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Chemical composition of essential oil / volatiles and fatty acids, and antimicrobial evaluation of two endemic Achillea (Asteraceae) species: *Achillea schischkinii* Sosn. and *Achillea teretifolia* Willd.

Gamze GÖGER [1*], Melisa Ergül GÖREL[2], Yasin Burak BİRTEK[2], Mehmet TEKİN[3], Gülmira ÖZEK[4]

1 Department of Pharmacognosy, Faculty of Pharmacy, Afyonkarahisar Health Sciences University, Afyonkarahisar, Türkiye
2 Faculty of Pharmacy, Trakya University, Edirne 22030, Türkiye
3 Department of Pharmaceutical Botany, Faculty of Pharmacy, Trakya University, Edirne 22030, Türkiye
4 Department of Pharmacognosy, Faculty of Pharmacy, Anadolu University, Eskişehir 26470, Türkiye

* Corresponding Author. E-mail: gamze.goger@afsu.edu.tr (G.G)

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ABSTRACT:

In the present study, phytochemical analyzes and investigations for *in vitro* antimicrobial activity of two endemic species *Achillea schischkinii* Sosn. and *A. teretifolia* Willd. were carried out. The essential oils and fatty acids as well as the different solvents extracts were obtained from aerial parts of the plants. Gas chromatography (GC) and Gas Chromatography/Mass Spectrometry (GC / MS) techniques were used to analyze the phytochemical composition of the volatiles and fatty acid methyl esters. *In vitro* antimicrobial evaluations of the plant extracts were determined against *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, *Candida albicans* ATCC 10231, *C. parapsilosis* ATCC 22019, *C. tropicalis* ATCC 750, and *C. glabrata* ATCC 90030 by microdilution method. The antifungal combination of infusion of *A. schischkinii* with fluconazole was determined by the checkerboard method againts *Candida* species.
Chemical compositions of *A. schischkinii* and *A. teretifolia* were determined as 1,8-cineol (25.2-30.4 %), and camphor (7.0-5.5 %). Antimicrobial evaluation of different extracts of *A. teretifolia*, were performed for the first time in this study. The MIC values were observed between at 156.25-5000 µg / mL for both of the plant extracts. The combination showed a synergistic effect (FIC ≤0.5) against *C. albicans* and *C. parapsilosis*.
Keywords: *Achillea teretifolia; Achillea schischkinii* essential oil; Fatty acids; Antimicrobial; Synergistic

1. INTRODUCTION

The epidemiological data indicate that major mycoses' incidence and prevalence are still a public health issue. Resistance to antifungal drugs has grown as a result of their expanded use. Finding new classes of antifungal from natural source, use of plant extracts or natural compounds with known antifungals might be great significance in yeast infection. Plants produce chemicals with a wide range of structures and chemical compositions, many of which have antimicrobial properties. When used alone, in combination, or in combination with known antimicrobial compounds to treat microbial infection, commonly used medicinal plants could be a valuable source of treatment [1]. For this purpose, a few examples of plant extracts combined with well-known antifungal drugs represented from the literature. The leaves of ethanol extract from *Ocimum basilicum* was combined with Amphotericin B against *Cryptococcus* species resulted with synergsim. And *Allium sativum* ethyl acetate extract was also combined with Amphotericin B and showed synergistic effect [2].

The largest family of vascular plants in the world, the Asteraceae, includes the genus *Achillea* L. There are roughly 115 taxa in this genus, which belongs to the Anthemideae Cass. tribe of the Asteraceae family. The medicinal properties of *Achillea* plants are recognized worldwide and used for their analgesic effect (against stomachache, abdominal pain, menstrual pain, birth pain), to eliminate flatulence of infants, for emmenagogic and wound healing properties and against diarrhea). Numerous *Achillea* species had anti-inflammatory and antimicrobial properties, according to the findings of the literature review [3, 4]. In

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addition, some *Achillea* species are used as perfume, pharmaceuticals, cosmetics, and food. Many *Achillea* extracts have properties that meet the requirements of the cosmetic industry. The most researched member of the genus is *Achillea millefolium* L., and in recent years there have been more studies on the dermatological advantages offered by *Achillea* species. The numerous secondary metabolites found in *Achillea* plants, such as flavonoids, phenolic acids, terpenes, guaianolides, phytosterols, fatty acids, and organic acids, are responsible for the health advantages of the extracts [5].

Achillea species are significant indigenous economic plants in Türkiye. According to recent studies, this genus is also widespread in Anatolia with 60 taxa, 31 of which are endemic for Anatolia [6]. *Achillea schischkinii* Sosn. and *A. teretifolia* Willd. species are endemic to Türkiye and according to IUCN criterions, classified as LR Ic (least concern) category by Ekim et al. [7].

Due to the presence of highly bioactive compounds, these species have medicinal uses [8]. The aerial parts of *A. teretifolia* are mostly used to treat skin and acne conditions as well as diseases of the digestive system [9]. Bali et al. reported that *A. teretifolia* extracts demonstrated anti-oxidant, cytotoxic and pro-apoptotic effects on the cancer cells [10].

The ethnopharmacological usage of infusion from *A. schischkinii* was reported againts hemorrhoid pains [11], The decoction was also used in Malatya but no knowledge was obtained for medical purposes [12]. Previous laboratory study was reported *A. schischkinii* and *A. teretifolia* have potential antioxidant and antimicrobial properties against to bacteria and yeast [13]. Since the number of antimicrobial activities on these species is low, it was aimed to study the antimicrobial activity of different extracts of *A. schischkinii* and *A. teretifolia*

The main point of the present work to determine chemical composition of essential oil, volatiles and fatty acids of *A. schischkinii* and *A. teretifolia*, respectively, to determine antimicrobial effects of the n-hexane, ethyl acetate and aqueous extracts, and a possible effect between infusion of *A. schischkinii* with fluconazole against *Candida* species.

2. RESULTS & DISCUSSION

2.1. Chemical composition of essential oil /volatiles oils

The compositions of the essential oil and volatiles of two endemic *Achillea* species were analyzed by GC-FID and GC/MS techniques (Table 1). The main compounds of *A. schischkinii* essential oil were found to be 1,8-cineol (25.2%) and *a*-terpineol (12.0%). The major volatile compounds of *A. teretifolia* were found to be borneol (38.9%), 1,8-cineol (30.4%), terpinen-4-ol (7.6%), and camphor (5.5%). Forty seven and eleven compounds were identified comprising 90.2% and 95.1% of *A. schischkinii* and *A. teretifolia* oils, respectively.

Ünlü *et al.* reported about the essential oil from *A. teretifolia* obtained by using steam distillation. The main components of the essential oil, 87.1% of whose composition was clarified, were found to be 1,8-cineol (19.9%), borneol (11.9%) and camphor (11.1%) [14].

Aslan *et al.* reported about the thirty-seven compounds identified in *A. teretifolia* oil and comprising of 83.53% of the total oil. The main compounds were found to be piperitone (21.4%), linalool (19.0 %), 1,8-cineol (6.8%), *a*-terpineol (5.9%), and borneol (4.3%) [15]. The essential oil obtained from the aerial parts of *A. teretifolia* was analyzed and the main components were determined as 1,8-cineole (34%), camphor (11%), terpinen-4-ol (8%), and *a*-thujone (5%) [16].

The essential oil obtained from the aerial part of *A. teretifolia* by water distillation was reported. Fourty-eight compounds were determined by defining 71.5% of the essential oil with GC-MS. The main compounds were found to be 1,8-cineol (16.1%), camphor (12.7%), *p*-cymene (10.6%), and terpinene-4-ol (6.1%) [17].

Table 1. Chemical composition of *Achillea schischkinii* essential oil and *Achillea teretifolia* volatiles

No	RRI	Compound	A	B
			%	%
1	1032	α-Pinene	0.5	-
2	1035	α-Thujene	tr	-
3	1076	Camphene	0.1	-
4	1093	Hexanal	0.3	-
5	1118	β-Pinene	0.7	-
6	1132	Sabinene	0.6	-
7	1176	α-Phellandrene	0.1	-
8	1188	α-Terpinene	0.1	-
9	1213	1,8-Cineole	25.2	30.4
10	1255	γ-Terpinene	0.2	-
11	1280	*p*-Cymene	4.4	-
12	1437	α-Thujone	-	2.2
13	1445	Filifolone	-	0.7
14	1451	β-Thujone	-	0.3
15	1499	α-Campholene aldehyde	0.2	-
16	1522	Chrysanthenone	-	2.9
17	1532	Camphor	7.0	5.5
18	1541	Benzaldehyde	0.2	-
19	1553	Linalool	0.5	-
20	1565	Linalyl acetate	0.3	-
21	1582	*cis*-Chrysanthenyl acetate	0.4	-
22	1586	Pinocarvone	0.5	-
23	1611	Terpinen-4-ol	5.7	7.6
24	1648	Myrtenal	0.2	-
25	1682	δ-Terpineol	1.3	-
26	1685	Isovaleric acid	0.4	-
27	1706	α-Terpineol	12.0	-
28	1709	α-Terpinyl acetate	-	3.1
29	1719	Borneol	-	38.9
30	1712	*cis*-Pinocarveol	0.6	-
31	1738	*p*-Mentha-1,5-dien-8-ol	0.2	-
32	1802	Cumin aldehyde	0.3	-
33	1804	Myrtenol	0.9	-
34	1805	α-Campholene alcohol	1.4	-
35	1823	*p*-Mentha-1(7),5-dien-2-ol	1.1	-
36	1845	*trans*-Carveol	1.7	-
37	1864	*p*-Cymen-8-ol	0.2	-
38	1921	α-Phellandrene epoxide	2.1	-
39	1948	*trans*-Jasmone	-	1.0
40	2008	Caryophyllene oxide	1.7	-
41	2050	(*E*)-Nerolidol	1.2	-
42	2074	Caryophylla-2(12),6(13)-dien-5-one	0.3	-

No	RRI	Compound	A	B
			%	%
43	2120	Zingiberenol (=*1,10-Bisaboladien-3-ol*)	1.2	-
44	2144	Spathulenol	2.2	-
45	2186	Eugenol	-	2.5
46	2187	*T*-Cadinol	1.8	-
47	2198	Thymol	1.2	-
48	2214	*ar*-Turmerol	0.3	-
49	2239	Carvacrol	1.4	-
50	2255	α-Cadinol	2.0	
51	2257	β-Eudesmol	1.3	
52	2308	(6*R*,7*R*)-Bisabolone	1.6	
53	2316	Caryophylla-2(12),6(13)-dien-5b-ol (=*Caryophylladienol I*)	1.3	
54	2365	(*Z*)-Methyl jasmonate	0.9	
55	2622	Phytol	2.4	
		Total	**90.2**	**95.1**
		Monoterpene hydrocarbons	6.7	0
		Oxygenated monoterpenes	64.6	92.6
		Sesquiterpene hydrocarbons	0	0
		Oxygenated sesquiterpenes	14.9	0
		Others	4.0	

RRI: Relative retention index experimentally calculated based on retention of *n*-alkanes; %, calculated from flame ionization detector data; A: *Achillea schischkinii*; B: *Achillea teretifolia*.
tr: trace

In our study, a high amount (38.9%) of borneol, 1,8-cineol (30.4%), terpinen-4-ol (7.6%), and camphor (5.5%) were found in the volatile oil of *A. teretifolia*.

The caryophyllene oxide (17.5%), spathulenol (9.1%), *p*-cymene (8.5%), and (*E*)-nerolidol (6.2%) were found to be the main components of *A. schischkinii* essential oil [18].

In our study, the caryophyllene oxide (1.7 %), (*E*)-nerolidol (1. 2%), spathulenol (% 2.2) and *p*-cymene (% 4.4) were reported in small quantities, while 1,8-cineol (25.2%) and *a*-terpineol (12.0%) were found to be main compounds of *A. schischkinii* essential oil.

The essential of *A. schischkinii* previously reported with 31 components. The two main compounds were 1,8-cineole (31.0%) and camphor (20.0%), along with trace amounts of caryophyllene oxide, spathulenol, and nerolidol detected (0.2%–1.1%) [19].

2.2. Fatty acids compositions

Fifteen compounds comprised of 97.5% of the oil of *A. teretifolia* (Table 2). The fatty acid profile of *A. teretifolia* was characterized by predomination of unsaturated fatty acids, namely linoleic (29.5%), oleic (20.9%), and linolenic (13.2%) acids. Saturated fatty acids were presented with palmitic (22.2%) and stearic (6.3%) acids. To our knowledge, the fatty acid composition of *A. teretifolia* was determined for the first time in this study.

Table 2. Chemical composition of *Achillea teretifolia* fatty acids

No	RRI	Compound	%
1	1390	Methyl octanoate	0.1
2	1194	Methyl decanoate (=*Methyl caproate*)	1.5
3	1810	Methyl dodecanoate (=*Methy laurate*)	0.2
4	2018	Methyl tetradecanoate (=*Methyl myristate*)	0.9
5	2095	Methyl pentadecanoate	0.2
6	2223	Methyl hexadecanoate (=*Methyl palmitate*)	22.2
7	2251	(*Z*)-9-Methylhexadecenoate (= *Methyl palmitoleate*)	0.2
8	2323	Methyl heptadecanoate	tr
9	2436	Methyl octadecanoate (=*Methyl stearate*)	6.3
10	2468	(*Z*)-9-Methyl octadecenoate (=*Methyl oleate*)	20.9
11	2468	(*Z,Z*)-9,12-Methyl octadecadienoate (=*Methyl linoleate*)	26.9
12	2509	Ethyl linoleate	2.6
13	2572	Methyl linolenate	13.2
14	2634	Methyl eicosanoate (=*Methyl arachidate*)	1.2
15	2842	Methyl docosanoate (=*Methyl behenate*)	1.1
		Total	**97.5**

The lipids of *A. schischkinii* were distinguished with a high abundance of the saturated fatty acids (>77.0%) (Table 3). About half of the lipids of *A. schischkinii* was comprised of palmitic (43.8%) acid. Nonadecanoic (18.3%), arachidic (7.8%), and stearic (7.1%) acids were other saturated fatty acids.

Table 3. Chemical composition of *Achillea schischinii* fatty acids

No	RRI	Compounds	%
1	2018	Methyl tetradecanoate (=Methyl myristate)	2.5
2	2223	Methyl hexadecanoate (= Methyl palmitate)	43.8
3	2255	Tricosane	4.6
4	2436	Methyl octadecenoate (=Methyl stearate)	7.1
5	2468	(*Z,Z*)-9,12-Methyl octadecadienoate (= Methyl linoleate)	10.9
6	2497	Methyl nonadecanoate	18.3
7	2572	Methyl (*Z,Z,Z*)-9,12,15-Octadecatrienoate (=Methyl linolenate)	4.9
8	2634	Methyl eicosanoate (= Methyl arachidate)	7.8
		Total	**99.9**

It was found that the fatty acids from the seeds of *A. schischkinii, A. lycaonica*, and *A. magnifica* contained palmitic acid. Stearic acid, myristic acid, and arachidic acid were the other main fatty acids in the saturated fatty acid fraction, and these acids were more abundant in *A. magnifica* [20].

The main fatty acids in the flower and leaves of *A. sivasica* were reported to be palmitic acid (22.2% and 17.3), and steraic acid (60.3% and 0.7%), respectively [21]. In our study, *A. schischkinii* contain methyl palmitate (43.8%) and methyl stearate (7.1%).

2.3. Antimicrobial activity (MIC, µg/mL)

Antimicrobial activities of the plant extracts obtained from *A. teretifolia, A. schischkinii,* and the essential oil of *A. schischkinii* were investigated. The antimicrobial activities of the extracts were given in Table 4. The *n*-hexane extract of *A. teretifolia* was found to have the same MIC value at 2500 µg/mL against *E. coli* and *S. aureus*. The MIC value was found at a lower concentration with 625 µg/mL for *C. albicans.* Ethyl acetate extract was found to be more effective than *n*-hexane extract. The MIC value for *E. coli* was found to be 312.5 µg /mL, however it was at 625 µg /mL for *S. aureus* and *C. albicans.*

Table 4: Minimum Inhibitory of Concentrations of *Achillea teretifolia* and *Achillea schischkinii* (MIC, µg / mL)

Extracts and standards	*Achillea teretifolia*			*Achillea schischkinii*		
	E. coli	*S. aureus*	*C. albicans*	*E. coli*	*S. aureus*	*C. albicans*
n-hexane	2500	2500	625	1250	2500	156.25
Ethyl acetate	312.5	625	625	1250	1250	312.5
Infusion	>2500	>2500	>2500	>2500	>2500	2500
Essential oil	-	-	-	625	312.5	5000
Ampicillin	>0.125	>0.125	_	>0.25	-	-
Moxifloxacin	0.125	>0.125	_	-	> 0.25	-
Clarithromycin	1	1	_	-	-	-
Fluconazole	_	_	0.25>			0.5

(-): not tested

The antimicrobial activity results of the extracts from *A. schischkinii* were found to be more effective against *C. albicans* than bacterial strains (Table 4). Hence, the extracts of *A. schischkinii* were evaluated with regard to the anticandidal activity against different *Candida* species, including *C. parapsilosis, C. tropicalis*, and *C. glabrata*. MIC value of *A. schischkinii* against the other *Candida* species were given in Table 5. The MIC values were found between 156.25- 312.5 µg / mL for *n*-hexane, and ethyl acetate extracts against all *Candida* species. The MIC value of infusion was found to be 1250 µg / mL for *C. glabrata*. The MIC values were observed between 2500-5000 µg / mL for other *Candida* species.

Table 5. Minimum Inhibitory of Concentrations of *Achillea schischkinii* against *Candida* species (MIC, µg / mL)

A. schischkinii extracts	*Candida albicans* **ATCC 10231**	*Candida parapsilosis* **ATCC 22019**	*Candida tropicalis* **ATCC 750**	*Candida glabrata* **ATCC 90030**
***n*-Hexane**	156.25	156.25	156.25	156.25
Ethyl acetate	312.5	312.5	312.5	312.5
Infusion	2500	5000	5000	1250
Essential oil	5000	2500	>5000	>5000
Fluconazole	8.0	2.0	2.0	32
Terbinafine	64	1.0	128	128

In literature, generally, the essential oil of *A. teretifolia* was investigated with regard to its antimicrobial activity [14, 16, 22]. Antimicrobial activity of different extracts obtained from the aerial parts of *A. teretifolia*, were evaluated for the first time in this study.

Antimicrobial activity of aerial parts of *A. schischkinii* and *A. teretifolia* were prepared water, methanol and chloroform extracts, and evaluated against a group of bacteria and yeasts, including *Bacillus, Staphylococcus Pseudomonas*, *Escherichia, Klebsiella, Enterobacter, Candida* and *Saccharomyces* by agar disc diffusion method. The extracts of *A. teretifolia* showed highest inhibitory effect against *P. aeruginosa* with the inhibition zone of 16 mm. The extracts of *A. schischkinii* had no anti-yeast activity against *S. cerevisiae* and *Candida* sp. and antibacterial activity against Gram positive or Gram negative bacteria [13]. In our study, the ethyl acetate extract of *A. teretifolia* was found more effective against *E. coli*. *A. teretifolia* was showed the same effect all tested strain with MIC=>2500 µg / mL (Table 4). *n*-Hexane extract of *A. schischkinii* was found effective against *Candida* species with MIC value of 156.25 µg / mL, whereas that of the ethyl acetate was 312.5 µg / mL. The infusion was only more effective against *C. glabrata* with MIC= 1250 µg / mL.

In another study, the chloroform extract of flowers of *A. teretifolia* were found active against *S. aureus* ATCC 43300, *S. aureus* ATCC 6538P, *Streptococcus epidermidis* ATCC 12228, *Salmonella typhimurium* CCM 5445 at MIC=50 µg/mL while, methanol extract of *A. schischkinii* showed inhibitory effect against *S. aureus* ATCC 43300 with MIC=162.5 µg/mL [23].

2.4. The combination of infusion of *A. schischkinii* with fluconazole

In this study, based on its ethnobotanical use, a combination study of the infusion was conducted. Hence, the antifungal combination evaluated for the first time for infusion of *A. schischkinii* with fluconazole. The results of antifungal combination interactions for infusion of *A. schischkinii* with fluconazole are summarized in Table 6. The checkerboard method was used to assess the antifungal effect of the combination against *C. albicans C. parapsilosis, C. tropicalis*, and *C. glabrata.* The FICI values were used to evaluate the synergistic and additive activity.

Table 6. Combination of *Achillea schischkinii* infusion extract with fluconazole against *Candida* species

Combination	Extract			Fluconazole			FICI	Result
	Alone	Combination	FIC	Alone	Combination	FIC		
Candida albicans	10000	2500	0.25	8	0.5	0.00625	0.31	**Synergistic**
Candida parapsilosis	20000	5000	0.25	2	0.25	0.125	0.37	**Synergistic**
Candida tropicalis	40000	5000	0.125	1	2	2	2.12	**Indifferent**
Candida glabrata	10000	2500	0.25	16	16	1.0	1.25	**Indifferent**

In our study, *A. schischkinii* combination of fluconazole showed synergic inhibitory effects against *C. albicans* and *C. parapsilosis* with FICI values calculated as 0.31 and 0.37, respectively. MIC of fluconazole decreased from 8 µg/mL alone to 0.5 µg/mL in the presence of extract of *A. schischkinii* against *C. albicans,* and decreased from 2 µg/mL alone to 0.25 µg/mL against *C. parapsilosis*. When the extract was combined with fluconazole, an independent effect (FIC >1-4) was observed against *C. tropicalis* and *C. glabrata.*

Azole antifungals inhibit ergosterol biosynthesis from cell membrane, while polyene antifungals have direct interactions with fungal cell membranes [24]. The antifungal drug fluconazole has restricted use because of its side effects and toxicity characteristic. However, its use in combination with plant extracts, which result in lower dosages, less toxicity, and fewer negative side effects, may be an alternative to restore its use [25].

Numerous studies in the literature have described the use of plant extracts in combination with antifungal drugs to combat various yeast and fungi. [2]. But we did not observed any antimicrobial evaluation on *A. schischkinii* extract with conventional antifungals. The results of a previous study showed that *A. millefolium*

was combined with other plant extracts against *S. aureus* ATCC 25923, *B. cereus* ATCC 7064, *L. monocytogenes* ATCC 7644, *E. coli* W3110, *A. baumannii* ATCC 19606, and *S. typhimurium* ATCC 14028 for both synergistic and antagonistic effects. [26]. This study examined possible antifungal drug, fluconazole and *A. schischkinii* interaction with synergistic or inhibitory activity against *Candida* species.

4. CONCLUSION

In the present study, the fatty acid composition of *A. teretifolia*, antimicrobial activity of aerial parts of different extracts from *A. teretifolia*, and *A. schischkinii*, and the antifungal combination of the infusion of *A. schischkinii* with fluconazole were evaluated for the first time. Discovering new classes of antifungals from natural materials, such as medicinal plants, is required due to the spread of multidrug-resistant fungi. Therefore, the absence of antagonistic effects in combination studies suggests that *A. schischkinii* and fluconazole may be effective against *Candida* or different species of dermatophytes in possible dermatological skin infections. However, the antimicrobial activity of the combinations should be investigated by working on different kind of bacteria and yeasts.

5. MATERIALS AND METHODS

5.1. Plant material

The specimens of *A. schischkinii* and *A. teretifolia* were collected during the flowering period from their natural populations in Sivas province of Türkiye. Localities of collected specimens: *A. schischkinii* were collected between Şerefiye to Suşehri, M. Tekin 1851, 19.06.2021 and *A. teretifolia* were collected between Zara and Şerefiye, M. Tekin 1849, 19.06.2021 (Fig. 1). Fresh samples and herbarium materials were used in each case for experimental analysis and morphological description which were identified by using the Flora of Turkey (DAVIS, 1975). The plant specimens were deposited in the Trakya University, Faculty of Pharmacy Herbarium.

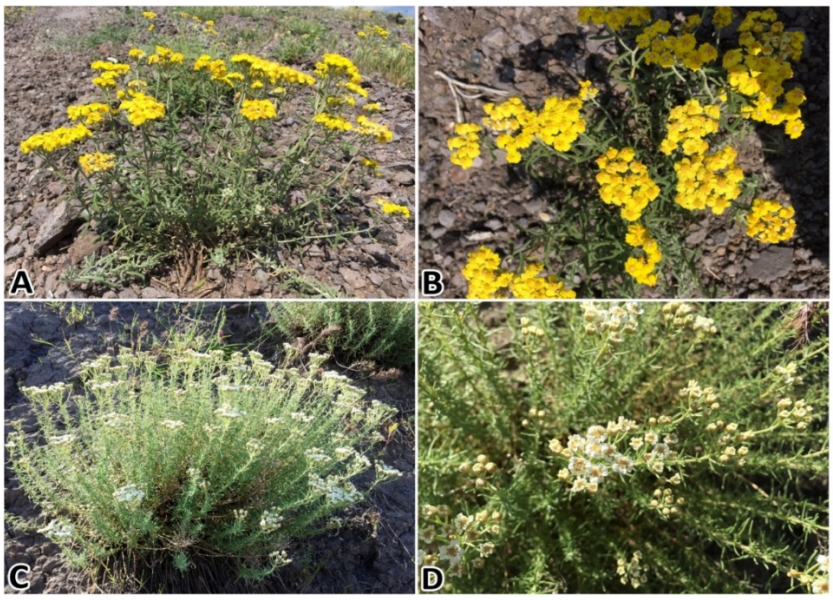

Fig. 1. General views in habitat of *A. schischkinii* (A-B) and *A. teretifolia* (C-D)

5.2. Preparation of extracts

The plant materials (40 g) were macerated with n-hexane and ethyl acetate (200 mL x 3), respectively, for 24 hours. The extracts were collected, filtered, and concentrated in a rotary evaporator. The dried extracts were maintained at 4 °C. Additionally, 20 g of the plant material was infused in 200 mL distilled water. The prepared infusion was lyophilized and stored under 4 °C.

5.3. Essential oil hydrodistillation

In order to obtain essential oil, the aerial parts of *A. schischkinii* (60.0 g) were hydrodistilled for three hours in the Clevenger apparatus [27]. The essential oil was stored at 4 °C.

5.4. Extraction of volatiles with microsteam distillation-solid phase microextraction (MSD-SPME)

The MSD-SMPE approach was used to extract the volatiles from aerial parts of *A. teretifolia* [21]. The ground aerial parts of the plant material (0.5 g) and 3 mL of water were added to a 25 mL round bottom flask. The flask was equipped with a capped Claisen distillation head, and a condenser set up for refluxing as opposed to distillation.

The SPME fiber assembly utilized the threaded plug. The volatiles were extracted using a manual SPME holder and "blue type" (65 µm) polydimethylsiloxane/divinylbenzene fiber. Prior to the experiment, the fiber was preconditioned at 250 °C for 10 min. 3 minutes were used for extracting the volatiles. Following trapping, the volatiles were analyzed using GC-MS/FID. The fiber was injected into the injection port (at 250°C) for 5 minutes, thermally desorbing the analytes from the fiber coating.

5.5. Gas chromatographic analysis

The Agilent technologies, 6890N GC, and 5975 GC/MSD system (USA) were used for the Gas Chromatographic studies. As previously reported, a helium carrier gas was employed with an HP-Innowax FSC column (0.25 µm film thickness, 0.25 mm, and 60 m) at a flow rate of 0.8 mL/min. [21]. In the current work, we used the MSD-SPME methodology in tandem to analyze the composition of volatiles in aerial parts of *A. teretifolia*, and the GC-MS/FID method to analyze the chemical composition of *A. schischkinii* essential oil gathered through hydrodistillation.

5.6. Fatty acids extraction

Total lipids from *A. schischkinii* and *A. teretifolia* were extracted using the lipid extraction kit. The procedure called for treating plant material (0.15 g) with a 3 mL solvent comprising of chloroform:methanol (2:1). After vortexing the mixture, 0.5 mL of the kit's aqueous buffer was added, and the sample was once more blended by vortexing. Following that, the extraction solution was put to a syringe system with a filter. The chloroform phase with total lipids was present in the eluted solvent. The total lipids were then separated into 200 mL portions and dried using a nitrogen stream in preparation for transesterification. After drying, 0.3 mL of n-hexane and 1 mL of BF3-MeOH solution were added. The mixture was reflux-heated for one hour at 95°C. Then, 1 mL of distilled water and 1 mL of n-hexane were added. The mixture was centrifuged at 500 x g for 5 min. The upper hexane layer was placed into a vial and then injected into the GC-MS and GC-FID systems

5.7. Identification of volatile constituents

Co-injection with standards, which were either obtained from natural sources or bought commercially, allowed the identification of the volatile components and fatty acid methyl esters. Additionally, MassFinder software 4.0 (Dr. Hochmuth Scientific Consulting, Hamburg, Germany), the Wiley GC/MS Library (Wiley, NY, USA), Adams Library, and NIST Library mass spectra were compared with those of the compounds to establish their identification. The "Başer Library of Essential Oil Constituents" database, which was created using the identical tools and procedures for chromatographic runs of pure compounds, was used internally to confirm the results To determine the samples' relative retention indices (RRI), a C8-C40 n-alkane standard solution was used from Fluka (Buchs, Switzerland). Isolated compounds expressed as a relative percentage.

5.8. Antimicrobial activity (MIC, µg/mL)

5.8.1. Microbial strains

The antimicrobial evaluation of the extracts was performed against *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, *Candida albicans* ATCC 10231, *C. parapsilosis* ATCC 22019, *C. tropicalis* ATCC 750, and *C. glabrata* ATCC 90030 with microdilution method previously published [28].

5.8.2.Materials

Standard drugs for antimicrobial research included ampicillin, moxifloxacin, clarithromycin, and fluconazole supplied from Sanovel Pharmaceutical Industry (Istanbul, Türkiye). Antimicrobial activity and checkerboard microdilution experiments were performed using RPMI-1640 medium with l-glutamine (Sigma), buffered pH 7 with 3-[N-morpholino]-propansulfonic acid (MOPS), and Mueller Hinton Broth (MHB, Sigma).

5.8.3.Determination of minimum inhibitory concentrations (MIC)

Different *Achillea* extracts were tested for antimicrobial activity. The extracts were prepared using water and 10% dimethyl sulfoxide (DMSO). Microdilution techniques were utilized to determine MIC values with a little modification [29, 30]. The initial two-fold dilution of the extracts resulted in a final concentration range of 2500 to 19.53 µg/mL. Standard antibacterial and antifungal drugs were used at a concentration of 64-0.125 µg/mL in DMSO and water. The cell suspensions of the tested microorganisms were prepared from fresh overnight cultures with 10^6 colony-forming units (CFU)/mL for bacterial strains and 1-2 x10^3 cells/mL for yeasts, respectively. After serially diluting the extracts in 96 well plates. Each microorganism solution was pipetted into each well and left to grow at 35 °C for 24 hours. Positive growth controls were carried out in wells devoid of antimicrobial compounds. MICs were determined as the lowest concentration which showed no pathogens growth.

5.8.4. The combination of infusion of A. schischkinii with fluconazole

The antimicrobial effect of combination infusion of *A. schischkinii* with fluconazole was determined by checkerboard method. The 10-by-7-well configuration was performed on 96-well plate. Seven serial dilutions of extract and ten serial dilution for fluconazole were prepared using RPMI medium as in the MIC test. The serial dilution of fluconazole (0.125-64 µg/mL in sterile medium) combined with serial dilutions of extract (19.53-2500 µg/mL in sterile medium).

A total of seven rows in 96-well plate, 200 µL aliquots of extract were serially diluted to next six rows in the horizontal direction. Similarly 200 µL aliquots of fluconazole was added in a vertical (column) orientation for anticandidal synergistic activity. Thus various concentrations of combinations of both extract and fluconazole were formed in the plate. A cell-seeding solution with a density of 1-2x10^3 CFU /mL was then made. Finally, we duplicated 96-well plates and added 50 µL of the dilution combination plates and the cell-seeding solution to each plate [31]. We appropriately seeded untreated and dead cell control wells, as well as a column and row of fluconazole or extract alone, as controls. The plates were incubated for 24 hours at 37°C. To the best of knowledge, this is the first study for combination of aerial parts of infusion extract of *A. schischkinii* with fluconazole. The fractional inhibitory concentration index (FICI), which was calculated using the formula below, was used to evaluate the combinations [32]:

$$\text{FIC of essential oil} = \frac{\text{MIC of essential oil in combination with antimicrobial drugs}}{\text{MIC of essential oil alone}}$$

$$\text{FIC of antimicrobial drug} = \frac{\text{MIC of antimicrobial in combination with essential oil}}{\text{MIC of antimicrobial drug alone}}$$

FICI = FIC of essential oil + FIC of antimicrobial drug

The types of effects were classified as follows:

FICIs: ≤0.5= synergistic; 0.5≤1 = additive; 1–4= indifferent; ≥4 = antagonistic

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