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Research Article

Determination of cytotoxic, antioxidant activities and LC/MS-MS profiles of three endemic *Verbascum* L. species

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ABSTRACT

The *Verbascum* genus includes many species used in folk medicine or the treatment of various diseases. In this study, the cytotoxic, antioxidant activity, and LC/MS-MS profiles of three *Verbascum* species, which are endemic in Eskişehir and its surroundings, were investigated.

The cytotoxic effects of methanol extract of *V.detersile*, *V. eskisehirensis,* and *V.gypsicola* species on the cervical (HeLa) and ovarian cancer (SKOV-3) cells were investigated using a colorimetric assay. The results indicated that cytotoxic effect was not observed after treatment of SKOV-3 cells with *Verbascum*. On the other hand, the cytotoxic activity of *V. detersile* was found to be 0.1910 mg/dL and 1.057 mg/dL for HeLa cells after 24 or 48 hours incubation with *V.detersile*, respectively.

The antioxidant activity was determined as 1,1-diphenyl-2-picrylhydrazyl (DPPH•) radical scavenging activity, trolox equivalent antioxidant capacity (TEAC assay), and also the total phenolic content of the samples was found. Total phenols were estimated as Gallic acid equivalents (GAE), expressed as mg Gallic acid/in 1g extract previously described by Singletton. LC/MS profiles separation and detection of phytochemicals of the extract were performed on a Shimadzu UPLC system consisting of a vacuum degasser, an autosampler (SIL20A Shimadzu Autosampler), a binary pump (LC20AD Shimadzu), an oven (CTO20A Shimadzu Column Oven) and DAD dedector (SPD M20A Shimadzu DAD Detector). In terms of antioxidant activity, *V. gypsicola* was found to have the least antioxidant activity among the three extracts, which is also correlated with the total amount of phenolic content in its content. In this way, it differs from other species. *V. detersile* exhibits a different chemical profile from the other two species with the iridoid catalpol derivatives it contains. Apigenin pentoside is the only flavonoid molecule detected in *V. detersille*.

Keywords: Antioxidant, LC/MS-MS, cytotoxicity, Scrophulariaceae, *Verbascum*

1. INTRODUCTION

The *Verbascum* L. (Scrophulariaceae) species are represented in the world with nearly 360 species [1]. In Turkey, the *Verbascum* genus consists of approximately 255 species, 200 of which are endemic (about 80% endemism rate) and it has 130 additional hybrid species [2].

Verbascum species are a popular herb with medicinal uses. In traditional Turkish folk medicine, these medicinal plants are used in the treatment of expectorant, stomachache, stomach ulcer, diabetes, hemorrhoid, rheumatism, and urinary tract infection [3-7]. It has been reported that they are used as respiratory disorders, expectorant, stomach tonic, dyspepsia, diarrhea, diuretic, snake bites, blood clotting of women after childbirth, wound disinfection, and sedative in Iran [8-11]. In addition, the Herbal Medicinal Products Committee (HMPC) reported that Mullein flowers (*Verbascum phlomoides*, *V. thapsus,* and *V. densiflorum*) can be used to soothe the throat in colds and dry cough [12].

In some studies on *Verbascum* has been found to have an antiviral [13], antibacterial [14], enzyme inhibitory activity [15], and wound healing properties [16]. It has also been reported that *V. pycnostachyum* species has a substantial cytotoxic effect against cervical (HeLa) and ovarian cancer (Skov-3) cell lines [17].

It is thought that free radicals may be effective in the formation mechanism of some common diseases and antioxidants prevent cellular damage by preventing these harmful effects [18]. Herbal secondary metabolites such as phenolics have a scavenging effect and so antioxidant activity against these harmful effects of free radicals. [19]. There are studies in the literature proving that some *Verbascum* contains phenolics and has an antioxidant effect [20-22, 29,30].

These and similar studies show that the Verbascum genus has antioxidant properties. However, the results of these species may vary depending on the extra batches, the individual in which they were grown, and the processing methods. Additionally, health effects related to antioxidants require further research.

In Turkey, *Verbascum detersile* Boisse & Heldr., *V. gypsicola* Vural & Aydoğdu and *V. eskisehirensis* Karavel., Ocak & Ekici known as "Zinemit", "Mermer sığırkuyruğu" and "Eski sığırkuyruğu" respectively [23]. In the present study, three endemic *Verbascum* were evaluated for their cytotoxic, antioxidant and LC/MS profiles.

2. MATERIALS AND METHODS

2.1. Plant materials

Information on the localities of three species is given in Table 1.

2.2. Preparation of Extracts

The dried air parts of *V. detersile*, *V. gypsicola,* and *V. eskisehirensis* species were macerated 3 times with 70% ethanol. The dry extracts were kept at +4°C after evaporation and lyophilization.

2.3. Cytotoxicity

2.3.1. Cell Culture and reagents

The human cervical adenocarcinoma (HeLa) and human ovarian adenocarcinoma (SKOV-3) cells were obtained from American Type Culture Collection (ATCC). HeLa cells were maintained in Eagle's Minimum Essential Medium (EMEM) (Sigma-Aldrich, UK) supplemented with 20% Fetal Bovine Serum (FBS) (Gibco, UK), 1% penicillin-streptomycin, and 4% sodium bicarbonate as adherent monolayers. SKOV-3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, UK) supplemented with 10% FBS and 1 % penicillin-streptomycin. The cell lines were routinely subcultured using 0,25 % trypsin-EDTA solution (Sigma-Aldrich, UK). Exponentially

Table 1. Locations of the studied *Verbascum* species

***Verbascum* Species**	**Locations**
V. detersile	B3: Eskişehir 05.07.2019 (ESSE 15614)
V. gypsicola	B4: Ankara 02.07.2019 (ESSE 15615)
V. eskisehirensis	B3: Eskişehir 01.06.2019 (ESSE 15616)

growing cultures were maintained in an incubator with a humidified atmosphere with 5% CO2/95 % air at 37°C.

2.3.2. Cytotoxicity assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] is a non-radioactive assay and measures the reduction of yellow MTT by mitochondrial succinate dehydrogenase. The reduction of MTT can only occur in metabolically active cells. The assay was performed as mentioned in Mosmann [24].

SKOV-3 and HeLa cells, which reached the appropriate density in flasks, were seeded in 96-well plates with $5x10^3$ cells in each well and incubated for 24 or 48 hours. After incubation, various concentrations (0.1; 0.2; 0.6; 1; 1.5; 2; 2.5; 3; 3.5 mg/dL) of *V. detersile*, *V. eskişehirensis* and *V. gypsicola* substances were added to the wells in 4 repetitions. After 24 and 48 hours, 20 µL of MTT dye (stock concentration of 5 mg/mL) was added to each well and then the wells were incubated for 3 hours. After 3 hours, the medium and MTT dye was completely withdrawn from the wells, 100 µL of DMSO was added to each well to dissolve the formazan crystals, and the plates were left in the shaker for 15 minutes. Spectrophotometric measurements of the plates taken from the shaker were performed at a wavelength of 540 nm in a Bio-Tek (ELx800) plate reader. The signal generated is directly proportional to the number of viable (metabolically active) cells in the well. Viability (%) is calculated using the following formula; Viability (%) = (Absorbance of the treated cells) / (Absorbance of the control wells) x 100.

2.3.3. Statistical analysis

The results were obtained from the MTT assay were expressed as mean ± SD. The significant differences were indicated as $p<0.05$ using one-way ANOVA. GraphPad Prism 7 software was used information of graphics.

2.4. Antioxidant activity

2.4.1. 1,1-Diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$) radical scavenging activity

In this analysis free radical scavenging activity was measured using the 1,1-Diphenyl-2-picrylhydrazyl (DPPH) method. Serial dilutions were prepared with half the concentrations of the previous one, resulting in stock stock solutions (4 mg / ml). DPPH (the same amounts) were added to the diluted solutions and the UV absorbance at 517 nm was measured after 30 minutes. The experiment, extract, and positive standard control were made in triplicate for BHT (Butile Hydroxytoluene). The averages of the absorbances were recorded for each concentration. The percentage of prevention was calculated via Equation 1. The IC_{50} value, which is the concentration of the test material inhibiting 50% of the free radical concentration, was determined as mg / mL using the Sigma Plot statistical program. Microplate dilution method of Kumarasamy was used for the assay [25].

2.4.2. Trolox equivalent antioxidant capacity (TEAC assay)

TEAC assay was performed as in our previous *Verbascum* publication [26]. This assay assesses the capacity of a compound to scavenge the stable ABTS radical in comparison tothe antioxidant activity of Trolox, a water-soluble form of vitamin E that is used as a standard. The blue-greenABTS was produced through the reaction of 7 mM ABTS with 2.5 mM sodium persulfate ($Na_2S_2O_8$) (final concentrations) in the dark at room temperature for 12-16 h before use. The concentrated ABTS solution was diluted with ethanol to a final absorbance of 0.8-0.7 at 734 nm. A 10 µl portion of extract was added to 990 µl of ABTS solution, and the reduction in absorbance was measured 1 min after addition of Trolox (final concentration 1-20 µM) and up to 40 min after addition of the extract. The stock solution of Trolox (2.5 mM) was prepared in ethanol. Absorbance was measured at 734 nm.

2.4.3. Quantitative Determination of the Total Phenolic Contents

Total phenols were estimated as Gallic acid equivalents (GAE), expressed as mg Gallic acid/in 1g extract previously described by Singletton [27]. The stock solutions of the extracts and gallic acid were prepared in methanol. In experiment, 20 µL of the sample (extract/ gallic acid), 1560 µL of ultrapure water and 100 µL FCR were mixed in the 96-deep wells by using 12-channel micropipetor. After 8 min incubation, 300 µL of sodium carbonate solution (20%) was added to the mixture and mixed again. The mixture was incubated during 2 h at 25°C in a dark. Then, 300 mL of the mixture was transferred into 96-well microplate and the absorbance values were measured at 760 nm.

2.5. LC/MS Profiles

LC-MS/MS analyses were carried out using a similar method as the previous study (26), with a slight modification, using Methanol as the organic solvent in gradient elution.

3. RESULTS AND DISCUSSION

The *Verbascum* genus includes many species used in folk medicine or the treatment of various diseases. In this study, the cytotoxic, antioxidant activity, and LC/MS-MS profiles of three *Verbascum* species, which are endemic in Eskişehir and its surroundings, were investigated.

3.1. Cytotoxicity Assay

The cytotoxic effects of all extracts were also examined on HeLa and SKOV-3 cancer cell lines. No effect was observed as a result of the treatment of *V. detersile* (after 24 or 48 hours), *V. eskisehirensis* (after 24 or 48 hours), and *V.gypsicola* (after 48 hours) with SKOV-3 cells. Therefore, statistical analyzes could not be performed. IC_{50} value could not be calculated since cell proliferation occurs in the 24 hour activation of the *V. gypsicola* extract (Figure 1; Table 2). Cytotoxic activity of *V. detersile* was found to be 0.1910 mg/dL and 1.057 mg/dL, respectively, for HeLa cells after 24 and 48 hours of dosing (Fig. 2).

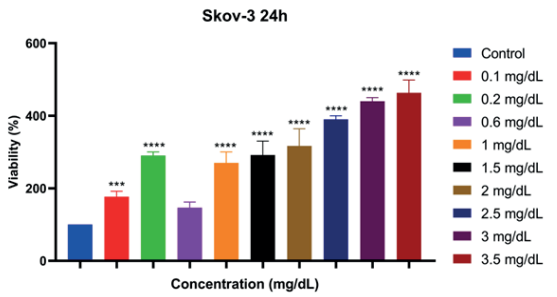

Figure 1. Cytotoxic activity of *V. gypsicola* in SKOV-3 cell lines at 24 h. Each data point is the average of three independent wells. Bars indicate mean ± standard deviation. All comparisons were made relative to untreated control cells (100% cell-viability).

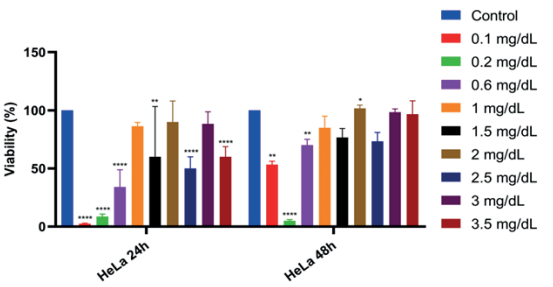

Figure 2. Cytotoxic activity of *V.detersile* in HeLa cell lines after 24 or 48 hours treatment. Each data point is the average of three independent wells. Bars show ± standard deviation. All comparisons were made against untreated control cells (100% cell viability). [based on control, * p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001].

Table 2. Cytotoxic activities (IC_{50}, mg/dL) of *V.detersile, V. eskisehirensis, V.gypsicola*

***Verbascum* Species**	**HeLa**		**SKOV-3**	
	24 h	**48 h**	**24 h**	**48 h**
V.detersile	0.1910	1.057	No effect	No effect
V. eskisehirensis	No effect	No effect	No effect	No effect
V.gypsicola	No effect	No effect	Proliferation	No effect

Table 3. Antioxidant activities of extracts of *Verbascum* species

	DPPH (IC_{50}, Mili molar)	TEAC (mM)	Total Phenolic Contents mg GAE / g extract
V. detersile	0.16	1.96	506.7
V. gypsicola	0.27	0.67	279.1
V. eskisehirensis	0.16	1.17	338.1
Gallic acid	0.002		

Table 4. Extracts composition of *Verbascum* species

Rt	m/z $[M-H]^-$	Fragments	Identification	Extract	Reference
12.4	401	269, 161	Apigenin pentoside	D	[28]
15.2	495	311, 209, 167	Unknown	G	
16.8	653	491, 377, 309,291, 187, 163, 145	p-Coumaroyl 6-O rhamnosylcatalpol	D	[28]
16.8	507	307, 145	Unknown	G	
17.6	623	461, 161, 135	Verbascoside	D,G	[28]
19.2	579	447, 285,151	Luteolin pentosyl-glucoside	E	[26]
20.6	447	285	Luteolin glucoside	E	[26]
20.9	695	533, 419, 333, 163, 145	p-Coumaroyl acetyl 6-O-rhamnosylcatalpol	D	[28]
21.3	461	369, 327, 285	Luteolin glucuronide	E	[26]
21.6	563	269	Apigenin pentosyl-glucoside	E	[26]
22.8	431	311, 269	Apigenin glucoside	E	[26]
23.4	683	637,445, 361	Unknown	D	
23.4	607	300, 284	Unknown	G	
23.5	461	445, 313, 297, 283,269, 255	Homoplantaginin	E	[29]
27.7	725	679, 487, 403, 311, 215, 163, 151, 147	Unknown	D	
28.7	285	175, 133	Luteolin	G	[26]

D: *V. detersile,* E: *V. eskisehirensis,* G: *V. gypsicola*; Rt: Retention time; m/z: mass-to-charge ratio

3.2. Cytotoxic Effects of Extracts

In cytotoxic studies, it was determined that none of the extracts showed any effect when treated with SKOV-3 cells, whereas only *V. detersile* extract was effective on HeLa cells. No previous cytotoxicity study has been found for this species. However, Küçük et al. conducted a study in 2016 with three different *Verbascum* sp. against HeLa and SKOV-3 cell types. They found that all *Verbascum* sp. extracts dose-dependently reduced cell viability in both HeLa and SKOV-3 cell types [17].

3.3. Antioxidant Activity

In the, the DPPH scavenging effect of the MeOH extract of *V. detersile* collected from Antalya has been reported as IC_{50}: 27 µg/mL [20].

The MeOH extract of *V. eskisehirensis* was reported as DPPH (IC_{50} 176.7 µg/mL) and TEAC assay (0.184 ± 0.08 mM) [26].

Various methods were used to evaluate the antioxidant activity.

According to the total phenolic content results, *V. detersile* was found to have the highest phenolic content among the three species studied. High phenolic content of *V. detersile* provided better results in antioxidant test systems than other extracts. Although the *V. detersile* and *V. eskisehirensis* activity results were the same in the DPPH radical scavenging effect and TEAC experiments, *V. detersile* showed a remarkable effect with antioxidant activity equivalent to approximately 2mM Trolox. In the DPPH radical scavenging effect, no extract was found as effective as the positive control gallic acid. In terms of antioxidant activity, *V. gypsicola* was found to have the least antioxidant activity among the three extracts, which is also correlated with the total amount of phenolic content in its content (Table 3).

In the LC-MS/MS analysis, it was determined that *V. eskisehirensis* was rich in luteolin derivatives and also contained apigenin derivatives. Among the three *Verbascum* species analyzed, *V. eskisehirensis* was the only species that did not contain verbascoside. In this way, it differs from other species. *V. detersile* exhibits a different chemical profile from the other two species with the iridoid catalpol derivatives it contains. Apigenin pentoside is the only flavonoid molecule detected in *V. detersille* (Table 4).

4. CONCLUSION

The *Verbascum* genus includes many species used in folk medicine or the treatment of various diseases. In this study, the cytotoxic, antioxidant activity, and LC/MS-MS profiles of three *Verbascum* species, which are endemic in Eskişehir and its surroundings, were investigated. The findings of this study provide useful information for breeding strategies, and for choosing the best species with high phenolic compound content to produce natural antioxidants for medical and pharmaceutical use. Pronounced antioxidant and rich bioactive compositions determined in this study reveal that *Verbascum* extracts might be a good source for natural health attributing sources.

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Ethical approval

Not applicable, because this article does not contain any studies with human or animal subjects.

Author contribution

Concept: SK, MS; Design: SK, MS; Supervision: SK, MS; Materials: SK, MS; Data Collection and/or Processing: SK, MS, FG, FÖ, ZS; Analysis and/or Interpretation: SK, MS, FG, FÖ, ZS; Literature Search: SK, MS, FG, FÖ, ZS; Writing: SK, MS, FG, FÖ, ZS; Critical Reviews: SK, MS, FG, FÖ, ZS.

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Conflict of interest

The authors declared that there is no conflict of interest.

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Research Article

Volatile components of *Ferulago aucheri* Boiss. (Apiaceae)

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ABSTRACT

Ferulago aucheri Boiss, which belongs to the Apiaceae family, is distributed from Türkiye to the Caucasus. Its chemical composition may vary depending on the region where it grows, climate conditions and topography. Essential oil components were determined using two different techniques (hydrodistilled and headspace-solid phase microextraction HS-SPME) with the species collected from Nallıhan district of Ankara, Türkiye. In the present study, essential oil and headspace volatiles of aerial parts of *Ferulago aucheri* Boiss. were analyzed by GC-GC/MS. Thirty-five components were characterized, representing 99.6% of the oil by hydrodistilled and thirty components were characterized, representing 98.7% by HS-SPME. In both techniques, the main substances were identified as α-pinene, limonene, and δ-3-carene. In previous studies, it has been observed that the main components are in different amounts, and some studies even have different main components..

Keywords: Apiaceae, essential oil, *Ferulago aucheri*, GC- GC/MS, HS-SPME

1. INTRODUCTION

The Apiaceae family, which is a cosmopolitan, is widespread in the temperate zone of the Northern Hemisphere with 466 genera and approximately 3.820 species in the world [1]. The *Ferulago* W. Koch (Apiaceae) includes about 49 species worldwide [2]. In Türkiye, the genus *Ferulago* consists of about 34 species, 19 of which are endemic [3].

Ferulago species are used in folk medicine as antidiabetic, aphrodisiac, cancer, dermal wounds, eye pains, enhancing body strength, menstrual regulator and sedative [4-8]. There have been extensive phytochemical studies conducted with the roots and aerial parts of *Ferulago* taxa, which have shown that they are extremely rich in coumarins. Many flavonoids, terpenoids and other metabolites have also been identified. In the literature, volatile components of the genus *Ferulago* have been investigated with different techniques (headspace solid phase microextraction, hydrodistillation, microdistillation) in many studies [9-18]. In addition, essential oils of some *Ferulago* species were showed antimicrobial activity [9,10,12,14,16,17]. Stated that *F. sylvatica* (Besser) Rchb. may have different chemical components in samples collected from diverse geographical districts [19].

Ferulago aucheri Boiss. is known as 'Yayla kişnişi', it is a perennial and endemic species [3, 20]. The aerial parts of *F. aucheri* contains aromatic components, coumarins and flavonoids [21].

This study aims to investigate and compare *F. aucheri* the essential oil and the headspace volatile components.

2. MATERIALS AND METHODS

2.1. Plant materials

F. aucheri was collected in July 2020, from Nallıhan in Türkiye. The specimen had been stored in Anadolu University, Faculty of Pharmacy Herbarium (ESSE: 15826).

2.2. Isolation and analysis of essential oil

The air dried and crushed aerial parts of *F. aucheri* (25 g) were hydrodistilled for three hours using Clevenger apparatus. The essential oil obtained was stored at 4°C in the dark until analyzed. The oil was analyzed by capillary GC and GC/MS using an Agilent GC-MSD system [22].

2.3. Headspace-solid phase microextraction (HS-SPME) method, GC/MS analysis and identification of components

A SPME (SUPELCO) device consisting of a fused silica fiber, coated with 65 µm Polydimethylsiloxane/ Divinylbenzene (PDMS/DVB) adsorbent (Blue) was used. The air dried and crushed aerial parts volatile components of *F. aucheri* were captured with HS-SPME fiber and analyzed by GC/MS [23].

Analysis of GC and GC/MS

GC/MS: The GC/MS analysis was performed with an Agilent 5975 GC-MSD system. Innowax FSC column (60m x 0.25mm, 0.25mm film thickness) was used with helium as carrier gas (0.8 mL/min.). The temperature of GC oven was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/ min, and kept constant at 220°C for 10 min and then programmed to 240°C at a rate of 1°C/ min. Split ratio was adjusted 40:1 (splitless for HS-SPME). The temperature of the injector was at 250°C. MS were taken at 70 eV. Mass range was from m/z 35 to 450.

GC: The GC analysis were performed with Agilent 6890N GC system equipped with a FID detector set at 300 °C. To obtain the same elution order with GC-MS, simultaneous auto-injection was performed on a duplicate of the same column applying the same operational conditions. Relative percentage amounts of the separated substances were calculated from FID chromatograms.

Table 1. Essential oil (A) and HS-SPME (B) components of *F. aucheri*

RRI	Components	A %	B %
1032	**α-Pinene**	**59.8**	**47.8**
1076	Camphene	0.3	-
1118	β-Pinene	1.9	0.8
1132	Sabinene	1.1	2.1
1138	Thuja-2,4(10)-dien	0.4	1.1
1159	**δ-3-Carene**	**5.4**	**11.4**
1174	Myrcene	1.8	2.0
1187	*o*-Cymene	0.2	-
1203	**Limonene**	**18.8**	**21.9**
1246	(*Z*)-β-Ocimene	1.9	1.3
1255	γ-Terpinene	0.1	-
1266	(E)-β-Ocimene	0.2	-
1278	*m*-Cymene	0.2	-
1280	*p*-Cymene	1.5	3.5
1286	Isoterpinolene	0.1	-
1290	Terpinolene	0.1	-
1382	*cis*-Alloocimene	-	1.8
1435	γ-Campholene aldehyde	-	0.1
1443	2,5- Dimethylstyrene	-	0.2
1452	*p*-Cymenene	-	0.2
1466	α-Cubebene	-	0.2
1499	α-Campholene aldehyde	0.3	0.6
1535	Pinocamphone	-	0.2
1586	Pinocarvone	0.1	0.2
1591	Bornyl acetate	0.1	-
1611	Terpinen-4-ol	0.1	-
1617	Lavandulyl acetate	0.1	-
1638	*trans*-p-Menth-2,8-dien-1-ol	0.1	0.1
1645	*cis*-Verbenyl acetate	-	0.6
1648	Myrtenal	0.2	0.3
1670	*trans*-Pinocarveol	0.7	0.5
1678	*cis*-p-Mentha-2,8-dien-1-ol	0.1	-
1684	*trans*-Verbenol	1.7	0.5
1725	Verbenone	-	0.5
1726	Germacrene D	0.2	-
1747	*p*-Mentha-1,5-dien-8-ol	0.4	-
1754	Carvone	0.3	0.2
1797	Myrtenol	0.2	0.1
1811	*trans*-*p*-Mentha-1(7),8-dien-2-ol	-	tr
1845	*trans*-carveol	0.5	0.3
1856	*m*-Cymen-8-ol	0.1	0.1
1864	*p*-Cymen-8-ol	0.1	0.1
1867	*cis*-Carveol	0.1	tr
2144	Spathulenol	0.4	-

RRI: Relative retention indices calculated against n-alkanes; % calculated from FID data; tr; Trace (<0.1 %).

Table 2. Previously reported volatile components of *F. aucheri* and this study

Localities	Methods	Plant parts	Main Components	References
Muğla	Microdistillation	Crushed fruits	α-Pinene (35.9%)	Başer et al. 2002
Antalya	Hydrodistillation	Crushed fruits	Limonene (43.1%), α-pinene (18.3%), myrcene (7.0%)	Başer et al. 2008
Manisa	Hydrodistillation	Crushed fruits	Germacrene D (25.7%), (2E, 6E)-farnesol (8.0%)	Başer et al. 2008
Ankara (Mülk)	Hydrodistillation	Root	α-Pinene (80.3%)	Cumhur 2019
Ankara (Mülk)	Hydrodistillation	Aerial parts	α-Pinene (28.7%), 2,5-dimethoxy-*p*-cymene (15.3%), limonene (10.9%) and bornyl acetate (6.1%)	Cumhur 2019
Ankara (Nallıhan)	Hydrodistillation	Aerial parts	α-Pinene (59.8%), limonene (18.8%) and δ-3-carene (5.4%)	This study
Ankara (Nallıhan)	HS-SPME	Aerial parts	α-Pinene (47.8%), limonene (21.9%) and δ-3-carene (11.4%)	This study

Identification of substances

The components of essential oils were detected by comparison of their mass spectra with those in the Baser Library of Essential Oil Constituents, Wiley GC/ MS Library, Adams Library, Mass Finder Library and confirmed by comparison of their retention indices. Alkanes were used as the reference agents while calculating relative retention indices (RRI). Relative percentage amounts of the separated substances were calculated from FID chromatograms. Table 1 shows the data of the analysis.

3. RESULTS AND DISCUSSION

In the present study, volatile components of *F. aucheri* were investigated. Although the amount of oil obtained in the hydrodistillation process is high, the high temperature applied during boiling of water causes some thermal reactions. As a result, artifact formation, hydrolysis and isomerization events occur. In the SPME method, the sample preparation, extraction and concentration stages are combined in a single solvent-free step [24]. The yield of essential oil was 0.5%. Thirty-five components representing 99.6% and thirty components representing 98.7% were characterized by essential oil and HS-SPME from the aerial parts, respectively.

The main components of the essential oil and HS-SPME were identified as α-pinene (59.8%, 47.8%), limonene (18.8%, 21.9%) and δ-3-carene (5.4%, 11.4%) respectively.

Table 3. Essential oil (A) and HS-SPME (B) components groups

Grouped substances	A %	B %
Monoterpene hydrocarbons	93.8	93.9
Oxygenated monoterpenes	5.0	4.0
Sesquiterpenes hydrocarbons	0.2	0.2
Oxygenated sesquiterpenes	0.4	-
Others	0.2	0.6
Total %	99.6	98.7

Monoterpene hydrocarbons (93.8%, 93.9%) and oxygenated monoterpenes (5.0%, 4.0%) were the main groups present in the oil and HS-SPME, respectively (Table 3).

Some components were identified only in the oil (such as *p*-mentha-1,5-dien-8-ol, spathulenol, camphene) and some in the HS-SPME technique (such as *cis*-alloocimene, *cis*-verbenyl acetate, verbenone) (Table 1). Major components of the volatiles of *F. aucheri* previously reported (Table 2). Studies on pinene, one of the main components obtained from *F. aucheri*, show that its biological activity is high and it is promising as a therapeutic agent [25]. There are also studies showing that it has antitumor and antiviral activity [25,26].

4. CONCLUSION

In conclusion, the volatile components of *F. aucheri* was investigated and it was found that the main components were quite consistent for the two

techniques. However, it is thought that the main components of this plant may vary according to the region. Biological activity studies can be performed due to the availability of major components.

Ethical approval

Not applicable, because this article does not contain any studies with human or animal subjects.

Author contribution

Concept: EO, FEK, MK, SK; Design: EO, FEK, MK, SK; Supervision: EO, FEK, MK, SK; Materials: EO, FEK, MK, SK; Data Collection and/or Processing: EO, FEK, MK, SK; Analysis and/or Interpretation: EO, FEK, MK, SK; Literature Search: EO, FEK, MK, SK; Writing: EO, FEK, MK, SK; Critical Reviews: EO, FEK, MK, SK.

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Conflict of interest

The authors declared that there is no conflict of interest.

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Research Article

HPLC method for simultaneous quantification of lumacaftor and ivacaftor bulk and pharmaceutical formulations

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ABSTRACT

In 2015, the Food and Drug Administration granted approval for the use of lumacaftor 200 mg and ivacaftor 125 mg in the treatment of cystic fibrosis patients who possess the F508del mutation, namely those who are 12 years of age or older. Since its approval, the medicine has been implemented in clinical settings, although the presence of numerous disputes, with the aim of mitigating disease symptoms and enhancing the overall quality of life. Given the existing gaps in the literature regarding the analysis of the amalgamation of these two active substances, a straightforward and practical HPLC approach has been devised in adherence to the guidelines outlined in the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) Q2(R1) document. To accomplish this objective, the process of separation was successfully carried out using a monolithic silica stationary phase (Chromolith High Resolution RP-18e, 100 mm × 4.6 mm i.d., Merck KGaA, Darmstadt, Germany). The separation process was conducted using a gradient mode. The initial composition of the mobile phase consisted of acetonitrile and a phosphate buffer solution with a concentration of 0.030 M and a pH of 3.5. The flow rate was recorded as 1.0 mL/min, and avanafil was used as an internal standard. The improved and verified approach has demonstrated successful application in bulk and pharmaceutical formulation evaluations when utilizing the ivacaftor/lumacaftor combination.

Keywords: Bulk form, HPLC, Ivacaftor, Lumacaftor, Pharmaceutical form

1. INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive genetic disease and commonual in Caucasians [1]. Although the incidence of the disease is reported to be 1 in 1/2000–3500 live births and the carrier rate is 1/25, it is known that the incidence of the disease varies between populations [2]. Cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations affect many organs and tissues, namely lungs, intestines, pancreas, sweat glands, causing organ secretions to become thick and. Even some clinical symptoms may be different, typical signs of cystic fibrosis comprise an increase in sweat salt level, decreased weight gain and growth, persistent cough, and recurrent infections of the lung [3]. The death is the most extreme outcome of the illness, which generally occurs due to respiratory failure; unfortunately, the average survival time is about 46 years for men and 41 years for women [4].

Until recently, symptomatic treatments (e.g., mucolytics, antibiotics, pancreatic enzymes, etc.) focusing on the consequences of the disease were applied. Although 30 years have passed since the discovery of the CFTR gene, there has been no significant development regarding gene therapy yet. However, in recent years, CFTR modulators that improve the activity of the CFTR gene have been developed, and very good clinical results have been obtained [5].

These molecules can partially eliminate the intracellular damage and/or malfunction of the CFTR protein and provide extraordinary improvements in the patient's quality of life. Clinical studies show that the molecules are safe and show mild or moderate side effects. However, these drugs are quite expensive (≈CHF 170,000/year/patient), and they work in a very limited range of patient profiles [6]. Additionally, the patient's age and clinical condition are also important. The mechanism of action of these molecules is not fully known, and they are produced by a single pharmaceutical company. These substances are ivacaftor (IVA), lumacaftor (LUMA), tezacaftor, and elexacaftor.

Thanks to their success in the therapy, agents capable of addressing the underlying CFTR deficiency have become a growing focus [7, 8] CF is a disease that can affect many systems and present with different clinical signs and symptoms in each patient. Clinical findings in CF vary depending on the age of the patient, the genetic mutation he carries, the severity of the disease, and the affected systems [9].

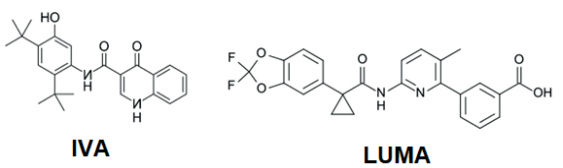

Figure 1. Molecular structures of IVA and LUMA

IVA given in Figure 1, is a medication used to treat mainly the G551D mutation which is responsible for 4–5% of CF cases [10]. Additionally, it is incorporated in the combination medications LUMA/IVA, tezacaftor/IVA, and elexacaftor/tezacaftor/IVA, which are administered to individuals with CF for therapeutic purposes [11]. IVA, developed by Vertex Pharmaceuticals in collaboration with the Cystic Fibrosis Foundation, is the pioneering medicine that targets the root cause of the disease rather than only addressing its symptoms. LUMA, given in Figure 1, is a medication used in combination with the fixed-dose combination product Orkambi [DB08820] for the treatment of CF in patients 6 years of age and older. LUMA/IVA, marketed as Orkambi®, is a medication that combines LUMA and IVA. It is prescribed to individuals with cystic fibrosis who possess two copies of the F508del gene.

Both LUMA and IVA are novel drugs that received approval from the FDA in 2015 and 2012, respectively [12, 13]. Phase studies are continuing at various stages to examine their effectiveness against different CF mutations. For this reason, there are not enough studies on IVA/LUMA simultaneous analyses. Table 1 provides an overview of the broad range and various practical applications of the approaches that have been developed and documented in the existing literature for IVA and LUMA analysis. However, today, the right to produce finished products still belongs to Vertex Pharmaceutical. When other pharmaceutical companies are granted production permits in the future, it is obvious that analytically competent methods will be needed for active substance or pharmaceutical formulation production studies. As can be seen in the table we created based on our best knowledge, the method developed for simultaneous quality control analyses of the two is one [18]. So there is a lack of fast, easy, and high accuracy and precision methods for the analysis of LUMA/IVA combination within the scope of pharmaceutical formulation studies. For this purpose, this study aimed to develop an HPLC method that analysts can use easily and conveniently.

Table 1. Data on methods developed for IVA and LUMA analysis

Compound	Linearity (µg/mL)	LOD/ULOQ (µg/mL)	LOQ/LLOQ (µg/mL)	wavelength	m/z	t_R (min)	Method	Sample	Reference
IVA	15 - 300	0.13	0.40	225 nm	-		HPLC	Tablet	[14]
IVA	0.01 - 10	2.50×10^{-3}	7.57×10^{-3}	-	392.49→393	6.2	LC-MS/MS	Saliva and plasma	[15]
IVA	1 -80	-	-	309 nm	-	6.2	HPLC		
IVA	62.5–312.5	-	-	254	-	3.1	HPLC	Tablet	[16]
IVA/LUMA	0.1-10	2.5×10^{-3}(IVA)	7.57×10^{-3} (IVA)		z 392.49 → 393	1.55 (IVA)	LC-MS/MS	Biological fluids	[17]
		6.08×10^{-4} (LUMA)	1.84×10^{-3} (LUMA)		m/z 452.40 → 453	2.3 (LUMA)			
IVA/LUMA	62.5-312.5 (IVA)	-	-	254		3.1 (IVA)	HPLC	Bulk and pharmaceutical	[18]
	100-500 (LUMA)					4.2 (LUMA)			
IVA/LUMA	0.01-10	0.01	10	-	393.20 → 337.10	2.64	LC-MS/MS	Sputum	[19]
					453.05 →131.10	2.56			
LUMA	0.5-20	0.08	0.2	216		5.3	HPLC	Orkambi®	[20]
IVA	0.55-20.22	0.20	0.08	247		8.3		Kalydeco®	[21]
	0.11-20.22	0.1×10^{-6}	1.0×10^{-6}	-	393.10→ 337.10; 319.15; 172.05	8.4	LC-MS/MS		

2. MATERIALS AND METHODS

2.1. Chemical and Reagents

IVA, avanafil (AVA, as internal standard) and LUMA (99.6%) purity TRC Company, Canada) were purchased. Chromatographically pure NaH_2PO_4 (99.8%) and $Na_2HPO_4 \cdot 2H_2O$ (99.6%) were purchased from Sigma-Aldrich Chemie GmbH (Seelze, Germany). Acetonitrile (ACN) and methanol of high chromatographic purity were acquired from Merck KGaA (Darmstadt, Germany).

2.2. Instruments

The analyses were conducted using a Prominence series HPLC system manufactured by Shimadzu (Kyoto, Japan). The system includes a DGU-20A5 online degasser, an LC-20AT tandem double submersible pump with a low-pressure gradient unit, and a SIL-20A autosampler. The CTO-10ASVP column oven, CBM-20A communication bus module, and SPD-M20A diode array detector are being used. The system's holding volume was determined to be 0.50 mL. The system underwent inspection, and chromatograms were processed using LCSolutions 1.11 SP1 software (Kyoto, Japan). The equipment includes an Explorer E12140 analytical balance manufactured by Ohaus in Nänikon, Switzerland, an RK 100H ultrasonic bath produced by Bandelin in Berlin, Germany, a RO 15 multi-point mixer made by IKA in Staufen, Germany, and an Eppendorf device from Hamburg. A centrifuge type 5810R, manufactured in Hamburg, Germany, was utilised for the processing of samples and solutions.

2.3. Chromatographic Parameters

The study employed a mobile phase gradient elution method. The initial composition of the mobile phase consisted of a 30 mM phosphate buffer with a pH of 3.5 and acetonitrile at a ratio of 3:97 (volume/volume). The gradient elution programme is shown in Table 2.

Liquid chromatographic separation was conducted using a second-generation C18-bonded monolithic silica column (Chromolith High Resolution RP-18e, 100 mm × 4.6 mm i.d., Merck KGaA, Darmstadt, Germany). The temperature of the column oven was adjusted to 30 °C, while the injection volume was set to 5 µL. The rate of flow is 1.0 millilitres per minute. The photodiode array detector was configured to operate at a specific wavelength of 220 nm. Real-time spectra were captured within the range of 190 to 380 nm. The data sampling frequency was set at 6.25 Hz, and a time constant of 0.080 was applied.

2.4. Preparation of Solutions

The buffer solutions were created by dissolving 1503.0 mg of NaH_2PO_4 and 503.5 mg of $Na_2HPO_4.2H_2O$ in 500 mL of water. The mixture was then sonicated for 5 minutes and filtered using a nonsterile membrane filter with a diameter of 47 mm and a pore size of 0.45 µm, manufactured by Sartorius in Germany.

The solvent utilised in all dissolving and dilution processes for standard solution preparation was a mixture of acetonitrile:water (25:75, *v*/*v*), by volume. For the preparation of stock solutions, precisely measured quantities of 5 mg IVA and 5 mg LUMA were individually placed into 10 mL volumetric flasks and then diluted to the desired volume. The proportion of IVA and LUMA quantities in both the samples and working solutions was established using this stock solution. To prepare the AVA solution, 5.0 mg of AVA standard was added to a 25 mL volumetric flask and then diluted with enough solvent to reach a final concentration of 100.0 µg/mL.

Table 2. The applied flow gradient elution program

Time (min)	ACN (%)
1.00	30
2.00	60
2.50	50
3.00	40
4.00	15
4.50	30
5.00	40
6.00	65
6.01	30
8.00	Stop

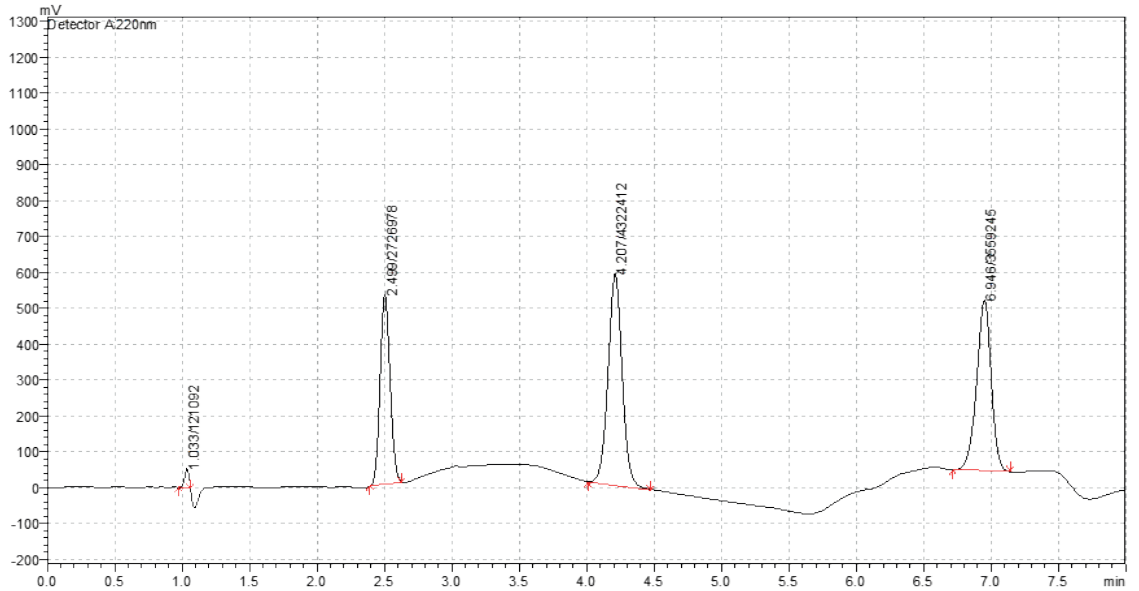

Figure 2. The chromatogram of standard solutions (C=150 µg/mL for IVA and LUMA, 100 µg/mL for AVA)

In addition, viable remedies for recuperation investigations were formulated using a pseudo composition of Orkambi® [20]. The needed dilutions were made with standard solution of active substance or IS.

2.5 Method validation

2.5.1. System Suitability Test

An evaluation of system compatibility was deemed essential in the development of the HPLC method to analyse the chromatographic performance of the HPLC apparatus. The asymmetry factor (As) and tailing factor (T) were calculated following the requirements of the United States Pharmacopoeia (USP) using the Shimadzu LCsolution v1.11 SP1 software.

2.5.2. Specificity

According to the ICH Q2(R1) guideline, it is advisable to employ an additional analytical process in order to compare the test outcomes for samples that are anticipated to contain impurities or degradation products. Hence, an analysis was conducted on the chromatograms and peaks of interest to ascertain the absence of any discernible positive or negative response to IVA, LUMA, and AVA. Furthermore, the assessment of peak purities was conducted by employing a photodiode array detector, which allowed for the examination of both the analyte and internal standard peaks. It was shown that these peaks could not be ascribed to multiple compounds simultaneously.

2.5.3. Linearity and Range

A linearity chart was constructed to encompass five different levels of IVA and LUMA concentrations, specifically 90, 120, 150, 175, and 200 µg/mL. The injection of each solution was performed in triplicate, and the resulting average values were deemed to be representative. The assessment of linearity was conducted using linear regression analysis, which included both intraday and interday repeats. The slope, intercept, correlation coefficient, confidence intervals for the slope, and the intercept at a 95% confidence level were computed. All statistical computations were performed using GraphPad Prism v6.0b (trial version).

2.5.4. Precision

Recovery tests were conducted in order to ascertain the precision of the methodology. The tablet samples were subjected to the addition of predetermined quantities of LUMA and IVA solutions, which were

carefully selected to represent low, medium, and high levels. Three sets of parallel items were prepared for each level. The spiked samples underwent reanalysis, and the mean recovery with standard deviation, expressed as a percentage of the blank spike solution (%RSD), was determined.

2.5.5. Accuracy

Precision investigations encompass both intraday and interday (sometimes known as intermediate) tests. The precision of the recommended approach was assessed by analysing standard solutions with a concentration of 120 µg/mL over three consecutive days. The data were subjected to statistical analysis, which involved calculating several measures such as the mean, standard error of the mean, standard deviation, %RSD, and confidence interval at a 95% confidence level. Furthermore, the analysis included an examination of the differences between groups on different days using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

2.5.6. Limitations of Detection and Quantification

The determination of the limit of detection (LOD) and limit of quantification (LOQ) was conducted in accordance with the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) standards, utilizing the standard deviation of the response and slope as key factors. In the context of Limit of Detection (LOD) and Limit of Quantification (LOQ), it is observed that the ratio of the standard deviation (σ) of the y-intercepts of the regression lines to the slope is multiplied by a factor of 3.3 for LOD and 10 for LOQ. These values of σ and slope were determined based on the analysis of combined data obtained from linearity experiments.

3. RESULTS AND DISCUSSION

The hydrophobicity profiles of IVA and LUMA molecules exhibit similarities, as indicated by their respective log P values of 5.6 and 5.8. This suggests that their retention in liquid chromatography and partitioning between phases are expected to be comparable. In light of this distinction, ACN was initially evaluated as an adjunctive organic modifier to water in the mobile phase. This choice was motivated by its favourable characteristics, including low absorption and low viscosity in the UV region, which facilitate enhanced mass transfer. The signals were monitored utilising a photodiode array detector operating at a wavelength of 220 nm. Satisfactory absorption was reported for both substances. In contrast, the compounds exhibit distinct molecular ionization properties. Specifically, IVA demonstrates a pK_a value of approximately 11.1, while LUMA exhibits a pK_a value of about 4.6 within the neutral to fundamental range. To enhance the regulation of pH-dependent ionization and retention for both chemicals, the mobile phase was buffered by employing a 0.030 M phosphate buffer that was adjusted to various pH values. Although the smoother peaks and elution within a 10-minute timeframe were seen, it is noteworthy that the two compounds exhibited distinct retention behaviors in response to alterations in the mobile phase's ACN content at a pH of 2. The chromatogram presented in Figure 1 depicts the standard solution.

A comprehensive analysis was conducted to compute the results of all system suitability tests (SST) as prescribed by the ICH Q2 (R1) guidelines for the optimized technique. The detailed findings are presented in Table 3. It is evident that all SST values fall within the prescribed limits and adhere to the parameters for chromatographic separation.

In order to conduct linearity and accuracy tests, the calculation involved determining the ratio between the peak area and retention time of each standard solution and the peak area and retention time of the IS solution contained in that particular solution. The utilization of peak normalization approach is crucial for systems that exhibit high sensitivity towards minor alterations or possess a significant matrix effect. By employing the suggested method, it is possible to achieve results of considerable accuracy and precision. Additionally, it facilitates the execution of method transfer, a crucial aspect in drug analysis, within more suitable ranges of values. Table 4 presents the data on linearity and precision that were acquired throughout the investigation.

Table 3. System suitability results (n = 3)

Parametre	LUMA	IVA	AVA	Recommended value
Retention time (min)	2.5	4.2	6.9	-
Retention time %RSD	0.06	0.3	0.5	RSD ≤1%
Repeatability of the peak area %RSD (n=6)	1.1	1.3	1.2	RSD ≤ 1%
Tailing factor (T)	1.2	1.3	1.2	T ≤ 2
Capacity factor (k)	2.6	2.1	2.2	2<k<10
Number of theoretical plate (N)	16289	30025	30258	N > 2000
USP Width	0.3	0.5	0.7	≤1
HETP (USP)	10.45	33.3	52.1	-

Table 4. Linearity and precision data

Parameter	IVA	LUMA
Linearity (µg/mL)	0.78	1.96
Slope (n=7)	-0.008	-0.006
Intercept (n=7)	0.9986	0.9968
LOD (µg/mL)	60 ng/ mL	50 ng/ mL
LOQ (µg/mL)	200 ng/mL	200 ng/mL
Slope (intra-day. k= 3)	0.99	2.21
Intercept (inter-day. k= 3)	-0.01	-0.01
Regression coefficient (inter-day. k= 3)	0.9940	0.99623
ANOVA	F (1,13) = 1.04	F (1,13) = 1.58
	P>0.05	P>0.05

Table 5. Recovery results (n=3)

		Precision		Accuracy	
	Added(µg/mL)	SD	RSD (%)	Recovery (%)	Bias (%)
IVA	120	0.2	0.9	98.6	-1.4
	150	0.1	0.5	98.8	-1.2
	175	0.1	0.4	98.4	-1.6
LUMA	120	0.1	0.9	98.4	-1.6
	150	0.1	0.8	96.4	-3.6
	175	0.3	1.9	97.8	-2.2

In order to conduct recovery tests, the researchers employed the conventional addition procedure and conducted a total of nine independent determinations at three distinct concentrations that encompassed the desired range. So, to achieve the desired objective, a patented formulation of Orkambi® was created, and subsequent recovery studies were conducted by introducing a specific quantity of a standard solution mixture to these tablets. The obtained results were given in Table 5.

In fact, in previous methods developed for IVA or LUMA analysis (one of the authors also contributed), stability studies have shown that the active ingredients are quite stable [20, 21]. However, in this study, stability studies were carried out meticulously because IVA and LUMA were mixed in the working solutions and ava contributed as IS. According to the results obtained, it can be seen in Table 6 that IVA/ LUMA are quite stable under operating conditions.

Table 6. Stability results of active substances (n=3)

	Added (µg/mL)	Short-term stability (48 h at room temper-ature)		Long-term stability (3 weeks -20°C)		Freezing-thawing stability (n=3)	
		Found (Mean)	RSD (%)	Found (Mean)	RSD (%)	Found (Mean)	RSD (%)
IVA	150.0	150.2	0.5	149.2	0.6	149.1	0.8
LUMA	150.0	150.3	0.7	150.1	0.6	149.3	0.9

4. CONCLUSION

Drug analysis is the backbone of the pharmaceutical industry. Each method developed contributes to the drug analysis of flour and sheds light on the process. HPLC has become the most important apparatus in this business today and has become indispensable. Although a new high-throughput technique is developed day by day and recommended to analysts, it does not seem possible to give up HPLC due to the convenience, high repeatability, and accuracy it provides.

In this study, a fast, highly accurate, and precise HPLC method was developed for the analysis of IVA/LUMA combinations in bulk and pharmaceutical formulations. Caftors are currently the most important therapeutic agents for CF diseases. They are also pioneer molecules in pharmaceutical chemistry because they contribute to the correction of the defective function of the CFTR gene. The importance of these molecules will increase day by day, and their analysis will be needed in almost all studies on them. In the method developed with this foresight, HPLC was preferred because it is an easily accessible and productive technique. The study is comprehensive, in accordance with the ICH Q2 (R1) guidelines, and will contribute to drug analysis.

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Ethical approval

Not applicable, because this article does not contain any studies with human or animal subjects.

Author contribution

Concept: SÖ, AE; Design: SÖ; Supervision: SL; Materials: SÖ; Data Collection and/or Processing: SÖ, SL, AE; Analysis and/or Interpretation: SÖ, AE; Literature Search: SÖ, SL; Writing: SÖ, AE; Critical Reviews: SL.

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Conflict of interest

The authors declared that there is no conflict of interest.

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Research Article

Synthesis of thiazole derivatives as cholinesterase inhibitors with antioxidant activity

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ABSTRACT

In the present research, we synthesized two unique series of thiazole compounds having 5-bromothiophene and 3-methylthiophene (**2a-2f**) in their structure. After that, spectroscopic methods were used to analyze the chemical compositions of the newly synthesized molecules. Then *in vitro* evaluation was done to determine acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) activity of the synthesized compounds using galantamine as reference standard. The compounds' antioxidant properties were assessed using DPPH radical scavenging and ferrous ion-chelating techniques. The results of the study showed weak anticholinesterase activity against AChE and BuChE enzymes for all the final compounds. The synthesized analogs also showed significant DPPH radical scavenging activities with IC_{50} values in the range of 29.16 ± 0.009 to 33.09 ± 0.004 µM (for DDPH) incomparison to standard gallic acid with IC_{50} = 31.13 ± 0.008 µM (for DDPH). Especially, compound **2c** showed the best antioxidant activity with IC_{50} value of 29.16 ± 0.009 µM.

Keywords: AChE, Antioxidant, BuChE, Thiazole

1. INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disease at the moment, which causes loss of memory, behavioral problems, and a reduction in cognitive function eventually leading to death [1,2]. The disease is most commonly caused by cholinergic hypothesis, Amyloid- β (Ab) plague formation, N-methyl-D-aspartate (NMDA) receptor (NMDAR) antagonism hypothesis, The accumulation of thin protein after their hyperphosphorylation, biometal dyshomeostasis, and oxidative stress [3-5].

Currently, the traditional "cholinergic hypothesis" is mostly agreed upon by academics [6]. According to the cholinergic hypothesis, the main biochemical features of AD are reported to be loss and dysfunctions of cholinergic transmission and reductions in acetylcholine neurotransmitters [7]. Cholinesterase enzymes encompass two distinct isozymes, namely AChE (E.C.3.1.1.7) and BuChE (E.C.3.1.1.8), which hydrolyze acetylcholine (ACh) and influence cholinergic neuron activity [8,9]. AChE hydrolyzes the neurotransmitter acetylcholine, which is present at cholinergic synapses, whereas BuChE co-regulates AChE's activity. Cholinesterase inhibitors increase the quantity of acetylcholine required for the neurotransmission process by counteracting the effects of these enzymes [10]. Four cholinesterase (ChE) inhibitors have so far received FDA approval for use in the treatment of AD (**Figure 1**): donepezil, tacrine, galantamine, and rivastigmine [11-12].

By scavenging and stabilizing free radicals, antioxidants are chemicals that lessen the oxidative damage caused by free radicals. Additionally, antioxidants have a protective effect on macromolecules such as proteins, nucleic

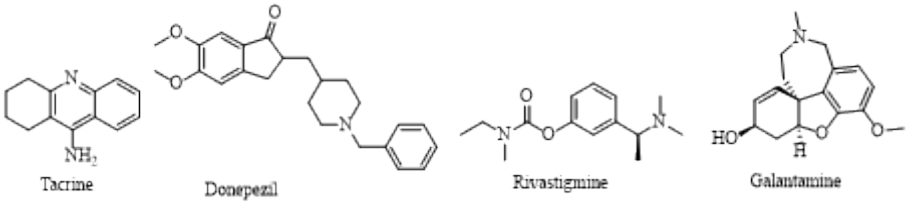


Figure 1. The structures of some commercially available AChE inhibitors.

acids and lipids. As a result, molecules with both cholinergic inhibitor and antioxidant properties provide advantages in the treatment of AD for potential therapeutic purposes [8].

In order to develop effective innovative drugs for the central nervous system, thiazole has been found as a potential scaffold. A number of thiazole derivatives are currently being investigated in clinical studies, and thiazole-based CNS medications are now used as therapeutic agents for a variety of CNS disorders [13-14].

In this study, thiazole derivatives were synthesized and their structure characterized using ^{1}H-NMR and ^{13}C-NMR, and HRMS. The compounds' antioxidant properties were assessed using Ferrous ion-chelating and DPPH Radical Scavenging methods. Furthermore, this study investigated these derivatives for *in vitro* inhibition on AChE and BuChE.

2. MATERIALS AND METHODS

2.1. Chemistry

Synthesis of 2-((3-Methyl/5-bromothiophene-2-yl)methylene)hydrazine-1-carbothioamide (1): In ethanol, 3-methylthiophene-2-carbaldehyde or 5-bromothiophene-2-carbaldehyde and thiosemicarbazide were dissolved. Following that, the mixture was refluxed for three hours. After the completion of reaction, the mixture was placed in an ice bath to chill down. The resultant precipitate was then removed by filtering.

Synthesis of Target Compounds (2a-2f): Ethanol was used to dissolve compound 1 and derivative of 2-bromoacetophenone. After that, the mixture was refluxed for four hours. After the reaction is complete, the mixture is placed in an ice bath to chill down. The resultant precipitate is removed by filtering. After chilling, the drying and crystallization of precipitates is done using ethanol.

4-(4-Cyanophenyl)-2-(2-((3-methylthiophenyl-2-yl)methylene)hydrazineyl)thiazole (2a): Yield: 75 %, M.P.= 248.6 °C. ^{1}H-NMR (300 Mega Hz, Dimethylsulfoxide-d_6): δ: 2.18 (3H, s, CH_3), 7.75-7.76 (1H, m, Aromatic CH), 7.81-7.82 (2H, m, Aromatic CH), 7.90 (1H, s, Aromatic CH), 8.11 (1H, s, CH=N), 8.31 (3H, d, *J*=8.60 Hz, Aromatic CH), 11.33 (1H, s, NH). ^{13}C-NMR (75 Mega Hz, Dimethylsulfoxide-d_6): δ= 15.59 (CH_3), 109.40, 120.43, 123.37, 125.18, 127.94, 128.79, 131.55, 132.31, 133.83, 136.39, 139.25, 152.08 (thiazole C), 170.14 (thiazole C). Calculated HRMS (m/z): [M+H] for $C_{16}H_{12}N_4S_2$: 325.0576; found: 325.0586.

4-(3,4-Dichlorophenyl)-2-(2-((3-methylthiophenyl-2-yl)methylene)hydrazineyl) thiazole (2b): Yield: 78 %, M.P.= 240.5 °C. ^{1}H-NMR (300 Mega Hz, Dimethylsulfoxide-d_6): δ: 2.27 (3H, s, CH_3), 7.01 (1H, s, Aromatic CH), 7.37 (1H, s, Aromatic CH), 7.72-7.80 (4H, m, Aromatic CH), 8.08 (1H, s, CH=N), 11.17 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): δ= 17.01 (CH_3), 102.65, 113.58, 119.19, 123.37, 124.70, 128.03, 129.84, 130.03, 132.31, 133.26, 136.58, 138.77, 151.03 (thiazole C), 170.42 (thiazole C). Calculated HRMS (m/z): [M+H] for $C_{15}H_{11}N_3O_2S_2Cl_2$: 367.9844; found: 367.9856.

4-(2,4-Difluorophenyl)-2-(2-((3-methylthiophenyl-2-yl)methylene)hydrazineyl) thiazole (2c): Yield: 76 %, M.P.= 200.2 °C. ^{1}H-NMR (300 Mega Hz, Dimethylsulfoxide-d_6): δ: 2.19 (3H, s, CH_3), 7.74 (1H, s, Aromatic CH), 7.88-7.90 (2H,

m, Aromatic CH), 8.03-8.05 (3H, m, Aromatic CH), 8.14 (1H, s, CH=N), 11.34 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): δ= 17.58 (CH_3), 108.45, 110.54, 112.54, 122.14, 124.04, 127.74, 128.80, 130.99, 132.40, 134.30, 135.54, 138.58, 150.18 (thiazole C), 170.23 (thiazole C). Calculated HRMS (m/z): [M+H] for $C_{15}H_{11}N_3F_2S_2$: 336.0435; found: 336.0446.

4-(4-Cyanophenyl)-2-(2-((5-bromothiophenyl-2-yl)methylene)hydrazineyl)thiazole (2d): Yield: 80 %, M.P.= 207.5 °C. ^{1}H-NMR (300 Mega Hz, Dimethylsulfoxide-d_6): δ: 6.89-7.05 (2H, m, Aromatic CH), 7.37 (1H, s, Aromatic CH), 7.82-7.92 (4H, m, Aromatic CH), 8.18 (1H, s, CH=N), 11.38 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): δ= 109.63, 120.14, 122.34, 125.08, 126.93, 127.54, 131.35, 132.20, 132.83, 135.29, 138.15, 152.11 (thiazole C), 170.18 (thiazole C). Calculated HRMS (m/z): [M+H] for $C_{15}H_9N_4S_2Br$: 388.9525; found: 388.9536.

4-(3,4-Dichlorophenyl)-2-(2-((5-bromothiophenyl-2-yl)methylene)hydrazineyl) thiazole (2e): Yield: 79 %, M.P.= 220.5 °C. ^{1}H-NMR (300 Mega Hz, Dimethylsulfoxide-d_6): δ: 6.94-7.08 (2H, m, Aromatic CH), 7.39 (1H, s, Aromatic CH), 7.89-7.94 (3H, m, Aromatik CH), 8.20 (1H, s, CH=N), 11.40 (1H, s, NH). ^{13}C-NMR (75 Mega Hz, Dimethylsulfoxide-d_6): δ= 109.18, 112.45, 118.57, 123.62, 125.78, 126.99, 129.80, 131.28, 133.44, 124.32, 136.58, 138.42, 150.28 (thiazole C), 170.12 (thiazole C).

4-(2,4-Difluorophenyl)-2-(2-((5-bromothiophenyl-2-yl)methylene)hydrazineyl) thiazole (2f): Yield: 74 %, M.P.= 197.2 °C. ^{1}H-NMR (300 Mega Hz, Dimethylsulfoxide-d_6): δ: 6.98-7.10 (2H, m, Aromatic CH), 7.42 (1H, s, Aromatic CH), 7.87-7.91 (3H, m, Aromatik CH), 8.18 (1H, s, CH=N), 11.42 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): δ= 110.42, 111.48, 114.63, 121.14, 123.44, 128.70, 128.84, 131.19, 132.44, 135.30, 136.62, 139.20, 150.24 (thiazole C), 170.22 (thiazole C). Calculated HRMS (m/z): [M+H] for $C_{14}H_8N_3F_2S_2Br$: 399.9384; found: 399.9394.

2.2. Assay for inhibition of cholinesterase enzyme

The ability of the synthesized compounds to inhibit the BuChE and AChE enzyme was examined. Ellman's modified spectrophotometric technique [15] was used to measure the inhibition potential of synthesized compounds against AChE and BuChE. Cholinesterase activity experiments were conducted using "equine serum BuchE" (EC 3.1.1.8, Sigma) and electric eel AChE (Type-VI-S, EC 3.1.1.7, Sigma) enzymes. The reaction's substrates were butyrylthiocholine chloride and acetylthiocholine iodide obtained from Sigma Aldrich at Saint Louis, USA. To test the cholinesterase activity, 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB, Sigma Aldrich at Saint Louis, United States America) was utilized. In a 96-well microplate, additional reagents were added in the following order: 50 mM Tris HCl buffer (pH 8.0), 6.8 mM DTNB, 10 µl of BuChE/AChE solution, and 2 µl of sample solutions and multichannel automated pipette obtained from Thermo Fisher Scientific, USA). Next, butyrylthiocholine chloride/acetylthiocholine iodide was added in 10 µl amount to start the reaction. The formation of the yellow 5-thio-2-nitrobenzoate anion, which results from the reaction of DTNB with thiocholines, was employed to track the acetylthiocholine iodide/butyrylthiocholine chloride hydrolysis. Using a 96-well plate, the following reaction was catalyzed by enzymes at a wavelength of 412 nm. The plate was obtained from Varioskan Flash, Thermo Scientific, USA). The incubation of microplate was done for 15 minutes at 27°C. Periodic test lasting 75 seconds was obtained. The Varioskan Flash software's SkanIt Software 2.4.5 RE was used to assess the measurements and computations. By comparing the sample reaction rates to those of the blank sample (DMSO and methanol) and applying the formula (E-S)/E x 100, the percentage of AChE and BChE inhibition was calculated. Three replicates of each experiment were conducted. Galantamine hydrochloride obtained from the Sigma-Al, USA has been utilized as a reference material.

In the formula;

E: the activity of the enzyme without the test sample.

S: the activity of the enzyme with the test sample.

2.3. Antioxidant Activity

2.3.1. Ferrous ion-chelating effect

Using Chua et al.'s (2008) approach, the ferrous ion-chelating impact of the reference compound and all the extracts was evaluated. In summary, 200 µL of a 2 mM $FeCl_2$ solution was used to incubate different dilutions of ethanol dissolved extracts (80%). Then, we added 5 mM ferrozine concentration of 5 mM ferrozine in 800 µL amount to the mixture to start the reaction, which was then allowed to stand for 10 minutes at room temperature. Using a spectrophotometer, the reaction mixture's absorbance was determined at 562 nm (Varioskan Flash, Thermo Scientific, USA) against ethanol (80%) as blank. The following formula was used to determine the ratio of inhibition of ferrozine-Fe^{2+} complex formation:

$$I\% = \left[\frac{A_{blank} - A_{sample}}{A_{blank}}\right] \times 100$$

where A_{sample} is the absorbance of the extracts/reference, and A_{blank} is the absorbance of the control reaction (which only contained $FeCl_2$ and ferrozine). In this test, butylated hydroxytoluene (BHT) and rutin served as the reference. Both were purchased from Sigma Aldrich in the USA. Three duplicate analyses were performed, and the average values with S.E.M. were reported as the results [16,17].

2.3.2. DPPH Radical Scavenging Activity

Radical-scavenging capacity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was screened using Blois's UV technique. Using this procedure, 20 µL of methanol was mixed with the compounds having 40 micro molar and 100 micro molar concentrations, as well as gallic acid. Then, in each solution180 µL of a 0.15 mM DPPH solution dissolved in methanol was added. Then incubation was done at room temperature for 20 minutes and amount of DPPH was measured at 520 nm (Varioskan Flash, Thermo Scientific, USA). The following formula was employed to determine radical scavenging capacity of DPPH.

$I\% = [(A_{control} - A_{sample})/A_{control}] \times 100$, where

$A_{control}$ = Absorbance of the control reaction

A_{sample} = Absorbance of the extracts/reference.

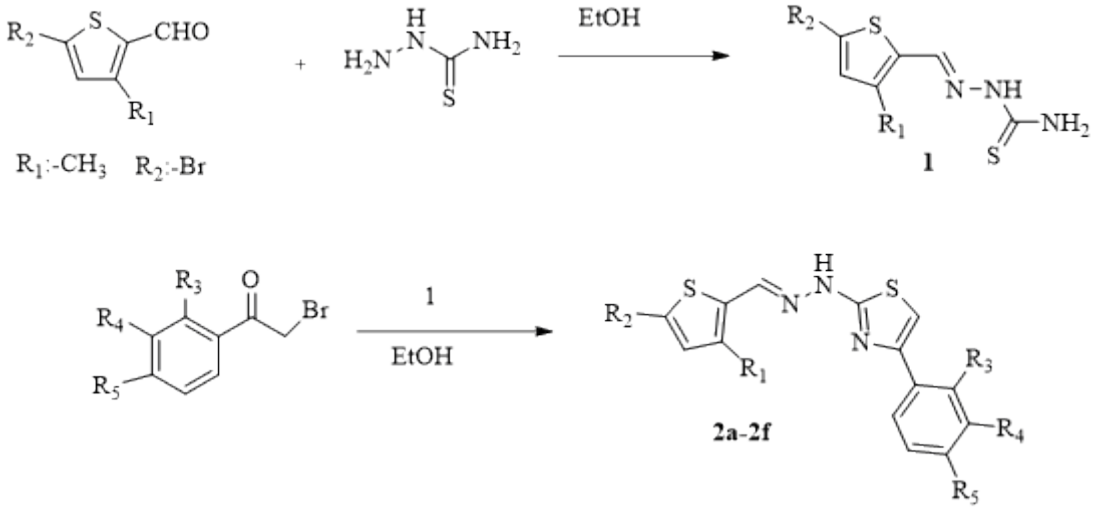
The experiments were performed as replicates of three, and the average was taken with standard error mean [18].

3. RESULTS AND DISCUSSION

3.1. Chemistry

In this study, three new thiazole derivatives were synthesized, as shown in **Scheme 1.** The synthesis of the compounds was carried out in two stages. At the first step, 3-methylthiophene-2-carbaldehyde or 5-bromothiophene-2-carbaldehyde compound was reacted with thiosemicarbazide and thiosemicarbazone compound was obtained. In the second step, the thiosemicarbazone compound obtained in the first step was reacted with 2-bromoacetophenone derivative compounds and thiazole derivative compounds were obtained. Thiazole compounds are made by the reaction between α-halo-ketones and thioamides, a procedure known as Hantzsch thiazole synthesis. The reaction is driven by the intense nucleophilic nature of the S in thioamides, which is enhanced by electron resonance from the amide group. Because halogen is a good leaving group, sulfur attacks the α-carbon of α-halo-ketones as a nucleophile instead of the nearby carbonyl group. This encourages the thiazole ring to form and cyclize.

The structure of the 4-(substitutedphenyl)-2-(2-((3-methyl/5-bromothiophenyl-2-yl)methylene) hydrazineyl)thiazole (**2a-2f**) derivatives were confirmed by using ^{1}H-NMR, ^{13}C-NMR, and HRMS. The main structure of the target compounds constitute 3-methylthiophene and thiazole rings. The proton of methyl group in 3-methylthiophene was detected at 2.18-2.27 ppm range as singlet. Hydrazine (CH=N) protons have been detected around 8 ppm. The signals belonging to aromatic protons were found at 7.01–8.31 parts per million.



Comp.	2a	2b	2c	2d	2e	2f
R_1	$-CH_3$	$-CH_3$	$-CH_3$	-H	-H	-H
R_2	-H	-H	-H	-Br	-Br	-Br
R_3	-H	-H	-F	-H	-H	-F
R_4	-H	-Cl	-H	-H	-Cl	-H
R_5	-CN	-Cl	-F	-CN	-Cl	-F

Scheme 1. Chemical structure and general procedure for the synthesis of the final compounds **2a-2f**.

The carbon of methyl in the 3-methylthiophene ring resonated at 15.59–17.58 parts per million when the compounds' ^{13}C-NMR spectra were analyzed. All of the masses matched the expected M+H values.

3.2. Cholinesterase Enzymes Inhibition Assay

Using galantamine as reference drug, the **2a-2f** were analysed for their inhibitory effect against AChE and BuChE were assessed by employing Ellman's technique. **Table 1** is a summary of the findings. To verify the outcomes, three separate tests were run in duplicate. The results of our study showed weak results against AChE and BuChE enzymes.

Table 1. % Cholinesterase inhibitory activities of the synthesized compounds **2a-2f** at 50 µM reaction concentrations

Comp.	AChE	BuChE
2a	15.59 ± 0.006	5.07 ± 0.004
2b	3.39 ± 0.001	8.72 ± 0.001
2c	11.16 ± 0.005	3.94 ± 0.005
2d	NA	7.71 ± 0.002
2e	NA	NA
2f	NA	11.70 ± 0.006
Gal HBr	97.89 ± 0.01	62.48 ± 0.01

Table 2. DPPH free radical-scavenging activity and ferric ion chelating effect (inhibition % ± S.E.M) of synthesized compounds at 50 µM and IC_{50} values (**µm**)

Comp.	DPPH	ION CHELATING	IC_{50} (DPPH) µm
2a	44.55 ± 0.002	NA	-
2b	29.66 ± 0.003	1.80 ± 0.007	-
2c	64.31 ± 0.005	6.41 ± 0.004	29.16 ± 0.009
2d	63.42 ± 0.004	2.11 ± 0.008	32.08 ± 0.007
2e	65.96 ± 0.021	3.91 ± 0.013	31.94 ± 0.011
2f	60.83 ± 0.003	3.11 ± 0.006	33.09 ± 0.004
Gallic Acid	70.29 ± 0.005	-	31.13 ± 0.008
RUTIN 50 µM	-	13.21 ± 0.007	-
BHT 50 µM	-	7.06 ± 0.009	-

3.3. Antioxidant Activity

Test compounds for DPPH free radical scavenging and Ferrous ion-chelating effect were set at the concentration of 50 µM. We used gallic acid for reference. Based on control activities, the percentage of all substances evaluated as antioxidants was estimated (**Table 2**). The results showed the antioxidant activity of 70.29±0.005 % for gallic acid, and of 64.31 ± 0.005 % for **2c** at the concentration of 50 µm. Therefore, the compound **2c** can behave as a potential antioxidant agent.

4. CONCLUSION

Three novel thiazole-based compounds were synthesized and their potential as antioxidant and AChE inhibitors therapy was assessed. The three compounds showed minimal activity against AChE enzyme while compound **2c** showed antioxidant activity comparable to the reference drug. Based on the results and non-significant activities of the synthesized compounds **2a-2f**, no further molecular docking and ADMET studies were performed for these compounds. However, in the future, this synthetic scheme can be employed to synthesize a new series of thiazole derivatives as a strong candidate for the symptomatic relief of Alzheimer's disease.

Ethical approval

Not applicable, because this article does not contain any studies with human or animal subjects.

Author contribution

Concept: UAÇ; Design: UAÇ; Supervision: UAÇ; Materials: AK, ZM, TE; Data Collection and/or Processing: UAÇ, ZM, TE; Analysis and/or Interpretation: UAÇ; Literature Search: AK; Writing: AK, ZM, TE, UAÇ; Critical Reviews: UAÇ.

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Conflict of interest

The authors declared that there is no conflict of interest.

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Research Article

Evaluation of volatile components of *Achillea millefolium* L. essential oil

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ABSTRACT

The genus *Achillea* (Asteraceae) is distributed in Europe and the Middle East and has approximately 140 endemic species. There are 40 *Achillea* sp. in the Turkish flora and 20 of them are endemic. This genus has a widespread area all over the world. Also, it has many different uses in traditionally, such as diarrhea, abdominal pain, hemorrhoids and wound healing. *Achillea* sp. is also widely used as food.

Achillea millefolium L. is known as "Yarrow" and has been used as a wound healer, diuretic, appetite stimulant and menstrual bleeding in Türkiye. The aerial parts of the plant are used in a variety of forms, including infusion, tincture, liquid extract, total extract, bath. It has also been stated that the essential oil of the plant is used in mouth sores and dental health due to its antimicrobial effect. It is also preferred in the treatment of aphtha and wounds in the mouth.

Within the scope of this project, essential oil of *A. millefolium* was obtained from European Pharmacopoeia (9th Edition) quality, supplied from German commercial source, with the Clevenger apparatus for 3 h. The chemical composition of the essential oil obtained was elucidated.

A total of 67 components were identified. Chamazulene (6.8%), caryophyllene oxide (5.8%), torilenol (5.6%), (*E*)-nerolidol (4.3%), borneol (4.0%) were found as major constituents of *A. millefolium* essential oil.

The original value of this study, aim of this study is to conduct a pharmacognosic examination of the European Pharmacopoeia quality *A. millefolium*, one of the *Achillea* sp. that is of great importance worldwide due to medicinal properties, especially herbal tea. With this study, the chemical composition of the volatile components of the *A. millefolium* was elucidated.

Keywords: Asteraceae, *Achillea millefolium* L., essential oil, GC, GC-MS

1. INTRODUCTION

Achillea millefolium L. is a perennial herbaceous species from the Asteraceae and it is considered one of the oldest medicinal plants. It is an erect herbaceous perennial plant that grows up to 50 cm tall, with a slender cropping rootstock throwing numerous roots and stolons with a blunt, succulent scale at each node. The leaves are 5–20 cm long, bipinnate or tripinnate, almost feathery, having varying degrees of hairiness (pubescence) and arranged spirally near the middle and bottom of the stem. The flowers are

typically white, but either pink or pale purple flowers with corymbose, ovoid, flat-topped heads at the end of stems and branches, having densely arranged petals in flattened clusters. Fruits are 2-mm, shiny, oblong achenes, with broadly winged margins and no pappus [1, 2]. *A. millefolium* can grow even at an altitude of 3500 meters above sea level. 130 species have been identified in Europe, Asia and America, 40 species in Türkiye and 19 species in Iran. It is one of the oldest known herbal medicines. It grows in clusters in sunny and warm environments, on narrow field paths, roadsides, meadows and sunny slopes. Flowering is observed between May and October, the flowers are generally white [3, 4]. It is popularly used for indigestion and colds. Dried flowering parts of this plant are used medicinally [1].

The phytochemical proporties of the *A. millefolium* has been revealed through studies conducted for many years. The various flavonoids such as apigenin, quercetin, kaempferol, naringenin, rutin and acacetin etc. [5-8]; phenolics such as *p*-coumaric acid, caffeic acid, ferulic acid etc. reported from different parts of *A. millefolium* [7-10]. In addition, mono- and sesquiterpenes reported as a major components from *A. millefolium* essential oils. *α*-Pinene, *β*-pinene, *β*-phellandrene [11], *α*-thujane, *α*-terpinene and *γ*-terpinene [12], camphene and limonene [13-14], and sabinene [14-16] are the monoterpenes identified in *A. millefolium* essential oil. (*E*)-*β*-caryophyllene [11, 15], *β*-cubebene, germacrene-D [17], are the sesquiterpenes hydrocarbons found in *A. millefolium* essential oils. Also, chamazulene which gives the its characteristic color-bluish [17] contains in *A. millefolium* essential oils.

A. millefolium is used as wound healer, diuretic, carminative, menstrual regulator, prevent stomach problems such as gastritis and ulcers, kidney stone reducer, blood and liver cleanser, anti-inflammatory, headache reliever and prostate treatment traditionally [1, 18]. Its has wide range of pharmacological activities such as antimicrobial [2], antihelmintic [19], antioxidant [20], antiinflammatory [21], antispazmodic [22], anticancer [23], antiulcer [24], hepatoprotective [25] activities.

In this study, it was aimed to reveal the phytochemistry of *A. millefolium* essential oil and its quality.

2. MATERIALS AND METHODS

2.1. Material

Pharma grade dried *A. millefolium* was obtained from German commercial source (Caesar & Loretz GmbH). Essential oil was obtained by hydrodistillation for 3 h with Clevenger apparatus. The yield of essential oil was found %0.025.

2.2. GC and GC-MS analyses

The essential oil was analyzed by GC using a Hewlett Packard 6890 system (SEM Ltd, Istanbul, Turkey) and an HP Innowax fused silica capillary column (FSC) (60 m x 0.25 mm Ø, with 0.25 µm film thickness) was used with nitrogen at 1 mL/min. Initial oven temperature was 60°C for 10 min, and increased at 4 °C/min to 220 °C, then kept constant at 220 °C for 10 min and increased at 1 °C/min to 240 °C. Injector temperature was set at 250 °C. Percentage compositions of the individual components were obtained from electronic integration using flame ionization detection (FID, 250 °C) (Demirci vd., 2015). Relative percentages of the separated compounds were calculated from FID chromatograms as cited in Table 1 [26].

GC-MS analysis was performed with a Hewlett-Packard GCD, system (SEM Ltd, Istanbul, Turkey) and Innowax FSC column (60 m x 0.25 mm, 0.25 µm film thickness) was used with Helium. GC oven temperature conditions were as described above, split flow was adjusted at 50 mL/min, the injector temperature was at 250 °C. Mass spectra were recorded at 70 eV. Mass range was from m/z 35 to 425 as previously reported [26].

Identification of the essential oil components were carried out by comparison of their relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) to series of *n*-alkanes. Computer matching against commercial (Wiley GC-MS Library, MassFinder

4.0 Library) [27, 28], and in-house "Başer Library of Essential Oil Constituents" built up by genuine compounds and components of known oils, as well as MS literature data [29] was used for the identification as also previously reported in detail [26].

3. RESULTS AND DISCUSSION

A. millefolium essential oil was analyzed by GC and GC/MS systems, simultaneously. A total of 67 components were identified in A. *millefolium* essential oil which accounted for 70.7%. Chamazulene (6.8%), caryophyllene oxide (5.8%), torilenol (5.6%), (*E*)-nerolidol (4.3%), borneol (4.0%) were found as major constituents of *A. millefolium* essential oil. The other constituents were given in Table 1.

When the compared the EP (9th edition), the standard for content of proazulenesis not less than 0.02%, expressed as chamazulene, calculated to the dried drug [30]. Our results were confirmed the quality of essential oil.

Many researchers investigated the chemical compositions of *A. millefolium* essential oil. In previous work of Kazemi et al. (2015), the major constituent was thymol (26.47%), followed by borneol (16.35%), limonene (14.53%), carvacrol (10.14%) and *α*-pinene (10.12%) [20]. Almadiy et al. (2016) were chamazulene (26.2%), *β*-pinene (16.6%), sabinene (9.2%), germacrene D (6.7%), and *β*-caryophyllene (5.9%) were the main compounds in *A. millefolium* essential oil [31]. Candan et al. (2003), analyzed the *A. millefolium* essential oil and 1,8-cineole (24.6%), camphor (16.7%), *α*-terpineol (10.2%), *β*-pinene (4.2%), and borneol (4.0%) were found as major components [32]. According to Jianu et al. (2016), chamazulene (16.37%) and germacrene D (15.38%) were found as the main components in the *A. millefolium* essential oil, the yield being 0.43% (v/w) [33]. Orav et al. (2005) identified 102 compounds from 19 different *A. millefolium* essential oils, grown in different regions of Europe. Chamazulene (0-42.0%), *α*-thujone (0-26.6 %), camphor (0.1-24.5 %), *β*-bisabolol (0-21.6 %) and *β*-pinene (0-20.3 %) were found as main components in different *A. millefolium* essential oils [34].

Table 1. Volatile components of *Achillea millefolium* L. essential oil

RRI[a]	Compound	%[b]
1032	*α*-Pinene	0.1
1035	*α*-Thujene	0.1
1093	Hexanal	0.1
1118	*β*-Pinene	0.2
1163	Isomenthone	0.1
1188	*α*-Terpinolene	0.1
1203	Limonene	trc
1213	1,8-Cineole	0.9
1244	2-Pentyl furane	0.2
1255	*γ*-Terpinene	0.2
1280	*p*-Cymene	0.5
1358	Artemisia ketone	0.2
1403	Yomogi alcohol	0.4
1430	*α*-Thujone	0.1
1450	2,5-Dimethyl styrene	tr
1451	*β*-Thujone	0.1
1452	*α,p*-Dimethylstyrene	0.1
1497	*α*-Copaene	0.2
1532	Camphor	2.8
1538	*trans*-Chrysanthenyl acetate	0.5
1553	Linalool	0.4
1586	Pinocarvone	0.3
1590	Bornyl acetate	0.6
1611	Terpinen-4-ol	1.3
1612	*β*-Caryophyllene	1.0
1648	Myrtenal	0.6
1706	*α*-Terpineol	1.3
1658	Sabinyl acetate	0.5
1664	*trans*-Pinocarveol	0.4
1709	*α*-Terpinyl acetate	1.0
1719	**Borneol**	**4.0**
1748	Piperitone	0.1
1764	*cis*-Chrysanthenol	0.3
1773	*δ*-Cadinene	0.5
1776	*γ*- Cadinene	0.3
1786	*ar*-Curcumene	0.1
1845	*(E)*-Anethol	1.4
1804	Myrtenol	0.2
1827	*(E,E)*-2,4-Decadienal	0.9
1868	*(E)*-Geranyl acetone	0.4
1941	*α*-Calacorene	0.4

RRI[a]: Relative retention indices calculated against *n*-alkanes;
%[b]: calculated from the FID chromatograms;
tr[c]: trace amount (< 0.1%).

Table 1. Continued

RRI[a]	Compound	%[b]
1945	1,5-Epoxy-salvial-4(14)-ene	0.3
1948	*trans*-Jasmone	0.4
1958	*(E)-β*-Ianone	0.2
2008	**Caryophyllene oxide**	**5.8**
2037	Salvial-4(14)-en-1-one	0.6
2050	***(E)*-Nerolidol**	**4.3**
2071	Humulen epoxide-II	0.6
2092	*β*-Oplopenone	1.6
2104	Viridifrolol	2.4
2131	Hexahydro farnesyl acetone	1.1
2144	Spathulenol	2.8
2179	3,4-Dimethyl-5-penthylidene-2(5H)-furanone	0.6
2187	*T*-Cadinol	1.3
2232	*α*-Bisabolol	0.7
2250	*α*-Eudesmol	0.1
2255	*α*-Cadinol	3.0
2257	*β*-Eudesmol	0.5
2265	Longiverbenone	0.5
2273	Selinen-11-en-4 *α*-ol	2.2
2278	**Torilenol**	**5.6**
2298	Decanoic acid	2.0
2300	Tricosane	0.7
2316	Caryophylladienol I	1.2
2400	Tetracosane	0.8
2430	**Chamazulene**	**6.8**
2600	Hexacosane	1.7
	Total	**70.7**

RRI[a]: Relative retention indices calculated against *n*-alkanes; %[b]: calculated from the FID chromatograms; tr[c]: trace amount (< 0.1%).

4. CONCLUSION

A. millefolium is used in the treatment of colds, dyspeptic diseases, mouth infections, and skin diseases. It also has many valuable biological activities such as antimicrobial, antioxidant and anticancer. Thanks to its traditional uses and biological activities, *A. millefolium* has an important place. Therefore, it has become important to elucidate its chemistry and reveal its quality for consumption. In conclusion, our results as found as similar in literature. It was confirmed that essential oil of *A. millefolium* phytochemical characterization and its quality.

Ethical approval

Not applicable because this article does not contain any studies with human or animal subjects.

Author contribution

Concept: BD; Design: BD; Supervision: BD; Materials: BD, GÖ; Data Collection and/or Processing: BD, GÖ, DK; Analysis and/or Interpretation: BD, GÖ, DK; Literature Search: GÖ; Writing: BD, GÖ; Critical Reviews: BD.

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Conflict of interest

The authors declared that there is no conflict of interest.

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Review Article

Pharmaceutical supply chain: The importance of outsourcing

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ABSTRACT

A vast web of organizations, companies, and teams involved in creating, researching, and producing drugs and associated products makes up the pharmaceutical sector. In this context, the term "supply chain" refers to the network of individuals, processes, information, and resources that transform raw materials and parts into finished goods and services before being supplied to customers. The pharmaceutical supply chain system, then, offers customers medications in the right amount, at the right time, with acceptable quality, and for the lowest feasible cost. Pharmaceutical companies are increasingly contracting out their supply chain activities due to the severe push to keep R&D expenses under control. Instead of being a cost-cutting measure, global supply chain outsourcing can be seen as a strategic competitive weapon that can improve positional advantage, offer production flexibility, and meet the ever-increasing expectations of final consumers. Global supply chain outsourcing is crucial for pharmaceutical firms to enhance performance and profit margins by leveraging core skills and resources beyond other strategies which can lower risk, increase flexibility, improve returns on capital, and improve a company's ability to respond to the needs of its shareholders and consumers. Even while there is widespread recognition of the alluring advantages of global outsourcing, many of the related concerns are frequently disregarded. In addition to briefly discussing risk assessment methods, the goal of this work is to offer manufacturer-focused insight into supply chain-related outsourcing concerns within the pharmaceutical industry.

Keywords: Outsourcing, Pharmaceutical supply chain, Risk assessment, Risk management, Risk mitigation

1. INTRODUCTION

The pharmaceutical industry is a complex system that includes the processes, businesses, and groups engaged in developing, discovering, and manufacturing medications and related goods [1]. However, the pharmaceutical sector is unique in that it differs from other industries because of having unique requirements for distributing and storing various kinds of products, as well as distinct laws and regulations regarding quality standards, safety and security, and perishability of products [2]. And once more, it varies from other industries and associated supply chains of goods because of its urgency, critical state, and importance, the requirement for high levels of regulation in storage and transportation safety, general regulatory requirements that precisely define the limits and boundaries, etc. [3].

The supply chain is a network of participants, procedures, data, and assets that moves raw materials and components into completed goods and services before delivering them to consumers [3]. Customers, vendors, middlemen, and outside service providers are all included [4]. It also encompasses all production, logistics, marketing, sales, product

design, financing, and information technology-related tasks [5].To provide value for consumers and stakeholders, supply chain management, or SCM, is described as the integration of critical business operations throughout the supply chain [6]. Supply and demand are, in fact, integrated within and between businesses through supply chain management, creating an effective business model [7].

The supply chain units of a typical pharmaceutical firm can be classified as primary and/or secondary manufacturing, pharmaceutical warehouses/ distribution channels, and hospitals. Global regulations create a broad framework; however, each of the aforementioned main units also has strictly limited regulations that vary from a regulatory body perspective as well as from nation to nation locally in certain aspects [3].

Worldwide, one of the top priorities is the provision of pharmaceuticals, including drugs, treatments, and related commodities. As such, managing the pharmaceutical supply chain is becoming increasingly important compared to other supply chain networks.

To benefit all parties involved, an effective pharmaceutical supply chain (PSC) provides clients with medicines in the appropriate quantity, at the correct time, with acceptable quality, and at the best possible price [8].

The PSC should maintain regulatory compliance to ensure better and best product quality, as pharmaceutical goods, i.e., drugs, vitamins, vaccines, etc., can only have one quality, which is the best quality that can be produced at that time [9]. The PSC system has particular challenges regarding data complexity and supply chain sufficiency, which require special consideration. The PSC as a whole is made up of the need for human resources, the management of warehouses, the absence of demand information, and inventory management, all. Additionally, because their supply chains contain several goods, markets, procedures, and intermediaries, pharmaceutical companies must manage a complicated network of supply networks. Consequently, the pharma industry requires a sophisticated, high-quality approach, information exchange capabilities, and effective supply chain networks to meet customer demands [10].

Considering all challenges and needs, a primary factor contributing to PSC network insufficiency is a lack of coordination between stakeholders and supply chain participants, which can reduce overall efficiency. To simplify the global health crisis and save lives, necessary steps should be taken to improve the delivery of pharmaceutical-based products in places that require them [11].

Due to many internal and external influences, organizations must have a systematic and disciplined approach to risk management and mitigation. The risks that businesses confront are increased by the markets' ongoing growth and change, rivals, rising technology, increasingly dynamic client requirements, a reduction in response times, and the strategic use of global outsourcing [12, 13]. Pharmaceutical companies involved in outsourcing agreements should pay more attention to risk management even though it is a crucial issue in global outsourcing because of supply chain strategies' expectations. Risk management is essential for the pharmaceutical sector, given the hazards connected to supply chain logistics outsourcing [14]. "Increased shareholder value, cost reduction, business transformation, improved operations, overcoming a lack of internal capabilities, competitive advantage, improved capabilities, increased sales, improved service, decreased inventory, increased inventory velocity and turns, mitigated capital investment, improved cash flow, and other tangible and intangible benefits have made global supply chain outsourcing an increasingly attractive strategic option for businesses" [9].

The hazards attached to outsourcing in the pharmaceutical industry are expanding along with it. These risks include mistakes that could result in FDA rejection, lengthy lead times, following Sarbanes-Oxley Act requirements, complying with regulations, and protecting private information. Global outsourcing's associated risks are being managed more extensively as supply-chain tactics for the pharmaceutical business change [14,15]. Reputable multinational pharmaceutical companies

are forming risk-sharing outsourcing partnerships to share management and financial obligations to reduce operational risks associated with the costlier and risky process of discovering and developing new medications. Outsourcing can assist businesses in growing their R&D pipelines and increase the likelihood that a treatment will eventually reach the launch phase by managing risk appropriately [16].

The risk associated with the pharmaceutical supply chain endangers patients' lives in addition to wasting resources. It is imperative for health systems to evaluate and put into practice methods aimed at managing the many stakeholders in the pharmaceutical supply chain. Nonetheless, a few review studies on enterprise risk management, supply chain logistics, quality assurance, cyber security, and counterfeiting have been done in the pharmaceutical business. This work aims to provide insight into supply chain-related risks of the pharmaceutical industry from a manufacturer's perspective while briefly` mentioning the risk assessment techniques. Additionally, it focuses on the value of outsourcing for the pharmaceutical industry in terms of challenges and benefits as a risk management option.

2. OUTSOURCING IN THE PHARMACEUTICAL INDUSTRY

Nowadays, companies are concentrating more on their core competencies and outsourcing functions in which they lack the experience to survive and prosper in the competitive global economy of today, maintain efficient cost structures and increase their top and bottom lines. The decreased number of new blockbuster medications in the pipeline and competition from generic companies have caused especially original drug-producer pharmaceutical companies to examine their operations more closely to increase profit margins and shareholder value [17]. Pharmaceutical outsourcing, in particular, is now more than ever a feasible strategic business choice to obtain a competitive edge. The most prominent explanations for the rise in pharmaceutical outsourcing include lower labour and infrastructure costs, less pressure on the company to focus on drug research and development, the switch to a variable cost model, and the opportunity to obtain novel technology and expertise by working with specialized outside providers [1,12]. It is no longer necessary or crucial to have pharmaceutical R&D developers these days. The expenses of developing pharmaceuticals may severely strain a company's resources.

2.1. Outsourcing Strategies in the Pharmaceutical Industry

Despite years of growth and profitability records, the pharmaceutical industry faces difficult times as a declining rate of novel chemically based small molecules produced conventionally and decreasing R&D productivity [18,19]. Therefore, with the need to penetrate unexplored markets and improve R&D productivity and efficiency, multinational pharmaceutical companies are outsourcing more and more of their activities to contract research organizations (CROs) in Asia, especially to two forthcoming players, China and India. Pharmaceutical companies not only outsource their R&D activities but also information technology, logistics, and manufacturing services to enhance their product pipeline and obtain a tactical edge over competitors [20]. So, pharmaceutical global outsourcing has developed into a successful business strategy that allows companies to outsource non-core operations to contract manufacturing organizations and/or contract research organizations to reorganize their distribution networks, spread risks, and focus on matters that are critical to their survival, competitive advantage, and long-term growth [16,20,21].

Pharmaceutical global outsourcing is growing due to several factors, the most important of which are the competitive global marketplace, increased productivity flexibility, achieving global manufacturing presence, expanding capacity, improved asset utilization, reduced capital asset investments, and improved product quality attained through cost reduction and better focus on core competencies which triggers time to market, cost advantage, risk management, and strategic focus [22].

The pharmaceutical and biotech industries are becoming more globalized, which supports outsourcing to take advantage of the market to get

a competitive edge. Pharmaceutical companies also have realized it is impractical to have every skill set needed for a particular business. Focusing on core capabilities is an efficient and effective strategy for organizations to achieve maximum value, as flexibility has become increasingly important in the market. In fact, the number of services and operations that can be outsourced to contract manufacturing and R&D companies has increased significantly as a result of the recent growth in pharmaceutical contract services [12,14,15].

Pharmaceutical companies have been actively reviewing their financial status and, at worst, pursuing mergers and acquisitions in recent years due to growing cost pressures and a survival instinct. The pharmaceutical industry's pursuit of global outsourcing can be attributed to several key factors, including the need to streamline the drug development cycle, expand a company's capacity, consolidate the industry, gain access to specialized therapeutic expertise, globalize the market across the United States, Europe, and Asia, and capitalize on new personalized therapy options [11,12].

2.2. Supply Chain Risks in The Pharmaceutical Industry and Position of Outsourcing

Access to healthcare systems, medicines/drugs, and related goods is accepted as a basic human right and one of the major concerns of the public, healthcare systems, and governments. In connection with the pharmaceutical industry, the supply chain is a primary part of the healthcare systems in distributing drugs to patients. Risks that directly affect supply chain systems can cause waste of allocated resources and deteriorate PSC performance. Also, waste of resources has a direct effect on patients' lives. Therefore, proper identification and risk analysis are crucial, as they could affect life/death conditions, in designing strategies to minimize the risks in the PSC.

According to the European Medicines Agency (EMA), which sets standards for Quality risk management and defines the related frame, "Risk is defined as the combination of the probability of occurrence of harm and the severity of that harm. However, achieving a shared understanding of risk management among diverse stakeholders is difficult because each stakeholder might perceive different potential harms, place a different probability on each harm occurring, and attribute different severities to each harm. About pharmaceuticals, although there are a variety of stakeholders, including patients and medical practitioners as well as government and industry, protecting the patient by managing the risk to quality should be considered of prime importance." [23]

One way to characterize supply chain risk is "any risk to the information, material and product flow from original suppliers to the delivery of the final product" [24]. A worldwide supply network is susceptible to several hazards [9]. These risks include disruptions in supply, delays in supply, variations in demand, price changes, and volatility in currency rates. Three categories of business risks are identified as arising from labour concerns in suppliers: "cost risk, operational risk, and reputational risk." In this way, "the ability to react rapidly to assure continuity" is the aim of supply chain risk management, or SCRM which is also a significant and essential component of a working pharmaceutical supply chain [25, 26]. Supply chain risk can also be described as "the process of identifying and managing supply chain risk utilizing a coordinated strategy among supply chain participants."[27]. To lower and manage the likelihood and effects of unfavourable events, it is crucial to recognize, evaluate, and rank every risk [28]. Concerning the supply chain literature from the standpoint of pharmaceutical enterprises, Two primary categories of supply chain can be distinguished regarding uncertainty and risk in the pharmaceutical industry as internal and external risks (Figure 1) [16].

Internal uncertainty and risks can be briefly defined as "the risk related to operations uncertainty and risk, financial uncertainty and risk, and quality-related uncertainty and risk", whereas internal operations uncertainty and risk may refer to "unexpected events, outcomes, and/or accidents during the internal processes, they mainly occur within pharmaceutical firms, such as errors in decision-making, quality issues, machine failure, mistakes, unexpected costs, etc." [16]. Since pharmaceutical products are highly regulated goods that fall under the jurisdiction of public regulatory bodies, quality-related uncertainty,

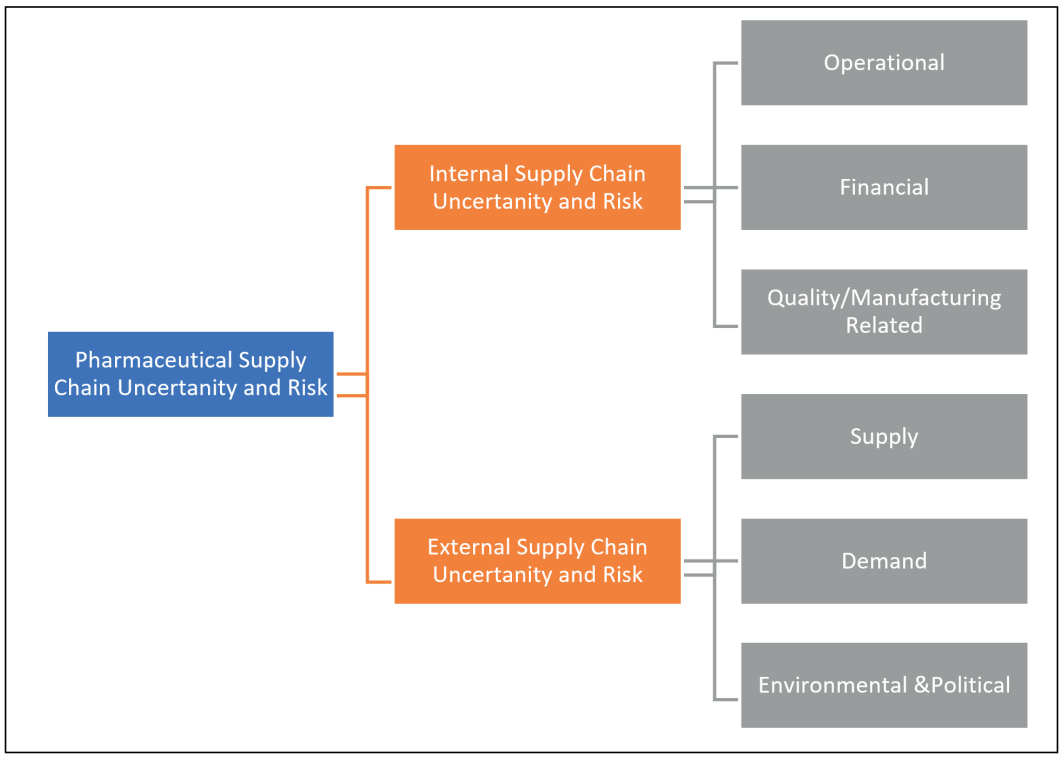

Figure 1. Types of supply chain uncertainty and risk from the pharmaceutical company perspective [16].

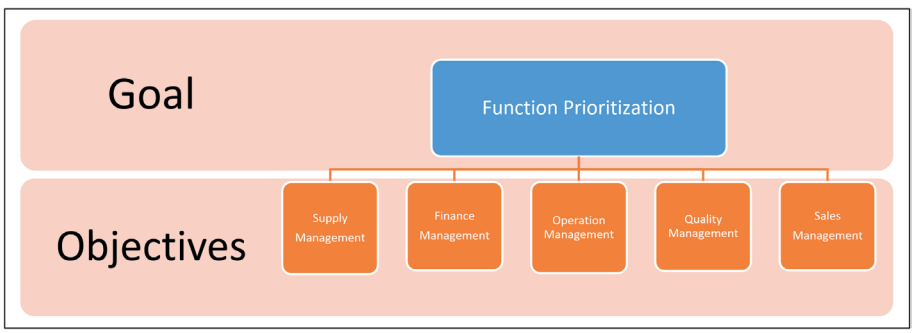

Figure 2. The hierarchy structure of PSC [31].

and risk have received increased attention due to the significance of the finished product and its direct impact on human life. Three primary components make up external uncertainty and risk as well: supply, demand, and environmental uncertainties and risks [29]. Furthermore, because poor nations often experience social, political, and economic unrest, the supply of medications is fraught with additional risks and vulnerabilities such as counterfeit drugs, unavailability of certain drugs, and so on. Over time, pharmaceutical companies may encounter a range of obstacles and hazards, including those related to operations, politics, economics, culture, environment, and ethnicity. [30]. The hazards associated with the pharmaceutical supply chain are examined by using the supply chain functions as a "hierarchy, which includes supply, finance, operation, quality, and sales management" (Figure 2).

Pharmaceutical companies are beginning to understand the possible financial benefits of contracting out non-core supply chain tasks to other parties. In addition to the benefits of outsourcing from a strategic, financial, organizational, and operational

standpoint, there are certain hazards associated with outsourcing that might have a negative impact on the partnerships [15]. The risks associated with outsourcing encompass a range of issues, such as possible inefficiencies in management, latent information asymmetry, loss of innovative capacity in logistics, hidden costs, reliance on third-party logistic providers, that can cause loss of control over them and also challenges assessing and tracking over their performance, and cultural alignment issues between participating firms [14]. Other issues and risks associated with pharmaceutical outsourcing can include but are limited to transaction costs, higher monitoring expenses, losing direct control over the launch of a product, losing internal competency and capacity, potentially losing crucial intellectual property, possible after-market competition, and higher costs associated with legal compliance and reputation [31].

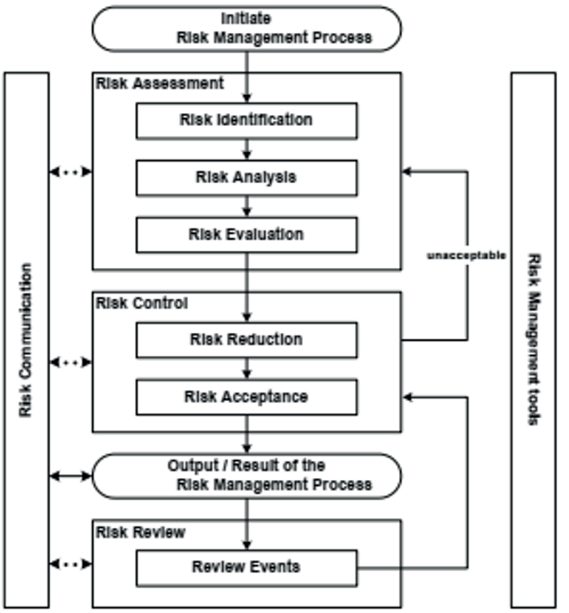

Figure 3. Overview of a typical risk management process of ICH Q9 [23].

2.3. Risk Management and Risk Mitigation Approaches in Pharmaceutical Supply Chain Outsourcing

The systematic process of assessing, controlling, communicating, and reviewing hazards is known as risk management. By using the right plans and tactics, risk management in supply chains can raise performance levels and lessen uncertainty and susceptibility [1, 32]. According to several suggestions, firms should manage risk according to a systematic framework that aids in supply chain risk identification, quantification, and mitigation. Despite the widespread recognition of supply chain risk management as a crucial topic within supply chain systems, the sector faces risks due to inadequate knowledge of supply chain risk management [11]. These risks are mostly caused by the gap between theory and practice. An outline for risk management is briefly schematized in Figure 3.

Risk management is initiated with a holistic approach to the system in which the below-mentioned steps can be followed;

i. Describe the issue and/or risk query, mentioning relevant presumptions that highlight the possibility of risk;

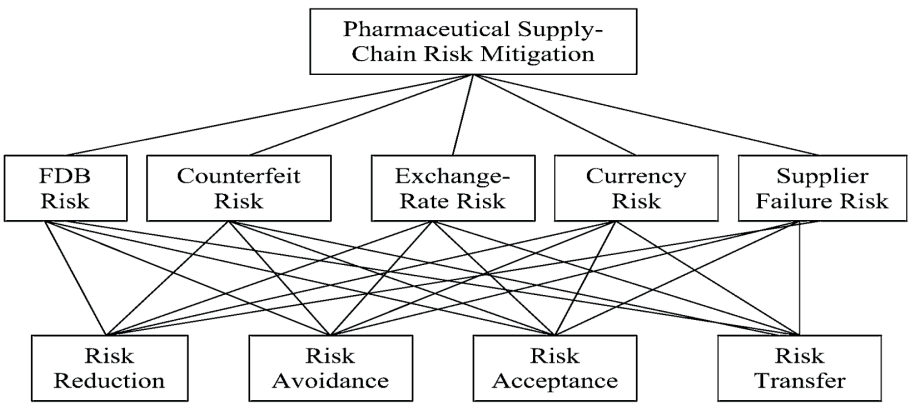

Figure 4. Decision hierarchy to mitigate PSC risk [47].

ii. Gather background data and/or information pertinent to the risk assessment regarding potential harm, hazard, or influence on human health; Identify a leader and necessary resources;
iii. Establish a schedule, deliverables, and suitable degree of decision-making for the risk management procedure.

This is followed by a stage called risk assessment, which entails identifying hazards, analysing and calculating the risks of being exposed to those dangers, and asking fundamental questions to determine the risk;

i. What might not work well (or wrong)?
ii. How likely is it that something will not work well (or wrong)?
iii. What are the repercussions (level of severity)?

The whole process is carried out step by step, which is schematized in Figure 3.

Supply-chain risk management is becoming a must for many forward-thinking pharmaceutical firms due to the surge in supply-chain risks in the industry and the pressure from regulatory bodies, evolving laws, customers, and fierce competition [1, 33].

Table 1. Recent publications related to risk management tools

Method	Contributions	Reference
AHP	Outlined a methodology for evaluating outsourcing risks in global PSCs.	[12]
AHP	Created a PSC risk assessment methodology focused on Iran.	[11]
A probability impact matrix based methodology	Evaluated the supply chain risks in the pharmaceutical industry's logistics.	[39]
Conceptual model	Created a framework to improve supply chain robustness within the Malaysian pharmaceutical sector.	[40]
Combined descriptive and application-based approach	Presented a risk-management strategy for PSCs that combines an application- and descriptive-based approach.	[41]
ELECTRE TRI	Suggested a method for assessing risks related to outsourcing PSC logistics.	[1]
Fuzzy AHP PROMETHEE	Assessed the PSCs' outsourcing risks.	[42]
A mathematical model	Emphasized the development of PSC resilience by taking capacity, inventory, and dual sourcing into account.	[43]
A two-stage stochastic programming model	Investigated supply chain design concerns to balance and reduce risks in PSCs.	[44]
Delphi model and AHP	Evaluated the relevant risks regarding PSC in the context of Bangladesh.	[45]
Data envelopment analysis (DEA) and fuzzy data envelopment analysis (FDEA) approaches	Assessed the impact of resilience indicators on PSC through DEA/FDEA and statistical methods in the veterinary organization.	[46]
Mixed-Integer Non-Linear Program Model Fuzzy	Evaluated risks and the uncertainty level of PSC process tools.	[47]
Fuzzy Cognitive Map	Evaluated risks related to PSC and distinguish the importance levels	[48]
Decision Making Evaluation and Laboratory-based Analytical Network Process	Risks involved in the generic medicine supply chain in Indonesia is assessed.	[33]
Ontologies and Fuzzy Quality Function Deployment	PSC risks associated with the transport and storage of finished products for export in Columbia were evaluated	[49]
2-Tuple ARAS-BWM approach	PSC risks faced during the COVID-19 period in Tunisia were evaluated and mitigation strategies discussed	[50]
Taguchi Orthogonal methodology	Uncertain variables associated with PSC risks were investigated and the effect of logistics evaluated	[32]
Pythagorean fuzzy AHP and Pythagorean fuzzy WASPAS method	Procurement risks and PSC risks are investigated	[51]

The latest risk management methodology, which is focused on the pharmaceutical supply chain system, is summarized in Table 1.

Depending on their vulnerabilities and circumstances, pharmaceutical companies in the public and private sectors confront varying supply chain risks and uncertainties, and it is challenging to offer a consistent strategy to reduce them. It is feasible, therefore, to put businesses' skills and assets to use in managing specific supply chain risk and uncertainty. A decision hierarchy to mitigate PSC risk is defined in Figure 4. In the frame of the decision hierarchy, regulatory-related issues, business-related issues, technical-related issues, and intellectual property are accepted as primary risk factors, therefore, reduction of risk avoidance from risks, acceptance of risks and transfer of risk proposed as direct risk mitigation approaches [34].

Global outsourcing's associated risks are being managed more extensively as supply-chain tactics for the pharmaceutical business change. "Developing new drugs carries more risks and is costlier than ever. Many leading pharmaceutical companies are entering into risk-sharing outsourcing partnerships to lower their operation risks by sharing management and financial responsibilities. Through proper risk management, outsourcing enables companies to expand their R&D pipelines and provide a greater chance for a product to launch, ultimately lowering overall business risks"[12]. The risks associated with global outsourcing include those related to intellectual property and proprietary confidentiality, technical expertise, capacity, resource availability, production risks, firm volatility, and management difficulties. Pharmaceutical companies must implement risk-reduction strategies to have successful outsourcing contracts.

Pharmaceutical companies benefit from outsourcing pharmaceutical products, but it's important to remember that hazards are involved, such as FDA rejection, longer lead times, noncompliance with regulations, and more. To prevent unanticipated resource waste that could significantly negatively influence patient health, a proper risk assessment and management strategy should be developed in compliance with regulatory guidelines when a decision to outsource is needed in the pharmaceutical supply chain [35].

3. CONCLUSION & FUTURE DIRECTIONS

Global pharmaceutical companies are facing more and more pressure to boost profit margins in an environment of diminishing R&D productivity, rising pricing pressure, and shifting regulatory constraints. Pharmaceutical companies are exploring consolidations through mergers and acquisitions and international outsourcing to address these issues. Pharmaceutical companies may afford to concentrate on their core skills, gain access to specialist knowledge, and increase cost-saving advantages that can increase shareholder value by forming strategic outsourcing partnerships. Global outsourcing of production and research and development activities has grown in importance in the pharmaceutical business in recent years due to the competitive nature of the modern market. Pharmaceutical companies have advanced up the outsourcing value chain from non-core to secondary core services [36].

Increased clinical trial complexity, regulatory requirements, data requirements, cost savings, market time, and therapeutic expertise access drive pharmaceutical outsourcing R&D. Global supply chain outsourcing efficiently utilizes labour, capital, technology, and resources [37]. Pharmaceutical companies see outsourcing their worldwide supply chain operations as a smart move that may provide flexibility, satisfy their expanding customer base, cut expenses, boost productivity, and increase profit margins. Supply chain operations can be globally outsourced, which enables businesses to utilize their core competencies and assets to far greater extents than possible with alternative approaches [38]. Companies can increase returns on capital, lower risk, increase flexibility, and become more responsive to the value requirements of shareholders and customers by implementing outsourcing strategies that are well-executed and based on core strengths.

Ethical approval

Not applicable because this article does not contain any studies with human or animal subjects.

Author contribution

Concept: ÖAD; Design: ÖAD; Supervision: ÖAD; Materials: ÖAD; Data Collection and/or Processing: ÖAD; Analysis and/or Interpretation: ÖAD; Literature Search: ÖAD; Writing: ÖAD; Critical Reviews: ÖAD.

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Conflict of interest

The authors declared that there is no conflict of interest.

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