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ORIGINAL ARTICLE

Comparison of the Essential Oils of *Ferula orientalis* L., *Ferulago sandrasica* Peşmen and Quézel, and *Hippomarathrum microcarpum* Petrov and Their Antimicrobial Activity

Ferula orientalis L., *Ferulago sandrasica* Peşmen ve Quézel ve *Hippomarathrum microcarpum* Petrov'un Uçucu Yağ ve Antimikrobiyal Etkilerinin Karşılaştırılması

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ABSTRACT

Objectives: To determine the chemical composition and antimicrobial activity of the essential oils of the aerial parts of *Ferula orientalis* L., roots of *Ferulago sandrasica* Peşmen and Quézel, and aerial parts of *Hippomarathrum microcarpum* Petrov.

Materials and Methods: Essential oils were analyzed by gas chromatography and gas chromatography/mass spectrometry. The antimicrobial activity of the essential oils was determined by bioautography assay.

Results: α-Pinene (75.9%) and β-pinene (3.4%) were the major components of the aerial parts of *F. orientalis*; with limonene (28.9%), α-pinene (15.6%), and terpinolene (13.9%) for *F. sandrasica*; and β-caryophyllene (31.4%) and caryophyllene oxide (23.1%) for the aerial parts of *H. microcarpum*. Essential oils from the aerial parts of *F. orientalis*, the roots of *F. sandrasica*, and the aerial parts of *H. microcarpum* were active against *Staphylococcus aureus* and *Candida albicans* strains. However, essential oils were not active against *Pseudomonas aeruginosa* or *Escherichia coli*.

Conclusion: The antimicrobial activities against *S. aureus* and *C. albicans* of these species may be attributed to the presence of the main components in the essential oils.

Key words: Antimicrobial, bioautography, *Ferula*, *Ferulago*, *Hippomarathrum*

ÖZ

Amaç: *Ferula orientalis* L.'nin toprak üstü kısımlarından, *Ferulago sandrasica* Peşmen ve Quézel'in köklerinden ve *Hippomarathrum microcarpum* Petrov'un toprak üstü kısımlarından elde edilen uçucu yağların içeriğini ve antimikrobiyal aktivitelerini belirlemektir.

Gereç ve Yöntemler: Bu çalışmada türlerden elde edilen uçucu yağların içerikleri gaz kromatografisi ve gaz kromatografisi/kütle spektrometresi ile analiz edilmiştir. Antimikrobiyal aktivite biyootografi yöntemiyle incelenmiştir.

Bulgular: Sırasıyla; α-pinen (%75.9) ve β-pinen (%3.4) *F. orientalis*'in toprak üstü kısımlarının; limonen (%28.9), α-pinen (%15.6) ve terpinolen (%13.9) *F. sandrasica*'nın köklerinin; β-karyofillen (%31.4) ve karyofillen oksit (%23.1) *H. microcarpum*'un toprak üstü kısımlarının ana bileşenleri olarak bulunmuştur. *F. orientalis*'in toprak üstü kısımlarından ve *F. sandrasica*'nın köklerinden elde edilen uçucu yağlar *Staphylococcus aureus* ve *Candida albicans* türlerine karşı etkili olduğu görülürken, *Pseudomonas aeruginosa* ve *Escherichia coli*'ye karşı etkisiz olduğu görülmüştür. *H. microcarpum*'un toprak üstü kısımlarının *P. aeruginosa*, *S. aureus, C. albicans* ve *E. coli*'ye karşı etkisiz olduğu tespit edilmiştir.

Sonuç: Bu türlerin *S. aureus* ve *C. albicans*'a karşı antimikrobiyal aktiviteleri uçucu yağlarında bulunan ana bileşenlerin varlığından kaynaklanabilir.

Anahtar kelimeler: Antimikrobiyal, biyootografi, *Ferula*, *Ferulago*, *Hippomarathrum*

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INTRODUCTION

The genus *Ferula* L. is a member of the family Apiaceae and has been found to be a rich source of gum resin.[1] *Ferula* species are known in Turkey as "çakşır", "asaotu", "kıngor", "heliz" etc.,[2] and *Ferula orientalis* is known as "heliz",[3] and they have been used as a carminative, sedative, laxative, antispasmodic, digestive, expectorant, diuretic, aphrodisiac, antiseptic, anthelmintic, analgesic,[4] and stimulant.[5] *Ferula* species have been found to contain sesquiterpenes and sesquiterpene coumarins.[6] Fresh peeling stems of *F. orientalis* L., known as "at kasnisi" are used by local people to give flavor to pickles.[5] It is 100-150 cm high, grows on rocky slopes at 1600-2900 m, and has distinguished yellow flowers, with a flowering time in late May and June.[7] *Ferulago* W. Koch. is represented by approximately 83 taxa throughout the world and is a perennial genus of Apiaceae.[8] *Ferulago* species are known as "çakşır", "şeytanteresi", and "kişniş" in Turkey and *Ferulago sandrasica* is known as "kuzu kişnişi".[2] Since ancient times *Ferulago* species have been used for the treatment of intestinal worms and hemorrhoids; as a tonic, aphrodisiac, digestive, and sedative; and against ulcers, snake bites, spleen diseases, and headache. These species have been found to contain coumarins, quinones, flavonoids, and sesquiterpenes.[9] *F. sandrasica* Peşmen and Quézel is an endemic glabrous species, 30-35 cm high; it grows on rocky serpentine slopes at 2000 m and its flowering time is in June and July.[7]

The genus *Hippomarathrum* link is a member of the family Apiaceae and it has five species. *Hippomarathrum* is an erect, much-branched perennial genus, 50-100 cm high, and distributed on rocky slopes and in fields. *Hippomarathrum microcarpum* is also used as food and is known as "çakşır" or "çaşır" by local people in Eastern Anatolia in Turkey.[10] The species of this genus have long been used as spices in ethnobotany.[11] *H. microcarpum* Petrov is a gray shrub with yellowish flowers[7] and it is reported that coumarins and furanocoumarins are found in the roots and fruits of the genus *Hippomarathrum*.[12] Essential oils or their components have been shown to exhibit antimicrobial, antiviral, antimycotic, antitoxigenic, antiparasitic, and insecticidal properties. It is considered that these characteristics are related to the function of these compounds in plants.[13]

The aim of the present study was to present and compare the chemical compositions of the essential oils of the aerial parts of *F. orientalis*, roots of *F. sandrasica*, and aerial parts of *H. microcarpum* growing wild in Turkey. We determined the chemical composition of the essential oils by gas chromatography (GC) and GC/mass spectrometry (MS) analysis and examined the antimicrobial activities of the essential oils by thin-layer chromatography (TLC)-bioautography assay. To the best of our knowledge, this is the first report on the chemical composition and antimicrobial activity of the essential oils in *F. orientalis*, *F. sandrasica*, and *H. microcarpum*.

MATERIALS AND METHODS

Plant material

The plant materials were collected from different parts of Turkey and were identified by Prof. Dr. Hayri Duman (Gazi University, Faculty of Science, Department of Biology) and the voucher specimens are kept in AEF (Herbarium of Ankara University Faculty of Pharmacy). The localities where these species were found are given in Table 1.

Table 1. Localities of the species

Species	Locality	Herbarium number
Ferula orientalis	B9: Between Ağrı and Erzurum, Mount Tahir, 2475 m, 13.07.2014	AEF 10966
Ferulago sandrasica	C2: Mount Sandras 3 km to Lake Kartal, Under *Pinus nigra* trees, in Muğla, 1675 m, 10.6.2013	AEF 26274
Hippomarathrum microcarpum	C5: Adana, south of Tufanbeyli, 13.07.2014	AEF 26699

Isolation of the essential oil

The roots and aerial parts were subjected to hydrodistillation for 3 h using a Clevenger-type apparatus in accordance with the method recommended in the European Pharmacopoeia. The oils obtained were dried in anhydrous sodium sulfate and stored in sealed vials at +4°C in the dark until analyzed and tested. All oils were pleasant smelling and transparent with a faint yellow and greenish color. The essential oil % yields of the aerial parts of *F. orientalis*, roots of *F. sandrasica*, and aerial parts of *H. microcarpum* were 0.022%, 0.019%, and 0.048%, respectively.

GC/MS analysis

GC/MS analysis was performed with an Agilent 5975 GC-MSD system. An Innowax FSC column (60 m×0.25 mm, 0.25 mm film thickness) was used with helium as carrier gas (0.8 mL/min). GC oven temperature 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 to 40:1 and injector temperature was set to 250°C. Mass spectra were recorded at 70 eV and mass range was from m/z 35 to 450.

GC analysis

GC analysis was performed with an Agilent 6890N GC system. The temperature of the flame ionization detector (FID) detector was 300°C. In order to obtain the same elution order as GC/MS, simultaneous auto-injection was done on a duplicate of the same column conforming with the same operational conditions. Relative percentage quantities of the separated compounds were calculated from FID chromatograms. The results of the analysis are given in Table 2. Identification of the essential oil components was performed by comparison of their relative retention times with those of authentic samples or by comparison of their relative retention index to series of *n*-alkanes. Computer matching against commercial sources[14,15] and the in-house "Başer Library of Essential Oil Constituents" established with genuine compounds and components of

known oils, alongside MS literature data,[16,17] was used for the identification.

Determination of antimicrobial compounds of the essential oils by TLC-bioautography assay

Chromatography was carried out on 0.2 mm silica gel 60 F_{254} aluminum sheet TLC plates. To the plates was applied 10 µL of essential oils with a minicaps capillary pipette. The plates were then developed with toluene:ethyl acetate, 93:7, as a mobile phase and another TLC plate for bioautography was prepared in parallel. After the development, the TLC plates were evaluated at UV 254 nm and 366 nm for determination of fluorescent compounds. Alcoholic vanillin–sulfuric acid reagent was used to visualize the separated compounds and they were heated for 3 min at 110°C.

Preparation of microorganisms and the TLC-bioautography assay

After TLC separation, the antimicrobial activity of the essential oils was determined by direct bioautography.[18,19] *Pseudomonas aeruginosa* ATCC 13388, *Staphylococcus aureus* ATCC BAA 1026, *Candida albicans* ATCC 24433, and *Escherichia coli* NRRL B-3008 strains were used for bioautography. Microbial suspensions were grown overnight in double strength Mueller-Hinton broth standardized to 10^8 CFU mL^{-1} (corresponding to McFarland no. 0.5). TLC plates were placed on nutrient agar plates and molten agar culture medium containing inocula was overlaid on the TLC plates and they were incubated at 37°C for 24 h. Then, by incubation, 2,3,5-triphenyl-2H-tetrazolium chloride solution was sprayed on the TLC plates. The treated plates were incubated at 37°C for 2 h and after incubation the inhibition zones were visible as pale spots against a red background.

RESULTS

Thirteen compounds were identified in the essential oil of the aerial parts of *F. orientalis*, representing 96.6% of the oil. α-Pinene (75.9%), β-pinene (3.4%), trans-verbenol (3.0%), and β-caryophyllene (2.5%) were the major components. The analysis on the roots of *F. sandrasica* resulted in the identification of 69 essential compounds representing 96.0% of the oil. Limonene (28.9%) was the most abundant compound in the essential oil, followed by α-pinene (15.6%), terpinolene (13.9%), camphene (2.6%), myrcene (2.8%), p-cymene (2.8%), and 2,3,6-trimethylbenzaldehyde (3.2%).

Twenty-one compounds were characterized in the oil of the roots of *H. microcarpum*, representing 98.7% of the oil. The major constituents were β-caryophyllene (31.4%), caryophyllene oxide (23.1%), bornyl acetate (9.1%), α-humulene (4.9%), germacrene D (4.2%), β-phellandrene (4.6%), α-pinene (3.0%), and caryophylla-2(12),6-dien-5β-ol (=caryophyllenol II) (3.0%). The essential oils obtained from these species did not show much qualitative and quantitative similarity. α-Pinene, camphene, β-pinene, limonene, β-phellandrene, p-cymene, and β-caryophyllene were the main compounds in the three species. Trans-verbenol was the main compound in the essential oils of *F. orientalis* and *F. sandrasica*. Thuja-2,4(10)-diene, pinocarvone, trans-pinocarveol, myrtenol, and cuparene were only found in the essential oils of the aerial parts of *F. orientalis*.

Sabinene, α-phellandrene, (Z)-β-ocimene, γ-terpinene, (E)-β-ocimene, terpinolene, α-copaene, bornyl acetate, α-humulene, germacrene D, δ-cadinene, caryophyllene oxide, and humulene epoxide-II were the main compounds in the essential oils of *F. sandrasica* and *H. microcarpum*. Caryophylla-2(12),6-dien-5β-ol (=*Caryophyllenol II*) was only found in the essential oils of the aerial parts of *H. microcarpum*. The composition of the essential oils obtained from these species and their relative percentages are given in Table 2.

The results for antimicrobial activity by bioautography showed that essential oils from the aerial parts of *F. orientalis* and roots of *F. sandrasica* were active against *S. aureus* and *C. albicans* strains. However, they were not active against the *E. coli* strain. Similarly, essential oil from the aerial parts of *H. microcarpum* was found to contain compounds active against *S. aureus* and *C. albicans*. The essential oil was more effective against *C. albicans* than against *S. aureus*. However, it did not have good activity against *E. coli*. The essential oils did not give any inhibition zone against *P. aeruginosa*. The TLC evaluation of the essential oils is shown in Figure 1.

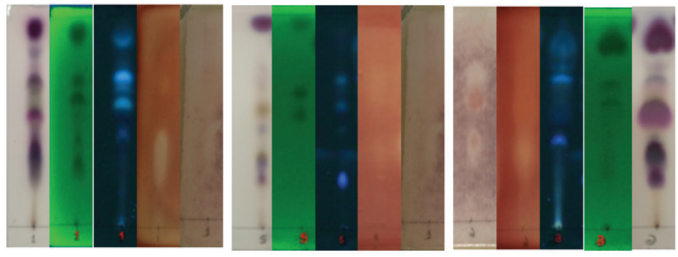

Figure 1. TLC separation of the essential oil from the *Ferula orientalis*, *Ferulago sandrasica*, and *Hippomarathrum microcarpum* on silica gel 60 F254

V/H_2SO_4: Vanillin/H_2SO_4 reagent, SA: *Staphylococcus aureus* ATCC 6538, CA: *Candida albicans* ATTC 90028, TLC: Thin-layer chromatography

DISCUSSION

Monoterpene hydrocarbons (P-cymene, myrcene, γ-terpinene, limonene, terpinolene, and (Z)-β-ocimene), oxygenated monoterpenes (carvacrol methyl ether, 2,5-dimethoxy-p-cymene, trans-chrysanthenyl acetate, cis-chrysanthenyl acetate, and ferulagone), aldehydes (like 2,3,6-trimethylbenzaldehyde, (E)-2-decenal, and octanal), alkane derivatives (hexadecanoic acid), sesquiterpene hydrocarbons (α-humulene, 4,6-guaiadiene, and 7-epi-1,2-dehydrosesquicineole), and oxygenated sesquiterpenes (like cubenol, humuleneepoxide II, and spathulenol) were the major components of some *Ferulago* species.

Some *Ferula* species contain monoterpene hydrocarbons [β-pinene, sabinene, camphene, β-phellandrene, and (E)-β-ocimene], alkane derivatives (nonane), sesquiterpene hydrocarbons (germacrene D, germacrene B, δ-cadinene, (Z)-

Table 2. Chemical composition of the essential oil of *Ferula orientalis*, *Ferulago sandrasica*, and *Hippomarathrum microcarpum*

RRI	Compound	*Ferula orientalis* %	*Ferulago sandrasica* %	*Hippomarathrum microcarpum* %
1032	α-Pinene	75.9	15.6	3.0
1072	α-Fenchene	0.6	0.3	-
1076	Camphene	3.4	2.6	1.5
1093	Hexanal	-	0.1	-
1118	β-Pinene	-	0.3	tr
1132	Sabinene	-	0.1	1.1
1135	Thuja-2,4(10)-diene	2.0	-	-
1151	δ-4-Carene	-	tr	-
1159	δ-3-Carene	-	0.1	-
1174	Myrcene	-	2.8	-
1176	α-Phellandrene	-	-	2.0
1188	α-Terpinene	-	0.2	-
1203	Limonene	1.4	28.9	1.9
1218	β-Phellandrene	1.3	0.6	4.6
1244	2-Pentyl furan	-	0.1	-
1246	(Z)-β-Ocimene	-	0.8	tr
1255	γ-Terpinene	-	1.9	tr
1266	(*E*)-β-Ocimene	-	tr	2.0
1280	*p*-Cymene	2.2	2.8	1.9
1290	Terpinolene	-	13.9	1.4
1294	1,2,4-Trimethyl benzene	-	0.1	-
1452	α,*p*-Dimethylstyrene	-	0.8	-
1468	*trans*-1,2-Limonene epoxide	-	0.3	-
1479	δ-Elemene	-	0.3	-
1497	α-Copaene	-	0.2	0.6
1532	Camphor	-	0.3	-
1538	*trans*-Chrysanthenyl acetate	-	0.2	-
1586	Pinocarvone	tr	-	-
1591	Fenchyl alcohol	-	1.3	9.1
1598	Camphene hydrate	-	0.1	-
1600	β-Elemene	-	0.6	-
1612	β-Caryophyllene	2.5	0.8	31.4
1614	Carvacrol methyl ether	-	1.3	-
1670	*trans*-Pinocarveol	2.0	-	-
1683	*trans*-Verbenol	3.0	-	-
1684	Isoborneol	-	1.3	-
1683	*trans*-Verbenol	-	0.2	-
1687	α-Humulene	-	0.3	4.9

Table 2. Continued

1704	γ-Muurolene	-	0.2	-
1706	α-Terpineol	-	1.8	-
1707	δ-Selinene	-	0.3	-
1719	Borneol	-	0.8	-
1726	Germacrene D	-	1.4	4.2
1742	β-Selinene	-	0.3	-
1744	α-Selinene	-	0.3	-
1751	Carvone	-	0.1	-
1773	δ-Cadinene	-	1.6	0.6
1776	γ-Cadinene	-	0.9	-
1779	(*E,Z*)-2,4-Decadienal	-	0.1	-
1786	*ar*-Curcumene	-	0.3	-
1796	Selina-3,7(11)-diene	-	0.4	-
1804	Myrtenol	1.6	-	-
1807	α-Cadinene	-	0.4	-
1827	(*E,E*)-2,4-Decadienal	-	0.4	-
1849	Cuparene	0.7	-	-
1864	*p*-Cymen-8-ol	-	0.2	-
1878	2,5-Dimethoxy-*p*-cymene	-	0.3	-
1918	β-Calacorene	-	tr	-
1941	α-Calacorene	-	0.1	-
1945	1,5-Epoxy-salvial(4)14-ene	-	0.2	-
2008	Caryophyllene oxide	-	0.3	23.1
2019	2,3,6-Trimethylbenzaldehyde	-	3.2	-
2037	Salvial-4(14)-en-1-one	-	0.1	-
2071	Humulene epoxide-II	-	0.1	2.4
2073	β-Caryophyllene alcohol	-	0.1	-
2080	Cubenol	-	0.6	-
2080	Junenol (=*Eudesm-4(15)-en-6-ol*)	-	0.2	-
2096	Elemol	-	0.1	-
2130	Salviadienol	-	0.2	-
2144	Spathulenol	-	0.1	-
2209	T-Muurolol	-	0.1	-
2255	α-Cadinol	-	0.5	-
2256	Cadalene	-	0.2	-
2269	Guaia-6,10(14)-dien-4β-ol	-	0.2	-
2369	Eudesma-4(15),7-dien-4β-ol	-	0.2	-
2392	Caryophylla-2(12),6-dien-5β-ol (=*Caryophyllenol II*)	-	-	3.0
	Total	96.6	96.0	98.7

RRI: Relative retention indices, calculated against *n*-alkanes, % calculated from flame ionization detector data, tr: trace (<0.1%)

β-farnesene, dehydrosesquicineole, and eremophilene), and oxygenated sesquiterpenes (germacrene D-4-ol, α-cadinol, shyobunone, epi-shyobunone, 6-epi-shyobunone, β-eudesmol, and α-eudesmol) were the major components of some *Ferula* species.

In addition, esters like bornyl acetate were major components of some *Ferula* and *Ferulago* species.[20]

Previous studies demonstrated that the major components of the essential oil of leaves from *F. sandrasica* were ocimene (30.5%), carene-δ-3 (27.4%), and α-pinene (17.8%).[19] Baser and Kırımer[20] studied 12 *Ferulago* species (*F. asparagifolia* Boiss., *F. aucheri* Boiss., *F. confusa* Velen, *F. galbanifera* (Mill.) W. D. J. Koch, *F. humilis* Boiss., *F. idaea* Özhatay and Akalın, *F. macrosciadia* Boiss. and Balansa, *F. mughlae* Peşmen, *F. sandrasica* Peşmen and Quézel, *F. silaifolia* (Boiss.) Boiss., *F. sylvatica* (Besser) Rchb., and *F. trachycarpa* Boiss.) growing in Turkey and that study showed that the major components of essential oils were 2,3,6-trimethylbenzaldehyde (38.9%) and myrcene (18.2%), α-pinene (35.9%), 2,5-dimethoxy-p-cymene (63.4%), α-pinene (31.8%) and sabinene (15.8%), (Z)-β-ocimene (32.4%), p-cymene (18.4%), carvacrol methyl ether (78.1%), α-pinene (25.4%), α-pinene (40.8%), trans-chrysanthenyl acetate (83.5%), p-cymene (45.8%), and (Z)- β-ocimene (30.7%).[21]

The major components of essential oils of some *Ferula* species were reported as phenol, 2-methyl-5-(1-methylethyl) (18.2%), cyclopropa [α] naphthalene-octahydro-tetramethyl (6.6%), and α-bisabolol (10.4%) (3); α-pinene (18.3%), β-pinene (50.1%), and Δ-3-carene (6.7%).[22] Comparing these results with previous studies of *F. orientalis* showed that the major components were nonane (45.6%) and 2-methyloctane (19.4%).[23] Furthermore, essential oils from the aerial parts of *F. orientalis* were obtained: β-phellandrene (24%), (E)-β-ocimene (14%), α-pinene (13%), α-phellandrene (12%), and dehydrosesquicineole (10%),[24] but α-pinene (75.9%) was a major component in our study.

Comparison of these results with previous studies of *Hippomarathrum boissieri* from Turkey[18] showed that the major component of the essential oils from both species was β-caryophyllene (31.4% for aerial parts oil of *H. microcarpum*, 25.6% for aerial parts oil of *H. boissieri*). Another study showed that the major components of essential oils of the leaf and flower of *H. microcarpum* were a-caryophyllene (26.4%), γ-muurolene (19.0%), and linalool (6.1%); and β-caryophyllene (18.5%), γ-muurolene (19.2%), thymol (6.9%), and linalool (5.9%), respectively.[10] The results gained in this investigation suggest that this chemical diversity may be useful in taxonomic classification.

There are not enough data on antimicrobial activity for these species. In a previous survey, the essential oil of *F. sandrasica* was tested against *E. coli* MC 400, *E. coli* ATCC 25922, *E. coli* 0157 H7, *Enterobacter colaecea* ATCC 23355, *Enterococcus faecalis* ATCC 19433, *P. aeruginosa* NRRL B-2679, *S. aureus* ATCC 25923, *S. aureus* ATCC 33862, *Bacillus cereus* NRRL B-3711, *Bacillus subtilis* ATCC 6633, *B. subtilis* NRRL B-209, *Bacillus licheniformis* NRRL B-1001, *Micrococcus luteus* NRRL B-1013, and *Listeria monocytogenes* ATCC 7644 by disk diffusion method. The results showed that the essential oil was active against all tested microorganisms.[18] It was previously reported that the essential oil of *H. microcarpum* was studied for antimicrobial and antifungal activity. The results showed that the essential oil of *H. microcarpum* had antimicrobial activity against *C. albicans* A117 and *S. aureus* ATCC-29213 but had no activity against *E. coli* A1 or *Pseudomonas* sp.[10] Our finding concur with this study.

Bioautography is a suitable method for evaluating essential oils because they contain mixtures of compounds. Therefore, there is a need for the detection of common antimicrobial compounds in essential oils. Additionally, this method is rapid, easy, economical, and inexpensive.[25] In the present study, our aim was the chemical characterization of the essential oils of *F. orientalis*, *F. sandrasica*, and *H. microcarpum* and the detection of antimicrobial activity of essential oils and their main components against some pathogenic bacteria and yeast by TLC-bioautography. The antimicrobial activity test performed against four different microorganisms showed that the essential oils were active against *S. aureus* and *C. albicans* strains; however, they were not active against *P. aeruginosa* or *E. coli* strains.

CONCLUSIONS

These data provide an abundance of information on the essential oil compositions of *F. orientalis*, *F. sandrasica*, and *H. microcarpum* and their antimicrobial activities against some pathogenic microorganisms. As far as we know, this is the first report on the antimicrobial activity of essential oils by TLC-bioautography. The antimicrobial activities against *S. aureus* and *C. albicans* of these species may be attributed to the presence of the main components in the essential oils. A comprehensive study should be conducted including the main compounds isolated from the essential oils or their combinations against different pathogenic microorganisms.

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

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