Compliance ensures that the patient obtains optimum benefit from drug therapy. Lack of compliance leads to negativities in the treatment process. From diagnosis to prescribing, from patient education to monitoring, every step of the process affects compliance. From the beginning of the treatment, the last contact of the patient is often the pharmacist. It is thought that compliance will increase if the pharmacist informs the patient correctly and adequately, determines and resolves the problems, and makes necessary suggestions (Toklu, 2010).

Patient compliance is the patient's ownership and maintenance of treatment. In history, Hippocrates (400 BC) was the first to notice that some patients were not taking their prescribed medication and later complained that the treatment was not working. Then in 1882, for the first time in modern medicine, Robert Koch referred to noncompliant patients with tuberculosis, the term patient non-compliance was coined in the 1970s to analyze why people did not comply with medical directives (Vrijens, 2012). Patient non-compliance was defined as "the extent to which an individual's behavior (in terms of taking medication, following diets, or implementing lifestyle changes) does not align with medical or health advice" (Ulmer, 1976). Before 1980, when the medical mandate was expanded, and physician authority increased, patient compliance was seen as a tool for understanding when and why medical efforts failed (Spencer, 2018).

Patient compliance has clinical and economic importance. Irregular, under, or excessive drug consumption will affect the economy and the clinic of the patient. Some drugs should be taken long after symptoms have disappeared, some drugs are dangerous when taken in excess, and many are ineffective unless certain critical medication levels are taken. Thus, it is well known that patient compliance with medication and other treatment regimens can have a profound effect on outcomes. Compliance and persistence affect treatment efficacy, treatment costs, adverse event rates, disease-related sequelae rates and severity, general health, and quality of life (Kadambi, 2012).

In this study, the importance of compliance to treatment for the patient to benefit from the treatment, and the effects of patient compliance were determined.

MATERIALS AND METHODS

This study is a decisive type of research. The forms with 5-point Likert-type questions created by the researchers were asked to community pharmacists via the Internet. A convenience sampling method was used.

One of the variables that are frequently tried to be measured in educational research is attitude. Attitude has been defined as a 'learned tendency to react positively or negatively to a particular object, situation, institution, concept or another person.' The most widely used one to measure the attitudes, tendencies, and views of individuals and groups is the Likert scale, which was developed as a simplified version of the Thurstone scale (Willoughby,1932).

The sample size of 26,748 pharmacies in Turkey was determined as 96 with a sampling error of 0.10 and a probability of occurrence of 0.5 (Çalıkuşu, 2021; Büyüköztürk, 2019). Expert opinion was taken about the questions prepared for the questionnaire, and a preliminary application was made. Ethical approval was obtained for the survey study with the decision of Ankara University Rectorate Ethics Committee dated 12.04 2021 and numbered 60.

RESULTS

Pharmacists also need to play a role in ensuring patient compliance. The results of the survey conducted to determine what community pharmacists do to increase patient compliance and

how much they care about this issue are given in the tables below. 110 pharmacists from different parts of Turkey participated in the survey. The data obtained in the research were analyzed with the SPSS ver. 25.0 program. The significance level (α) was determined as 0.05 in the analyzes made in the study. The Cronbach-alpha reliability coefficient for the developed scale was found to be 0.847. General information about the participants is given in Table 1.

Table 1. General Information About the Participants

Age	Frequency	Percent
30 and below	43	39.1
31-40	23	20.9
41-50	15	13.6
50 and above	29	26.4
Professional Experience (Year)		
10 and below	60	54.5
11-20	18	16.4
21-30	12	10.9
30 and above	20	18.2
Total	**110**	

The average age of the pharmacists participating in the survey is 39 years, and the average time spent by community pharmacies is 14 years. The highest participation in the study was from the age group of 30 and below; the lowest participation was from the age group of 41-50. In terms of professional experience, the highest participation was found at 54.5% with 10 and below, and the lowest was 10.9% with 21-30 years. In Table 2, the results of the participants' attitudes towards increasing drug compliance are given.

Table 2. Results on the Attitudes of Community Pharmacists Towards Improving Patient Compliance with Medication

	Average	Standard Deviation
1) I provide education about the disease and medication to increase the patient's commitment to treatment.	4.02	1.031
2) To increase the commitment of my patients to treatment, I contact their physicians.	3.30	1.216
3) I test whether my patients use their previous medications properly.	4.06	0.989
4) I examine the reasons for my patients who do not use their medications correctly.	3.93	1.055
5) I give written information notes to my patients as well as verbal information about their medications.	4.08	1.142
6) I test patients' understanding of what I tell them about their medication by asking questions.	4.08	1.033
7) I want my patients to inform me from time to time about the use of their medications.	3.45	1.224
8) I congratulate and reward my patients who use their medicines rationally.	2.14	1.208
9) I monitor my patients in their drug use processes.	3.34	1.191
10) I care that the patient is satisfied with the service I provide.	4.68	0.676
11) I create opportunities for my patients to reach me quickly.	4.60	0.780
12) I think a mobile application where my patients will always reach me would be beneficial.	3.58	1.480
13) I discuss the treatment results of my patients' medications with them.	3.83	1.108
14) I stay in contact with my patients more when using multiple drugs.	3.75	1.127
15) My one-to-one care with the patient increases the patient's drug compliance.	4.66	0.707

Analysis of Variance (ANOVA) test was applied to analyze the pharmacists' responses to improve patient drug compliance in treatment. The test results are shown in Table 3.

Table 3. ANOVA Test Results

Age Groups	N	Average Score	Standard Deviation	Sig. (2-tailed)
30 and below	43	59.1628	6.88660	0.001*
31-40	23	50.5652	10.21063	
41-50	15	59.8000	9.20559	
50 and above	29	59.3793	9.09636	
Professional Experience by year				
10 and below	60	55.7833	9.34479	0.180
11-20	18	59.7222	7.27450	
21-30	12	60.5000	7.97154	
30 and above	20	58.9000	10.34103	
Total	**110**			

According to the ANOVA test results;

- ✓ While there is no significant difference between professional experience by years, a significant difference ($p<0.05$) was found between age groups.
- ✓ It has been determined that pharmacists in the age group of 30 and below, 41-50, and 50 and above try to significantly increase the patient's drug compliance in treatment compared to pharmacists in the age group of 31-40.

DISCUSSION

In different languages, terms such as commitment, compliance, and cooperation are used instead of 'patient compliance'. Definitions include terms that can mean obedience or shelve collaboration with the patient. In this study, the word 'compliance' is based.

The patient's non-compliance with drug use is significant as it affects the patient's quality of life, the clinic of the disease, and the economy (Nasseh, 2012). In addition, it is thought that pharmacists have a great responsibility to increase the drug compliance of the patients. They have competencies related to rational drug use in the national core education program. There are also many studies on the pharmacist's role in rational drug use (Toklu, 2015; Pehlivanlı, 2021; Khalil, 2021).

74.6% of the pharmacists who participated in the survey stated that they gave information to the patient about medicine and disease. In the studies conducted, it was determined that patient compliance increased when the pharmacist gave information to the patients (Goggin, 2010; Jimmy, 2011; Darbishire, 2018). It can be said that the attitudes of most pharmacists in Turkey in providing education to patients about their medicines have a positive effect on patient compliance. 71.8% of the pharmacists stated that they gave written information notes to their patients along with verbal information about their drugs, and 75.4% even indicated that they tested their patients' understanding of the drugs. Underneath this, it means that community pharmacists are willing to provide education to their patients, and there is no doubt that compliance will increase with the teaching of patients (López Cabezas, 2006). In particular, pharmacists or clinical pharmacists who have gained competence in patient compliance during their undergraduate education will have an essential role in increasing patient compliance (Savaş, 2020).

44.5% of community pharmacists said they monitor their patients' drug use processes. 63.6% of the pharmacists indicated that they provided the opportunity to discuss the treatment results with the patient. Thus, patient participation can also

be provided. In community pharmacies, 90.9% of pharmacists state that one-to-one care with patients increases their drug compliance, prevents recurrence of hospitalizations, and provides economic savings (López Cabezas, 2006). Some studies show that patients experience severe illnesses and hospitalizations again, as well as financial losses because they do not take their medications as prescribed (Giannetti, 2016; Su, 2019; Sokol, 2005; Colom, 2003; Fallowfield, 2009; Hughes, 2007).

In our study, 40.9% of community pharmacists contact patients' physicians only to increase patient compliance. Studies are emphasizing the importance of physician-pharmacist collaboration to improve patient compliance (Krummenacher, 2011). Factors affecting patient compliance have been determined such as socioeconomic factors, factors related to the healthcare team and the system, factors related to the situation, factors related to treatment, and patient-related factors (Kardas, 2013).

69.1% of pharmacists are analyzing the reason for this behavior in patients who do not use their medicines correctly. This approach will improve the factors affecting patient compliance. It will raise the awareness of the pharmacist to increase patient compliance. 49.1% of community pharmacists participating in the survey stated that they want their patients to inform them about the use of their medicines from time to time. It is thought that patient follow-up by the pharmacist is an attitude that will increase patient compliance (Jimmy, 2011).

12.8% of pharmacists stated that they congratulated and rewarded their patients who used compatible and rational drugs. There are studies stating that motivational interviews increase patient motivation and increase the permanence of compliance (Krummenacher, 2011). To improve this rate, it would be beneficial for the pharmacist to give importance to motivational interviews.

Almost all community pharmacists (94.6%) care about the patients' satisfaction with the service they provide. 90% of pharmacists stated that communication tools such as the telephone provide convenience for the patient to reach them. In addition, only 55.4% of pharmacists believe that patients can get them more easily through a mobile application. From this point of view, community pharmacists are people who are easily called by patients most of the time.

One of the reasons for the patient's noncompliance with treatment is the use of multiple drugs (Kardas, 2013). According to the survey results, 60% of the community pharmacists stated that they spend more time with their patients who use multiple drugs.

With the development of technology, different suggestions and measures are also emerging. Used by pharmacists and physicians in the US, the DirectRx program develops plans, programs, and software designed to track and improve patients' compliance rates while motivating patients' results to provide feedback to their physicians and pharmacists. These systems continuously monitor compliance and permanence, resulting in better outcomes, enabling early detection of compliance problems and appropriate intervention (DirectRx Pharmacy, 2020).

Patient compliance does not depend only on the pharmacist, it occurs with many factors. The physicians also have responsibilities. It is crucial to apply the drug supply process recommended by the World Health Organization (Toklu, 2015).

As a result, high patient compliance increases the quality of life, prolongs the lifetime, and provides financial savings to the health system. To improve patient compliance, the pharmacist should be in effective communication with the patient, provide information about patients' drugs within the framework of the principles of rational drug use, test that the information is understood by the patient, and permanent with small information notes or labels to be attached on the drug box, if necessary, and areas, where the patient can easily reach the pharmacist should be created.

In this study, it is seen that community pharmacists make an effort to increase the drug compliance of patients. There was no significant difference between the age and professional experience of the pharmacist in improving patient compliance. However, pharmacists need to work more systematically to improve patients' drug compliance. It is thought that the concept of drug compliance is frequently included in the education curriculum of Pharmacy Schools. Still, the necessary educational content and learning opportunities are not sufficient to increase it. Motivational interviewing, which is especially important in the literature, can be included in the Pharmacy Schools' curriculum. Thus, drug compliance can be achieved by increasing patient motivation.

CONFLICT OF INTEREST

All the authors of this article declared no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Data collecting, experimenting, data analysis and interpretation (FÇ). Research concept and design, data, data analysis, and interpretation, manuscript draft, final approval (MÇ). Research concept and design, data, data analysis, and interpretation, final approval (GÖ).

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RESEARCH ARTICLE

Development of Innovative Cosmetic Formulations to Help Fungal Treatment and Testing the Efficiency of Formulations

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Development of Innovative Cosmetic Formulations to Help Fungal Treatment and Testing the Efficiency of Formulations

SUMMARY

In the fungus of hands and toenails, the thickening of the nail and its yellow color is the first signs of attention. The fungus of hands and toenails is mainly caused by Trichophyton rubrum dermatophyte. They have antifungal properties due to the components of lavender oil, geranium oil, and tea tree oil structures. Oral antifungal agents used the treatment of nail fungus can cause serious side effects, especially the liver. Therefore; topical applications have been given importance in recent years. However; in topical applications, antifungal agents have difficulties sending to the target area. Therefore; nanoemulsion technology was preferred in the study. Nanoemulsion formulations of essential oils were prepared using the ultrasonication method. Centrifugal and thermal tests were applied as preliminary stability to the formulations, the pH value, viscosity, droplet size, and polydispersity index of the formulations passing this step were measured, and organoleptic controls were performed. Antifungal efficacy and release studies were performed on the formulations F4P3-I (pelargonium), F4P3-L (lavender), F4P3-C (tea tree), and F4P3-K (mixture), which were successful as a result of all the tests. According to the study, it was concluded that F4P3-I, F4P3-L, F4P3-Ç, and F4P3-K formulations might help in the treatment of fungi.

Key Words: *Lavender oil, geranium oil, tea tree oil, nanoemulsion, ultrasonication*

Mantar Tedavisine Yardımcı Yenilikçi Kozmetik Formülasyonların Geliştirilmesi ve Formülasyonların Etkinliğinin Test Edilmesi

ÖZ

El ve ayak tırnaklarındaki mantarlarda genellikle tırnağın kalınlaşması ve sarı bir renk alması ilk dikkat çeken belirtilerdir. El ve ayak tırnaklarındaki mantara çoğunlukla Trichophyton rubrum dermatofiti neden olmaktadır. Lavanta yağı, ıtır yağı ve çay ağacı yağı yapılarında bulunan bileşenlerden dolayı antifungal özelliğe sahiptirler. Tırnak mantarı tedavisinde kullanılan oral antifungal ajanlar özellikle karaciğer üzerinde ciddi yan etkilere yol açabilmektedir. Bu yüzden son yıllarda topikal uygulamalara önem verilmiştir. Ancak topikal uygulamalarda da antifungal ajanların hedef bölgeye gönderilmesinde zorluklar yaşanmaktadır. Bu nedenle, çalışmada nanoemülsiyon teknolojisi tercih edilmiştir. Ultrasonikasyon yöntemi kullanılarak uçucu yağların nanoemülsiyon formülasyonları hazırlanmıştır. Formülasyonlara ilk önce santrifüj ve termal testler uygulanmıştır ve stabil kalan formülasyonların pH değeri, viskozite, damlacık boyutu ve polidispersite indeksi ölçülmüştür ve organoleptik kontrolleri yapılmıştır. Tüm testler sonucunda başarılı olan F4P3-I (ıtır), F4P3-L (lavanta), F4P3-Ç (çay ağacı) ve F4P3-K (karışım) formülasyonlarında antifungal etkinlik ve geçiş çalışmaları gerçekleştirilmiştir. Çalışmaya göre F4P3-I, F4P3-L, F4P3-Ç, F4P3-K formülasyonlarının mantar tedavisine yardımcı olabileceği sonucuna varılmıştır.

Anahtar Kelimeler: *Lavanta yağı, ıtır yağı, çay ağacı yağı, nanoemülsiyon, ultrasonikasyon*

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INTRODUCTION

Various methods are used in the treatment of hands and toenails fungus. These methods include chemical or mechanical removal of nails, treatment with systemic antifungal drugs, treatment with topical nail polishes, or a combination of these (Denning, 1995). In most cases, oral antifungal agents such as terbinafine, itraconazole, and fluconazole are used because they penetrate the nail bed and nail plate. However, the common side effects of these drugs include headaches, gastrointestinal symptoms, nausea, and rashes (Kreijkamp-Kaspers, 2017). As a topical treatment, amorolfin, ciclopirox, etc., products that are usually in solution form are used. Topical antifungal agents have the advantage of causing fewer side effects, while their effectiveness is limited, and treatment times are extended due to low nail plate penetrations (Elewski, 2013).

In recent years, nanoemulsion technology has been used in cosmetics, medicines, paints, etc. Nanoemulsions are oil-in-water (o/w) or water-in oil (w/o) emulsions with average droplet diameters ranging from 50-1000 nm (Shah, 2010). Nanoemulsions have several advantages, including high tolerability, rapid biodegradation, high bioavailability, good stability, and reasonable skin penetration rates of the active agents (Tadros, 2004; Aboofazeli, 2010). High energy or low energy methods are used to prepare of nanoemulsions (Anton, 2009). In high-energy methods, ultrasonics, microfluidizers, and high-pressure homogenizers are used (Graves, 2005; Mason, 2006; Jafari, 2007). In low-energy, phase inversion temperature, and phase inversion composition methods are used (Marszall, 1975; Shinoda, 1986).

This study aims develop formulations in the form of nanoemulsions, which can provide a more effective penetration of the active substances from the nail in the treatment of nail fungus and to examine their antifungal activity and release profiles with *in vitro* experiments.

MATERIAL AND METHODS

Materials

Lavandula angustifolia oil, *Melaleuca alternifolia* oil and *Pelargonium graveolens* oil were purchased Herbarom Laboratoire (Aouste-sur-Sye, France). Geogard Ultra is obtained from Lonza Group AG (Basel, Switzerland). Pluronic® F68 was obtained from BASF (Ludwigshafen, Germany). Transcutol® HP is obtained from Gattefosse (Lyon, France). Tween 20® (Polysorbate 20) and RPMI-1640 medium; were purchased from Merck KGaA (Darmstadt, Germany). *Trichophyton rubrum*, ATCC 28188, is purchased from ATCC (Virginia, USA). Ketoconazole is purchased from Liofilchem, Inc. (Italy). All organic solvents and other chemicals were analytical grade and obtained from Merck KGaA.

Preparation Method for Nanoemulsion Formulations

The essential oils are added to Transcutol® HP. Minimum inhibition concentrations (MIC) of essential oils against *Trichophyton rubrum* dermatophyte have been found in the literature and used in formulations at this rate (Shin, 2004). The oil phase is added into the water phase, which comprised a Pluronic® F68 and Geogard Ultra mixture at the same temperature (20 °C). To see whether the pre-mixing process affects on the characterization of nanoemulsion formulations, only half of the formulations had an ultrasonication method and the other half was pre-mixing and ultrasonication.

In the pre-mixing process, the formulations were mixed under constant stirring (8,100 rpm) and temperature (20°C) with an Ultra-Turrax (IKA T-25 Digital, Germany) for 5 m. In the ultrasonication process, the formulations were sonicated using a Hielscher UP200Ht probe-type sonicator (Hielscher®, Teltow Germany) at 50% amplitude level for 20 and 30 minutes to obtain a nanoemulsion.

Table 1. Preparation of Nanoemulsion Formulations of Essential Oils

Ingredients	F4P3-L (%)	F4P3-I (%)	F4P3-Ç (%)	F4P3-K (%)
Lavandula angustifolia oil	0.05	-	-	-
Melaleuca alternifolia oil	-	-	0.1	-
Pelargonium graveolens oil	-	0.05	-	-
Mixture (Lavender, Tea Tree Oil, Geranium)	-	-	-	0.2
Pluronic® F68	0.033	0.033	0.067	0.133
Transcutol® HP	0.067	0.067	0.133	0.267
Geogard Ultra	0.75	0.75	0.75	0.75
Water	99.1	99.1	98.95	98.65

Characterization of Nanoemulsion Formulations

Dynamic light scattering, called Photon Correlation Spectroscopy (PCS), is used to analyze fluctuations in the scattering intensity of droplets and particles due to Brownian motion (Ruth, 1995). Nanoemulsion droplet size, polydispersity, and zeta potential can be evaluated by PCS using a particle size analyzer. The polydispersity index indicates the quality or homogeneity of the dispersion (Li, 2011).

Particle Size and Zeta Potential Measurements

The mean diameter, polydispersity index (PI), and zeta potential of each sample were obtained using a Malvern Zetasizer Nano ZS (Malvern Instruments, U.K.) at 25°C. Before all measurements, the formulations were diluted with distilled water 1/100 in the flask.

***In Vitro* Release Study**

Active components (Linalyl acetate, Terpinene oil, Citronellol) of essential oils released from nanoemulsion formulations were performed using the dialysis bag technique. The dialysis bags (MWCO: 12-14 kDa, Spectrum Laboratories, Inc., CA) were soaked and preconditioned before the experiment. The required amount of formulation (10 ml) was placed into the preconditioned dialysis bag. Then, the dialysis bag is put in 150 ml phosphate-buffered saline (pH: 7.4) and incubated in a thermostatic reciprocating shaker maintained at 37 ± 0.5°C and continuously shaken at 300 rpm. An aliquot of 1 ml of release medium was withdrawn at predetermined time intervals (0.50, 1, 2, 3, 4, 5, 6, 7, and 8 h) and replaced immediately with the same volume of fresh medium to maintain the sink conditions. The concentration of active components in the aliquot was quantified using gas chromatography-mass spectrometry (GC/MS).

GC/MS Analysis

A pharmacopeia method is used for the GC/MS analysis of active components of essential oils during release studies. For this purpose, an Agilent Cary 60 system (Agilent Technologies, California, USA) consisting of an HP-5ms Ultra Inert, 30 m x 250 µm x 0,25 µm column compartment. The chromatograms were monitored and integrated using Agilent ChemStation software. While preparing the samples, 5 ml sample was diluted in 1.5 ml water: acetone mixture, and 2 ml sample were injected. Helium gas with a flow rate of 1.1 ml / min and a split ratio of 10:1 was used as the carrier gas. The injector temperature is 220°C. The initial and end temperatures of the analysis are 60°C and 260°C, respectively. The temperature increase rate was 3°C/min and the total analysis time took 66.6 min.

Disk Diffusion Method

The antifungal efficacy of the formulations against *Trichophyton rubrum* ATCC 28188 standard strain *in vitro* was evaluated using the disc diffusion method. For this purpose, a mixture of 200 ml of 2% glucose and 2% agar was sterilized in an autoclave, then added to RPMI 1640 medium (Applichem, Darmstadt, Germany) and distributed in Petri dishes. The inoculum of the microorganism used in the experiment was

prepared by CLSI (Clinical and Laboratory Standards Institute) M38-A criteria. The dermatophyte conidia suspension used in the study was prepared using 5 ml of sterile 0.9% saline.

The concentration of dermatophyte conidia suspension is 1.5×10^6. The prepared conidia suspension was spread on the surface of Petri dishes containing sterile RPMI 1640 medium with 2% glucose. The Petri dishes were left to dry under aseptic conditions for 15 minutes. Then, 10 µl of formulation impregnated discs were added to the Petri plates. Discs containing 10 µg Ketoconazole (Liofilchem) are included in the study as a control. Petri dishes were incubated at 25°C for 4-7 days. The activity is evaluated by measuring the inhibition zones formed around the discs at the end of the incubation.

RESULTS AND DISCUSSION

In this study, nanoemulsion formulations of essential oils were successfully prepared using the ultrasonication method with the mixture of Pluronic® F68, Transcutol® HP, *Lavandula Angustifolia* oil, *Melaleuca alternifolia* oil, and *Pelargonium graveolens* oil. According to the literature research, studies have been found on pre-mixing while creating nanoemulsion formulations with this method (Hosseini, 2015; Carpenter, 2016). The formulations that were applied the pre-mixing process could not be stable. It is seen that when the ultrasonication process time increased, the particle size and polydispersity index (PDI) value of formulations decreased. When the droplet size and PDI values are examined, it was found that formulations with 30 minutes ultrasonication process, oil phase: surface- active substance ratio of 1:2 and Pluronic® F68 as surface-active substance and Transcutol® HP as co-surfactant were more suitable. The average particle size and polydispersity index of the formulations are shown in Table 2. The smallest average size (108.20 nm) was observed in the F4P3-K formulation. The PDI values of the formulations were lower than 0.3. PDI≤0.3 indicates excellent homogenous distribution (Salouti, 2014). The zeta potentials of the formulations are negative.

Table 2. Particle Size, PDI (polydispersity index), and Zeta Potential Values of the Formulations

Formulation code	Particle size (nm)	PDI	Zeta Potential
F4P3-L	182.4 ± 1.5	0.182 ± 0.05	-24,7 ± 0.79
F4P3-I	141.2 ± 0.9	0.220 ± 0.07	-29,8 ± 0.66
F4P3-Ç	188.1 ± 1.0	0.166 ± 0.04	-30,4 ± 1.22
F4P3-K	108.2 ± 0.3	0.111 ± 0.03	-37,2 ± 1.64

Antifungal activity test of nanoemulsion formulations that passed the long-term stability tests against *Trichophyton rubrum* was performed by disk diffusion method. The inhibition zone diameters of the formulations are given in Table 3.

Table 3. Inhibition Zone Diameters of the Formulations

Formulation code	Inhibiton Zone Diameter (mm)
F4P3-L	13,8
F4P3-I	12,9
F4P3-Ç	10,0
F4P3-K	11,0

Antifungal activity of nanoemulsion formulations of *Lavandula Angustifolia* oil, *Melaleuca alternifolia* oil, and *Pelargonium graveolens* oil has shown. However, the mixture of these oils did not show the expected antifungal activity.

CONCLUSION

Stability and characterization studies of nanoemulsion formulations have been successfully performed. The release of nanoemulsion and pure essential oil formulations was carried by the dialysis bag method in *in vitro* conditions. Figures 1-3 show the release profiles of nanoemulsion and pure formulations of essential oils. Active components of essential oils are examined in the release study. The release of the nanoemulsion formulation, which is a mixture of 3 essential oils from the membrane, is not examined due to having more than one essential oil. The antifungal efficacy test of nanoemulsion formulations against *Trichophyton rubrum* dermatophyte was performed by disc diffusion method under *in-vitro* conditions. The inhibition zone diameter of F4P3-L (lav-

ender nanoemulsion), F4P3-I (geranium nanoemulsion), F4P3-Ç (tea tree oil nanoemulsion), F4P3-K (lavender + geranium + tea tree oil nanoemulsion), and Ketoconazole (positive control) was 13.8, 12.9, 10.0, 11.0, 21,4 mm, respectively. While nanoemulsion formulations of lavender, geranium and tea tree oil showed antifungal activity, the nanoemulsion formulation in the form of the mixture (lavender + geranium + tea tree oil) did not show the expected antifungal activity.

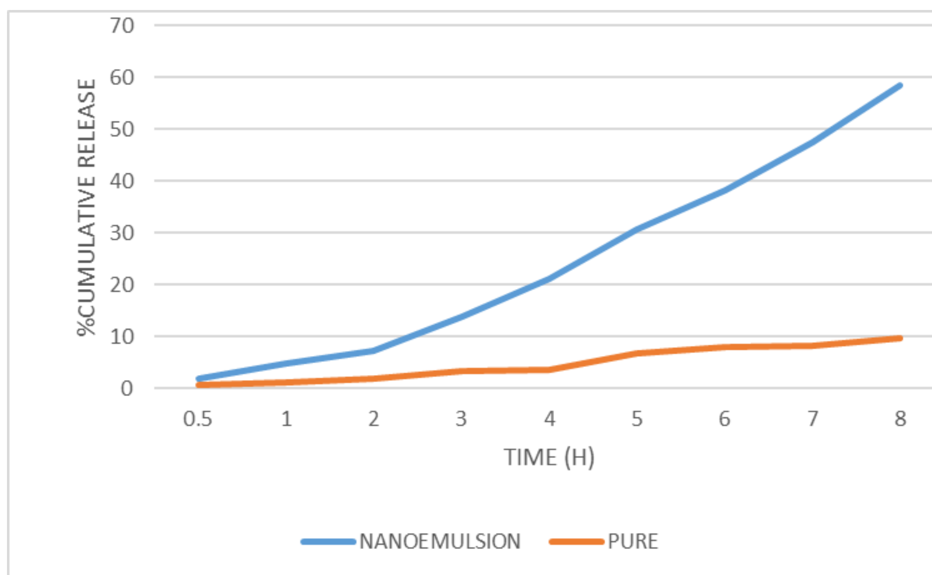

Figure 1. The cumulative release of nanoemulsion and pure lavender oil

Figure 1 shows the time-dependent release of nanoemulsion and pure lavender oil. The time-dependent release profile is determined according to the linalyl acetate substance, which is the significant component of lavender essential oil. After 8 hours, the nanoemulsion linalyl acetate component passed about 8.5 times more than its pure form.

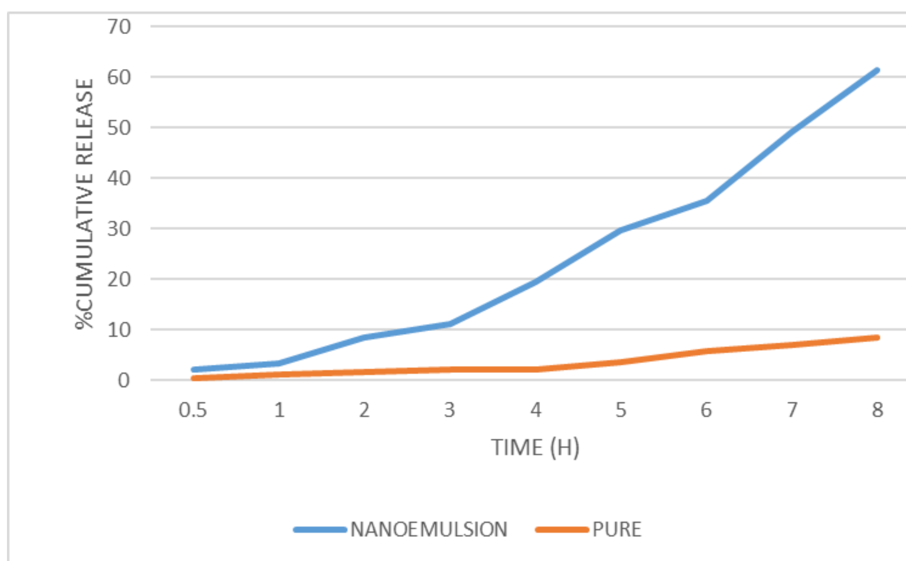

Figure 2. The cumulative release of nanoemulsion and pure tea tree oil

Figure 2 shows the time-dependent release of nanoemulsion and pure tea tree oil. The time-dependent release profile is determined according to the terpinenol substance, which is the significant component of tea tree essential oil. After 8 hours, the nanoemulsion terpinenol component passed about 7.5 times more than its pure form.

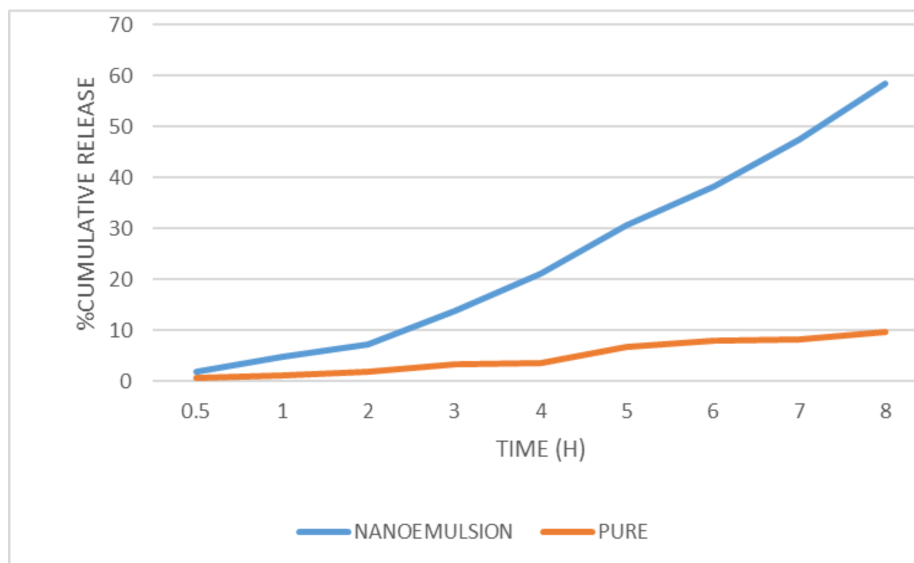

Figure 3. The cumulative release of nanoemulsion and pure geranium oil

Figure 3 shows the time-dependent release of nanoemulsion and pure geranium oil. The time-dependent release profile is determined according to the citronellol substance, which is the significant component of geranium essential oil. After 8 hours, the nanoemulsion citronellol component passed about six times more than its pure form.

As a result of all studies, it was concluded that F4P3-L, F4P3-I, F4P3-K, and F4P3-Ç formulations in nanoemulsion form could penetrate deeper and have better antifungal effects than their pure form.

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AUTHOR CONTRIBUTION STATEMENT

Initial literature survey, experimental design, data acquisition and analysis, interpretation of result, writing and revision of the manuscript (FGY). Initial literature survey, experimental design, sourcing for materials, laboratory work, data acquisition and analysis, interpretation of result (CA). Initial literature survey, experimental design, data acquisition and analysis, interpretation of result (BÖÇ). Initial literature survey, experimental design, sourcing for materials, laboratory work, data acquisition and analysis, interpretation of result (EMK).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Novel (p-Tolyl)-3(2H)-Pyridazinone Derivatives Containing Substituted-1,2,3-Triazole Moiety as New Anti-Alzheimer Agents: Synthesis, *In vitro* and *In silico* Assays

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Novel (p-Tolyl)-3(2H)-Pyridazinone Derivatives Containing Substituted-1,2,3-Triazole Moiety as New Anti-Alzheimer Agents: Synthesis, In vitro and In silico Assays

SUMMARY

Alzheimer's disease (AD) is a chronic neurodegenerative disease that is the most common cause of dementia. The risk of developing the disease increases with age. When the histopathology of the disease is examined, senile amyloid plaques, neurofibrillary tangle formation, synapse-neuron loss, and marked atrophy in the brain are detected. The decrease in the level of choline acetyltransferase, which is responsible for the synthesis of acetylcholine in Alzheimer's disease, is 58-90%. There is a great need for new drugs that target the basis of the cause of the disease, as existing drugs cannot stop the progression of the disease. In this study, triazole-pyridazinone derivative compounds showing acetylcholinesterase inhibition were synthesized and their inhibitions were investigated. Compound 6e exhibited the strongest inhibitory effect with a Ki value of 0.049 ± 0.014 µM (Tacrine Ki= 0.226 ± 0.025 µM). In addition, in silico studies were applied for all compounds.

Keywords: *3(2H)-pyridazinone, acetylcholinesterase, molecular docking*

1,2,3-Triazol Uygulaması Yeni Anti-Alzheimer (P-Tolil)-3(2H)-Piridazinon Türevleri: Sentez Çalışmaları, in vitro ve in siliko Analizleri

ÖZ

Alzheimer hastalığı (AH), demansın en yaygın nedeni olan kronik nörodejeneratif bir hastalıktır. Hastalığa yakalanma riski yaşla birlikte artar. Hastalığın histopatolojisi incelendiğinde senil amiloid plakları, nörofibriler yumak oluşumu, sinaps-nöron kaybı ve beyinde belirgin atrofi saptanır. Alzheimer hastalığında asetilkolin sentezinden sorumlu olan kolin asetil transferaz düzeyindeki azalma %58-90'dır. Mevcut ilaçlar hastalığın ilerlemesini durduramadığından, hastalığın temel nedenini hedef alan yeni ilaçlara büyük ihtiyaç vardır. Bu çalışmada asetilkolinesteraz inhibisyonu gösteren triazol-piridazinon türevi bileşikler sentezlenmiştir ve enzim inhibisyonları araştırılmıştır. Bileşik 6e, 0.049 ± 0.014 µM Ki değeri ile en güçlü inhibitör etkiyi göstermiştir (Takrin Ki= 0.226 ± 0.025 µM). Ayrıca sentezlenen tüm bileşikler için in siliko çalışmalar yapıldı.

Anahtar Kelimeler: *3(2H)-piridazinon, asetilkolinesteraz, moleküler yerleştirme*

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INTRODUCTION

Alzheimer's disease (AD) is an age-related progressive neurodegenerative disease that is characterized by cognitive impairment, has a high incidence in the elderly, and gradually results in death (Arora, Alfulaij, Higa, Panee, & Nichols, 2013; Daulatzai, 2016; Kumar, Kumar, Keegan, & Deshmukh, 2018). Today, around 50 million people worldwide, especially older people, suffer from dementia. According to estimates, the number of patients between 2040 and 2050 is reported to be approximately 130 million. Most dementia cases (70%) are due to AD (Cummings, Lee, Zhong, Fonseca, & Taghva, 2021; Taudorf, Nørgaard, Waldemar, & Laursen, 2021). Histopathologically, AD is characterized by loss of cholinergic neurons, amyloid b (Ab) peptide deposits (plaques), and intracellular neurofibrillary tangles of tau protein. Therefore, they have become important drug development targets (Cummings et al., 2021; Huang, Chao, & Hu, 2020; Taudorf et al., 2021). Currently, there is no treatment available to cure AD and stop its progression. The drugs are used to reduce the problems that occur in patients' ability to understand and comprehend behavioral findings (Srivastava, Ahmad, & Khare, 2021). When the brain tissues of AD patients were examined, it was shown that there was a decrease in acetylcholine synthesis and release and a decrease in acetylcholine transferase activity (Fani Maleki et al., 2020; Koronyo-Hamaoui et al., 2020; Zhang et al., 2020). For this purpose, acetylcholinesterase inhibitors have been developed to increase the residence time of acetylcholine in the synaptic gap (Birks, 2006).

In our previous studies, we synthesized a series of 3(2*H*)-pyridazinone derivatives and it has been reported that have been anticholinesterase biological activities (Bozbey et al., 2020; Çöl, Bozbey, Türkmenoğlu, & Uysal, 2022b; Özçelik, Özdemir, Sari, Utku, & Uysal, 2019). Likewise, the anticholinesterase effects of triazole structures have also been widely reported in the literature (Hosseini, Pourmousavi, Mahdavi, & Taslimi, 2022; Krasiński et al., 2005; Mina Saeedi et al., 2021; M. Saeedi et al., 2017). In the light of all this information, the combination of two bioactive compounds, triazole and pyridazinone, can be considered a significant strategy for drug design and discovery. Within the scope of our study, five novel triazole ring-substituted derivatives were synthesized, evaluated their acetylcholinesterase (AChE) enzyme inhibition, and *in silico* studies were performed.

MATERIALS AND METHODS

Chemistry

Unless otherwise noted, all of the reagents were commercial quality and were used without purification. The progress of reactions and the purity of the compounds were monitored by TLC using silica gel plates (250 µm, F_{254}) under UV light. NMR spectra were recorded on an Agilent Varian Mercury 400 MHz (^{1}H, 400 MHz; ^{13}C, 100 MHz), in $CDCl_3$ and DMSO-d_6 (internal standard tetramethylsilane). Chemical shifts ($\boldsymbol{\delta}$) are expressed as parts per million (ppm) downfield from tetramethylsilane (TMS) and the coupling constants (*J*) quoted in Hertz. Splitting patterns have been designated as follows: s (singlet), d (doublet), t (triplet) and m (multiplet), br (broad). The IR Spectra were recorded on Thermo Scientific Nicolet 6700 ATR/Fourier transform infrared spectrophotometer. High-resolution mass spectra data (HRMS) were collected in sing a Waters LCT Premier XE Mass Spectrometer (high sensitivity orthogonal acceleration time flight instrument) operating in the ESI (+) method, also coupled to an AQUITY Ultra Performance Liquid Chromatography system (Waters Corporation, Milford, MA, USA).

General procedure for the preparation of compounds (1) and (2)

The substituted 4'-methylacetophenone (1 mmol), glyoxylic acid monohydrate (1 mmol), and acetic acid (2 mL) were heated and stirred under reflux for 5 h. After the reaction was completed, the reaction mix-

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ture cooled down to room temperature, and 20 mL of water and ammonium hydroxide solution (25%) were added until the medium pH became 8. Then the reaction mixture was extracted with dichloromethane (3x20 mL). To the aqueous layer was added hydrazine hydrate (10 mmol) and the reaction mixture was refluxed for 3 h. After completion of the reaction, the reaction mixture cooled down to room temperature. The resulting white precipitate was filtered and recrystallized from ethanol (Xu et al., 2016).

General procedure for the preparation of compound (3)

A solution of **(2)** (1 mmol), K_2CO_3 (3 mmol), and ethyl 3-bromopropionate (3 mmol) in acetone (20 mL) was heated and stirred under reflux for 12 h. After the reaction was completed, the reaction mixture cooled down to room temperature. The resulting mixture was poured into ice-water, filtered off, and washed thoroughly with water. The resulting precipitate **(3)** was recrystallized from ethanol (Mantu, Luca, Moldoveanu, Zbancioc, & Mangalagiu, 2010).

General procedure for the preparation of compound (4)

To a solution of **(3)**, (1 mmol) in ethanol (20 ml) was added hydrazine hydrate (5 mmol). The reaction mixture was heated under reflux for 1 h. The precipitate obtained was washed thoroughly with water, dried, and recrystallized from methanol (Hassanien, 2003).

General procedure for the preparation of compounds 5, 6 (a-e)

A reaction mixture of appropriate hydrazide **(4)** (1 mmol) and aryl isothiocyanate (1 mmol) in ethanol (25 mL) was heated under reflux for 5 h and the progress of the reaction was monitored by thin layer chromatography. Next, the solution was cooled and the solid formed was filtered off, washed with diethyl ether, dried, and crystallized from ethanol. After, synthesized derivatives of thiocarbazide (1 mmol) were dissolved in the 2% solution of sodium hydroxide (20 mL) and heated under reflux for 4 h. After cooling, the solution was neutralized with dilute HCl. The precipitate was filtered off and then recrystallized from ethanol (Hassanien, 2003; Onkol et al., 2008).

2-(2-(4-phenyl-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-3-yl)ethyl)-6-(*p*-tolyl)pyridazin-3(2*H*)-one (6a)

White solid (52% yield), R_f 0.53 (CH_3OH-$CHCl_3$ 9:1), mp 125-127˚C, ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 11.72 (s, 1H, -NH), 7.58 (d, 1H, J=8.0 Hz, 5-CH), 7.51 (d, 2H, J=8.0 Hz, 2›- & 6›-CH), 7.48-7.39 (m, 3H, Ph-H), 7.34 (d, 2H, J=8.0 Hz, Ph-H), 7.24 (d, 2H, J=8.0 Hz, 3'- & 5'-CH), 6.92 (d, 1H, J=8.0 Hz, 4-H), 3.75 (t, 2H, J=4.0 Hz, $-CH_2-$), 2.98 (t, 2H, J=4.0 Hz, $-CH_2-$), 2.39 (s, 3H, $-CH_3$), ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 160.82 (5"-C), 145.93 (3-C), 141.01 (3"-C), 133.37 (6-C), 130.99 (4'-C), 130.36, 130.11, 129.76, 127.27 (Ph-C), 129.86 (2'-, 3'-, 5'- & 6'-C), 125.90 (4- & 5-C), 47.12 ($-CH_2-$), 26.55 ($-CH_2-$), 21.85 ($-CH_3$), FT-IR (neat, cm^{-1}) 3220.20, 2948.89, 1635.87 (C=O), 1573.71, 1416.03, 1125.45 (C=S), MS m/z (ESI) calcd for $C_{21}H_{19}N_5OS$ ($M+H^+$) 390.1389, found 390.1395.

2-(2-(4-(4-bromophenyl)-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-3-yl)ethyl)-6-(*p*-tolyl)pyridazin-3(2*H*)-one (6b)

White solid (42% yield), R_f 0.55 (CH_3OH-$CHCl_3$ 9:1), ^{1}H NMR (400 MHz, DMSO-d_6) δ_H 13.02 (s, 1H, -N*H*), 7.95 (d, 1H, *J*=8.0 Hz, 5-C*H*), 7.65 (d, 4H, *J*=8.0 Hz, 2'-, 6'-C*H* & Ph-*H*), 7.28 (d, 4H, *J*=8.0 Hz, 3'-, 5'-C*H*, Ph-*H*), 6.94 (d, 1H, *J*=8.0 Hz, 4-*H*), 4.19 (t, 2H, *J*=4.0 Hz, $-CH_2-$), 2.91 (t, 2H, *J*=4.0 Hz, $-CH_2-$), 2.34 (s, 3H, $-CH_3$), ^{13}C NMR (100 MHz, DMSO-d_6) δ_C 167.53 (5"-*C*), 159.11 (3-*C*), 148.08 (3"-*C*), 144.03 (6-*C*), 139.42 (4'-*C*), 135.99, 132.18, 130.88, 129.97 (Ph-*C*), 126.17 (2'-, 3'-, 5'- & 6'-*C*), 121.42 (4- & 5-*C*), 48.99 ($-CH_2-$), 25.32 ($-CH_2-$), 21.32 ($-CH_3$), FT-IR (neat, cm^{-1}) 3184.37, 3021.94, 1648.16 (C=O), 1579.49, 1439.92, 1164.97 (C=S), MS *m/z* (ESI) calcd for $C_{21}H_{19}N_5OSBr$ ($M+H^+$) 468.0494, found 468.0494.

2-(2-(5-thioxo-4-(*p*-tolyl)-4,5-dihydro-1*H*-1,2,4-triazol-3-yl)ethyl)-6-(*p*-tolyl)pyridazin-3(2*H*)-one (6c)

White solid (61% yield), R_f 0.59 (CH_3OH-$CHCl_3$ 9:1), ^1H NMR (400 MHz, DMSO-d_6) δ_H 13.60 (s, 1H, -N*H*), 7.93 (d, 1H, *J*=8.0 Hz, 5-C*H*), 7.63 (d, 2H, *J*=8.0 Hz, 2'- & 6'-C*H*), 7.31 (d, 2H, *J*=8.0 Hz, 3'-, 5'-C*H*), 7.26 (d, 4H, *J*=8.0 Hz, Ph-*H*), 6.94 (d, 1H, *J*=8.0 Hz, 4-*H*), 4.20 (t, 2H, *J*=4.0 Hz, -CH_2-), 2.96 (t, 2H, *J*=4.0 Hz, -CH_2-), 2.47 (s, 3H, -CH_3), 2.34 (s, 3H, -CH_3), ^{13}C NMR (100 MHz, DMSO-d_6) δ_C 168.41 (5"-*C*), 159.33 (3-*C*), 150.26 (3"-*C*), 144.38 (6-*C*), 139.92 (4'-*C*), 139.72, 132.00, 131.67, 130.81, 130.47, 130.01 (Ph-*C*), 128.78, 128.41 (2'-, 3'-, 5'- & 6'-*C*), 126.35 (4- & 5-*C*), 48.38 (-CH_2-), 25.22 (-CH_2-), 21.50 (-CH_3), 21.45 (-CH_3), FT-IR (neat, cm^{-1}) 3145.81, 3043.49, 2918.67, 2851.42, 1654.35 (C=O), 1586.50, 1412.93, 1127.07 (C=S), MS *m/z* (ESI) calcd for $C_{22}H_{21}N_5OS$ ($M+H^+$) 404.1545, found 404.1541.

2-(2-(4-(2-methoxyphenyl)-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-3-yl)ethyl)-6-(*p*-tolyl)pyridazin-3(2*H*)-one (6d)

White solid (38% yield), R_f 0.53 (CH_3OH-$CHCl_3$ 9:1), ^1H NMR (400 MHz, DMSO-d_6) δ_H 13.67 (s, 1H, -N*H*), 7.97 (d, 1H, *J*=8.0 Hz, 5-C*H*), 7.66 (d, 2H, *J*=8.0 Hz, 2›- & 6›-C*H*), 7.52 (t, 1H, *J*=8.0 Hz, Ph-*H*), 7.32-7.25 (m, 4H, 3'-, 5'-C*H* & Ph-*H*), 7.10 (t, 1H, *J*=4.0 Hz, Ph-*H*), 7.24 (d, 2H, *J*=8.0 Hz, 3'- & 5'-C*H*), 6.97 (d, 1H, *J*=8.0 Hz, 4-*H*), 3.76 (s, 3H, -OCH_3), 2.89 (m, 2H, -CH_2-), 2.78 (m, 2H, -CH_2-), 2.34 (s, 3H, -CH_3), ^{13}C NMR (100 MHz, DMSO-d_6) δ_C 168.49 (5"-*C*), 159.15 (3-*C*), 154.97 (Ph-*C*-OCH_3), 150.33 (3"-*C*), 144.12 (6-*C*), 139.55 (4'-*C*), 131.88, 129.96, 122.18, 121.27 (Ph-*C*), 126.14 (2'-, 3'-, 5'- & 6'-*C*), 113.31 (4- & 5-*C*), 56.37 (-OCH_3), 48.17 (-CH_2-), 24.66 (-CH_2-), 21.29 (-CH_3), FT-IR (neat, cm^{-1}) 3037.70, 2919.72, 1648.05 (C=O), 1581.69, 1463.50, 1155.46 (C=S), MS *m/z* (ESI) calcd for $C_{22}H_{21}N_5O_2S$ ($M+H^+$) 420.1494, found 420.1496.

2-(2-(5-thioxo-4-(4-(trifluoromethoxy)phenyl)-4,5-dihydro-1*H*-1,2,4-triazol-3-yl)ethyl)-6-(*p*-tolyl)pyridazin-3(2*H*)-one (6e)

White solid (69% yield), R_f 0.49 (CH_3OH-$CHCl_3$ 9:1), ^1H NMR (400 MHz, DMSO-d_6) δ_H 13.08 (s, 1H, -N*H*), 7.92 (d, 1H, *J*=8.0 Hz, 5-C*H*), 7.64 (d, 2H, *J*=8.0 Hz, 2'- & 6'-C*H*), 7.41 (d, 4H, *J*=8.0 Hz, Ph-*H*), 7.26 (d, 2H, *J*=8.0 Hz, 3'-, 5'-C*H*), 6.92 (d, 1H, *J*=8.0 Hz, 4-*H*), 4.20 (t, 2H, *J*=4.0 Hz, -CH_2-), 2.89 (t, 2H, *J*=4.0 Hz, -CH_2-), 2.34 (s, 3H, -CH_3), ^{13}C NMR (100 MHz, DMSO-d_6) δ_C 167.33 (5"-*C*), 159.07 (3-*C*), 147.54 (3"-*C*), 143.97 (6-*C*), 139.35 (4'-*C*), 136.64, 132.03, 130.56, 129.90 (Ph-*C*), 130.82 (-OCF_3), 126.18 (2'-, 3'-, 5'- & 6'-*C*), 121.46 (4- & 5-*C*), 49.32 (-CH_2-), 25.42 (-CH_2-), 21.29 (-CH_3), FT-IR (neat, cm^{-1}) 3038.88, 2926.27, 1654.81 (C=O), 1585.96, 1442.30, 1154.85 (C=S), MS *m/z* (ESI) calcd for $C_{22}H_{18}F_3N_5O_2S$ ($M+H^+$) 474.1212, found 474.1211.

Acetylcholinesterase (AChE) Enzyme Inhibition Studies

AChE enzyme was supplied ready-made. AChE enzyme activity was determined according to the method performed by Ellman's et al. (Ellman, Courtney, Andres, & Featherstone, 1961). The AChE enzyme has two substrates, DTNB [(Ellmans Reagent) 5,5-dithio-bis-(2-nitrobenzoic acid)] and acetyltiyocholiniodate. Thiocholine is formed because of the hydrolysis of the substrates. The thiocholine formed reacts with DTNB and forms the yellow 5-thio-2-nitrobenzoate anion. This molecule gives maximum absorbance at 412 nm wavelength (Shirinzadeh, Dilek, & Alım, 2022). A percent activity versus inhibitor concentration graph was drawn for the designation of the inhibition efficacy of each of the new derivatives on the AChE enzyme. The IC_{50} values were obtained from these graphs. For the calculation of K_i values, three different of these compounds concentrations and five substrate concentrations were used. The study also included an inhibition graph of the most effective

compound which was drawn. The same procedures were performed for Tacrine, the standard inhibitor of the AChE enzyme, and both IC_{50} and K_i values were calculated.

Molecular Docking

The interaction of compound **6e** with the AChE enzyme was investigated in molecular docking with *in silico* approaches. Molecular docking studies were applied to determine the amino acid residues in the active site of compound **6e** and the reference compound Tacrine and to calculate the binding parameters. Schrödinger 2021-2 software (Schrödinger Release 2021-2: Glide) was used in all docking studies to investigate the binding mode.

Molecular docking procedures specified in previous studies were applied (Anil, Aydin, Demir, & Turkmenoglu, 2022; Çöl, Bozbey, Türkmenoğlu, & Uysal, 2022a). The possible conformations of the studied compound were optimized using the "Ligand preparation wizard" program of Schrödinger 2021-2 (Schrödinger Release 2021-2: LigPrep). Possible tautomeric states of pH 7.0 ± 2.0 in the Epic ligand preparation portion were used to generate a net negative substitution change that varied in each case.

The AChE crystal structure was obtained from the protein database (https://www.rcsb.org/structure) and the crystal structure with PDB code number 1ACJ (Harel et al., 1993) was used. The crystal structure was prepared with the "Protein Preparation Wizard" interface of Schrödinger 2021-2 software (Schrödinger Release 2021-2: Protein Preparation Wizard; Epik). It was prepared by sequential processes such as deletion of water molecules, the addition of missing side chains and hydrogen atoms, protonation states, assignment of partial charges, optimization, and minimization using the OPLS-2005 force field.

Prime MM/GBSA (Schrödinger Release 2021-2: Prime)analysis was used to calculate ligand binding energies using the OPLS_2005 force field and the VSGB solvent model. MM/GBSA analysis was applied to determine the free binding energy of compound **6e** and the AChE crystal structure by molecular docking.

RESULTS AND DISCUSSION

Chemistry

In this study, five new AChE enzyme inhibitor compounds were synthesized (Figure 1) according to the general synthesis method outlined in Figure 2 and their anticholinesterase activities were examined. In the synthesis of the compounds, the 3(2*H*)-pyridazinone ring, which our study group worked on, was chosen as the main structure. In previous studies, hydrazone derivatives and sulfonyl hydrazide derivatives bearing pyridazinone ring were studied. In this study, these structures were modified and the triazole ring system was added to the general structure.

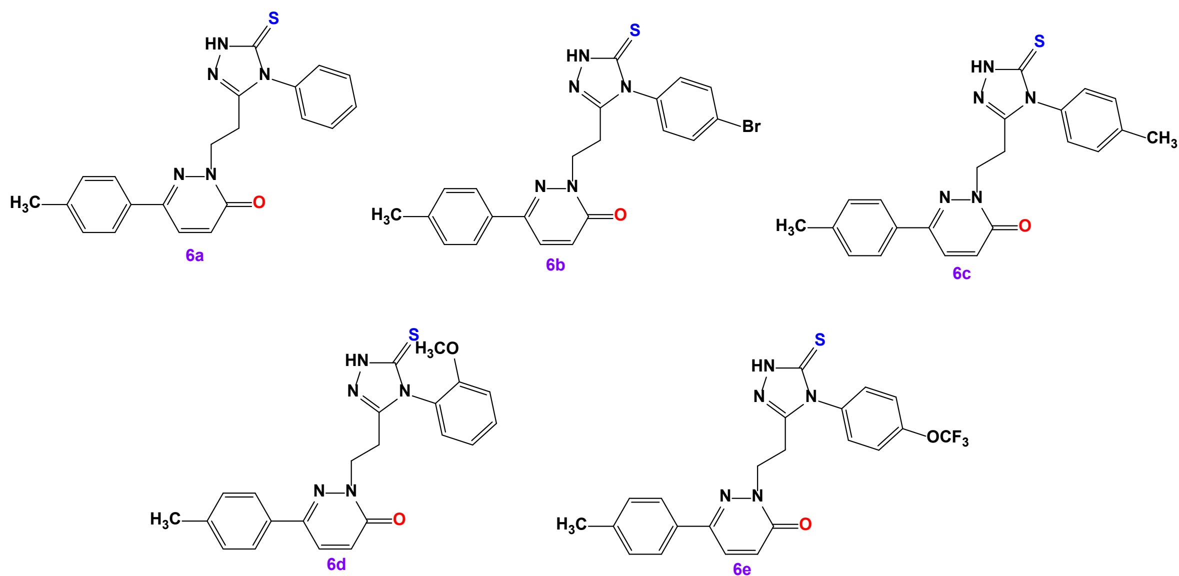

Figure 1. Synthesized compounds

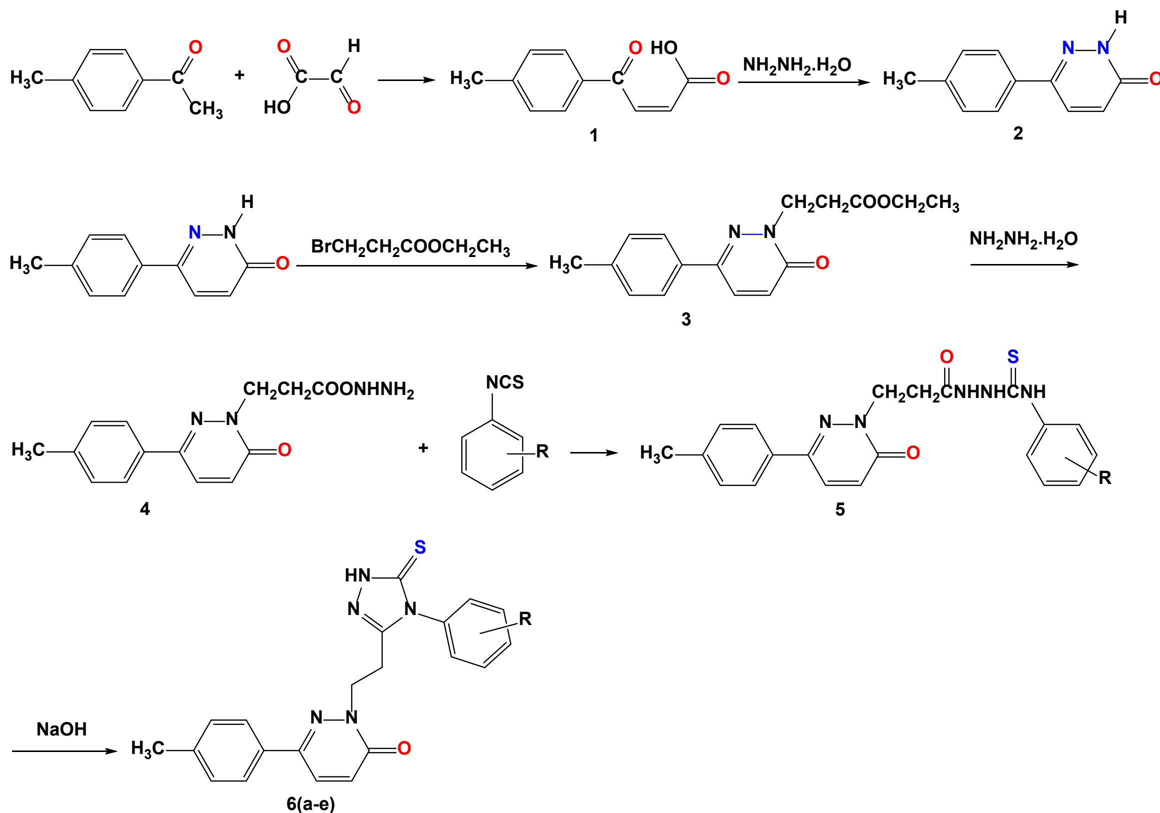

Figure 2. General synthesis method

The White-colored compounds **6**(**a-e**) were synthesized in yields ranging from 56-37% in general. The range of 13.67-11.72 ppm in the ^{1}H NMR spectrum showed triazole-N*H* peaks. Bridge of triazole and pyridazinone rings -CH_2CH_2- multiplet or triplet peaks were generally observed near 4.2-2.8 ppm in proton NMR (Figure 3). In the ^{13}C NMR spectrum, the pyridazinone ring carbonyl carbon supported structure accuracy in the range of 159.33-145.93 ppm. The specific thiocarbonyl group of the triazole ring was observed in the range of 168.49-160.82 ppm (Figure 4). In the FT-IR spectra, newly synthesized compounds **6**(**a-e**) exhibited characteristic ν(C=O) bands at 1654-1635 cm^{-1} for pyridazinone rings. The ν(N—H) stretching bands of the triazole rings were centered at 3220-3145 cm^{-1}. In addition, characteristic ν(C=S) bands were observed at 1164-1125 cm^{-1} (Figure 5).

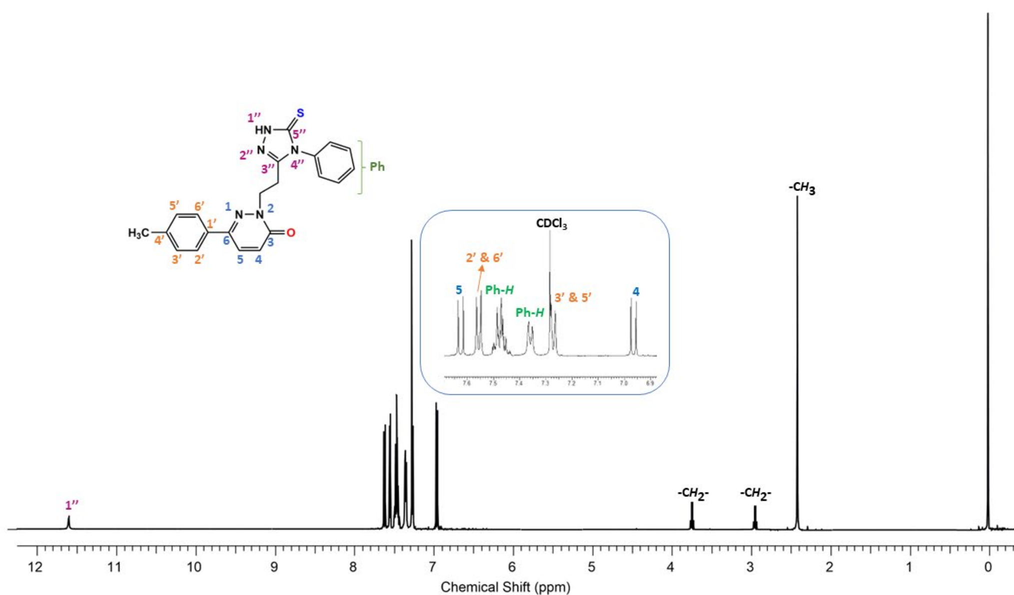

Figure 3. ^{1}H-NMR spectrum of compound **6a**

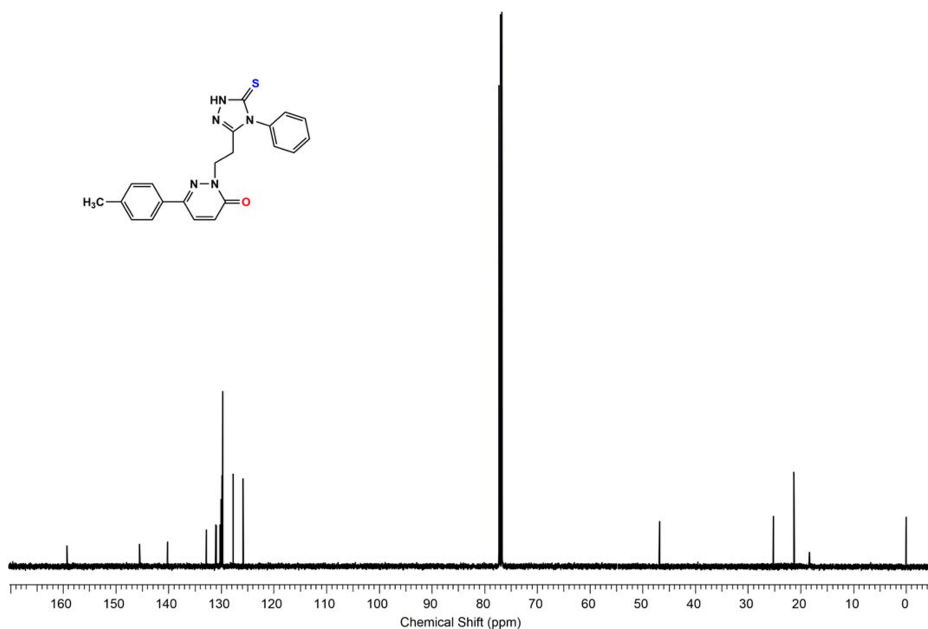

Figure 4. ^{13}C-NMR spectrum of compound **6a**

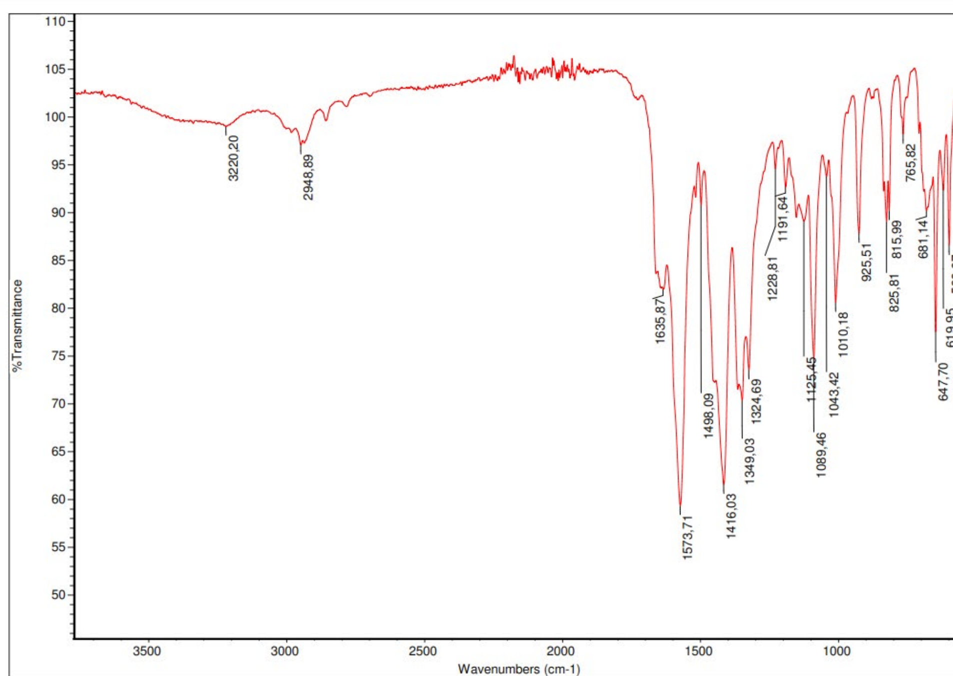

Figure 5. ATR-IR spectrum of compound **6a**

Biological evaluation and structure-activity relationship

IC_{50}, K_i, and inhibition types of five newly synthesized triazole-pyridazinone derivative compounds were determined. These values of the compounds are given in Table 1 and the IC_{50} graph and Lineweaver-Burk graph (B) of **6e** and Tacrine for AChE (Figure 6 and Figure 7). The IC_{50} values of the compounds were found as 0.310-0.592 µM and Ki values as 0.049 ± 0.014 - 0.484 ± 0.090 µM. The IC_{50} value of Tacrine, which we used as the reference compound, was determined as 0.519 µM and the Ki value as 0.226 ± 0.025 µM. When the contribution of the substituents to the structure was examined, the Ki value of the non-substituted compound **6a** was determined as 0.163 ± 0.017 µM. Compared to the non-substituted derivative (**6a**), 4-OCF_3 (**6e**) and 4-CH_3 (**6c**) groups in the structure increased the inhibitory effect, while the 4-Br (**6b**) and 2-OCH_3 (**6d**) groups decreased the inhibitory effect. The compound with the strongest inhibitory effect compared to Tacrine is compound **6e** with a Ki value of 0.049 ± 0.014 µM. These compounds were followed by **6c** and **6a** with Kı values of 0.098 ± 0.009, and 0.163 ± 0.017. The effect of the substituents on AChE activity was **6e** (4-trifluoromethoxy derivative) > **6c** (4-methyl derivative) > **6a** (non-substituted) > **6d** (2-methoxy derivative) > **6b** (4-bromo derivative). The inhibition type of compound **6a** was determined competitively as the reference compound Tacrine. In summary, especially the trifluoromethoxy substitute positively affected the activity.

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Table 1. The IC_{50} values, K_i constants and inhibition types were determined for **6(a-e)** molecules having inhibitory effects on AChE.

Compound	AChE			
	IC_{50} (μM)	R^2	K_i (μM)	Inhibition Type
6a	0.474	0.9714	0.163 ± 0.017	Competitive
6b	0.551	0.9899	0.484 ± 0.090	Noncompetitive
6c	0.523	0.9713	0.098 ± 0.009	Uncompetitive
6d	0.592	0.9932	0.413 ± 0.093	Noncompetitive
6e	0.310	0.9747	0.049 ± 0.014	Uncompetitive
Tacrine	0.519	0.9257	0.226 ± 0.025	Competitive

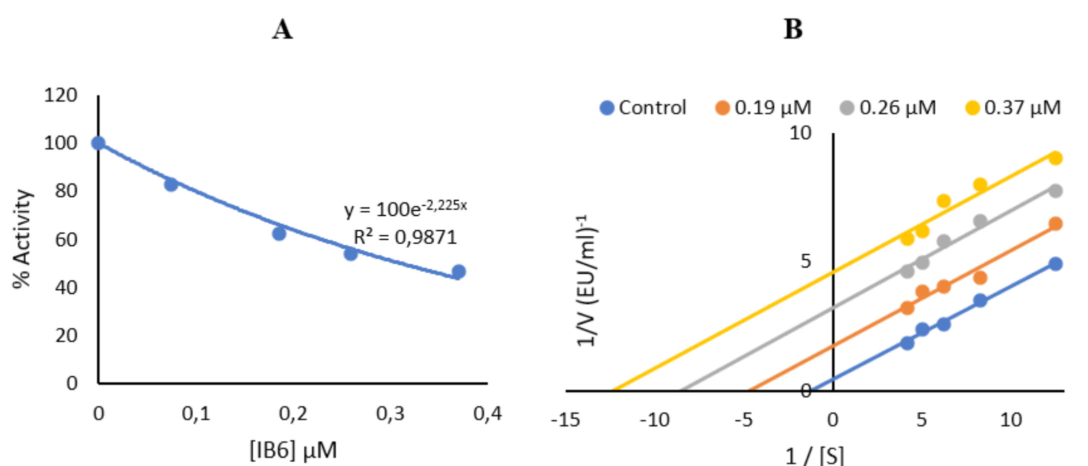

Figure 6. IC_{50} graph (A) and Lineweaver-Burk graph (B) of **6e** for AChE.

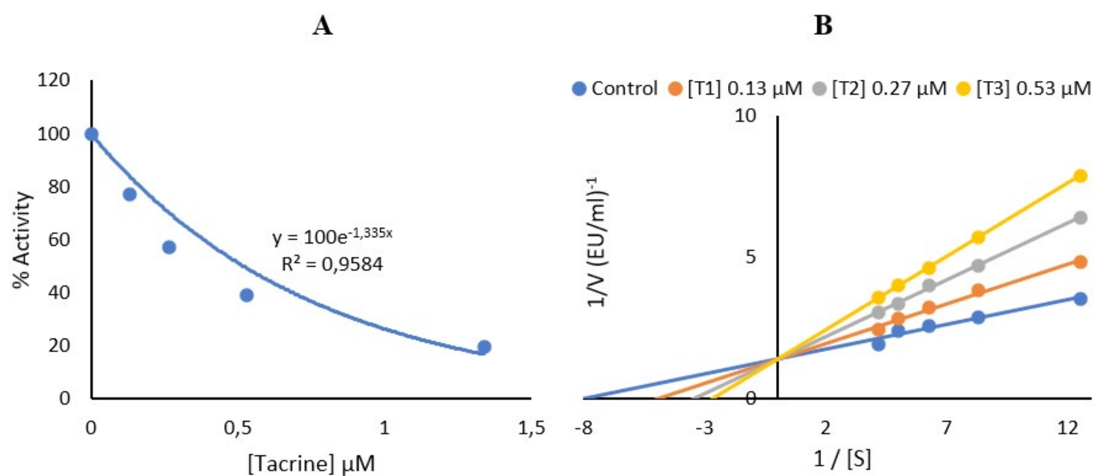

Figure 7. IC_{50} graph (A) and Lineweaver-Burk graph (B) of **Tacrine (TAC)** for AChE.

Molecular Docking

For the theoretical evaluation of the experimental activity of compound **6e** on AChE, binding interactions were determined by *in silico* approaches. Therefore, the crystal structure of the AChE enzyme (PDB ID: 1ACJ (Harel et al., 1993)) was used as the primary target for this study. The values of molecular docking results of **6e** and Tacrine compounds interacting with AChE, respectively, *in silico* approaches, were presented in Table 2.

Table 2. Binding parameter values as a result of *in silico* interaction of **6e** and Tacrine compounds with AChE crystal structure (PDB ID: 1ACJ (Harel et al., 1993)).

Parameters	6e	Tacrine
ΔG_{Bind} (kcal/mol)	-46.05	-32.14
Glide score (kcal/mol)	-8.850	-5.673
Docking score (kcal/mol)	-8.850	-5.673
Glide energy (kcal/mol)	-46.733	-32.657
Glide emodel (kcal/mol)	-44.843	-46.952

While designing compounds that can be new effective drug candidates, the results of the reference compounds used in the experimental activity are always taken into consideration. Therefore, molecular docking results of the compound synthesized with the reference compound with which the target interacts are important *in silico* approaches. Molecular docking results were none other than the binding parameter values shown in Table 2.

While the free binding energy of the binding parameters is -46.05 kcal/mol for compound **6e,** this value is -32.14 kcal/mol for Tacrine. When the glide score and docking score values are compared, it can be said, according to Table 1 that the docking score value of compound **6e** (-8.850 kcal/mol) is much better than the result of Tacrine (-5.673).

In addition, Glide energy and Glide emodel values, which are other important binding parameter values, were –46.733 kcal/mol and -44.843 kcal/mol for compound **6e**, respectively. These values were better than Tacrine.

The interactions with amino acid residues in the binding sites between the target and the ligand are as important as the binding parameters in the calculations for *in silico* approaches.

The 2D interaction diagram of **6e** and Tacrine compounds with the AChE crystal structure activated in molecular docking is presented in Figure 8. Figure 8(A) shows the amino acids in the active binding site in the 2D interaction diagram of **6e**, while Figure 8(B) shows the 2D interaction diagram of the PDB ID: 1ACJ (Harel et al., 1993) crystal structure interacting with the reference compound Tacrine.

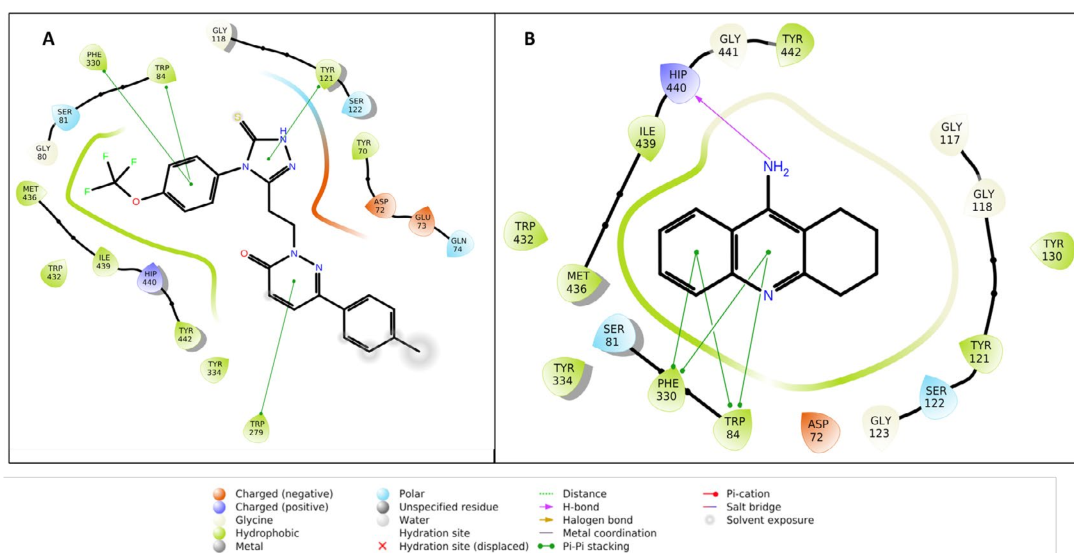

Figure 8. (A) 2D interaction diagram of **6e** compound with 1ACJ. (B) 2D interaction diagram of Tacrine with 1ACJ.

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Figure 9 shows the 3D surface model structure of compound **6e** docked in the main groove of the 1ACJ crystal structure and the interaction of amino acid residues in its active binding site.

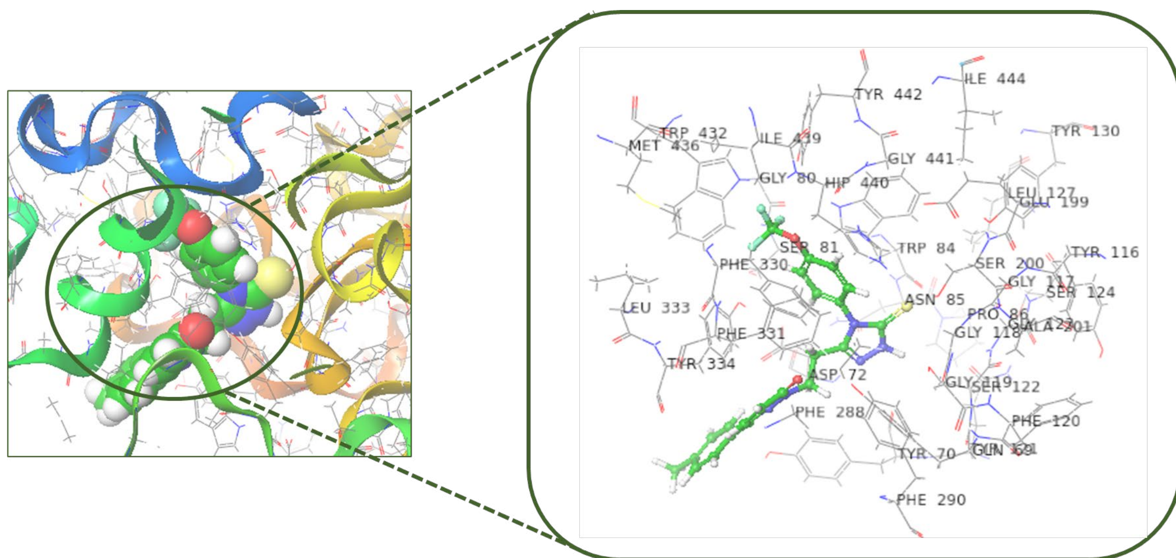

Figure 9. (A) Molecular docked structure of **6e** compound to 1ACJ crystal structure. (B) Amino acid residues at the binding site in the interaction of 1ACJ and **6e**.

In Figure 8(A), it was determined that compound **6e** interacts with the most important residues Trp 84, Phe 330 amino acid residues in this active site, in the binding site of AChE, and π- π interaction. In addition, the presence of π- π interaction of compound **6e** with Tyr 121 and Trp 279 is shown in Figure 8(A). Compound **6e** also appears to be well docked in the main cavity of the crystal structure. In Figure 8 (B), it is understood that the Tacrine compound makes **π-π** interaction with Trp84 and Phe 330, and hydrogen bond interaction with Hip 440. Molecular docking studies have been applied to understand the mechanism of action of **6e**, a compound that has the potential to act on AChE experimentally. For this reason, it can be said that the docking results of compound **6e** are better than Tacrine, the reference compound interacting with the target structure.

CONCLUSION

In summary, *in vitro* enzyme inhibitor activities were investigated by synthesizing five new compounds that we expect to have AChE enzyme inhibitor effects. The contribution of these groups to the activity was investigated by using non-substituted isothiocyanate, bromo, fluoro, methyl, methoxy and trifluoromethoxy substituted isothiocyanate derivatives in different positions in the synthesis of the derivatives. All synthesized derivatives **6(a-e)** were elucidated by spectroscopic analysis. Of the substituted pyridazinone derivatives, the p-trifluoromethoxy substituted derivative (**6e**) was found to be more potent against AChE inhibition. The molecular docking studies of the experimentally active compound **6e**, one of the compounds whose effects on the AChE enzyme were investigated, were investigated on the target crystal structure. It was noteworthy that Tacrine, the reference compound on the AChE enzyme, and compound 6e had similar binding sites and parameters. The results of the effect of compound 6e on the AChE enzyme, according to molecular docking with *in silico* approach, are promising.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

İ.B.M.; Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Visualization, G.T.Ö.; Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Visualization, B.T.; Methodology, Resources, Writing – review & editing, Visualization

Ş.G.; Resources, Writing – review & editing, Visualization, E.D.; Resources, Writing – review & editing, Visualization

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RESEARCH ARTICLE

Development and Radiolabeling Evaluation of ^{177} Lutetium-Tedizolid

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Development and Radiolabeling Evaluation of ^{177}Lutetium-Tedizolid

SUMMARY

Infection diseases is still one of the major health problems all around the world. Early diagnosis and differentiation of infection from other pathological conditions such as cancer and inflammation play a critical role in treating the infection in acute stages. Imaging techniques using in the infection diagnosis present some advantages such as, the ability to image the whole body, the detection of focal location and stage, and following up on infection. Although various antibiotics can be used in the treatment, there are some problems including serious side effects of antibiotics or the development of antimicrobial resistance in the clinics. In our study, tedizolid, a second-generation oxazolidinone antibiotic, was radiolabeled with ^{177}Lu radionuclide to develop a theranostic agent against gram-positive bacterial infections. The radiolabeling was performed under room conditions, and labeling efficiency and stability were evaluated by paper chromatography and HPLC. The optimum incubation period was found as 60 min to obtain high radiolabeling efficiency. Different mobile and stationary phases in paper chromatography were tested to determine the radiochemical impurities in ^{177}Lu-TDZ solution, and ITLC-SG was found to be proper as the stationary phase. In addition, ammonium hydroxide: methanol: water, and DTPA solutions were chosen as mobile phases. In the HPLC chromatogram, two different peaks were observed depending on the retention times of the free ^{177}Lu and ^{177}Lu-TDZ complex. Unfortunately, over 80% purity values were not obtained in the results of radiolabeling stability analyses; therefore, the addition of a chelating agent in the radiolabeling condition was suggested to increase the stability.

Key Words: *lutetium-^{177}, tedizolid, theranostic, infectious diseases*

^{177}Lutetium-Tedizolid'in Geliştirilmesi ve Radyoişaretlenmesi

ÖZ

Enfeksiyon hastalıkları hala dünyada temel sağlık problemlerinden birini oluşturmaktadır. Enfeksiyonun erken aşamalarda teşhisi ve kanser veya inflamasyon gibi diğer patolojilerden ayrımı, enfeksiyonu akut aşamalarda tedavi etmede kritik rol oynamaktadır. Enfeksiyon teşhisinde kullanılan görüntüleme yöntemleri tüm vücut görüntüsü alabilme, enfeksiyonu odağını ve evresini tespit edebilme ve hastalığı izleyebilme gibi avantajlara sahiptir. Enfeksiyon tedavisinde çeşitli antibiyotikler kullanılmasına rağmen, klinikte antibiyotiklerin ciddi yan etkileri ve antimikrobiyal direnç gelişimi gibi problemler mevcuttur. Çalışmamızda enfeksiyon için teranostik bir ajan geliştirme amacıyla gram pozitif bakterilere karşı etkili ikinci nesil oksazolidinon antibiyotiği olan tedizolid, ^{177}Lu radyonüklidi ile radyoişaretlenmiştir. Radyoişaretleme, oda koşullarında gerçekleştirilmiş ve işaretleme etkinliği ile stabilitesi, kağıt kromatografisi ve HPLC ile değerlendirilmiştir. Yüksek radyoişaretleme verimi elde etmek için optimum inkübasyon süresi 60 dakika olarak bulunmuştur. ^{177}Lu-TDZ çözeltisindeki radyokimyasal safsızlıkları belirleme amacıyla kağıt kromatografisi için farklı mobil ve sabit fazlar test edilmiş ve sabit faz olarak ITLC-SG uygun bulunmuştur. Ayrıca amonyum hidroksit: metanol: su ve DTPA çözeltileri mobil faz olarak seçilmiştir. HPLC kromatogramında serbest ^{177}Lu ve ^{177}Lu-TDZ kompleksinin alıkonma sürelerine bağlı olarak iki farklı pik gözlenmiştir. Ne yazık ki, radyoişaretleme stabilitesi testlerinin sonuçlarında %80'in üzerinde saflık değerleri elde edilememiştir, bu nedenle radyoişaretleme ortamına şelat yapıcı ajan eklenmesi önerilmiştir.

Anahtar Kelimeler: *lutesyum-^{177}, tedizolit, teranostik, enfeksiyon hastalıkları*

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INTRODUCTION

Infection is still one of the major health problems worldwide in view of the global coronavirus disease 2019 (Covid-19). Despite acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes Covid-19, according to the literature, secondary or concurrent bacterial infections, including *Staphylococcus aureus (S. aureus)* co-infections, have been detected in many patients with SARS-CoV-2 (Singh, Upadhyay, Reddy, & Granger, 2021). In addition to SARS-CoV-2, many viral respiratory pathogens (e.g., orthomyxoviruses) are the leading cause of seconder severe bacterial infections such as staphylococcal pneumonia (Mulcahy & McLoughlin, 2016). Hospital-acquired bacterial pneumonia (HABP) and ventilator-associated bacterial pneumonia (VABP) are remarkable burdens on the healthcare system due to mortality rate and the development of antibiotic resistance (Bart, Rubin, Kim, Farley, & Nambiar, 2020). *S. aureus* is one of the commonly identified pathogens in patients with HABP and VABP, and it also can cause various infections such as gastroenteritis, meningitis, osteomyelitis, septic arthritis, or urinary tract infections (Jones, 2010; Tong, Davis, Eichenberger, Holland, & Fowler, 2015). Different antibiotics (e.g., vancomycin, trimethoprim-sulfamethoxazole, linezolid, or teicoplanin) can be chosen depending on methicillin-resistant (MR) or infection syndromes in the management of *S. aureus* infections (Tong et al., 2015). Although vancomycin and linezolid are the first-line therapeutic agents in the treatment of pneumonia caused by MR *S. aureus*, they cause serious side effects such as nephrotoxicity and myelosuppression, respectively (Liu et al., 2011). Moreover, the development of antimicrobial resistance is increasing day by day. In addition to therapy of infection, diagnosis possesses a crucial role in the selection of an appropriate therapeutic agent and treating the infection in acute stages. The infection diagnosis comprises the investigation of patients' samples, the evaluation of patients' symptoms, laboratory tests, and imaging techniques. Imaging techniques present advantages, such as the ability to image the whole body, the detection of focal location and stage, and the following up of infection. However, they may be inadequate in the differentiation of infection from other pathological cases such as cancer or inflammation. Therefore, the development of effective treatment and imaging options for *S. aureus* is critical for avoiding the progression of infection and decreasing infection-related deaths.

Among imaging techniques, nuclear medicine techniques (gamma camera, positron emission tomography (PET), and single-photon emission tomography (SPECT)) are advantageous systems due to their ability to image the whole body, provide three-dimensional functional and physiological images, and use with other imaging techniques such as magnetic resonance imaging (MRI) or computed tomography (CT) as hybrid systems (PET/CT, PET/MRI, SPECT/CT) (Wu & Shu, 2018). Radiopharmaceuticals, used for diagnosis or therapy of various diseases, contain pharmaceutical parts and radioactive isotopes. They can be used in the diagnosis and therapy of various diseases including infection depending on the radioactive decay type of radionuclides. Infection can be imaged using different radiopharmaceuticals such as ^{67}Ga-citrate, ^{99m}Tc-diphosphonates, ^{99m}Tc/^{111}In l-white blood cells, ^{111}In-biotin in nuclear medicine clinics (Karpuz & Silindir-Gunay, 2022). Radionuclides (e.g., ^{99m}Tc, ^{18}F, ^{68}Ga, ^{123}I, ^{67}Ga, ^{201}Tl), which emit gamma (γ) or positron (β^+) rays, use for imaging purposes, while alpha- *(α) or beta-* (negatron, β^-) emitting ones (e.g., ^{213}Bi, ^{225}Ac, ^{223}Ra, ^{90}Y) are therapeutic radionuclides (Kassis, 2008; Velikyan, 2014). In addition, some radionuclides, that emit both γ or β^+ and α or β^-, are referred to as theranostic, and they are used for imaging and therapy purposes. Although ^{99m}Tc radiopharmaceuticals are widely used in nuclear medicine clinics, the development of new radiopharmaceuticals with different radionuclides is required because of the shortage of ^{99m}Tc. The theranostic approach, the combination of therapy and imaging, presents some ad-

vantages, including monitoring the pharmacokinetic/biodistribution profile and the targeting ability of a therapeutic agent, and rapid diagnosis and therapy. Radiopharmaceuticals, containing ^{131}I, ^{177}Lu, ^{111}In, or ^{64}Cu as a radionuclide part, can be used as theranostic molecules (Gutfilen, Souza, & Valentini, 2018; Levine & Krenning, 2017). ^{177}Lu is one of the most widely used theranostic radionuclides due to its gamma photons and beta particulates. The studies regarding ^{177}Lu labeled radiopharmaceuticals have increased in recent years thanks to its advantages such as long physical half-live and large-scale production. $^{177}Lu^{+3}$ can conjugate with various molecules (e.g., antibodies, peptides, glycoproteins, hydroxyapatite minerals) due to its empty s, p, and d orbitals (Banerjee, Pillai, & Knapp, 2015). Moreover, some ^{177}Lu-radiopharmaceuticals containing antibiotic molecules like kanamycin, bleomycin, benzylpenicillin, sulfadiazine, and colistin were developed in the literature (Akbar et al., 2017; Karpuz et al., 2022; Naqvi et al., 2017; Shahzad et al., 2017; Yousefnia et al., 2010b)

Tedizolid phosphate (TDZ), a second-generation oxazolidinone, was developed for linezolid-resistant *S. aureus* infections. The minimum inhibitory concentration of TDZ has found 8-fold lower compared to linezolid. TDZ, a prodrug, binds bacterial 23S ribosome initiation complex and 50S ribosome subunit to prevent the formation of 70S ribosome complex after its activation by phosphatase in plasma (Cada, Ingram, & Baker, 2014). Although it was approved by the US Food and Drug Administration (FDA) for the treatment of acute bacterial skin and skin structure infection in 2014, its *in vitro* antibacterial effect was also evaluated for tuberculosis, pneumonia, or gas gangrene in the literature (Bryant, Bayer, Aldape, McIndoo, & Stevens, 2020; Srivastava, Cirrincione, Deshpande, & Gumbo, 2020; Wunderink et al., 2021). Although no study has been performed in the literature about radiolabeling of TDZ, linezolid was radiolabeled with ^{18}F to image *Mycobacterium tuberculosis*-infected lungs (Mota et al., 2020).

In light of the information above, in our study, a pre-radiolabeling process of TDZ with ^{177}Lu was researched to obtain a theranostic radiopharmaceutical against gram-positive bacterial infections. The study's novelty is to radiolabel TDZ with ^{177}Lu, and evaluate the radiolabeling efficiency and stability of the ^{177}Lu-TDZ complex.

MATERIALS AND METHODS

Materials

TDZ was obtained from Wuhan Vanzpharm Inc. (Wuhan, China). $^{177}LuCl_3$ was obtained from Eczacıbaşı-Monrol Nuclear Products. Diethylenetriamine-pentaacetic acid (DTPA), ethylenediamine-tetraacetic acid (EDTA), ammonium hydroxide, and ammonium acetate were purchased from Sigma-Aldrich. Acetonitrile, and instant thin layer chromatography-silica gel (ITLC-SG) and whatman chromatography papers were obtained from Merck and Agilent, respectively.

Methods

Characterization of TDZ

The characterization studies were performed by ultraviolet spectrum (UV) and Fourier transform infrared (FTIR) analyses to determine TDZ and control its quality. UV spectrum of TDZ in distilled water was obtained between 100 and 400 nm to determine the wavelength of maximum absorption (λ_{max}) using a spectrophotometer (BMG Labtech-Clariostar plus). FTIR analysis was performed using a potassium bromide tablet in the 4000 to 650 cm^{-1} range by a spectrometer (Perkin Elmer Spectrum 100 FT-IR).

Radiolabeling study

To radiolabel TDZ with ^{177}Lu, 1 mL of $^{177}LuCl_3$ solution with 1 mCi radioactivity was added to the TDZ % 0.9 saline solution with 2.5 mg.mL^{-1} concentration. Different incubation periods (5, 30, and 60 min) and filtration effects were evaluated to determine the optimal radiolabeling condition TDZ with ^{177}Lu. For this purpose, radiolabeled mixtures were incubated for 5, 30, and 60 min at room temperature. In addition, after the separation of radiolabeled TDZ

solution into two samples, one of the samples was filtered through a cellulose nitrate membrane filter having a 0.22-μm pore size using a syringe.

Radiolabeling efficiency of ^{177}Lu-TDZ

After the radiolabeling studies, the percentages of radiochemical purity (RCP %) were tested by using paper chromatography for the evaluation of the filtration effect on radiolabeling efficiency, and the determination of the optimum incubation period.

To obtain the RCP % values, ^{177}Lu-TDZ complex was applied to stationary phases, and the radioactivity values at the origin and front were measured by a dose calibrator (Biodex Atomlab) at the end of the development of mobile phases. Different stationary (ITLC-SG and Whatman 3MM) and mobile phases (Ammonium hydroxide: methanol: water solution (1:20:20, v:v), Ammonium acetate: methanol solution (1:1, v:v), DTPA solution (10 mM), and EDTA solution (50 mM)) which are given in Table 1, were used. The RCP % values of ^{177}Lu-TDZ were calculated by using the following equation:

$$RCP(\%)\frac{Lu-177-TDZradioactivity}{Lu-177-TDZradioactivty+FreeLu-177radioactivity}\times 100$$

Radiolabeling stability of ^{177}Lu-TDZ

The radiolabeling stability of the ^{177}Lu-TDZ complex was evaluated by paper and radio-high pressure liquid chromatography (Radio-HPLC). To that end, RCP % values of the filtered ^{177}Lu-TDZ complex in saline solution were calculated at 0, 1, and 7 days after the radiolabeling procedure.

In the radio-HPLC study, 10 μL volume of ^{177}Lu-TDZ complexes were injected in an HPLC system fitted with a NaI Gamma detector, C18 (Inerstsil ODS-3 GL Science 150 × 3 mm) column, the photodiode array (PDA), and LC Solution software data analyzer (Shimadzu SCL-10A). Elution was obtained using the following gradient steps of solvents A (ultrapure water) and B (acetonitrile) 90:10 for 5 min, then 80:20 for 5 min, then 95:5 for 5 min, and then 90:10 for 5 min at a flow rate of 50 μL.min^{-1}. Analyses were performed at room temperature.

Statistical Analysis

All experiments were performed in triplicates. Data are expressed as the mean ± the standard deviation of the mean (SD). Differences were examined with Student's t-test using GraphPad Prism 6 program, and statistical significance was set at $p < 0.05$ for all data.

RESULTS AND DISCUSSION

Characterization Results of TDZ

The quality control of drug substances and excipients should be performed to check if the package ingredients obtained from the company are suitable before the preparation of pharmaceutical formulations including radiopharmaceuticals. Therefore, TDZ, used as the pharmaceutic part of the radiolabeled complex, was successfully identified by the UV and FTIR analyses. In the UV analysis, the maximum absorption peak of TDZ was obtained at 290 nm wavelength, as seen in Figure 1. This finding is in agreement with the study in the literature (Yang, Tian, Liu, & Huang, 2018).

The vibrations of different functional groups and bonds obtained by FTIR are founded as compatible with the molecular structure of TDZ, as seen in Figure 2A and Figure 2B. The vibration of C=C stretching and C-C stretching in the phenyl ring of TDZ was detected at 1621 cm^{-1}, similar to the literature (Paczkowska-Walendowska et al., 2020). C=O, C-O, C-C-N, C-C-C, and C-N-C bonds in the oxazolidinone group were shown in 1742, 1090, and 1020 cm^{-1}. The characteristic vibrations of (pyridine-3-yl)phenyl-3-fluoro containing C-C-N bending, C-F, and C-O bands were detected at 1405, 1276, and 1200 cm-1, which is in

agreement with a study (Michalska, Mizera, Lewandowska, & Cielecka-Piontek, 2016). Furthermore, the vibration of the phosphate group ($H_2PO_4^-$) in TDZ is determined in 1214, 1156, 1078, 942, and 883 cm^{-1} of spectra. These characteristic peaks of the phosphate group were found in similar ranges of spectra to the previous study in the literature (Klähn et al., 2004).

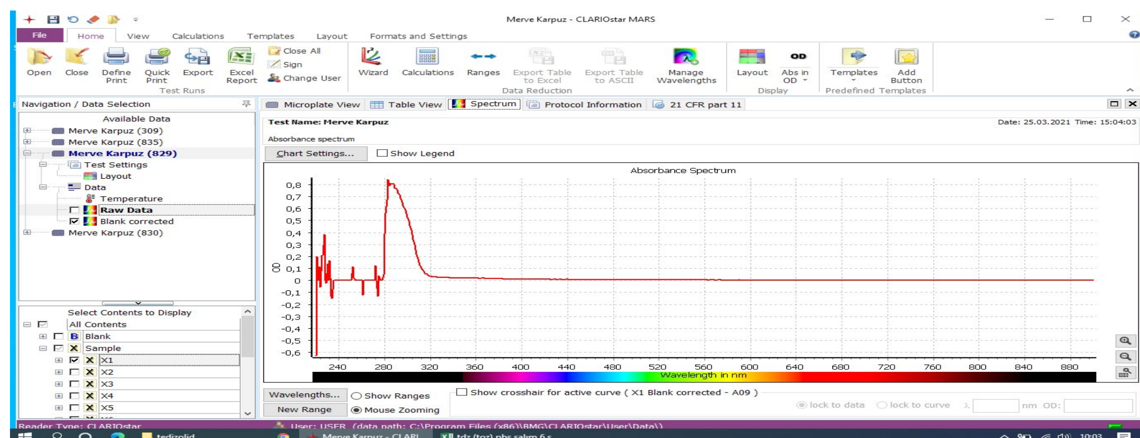

Figure 1. Absorbance spectrum of TDZ.

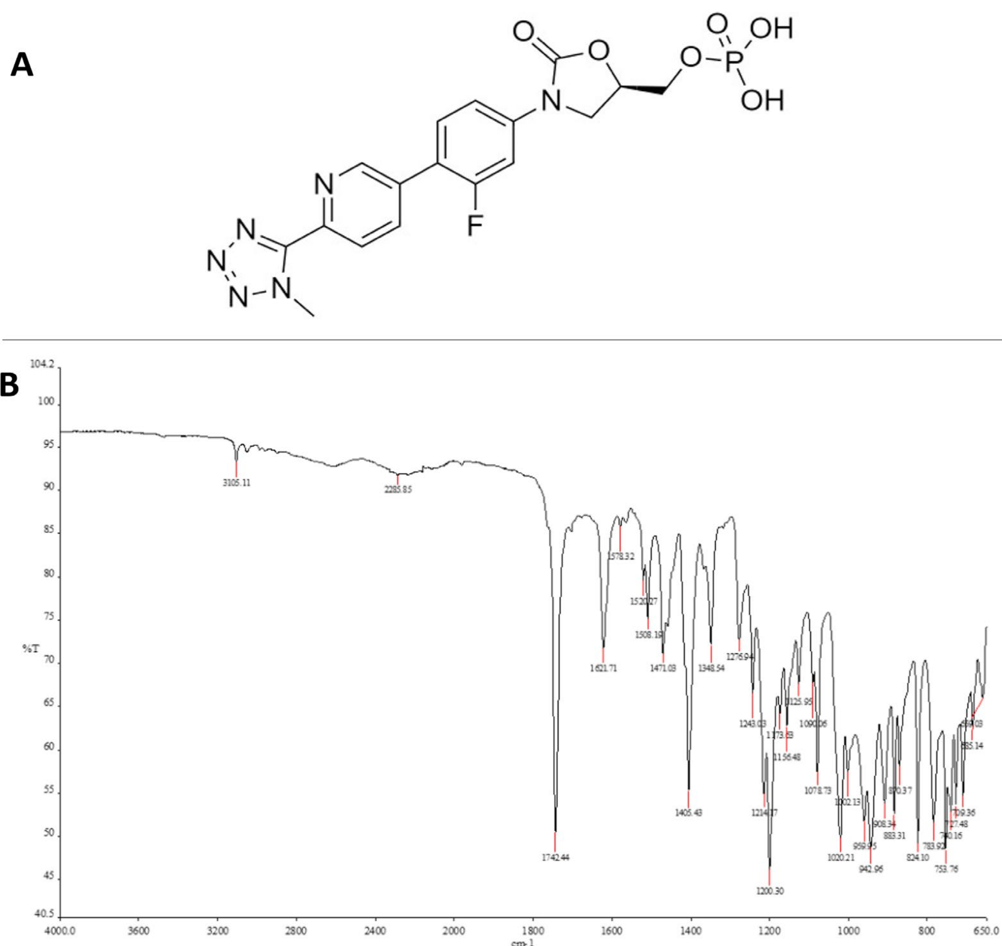

Figure 2. (A) Molecular structure of TDZ (Drawn by using ChemDraw), (B) FTIR spectra of TDZ.

Radiolabeling Study

TDZ was successfully radiolabeled with ^{177}Lu, and the radiochemical purity of the ^{177}Lu-TDZ complex was calculated. After the radiolabeling procedure, radiolabeled complexes should be tested to detect radiochemical impurities such as free radionuclides. Therefore, the amount of free $^{177}Lu^{+3}$ as the main radiochemical impurity should be calculated for ^{177}Lu radiopharmaceuticals (Banerjee et al., 2015). To separate free $^{177}Lu^{+3}$, different mobile phases were used. The location of the radiolabeled complex in the chromatography system was found variable depending on using various stationary and mobile phases. The presence of complex ^{177}Lu with DTPA and EDTA caused the elution of free ^{177}Lu to the front, while free ^{177}Lu remained at the origin in the elution system using ammonium hydroxide: methanol: water or ammonium acetate: methanol mobile phases. The Rf value of the free ^{177}Lu was found as 0.8 and 1.0 in DTPA and EDTA mobile phases, respectively. In addition, the Rf value of ^{177}Lu-TDZ complex was calculated as 0.8 for both ammonium hydroxide: methanol: water and ammonium acetate: methanol mobile phases. These results agree with the study in which the Rf value of free $^{177}Lu^{+3}$ was reported as 0.9 and 0.1 in DTPA solution and ammonium acetate: methanol solution, respectively (Yousefnia et al., 2010a). In the other two studies using ammonium acetate: methanol as a mobile phase, it was reported that free $^{177}Lu^{+3}$ remained at the origin (Akbar et al., 2017; Xu et al., 2019). Also, EDTA solution was used for the detection of radiochemical impurities such as free $^{177}Lu^{+3}$ at the front of the plate (Naqvi et al., 2017).

Radioactivity values for different incubation times measured at the end of the development of mobile phases in the chromatography system are given in Table 1. The radioactivity values of the ^{177}Lu-TDZ complex were found to be statistically higher in the ammonium hydroxide: methanol: water and ammonium acetate: methanol solutions than that of DTPA and EDTA solutions due to the ^{177}Lu-DTPA/EDTA complex formation ($p<0.05$). Therefore, the radiochemical impurities were separated using two different solvent systems to calculate the RCP % values of ^{177}Lu-TDZ, and RCP % values were found between 48-87 %, as seen in Table 2. In the literature, similar to our findings, it was reported that the possible ^{177}Lu-DTPA/EDTA complex formation caused the low RCP % values (Yousefnia et al., 2010a). Different incubation periods were tested to obtain high RCP %, and the highest RCP % values (approximately 90%) were obtained in 60 min incubation period, which is in agreement with the incubation time of a ^{177}Lu labeling study in the literature (Hu et al., 2002). In addition, for 30 min incubation period, higher RCP % values were obtained in the chromatography system using ammonium hydroxide: methanol: water and DTPA solutions in a mobile phase ($p < 0.05$). Thus, 60 min and ammonium hydroxide: methanol: water and DTPA solutions were chosen as an optimal incubation time and a mobile phase for the radiolabeling and evaluation of radiolabeling efficiency studies.

Table 1. The radioactivity values at the origin and front of the plate for the ^{177}Lu-TDZ complex in different phases and incubation times.

Mobile phases/ incubation time (min)	**Ammonium hydroxide: methanol: water solution**			**Ammonium acetate: methanol solution**			**DTPA solution**			**EDTA solution**		
	5	**30**	**60**	**5**	**30**	**60**	**5**	**30**	**60**	**5**	**30**	**60**
Origin	0.25 ± 0.08	0.71 ± 0.39	0.145 ± 0.08	0.04 ± 0.03	0.98 ± 0.08	0.05 ± 0.03	0.23 ± 0.16	1.59 ± 0.59	1.69 ± 0.61	0.08 ± 0.07	5.17 ± 0.86	2.8 ± 0.42
Front	3.26 ± 0.06	2.79 ± 0.47	0.65 ± 0.06	1.39 ± 0.06	3.75 ± 0.42	0.87 ± 0.03	2.06 ± 0.37	4.88 ± 3.58	1.145 ± 0.30	1.75 ± 0.37	7.09 ± 0.44	1.22 ± 0.28

Table 2. The RCP% values of ^{177}Lu-TDZ.

Mobile phases/ incubation times (min)	Ammonium hydroxide: methanol: water solution and DTPA solution			Ammonium acetate: methanol solution and EDTA solution		
	5	30	60	5	30	60
RCP % of ^{177}Lu-TDZ complex	76.8 ± 4.5	66.8 ± 3.9	87.1 ± 2.3	80.3 ± 1.5	48.9 ± 2.2	86.1 ± 4.2

The main administration route for radiopharmaceuticals is intravenous injection because of the physical half-life of radionuclides. Therefore, they should be sterilized by filtration using a membrane filter with a 0.22 μm pore size before the injection (Aerts et al., 2014). After the radiolabeling study, the radiolabeled complex was filtrated, and the pH value was evaluated. The pH values of both filtrated and un-filtrated ^{177}Lu-TDZ complexes were found to be 4.5, which is the optimal pH for the radiolabeling with ^{177}Lu, according to the literature (Breeman, De Jong, Visser, Erion, & Krenning, 2003; Shahzad et al., 2017; Xu et al., 2019). The filtration effect on RCP % values and radiolabeling stability was evaluated, and the results are given in Figure 3. No statistically significant difference was observed between RCP % values of filtrated and un-filtrated ^{177}Lu-TDZ solutions on the 0th and 1st day ($p \geq 0.05$). However, the RCP % value of filtrated solution significantly decreased on the 4th and 7th days after radiolabeling ($p < 0.05$). In our study, the radiolabeled complex was filtrated after the preparation. However, its radiochemical purity was found to be significantly lower compared to the un-filtrated complex. Therefore, it can be suggested that ^{177}Lu-TDZ solutions should be filtrated just before the administration to avoid this undesirable decrease in radiolabeling stability.

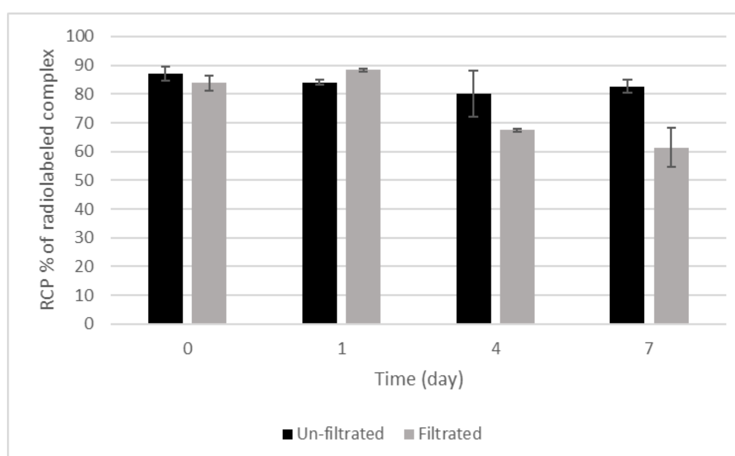

Figure 3. RCP % values of filtrated and un-filtrated radiolabeled complex (n=3).

Radiolabeling Stability

The separation of radionuclide from the pharmaceutical part is a critical problem since it causes the radionuclide can not to reach the targeted tissue. In addition to obtaining inadequate radioactive signals from targeted tissue, this separation gives rise to the uptake of unnecessary radioactivity in the healthy tissues due to free radionuclide localization. Therefore, the evaluation of radiolabeling should be performed (de Blois, de Zanger, Chan, Konijnenberg, & Breeman, 2019). In our study, the RCP % value of ^{177}Lu-TDZ was calculated by the paper chromatography and HPLC 7 days after radiolabeling to evaluate the labeling stability of ^{177}Lu-TDZ in saline at room temperature. The RCP % values obtained from paper chromatography and HPLC are given in Table 3. Although over 80% RCP % value was calculated by paper chromatography at the beginning of the radiolabeling stability study, low-

er RCP % values were obtained from HPLC. In addition, according to the results of both HPLC and paper chromatography analyses, it was found that RCP % values significantly decreased on the 7th day ($p < 0.05$). In the literature, it was reported that the use of chelating agents such as DTPA and 1,4,7,10-tetraazacyclododecan-N,N´,N´´,N´´´-tetraacetic acid (DOTA) increased the radiolabeling stability in the labeling process of ^{177}Lu (Kang et al., 2015; Watanabe, Hashimoto, & Ishioka, 2015).

Furthermore, the HPLC chromatograms of free ^{177}Lu and ^{177}Lu-TDZ complex are given in Figure 4. Free ^{177}Lu chromatogram is presented in Figure 4A, and its retention time is 2.45 minutes. The second chromatogram showed a ^{177}Lu-CMS peak with 10.8 minutes retention time in addition to the peak of free ^{177}Lu displayed.

Table 3. RCP % values of filtrated ^{177}Lu-TDZ obtained from HPLC and paper chromatography.

Time (days)	HPLC	Paper chromatography
0	76.5 ± 6.9	83.9 ± 2.6
1	80.2 ± 7.7	67.4 ± 0.4
7	67.8 ± 5.7	61.5 ± 6.9

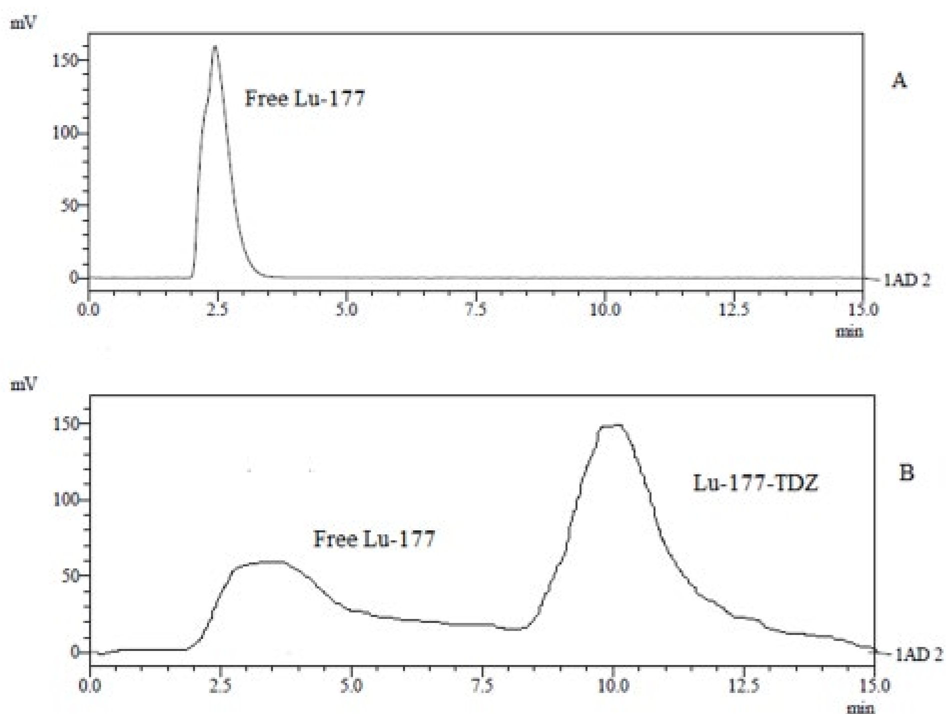

Figure 4. HPLC chromatograms of free ^{177}Lu (A) and ^{177}Lu-TDZ (B).

CONCLUSION

Globally, infection is still one of the significant health problems in view of Covid-19. The diagnosis and differentiation of infection from other pathologies is critically important to choose the appropriate treatment options, start the treatment in acute stages, and follow the progression of the disease. Infection can be clinically diagnosed by biopsy, imaging techniques, biochemical tests, or symptomatic evaluation of patients. Imaging techniques present advantages such as their applicability to obtain whole-body images, ability to detect unknown infection focally, and

low risk compared to biopsy. In imaging systems, nuclear medicine techniques have been preferred due to providing functional and physiological images from tissues in addition to anatomical information. Therefore, the development of sensitive and specific radiopharmaceuticals is essential to image infection at molecular stages. Although infection treatment can be performed by the use of various antibiotic molecules, the severe side effects of antibiotics and the development of antimicrobial resistance limit treatment efficiency. Theranostic systems, consisting of therapeutic and diagnostic agents, have been become crucial in recent years due to their advantages.

Hence, in our study, a theranostic radiopharmaceutical, containing TDZ and ^{177}Lu, was developed to image SPECT or gamma camera and treat the infection. In the experimental part, different incubation periods and chromatographic conditions were evaluated, and 60 min of incubation period was selected as the optimum time to obtain high RCP. In addition, ITLC-SG and ammonium hydroxide: methanol: water and DTPA solutions were chosen as the stationary and mobile phases, respectively, to detect amounts of impurities. Although over 80 % RCP was achieved in the radiolabeling efficiency study, the radiolabeling efficiency of the filtration complex significantly decreased over time. Therefore, the radiolabeled complex should be filtrated just before the administration to avoid this undesirable decrease in radiolabeling stability. Furthermore, in the HPLC chromatogram, two different peaks were observed depending on retention times of the free ^{177}Lu and ^{177}Lu-TDZ complex, but the radiolabeled complex did not exhibit stability for 7 days. Therefore, the addition of a chelating agent in radiolabeling conditions was suggested to increase the radiolabeling efficiency and stability. Thus, ^{177}Lu-TDZ can be a promising radiopharmaceutical in the treatment and imaging of difficult-to-reach and difficult-to-treat infections such as joint infections.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

All the authors of this article declared no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Conception and design (Karpuz, M., Ozgenc, E., Atlihan-Gundogdu, E.), data collection and processing (Ozgenc, E., Atlihan-Gundogdu, E, Burak Z), analysis and interpretation (Karpuz, M., Ozgenc, E., Atlihan-Gundogdu, E), literature search (Karpuz, M.), preparing the study text (Karpuz, M.), critical reviews (Atlihan-Gundogdu, E., Burak, Z).

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RESEARCH ARTICLE

Phytochemical Analysis and Screening of Acetylcholinesterase and Carbonic Anhydrase I and II Isoenzymes Inhibitory Effect of *Heptaptera triquetra* (Vent.) Tutin Root

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Phytochemical Analysis and Screening of Acetylcholinesterase and Carbonic Anhydrase I and II Isoenzymes Inhibitory Effect of Heptaptera triquetra (Vent.) Tutin Root

SUMMARY

Alzheimer's disease (AD) is characterized by progressive memory loss, deterioration of other cognitive functions, and inability to perform activities of daily living. Inhibiting the acetylcholinesterase (AChE) enzyme causes Ach accumulation in cholinergic synapses. This situation is expected to increase cognitive functions. Carbonic anhydrase enzymes (CAs) are ubiquitous in all living organisms. They have crucial physiological and pathological roles. CA inhibitors (CAIs) bind to catalytic zinc ions in the active site of CA isoenzymes and block their activity. The clinical use of CAIs had been established as antiglaucoma, anticonvulsant agents, diuretics, and anti-obesity drugs, in managing mountain sickness, gastric and duodenal ulcers, neurological disorders, osteoporosis, and tumors. To evaluate the bioactive profile of Heptaptera triquetra root, isolation studies, AChE, and human carbonic anhydrase (hCA) I and II inhibitory activities were performed. According to isolation studies, one fatty acid, coniferyl palmitate (1); four sesquiterpene coumarins, umbelliprenin (2), badrakemin acetate (4), colladonin (5), karatavicinol (6); and two sterols, stigmasterol (3a), β-sitosterol (3b) were isolated. All isolated compounds showed high potency against all enzymes (except badrakemin acetate for AChE) compared to standards. Umbelliprenin (2) with an IC50 value of 31.500 nM against hCA I, colladonin (5) with an IC50 value of 36.473 nM against hCA II and stigmasterol (3a), and β-sitosterol (3b) mixture with an IC50 value 9.000 nM against AChE demonstrated the best activity.

Key Words: *Heptaptera triquetra, Apiaceae, enzyme inhibition, acetylcholinesterase, carbonic anhydrase, isolation*

Heptaptera triquetra (Vent.) Tutin Root'un Asetilkolinesteraz ve Karbonik Anhidraz I-II Enzimlerini İnhibe Edici Etkilerinin Taranması ve Fitokimyasal Analizi

ÖZ

Alzheimer hastalığı (AH), ilerleyen hafıza kaybı, diğer bilişsel işlevlerde bozulma ve günlük yaşam aktivitelerini yerine getirememe ile karakterizedir. Asetilkolinesteraz (AChE) enziminin inhibe edilmesi, kolinerjik sinapslarda Ach birikimine neden olur. Bu durumun bilişsel işlevleri artırması beklenir. Karbonik anhidraz enzimleri (CA'lar) tüm canlı organizmalarda bulunur. Çok önemli fizyolojik ve patolojik rolleri vardır. CA inhibitörleri, CA izoenzimlerinin aktif bölgesindeki katalitik çinko iyonuna bağlanır ve etkilerini inhibe eder. CA I'lerin klinik kullanımı, dağ hastalığı, mide ve duodenum ülserleri, nörolojik bozukluklar, osteoporoz ve tümörlerin tedavisinde, antiglokom, antikonvülsan, diüretik ve antiobezite ilaçları olarak belirlenmiştir. Heptaptera triquetra kökünden hazırlanan diklorometan ekstresinin biyoaktif profilini değerlendirmek için izolasyon çalışmaları, AChE, insan karbonik anhidraz (hCA) I ve II inhibitör aktivite tayinleri yapılmıştır. İzolasyon çalışmalarına göre, bir yağ asidi, koniferil palmitat (1); dört seskiterpen kumarin, umbelliprenin (2), badrakemin asetat (4), kolladonin (5), karatavisinol (6); ve iki sterol, stigmasterol (3a), β-sitosterol (3b) izole edilmiştir. İzole edilen tüm bileşikler, standartlarla karşılaştırıldığında (AChE için badrakemin asetat hariç) tüm enzimlere karşı yüksek etki göstermiştir. hCA I'e karşı 31.500 nM IC50 değerine sahip umbelliprenin (2), hCA II'ye karşı IC50 değeri 36.473 nM olan kolladonin (5) ve AChE'ye karşı IC50 değeri 9.000 nM olan stigmasterol (3a) ve β-sitosterol (3b) karışımı en iyi aktiviteyi göstermiştir.

Anahtar Kelimeler: *Heptaptera triquetra, Apiaceae, enzim inhibisyonu, asetilkolinesteraz, karbonik anhidraz, izolasyon*

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INTRODUCTION

The genus *Heptaptera*, which belongs to the Apiaceae family, is represented by ten species in the world and is naturally grown from Europe to the Middle East, including Italy, the Balkans, Turkey, Syria, and Palestine (IPNI, 2022). It is represented by four species in Turkey, named *H. anisoptera, H. anatolica, H. cilicica*, and *H. triquetra*, one of which (*H. cilicica*) is endemic (Güner et al., 2012). *Heptaptera* species are rich in sesquiterpene coumarins (Appendino et al., 1992a; 1992b; 1993). In previous studies it has been reported that several sterols and coumarins have exhibited potent inhibitory activity against acetylcholinesterase (AChE) and human carbonic anhydrase (hCA) enzymes (Aydın et al., 2019; Pereira et al., 2016; Sepheri et al., 2020; Supuran, 2020; Şenol et al., 2010).

Alzheimer's disease (AD) is a wide spread dementia disease that occurs due to the decrease of neurotransmitters in the brain, and the neurotransmitter that shows the most reduction in this condition is acetylcholine. AD has no definitive cure. Current treatments are aimed at eliminating the symptoms of the disease. For this purpose, AChE inhibitors such as tacrine, donepezil, and rivastigmine are used. Since these drugs cause side effects such as hepatotoxicity and gastrointestinal disorders, safe, effective, and especially natural AChE inhibitors have gained more importance recently (Göçer et al., 2013).

Carbonic anhydrase (CA) is a pH-regulating enzyme in all living elements. This enzyme is found in many living systems (Küçük and Gulcin, 2016). It plays a role in various pathologic and physiological effects, including neurological disorders, fluid balance, pH regulation, bone resorption, carboxylation reactions, glaucoma, calcification, osteoporosis, cancer, and tumor production (Gulcin and Beydemir, 2013; Nar et al., 2013). CA plays a critical role in long-term synaptic transformation and is related tomental retardation, AD. There is evidence that CAII is increased in the AD brain (Jang et al., 2010). hCA isoenzymes are critical therapeutic targets. Its inhibitors and activators are currently used as drugs. hCA I can be used as an indicator to differentiate autoimmune hemolytic anemia from other types of anemia (Akıncıoğlu et al., 2014; Çoban et al., 2009). hCA II, implicated as a biomarker of stromal tumors, is required to maintain ion transport in erythrocytes and its deficiency syndrome causes renal tubular acidosis and osteoporosis, a marble brain disease or Guibaud-Vainsel syndrome. hCAs are inhibited by two groups of compounds. The first group is metal complex-forming anions, and the second group is sulfonamides and their isosteres. The most potent organic inhibitors are aromatic and heteroaromatic sulfonamides ($R\text{-}SO_2\text{-}NH_2$). Acetazolamide is used to treat glaucoma, altitude sickness, and benign intracranial hypertension (Akbaba et al., 2014; Gökçen et al., 2017).

According to a clinical study, carbonic anhydrase-II levels in plasma and brain hippocampus of Alzheimer's patients increased 1.24 times compared to healthy individuals. It has been shown that there is a definite relationship between plasma carbonic anhydrase-II levels and AD (Jang et al., 2010). It is known that there are many factors in the pathophysiology of AD (Öztürk-Karan, 2009). Therefore, it is thought that new treatment targets will develop through multiple approaches (Akdağ et al., 2019). *Heptaptera triquetra* (Vent.) Tutin is known as the "Üçgen Çakşır" in Turkey (Güner et al., 2012). The plant is erect, perennial, and 65-120 cm tall (Davis et al., 1988). It is naturally grown in Bulgaria, and Turkey-in-Europe (POWO, 2022). According to previous studies, *H. triquetra* exhibited high antioxidant activity (Şenol et al., 2010) and contained coumarins (Simova et al., 1986). In our earlier study, coumarin derivatives isolated from the root of *Heptaptera* cilicica displayed potent activity against AChE (Özbek et al., 2018). Additionally, coumarins have intense AChE and CA enzymes inhibitory activity (Karakaya et al., 2020). In light of this information, we aimed to investigate the possible inhibition effects of *H. triquetra* root extract and its isolated compounds against AChE, hCA I and II

enzymes linked to AD, known as a prevalent disease.

MATERIALS AND METHODS

General Experimental Procedure

1D (^{1}H-NMR, ^{13}C-NMR, and DEPT) and 2D (COSY, HMQC, and HMBC) NMR spectra were derived using Varian Mercury Plus (400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR) spectrometers, with TMS as an internal standard. These instruments were in the Faculty of Science at Ataturk University. HRESIMS data were recorded using an Agilent 6530 Accurate-Mass apparatus and AB Sciex TripleTOF 4600 (Agilent Technologies Inc., California, USA) at the Ataturk University East Anatolia High Technology Application and Research Center (DAYTAM). Open column chromatography (CC) was carried out using silica gel 60 (0.063–0.2 mm) (Merck, Germany), and solvents were purchased from Sigma-Aldrich (USA). TLC was performed using precoated silica gel 60 F_{254} plates (Merck), and the spots were visualized by spraying with 1% vanillin solution in concentrated sulfuric acid, followed by heating at 110 °C. All commercially available reagents were purchased from Sigma-Aldrich (USA) were used for bioactivity assays.

Plant Material

The roots of *Heptaptera triquetra* were collected in Tekirdağ City, Turkey (A1, 12 km from Saray, right side of the road, under oak forest, 202 m). The plant material was authenticated by Assoc. Prof. Gülderen Yılmaz. A voucher specimen (No. AEF 23723) was deposited at Ankara University Faculty of Pharmacy Herbarium (AEF), Ankara, Turkey.

Extraction and Isolation

H. triquetra roots (463.30 g) were dried in the open air in a shaded location, powdered, and extracted in 1250 mL of dichloromethane for -three h at 40 °C using a mantle heater and reflux cooler. The filtered extracts were concentrated to dryness in a rotary evaporator at 40 °C and 120 rpm. The dichloromethane extract (16.66 g of total extract) was fractionated using silica gel CC (70–230 mesh, 75 g) with *n*-hexane:ethyl acetate (EtOAc) (100:0→0:100, v/v) to yield two fractions, *Fr. 1* and *Fr. 2*. *Fr. 1* (7.95 g) was further fractionated using silica gel CC with *n*-hexane:EtOAc (100:0→55:45, v/v) to obtain subfractions *Frs. 1.1–12*. Compound **1** (39 mg) was precipitated from *Fr. 1.4* (129.6 mg). Compound **2** (332 mg) was precipitated from *Fr. 1.6* (612 mg). Compound **3** (24 mg) was precipitated from *Fr. 1.7* (395 mg). *Fr. 1.11* (892.7 mg) was purified using a Sephadex LH-20 CC (50 g) with CH_2Cl_2:MeOH (25:75, v/v) to yield compound **4** (38.9 mg). *Fr. 2* (6.23 g) was further fractionated using silica gel CC with *n*-hexane:EtOAc (100:0→0:100, v/v) to obtain subfractions *Frs. 2.1–8*. Compound **5** (539 mg) was precipitated from *Fr. 2.4* (1.16 g). *Fr. 2.6* (448 mg) was purified using a Sephadex LH-20 CC (50 g) with CH_2Cl_2:MeOH (25:75, v/v) to obtain *Fr. 2.6.1*. *Fr. 2.6.1* (302.7 mg) was purified using a Sephadex LH-20 CC (50 g) with MeOH to obtain *Fr. 2.6.1.1*. *Fr. 2.6.1.1* (138.1 mg) was again purified using a Sephadex LH-20 CC (50 g) with water (H_2O):MeOH (10:10→0:20, v/v) to obtain *Fr. 2.6.1.1.1*. Compound **6** (13.8 mg) was precipitated from *Fr. 2.6.1.1.1* (71.6 mg).

Carbonic Anhydrase Activity Assay

Fresh human blood erythrocytes for carbonic anhydrase I and II isoenzymes were purified using the Sepharose-4B-L-Tyrosine-sulfanilamide affinity chromatography technique (Gocer et al., 2016). CA activities of the isoenzymes were determined by spectrophotometric measurements at 348 nm (Verpoorte et al., 1967). Acetazolamide was used as positive control (Burmaoğlu et al., 2019; Hisar et al., 2005).

Anticholinergic Assay

The AChE inhibitory effect of dichloromethane extract and isolated compounds were determined by Ellman's method (1961) as given in previous studies (Eruygur et al., 2019; Polat Köse et al., 2015). AChE was commercially purchased and obtained from electric eel (*Electrophorus electricus*). 5,5′-Dithiobis(2-nitrobenzoic acid) and acetylthiocholine iodide (AChI) were used as a substrate for the cholinergic reaction. Tacrine was used as the positive control (Erdemir et

al., 2019; Lolak et al., 2020).

RESULTS AND DISCUSSION

The dichloromethane extract of the roots of *H. triquetra* afforded one fatty acid, coniferyl palmitate **(1)** (Lee et al., 2004); four sesquiterpene coumarins, umbelliprenin **(2)** (Appendino et al., 1994), badrakemin acetate **(4)** (Eshbakova et al., 2009), colladonin **(5)** (Appendino et al., 1992a), karatavicinol **(6)** (Ahmed, 1999); and two sterols, stigmasterol **(3a)** (Woldeyes et al., 2012), β-sitosterol **(3b)** (Güvenalp et al., 2009).

Coniferyl palmitate, umbelliprenin, badrakemin acetate, karatavicinol, stigmasterol, and β-sitosterol were firstly isolated from this species. Coniferyl palmitate, stigmasterol, and β-sitosterol were not reported in this genus before. Colladonin was previously reported in *H. triquetra* (syn.: *Colladonia triquetra*) (Simova et al., 1986). Chemical structures of all isolated compounds from *H. triquetra* are given in Figure 1, and NMR data arepresentedin Tables 1-3. The HRESIMS, 1D-, and 2D-NMR spectra of the compounds are available in Supporting Information.

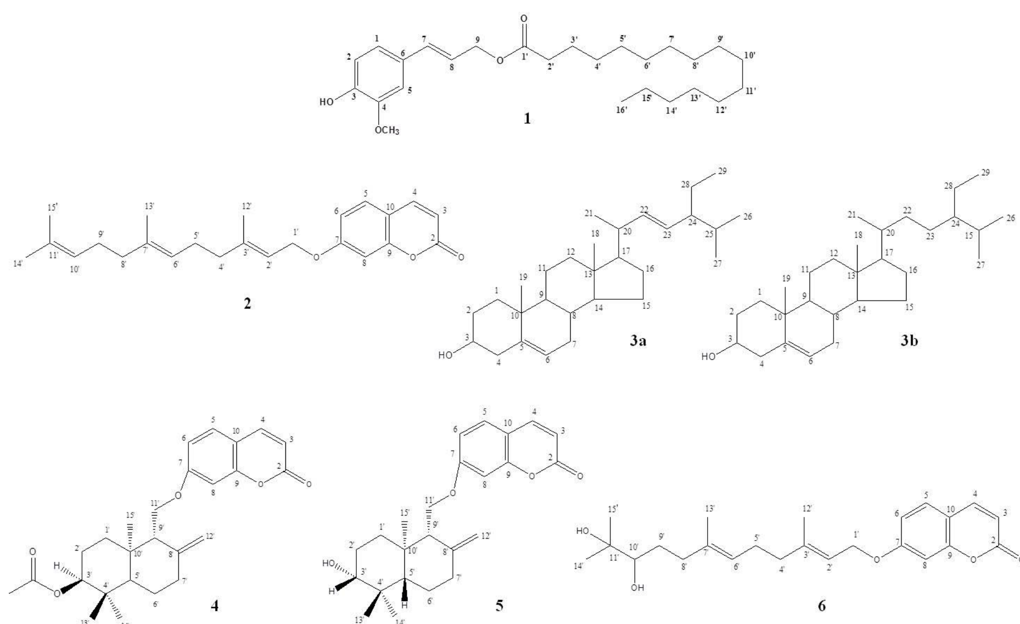

Figure 1. Chemical structures of isolated compounds (**1**-**6**)

Table 1. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) data of coniferyl palmitate **(1)** in $CDCl_3$ (δ in ppm, *J* in Hz).

Position	Coniferyl palmitate (1)	
	δ_H	δ_C
1	6.92 (*d*, 2.3)	108.32
2	6.88 (*dd*, 8.0/2.3)	114.44
3		145.87
4		146.62
5	6.87 (*d*, 8.0)	120.63
6		128.81
7	6.56 (*d*, 15.8)	134.37
8	6.19 (*dt*, 15.8/6.6)	120.91
9	4.72 (*dd*, 6.56/0.8)	65.14
1’		173.83
2’	2.35 (*t*, 7.5)	34.39
3’	1.65 (*m*)	24.99
4’	1.22-1.36 (*m*)	29.18-29.71
5’	1.22-1.36 (*m*)	29.18-29.71
6’	1.22-1.36 (*m*)	29.18-29.71
7’	1.22-1.36 (*m*)	29.18-29.71
8’	1.22-1.36 (*m*)	29.18-29.71
9’	1.22-1.36 (*m*)	29.18-29.71
10’	1.22-1.36 (*m*)	29.18-29.71
11’	1.22-1.36 (*m*)	29.18-29.71
12’	1.22-1.36 (*m*)	29.18-29.71
13’	1.22-1.36 (*m*)	29.18-29.71
14’	1.22-1.36 (*m*)	31.94
15’	1.22-1.36 (*m*)	22.71
16’	0.90 (*t*, 6.6)	14.15
O-CH_3	3.91 (*s*)	55.88

Table 2. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) data of sesquiterpene coumarins in $CDCl_3$ (δ in ppm, *J* in Hz).

Position	Umbelliprenin (2)		Badrakemin acetate (4)		Colladonin (5)		Karatavicinol (6)	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
2		161.32		161.24		161.26		161.39
3	6.24 (*d*, 9.5)	112.94	6.23 (*d*, 9.5)	113.01	6.23 (*d*, 9.5)	112.98	6.25 (*d*, 9.4)	112.95
4	7.63 (*d*, 9.5)	143.47	7.63 (*d*, 9.5)	143.46	7.63 (*d*, 9.5)	143.46	7.64 (*d*, 9.4)	143.52
5	7.36 (*d*, 8.5)	128.69	7.37 (*d*, 8.2)	128.77	7.36 (*d*, 8.9)	128.76	7.37 (*d*, 8.5)	128.71
6	6.85 (*dd*, 8.5/2.4)	113.22	6.83 (*dd*, 8.5/2.3)	113.14	6.83 (*dd*, 8.9/2.4)	113.10	6.85 (*dd*, 8.5/2.3)	113.36
7		162.16		162.13		162.22		162.12
8	6.82 (*d*, 2.4)	101.58	6.80 (*gs*)	101.27	6.82 (*gs*)	101.33	6.82 (*d*, 2.3)	101.55
9		155.87		155.90		155.90		155.86
10		112.42		112.48		112.47		112.46
1'	4.60 (*d*, 6.6)	65.48	1.53 (*dd*, 12.8/3.3) 1.78 (*m*)	36.83	1.45 (*m*) 1.79 (*m*)	37.19	4.61 (*d*, 6.4)	65.55
2'	5.47 (*td*, 6.6/1.1)	118.43	1.63 (*ddd*, 25/13.1/3.3) 1.76 (*m*)	24.10	1.65 (*ddd*, 24.8/12.9/3.3) 1.77 (*m*)	27.68	5.46 (*t*, 6.4)	118.69
3'		142.38	4.54 (*dd*, 11.50/4.2)	80.33	3.30 (*dd*, 11.6/4.3)	78.50		142.00
4'	2.12 (*m*)	39.52		38.05		38.81	2.15 (*m*) 2.11 (*m*)	39.38
5'	2.14 (*m*)	26.13	1.26 (*dd*, 12.4/2.5)	54.38	1.15 (*dd*, 12.4/2.6)	54.30	2.14 (*m*) 2.17 (*m*)	25.97
6'	5.09 (*m*)	123.49	1.43 (*ddd* 25.8/12.9/4.2) 1.74 (*m*)	23.27	1.47 (*m*) 1.75 (*m*)	23.45	5.18 (*t*, 6.4)	124.28
7'		135.58	2.11 (*dt* 4.2/2.1) 2.45 (*dq*, 13.1/2.2)	37.28	2.10 (*td*, 13.02/4.56) 2.46 (*ddd*, 13.1/4.1/2.3)	37.42		135.48
8'	1.97 (*m*)	39.67		146.11		146.28	2.29 (*m*) 2.06 (*m*)	36.75
9'	2.04 (*m*)	26.69	2.23 (*gt*, 5.24)	54.65	2.21 (*dd*, 6.3/4.9)	54.76	1.61 (*m*) 1.40 (*m*)	29.65
10'	5.08 (*m*)	124.30		38.66		39.18	3.35 (*dd*, 9.2/1.2)	78.13
11'		131.33	4.17 (*dd*, 9.7/6.7) 4.20 (*dd*, 9.7/4.2)	65.64	4.15 (*dd*, 9.6/6.8) 4.21 (*dd*, 9.6/4.2)	65.67		72.98
12'	1.77 (*s*)	16.78	4.91 (*s*) 4.52 (*gs*)	107.97	4.54 (*s*) 4.92 (*s*)	107.82	1.76 (*s*)	16.70
13'	1.60 (*s*)	16.04	0.90 (*s*)	28.29	1.03 (*s*)	28.34	1.62 (*s*)	15.91
14'	1.68 (*s*)	25.71	0.88 (*s*)	16.65	0.81 (*s*)	15.53	1.20 (*s*)	26.41
15'	1.59 (*s*)	17.69	0.86 (*s*)	15.39	0.85 (*s*)	15.35	1.16 (*s*)	23.29
C=O				170.97				
OCH_3			2.06 (*s*)	21.32				

Table 3. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) data of sterols in $CDCl_3$ (δ in ppm, *J* in Hz).

Position	Stigmasterol (3a)		*β*-sitosterol (3b)	
	δ_H	δ_C	δ_H	δ_C
1		37.26		37.26
2		26.07		31.66
3	3.55 (*m*)	71.81	3.55 (*m*)	71.81
4		42.30		42.30
5		140.76		140.76
6	5.36 (*gd*, 5.1)	121.72	5.36 (*gd*, 5.1)	121.72
7		31.66		31.90
8		31.91		31.90
9		50.15		50.13
10		36.51		36.51
11		24.31		21.22
12		39.68		39.78
13		42.32		42.22
14		56.77		56.87
15		25.42		24.31
16		29.83		28.25
17		55.95		56.06
18	0.70 (*s*)	12.26	0.71 (*s*)	12.05
19	1.01 (*s*)	19.40	1.01 (*s*)	19.40
20		40.51		36.15
21	1.03 (*s*)	21.09	0.94 (*d*, 6.6)	19.04
22	5.17 (*dd*, 15.1/8.6)	138.33		33.94
23	5.03 (*dd*, 15.1/8.6)	129.28		26.07
24		51.24		45.83
25		31.90		29.15
26	0.82 (*d*, 7.1)	18.99	0.82 (*d*, 7.1)	19.83
27	0.81 (*d*, 7.0)	19.04	0.81 (*d*, 7.0)	19.40
28		28.25		23.07
29	0.87 *(signal overlap)*	11.97	0.85 *(signal overlap)*	11.99

The dichloromethane extract and isolated compounds were evaluated to determine their human carbonic anhydrase I and II (hCA I and II), and acetylcholinesterase (AChE) enzyme inhibitory activities. Acetazolamide and tacrine were used as the positive controls, respectively (Gülçin et al., 2016). The dichloromethane extract exhibited activity with a 20.382 ng/μL IC_{50} value against hCA I, 21.656 ng/μL IC_{50} value against hCA II, and 5.634 ng/μL IC_{50} value against AChE.

Table 4. The inhibition effects of isolated compounds on human carbonic anhydrase I and II isoforms (hCA I and II) and acetylcholinesterase (AChE) enzyme.

Samples	IC_{50} (nM)						K_i (nM)		
	hCA I	r^2	hCA II	r^2	AChE	r^2	hCA I	hCA II	AChE
Coniferyl palmitate (1)	53.307	0.9606	40.500	0.9923	10.191	0.9901	59.412±10.86	117.343±19.41	4.538±0.59
Umbelliprenin (2)	31.500	0.9707	38.500	0.9832	11.550	0.9817	82.647±4.26	87.427±13.60	5.918±1.49
Stigmasterol and *β*-Sitosterol (3)	63.000	0.9734	59.300	0.9709	9.000	0.9770	83.183±8.87	74.560±8.88	3.806±0.27
Badrakemin acetate (4)	40.764	0.9634	69.300	0.9983	19.250	0.9840	32.963±4.49	93.333±11.06	6.631±1.11
Colladonin (5)	43.312	0.9629	36.473	0.9872	10.360	0.9840	60.560±10.61	38.571±5.72	2.771±0.95
Karatavicinol (6)	63.000	0.9836	53.412	0.9610	13.326	0.9847	65.155±9.86	49.026±6.37	3.156±0.92
Acetazolamide*	99.000	0.9959	87.954	0.9909	-	-	82.4079±10.45	159.597±9.05	-
Tacrine**	-	-	-	-	15.0652	0.9766	-	-	3.456±0.29

*Acetazolamide is a standard inhibitor of hCA I and II.

**Tacrine is a standard inhibitor for AChE.

For the hCA I isoenzyme, IC_{50} values were found as 53.307 nM for coniferyl palmitate, 31.500 nM for umbelliprenin, 63.000 nM for stigmasterol and *β*-sitosterol mixture, 40.764 nM for badrakemin acetate, 43.312 nM for colladonin, and 63.000 nM for karatavicinol. For the hCA II isoenzyme, IC_{50} values were found as 40.500 nM for coniferyl palmitate, 38.500 nM for umbelliprenin, 59.300 nM for stigmasterol and *β*-sitosterol mixture, 69.300 nM for badrakemin acetate, 36.473 nM for colladonin, and 53.412 nM for karatavicinol. The IC_{50} values of acetazolamide were determined as 99.000 nM against hCA I and 87.954 nM against hCA II. Regarding the K_i values of the isolated compounds (**1**-**7**) and the positive control (Table 4), remarkable activities against hCA I and hCA II in the ranges of 33.0-83.2 nM and 38.6-117.3 nM, respectively, were obtained.

The inhibitory effect of *H. triquetra* root against hCA I and II is reported here for the first time.As well as it is the first study for the *Heptaptera* genus. Coumarins are among the most isoform-selective CA inhibitors. They undergo hydrolysis of the lactone ring mediated by the esterase activity of CA. The resulting 2-hydroxy-cinnamic acids then bind to a particularpart of the enzyme's active site (Supuran, 2020). In a previous study, umbelliprenin was found to be apotent compound with a Ki value against CA XII of 5.8 nM when compared with acetazolamide and demonstrated high selectivity forthe CA I/II isoforms as in our study (Fois et al., 2020). According to our knowledge, it is the first study to evaluate hCA I and II enzymes inhibitory activity of other sesquiterpene coumarins such as badrakemin acetate, colladonin, and karatavicinol. As well as it is the first evaluation for coniferyl palmitate. In previous studies, *β*-Sitosterol has not inhibitedhCA I and II (Aydın, 2020; Saleem et al., 2019), while stigmasterol has displayed potent activity (Aydın et al., 2019). It shows that the activity is due to stigmasterol.

IC_{50} values of AChE were 10.191 nM for coniferyl palmitate, 11.550 nM for umbelliprenin, 9.000 nM for stigmasterol and *β*-sitosterol mixture, 19.250 nM for badrakemin acetate, 10.360 nM for colladonin, and 13.326 nM for karatavicinol. The IC_{50} value of tacrine was determined as 15.0652 nM against AChE (Table 4). In a study, the methanolic and ethyl acetate extracts prepared from *H. triquetra* aerial part and root were evaluated against AChE, and none of them showed inhibitory activity when compared with galantamine (98.88%) (Şenol et al., 2010). In our study, the isolated compounds from dichloromethane extract were effective against AChE. It shows that this effect is due to nonpolar compounds. In our previous study, umbelliprenin exhibited significantly high in-

hibitory potency against AChE (IC_{50} = 5.86 μM) as in our current study (Guvenalp et al., 2017). In another study, umbelliprenin, and colladonin were isolated from the roots of *Ferulago campestris* and showed moderate AChE inhibitory activity (Dall'Acqua et al., 2010). This is the first report on the AChE inhibitory activity of coniferyl palmitate. Additionally, as in our study, *β*-sitosterol isolated from acetone extract of the roots of *Salvia syriaca* exhibited high AChE activity with a 34.3 μg/mL IC_{50} value (Bahadori et al., 2016).

The evaluation of the bioactivity and determining the phytochemical content of *Heptaptera triquetra* had great importance. The dichloromethane extract of the root and isolated compounds were evaluated for their bioactivities on some metabolic enzyme's inhibitory properties related to several global diseases. The results indicated that all the isolated compounds were effective against hCA I, hCA II, and AChE. The main compounds, sesquiterpene coumarins, stigmasterol and *β*-sitosterol, were found as responsible for the inhibitory activities. Current studies show that enzyme inhibition is becoming a key target in the treatment or management of many common and global diseases. (Bayrak et al., 2019; Biçer et al., 2019). These findings suggest the potential of *H. triquetra* and its compounds as novel therapeutic candidates and herbal medicines for the treatment of glaucoma and AD.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

AUTHOR CONTRIBUTION STATEMENT

AÇK, HÖ, ZG: developing hypothesis; AÇK, HÖ, ZB, GY, CK: experimenting; AÇK, HY: preparing the study text; ZG, İG: reviewing the text; AÇK, HÖ, ZG, HY: analysis and interpretation of the data; AÇK, HÖ, HY, ZG: literature research.

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REVIEW ARTICLES

The Effects of Gender in Neurological Disorders: A Special Focus on Autism Spectrum Disorders and Thiomersal Toxicity

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The Effects of Gender in Neurological Disorders: A Special Focus on Autism Spectrum Disorders and Thiomersal Toxicity

SUMMARY

Estrogen and testosterone serve as the main sex hormones in humans. In addition, they have many different functions in terms of metabolic, and body defense. Gender differences lead to various social, economic, physiological, and pathological outcomes. Gender differences may also cause different toxicokinetics for metalslike mercury.Mercury exposure is suggested to cause neurological disorders. Thiomersal which is one of the most widely used preservatives particularly in vaccines, consists of approximately 50% mercury by weight. Autism spectrum disorders (ASD) have been associated with thiomersal exposure from vaccines in the last decades. Recent studies show that the incidence of ASD is higher in boys compared to girls. However, studies and discussions continue in this area. It is thought that this situation may be related to the difference in the toxicokinetics of mercury in different genders. There are concerns that ASD in girls may have a distinct phenotype than boys. The studies are now focused on whether there is an overlook due to the difficulty of diagnosing in females or it is more common in males due to physiological and hormonal reasons or not. In this review, we evaluated the frequency of ASD in different genders, the association between thiomersal and ASD and whether thiomersal exposure from vaccines could be an underlying factor of ASD in boys or not.

Key Words: *Autism, gender, neurodevelopmental disorders, mercury, thiomersal*

Nörolojik Bozukluklarda Cinsiyetin Etkileri: Otizm Spektrum Bozuklukları ve Tiyomersal Toksisitesine Özel Bir Bakış

ÖZ

Östrojen ve testosteron, insanlarda ana seks hormonlarıdır. Ayrıca metabolizmanın düzenlenmesi ve vücut savunması açısından da çok farklı işlevleri vardır. Cinsiyet farkı, farklı sosyal, ekonomik, fizyolojik ve patolojik sonuçlara yol açar. Metallerin farklı cinsiyetlerde farklı toksikokinetiğe sahip olabileceği, cinsiyet farkının özellikle cıva kaynaklı nörolojik bozukluklarda önemli olabileceği bilinmektedir. Tiyomersal, özellikle aşılarda koruyucu olarak kullanılan organik bir cıva bileşiğidir ve tiyomersalin ~%50'si cıvadan oluşur. Otizm spektrum bozuklukları (OSB), son yıllarda aşılardan tiyomersal maruziyeti ile ilişkilendirilmiştir. Son araştırmalar, OSB insidansının erkeklerde kızlara göre daha yüksek olduğunu göstermektedir. Ancak, bu alanda çalışmalar ve tartışmalar devam etmektedir. Bu durumun farklı cinsiyetlerde cıvanın toksikokinetiğinin farklı olmasıyla ilişkili olabileceği düşünülmektedir. Kızlarda OSB'nin erkeklerden farklı bir fenotipe sahip olabileceğine dair endişeler vardır. Araştırmalar OSB'nin kadınlarda tanı koymanın zorluğundan kaynaklanan bir gözden kaçma mı yoksa fizyolojik ve hormonal nedenlerle erkeklerde mi daha sık görüldüğü üzerine odaklanmıştır. Bu derlemede, farklı cinsiyetlerde OSB sıklığını, tiyomersal ile OSB arasındaki ilişkiyi ve aşılardan tiyomersal maruziyetin erkek çocuklarda OSB'nin altında yatan bir faktör olup olamayacağının değerlendirilmesi amaçlanmıştır.

Anahtar kelimeler: *Otizm, cinsiyet, nörogelişimsel bozukluklar, cıva, tiyomersal*

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INTRODUCTION

Estrogen and testosterone are the leading sex hormones that determine the differences in the female and male bodies. It is well known that they have led to their different-sex characteristics. The World Health Organization (WHO) has clearly expressed the difference between gender and sex. Gender describes the roles, behaviors, activities and opportunities that communities and societies deem appropriate for women and men. In contrast, sex refers to the characteristics that are biologically determined (WHO, 2019a). In neurological and behavioral studies, this term is of great importance when gender predisposition evaluations are made as evaluating individuals from all aspects, not only physiologically and biologically, will yield more accurate results.

Various studies are showing that there may be gender differences in the metabolism and toxicity of heavy metals such as mercury, nickel or cadmium. These differences are thought to be related to exposure dose, period and duration. The excretion and accumulation of these particular metals in female and male bodies are clearly different. This phenomenon is related to the difference in the activity and amounts of xenobiotic-metabolizing enzymes in different sexes (Vahter, 2007).

As the number of males in children diagnosed with Autism spectrum disorder (ASD) is strikingly high, other than biotransformation, the role of sex hormones should be discussed (Halladay, 2015; Van Wijngaarden-Cremers, 2014; Hiller, 2014; Hanen Center, 2017). Studies suggest that the predominance of estrogen and testosterone has both advantages and disadvantages for the brain. It is emphasized that estrogens play a protective role in the female brain due to their antioxidant effects. Estrogens have vasoprotective and neuroprotective effects (Kenchappa, 2004). In various *in vitro* studies, estradiol was shown to protect neurons against oxidative stress at pharmacological and physiological concentrations (Behl, 1995,1997,1999). However, high levels of testosterone in the male brain may lead to oxidative stress as testosterone can decrease antioxidant levels in the brain and neurons, and this effect leads to neurodegeneration (Branch, 2009; Holmes, 2016; Son, 2016). On the other, low testosterone levels have been associated with various diseases, such as premature aging, diabetes, obesity, sexual dysfunction and stroke (Shores, 2018). Although estrogen seems to be protective, due to physiological reasons, the phenotype of autism in girls may differ from boys. Moreover, the diagnosis of girls with ASD may be overlooked by the current diagnostic criteria (Halladay, 2015; Van Wijngaarden-Cremers, 2014; Hiller, 2014; Hanen Center, 2017).

Heavy metals can cause oxidative stress in the central nervous system (CNS). The presence of high levels of testosterone in the male brain may aggravate the deterioration of oxidant/antioxidant balance. Therefore, it is suggested that males may be more sensitive to neurotoxicity caused by heavy metals. Various epidemiological studies and animal studies support this suggestion (Vahter, 2007; Niedhammer, 2000). However, industrial studies, such as the studies on methyl mercury, are mainly conducted on male workers, and this may cause bias (Niedhammer, 2000). In recent years, exposure to thiomersal, a vaccine preservative that consists of ethyl mercury, has been increasingly associated with neurological diseases such as ASD, although there are contradictory reports as well (Hurley, 2010).

The high incidence of ASD in boys compared to girls (1: 4-8) has also suggested that gender difference may be an essential factor in thiomersal toxicity (Halladay, 2015; Moseley, 2018; Lai, 2015; NAS, 2019; Loomes, 2017; Stamova, 2011; Gallagher, 2010a; Gallagher, 2010b).

First, this relationship has not been fully proven, and more mechanistic studies are needed to clarify whether or not an association is present. However, there are many question marks on the association between ASD and thiomersal exposure. Concerning

all the available data, this review will focus on the frequency of ASD in different genders, the possible association between thiomersal and ASD, and whether thiomersal exposure from vaccines could be an underlying factor of ASD in boys or not. We will mainly explain gender differences in neurological disorders, the possible underlying mechanisms, gender differences in thiomersal toxicity and the different effects of thiomersal in males and females.

Testosterone and estrogens

Estrogen and testosterone, which serve as the main sex hormones in humans, perform their functions by binding to specific steroid hormone receptors in different cell compartments, such as the nucleus, cytosol, and cell membrane. The long or short-term effects of sex hormones are determined by the location to which they are attached and the downstream gene activation (Cohen-Bendahan, 2005; Knickmeyer, 2006; Arnold, 2009).

Sex hormone receptors are present in different cell types. However, these receptors show differential general brain expression in prenatal, postnatal, and adult brains. Therefore, the levels of sex hormones and their interactions may differ in specific periods of life, and their effects may differ between two genders (Mccarthy, 2008; Tobet, 2009; Reddy, 2014).

Testosterone, the primary male sex hormone that performs male reproductive functions, was discovered in the 1930s (Shores, 2018). Testosterone is produced by the testicles of men (by Leydig cells), the ovaries (by ovarian follicular cells) and adrenal glands of females. This androgen is carried by sex hormone-binding globulins (SHBG) and albumin. SHBG levels increase with age in men. Therefore, the free testosterone levels decrease and reach the lowest levels in men after 60 years (Iqbal, 1983). Testosterone has various effects on different tissues, organs and systems. The free testosterone can cross the blood-brain barrier and thereby can affect neurons (Białek, 2004).

Low testosterone levels are associated with various diseases such as premature aging, obesity, sexual dysfunction, diabetes and stroke (Shores, 2018). High testosterone levels can be harmful to the cardiovascular system (Xie, 2017). Testosterone may decrease antioxidant levels and therefore can increase oxidative stress within the brain. However, the type of oxidation (lipid peroxidation, protein oxidation or both) may vary depending on the parts and conditions (such as stress) in the brain (Son, 2016). Moreover, testosterone has been reported to increase neurotoxicity (induced by oxidative stress in rats), subsequently leading loss of dopaminergic neurons and finally neurodegeneration (Holmes, 2016).

Orchiectomy may increase oxidative stress in the brain. It has been established that castration in male mice may cause loss of dopaminergic neurons in the *striatum* and *substantia nigra* and subsequently may lead to stimulation of Parkinson's disease (PD)-related pathogenesis (Khasnavis, 2013). In addition, high testosterone levels have been shown to be associated with cognitive decline, possibly due to increased oxidative stress, in male patients (Holmes, 2016). All these data may explain the interaction between testosterone and neurodegenerative diseases.

In females, estrogens are produced primarily by the ovaries, and by the placenta during pregnancy, while estrogens are secreted by the adrenal glands and the testes in males. Estrogens are known for their vasoprotective and neuroprotective effects and the incidence of neurodegenerative disorders in females is generally lower than that of males. They exhibit a different mechanism of action in pharmacological and physiological concentrations (Kenchappa, 2004; Son, 2016). In various studies, estradiol has been reported to inhibit lipid peroxidation, protect the neurons against *in vitro* oxidative stress and glutamate-induced excitotoxicity at the pharmacological concentrations (Behl, 1995,1997,1999). At physiological concentrations, estrogens affect estrogen receptors (ERs) by stimulating or suppressing gene expression

(Kenchappa, 2004).

Gender differences in immune responses

Researchers have sought a link between gender and immune responses. The prevalence of certain infectious diseases, as well as the difference in the resistance in two genders, were evaluated by several studies. Females can exhibit more robust immune responses than males. It is suggested that males are more susceptible to a wide range of diseases and infections. Differences in this peripheral immune response may also play a role in the development of autoimmune diseases (McCombe, 2009; Kivity, 2010). It is known that events such as the overproduction of pro-inflammatory cytokines play a role in the onset and progression of various neurological diseases and neurodegenerative disorders. Inflammatory mediators are synthesized in neurodegeneration sites in stroke (De Simoni, 2002; Marquardt, 2005), multiple sclerosis (MS) (Silberberg, 2001), amyotrophic lateral sclerosis (ALS) (Barbeito, 2004), Alzheimer's disease (AD) (Grammas, 2001; Moore, 2002) and PD (Hirsch, 2005). Inhibition of neuroinflammation by steroids or non-steroidal drugs decreases neurodegeneration (Kurkowska-Jastrzebska, 2004; Fahrig, 2005). Immune function and inflammatory processes in the brain can be affected by sex steroids, especially 17β-estradiol (Lei, 2003; Ospina, 2003).

The main effects of estrogens on neuroinflammation are (Mor, 1999; Baker, 2004):

- ✓ Reducing the activation of the neuroinflammatory cascade at the cellular level
- ✓ Preventing the progression of the inflammatory response with these effects
- ✓ Inhibiting the release of molecular factors.

The neuroprotective effects of estrogens are directly related to their immunomodulatory effects. Therefore, the gender difference in immune responses against different biological and chemical agents outstands as a crucial topic to investigate (Olsen, 1996; Gaillard, 1998; Klein, 2000; Bouman, 2005).

Gender differences in the male and female brain

Studies on the morphology and functions of male and female brains show that some brain structures are sexually dimorphic. Brain and related regions show biochemical, functional and anatomical differences between genders (Ruigrok, 2014). Anatomical differences include changes in size and weight, gray matter/white matter ratio, and structural differences in various regions of the brain. For example, the male brain is heavier than the female brain, and the head circumference of men is larger than females. However, when this difference is proportioned to body weight, it is determined that there is no relative difference (Zaidi, 2010). When the gray and white matter ratios are analyzed, gray matter is higher in the male brain, and white matter is higher in the female brain (Allen, 2003). In addition, many neurochemical sexual dimorphisms include neurotransmitter systems and anatomical differences. All these changes cause different responses of the brain to neurological diseases. Moreover, treatment of certain neurological and physiological disorders can differ between two genders (Cahill, 2006).

Gender differences in neurological disorders

Progressive and gradual impairments in functions such as movement, motivation and memory because of structural changes in neurons or irreversible loss of neurons are defined as "neurodegeneration" (Kovacs, 2016). In the development of neurodegeneration, biological processes like oxidative stress, mitochondrial impairments, endoplasmic reticulum stress, neuroinflammation, production and accumulation of misfolded proteins, and excitotoxicity are observed (Dong, 2009; Doyle, 2011; Jellinger, 2010).

The brain is highly sensitive because of the irreplaceable and nonrenewable nature of the neurons and is more vulnerable than other tissues and organs to destructive processes such as oxidative stress, as it contains highly peroxidizable fatty acids, as a lip-

id-rich organ, and has limited antioxidant enzyme activity (Angelova, 2015; Son, 2016). In addition, non-coding RNAs, genetic mutations [in PD genes (PARK1, PARK4, PARK8), Presenilin-1 (PS1), Presenilin-2 (PS2), Apolipoprotein E (APOE); in genes related to frontotemporal dementia (FTD)] and environmental factors (such as pesticides, fungicides, addictive drugs, heavy metals, viruses) may play a role in the development of neurodegeneration (Salta, 2017). Neurological and neurodegenerative diseases may be caused by not only physiological disorders occurring in the brain but also by various conditions affecting the general health of patients. These disorders occur because of gradual damage caused by the rapid and irreversible loss of critical cognitive and motor functions in neurons. Recent reports of WHO emphasize that neurological disorders affect more than 1 billion people throughout the globe (WHO, 2007).

This high incidence attracted the interest of the investigators to focus on the effects of gender on the progression of these diseases (Yanguas-Casás, 2017). The development, structure, function, and biochemistry of the adult brain vary significantly by gender, due to the differences in gender-determining genes and fetal hormonal programming. Gender-specific differences in the anatomic structure of a healthy human brain are likely to lead to alterations in the pathology, progression, and severity of various diseases in two different genders. Moreover, these differences may also alter the susceptibility of different genders to specific neurological conditions (Cahill, 2006; Cosgrove, 2007; Gillies, 2010; McCarthy, 2012). The discovery of the sexual dimorphisms in the brain is crucial for understanding the significance of sex at different phases of neurological diseases and disorders (Yanguas-Casás, 2017).

Several neurological disorders have a striking gender bias in incidence, prevalence, and progression (Hanamsagar, 2016). AD has a higher prevalence (1.6-3: 1) in women over 65 years. AD also causes more and faster cognitive impairment in women (Seshadri, 1997; Plassman, 2011; Irvine, 2012). The specific pathogenic mechanism underlying the higher incidence of AD in women is that at a younger age (before menopause), the mitochondria are protected against amyloid β toxicity due to estrogen. Thus, mitochondria generate less reactive oxygen species (ROS), and apoptotic signals are less in females compared to males. However, at older ages (after menopause), as estrogen levels decrease, all this advantage is lost. On the other hand, the incidence of PD in men is higher (2 - 3.5: 1) than in women (Viña, 2010). However, the differences in symptoms and cognitive effects of PD between men and women have not been extensively studied (Miller, 2010). It is suggested that PD progresses more slowly in women than in men (Baldereschi, 2000; Elbaz, 2002). When autoimmune diseases are analyzed, women experience more MS than men (2-3: 1), but the disease progresses more slowly in men (Confavreux, 2003; Voskuhl, 2012). In the case of motor neuron diseases such as ALS, the incidence is higher in men (1.6: 1). Although the onset of the disease starts earlier in men, ALS is more fatal in women than men (del Aguila, 2003; McCombe, 2010). Mood-related disorders (such as depression or anxiety disorders) are more common in women (2: 1). In addition, symptoms are more severe, and women show a higher incidence of subclinical depression (Nolen-Hoeksema, 1994; Altemus, 2014). On the other hand, in attention deficit hyperactivity disorder (ADHD), males show a higher prevalence (3: 1). In addition, men experience more severe deficiencies in motor skills and more distraction than women (Cole, 2008; Bálint, 2009; Catalá-López, 2012; Willcutt, 2012). Furthermore, males have a higher incidence in schizophrenia (1.4: 1) compared to females, and the onset of the disease is earlier in males vs. females. Men also have a weak prognosis with severe symptoms and respond more negatively to antipsychotics than women (McGrath, 2008; Goldstein, 2013). Many neurodevelopmental disorders, including autism, dyslexia, ADHD, and early-onset persistent antisocial behavior, are more common in male individuals

compared to female individuals (Rutter, 2003).

Autism spectrum disorders, causes and their prevalence

Autism, Asperger's syndrome and pervasive developmental disorder not otherwise specified (PDD-NOS) are gathered under the name "ASD". Although it was first defined in the USA and Europe in the 1940s and entered the medical literature, it is thought that the ASD profile was known a few centuries ago with references to fictional and historical individuals (Wing, 2002). Its importance was neglected since it was very rare in the 1980s (5 out of 10000 people) (Gillberg, 1991). Today, it is ranked second after mental retardation among the most common developmental severe disabilities in the United States (Bhasin, 2006; Yeargin-Allsopp, 2003). In 2021, the Centers for Disease Control and Prevention (CDC) reported that approximately 1 in 44 children (1 in 27 boys identified with autism while 1 in 116 girls identified with autism) in the U.S. is diagnosed with an autism spectrum disorder (ASD), according to 2018 data (Autism Speaks, 2022).

ASDs are generally characterized by disorders in mutual social interaction and communication, absence of creative play ability, and recurrent stereotypical behavior and interests. These behavioral changes do not occur equally in all individuals with ASD. Individuals with Asperger's disorder do not experience a significant speech delay and have above-average cognitive skills. Individuals with PDD-NOS show anomalies, especially in core social behavior. The most accepted diagnostic methods for ASD diagnosis are standard face-to-face interviews and direct observation (Newschaffer, 2007). In the literature, it is stated that in children diagnosed with ASD, there are insufficiencies regarding eye-to-eye communication, understanding social stimuli, using body language, facial expressions, and these children display problematic behaviors due to these insufficiencies (Keller, 2014). In the first six months of life, they do not exhibit sounding behaviors such as smiling and gurgling like healthy newborns. Delay in speech is often the first symptom that attracts attention in the families of children with ASD (Keller, 2014; Volkmar, 2017). With these methods, clinicians reliably detect deficiencies in social interaction and communication. In recent years, as ASD has more incidence than previously thought, studies have increased in routine clinical practices to improve existing tools for diagnosis and develop new tools (Lord, 2000; Newschaffer, 2007).

Although ASDs are known to be a sum of powerful neurobiological and genetic impairments, the factors causing these conditions are still not well known, and there are many different hypotheses discussed in the literature (Stodgell, 2001; Scott, 2002). Genetic factors are significant in autism development. Because of studies conducted on twins, the average heritability of autism is estimated to be 90%. Today, autism is considered a heritable and multi-factor psychiatric disorder that does not follow the classic Mendelian pattern (Lichtenstein, 2010). It is also thought that different features in autism may result from various genes associated with separate brain regions (Happe, 2006). Despite the importance of genetic factors, environmental factors are also important in autism. Indeed, epidemiological studies have identified numerous correlations between nongenetic influences and ASD. Several drugs, toxic chemicals, and metabolic and nutritional factors are suggested to increase the risk of autism in epidemiological studies. The risk of autism is higher when the exposure happens during the prenatal period. Moreover, immunologic risk factors, including maternal infections during pregnancy, autoantibodies to fetal brain proteins, and familial autoimmune disease, have consistently been observed across multiple studies, as have immune abnormalities in individuals with ASD (Matelski, 2016). Studies are showing the association of many environmental factors such as heavy metals, pesticides, endocrine-disrupting chemicals (EDCs) and some drugs with various neurological diseases, including autism. Antidepressant exposure in the prenatal period, especially exposure to selective serotonin reuptake inhib-

itors (SSRIs), has been shown to increase the risk of autism in children (Andrade, 2017; Croen, 2011; He, 2022; Ijomone, 2020, Kobayashi, 2016; Moosa, 2018). Exposure to various toxicants, including pesticides and EDCs such as polychlorinated biphenyls (PCBs), and polybrominated diphenyl ethers (PBDEs), can have detrimental consequences on neurodevelopmental processes (Newschaffer, 2007). In a recent meta-analysis, old parental age, maternal gestational bleeding and gestational diabetes are suggested to be the most important factors in the development of autism (Gardener, 2009). Causes of ASD are summarized in Figure 1.

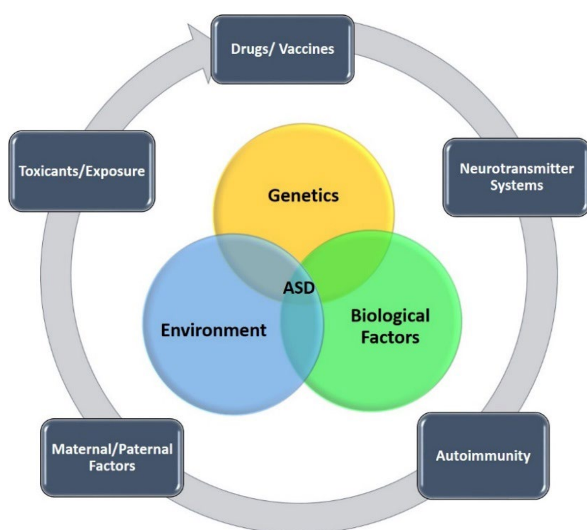

Figure 1. Causes of ASD.

According to the estimation of WHO, one in 160 children has ASD. This estimate represents the average reported prevalence obtained from several studies. However, some well-controlled studies reported substantially higher numbers. In addition, WHO stated that the prevalence of ASD in many low- and middle-income countries was so far not known (WHO, 2019b). In the USA, about one in 54 children has been identified with ASD, according to estimates from the Center for Disease Control and Prevention (CDC)'s Autism and Developmental Disabilities Monitoring (ADDM) Network (CDC, 2020). Over the past 50 years, the data obtained from different epidemiological studies show that the prevalence of ASD is increasing globally. This increase can be partly due to the improved awareness, expansion of diagnostic criteria, better diagnostic tools and improved reporting. However, there may still be unknown or uninvestigated factors that lead to this high prevalence (WHO, 2019b). In a large sample of children in the UK, the ASD prevalence was reported to be 61.9 per 10,000. In this group, the estimated prevalence of autism is 21.6, and the estimated prevalence of Asperger syndrome is 16.6 (Williams, 2008). Data obtained by screening more than 55,000 children aged 9 to 10 showed that the ASD prevalence was 77.2 per 10,000, and the autism prevalence was 38.9 per 10,000 in UK (Baird, 2006). In a report from the Danish Psychiatric Center Records, data from children under the age of 10 showed that the prevalence of autism was 11.8 per 10,000 children, and the estimated Asperger syndrome prevalence was 4.7 per 10,000 children under 10 (Lauritsen, 2004). In France, it is stated that approximately five out of every 10,000 children have autism (Fombonne, 1997).

Worldwide studies published after 2000 estimated that the prevalence of autism was between 16.8 and 40.5 per 10,000 people (CDC, 2018). In different studies that analyzed data from 11 years from 1997 to 2008, the prevalence of autism in children aged 3 to 17 was reported to be 47 per 10,000 (Baird, 2000; Bertrand, 2001; Chakrabarti, 2005; Boyle, 2011). There is no detailed research on the prevalence of ASD in Turkey. According to Autism Platform data, due to lack of healthy statistics, the estimated number of individuals with autism and the number of children (age:0-14) with autism has been reported to be approximately 550,000 and 150,000, respectively (Tohum, 2013).

Diagnostic criteria and effects of gender for autism spectrum disorders

Although autism symptoms can be detected in the first 12-18 months of life, a precise diagnosis is usually made between 24 to 36 months. Even in some cases, diagnosis can be made just before adulthood (Filipek, 1999). The most important disorders accompanying the differential diagnosis are childhood

schizophrenia, mental retardation, language skill disorders, psychosocial deprivation, and disintegrative psychosis (Sadock, 2000). American Psychiatric Association created a guide called "Diagnostic and Statistical Manual of Mental Disorders (DSM-5)" to diagnose mental disorders. According to DSM-5, the criteria for diagnosis are (NIH, 2018):

- ✓ Difficulties in communicating and interacting with people
- ✓ Having limited interests and showing repetitive behaviors
- ✓ Symptoms that interfere with the proper functioning of education, work, and survival skills

Autism has been described as a manifestation of the "extreme male brain". The extreme male brain hypothesis was initially based on certain factors that determine the brain "as a male brain type" or "a female brain type" during fetal life. Later, it was redefined that the male brain is more advanced in 'systematizing' and the female brain is more advanced in 'empathizing' (Baron-Cohen, 2002). Some researchers suggest that this theory not only helps to identify the causes of autism but also is very important in recognizing and correctly guiding the abilities and behaviors of children and adults with autism (Bartley, 2006). When a person's ability to systematize is unspoiled or excellent compared to normal, but his ability to empathize is impaired, it is the point where autism occurs (Lawson, 2004). It has been reported that individuals with ASDs perform badly in tests such as "Reading the Mind in the Eyes" where female subjects are generally superior to male subjects. However, male subjects are generally superior to female subjects in tests such as Embedded Figures Task (Jolliffe, 1997; Baron-Cohen, 2001). Two consistent findings from gender-related ASD studies have been reported in the literature. The first finding is that ASDs have a higher incidence and prevalence (4: 1) in men than in women, and this rate is increased in higher functional individuals (8-9: 1) (Mandy, 2012). The second is that women with ASD have lower intelligence than men with ASD (Lord, 1985; Volkmar, 1993). In addition, some researchers have stated that women with ASD show less serious repetitive/restricted behaviors and interests (RRBIs) (Fombonne, 2003; Hattier, 2011; Hiller, 2016). Frazier et al. (2014) investigated the effects of gender on ASD symptoms by analyzing the data from 304 females and 2114 males (age: 4-18 years) obtained from Simons Simplex Collection. They evaluated the relationship between gender and autism symptoms, cognitive and motor functions, and adaptive behavior problems. The researchers observed that women had fewer RRBIs without a consistent difference in social communication symptoms (Frazier et al., 2014). In a study conducted in North Carolina, a large sample group (384 boys and 91 girls, age: 3-8 years) of children with learning disabilities and ASD were recruited. It was reported that girls showed similar difficulties in social connections and communicational abilities, but less repetitive and stereotypical behavior (RSB) compared to boys (Lord, 1982). On the other hand, contradictory results were obtained from a study conducted by Hartley (2009). Between 2003 and 2007, 499 young children aged 18–47 months (157 males and 42 females) were transferred to the interdisciplinary autism clinic in a medical hospital in the northwestern USA. 199 (58.9%) of these children were diagnosed with ASD. They investigated gender differences by analyzing autistic symptoms, behavioral problems and learning scales. The researchers found that girls with ASD have fewer RSBs and worse communication disorders compared to boys (Hartley, 2009). In another study, Carter (2007) evaluated 90 children (22 girls and 68 boys) between 18 and 33 months. Researchers reported that the development profile of ASD showed gender-specific differences. Although they did not find any difference in RSBs between genders, they observed that social communication skills in females with ASD were lower than males. The researchers recruited 30 right-handed pre-menopausal women and 30 men with autism (18-49 years). The clinical diagnosis was officially made by a psychiatrist or clinical psychologist in the UK. Women were found to have more se-

vere symptomatology in social interaction, communication and RSBs, and they show less behavioral autistic features during interpersonal interaction (Lai, 2013). McLennan (1993) studied autistic behavior in 21 males and 21 females (between ages of 6-36 years) with ASD and reported that females had milder difficulties in social interests and communication abilities than males. In early childhood, females were reported to behave more successfully than males in mutual social interaction and communication. Studies were also conducted to examine the gender effect on ASD-related features; however, consistent findings have not been obtained. In studies on emotional difficulties accompanying ASD, it has been reported that women with ASD have higher (Hartley, 2009), lower (Mclennan, 1993) or similar (Lai, 2013) internalization problems. Studies investigating gender differences in brain structure regardless of the severity of RRBIs reported alterations between males and females. It was stated that there was a gender-specific white matter connection and the functional connection of the frontal lobe changes in males unlike females (Mclennan, 1993; Lai, 2013; Irimia, 2017). In a study, the researchers recruited 25 females and 25 males (7-13 years) with ASD and 19 females and 19 males as control. Subjects were identified from the National Database for Autism Research, a publicly available research repository in the USA. Researchers analyzed symptom severity and structural imaging data from Autism Brain Imaging Data Exchange using multivariate pattern analysis. Gender differences in the anatomy of the brain associated with RRBIs in the Autism Brain Imaging Data Exchange dataset were evaluated. Investigators reported that gray matter in motor areas could vary between boys and girls with ASD. In addition, RRBIs in girls were associated with the increased gray matter of the motor cortex, additional motor area and Crus 1 subsection of the *cerebellum*, while in boys, RRBIs only correlated with the right putamen. Brain anatomy and RRBIs can develop differently in different genders, suggesting that the behaviors that develop in ASD may occur *via* different pathways (Supekar, 2015). Researchers used a group of twins to study the relationship in the brain anatomy and RRBIs between two genders. In 75 twins (n = 150, 62 females, 88 males) with ASD (n = 32 twins, 20 males and 12 females) and other neurodevelopmental disorders (n = 25 twins, 16 males and 9 females), they investigated the relationship of RRBIs with the cortical volume, surface area and thickness of different networks in the brain (neocortical, subcortical and cerebellar regions). The investigation revealed a within-pair relationship between the increased thickness of the right intraparietal sulcus and decreased volume of the right orbital gyrus only in females and RRBI symptoms. Researchers have shown that structural changes related to RRBIs in frontoparietal networks occur in females, while striatal networks are more affected in males. It was suggested that the autism symptoms are affected by gender differences, which may be due to alterations in brain structure (van't Westeinde, 2019). Researchers examined the relationship between ASD and gender differences in high-functioning children with ASD between the ages of 3 and 18 (n = 325, 52 females). It was stated that there was no effect of gender on IQ scores. Through the parental report and observation of researchers, females were reported to exhibit fewer RRBIs compared to their male equivalents. Teachers reported that males with ASD have more externalization and social problems than females (Mandy, 2012). In a study, mutation burden analysis on corresponding candidate genes using the Transmission and *de novo* association test (TADA) model was performed. Researchers collected DNMs from 5748 ASD trios (4783 male probands and 965 female probands, 4-18 years) and 1911 control trios (900 unaffected brothers and 1011 unaffected sisters) from published studies. Researchers found that the prevalence of functional delayed-non-match to sample (DNMs) was significantly higher in women compared to men. This finding suggests that a higher genetic burden should occur in women to reach a diagnosis. One hundred seventy-four candidate genes (60 shared, 91 male-spe-

cific and 23 female-specific genes) were primarily examined in the study. Sodium voltage-gated channel alpha subunit 2 (SCN2A), an important autism-related gene due to voltage-gated sodium channel activity and ion channel activity, chromodomain helicase DNA binding protein 2 (CHD2) and phosphatase and tensin homolog (PTEN) (associated with dysfunction of estrogen dihydrotestosterone), the most important unique male-specific gene lysine demethylase 5B (KDM5B, associated with chromatin organization and associated with recessive developmental disorders), forkhead box protein P1 (FOXP1, another male-specific gene associated with androgen receptor signaling), transcription factor 4 (TCF4, the female-specific gene associated with autism and nuclear regulation of androgen receptor activity) were among the genes that were studied. It has been reported that all these genes were co-expressed significantly less in males compared to females. Analyses of different genetic components revealed evidence to support the protective effect of the female gender in ASD, and the researchers stated that more studies were needed to understand the effects of gender in ASD (Zhang, 2020).

Thiomersal

Thiomersal is an organic mercury compound containing approximately 50% ethyl mercury by weight. The chemical structure of thiomersal is shown in Figure 2. It has been used in some vaccines since the 1930s. The aim of the use of thiomersal in vaccines, cosmetic products and drugs is to prevent bacterial and fungal contamination. It is more likely to confront contamination, particularly in multi-dose vials of vaccines where repeated doses are withdrawn from the same vial (Geier, 2015).

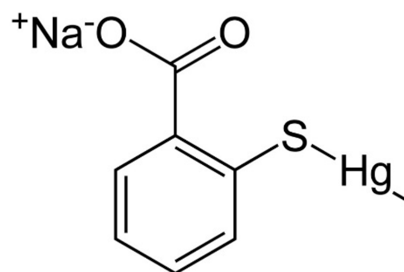

Figure 2. The chemical structure of thiomersal

In addition to vaccines, some cosmetic products and drugs (creams, eye drops, etc.) also contain thiomersal (Fonacier & Boguniewicz, 2016). In recent years, the presence of thiomersal in some vaccines recommended in routine immunization, such as hepatitis B and some influenza vaccines, has raised some health concerns. In systematic reviews and meta-analyses, mercury exposure has been associated with neurodevelopmental diseases (Jafari, 2017; Sulaiman, 2020; Yoshimasu, 2014). Several meta-analysis reports suggest that thiomersal exposure in infancy increases the risk of neurodevelopmental disorders such as ASD, ADHD and tic disorders (Dorea, 2018; Taylor, 2014; Yoshimasu, 2014). In addition, it was suggested that exposure to mercury during the developmental period could cause learning disorders and behavioral abnormalities like in autism. Some researchers indicate that early-life thiomersal exposure may be an essential factor for the development of ASD (Yassa, 2014; Pletz, 2016). There is evidence that the effects of toxic metals on health can occur differently in men and women due to their toxicokinetics, modes of action, and sensitivity. Generally, only male animals are used for experimental toxicological studies. Therefore, in many studies, the effects of gender-specific differences (such as specific hormone interactions and mechanism of action in two different genders) are usually neglected. Moreover, gender differences were not seriously considered in most of the environmental health risk assessment and toxicity studies until the last decades. Today, data for men and women are reported separately in epidemiologi-

cal studies though they are still very few (Niedhammer, 2000; Vahter, 2007).

Due to the epigenetic effects of sex hormones, differences can occur in brain function in different genders. In recent years, human and animal studies have revealed that gender differences in neurotoxicity are more common than expected. Recognition of gender-specific symptoms, observance of risk factors, and knowing that gender can make the person more vulnerable to the toxicity of certain metals and chemicals are extremely important for effective prevention and treatment strategies (Patočka, 2014). Mercury enters the environment because of the processing of fossil fuels, compounds used in industry and agriculture, and waste. The primary source for this metal is volcanic activity. The transition from soil to plant is limited, and trace amounts of mercury can be found in some edible mushrooms. However, in aquatic environments, there are very high concentrations of mercury in fish, marine mollusks and shellfish. Exposure to methyl mercury often occurs by consuming seafood, especially fish and marine mammals caught in their natural habitats (NRC, 2000; Patočka, 2014). There are studies showing differences in methyl mercury metabolism in different genders in both humans and experimental animals. However, the results are contradictory. For instance, mercury analysis was performed on human kidney cortex biopsy samples, and three times higher concentrations were detected in women than in men (Barregård, 1999). In animal studies, it has been found that after mercury exposure, male rats have significantly higher levels of mercury in their kidneys and brains compared to female rats. Moreover, females have been reported to eliminate mercury from their bodies more quickly than males. Although urinary excretion was specified as a more minor pathway for mercury clearance, sexual differences were also reported for this elimination route. While males excrete about 3.2% of the dose with urine, this rate has been reported as 7.5% in females. Urinary cumulative excretion of organic Hg accounted for 1.8% of the amount in males and 5.3% of the amount in females (Thomas, 1986; 1987). On the other hand, after mercury exposure, mercury levels in both blood, brain and muscles were significantly lower in male mice than in female mice. In contrast mercury kidney accumulation was significantly higher in male mice than in female mice. Moreover, the toxicokinetics of methyl mercury in male and female mice was found to be markedly different (Nielsen, 1991).

In vitro and *in vivo* studies have shown that interactions with sulfhydryl groups, microtubule destabilization, changes in intracellular calcium levels, and formation of ROS are critical mechanisms at the onset of methyl mercury neurotoxicity (Sarafian, 1991; Fredriksson, 1993; Atchison, 1994; Daré, 2000; Usuki, 2001; Carrillo, 1992; Borrás, 2003). It has also been reported that estrogen can provide additional protection against oxidative stress by inducing the synthesis of protective molecules through the activation of estrogen receptors (Behl, 1995; Singer, 1998; Olivieri, 2002). Therefore, the role of gender differences in chronic methyl mercury toxicity can also be explained in part by oxidative stress.

Researchers investigated the levels of mercury in newborns and mothers and the association between prenatal exposure to mercury and the neurobehavioral development of newborns in Zhoushan City of Zhejiang Province, China. Four hundred and eight surveys were conducted; 405 hair samples from mothers and 406 umbilical cord samples were collected, and behavioral neurological evaluations were performed on 384 newborns. In cord samples and maternal hair samples, mercury levels were determined as 5.58 mg/L (range: 3.96-7.82 mg/L) and 1246.56 mg/kg (range: 927.34-1684.67 mg/L), respectively. In 70% of newborns, mercury levels exceeded the reference dose (RfD = 5.8 mg/L) reported by Environmental Protection Agency (EPA). While the increase in prenatal mercury exposure was associated with a decrease in behavioral ability in men, this relationship was not observed in women (Gao, 2007).

A comparative study was designed to evaluate the toxicities induced by methylmercury and ethyl mercury, as well as by their complexes with cysteine in the C6 rat glioma cell line. Both of the organic mercury compounds markedly induced cytotoxicity. Significant cytotoxicity was also observed when cells were treated under the same conditions with methyl mercury-S-Cys and ethyl mercury-S-Cys, but the respective EC50 values were markedly higher. L-methionine significantly protected against the toxicities induced by both complexes. However, no protective effects of L-methionine were observed against toxicities of organic mercury compounds. Although it has not been fully elucidated how methyl and ethyl mercury enter the brain and generate its toxicity, its high affinity for thiols and selenols is suggested to be important in this regard (Zimmermann, 2013). It is assumed that the transition of the methyl mercury from the blood to the brain usually occurs by simple diffusion (Simmons-Willis, 2002). However, some studies have shown that this transport is in the form of a methyl mercury-cysteine (MeHg-S-Cys) complex with the L-type neutral amino acid carrier (LAT) system (Clarkson, 2007; Yin, 2008). When MeHg-S-Cys is applied in different cell lines, over-expression of LAT-1 (an important LAT subtype) has been reported to increase the uptake of mercury, the breakdown of LAT-1 reduces MeHg-S-Cys uptake and weakens its cytotoxicity (Simmons-Willis, 2002; Yin, 2008). In addition, *in vivo* studies have shown that administration of the MeHg-S-Cys complex results in a significant increase in mercury accumulation in the brain (cortex and cerebellum) and liver in mice compared to methyl mercury administration (Roos, 2010). Although methyl mercury and ethyl mercury are closely related chemically, and they both can cause similar damage to the brain at toxic doses and thiomersal has been shown to cause significant neurotoxicity *in vitro* and *in vivo*, it is anticipated that ethyl mercury has a shorter half-life compared to methyl mercury and is metabolized to inorganic mercury faster (Barregard, 2011). Although it was suggested that ethyl mercury compounds did not cross the blood-brain barrier, a systematic study on different articles in the literature indicated that ethyl mercury compounds, including thiomersal, can cross the blood-brain barrier and exposure to ethyl mercury-containing compounds (intravenously, intraperitoneally, topically, subcutaneously, intramuscularly, or intranasally administered) results in accumulation of mercury in the brain (Kern, 2020). Moreover, it was suggested that thiomersal crossed the barrier in both directions, with a slight accumulation in the basolateral, brain-facing compartment, after simultaneous incubation in both compartments (Lohren, 2016).

Gender differences in thiomersal toxicity

There is evidence that the harmful effects of toxic metals on health occur differently in men and women due to toxicokinetics, mode of action and sensitivity differences, but data are limited.

Researchers tested the assumption that exposure to thiomersal during the perinatal period disrupts CNS development and especially the cerebellum due to oxidative stress. Spontaneous hypertensive (SH) or normal Sprague-Dawley (SD) rats were given thiomersal (200 **μg**/kg) during pregnancy (between gestational days G10-G15) and lactation (P5-P10), evaluation was made by researchers to determine auditory and motor functions in male and female newborn rats. In SH rats exposed to thiomersal, the rollover time on P4 decreased by 59% in male offspring and only 13% in females. Testing of auditory functions was performed with a startle response. In SH rat pups exposed to thiomersal, the startle response measured on P14 decreased by 12.2% in males, not in females. In SD rats, male offspring showing startle response decreased by 27.8% and female offspring by 19.2%. It has been reported that perinatal exposure to thiomersal caused a significant decrease in cerebellar type-2 iodothyronine deiodinase (DIO2) activity by 60.9% in male SH rats but not in females. The data obtained in

the study showed that perinatal thiomersal exposure had adverse neurodevelopmental effects that seemed to be both strain and gender-dependent (Sulkowski, 2012). As the exposure to thiomersal was 200 **μg**/kg, these levels seem to be comparable to the amount of thiomersal received from one dose of a vaccine. However, chronic exposure to thiomersal cannot be observed after vaccine application, although humans are chronically exposed to thiomersal from other sources.

Researchers applied thiomersal *via* postnatal injections at different doses (12, 240, 1440, 3000 μg/kg) to young adult Wistar rats (n=4 in each group) on postnatal days 7, 9, 11 and 15 in four equal doses. An open field test was performed to evaluate general locomotor activity and anxiety in rats on the 30th day after birth. It has been reported that a significant reduction in overall locomotor activity was observed in all doses in thiomersal-treated male rats. A similar effect was recorded only at the highest dose in female rats. This finding indicated that male rats are more prone to the neurotoxic impacts than females (Olczak, 2011).

CONCLUSION

It is clearly known that females and males are quite different in both biological and social aspects and can react differently to the pathological and psychological conditions they encounter. The metabolism and toxicity of the metals and chemicals may have different effects on females and males depending on the enzymes, hormones and immune system. It can be stated that this situation is even more evident in terms of neurotoxicity and neurodevelopment. Neurological diseases such as ASD, which including behavioral changes, are seen in men at a much higher frequency. However, it is unclear whether this bias is due to physiological differences or problems in planning studies and determining diagnostic criteria. Concerns have increased over the last years that the criteria used in the diagnosis of ASD do not openly include the ASD phenotype in girls. In addition, studies on metal exposure examine occupational exposure and have been performed on men. This makes it difficult to make a clear comment about females. Considering all these data, new studies should be planned, and perhaps new criteria suitable for the phenotype in girls should be considered for the diagnosis of ASD. Physiatrists should provide new evaluation scales to accurately recognize the cases that are avoided attention in girls and make the correct diagnosis. New *in vitro* and *in vivo* test methods should be developed in order to show how male and female brains respond differently to different environmental chemicals or vaccine ingredients. Recently, we have created an *in vitro* test system to test how male and female neurons respond differently to thiomersal. This system can also be used for future research to investigate the toxic effects of mercury compounds in different genders (Erdemli-Köse, 2021).

The concern that thiomersal exposure may lead to ASD has caused hazardous consequences such as vaccine hesitancy and rejection. We have observed that vaccine rejection has caused an unending COVID-19 pandemic, and the world has experienced extraordinary economic and spiritual collapses. Although childhood vaccines used in USA and Turkey do not contain thiomersal as a preservative, parents may still be hesitant, and they may prevent their children from getting the necessary childhood vaccination. If vaccine hesitancy or rejection spreads in a wave, the immunization processes of the population will be significantly disrupted. There are not any studies in the literature that suggest ASD is entirely associated with thiomersal exposure. Therefore, more studies that are mechanistic are needed to clarify the association between thiomersal exposure and neurological conditions like ASD.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTION STATEMENT

Selinay Basak ERDEMLI-KOSE, Aylin BALCI-OZYURT and Anil YIRUN searched the Scopus, Web of Science, PubMed and Google Scholar databas-

es for the relevant articles that are cited in this review. Selinay Basak ERDEMLI-KOSE was responsible for the thiomersal section. Aylin BALCI-OZYURT was responsible for the autism section. Anil YIRUN was responsible for drawing the figures and referencing. Pinar ERKEKOGLU reviewed the manuscript and wrote the "Conclusion" section.

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REVIEW ARTICLES

Biyoparçalanır Doğal ve Sentetik Polimerlerin Yara Örtülerinde Kullanımı

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Use of Biodegradable Natural and Synthetic Polymers in Wound Dressing

SUMMARY

Wound healing is an extremely complex process consists of hemostasis, inflammation, cell proliferation and scar tissue remodeling phases. Delay or disruption of this process causes not only important health problems which impair the quality of patients' life but also a socio-economic burden for health systems. Thus various wound dressing designs are being developed that can meet the needs for different wound types and healing phases in order to ensure effective wound management. Natural or synthetic biodegradable polymers with adaptable flexible properties are considered as alternative biomaterials for wound healing applications. While natural polymers are preferred due to their biocompatibility, similarity to the extracellular matrix and cellular interactions, synthetic degradable polymers are widely used because of their low immunogenicity and ability to be synthesized in line with determined specifications. Wound dressings made of biodegradable polymers do not require a second procedure for removal. Also minimizing contact with the wound area in the treatment reduces the risk of infection and increases patient compliance. Polymeric wound dressings which play an active role in wound healing by keeping the wound environment moist and preventing the formation of microbial biofilms, could be in alginate, hydrogel or hydrocolloid structure, film or foam form. Also there are tissue engineering products made of polymeric scaffolds which keratinocytes and fibroblast cells are cultured on. In this article, the properties of biodegradable polymers used in wound dressings and their role in wound healing are summarized; ffilm, foam, hydrogel, hydrocolloid, alginate dressings and tissue engineered skin substitutes are reviewed and commercialized biodegradable polymeric wound dressings have been mentioned.

Key Words: *Wound healing, wound management, biodegradable polymers, natural and synthetic polymers, bioactive wound dressings.*

Biyoparçalanır Doğal ve Sentetik Polimerlerin Yara Örtülerinde Kullanımı

ÖZ

Yara iyileşmesi; hemostaz, enflamasyon, hücre çoğalması ve skar dokusunun yeniden şekillendirilmesi aşamalarından oluşan son derece karmaşık bir süreçtir. Bu sürecin gecikmesi ya da bozulması, hastaların yaşam kalitesini düşürerek önemli sağlık sorunlarına yol açmanın yanı sıra, sağlık sistemleri için de sosyo-ekonomik bir yük getirmektedir. Bu nedenle, etkili yara iyileşme sürecinin sağlanabilmesi için farklı yara tiplerine ve iyileşme fazlarına yönelik ihtiyaç duyulan gereksinimleri karşılayabilecek çeşitli yara örtüsü tasarımları geliştirilmektedir. Uyarlanabilen esnek özelliklere sahip, doğal ya da sentetik biyoparçalanır polimerler ise yara iyileştirme uygulamaları için alternatif biyomalzemeler olarak değerlendirilmektedir. Doğal polimerler biyouyumlulukları, ekstraselüler matrikse benzerlikleri ve hücresel etkileşimleri nedeniyle tercih edilmekteyken sentetik parçalanır polimerler düşük immünojenite göstermeleri ve belirlenen spesifikasyonlar doğrultusunda sentezlenebilme üstünlükleri açısından yaygın olarak kullanılmaktadır. Biyoparçalanabilir doğal ya da sentetik polimerlerden üretilen yara örtülerinin çıkarılmaları için ikinci bir işlem gerekmemekte, tedavide yara bölgesiyle temasın en aza indirgenmesi enfeksiyon riskini de azaltmakta ve hasta uyuncunu artırmaktadır. Yara çevresini nemli tutarak yara iyileşmesinde aktif bir rol oynayan, mikrobiyal biyofilmlerin oluşmasını önleyen polimerik yara örtüleri aljinat, hidrojel ya da hidrokolloid yapıda, film ya da köpük formunda hazırlanabilmektedir, ayrıca, keratinosit ve fibroblast hücrelerinin kültüre edildiği polimerik iskelelerden oluşan doku mühendisliği ürünleri de bulunmaktadır. Bu makalede, yara örtülerinde kullanılan biyoparçalanır polimerlerin özellikleri ve yara iyileşmesindeki rolleri özetlenmekte, biyoparçalanır polimerlerden üretilen film, köpük, hidrojel.., hidrokolloid, aljinat yara örtüleri ve doku mühendisliği ürünleri, deri ikameleri, incelenmekte ve ticarileştirilmiş örneklerine yer verilmektedir.

Anahtar kelimeler: *Yara iyileşmesi, yara tedavisi, biyoparçalanır polimerler, doğal ve sentetik polimerler, biyoaktif yara örtüleri.*

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GİRİŞ

Yara; fiziksel, termal ya da kimyasal etkenler sonucunda veya altta yatan fizyolojik bir durumun varlığında ciltteki, iç organlardaki normal anatomik bütünlüğün ve fonksiyonun bozulması olarak tanımlanmaktadır (Dhivya ve ark., 2015; Mayet ve ark., 2014).

Yara iyileşmesi doku hasarının meydana geldiği andan itibaren başlayan; doku bütünlüğünü ve homeostazı yeniden sağlamak, aktive ve koordine etmek için çeşitli hücre tiplerini (bağışıklık hücreleri, endotelyal hücreler, keratinositler ve fibroblastlar) ve yolaklarını içeren dinamik ve karmaşık bir fizyolojik süreçtir. Birbiriyle iç içe geçen 4 farklı aşamadan oluşur; i) koagülasyon ve hemostaz fazını (ii) enflamasyon (iii) yeni dokuların ve kan damarlarının oluştuğu proliferasyon ve (iv) yeni dokuların yeniden şekillendirilmesinin gerçekleştiği matürasyon fazları izler. Sürecin gecikmesi ya da bozulması uzun süreli ve aşırı enflamasyona, kalıcı enfeksiyonlara, ilaca dirençli mikrobiyal biyofilmlerin oluşmasına ve dermal/epidermal hücrelerin onarıcı uyaranlara yanıt vermemesine neden olmaktadır (Summa ve ark., 2018). Etkili yara yönetiminin sağlanamadığı durumlarda anormal yara iyileşmesi (keloid, hipertrofik skar oluşumu vb.) görülebilir, yara akut fazdan kronik faza geçebilir. İyileşmeyen yaraların, yaşam kalitesini düşüren önemli sağlık sorunlarına yol açmasının yanı sıra sağlık sistemleri için sosyo-ekonomik bir yük getirdiği belirtilmektedir (Man ve Hoskins, 2020).

1962'nin ortalarına kadar önem verilmeyen yara iyileştirme araştırmaları, Winter'ın (1962) tasarladığı yara filminin ya da "örtüsü"nün, domuz derisinde geliştirilmiş yara modelinde epitelizasyon hızını, açık kalan yaraya göre iki kat artırdığını ortaya koyduğu çığır açan çalışmasının ardından hız kazanmıştır (Kamoun ve ark., 2017).

Daha önce yara yönetiminde farklı derecelerde absorpsiyon kapasitesine sahip doğal ya da sentetik bandajlar, hidrofil pamuk, gazlı bezler ve sargı bezleri, yara eksüdasının (sıvısının) buharlaşmasına izin vererek yarayı kuru tutmak ve bakterilerin neden olabileceği enfeksiyonlardan yara bölgesini korumak için kullanılmaktayken artık epitel hücrelerinin hareket etmesine izin veren, yara çevresini nemli tutan, oksijen sirkülasyonunu sağlayan ve bakteriyel yükü azaltan interaktif ve biyoaktif yara örtüleri klinikte yer almaktadır (Boateng ve ark., 2008).

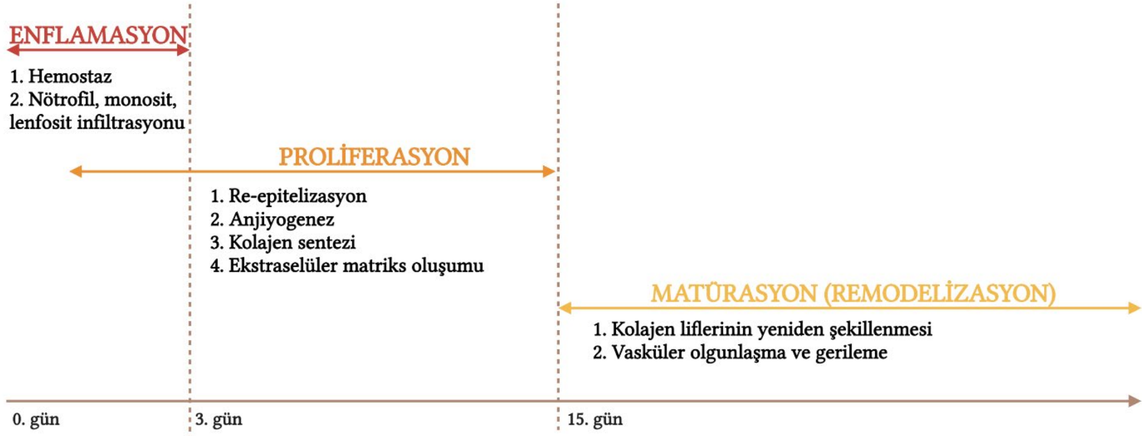
Hem akut hem de kronik yaraların iyileşmesinde destekleyici, aktif bir rol oynayan biyoparçalanır, biyouyumlu, gelişmiş terapötik yara iyileştirme tasarımları ise yeni nesil yara bakımı için ihtiyaç duyulan gereksinimleri karşılamakta ve umut verici bir yaklaşımı temsil etmektedir (Summa ve ark., 2018).

Fonksiyonlarını tamamladıktan sonra implantasyon bölgesinde *in vivo* olarak parçalanan ve resorpsiyona uğrayarak biyolojik sistemden uzaklaştırılan polimerlerin biyomalzemelerde kullanılması, revizyon cerrahisini ve buna bağlı olarak gelişebilen enfeksiyon riskini ortadan kaldırmaktadır. Çıkarılması gereken bazı yara örtülerinde görüldüğü gibi yeni oluşan cilt katmanlarının tipik "kopması" meydana gelmez ve kozmetik açıdan daha iyi sonuçlar elde edilir. Doku reaksiyonu ve enflamatuvar yanıt biyoparçalanmayan sistemlerle karşılaştırıldığında tartışılır düzeydedir. Bununla birlikte hasta konforu ve uyuncu sağlanmaktadır (Anju ve ark., 2020; Las Heras ve ark., 2020).

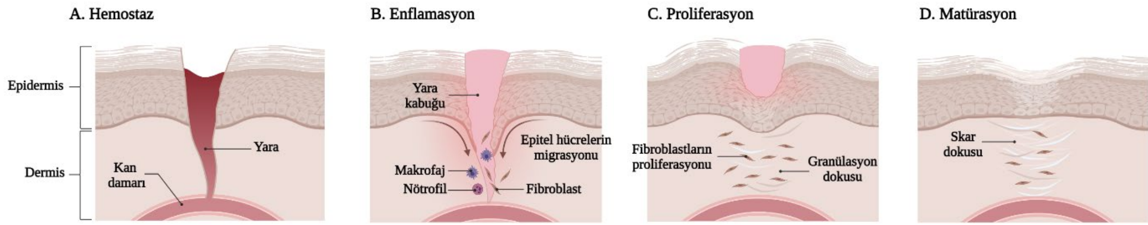
Bu makalede; yara örtülerinde kullanılan biyoparçalanır polimerler özetlenecek, piyasadaki biyoparçalanır polimerlerden üretilen film, köpük, aljinat, hidrojel, hidrokolloid yara örtülerinin özelliklerine ve yara iyileştirmedeki rollerine yer verilecek, ayrıca, doku mühendisliği ürünleri üzerinde de durulacaktır.

YARA İYİLEŞMESİ

Yaralanmayı izleyen süreçte yara iyileşmesi, koordineli bir şekilde gerçekleşen ve kısmen birbiri ile örtüşen fazlardan meydana gelir (Şekil 1., Şekil 2.)



Şekil 1. Yara iyileşme fazlarının zaman çizelgesi. BioRender.com ile oluşturuldu.



Şekil 2. Yara iyileşmesinin şematik olarak gösterimi. BioRender.com ile oluşturuldu.

Hemostaz, başlangıç aşamasını oluşturur ve kanamanın kontrol altına alınması için vazokonstriksiyon ve koagülasyon sisteminin aktivasyonu gerçekleşir. Trombosit kaynaklı büyüme faktörü (PDGF), dönüştürücü büyüme faktörü beta (TGF-β) ve epidermal hücre proliferasyonunu uyaran epidermal büyüme faktörü (EGF) salgılanır (Broughton ve ark., 2006). Enflamatuvar faz ve fibroblast proliferasyonu gibi yara iyileşmesinin farklı basamaklarında rol oynayan granülosit makrofaj koloni stimule edici faktör de (GM-CSF) keratinositler, endotel hücreleri ve lökositler tarafından üretilir (Memişoğlu ve ark. 1997).

Kanama kontrol altına alındığında, 2-4 saat içerisinde enflamatuvar hücrelerin yara bölgesine infiltrasyonu izlenir. Nötrofiller yara bölgesindeki istilacı mikroorganizmaları ve hücresel kalıntıları temizlerken daha fazla hasara neden olabilecek proteazlar ve reaktif oksijen türleri (ROS) üretebilirler. Makrofajlar, yara iyileşmesinin ilerlemesi için görevlerini tamamlayarak apoptoza uğrayan nötrofillerin de dahil olduğu apoptotik cisimleri fagosite ederler ve doku rejenerasyonunu desteklemek için keratinositleri, fibroblastları ve anjiyogenezi uyarırlar. T-lenfositler, makrofajlardan sonra yara bölgesine göç ederler ve geç proliferasyon/erken matürasyon fazında pik yaparlar (Guo ve DiPietro, 2010; Memişoğlu ve ark. 1998). CD4+ hücreleri (T-yardımcı hücreleri) maksimum hızda yara kapanmasını sağlayarak yara iyileşmesinde olumlu bir rol oynar (Boyce ve ark., 2000).

Enflamasyon fazını takip eden proliferasyon fazı epitelizasyon, anjiyogenez, granülasyon dokusunun oluşumu ve kolajen birikimiyle karakterizedir. Yara

yüzeyi boyunca keratinositler migrasyon ve çoğalma gösterir. Vasküler endotelyal büyüme faktörü (VEGF), yeni kan damarlarının gelişimini uyarır. Fibroblastlar, kolajen biyosentezini ve kolajen lifleri arasındaki çapraz bağlanma derecesini organize ederek yaranın gerilme gücünü belirler. Ayrıca, granülasyon dokusunun kritik unsurları olan elastin üretiminden ve ekstraselüler matriksin (ESM) oluşumundan sorumludur (Janis ve Harrison, 2014; Gantwerker ve Hom, 2012).

Yara açıklığının kapanmasıyla birlikte, yıllarca sürebilen ve cilt dokusunun olgunlaşmasını içeren remodelizasyon fazı başlar. Kılcal damarların gelişimi durur ve vasküler yoğunluk normale döner. Yaranın yeniden şekillenmesi ilerledikçe daha sıkı yapıdaki tip I kolajen, granülasyon dokusundaki tip III kolajenin yerini alır. Bu süreç, eşzamanlı olarak gerçekleşen tip I kolajen sentezi ve tip III kolajen lizizinin bir sonucudur ve bunu ESM'nin modifikasyonu izlemektedir (Rodrigues ve ark., 2019).

YARA İYİLEŞMESİNİ ETKİLEYEN FAKTÖRLER

Yara iyileşmesinde biyolojik sistemler arasındaki bu koordineli etkileşimler çeşitli sistemik ve lokal faktörlerden etkilenir. Venöz yetmezlik, enfeksiyon, ölü veya nekrotik dokuların varlığı, tekrarlayan travmalar ve hipoksi gibi lokal faktörler iyileşme süresini uzatabilir. Bireyin hastalık durumuyla birlikte kullanılan ilaçlar, yaş, cinsiyet, hormonlar, stres ve beslenme ise yara iyileşmesini etkileyen sistemik faktörler arasında gösterilmektedir. (Guo ve DiPietro, 2010).

Doku hasarının şiddetli ya da kronik olduğu ve hem parankimal hücrelerin hem de dokunun stromal çerçevesinin zarar gördüğü durumlarda iyileşme rejenerasyon (kaybedilen doku bileşenlerinin aynı şekilde tekrar kazanılması) ile gerçekleştirilemeyebilir. Bu koşullar altında ana iyileşme sürecinde yara, kolajen ve diğer ESM bileşenlerinin birikmesiyle onarılır. Onarım, bir dokuyu eski haline getirmektense "yamalayan" fibroproliferatif bir yanıttır ve aşırı miktarda kolajen birikimi hipertrofik skar ya da keloid oluşmasına neden olmaktadır. Hipertrofik skarların ve keloidlerin tedavisi zordur ve meydana gelmeden önlenmeleri en iyi tedavi yaklaşımı olarak kabul edilmektedir. Bu nedenle yara yönetiminin amacı, yarayı en az skar oluşumu ile mümkün olan en kısa sürede iyileştirmektir (Karakaş ve ark., 2012).

YARA TEDAVİSİNDE KULLANILAN YARA ÖRTÜLERİ

Yara örtüleri; bitkilerin, hayvan yağlarının, balın yara yüzeyine ham uygulamalarından doku mühendisliği ile üretilen yapı iskelelerinin yara bakımında kullanılmasına kadar yıllar içerisinde geliştirilmiştir. Yara çevresi temizliğinin öneminin farkına varılması ve cerrahideki iyi aseptik uygulamaları yara yönetiminde kullanılan malzemelerin de değişmesine yol açmıştır Günümüzde yara iyileşmesini desteklemek amacıyla yara çevresinin nemli tutulmasında, büyüme faktörlerinin yara bölgesine uygulanmasında, vitaminlerin ve antibakteriyel özellik gösteren maddelerin kontrollü salımının sağlanmasında ve iyileşmesi zor olan kronik yaralar için hücre ve doku yenilenmesine yardımcı olması hedeflenen daha gelişmiş uygulamalarda doğal ve sentetik polimerler kullanılmaktadır (Boateng ve ark., 2008). Şekillendirilebilir polimer kimyası, ihtiyaçlar doğrultusunda istenilen mekanik ve fiziksel özelliklere sahip malzemelerin üretilebilmesine olanak tanımaktadır. Medikal teknolojideki güncel yaklaşım ise biyoparçalanmayan polimerlerin yerine biyoparçalanır polimerleri koyma eğilimindedir (Anju ve ark., 2020; Ulery ve ark., 2011).

Kasım 1999'da yara örtülerinin tanımlanmasını ve sınıflandırılmasını yayınlayan Amerikan Gıda ve İlaç Dairesi (FDA); i) harici kullanım için resorbe olmayan gazlı bez/sünger yara örtülerini, ii) hidrofilik yara örtülerini, iii) oklüzif yara örtülerini ve iv) hidrojel yanık ve yara örtülerini sınıf I tıbbi cihaz olarak değerlendirmekteyken normal yara iyileşme sürecini hızlandırmayı, tam kalınlıkta deri greftlerinin yerini alabilmeyi (ör., yapay deri ikameleri) veya tam kalınlıktaki (3. derece) yanıkları tedavi etmeyi amaçlayan yara örtülerini sınıf III tıbbi cihaz kategorisinde tutmaktadır (FDA, 2016).

Tasarımlarında kanamayı durdurma, enfeksiyonu azaltma, yara eksüdasını absorplayabilme, debridmanı destekleme, kullanım kolaylığı sağlama, biyolojik olarak parçalanabilme, kolay sterilize edilebilme, toksik olmama, iyi su buharı ve gaz geçirgenliğine sahip olma gibi özelliklerin göz önünde bulundurulduğu yara örtüleri; pasif yara örtüleri, interaktif yara örtüleri, biyoaktif yara örtüleri olarak sınıflandırılmaktadır (Sezer ve Cevher, 2011).

Pasif yara örtüleri, gazlı bez ya da sargı bezi gibi, yara tedavisinde yaranın üzerini kapatarak dış ortamla temasını engelleyen, kanamayı kontrol eden ve yara eksüdasının buharlaşmasına izin vererek yarayı kuru tutan ürünleri kapsamaktadır. İnteraktif yara örtüleri ise pasif yara örtülerinin aksine, yara çevresinde nemli ortam sağlayarak re-epitelizasyonu iyileştiren, su buharı ve gaz geçirgenliği olan ve mikrobiyal enfeksiyonlara karşı bariyer görevi gören iyi mekanik özelliklere sahip ürünlerdir. Biyoaktif yara örtüleri, doku mühendisliği ürünlerini de içermekte ve normal yara iyileşme sürecinde ve yeni doku oluşumunda rol oynamaktadır. Ayrıca yara örtüleri, yaradaki işlevlerine (debridman, antibakteriyel, oklüzif, absorban, adezif), üretimlerinde kullanılan materyale (hidrokolloid, aljinat, kolajen) ve formlarına (merhem, film, köpük, jel) göre de gruplandırılabilir (Boateng ve ark., 2008; Alven ve ark., 2020).

İlerleyen bölümde biyoparçalanır polimerlerden üretilen film, köpük, hidrokolloid, aljinat ve hidrojel yara örtülerine yer verilmekte ve Tablo 1'de de üstünlükleri, sakıncaları ve klinikteki kullanımları özetlenmektedir.

YARA ÖRTÜLERİNDE KULLANILAN BİYOPARÇALANABİLİR POLİMERLER

Biyoparçalanma terimi tipik olarak parçalanmanın biyolojik varlıkları ve ortamları içerdiği durumlar için kullanılmaktadır. Vücut sıvıları, hücresel aktiviteler, enzimatik reaksiyonlar gibi faktörlerle *in vivo* olarak parçalanabilen polimerlerin, biyouyumlu parçalanma ürünlerine sahip olmaları ve parçalanma ürünlerinin resorpsiyona uğrayarak biyolojik sistemden uzaklaştırılabilmeleri gerekir (Tuncay ve Çalış, 1999). Materyalin parçalanma süresi, iyileşme veya rejenerasyon süreciyle senkronize olmalı, parçalanmayla materyalin mekanik özelliklerinde meydana gelen değişiklikler iyileşme veya rejenerasyon sürecini etkilememelidir (Nair ve Laurencin, 2007).

Biyoparçalanır doğal polimerik yara örtülerinin tasarımında alginat, kolajen, dekstran, hyaluronik asit, jelatin gibi polimerler kullanılırken biyoparçalanır sentetik polimerik yara örtüleri bunlarla sınırlı olmamakla birlikte poli(laktik asit) (PLA), poli(laktik-ko-glikolik asit) (PLGA), poli(glikolik asit) (PGA), poli(ε-kaprolakton) (PCL) ve poliüretan polimerlerini içermektedir (Mir ve ark., 2018).

Yara örtülerinde kullanılan biyoparçalanabilir doğal polimerler

Biyouyumlulukları, biyoparçalanabilirlikleri, ESM'ye benzerlikleri ve iyileşme yanıtını koordine eden hücrelerle etkileşimleri nedeniyle doğal polimerler, yara mikro-çevresini daha iyi taklit etmek ayrıca yapısal ve fonksiyonel iyileşmeyi uyarmak için cilt yenilenmesinde ve doku onarımında sıklıkla kullanılmaktadır. Antibakteriyel, antienflamatuvar, nemlendirici ya da hemostatik özelliklere de sahip olabilen doğal polimerlerin sayısız üstünlüğü bulunmaktadır (Mogoşanu ve Grumezescu, 2014; Gruppuso ve ark. 2021).

Ancak bu gruptaki polimerlerin büyük ölçekteki ticari üretim için sınırlı olmaları, kaynağa bağlı olarak varyasyonlarının bulunması, mekanik özelliklerinin ve degradasyon hızlarının kontrol edilmesinin güç olması ve saflaştırılmalarındaki zorluk sakıncaları olarak gösterilmektedir. İmmünolojik yanıt oluşturma ve mikrobiyal yük taşıma riskleri de göz ardı edilememektedir (Song ve ark., 2018; Tuncay ve Çalış, 1999). Bunların yanı sıra kitosan, kolajen, jelatin gibi polimerlerin hayvan doku bileşenlerinden elde edilmesi etik açıdan değerlendirilmesi gereken bir durumdur (Phelan ve Council, 2019).

Kolajen

Kolajen, tekrarlayan aminoasit dizilerinden oluşan proteinlerin üçlü sarmal yapısıdır. Son derece dinamiktir, gerekli fizyolojik işlevler için sürekli olarak yeniden şekillenmektedir. ESM'nin ana bileşenidir ve

yara iyileşme sürecinde pıhtılaşmanın indüklenmesinden yara izinin oluşumuna ve görünümüne kadar rol oynamaktadır (Song ve ark., 2018; Arif ve ark., 2021). Yaralanmayı takip eden enflamatuvar fazda, kolajenin degradasyonu sonucu oluşan Arg-Gly-Asp (RGD) tripeptidi makrofajlar için kemotaktik, fibroblastlar içinse mitojeniktir ve granülasyon dokusunun oluşumunu desteklemektedir (Bellis, 2011).

Genellikle, sığır veya domuz derisi ve tendonlarından elde edilmektedir; ancak hayvansal kaynaklı kolajenin saflaştırılması için yüksek maliyet gerektirmesi, değişen fizikokimyasal ve parçalanma profili ve bulaşıcı hastalık etkenlerini taşıma riskine karşı araştırmalar, rekombinant insan kolajeni üretebilen sistemler üzerine yönelmektedir (An ve ark., 2014).

Doku mühendisliği uygulamalarında cilt yapısına benzer modeller oluşturmak amacıyla kullanılmaktadır. Piyasada sünger, film ve hidrojel formlarında bulunan kolajen yara örtülerinin üçüncü derece yanıklar ve kuru yaralar için kontrendike olduğu ayrıca antimikrobiyal aktivite göstermediği de belirtilmektedir (Sahana ve Rekha, 2018).

Aljinat

Kahverengi alglerin (Macrocystis pyrifera, Laminaria hyperborea, Ascophyllum nodosum) hücre duvarlarında doğal olarak bulunan anyonik bir polisakkarittir. Aljinik asit ve tuzlarının yanı sıra aljinik asit türevleri de "aljinat" olarak adlandırılmaktadır. β-D(1-4)-mannuronik asit (M) ve α-L(1-4)-guluronik asit (G) monomerlerinden oluşur. M-bloklarının ve G-bloklarının yinelenmesiyle (MMMGGG) diblok kopolimer yapısında olabileceği gibi MG-bloklarının tekrarlamasıyla (MGMGMG) ardışık kopolimer yapısında da olabilir. M ve G bloklarının dağılım paterni, polimerin viskozitesini, jel oluşturma kapasitesini ve sıvı absorplayabilme yeteneğini etkilemektedir (Szekalska ve ark., 2016).

Biyouyumlu olması, hemostatik etki göstermesi, uygun maliyet gerektirmesi, iki değerlikli katyonların (Ca^{2+}) varlığında iyon değişimiyle yara yüzeyinde hidrojel oluşturarak yara iyileşmesi için gerekli olan nemi sağlaması ve yüksek derecede su/vücut sıvılarını absorplayabilme kapasitesi nedeniyle yara örtülerinin üretilmesinde yaygın olarak kullanılmaktadır (Cai ve ark., 2018).

Kitosan

Kitosan, deniz kabuklularının dış iskeletinde bulunan kitinin N-deasetilasyonu ile elde edilen, β-1,4-bağlı D-glukozamin (GlcN) ve N-asetil-D-glukozamin (GlcNAc) monomerlerinin farklı oranlarından oluşan lineer yapıda, polikatyonik bir aminopolisakkarittir (Colobatiu ve ark., 2019). N-asetil-D-glukozamin birimlerinin, polimerin toplam birim sayısına (D-glukozamin ve N-asetil-D-glukozamin birimleri) oranı kitosanın "asetilasyon derecesi"ni belirlemektedir. Deasetilasyon reaksiyonunun koşulları değiştirilerek farklı asetilasyon derecesine sahip polimerlerin üretimi sağlanabilmektedir. Asetilasyon derecesi, kitosanın çözünürlüğünü, kristalliğini, yük yoğunluğunu ve enzimatik bozunma hızını etkileyen bir parametre olarak kabul edilir (Lim ve ark., 2008). Keratinositlerin ve fibroblastların adezyonunun ve proliferasyonunun asetilasyon derecesinden etkilendiği de bildirilmektedir (Çakmak ve ark., 2009).

Yapısında bulunan amin ($-NH_2$) ve hidroksil (-OH) fonksiyonel gruplarının çapraz bağlama maddeleriyle reaksiyonu sonucu farklı özellikler kazandırılabilmekte, amin gruplarının, pH 6,3'ün altında amonyum gruplarına dönüşmesinden yararlanılarak pH'ya duyarlı malzemeler üretilebilmektedir (Pellá ve ark., 2018). Bunların yanı sıra, kitosanın antitrombojenik özellikler sunan hemostatik bir madde gibi davranması, viral ve bakteriyel enfeksiyonlara karşı konağın bağışıklık sistemini uyarması, düşük toksisite göstermesi (Çakmak ve ark., 2009), güçlü adezif özelliği, film oluşturma kapasitesi de yara iyileştirmede geniş bir uygulama alanı sağlamaktadır (Huang ve Fu, 2010).

Hyalüronik Asit

ESM'nin yapısında bulunan hyalüronik asit, D-glukuronik asit ve N-asetil-D-glukozamin birimlerinden oluşan, *in vivo* moleküler ağırlığı 2×10^4-1×10^8 Dalton arasında değişebilen lineer bir glikozaminog-

likandır. Fizyolojik pH'da, hyalüronan veya hyalüronat olarak adlandırılan tuz formundadır (Fallacara ve ark., 2018).

Molekül ağırlığı biyolojik fonksiyonlarını etkileyen temel faktördür ve yapısı, farklı moleküler ağırlıklarda üretilebilmesine olanak tanımaktadır. Düşük moleküler ağırlıktaki hyalüronik asit pro-anjiyogeniktir ve ESM'nin yeniden şekillenmesinde yer alan büyüme faktörlerinin yanı sıra pro-enflamatuvar sitokinlerin salımını da uyarmaktadır. Yüksek moleküler ağırlıktaki hyalüronik asit ise enflamatuvar hücrelerin migrasyonunu ve enflamatuvar sitokinlerin seviyesini kontrol ederek antienflamatuvar etki göstermektedir. İçerdiği karboksil ve hidroksil grupları, hyalüronik asidin hidrofilik özellikte olmasını, yara eksüdasını absorbe etmesini ve hücre adezyonunu güçlendirmesini sağlamaktadır (Graça ve ark., 2020). Ayrıca, yüksek higroskopikliği sayesinde doku hidrasyonunu düzenlemede kilit bir rol oynadığı belirtilmektedir (Longinotti, 2014).

Jelatin

Kolajenin asit ya da alkali hidrolizinden türetilen tek sarmallı bir protein olan jelatin kolaylıkla degradasyona uğrayabilmektedir. B tipi jelatin iyi biyouyumluluk özellikleri gösterirken A tipi jelatin viskoz çözeltiler oluşturmaktadır (Arif ve ark., 2021). Jelatinin mekanik ve termal özellikleri, çapraz bağlanma derecesine, kolajen kaynağına ve ekstraksiyon yöntemine bağlı olarak değişebilmektedir (Song ve ark., 2018).

İçerdiği peptit dizileri hücrelerdeki integrin reseptörlerinin tanınmasını sağlayarak hücrelerin adezyonunu destekler. Ayrıca, cildin yenilenmesi için gerekli olan nanolif yapıları oluşturma eğilimindedir. Nanolifler, geniş spesifik yüzey alanına, yüksek gözenekliliğe ve iyi geçirgenliğe sahip olmaları nedeniyle ESM'yi taklit edebilir (Zheng ve ark. 2018). Doku mühendisliğinde yapı iskele bileşeni olarak kullanılan jelatin, iskelelerde hücrelerin migrasyonunu, yayılmasını ve proliferasyonunu önemli ölçüde iyileştirmektedir. Degradasyon kinetiği ve jelleşme özellikleri ise ilaç taşıyıcı sistemlerin geliştirilmesinde çok yönlülük sunmaktadır (Su ve Wang, 2015).

Dekstran

Dekstran, α-1,6-bağlı D-glukoz monomerlerinden oluşan dallı yapıda bir polisakkarittir. Toksik olmaması, değiştirilebilir fonksiyonel gruplarının bulunması ve kanıtlanmış klinik güvenliliğiyle doku mühendisliği alanında yapılan çalışmalarda dikkat çekmektedir (Zhao ve Jalili, 2022).

Büyüme faktörü salgılanmasını ve hücre proliferasyonunu uyaran fibroblastlar üzerindeki spesifik glukan reseptörleri ile etkileşir bu nedenle biyouyumluluğu, biyoparçalanbilirliği ve hidrofilisitesinin yanı sıra yara tedavisinde ESM'deki glikozaminoglikanların bir analoğu olarak da kabul edilir (Gruppuso ve ark., 2021). Kullanıldıkları yara örtüsü tasarımlarında su buharı iletimini ve şişme kapasitesini artırdıkları (Kamoun ve ark., 2017), endotel hücrelerinin yara bölgesine migrasyonunu kolaylaştırdıkları ve tedavi sırasında hızlı neovaskülarizasyonu destekledikleri belirtilmektedir (Sun ve ark., 2011).

Yara örtülerinde kullanılan biyoparçalanır sentetik polimerler

Sentetik parçalanır polimerler ise doğal polimerlere göre düşük immunojeniteye sahiptir. Belirlenen spesifikasyonlar (molekül büyüklüğü, yük, hidrofobisite ve ilaç yükleme kapasitesi) doğrultusunda sentezlenebilmeleri, seriden seriye tekdüzeliğin sağlanabilmesi (Vaid ve ark., 2020), hazırlık ve saklama süresince mekanik dayanıklılıklarını koruyabilmeleri sentetik polimerlerin üstünlükleri arasında sayılmaktadır.

Ancak hücresel etkileşimleri desteklemediklerinden daha uzun iyileşme süresi gerekebilir. Parçalanma ürünlerinin toksisitesi değerlendirilmelidir. Kötü biyouyumluluk gösterebilirler bu da ciddi enflamatuvar yanıtların oluşmasına neden olabilir (Gruppuso ve ark., 2021).

Poliesterler

Vücuttan atılabilmeleri için molekül ağırlıklarının ortalama 50.000 Daltondan az olması gereken poliester grubu polimerler düşük toksisite gösterirler (Ulery ve ark. 2011). FDA tarafından onaylı poliesterler PLA,

PGA, poli(hidroksi bütirat) (PHB) ve PCL ticari olarak kolaylıkla temin edilebilmekte ve biyomedikal uygulamalarda yaygın olarak kullanılmaktadır. Başlangıç materyali olarak FDA onaylı polimerlerin tercih edilmesi, daha hızlı bir pazara çıkma olanağı sağlamaktadır (Arif ve ark. 2021).

Poliesterlerin fizyolojik sistemlerde şişme davranışlarını etkileyen hidrofobik profilleri, PGA hariç olmak üzere, tıbbi uygulamalarda bir sakınca oluşturmaktadır. Çoğu poliesterin yüzey enerjisi, hidrofilik olmayan karakterlerinin bir sonucu olarak oldukça düşüktür, bu da zor ıslanabilirliğe ve yavaş parçalanmaya neden olmaktadır. Zor ıslanabilirlik ise biyouyumluluğu olumsuz yönde etkilerken implant materyali ile canlı doku arasında da yetersiz etkileşime yol açmaktadır. PGA, bulk erozyonla vücut tarafından metabolize edilebilen ve idrarla atılabilen ya da Krebs döngüsünde karbondioksit ve suya dönüştürülebilen glisin ve glikolik asite parçalanır. Bu nedenle immün ya da toksik yanıt oluşturması beklenmez ancak parçalanma hızı yüksek olduğunda ortaya çıkan asidik parçalanma ürünleri kullanımlarını kısıtlar. Metil gruplarının varlığı nedeniyle PGA'dan daha hidrofobik olan PLA, daha yavaş bir parçalanma hızına sahiptir. Glikolik asidin aksine laktik asidin optik olarak aktif iki formu (L-laktik asit ve D-laktik asit) bulunmaktadır. Optik olarak aktif monomerlerin polimerizasyonuyla poli(L-laktik asit) (PLLA) veya poli(D-laktik asit) (PDLA) elde edilmektedir. PLLA moleküler ağırlığına bağlı olmak üzere yaklaşık %37 kristalliktedir. PGA ile karşılaştırıldığında PLLA daha yavaş biyoparçalanmaktadır. PLGA kopolimeri ise biyouyumluluk, mekanik dayanıklılık ve istenen şekil ve boyutlarda manipüle edilebilme gibi birçok önemli özelliğe sahiptir. Üç boyutlu yapı iskelelerinde hücre adezyonunu ve proliferasyonunu sağlamaktadır. PCL, nispeten ucuz bir monomer olan "ε-kaprolakton" kullanılarak halka açılma polimerizasyonu ile sentezlenen yarı kristal formda bir diğer poliesterdir. 55–60 °C'lik düşük erime sıcaklığı (Tm) ve -60 °C'lik camsı geçiş sıcaklığı (Tg) polikaprolaktonun kolayca işlenmesini sağlar (Nair ve Laurencin, 2007; Vaid ve ark., 2020). Bu özellikleri onu absorplanabilir liflerin eğirme yöntemi için alternatif bir madde haline getirir. Elektroeğrilmiş PCL lifleri, ESM'nin lifli yapısını taklit edebilmektedir (Mir ve ark., 2018).

Poliüretan

Poliüretanlar, yaygın olarak yara örtüsü malzemelerinin üretiminde kullanılan başka bir polimer sınıfıdır; eksojen yolla mikroorganizmaların bulaşmasına karşı bir bariyer görevi görme, oksijen değişimini destekleme, su buharı iletimini kontrol etme ve epitelizasyonu hızlandırma özellikleri nedeniyle potansiyel yara örtüsü malzemeleri olarak değerlendirilmektedir (Khil ve ark., 2003). Ancak yara bölgesi çevresindeki nemli ortamı desteklediklerinden ağır eksüdalı yaralarda kullanımları uygun bulunmamaktadır (Arif ve ark. 2021).

BİYOPARÇALANIR POLİMERİK YARA ÖRTÜLERİ

Film yara örtüleri

Bir yüzünde adezif poliüretan katman bulunan film yara örtüleri, su buharının, O_2 ve CO_2 gazlarının geçişine izin veren ancak bakterilere karşı bir bariyer oluşturan şeffaf membranlardır. Son derece elastik ve esnektirler bu nedenle istenilen şekle uyarlanabilirler. Şeffaf oldukları için çıkarılmadan da yara iyileşmesinin takip edilebilmesine olanak tanırlar. Sınırlı absorpsiyon kapasitesine sahip olmaları yüksek eksüdalı yaralarda kullanımlarını sınırlar. Yüzeysel ya da az eksüdalı yaralar için tercih edilirler (Dhivya ve ark., 2015).

Saf hyalüronik asidin esterleştirilmiş versiyonu olan HYAFF®'dan üretilen Hyalosafe® film yara örtüsü (Şekil 3.) (Longinotti, 2014), yüzeysel yaraları örtmek ve yara çevresinde nemli bir ortam sağlamak için tasarlanmıştır. 15 x 10 cm boyutlarında steril, esnek ve şeffaftır. Doğrudan lezyon bölgesine uygulanabilmekte ve herhangi bir araç gerektirmeden sabitlenebilmektedir. Hyalosafe® film yara örtüsünün kısmi kalınlıkta yüz yanığı olan hastalara uygulandığı klinik çalışmada, hastaların büyük çoğunluğunda skar oluşumu meydana gelmeden ortalama 9 günde iyileşme

sağlandığı kaydedilmiştir. Ayrıca, ağrı duyusunda belirgin bir azalma gözlemlendiği belirtilmiştir. Ürünün bir kez uygulanması ve rezorbe olması tekrar yara örtüsü uygulama ihtiyacını ortadan kaldırdığı için yara bölgesi ile temasın önlenerek enfeksiyon riskinin azaltıldığı raporlanmıştır. Hastanede kalış süresini kısaltılması ve pansuman gerektirmeme üstünlüğü de bulunmaktadır (Yildirim ve ark., 2019).

Şekil 3. Tipik bir film yara örtüsü (Hyalosafe®, Anika Therapeutics S.r.l., Abano Terme, İtalya), (Longinotti, 2014).

Genellikle travma ve ortopedik cerrahide klinik kullanım için piyasada bulunan diğer biyoparçalanabilir yara örtüsü laktik asit-kaprolakton kopolimer bazlı Topkin®'dir (Biomet Merck GmbH). Topkin®, ağrılı olabilen yara örtüsü değişimini en aza indirgemek ve steril koşullar altında yüzeysel ve enfekte olmayan yaraları geçici olarak kapatmak için geliştirilmiştir. Biyoparçalanma, kopolimerin, D ve L-laktik aside ve 6-hidroksikaproik aside hidroliziyle yaklaşık 4 hafta içerisinde gerçekleşir. L-laktik asit Cori döngüsünde glukoza dönüştürülebilirken, D-laktik asit renal yolla ya da CO_2 şeklinde akciğerlerden atılabilmektedir (FDA, 2003). pH'nın hidrolitik reaksiyon sırasında asit aralığına doğru kayması bakteriyel çoğalmanın azalmasına ve bununla birlikte epitel hücrelerinin proliferasyonunun artmasına neden olur. Yüksek lokal laktat konsantrasyonu ise kolajen sentezini uyarır (Jürgens ve ark., 2006).

Köpük yara örtüleri

Köpük yara örtüleri sünger formunda da işlenebilen (Maye ve ark., 2014), tipik olarak poliüretan bazlı gözenekli materyallerdir. Son derece absorban özellikte olan köpüklerin absorpsiyon kapasiteleri yapılarına, kalınlıklarına ve gözenek boyutlarına bağlı olarak kontrol edilebilmektedir. Otolitik debridmanı uyaran aynı zamanda su buharı ve gaz alışverişine izin veren köpük yara örtüleri, yara çevresinde nemi korumakta ve termal izolasyon sağlamaktadır. Ülserde, deri transplantlarında ya da orta-ağır eksüdalı yanıklarda uygulanmaktadırlar.

Üretimlerinde doğal polimerlerin de kullanılmaya başlanmasıyla köpük yara örtülerine benzersiz özellikler kazandırılmıştır. Poliüretan kompozitleriyle, uygulanan kuvvet altında esneklik ve deforme olabilirlik gibi poliüretanın niteliklerini korurken daha iyi dayanıklılık ve ek biyoaktivite elde edilmektedir (Boateng ve ark., 2008; Gruppuso ve ark., 2021). Namviriyachote ve arkadaşları, gümüş nanopartikülleri ile fonksiyonelleştirdikleri poliüretan köpük yara örtüsünü nişasta, yüksek moleküler ağırlıklı kitosan ve jelatin ile birleştirerek köpüğün sertliğini ve gözenekliliğini artırdıklarını bildirmişlerdir (Namviriyachote ve ark., 2020). Bužarovska ve arkadaşları da poliüretanın mekanik özelliklerini, ZnO nanoparçacıklarının antimikrobiyal karakteri ve yara hücre adezyonunu ve büyümesini destekleme yetenekleri ile geliştirmek amacıyla termoplastik poliüretan/ZnO nanokompozit köpükleri üretmişlerdir. 10-60 μm arasında boyutlara sahip birbirine bağlı gözeneklerden oluşan nanokompozit yapı, 8,9 mg/cm^2'ye kadar su buharı iletim hızına (WVTR) izin vermektedir (Bužarovska ve ark., 2019).

Köpüklerin çok yönlülüğü; yoğunluk ve gözenek morfolojisi açısından yapılarının ve sistemdeki çoklu fazların bileşimi, şekli ve miktarı açısından da formülasyonlarının istenilen şekilde tasarlanabilmelerine dayandırılmaktadır. Sistemin hafifliği veya yoğunluğu, gözenek boyutu ve dağılımı, açık ya da kapalı gözenekliliği köpüklere özel nitelikler kazandıran tipik yapısal özelliklerdir (Trucillo ve Di Maio, 2021).

Ticarileştirilen CuraSpon® (CuraMedical B.V) (Şekil 4.), yüksek oranda saflaştırılmış jelatin köpükten üretilmiştir. Tek tip gözenek yapısına sahiptir. Kanamayı kontrol etmek için kullanılan ligasyon, dikiş atma veya diğer geleneksel yöntemlerin kullanışsız ya da etkisiz olduğu cerrahi prosedürlerde, kılcal, venöz ve küçük arteriyel kanamalarda hemostazın sağlanması için uygulanmaktadır. Jelatin sünger matriksi trombositleri aktive etmekte, trombositlerin yüzey karakterlerini değiştirirken aynı zamanda agregasyonlarını destekleyen bir dizi madde salgılamalarına ve bu sayede fibrin oluşumu için katalizör görevi görmelerine katkıda bulunmaktadır. 4 hafta içerisinde tamamen emilmektedir (Trucillo ve Di Maio, 2021; CuraMedical, 2017; CuraMedical, t.y.).

Şekil 4. CuraSpon® köpük yara örtüsü (CuraMedical B.V, Hollanda), (CuraMedical, 2017).

Hidrojel yara örtüleri

Hidrojeller, polimer zincirleri arasındaki boşluklarda çok miktarda su tutarak şişebilme özelliğine sahip üç boyutlu çapraz bağlı ağ yapılardır. Elastikiyetlerinin doğal dokulara benzerliği, yara yüzeyini soğutarak ağrıyı azaltmaları, yara için nemli bir ortam sağlamaları, gözenekli yapıları sayesinde yara eksüdasını absorplayabilmeleri, gaz alışverişine izin vererek anaerob bakterilerin çoğalmasını önlemeleri yara iyileştirme uygulamalarında akademinin ve endüstrinin ilgisini çekmektedir (Asadi ve ark., 2020; Bagher ve ark., 2020; Cascone ve Lamberti, 2020). Ayrıca, hidrojel matriksine antienflamatuvar ilaçlar (Kong ve ark., 2019) ya da büyüme faktörleri yüklenerek ek biyoaktivite oluşturulabilmektedir (Obara ve ark., 2003).

Biyoparçalanır hidrojel yara örtüleri kimyasal ya da fiziksel çapraz bağlı hyalüronik asit, kitosan, selüloz, aljinat, kolajen ve jelatin makromoleküllerinden üretilebilir (Francesko ve ark., 2018; Mogoşanu ve Grumezescu, 2014; Murray ve ark., 2019). Fiziksel olarak çapraz bağlı hidrojeller, pH'nın, sıcaklığın, hidrojen bağ etkileşimlerinin ve iyonik kuvvetlerin değiştirilmesi ile modüle edilebilir (Annabi ve ark., 2014). Bu sistemler, kimyasal modifikasyona gerek duyulmadan ya da bir çapraz bağlayıcı kullanılmadan oluşturulabilir, bu nedenle *in vivo* uygulamalarda güvenli olarak değerlendirilmektedir. Ancak zayıf mekanik özellik gösterdikleri ve hızlı parçalandıkları için gözenek büyüklükleri, jelleşme süreleri ve parçalanma profilleri dahil olmak üzere yapı değişkenlerini kontrol etmek güçtür. Bu durum ürünlerin tasarım esnekliğini de kısıtlamaktadır. Fiziksel olarak bir arada duran materyallere göre kimyasal çapraz bağlı hidrojeller daha iyi mekanik özelliklere sahiptir. Bununla birlikte, çapraz bağlanmada polimer ön aktivasyonunu sağlayan ek bir adımın uygulanması ya da bir çapraz bağlayıcının kullanılması, artık kimyasalların ve organik çözücülerin sitotoksik etki göstermesine neden olabilir (Francesko ve ark., 2018).

İyileşme sürecinin tüm aşamalarında (hemostaz, iltihaplanma, hücre göçü/çoğalması ve olgunlaşma) kullanılabilen hidrojeller elastik yaprak, amorf jel ya da film şeklinde hazırlanabilir. Yaprak formundaki hidrojeller yara çevresine uygun şekilde kesilip uygulanabilirler. Primer örtü olarak kullanılabilir ve adezif ikincil bir örtü gerektirmezler (Boateng ve ark., 2008). Piyasada bulunan Helix3-cm® (Amerx Health Care Corporation) %100 sığır kolajenin üçlü-sarmal liflerinden oluşan ince yaprak şeklinde, yarı şeffaf bir yara örtüsüdür. Yara iyileşme sürecinin daha iyi gözlemlenmesini sağlar ve yara çevresini nemli tutar. Granülasyon dokusunun oluşmasını ve epitelizasyonun gelişmesini destekler. Yanıkların, yaraların, kabarcıkların ve ülserlerin tedavisinde topikal ilaçlarla kombine bir şekilde de kullanılabilir (Cascone ve Lamberti, 2020; Amerx Health Care, t.y.).

Ayrıca polimerizasyon yöntemlerindeki gelişmeler, yaraya doğrudan uygulanabilen ve derin ya da anormal şekilli yaraları tam kapatabilen enjekte edilebilir hidrojellerin üretilmesine olanak sağlamaktadır. Enjekte edilebilir hidrojeller belirli uyaranlar altında *in situ* olarak soldan jele dönüşmektedir (Murray ve ark., 2019; Qiu ve ark., 2021). Deng ve arkadaşları altın nanokompozitleri ile yüklenen oksitlenmiş sodyum aljinat ve modifiye edilen jelatinden oluşan enjekte edilebilir biyomimetik hidrojellerin yara iyileşmesini hızlandırdığını ve bakteriyosidal etki gösterdiğini raporlamıştır (Deng ve ark., 2021). Qu ve arkadaşlarının yaptığı bir çalışmada da N-karboksietil kitosan ve oksitlenmiş hyalüronik asit greft edildiği anilin tetramerinden üretilen iletken özellikteki enjekte edilebilir hidrojellerle tam kalınlıktaki cilt kaybı modelinde granülasyon dokusu gelişiminin iyileştirildiği, anjiogenezin ve kolajen birikiminin desteklendiği belirtilmiştir (Qu ve ark., 2019).

Aljinat yara örtüleri

Aljinat yara örtüleri genel olarak ameliyat sonrası enfekte yaralar ve bacak ülserleri dahil olmak üzere eksüdalı yaraları tedavi etmek için kullanılmaktadır. Dondurarak kurutma ile hazırlanan gözenekli tabakalar ya da yara boşluklarına doldurulabilen lifli yapılar şeklinde bulunmaktadır (Mir ve ark., 2018).

Aljinat yara örtüleri iki değerlikli katyonların varlığında iyonotropik jelleşmeye uğrar. Kalsiyum iyonlarının yara yatağında bulunan sodyum iyonları ile yer değiştirmesi sonucu oluşan hidrofilik jel, yara çevresindeki salgıları sınırlandırır ve bakteriyel kontaminasyonu en aza indirir. Aynı zamanda doku rejenerasyonunu uyaran nemli bir ortam yaratır. Bunlara ek olarak, salınan kalsiyum iyonları koagülasyon kaskadında da aktif bir rol oynar. Sıvılara maruz kaldıktan sonra daha esnek jeller oluşturan mannuronik asit açısından zengin (Sorbsan™); diğeri ise hidratlandığında daha güçlü jel yapısı oluşturan guluronik asit açısından zengin (Kaltostat®) yara örtüleri olmak üzere iki farklı formülasyonda tasarlanır (Boateng ve ark., 2008; Gruppuso ve ark., 2021). Formülasyondaki guluronik/mannuronik asit oranına bağlı olarak aljinatlar kendi ağırlıklarının 15-20 katı kadar sıvıyı emebilmektedir. Ayrıca diğer yara örtüsü gruplarında olduğu gibi, aljinat yara örtülerinin de aljinat/hidrokolloid, aljinat/hidrojel ve aljinat/kolajen gibi farklı kombinasyonları bulunmaktadır (Jones, 1999).

Orta, ağır derecede eksüdalı kronik ve akut yaralar ve hafif kanamalı yaralar için birincil yara örtüsü olarak geliştirilen Kaltostat® (Convatec), aljinat (% 80 kalsiyum aljinat, % 20 sodyum aljinat) bazlı bir yara örtüsüdür. Eksüdadaki sodyum iyonları ile yara örtüsündeki kalsiyum iyonları yer değiştirmekte ve bu değişim sayesinde yara örtüsü kuru fibröz yapıdan dereceli olarak jele dönüşmektedir. Aynı zamanda hemostat özelliğe sahiptir ve hafif kanamalı yaralarda pıhtılaşmayı desteklemektedir (Cascone ve Lamberti, 2020; Convatec, t.y.).

Kalsiyum aljinat bazlı Sorbsan™, Algisite™ M, Algosteril® ve Curasorb™ yara örtüleri ise genellikle venöz ya da diyabetik ülserlerin tedavisinde kullanılmaktadır (Gruppuso ve ark., 2021).

Aljinat yara örtülerinin her ne kadar biyolojik olarak parçalanabilir olduğu belirtilse de bu materyaller çok yavaş emildiğinden dokularda uzun süre bırakıldığında yabancı cisim reaksiyonuna neden olabilir (Jones, 1999). Etkili bir şekilde fonksiyonlarını yerine getirmek için neme ihtiyaç duyduklarından kuru yaraların ve sert nekrotik dokuların iyileştirilmesinde kullanılmazlar. Bu yara tiplerinde yüksek hacimde sıvıyı tutarak ciltte kuruluğa ve hastada yanma hissine neden olabilirler (Gruppuso ve ark., 2021).

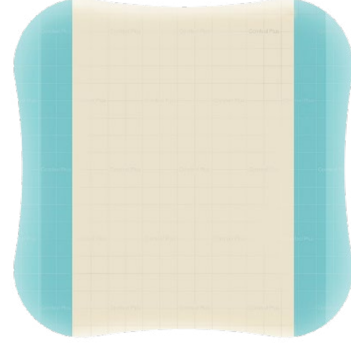
Hidrokolloid yara örtüleri

Yara çevresinin nemli tutulması için kullanılan yara bakım ürünlerinde hidrojellerle birlikte hidrokolloidler de bulunmaktadır. “Hidro” eki ile adlandırılmış olsalar da hidrokolloidler nem içermez, bunun yerine yara yüzeyinde bir “mühür” oluşturarak cildin neminin buharlaşmasını önlerler (Beldon, 2010).

Yara iyileşmesinde yaygın olarak kullanılan hidrokolloid yara örtüleri elastomerler ve adezifler gibi diğer bileşenlerle birlikte koloidal malzemelerden (jelleştirici maddelerden) elde edilen ürünlerdir. Tipik jelleştirici maddeler arasında karboksimetil selüloz, jelatin ve pektin gösterilebilir (Boateng ve ark., 2008).

Kalın ve adezif özelliğe sahip jelleşebilir tabaka esnek ve su geçirmeyen film/yaprak üzerine lamine edilmektedir (Mir ve ark., 2018).

Su buharına karşı geçirimsiz olan hidrokolloid yara örtülerinin yara eksüdasının absorpsiyonuyla birlikte fiziksel yapısı değişmekte ve hidrokolloidler su ve hava için giderek daha geçirgen hale gelmektedir (Beldon, 2010). Hidrokolloid yara örtülerinin sıvıyı hapsetme kapasitesi ise örtünün fizikokimyasal özellikleri ve tasarımı gibi birçok faktöre bağlıdır (Thu ve ark., 2012). İlk hidrokolloid yara örtüleri (1982–83), eksüdanın fazla olduğu yaraların tedavisinde iyi bir performans gösterememiş olsa da yüksek seviyede eksüdalı yaralar için revize edilmiş formülasyonlar geliştirilmiştir (Agarwal ve ark., 2011). Örnek olarak aljinat içeren hidrokolloidler verilebilir (Mir ve ark., 2018). Comfeel® Plus Ulcer (Coloplast) (Şekil 5.) sentetik, elastik ve yapışkan bir kütle içinde enkapsüle edilmiş nemi absorbe eden sodyum karboksimetil selüloz parçacıklarından oluşur. Emilimi artırmak için yara örtüsüne kalsiyum aljinat eklenmiştir. Üst tabakası mikroorganizmaların ve suyun yara bölgesine girişini engelleyen yarı geçirgen poliüretan filmden oluşmaktadır. Bacak ülserleri, basınç ülserleri, yüzeysel yanıklar, ameliyat sonrası yaralar ve cilt sıyrıkları dahil olmak üzere düşük-orta derecede eksüdalı yaraların tedavisinde kullanılabilmektedir. Yara eksüdasıyla temas ettiğinde eksüdayı absorplayan ancak yaraya yapışmayan viskoz bir jel oluşturmaktadır (Coloplast, t.y.).



Şekil 5. Comfeel® Plus Ulcer (Coloplast A/S, Danimarka), (Coloplast, t.y.).

Hidrofiber teknolojisi ile geliştirilen Aquacel® Ag (Convatec Inc.) ise gümüş içeren % 100 sodyum karboksimetilselülozdan oluşan, eksüda ile temas ettiğinde jele dönüşerek nemli bir ortam sağlayan ve yara iyileşmesini kolaylaştırdığı gösterilen hidrokolloid bir yara örtüsüdür. Bütünlüğünü kaybetmeden ağırlığının 30 katına kadar sıvıyı absorplayabildiği belirtilmektedir. Aquacel® Ag yara örtüsünün, total eklem artroplasti ameliyatlarının yaklaşık üçte birinde rapor edilen yara bölgesinde su toplanması, enfeksiyon riski ve gecikmiş iyileşme gibi komplikasyonlar üzerindeki etkisinin değerlendirildiği klinik çalışmada hem elde edilen sonuçlar hem de hasta memnuniyeti açısından geleneksel gazlı bez yara örtüsü ile gerçekleştirilen tedaviden daha üstün olduğu raporlanmıştır (Akdogan ve Atilla, 2020).

Tablo 1. Film, köpük, hidrojel, aljinat ve hidrokolloid yara örtülerinin üstünlükleri, sakıncaları ve klinikteki kullanımları (Gruppuso ve ark., 2021; Leveriza-Oh ve Phillips, 2021).

Yara Örtüsü	Üstünlükleri	Sakıncaları	Klinik Kullanım
Film	• Şeffaf • Bakteriler için bariyer oluşturur • Adezif olduğu için ikincil bir yara örtüsü ile sabitlenmesi gerekmez • İyi gaz geçirgenliğine sahip	• Yaraya yapışabilir • Ağır eksüdalı yaralarda sıvı birikmesine neden olabilir	• Yüzeysel yara ve yanıklar • Enfekte yaralar • Minimal eksüdalı kısmi kalınlıktaki yaralar
Köpük	• Yüksek miktarda eksüdayı absorbe etme yeteneği bulunur • Otolitik debridmanı destekleyicidir • İyi su buharı ve gaz alışverişi sağlar	• Opak • Zayıf adezif özelliktedir • Kuru yaralarda kullanışsızdır	• Ülser • Deri nakli • Hafif yanıklar • Orta-ağır eksüdalı yaralar
Hidrojel	• Yüksek derecede eksüdayı absorplayabilme kapasitesine sahiptir • Kolay işlenebilirlik özelliktedir • Yaraya direkt uygulanabilir • Nekrotik doku otolizine yardımcı olur • Oksijen değişimine izin verir • Uygun maliyet	• Zayıf mekanik dayanıklılık gösterir • Bakterilere karşı etkisiz bariyer oluşturur • Ciltte maserasyon gerçekleşebilir • İkincil yara örtüsü kullanımı gerektirebilir	• Kuru yaralar • Orta derecede eksüdalı yaralar • Derin yaralar
Aljinat	• Yüksek düzeyde absorban • Hemostatik • Yaralara yapışmaz • Toksisite göstermez • Eksüda varlığında jel oluşturma yeteneği bulunur	• Kuru yaralar için endike değil • Jel hoş olmayan kokuya neden olabilir • İkincil yara örtüsü gerekebilir	• Kanamalı yaralar • Enfekte yaralar • Cerrahi yaralar • Ağır eksüdalı yaralar
Hidrokolloid	• Otolitik debridmanı destekler • Anjiyogenezi arttırır • Absorban • Bakteriyel ve fiziksel bariyer oluşturur • Nem regülasyonu sağlar	• Opak • Yüksek maliyet gerektirebilir • Gazlara karşı geçirimsiz	• Basınç ülserleri • Venöz ülserler • Akut cerrahi yaralar

DOKU MÜHENDİSLİĞİ YAKLAŞIMIYLA ÜRETİLEN DERİ İKAMELERİ

Cilt onarımında, fibroblastlar ve diğer hücreler granülasyon dokusunu oluşturmak için yaralanmadan kaynaklanan boşluğu ESM ve yeni kan damarları ile doldururken keratinositler boşluğun üzerini kapatır. Ancak epidermis ve dermis hasarının geniş çaplı olduğu yara ve yanıklarda cilt, restorasyonu için gerekli olan hücrelerden genellikle yoksundur ve ESM yapısı bozulmuştur. Bu nedenle çok daha karmaşık bir tedaviye gereksinim duyulmaktadır (Böttcher-Haberzeth ve ark., 2010; Vig ve ark., 2017).

Re-epitelizasyon için kendi kendini yenileyen keratinosit kök hücrelerini ve minimum yara izi oluşumu için uygun selüler ve aselüler bileşenleri içeren deri ikamelerinin geliştirilmesi bu açıdan yara tedavisinde kilometre taşlarından biri olmuştur (Böttcher-Haberzeth ve ark., 2010). Vücutta kimyasal olarak parçalanan ve emilime uğrayarak resorbe edilen aynı zamanda doğru bir şekilde doku entegrasyonunu sağlayan biyomalzemelerin kullanılmasıyla birlikte de oldukça ilerleme kaydedilmiştir (Anju ve ark., 2020).

Doku mühendisliği temel olarak canlı hücrelerle ekilmiş doğal ya da sentetik biyoparçalanabilir bir iskelenin kültüre alındıktan sonra *de novo* doku oluşumunu indüklemesine dayalıdır (Lo ve ark., 2014). Hücre kaynağı, otolog (hastanın kendi hücreleri), allojenik (bir donörden elde edilen insan hücreleri) ya da ksenojenik (hayvan kaynaklı hücreler) olabilir. İskeleler ise doğal ESM'lere benzer şekilde, hücrelerin

çoğalmasını ve farklılaşmasını desteklemek amacıyla kullanılan yapay ESM'lerdir (Akter, 2016). İskele, hücrelerin yüzeylere yapışmalarını sağlamak için biyouyumlu olmalı ve biyoparçalanarak hücrelerin kendi ESM'lerini oluşturabilmelerine izin vermelidir. Üç boyutlu (3D) ve gözenekli formda tasarlanan iskelelerin, hücrelerin büyüklüğü ve hücre migrasyonu gereksinimleri nedeniyle, ideal gözenek boyutu yaklaşık 100 µm'dir, ancak kılcal damarların oluşması için 300 µm'den daha büyük gözenek boyutları tercih edilmektedir.

İskele yapılarının üretiminde PLLA, PGA ve PLGA dahil olmak üzere çok sayıda sentetik polimer kullanılmaktadır. Sentetik polimerler, istenilen özelliklerde üretilebilmeleri, kopolimer bileşimlerinin değiştirilebilmesi ve parçalanmalarının kontrol edilebilmesi açısından oldukça başarı göstermiş olsa da biyoaktivitenin azalması nedeniyle vücuttan reddedilme riskileri de göz önünde bulundurulmalıdır. Ayrıca, PLLA ve PGA'nın hidrolizle karbondioksite parçalanarak lokal pH'yı düşürmeleri, hücre ve doku nekrozuna yol açabilmektedir. Sentetik polimer bazlı iskelelerin aksine doğal polimerlerden üretilen iskele materyalleri biyolojik olarak aktiftir. Kolajen, kitosan, jelatin, fibrin, hyalüronik asit ya da aljinat bazlı iskeleler mükemmel hücre yapışmasını sağlamakta ve hücrelerin gelişmesini desteklemektedir (Dixit ve ark., 2017; O'brien, 2011).

Deri rekonstrüksiyonu için kullanılan biyomateryaller genel olarak toksik ya da immünojenik olmamalı, aşırı enflamasyona yol açmamalıdır. Yer değiştirecekleri derinin anatomik ve mekanik özelliklerine benzer özelliklere sahip olmalı, yara yüzeyinden sıvı ve ısı kaybını önlemeli ve yarayı enfeksiyondan korumalıdır. Bunlarla sınırlı olmamakla birlikte biyomateryalin uygun maliyetli olması, kolaylıkla temin edilebilmesi ve uzun raf ömrüne sahip olması da önemli bir üstünlük yaratacaktır (Shevchenko ve ark., 2010).

Yaraların heterojen doğası nedeniyle "herkese uyan" bir tedavi seçeneğinin olmaması, ayrıca FDA'nın, doku mühendisliği ile üretilmiş deri ikamelerini sınıf III tıbbi cihaz olarak değerlendirmesine rağmen biyolojik standartlarda tutması geri ödeme sorunlarına yol açarak ürünlerin klinikteki kullanımlarını etkilemiş olsa da teknolojideki sürekli ilerlemenin gelecekte yeni yara iyileştirme tedavileri ile sonuçlanacağı ve doku mühendisliği ürünlerinin pazar paylarının artacağı öngörülmektedir (Li ve ark., 2009, Murray ve ark., 2019).

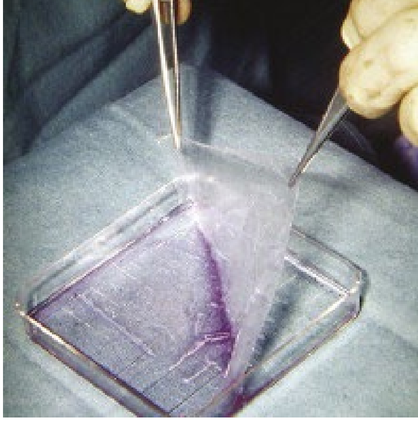
Devam eden bölümlerde ticari olarak temin edilebilen ve klinikte kullanılan deri ikameleri anatomik yapı sınıflandırmasına göre (dermal, epidermal ve dermal-epidermal (kompozit)) (Shevchenko ve ark., 2010) (Şekil 6.) detaylandırılmış ve Tablo 2'de özetlenmiştir (Vig ve ark., 2017).

Epidermal deri ikameleri

1970'lerdeki ilk başarılı keratinosit kültürüyle birlikte keratinositlerin allogreftleri ve otogreftleri yanıkların tedavisinde uygulanmaktadır. Son derece farklılaşmış epitel hücreleri olan keratinositler, dış çevreye karşı koruyucu bir tabaka meydana getirmekte ve dehidrasyonu önlemeye yardımcı olmaktadır (Murray ve ark., 2019). Keratinositler hücre tabakaları olarak hazırlanabilir ancak doğal ya da sentetik polimer iskeleleri üzerine kültüre edilmesiyle keratinositlerin kültürleme süresi azaltılırken sistemin mekanik özellikleri iyileştirilerek uygulama kolaylığı sağlanmaktadır. Ayrıca bakteriyel enfeksiyonlara ve dehidrasyona karşı daha iyi bir bariyer elde edilmektedir.

Ticari olarak temin edilebilen epidermal cilt ikameleri arasında, Fidia Advanced Biopolymers tarafından tasarlanan ve Bristol-Myers Squibb Company'nin bir parçası olan ConvaTec'e üretim ve dağıtım hakları verilen Laserskin® (Vivoderm®) (Şekil 7.) bulunmaktadır (Shevchenko ve ark., 2010; Zeng ve ark., 2017). Ürün, mikrodelikli hyalüronik asit benzil esteri matriksi üzerine kültürlenen otolog keratinositlerden oluşur. Hyalüronik asidin esterleşme derecesinin (%75-100) değiştirilmesiyle, sistemin parçalanma kinetiğini kontrol etmek mümkündür. Mikrodelikli yapısı keratinositlerin destek materyalinden yara yatağına doğru göç etmesine izin verir. Laserskin® (Vivoderm®) absorplandıktan sonra lezyon bölgesinde sadece yeni oluşan doku kalır. Venöz, diyabetik ve yatak yaraları-

nın ve II. ve III. derece yanıkların tedavisindeki potansiyel kullanımına atıfta bulunulmaktadır (Soranzo ve ark., 1996).



Şekil 7. Laserskin® (Fidia Advanced. Biopolymers, Padua, İtalya), (Zeng ve ark., 2017).

Bioseed-S (BioTissue Technologies GmbH) epidermal cilt ikamesi ise fibrin yapıştırıcı (Tissucol Duo S Immuno) içerisinde süspanse edilmiş otolog keratinositlerden oluşur. İyileştirilmiş hemostaz sağlar ve hücrelerin tutunmasını destekler. Genellikle tedaviye dirençli kronik venöz bacak ülserlerini tedavi etmek için kullanılmaktadır (Przekora, 2020; Shahrokhi ve ark., 2014).

Dermal deri ikameleri

Dermal deri ikameleri, tip I ve tip III kolajen bazlı ESM'den ve fibroblast hücrelerinden oluşan dermis tabakasının fonksiyonlarını yerine getirmek amacıyla tam kalınlıktaki yara ve yanıkların tedavisinde kullanılmaktadır. Dermisin yüksek oranda vaskülarize bir yapı olmasından dolayı yara tedavisinde kullanılacak dermal deri greftlerinin, iyi hücre migrasyonunu ve yeni kan damarları oluşumunu sağlaması için yüksek makrogözenekliliğe (gözenek çapı >100 µm) sahip olması gerekir.

Dermal deri ikameleri üretim maliyetlerini azaltmak için genellikle herhangi bir hücre dahil edilmeden biyomateryal bazlı matrikslerden hazırlanmaktadır. Bu nedenle, aselüler dermal yapıların birincil rolü uygulandıktan sonra canlı organizmada fibroblastların ve endotel hücrelerinin göçü ve infiltrasyonu için iskele görevi görmektir. Ayrıca selüler dermal deri ikameleri ile karşılaştırıldığında aselüler dermal deri ikamelerinin klinik kullanım onayı alması çok daha kolaydır. Bununla birlikte, iyileşmeyi hızlandırmak için transplantasyondan önce in vitro olarak deri fibroblastları ile ekilebilen çok sayıda selüler dermal deri ikameleri de bulunmaktadır (Przekora, 2020; Shahrokhi ve ark., 2014).

Hem yanıklarda hem de kronik yaralarda kullanılan Dermagraft™ (Advanced Tissue Sciences ve La Jolla), kriyoprezerve edilmiş insan yenidoğan fibroblastları içeren biyoabsorplanabilir poliglaktin (PGA ve PLA) iskelesinden oluşur. Aseptik koşullarda steril bileşenlerle üretilir. 150 µm-220 µm arasında değişen gözenek büyüklüğüne sahiptir. Canlılığını koruyan allojenik fibroblast hücrelerinden üretildiği için hastanın hücrelerinin bir yarayı kapatacak kadar çoğaltılmasına gerek yoktur, hemen uygulanabilir. 3-4 hafta içerisinde emilir. Üretiminin maliyetli olması bir sakınca olarak değerlendirilse de başarılı olunursa tek greft yeterli olmaktadır. Dermagraft™'ın kronik diyabetik ayak ülseri tedavisinde kullanımı FDA tarafından onaylanmıştır (Murray ve ark., 2019; G. K. Naughton ve B. A. Naughton, 1990; FDA, 2001).

Hyalograft 3D™ (Fidia Advanced Biopolymers) bir diğer biyoparçalanan dermal deri ikamesidir. Yara iyileşme süreci boyunca gerekli büyüme faktörlerini/sitokinleri salgılayan otolog fibroblastlar üç boyutlu hyalüronik asit iskelesine kültüre edilir. Hyalograft 3D™, derin yanıklar ve ayak ülserlerinin tedavisi için kullanılmaktadır (Dixit ve ark., 2017). Bağışıklık tepkisini en aza indiren otolog hücrelerin yaraya uygulanması üstünlük olarak değerlendirilebilir ancak hücrelerin hastadan alınabilmesi için uygun bir donör bölgenin olması gerekir. Ayrıca in vitro hücre kültüründe yeterli sayıda hücrenin elde edilmesi de zaman almaktadır (Murray ve ark., 2019).

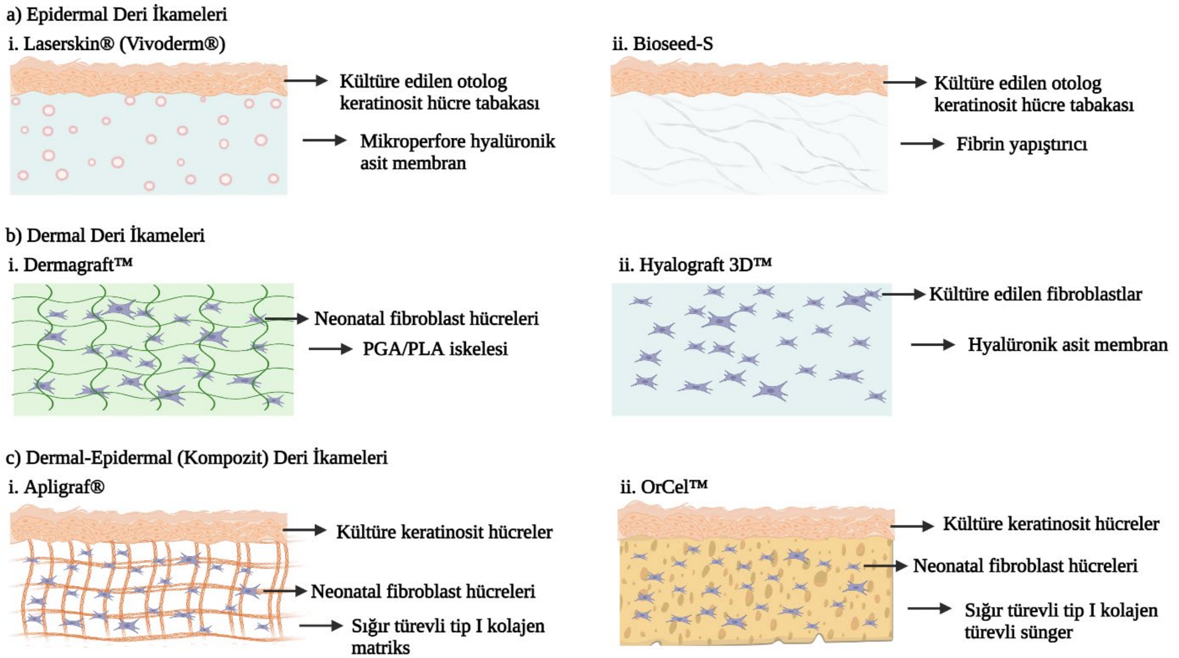
Dermal-epidermal (kompozit) deri ikameleri

Gelişmiş deri ikameleri olarak kabul edilen dermal-epidermal deri ikameleri, hem epidermisin hem de dermisin doğal yapısını taklit eder. Sentetik ya da doğal metaryallerden üretilen iskelelerde fibroblast ve keratinosit hücreleri birleştirilmektedir. Bu sayede

konak hücrelerini uyarmak ve iyileşme sürecini hızlandırmak için büyüme faktörlerinin ve sitokinlerin salgılanması ve ESM'nin sentezlenmesi sağlanır. Ticari olarak temin edilebilen dermal/epidermal ikameler arasında Apligraf® ve OrCel™ bulunmaktadır (Dong ve Gurtner, 2018).

Cilt yapısını ve epidermisle dermis arasındaki fizyolojiyi daha iyi taklit etmek için geliştirilen Apligraf® (Organogenesis), üst tabakasında stratum korneuma benzer, çevresel etkenler için bir bariyer görevi gören insan neonatal epidermal keratinosit katmanlarından ve dermal tabakasında sığır türevli tip I kolajen matriks içerisinde prolifere olabilen neonatal dermal fibroblastlardan oluşmaktadır (Murray ve ark., 2019). Kolajen konsantrasyonu ve hücre sayısı, doku eşdeğerinin çapını ve kalınlığını optimize etmek için kontrol edilebilir (Kemp ve ark., 1996). Apligraf® FDA'den sınıf III tıbbi cihaz olarak pazar öncesi onay almıştır. Diyabetik ayak ülseri bakımında ve 1 aydan uzun süreli venöz yetmezlik nedeniyle enfekte olmayan kısmi ve tam kalınlıktaki deri ülserlerinin tedavisinde endikedir (FDA, t.y.).

Benzer şekilde bir diğer çift katmanlı cilt ikamesi OrCel™ (Ortec International), insan allojenik deri hücrelerinin tip I sığır kolajen süngerinde kültürlendiği selüler matriksten oluşmaktadır. Yenidoğan dermal fibroblastları sığır türevi tip I kolajen matriks süngerinin poröz kısmında kültürlenirken aynı donörden alınan keratinositler, kolajen matriksinin gözeneksiz kısmında kültürlenmektedir. Matriks, yara iyileşme sürecinde emilir ve üreticiye göre allojenik hücrelerden gelen DNA uygulamadan 2-3 hafta sonra analizlerde belirlenememektedir (Murray ve ark., 2019; Abou Neel ve ark., 2013). Kalınlığı yaklaşık olarak 0,8 mm'dir bu nedenle epidermal greftlerden daha sağlamdır ve uygulaması kolaydır (Eisenberg, 2000).



Şekil 6. Doku mühendisliği yaklaşımıyla üretilen deri ikameleri. (a) Epidermal Deri İkameleri: i. Laserskin® (Vivoderm®) ii. Bioseed-S, (b) Dermal Deri İkameleri: i. Dermagraft™ ii. Hyalograft 3D™, (c) Epidermal/Dermal Deri İkameleri: i. Apligraf® ii. OrCel™. BioRender.com ile oluşturuldu.

Tablo 2. Ticari olarak temin edilebilen deri ikameleri (Vig ve ark., 2017).

Deri İkamesi	Ticari Adı	Üretici	Materyal/Bileşenler
Epidermal	Laserskin® (Vivoderm®)	Fidia Advanced Biopolymers Srl	Mikroperforeli hyalüronik asit membranda kültürlenen otolog keratinosit hücreleri
	Bioseed-S	BioTissue Technologies GmbH	Fibrin yapıştırıcı içerisinde kültüre edilen otolog keratinosit hücreleri
Dermal	Dermagraft™	Advanced BioHealing, Inc	Kriyoprezerve edilmiş insan yenidoğan fibroblastları içeren biyoabsorplanabilir poliglaktin iskelesi
	Hyalograft 3D™	Fidia Advanced Biopolymer	Hyalüronik asit membranına kültürlenmiş fibroblastlar
Dermal-Epidermal (Kompozit)	Apligraf®	Organogenesis Inc.	İnsan neonatal fibroblastları ve keratinositleri ile kültürlenmiş sığır türevli kolajen matrisi
	OrCel™	Ortec International Inc.	İnsan neonatal fibroblastları ve keratinositleri ile kültüre edilen tip I kolajen süngeri

SONUÇ

Yaraların heterojen yapısı nedeniyle her hastaya uyan bir tedavi yaklaşımının olmaması, yara yönetiminde farklı fiziko-kimyasal özelliklere sahip yara örtülerinin kullanımını da beraberinde getirmektedir. Doku onarımı sürecinin aydınlatılması ve teknolojideki sürekli ilerleme; biyoparçalanır, biyouyumlu, gelişmiş terapötik yara iyileştirme tasarımlarının da klinikte yer edinmesini sağlamıştır.

İyileşme sürecini destekleyebilen ve antimikrobiyal, antienflamatuvar ve antioksidan özellik gösterebilen biyoparçalanır doğal, sentetik ya da hibrid polimerlerin yara örtülerinin üretilmesinde kullanımıyla birlikte yara yönetimi daha etkin olarak yürütülebilmekte, hastanede kalış süresi kısaltılabilmektedir. Hemostatik bir bileşen gibi davranabilen polimerlerin kanamalı yaralarda, yüksek derecede absorban özellikteki polimerlerin de ağır eksüdalı yaralarda uygulanabilen yara örtüsü tasarımları geliştirilmektedir. Tasarımlar film, köpük, hidrojel, aljinat ya da hidrokolloid yara örtüleri kategorisinde sınıflandırılabilmektedir.

Yara çevresinde nemli bir ortam oluşturarak yara iyileşmesi için gerekli ortamı sağlayan biyoaktif yara örtüleri biyoparçalanarak yara örtüsü değişimini ve yara yüzeyi ile teması en aza indirgemekte bu sayede enfeksiyon riskini de azaltmaktadır. Ayrıca gazlara karşı geçirgen özellik gösterdikleri için anaerob bakterilerin çoğalmasını sınırlandırabilecekleri göz önünde bulundurulmalıdır. Bu ürünler geleneksel yara örtülerine karşı üstün olarak değerlendirilse de sakıncaları arasında yabancı cisim reaksiyonuna neden olabilecekleri, ağır eksüdalı yaralarda yeterli miktarda sıvıyı absorbe edemeyebilecekleri bildirilmektedir.

Deri ikameleri ise son derece gelişmiş, tam kalınlıktaki yara ve yanıkların tedavisinde ön plana çıkan tıbbi cihazlardır. Ancak implantasyondan sonra deri ikamelerinin mekanik özelliklerinde ani kayıpların olması ya da yerlerini bırakacakları biyolojik doku oluşmadan parçalanmaları klinikteki kullanımlarını etkileyen nedenler arasında gösterilebilmektedir.

Gelecekte ideal kopolimerler ya da sentezlenecek yeni polimerler sayesinde üstün dayanıklılığa sahip deri ikamelerinin üretilmesi, kişiselleştirilmiş yara iyileştirme malzemelerinin tedavide kullanılması, kök hücre teknolojisiyle bütünleştirilerek tıbbi cihazların biyoaktif özelliklerinin geliştirilmesi çok yönlü ve işlenebilir özellikteki polimerlerden oluşan yara örtülerinin farklı yara tiplerine uygulanabilir olması öngörülmektedir.

KISALTMALAR

ECM; Ekstra Selüler Matriks

EGF; Epidermal Büyüme Faktörü

FDA; Amerikan Gıda ve İlaç Dairesi

GM-CSF; Granülosit Makrofaj-Koloni Stimule Edici Faktör

PCL; Poli(ε-kaprolakton)

PDGF; Trombosit Kaynaklı Büyüme Faktörü

PDLA; Poli(D-laktik asit)

PGA; Poli(glikolik asit)

PLA; Poli(laktik asit)

PLGA; Poli(laktik-ko-glikolik asit)

PLLA; Poli(L-laktik asit)

ROS; Reaktif Oksijen Türleri

TGF-β; Dönüştürücü Büyüme Faktörü-Beta

WVTR; Su Buharı İletim Hızı

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