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Original Scientific Paper

Chemical analysis and biological activity of the essential oils and extracts of two liverwort species growing in Turkey

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ABSTRACT:
The aim of this study was to investigate the chemical composition of the essential oils (EOs) from two *Diplophyllum* species and to evaluate their bioactivity potential [antimicrobial, antioxidant, anti-urease, anti-xanthine oxidase (XO)], and phenolic compounds. The analysis of *Diplophyllum albicans* and *Diplophyllum taxifolium* permitted the identification of 62 components, comprising ≥ 99.6% of the total EO composition. The major components found in these liverwort species were β-patchoulene, β-santalene, β-himachalene, and cubebol. The antimicrobial assays showed that the solvent extracts (*n*-hexane and methanol) from these liverwort species exhibited weak to moderate antimicrobial activity. In addition, the methanol extracts of these liverwort species also exhibited moderate to high antioxidant potential. The enzyme inhibitory effects of the species were determined using urease and XO for the methanol extracts. Generally, the methanol extracts of *D. albicans* and *D. taxifolium* exhibited powerful urease inhibition with IC_{50} values of 9.711 ± 0.058 and 6.304 ± 0.099 μg/mL, respectively, compared to the IC_{50} value of 26.124 ± 0.077 μg/mL for the standard (acetohydroxamic acid). Moreover, the HPLC-UV detection method showed that the analysed methanol extracts of these liverwort species contained only catechin and benzoic acid. These findings suggest that the analysed liverwort species possess antioxidant and urease inhibition, thus indicating the potential to explore new bioactive molecules.

Keywords:
Diplophyllum taxifolium, *Diplophyllum albicans*, oil composition, GC/FID/MS, biological evaluation, phenolic constituents, HPLC-UV

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INTRODUCTION

Bryophytes are a specific group of the oldest green land plants and are grouped into three classes: Bryophyta (mosses; 14,000 species), Marchantiophyta or Hepaticae (liverworts; 6000 species), and Anthocerotophyta (hornworts; 300 species) (Gauvin-Bialecki *et al.* 2010; Asakawa & Ludwiczuk 2018). They consist of approximately 25,000 species, found in different ecosystems (soil, trees, rocks, lakes, and rivers) everywhere in the world, with the exception of the sea (Pannequin *et al.* 2017).

The species *Diplophyllum albicans* (L.) Dumort. and *Diplophyllum taxifolium* (Wahlenb.) Dumort. are members of the Scapaniaceae family (liverworts). The liverworts of the genus *Diplophyllum* are represented by 2 taxa in Turkey. It has been demonstrated that almost all liverworts contain oil bodies, which are used as biological markers for Marchantiophyta (Hepaticae) clas-

sification. These oils are a source of volatile compounds (Asakawa 2004, 2007). For this reason, liverworts have been specially studied for their chemical properties because of the abundance of bioactive mono-, sesqui-, di-terpenoids, or lipophilic aromatic compounds, and bibenzyls (Asakawa 1999; Saritaş *et al.* 2001; Adio *et al.* 2004; Wang *et al.* 2016). No references concerning their use as foods for humans have been found (Asakawa *et al.* 2013a). Sesquiterpenoids have been shown to display a variety of significant biological properties, such as cytotoxic, antifungal, piscicidal qualities, and plant growth inhibitory activity (Lorimer *et al.* 1997; Komala *et al.* 2010; Dey & Mukherjee 2015). A previous study showed that the EO isolated from *Scapania undulata* contained sesquiterpene hydrocarbons, among which (+)-helminthogermacrene, (-)-*cis*-β-elemene, (+)-β-isolongibornene, and (-)-perfora-1,7-diene were new natural compounds (Adio *et al.* 2004). Recent analyses of the chemical profile of *D. albicans* reported an abundance of sesquiterpenoids with related compounds belonging to the *ent*-eudesmanolides group (Ohta *et al.* 1977; Adio & Konig 2007). Also, the EO isolated from *D. albicans* was reported to indicate important cytotoxic activity against human epidermoid carcinoma (Ohta *et al.* 1977). Only one study dealing with the terpenoids from *D. taxifolium*, which were isolated in EtOH extracts, has been reported (Asakawa *et al.* 2013b; Wang *et al.* 2016). Also, most research into the chemistry of liverworts emphasised the terpenoid compounds, especially of sesquiterpenoids, while studies on the EO composition of *D. taxifolium* are still incomplete (Wang *et al.* 2016). Moreover, the biological properties (antimicrobial, antioxidant, urease, and xanthine oxidase (XO) inhibitory effects) of *Diplophyllum* species, such as *D. albicans* and *D. taxifolium*, have not been reported to date and there is very little information concerning the EO of *Diplophyllum* species. Within this framework, more research is needed on the chemical components and biological activities of the EOs of *Diplophyllum* species.

The present study was designed to analyse the chemical components of the EOs of the liverworts *D. albicans* and *D. taxifolium* using the GC/FID/MS (Gas Chromatography/Flame Ionization Detection/Mass Spectrometer) method. Furthermore, we determined the antibacterial activity of these EOs and solvent extracts (*n*-hexane and methanol) by determining the minimum inhibitory concentrations (MIC) and evaluated the antioxidant, and certain enzyme inhibition activities (urease and XO), as well as the phenolic compounds of the methanol extracts of two *Diplophyllum* species for the first time.

MATERIAL AND METHODS

Plant material and chemicals. The fresh herbs of *Diplophyllum albicans* (L.) Dumort. and *Diplophyllum taxifolium* (Wahlenb.) Dumort. were collected in the region of Gümüşhane (Turkey: N 40°42'; E 38°57') in May 2020 (spring season). The collection was carried out at 1765 m above mean sea level. These liverworts were identified by Prof. Dr. Nevzat Batan in the Karadeniz Technical University (Uyar & Çetin 2004; Fedosov & Ignatova 2009). Voucher specimens and related information have been deposited at the Herbarium of the Biology Department (KTUB; 1612 and 1613, respectively) at the university. All the used chemicals and solvents were obtained from Sigma-Aldrich if not indicated otherwise.

Isolation of the EOs. The freshly collected liverworts of *D. albicans* and *D. taxifolium* were dried and cut into smaller pieces prior to use. Then, the EOs of *D. albicans* and *D. taxifolium* were extracted by the conventional hydro distillation (HD) method in a modified Clevenger-type apparatus with a cooling bath (-15°C) system, using 80 g dried samples for 4 h. The oils were dissolved in 0.5 mL *n*-hexane (HPLC grade), dried over anhydrous Na_2SO_4, and then filtered and stored in the dark at -20°C until ready for analysis and testing.

Solvent (*n*-hexane and methanol) extracts. The air-dried ground (20 g) plant material of *D. albicans* and *D. taxifolium* was extracted with *n*-hexane and methanol (1 L × 3, each for one day) at room temperature. The crude extracts were obtained after the *n*-hexane and methanol (MeOH) extracts were concentrated in a rotary evaporator.

Characterization of the chemical composition of the EOs. The EO samples were analysed by using a GC/FID/MS (QP2010, Kyoto, Japan) equipped with a Restek Rxi-5MS capillary column (60 m × 0.25 mm i.d., 0.25 µm film thickness). Mass spectrometry (MS) was performed in the EI (Electron ionization) mode at an ionisation energy of 70 eV and the MS data were achieved in a full scan range (*m/z* of 40–450). The column temperature was programmed to remain at 60°C for 2 min, increasing to 240°C at a rate of 3°C/min, and maintained at 250°C for 4 min. The carrier gas was helium with ultra-high purity (> 99.999%) at 1.0 mL/min. The injection volume was 0.2 µL of 1% *n*-hexane solution in a split mode (10%) (Özgenç *et al.* 2017; Çelik *et al.* 2021).

The identification of each compound was performed from their GC retention indices (RIs) achieved with references for a series of *n*-alkanes (C_6–C_{30}) (Supelco USA) on a Restek Rxi-5MS column (Adams 2004; Kahriman *et al.* 2011; Tosun *et al.* 2015; Çelik *et al.* 2021; Jugran *et al.* 2021). The mass spectra data were compared with other published MS literature data and the FFNSC 2 (Mondello 2011) and NIST (NIST 2005) mass spectral database of the GC/MS system.

Antibacterial assay (MIC). The antibacterial activity of the EO and solvent extracts (*n*-hexane and MeOH)

was determined against eight bacterial strains and two yeasts, namely *Escherichia coli* (ATCC 25922), *Yersinia pseudotuberculosis* (ATCC 911), *Pseudomonas aeruginosa* (ATCC 43288), *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Bacillus cereus* (709 Roma), *Mycobacterium smegmatis* (ATCC 607), *Candida albicans* (ATCC 60193), *Candida tropicalis* (ATCC 13803) and *Saccharomyces cerevisiae* (RSKK 251) using agar well diffusion and minimal inhibition concentration (MIC) assays. The tested microorganisms were obtained from the Refik Saydam Hıfzıssıhha Institute (Ankara, Turkey) as previously report in other studies (Barry 1999; Tosun *et al.* 2014; Çelik *et al.* 2021). Cultures of the microorganisms were grown in Mueller-Hinton broth (MH) (Difco, Detroit, MI) at pH 7.3 and buffered Yeast Nitrogen Base (Difco, Detroit, MI) at pH 7.0, respectively. The test plates were then incubated at 35°C for 18–24 h. *Mycobacterium smegmatis* was incubated using Brain Heart Infusion broth (BHI) (Difco, Detroit, MI) at 35°C for 48–72 h (Woods *et al.* 2003). After incubation, the MIC was defined as the lowest concentration which displayed no growth. Ampicillin, streptomycin, and fluconazole were used as the standard, while pure DMSO was utilised as the solvent control. All the determinations were recorded in triplicate. The EOs were dissolved in *n*-hexane (76.500–78.500 µg/mL), and the solvent extracts in dimethyl-sulphoxide (99.0%) (DMSO) to prepare the extract solution within the range of 26.600–107.200 µg/mL.

Determination of antioxidant capacity. The total phenolic content (TPC) of the methanolic extract of *D. albicans* and *D. taxifolium* was analysed using the Folin–Ciocalteu assay at 760 nm, with gallic acid as the standard (Singleton & Rossi 1965). The TPC was calculated as mg of gallic acid equivalents (GAE) per g pure extract.

The ferric reducing antioxidant capacity of the methanol extract of *D. albicans* and *D. taxifolium* was determined according to the method proposed by Benzei and Strain (Benzie & Strain 1999). $FeSO_4.7H_2O$ (Sigma Chemical Co, USA) was used for the calibration curve. The FRAP values were determined as µmol $FeSO_4.7H_2O$ equivalent per g pure extract.

The DPPH radical scavenging activity of the methanolic extract of *D. albicans* and *D. taxifolium* was measured according to the literature (Brand-Williams *et al.* 1995). The radical scavenging activity was measured by using Trolox (Sigma Chemical Co, USA) as the standard and all the DPPH values are calculated as IC_{50}, the concentration of the samples which causes 50% DPPH radical scavenging.

Urease and XO inhibition assay. The urease inhibitory effects of the methanolic extracts of *D. albicans* and *D. taxifolium* were evaluated according to previous reports (Weatherburn 1967; Baltaş *et al.* 2016; Baltaş 2017; Kantar *et al.* 2018). Acetohydroxamic acid (AA) was used as the positive control.

The xanthine oxidase (XO) inhibitory effects of the methanolic extracts of *D. albicans* and *D. taxifolium* were determined according to the literature (Hayashi *et al.* 1988; Kantar *et al.* 2015; Okan *et al.* 2019). Allopurinol (Sigma-Aldrich, St. Louis, MO) was used as the reference component.

All the experiments were performed in triplicate in order to calculate standard deviation. To calculate the IC_{50} values, various concentrations of each extract and the standard were measured under similar reaction conditions. The concentrations of those extracts which inhibited substrate hydrolysis [urease and xanthine oxidase (XO)] by 50%, IC_{50} (µg/mL) were determined from the dose-response curve.

Determination of HPLC-UV analyses. The HPLC-UV analyses of the phenolic compounds were carried out using the Thermo Scientific Dionex Ultimate™ 3000 system (Thermo Scientific, Bremen, Germany), by means of a gradient programme with two solvent systems (Sahin *et al.* 2019). Gallic acid, protocatechuic acid, protocatechuic aldehyde, *p*-hydroxybenzoic acid, catechin, chlorogenic acid, vanillic acid, caffeic acid, syringic acid, epicatechin, vanillin, *p*-coumaric acid, syringaldehyde, rutin, ferulic acid, benzoic acid, rosmarinic acid, and quercetin were analysed at 280 nm (Karaçelik & Sahin 2021).

RESULTS AND DISCUSSION

Chemical composition of the EOs of *D. albicans* and *D. taxifolium*. In this study, the EO of two liverworts, *D. albicans* and *D. taxifolium,* were extracted by conventional hydro distillation for 4 h to yield characteristically yellow oil in the range of 1.05% (w/w) and 0.53%, respectively. The chemical analyses of the EOs resulted in the identification of sixty-two compounds from *D. albicans* and *D. taxifolium*, which accounted for more than 99% of the total composition. In order to facilitate the comparison of the chemical composition of the EOs, they were separated into nine classes: monoterpene hydrocarbon, oxygenated monoterpene, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, terpene related compounds, aldehydes, ketone, alcohols, and others, as shown in Table 1. The composition of the two liverwort EOs mainly consisted of sesquiterpene hydrocarbons (53.4–68.3%), followed by oxygenated sesquiterpenes (26.6–39.4%) and terpene related compounds (0.4–3.5%). The major compounds (> 10%) of these liverwort species were β-patchoulene (**22**; 35.1% and 19.5%, respectively), followed by β-santalene (**34**; 4.4% and 23.3%, respectively), β-himachalene (**43**; 17.9% in *D. albicans*), cubebol (**45**; 15.3% in *D. taxifolium*), (*Z*)-β-elemonene (**50**; 11.8% in *D. albicans*) and cedrol (**51**; 14.0% in *D. taxifolium*) (Fig. 1).

Table 1. The contents and chemical composition of the essential oils from two liverworts, *Diplophyllum albicans* and *Diplophyllum taxifolium,* collected from the Gümüşhane province, Turkey, harvested in the spring season (May 2020).

		Content[b](%)			
No	Compounds[b]	A[a]	B[a]	$RI_{exp.}$[c]	$RI_{lit.}$[d]
1	Hexanal	1.5	1.1	817	802
2	Heptanal	0.4	0.1	909	902
3	α-Pinene	0.2	0.1	944	939
4	2(*E*)-Heptenal	-	0.1	962	959
5	Benzaldehyde	0.1	-	970	960
6	1-Octene-3-ol	-	0.2	982	979
7	3-Octanone	-	0.1	990	984
8	2-Amylfuran	0.3	0.1	996	991
9	Octanal	0.1	0.1	1006	999
10	Benzene acetaldehyde	0.2	0.1	1050	1042
11	2(*E*)-Octenal	0.1	0.1	1062	1055
12	Octanol	-	0.1	1071	1068
13	Nonanal	0.4	0.3	1106	1101
14	2(*E*)-Nonenal	-	0.1	1163	1162
15	Decanal	0.1	0.1	1208	1202
16	2(*E*)-Decenal	0.2	0.1	1265	1264
17	2(*E*),4(*Z*)-Decadienal	-	0.1	1298	1293
18	Undecanal	0.1	0.1	1310	1307
19	2(*E*),4(*E*)-Decadienal	0.1	0.2	1321	1317
20	Bicycloelemene	1.1	1.0	1348	1333
21	2-methyl-Undecanal	0.1	0.1	1365	1368
22	β-**Patchoulene**	35.1	19.5	1388	1381
23	α-Duprezianene	0.1	-	1389	1389
24	β-Elemene	2.0	1.0	1402	1391
25	(*Z*)-Isoeugenol	0.6	-	1420	1407
26	α-Gurjunene	0.1	0.3	1423	1410
27	β-Cedrene	-	0.7	1427	1421
28	β-Ylangene	0.2	-	1428	1421
29	(*E*)-α-Ionene	0.4	0.2	1434	1430
30	γ-Elemene	0.5	0.4	1438	1437
31	α-Guaiene	-	0.2	1443	1440
32	Aromadendrene	0.4	-	1450	1441
33	α-Himachalene	2.5	1.0	1454	1451
34	β-**Santalene**	4.4	23.3	1465	1460
35	α-Acoradiene	0.1	-	1476	1466
36	Thujopsadiene	-	0.8	1477	1468
37	β-Chamigrene	1.0	0.5	1482	1478
38	β-Selinene	-	3.6	1491	1490
39	(*Z*)-β-Guaiene	0.1	0.6	1496	1493
40	α-Selinene	1.8	-	1501	1498
41	α-Muurolene	-	0.4	1503	1500
42	Epizonarene	0.7	-	1504	1502
43	β-**Himachalene**	17.9	-	1513	1505
44	β-Bisabolene	0.1	-	1516	1506
45	**Cubebol**	-	15.3	1517	1515
46	cis-Calamenene	0.2	0.1	1549	1540
47	Ledol	-	0.1	1571	1569
48	Himachalene epoxide	2.2	-	1584	1580
49	Globulol	-	4.4	1587	1585
50	(***Z***)-β-**Elemonene**	11.8	-	1594	1590
51	**Cedrol**	4.5	14.0	1601	1601
52	Khusimone	-	3.3	1606	1604
53	Humulene epoxide II	3.4	2.0	1613	1608
54	cis-Isolongifolene	1.0	-	1616	1613
55	1-epi-Cubenol	0.6	-	1630	1629
56	Gossonorol	0.6	-	1639	1637
57	Himachalol	1.8	1.9	1656	1654
58	epi-β-Bisabolol	-	1.5	1674	1672
59	(*Z*)-Apritone	0.3	-	1693	1690
60	Germacrone	0.3	-	1699	1694
61	Curcuphenol	0.1	0.1	1725	1718
62	iso-Longifolol	-	0.1	1740	1730

Grouped compounds	Content[b] (%)	
	A[a]	B[a]
Monoterpene hydrocarbon	0.2	0.1
Oxygenated monoterpene	0.6	-
Sesquiterpene hydrocarbons	68.3	53.4
Oxygenated sesquiterpenes	26.6	39.4
Terpene related compounds	0.4	3.5
Aldehydes	3.4	2.7
Ketone	-	0.1
Alcohols	-	0.3
Other	0.3	0.1
Total	99.8	99.6

[a] A: *D. albicans* and B: *D. taxifolium;*[b]the compounds are listed in the order of their elution using a Restek Rxi-5MS capillary column (60 m × 0.25 mm, 0.25 μm film thickness); [c]$RI_{exp.}$: the linear retention indices calculated against *n*-alkanes(C_6-C_{30}); %: calculated from FID data; MS: tentatively identified based on computer matching of the mass spectra with those of the FFNSC 2 and NIST libraries and comparison with literature data; [d]$RI_{lit.}$: the relative retention indices for the essential oil compounds according to Adams (2004); the main constituents are in bold; (-): not observed.

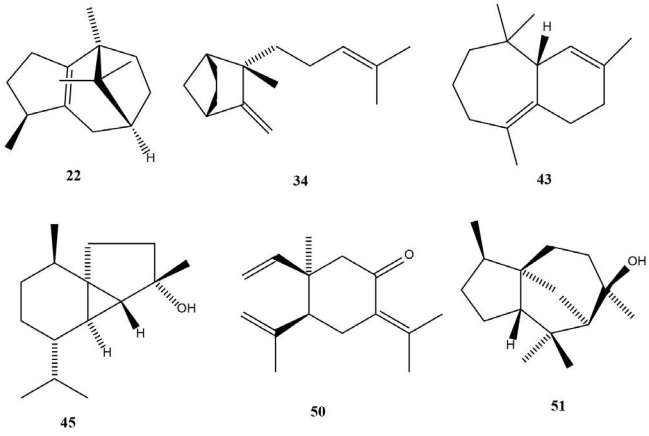

Fig. 1. The chemical structures of some major compounds identified in the EOs from *Diplophyllum albicans* and *D. taxifolium*

Our results are completely supported by Ohta *et al.* (1977), and Adio & König (2007), who reported that sesquiterpene hydrocarbons were the main compounds of the genus *Diplophyllum* and that these compounds were related to the *ent*-eudemanolides group. Similarly, the ethanol extracts of *D. taxifolium* (Wahlenb.) Dumort. were also found to have a predominance of *ent*-eudesmone sesquiterpene lactones (Asakawa *et al.* 2013b; Ludwiczuk & Asakawa 2017). On the other hand, the components in plants differ as subspecies change within the same species. According to the literature, Fan *et al.* (2019) reported the isolation of three new diterpenoids from the Chinese liverwort *Diplophyllum apiculatum* (A. Evans) Stephani. The GC/MS analyses of the EOs of the Belgian liverwort *Scapania undulata* (L.) Dum. showed the main cadinene-type sesquiterpenoids accompanied by scapanol, *ent*-tau-muurolol, and (+)-4-muurolen-6α-ol (Nagashima *et al.* 1995). Moreover, another study related to the EO of the liverwort *Plagiochilo bifaria* mentioned sesquiterpenes as the main compounds, which also corresponds to our results (Hackl *et al.* 2006).

A total of 45 EO components were identified in *D. albicans*, the five most abundant of which_were β-patchoulene (**22**; 35.1%), β-himachalene (**43**; 17.9%), (*Z*)-β-elemonene (**50**; 11.8%), cedrol (**51**; 4.5%) and β-santalene (**34**; 4.4%). When we review the EO studies concerned with this species, both sesquiterpene hydrocarbons (68.3%) and oxygenated sesquiterpenes (26.6%) were identified as the main classes, being at trace level of the other classes. Our results are similar to those previously reported (Ohta *et al.* 1977; Adio & König 2007; Asakawa *et al.* 2013b; Ludwiczuk & Asakawa 2017).

Forty-five components were also identified in the EO composition of *D. taxifolium* and the four major compounds (> 10%) of this liverwort species were β-santalene (**34**; 23.3%), β-patchoulene (**22**; 19.5%), cubebol (**45**; 15.3%) and cedrol (**51**; 14.0%). *D. taxifolium* EO was dominated by sesquiterpene hydrocarbons (53.4%), in addition to significant amounts of oxygenated sesquiterpenes (39.4%). Wang *et al.* (2016) identified two diterpenoids from *D. taxifolium* using the isolation method. To the best of our knowledge, the EO composition of *D. taxifolium* has been studied for the first time in this study.

The results of this study of the EO of *D. albicans* and *D. taxifolium* species identified β-patchoulene (**22**; 35.1% in *D. albicans* and 19.5% in *D. taxifolium*) as the major component among sesquiterpene hydrocarbons. The EO of *D. taxifolium* contained the highest amount of (> 10%) β-santalene (**34**; 23.3%), followed by cedrol (**51**; 14%), while the EO of *D. albicans* contained the lowest quantity (< 5%) of cedrol (**51**; 4.5%), followed by β-santalene (**34**; 4.4%). The other sesquiterpene hydrocarbons present in significant amounts were β-elemene, γ-elemene, α-himachalene, and β-chamigrene. Also, the EO profiles of these species change depending on when they were collected, the soil content, and the weather.

Antimicrobial activity of the EOs and solvent extracts (*n*-hexane and methanol) of *D. albicans* and *D. taxifolium*. The *in vitro* antimicrobial activity of the EOs and solvent extracts (*n*-hexane and methanol) of *D. albicans* and *D. taxifolium* were tested against ten different microorganisms (strains of bacteria, yeast, and fungi) according to the minimal-inhibitory-concentration (MIC), which is listed in Table 2 (Barry *et al.* 1999; Woods *et al.* 2003; Çelik *et al.* 2021) (yields: 0.83 g, 4.15%: in the methanol extract of *D. albicans*; yields: 0.12 g, 0.61%: in the *n*-hexane extract of *D. albicans*; yields: 0.25 g, 1.26%: in the MeOH extract of *D. taxifolium*; yields: 0.20 g, 1.02%: in the *n*-hexane extract of *D. taxifolium*). According to the literature, the antimicrobial activity of the *Diplophyllum* genus has not been previously reported. Our data showed weak to moderate antimicrobial activity against the tested bacteria, with the MIC values in the range of 41–5360 μg/mL. According to these results, the EO and solvent extracts (*n*-hexane and methanol) from *D. albicans* and *D. taxifolium* showed no antimicrobial activities against Gram-negative bacteria (*Escherichia coli*, *Yersinia pseudotuberculosis,* and *Pseudomonas aeruginosa*), with the exception of the *n*-hexane extract of *D. albicans*. In addition, *Staphylococcus aureus*, *Bacillus cereus*, *Mycobacterium smegmatis*, *Candida albicans*, and *Saccharomyces cerevisiae* (Gram-positive bacteria, acido-resistant mycobacterium, and yeast-like fungi) proved to be more resistant to the EO and solvent extracts (*n*-hexane and methanol) of *D. albicans* and *D. taxifolium*. In general, the hexane extracts (41–2845 μg/mL) exhibited better antibacterial activity than the methanol extracts (161–5360 μg/mL) and EOs (683–3825 μg/mL). In particular, the highest antimicrobial activity was detected in the *n*-hexane extracts of *D. albicans* against Gram-positive bacteria, acido-resistant mycobacterium, and yeast-like fungi with

Table 2. Screening for the antimicrobial activity of the essential oil and solvent extracts of *Diplophyllum albicans* and *D. taxifolium*

Samples		Stock Sol. μg/mL	Microorganisms and minimal inhibition concentration (MIC, μg/mL)									
			Ec	Yp	Pa	Ef	Li	Sa	Bc	Ms	Ca	Sc
Essential oils	A	76.500	-	-	-	-	-	3825	1912	683	3825	1912
	B	78.500	-	-	-	-	-	3925	1962	1905	1962	981
Hexane extracts	A	26.600	-	-	1330	-	-	332	332	83	83	41
	B	56.900	-	-	-	-	-	2845	2845	177	2845	2845
Methanol extracts	A	107.200	-	-	-	5360	5360	1340	1340	1340	-	5360
	B	103.400	-	-	-	5170	5170	323	161	161	5170	5170
Ampicillin		10	10	10	>128	10	10	35	15			
Streptomycin		10								4		
Fluconazole		5									<8	<8

A: *D. albicans* and **B:** *D. taxifolium;* **Ec:** *Escherichia coli* **(ATCC 25922), Yp:** *Yersinia pseudotuberculosis* **(ATCC 911)**, **Pa:** *Pseudomonas aeruginosa* **(ATCC 27853)**, **Sa:** *Staphylococcus aureus* **(ATCC 25923)**, **Ef:** *Enterococcus faecalis* **(ATCC 29212)**, **Li:** *Listeria* monocytogenes **(ATCC 43251)**, **Bc:** *Bacillus cereus* **(709 Roma)**, **Ms:** *Mycobacterium smegmatis* **(ATCC607)**, **Ca:** *Candida albicans* **(ATCC 60193)**, **Sc:** *Saccharomyces cerevisiae* **(RSKK 251)**, Amp.: Ampicillin, Strep.: Streptomycin, Flu.: Fluconazole, -: no activity in the tested concentrations.

Table 3. The total phenolic content and antioxidant activity of the methanol extracts of *Diplophyllum albicans* and *D. taxifolium*

Methanolic extracts	TPC[a]	FRAP[b]	DPPH[c,d] IC_{50} value
D. albicans	16.804 ± 0.356	123.545 ± 1.732	1.528 ± 0.017
D. taxifolium	23.423 ± 1.413	95.042 ± 1.249	1.307 ± 0.016
Trolox			0.001 ± 0.000

[a]The total phenolic content was expressed as mg GAE per gram of pure extract. [b]The FRAP value was expressed as μmol $FeSO_4.7H_2O$ per gram of pure extract. [c]Each SC_{50} value is reported as the mean value ± standard deviation (SD), calculated from a triplicate analysis of the samples.[d]The concentration of the test sample (mg/mL) required to produce 50% DPPH radical scavenging.

Table 4. Enzyme inhibition values of the methanolic extracts of *Diplophyllum albicans* and *D. taxifolium*

Sample	Inhibition of urease and XO activity % ± SD[a]	
	IC_{50} for urease [μg/mL]	IC_{50} for XO[μg/mL]
D. albicans	9.711 ± 0.058	14.721 ± 0.135
D. taxifolium	6.304 ± 0.099	10.355 ± 0.103
Acetohydroxamic acid[b]	26.124 ± 0.077	--------
Allopurinol[c]	--------	0.542 ± 0.009

[a]Standard deviation (n = 3), [b]Reference for urease inhibition; [c]Reference for XO inhibition.

MIC values of 41–332 μg/mL. Furthermore, the methanol extracts of *D. albicans* and *D. taxifolium* exhibited antibacterial activity against *Enterococcus faecalis* and *Listeria monocytogenes* (Gram-positive bacteria), with an MIC of 5170–5360 μg/mL. Only the methanol extract of *D. albicans* displayed no activity against *C. albicans*.

The high percentages of sesquiterpenes (hydrocarbons and oxygenated) could be responsible for the antimicrobial effects of these EOs and solvent extracts (*n*-hexane and methanol) (Vasconcelos *et al.* 2020). Similarly, Selles *et al.* (2013) reported that being rich in oxygenated sesquiterpenes, the EOs of *Anacvclus pyrethrum* L. exhibited activity against *Candida albicans* and *Staphylococcus aureus* bacteria strains. Another study performed by Bukvički *et al.* (2012) demonstrated that the presence of a high content of sesquiterpene hydrocarbons could be responsible for improved antimicrobial activity. Previous research on the antimicrobial activity of nine liverwort species from South Africa indicated that most of them have significant antimicrobial potential (Linde *et al.* 2016). Furthermore, the antimicrobial activity of the EOs and solvent extracts (*n*-hexane and methanol) of *D. albicans* and *D. taxifolium* was also found to be slightly different. These findings may be related to the different phytochemical compositions of the examined species.

Total phenolic content and antioxidant activity of the methanol extracts of *D. albicans* and *D. taxifolium*. The antioxidant activity of the methanol extracts obtained from *D. albicans* and *D. taxifolium* were determined by three *in vitro* methods (TPC (total phenolic content), FRAP (ferric reducing antioxidant power), and DPPH (2,2-diphenyl-1-picrylhydrazyl). The results of the anti-

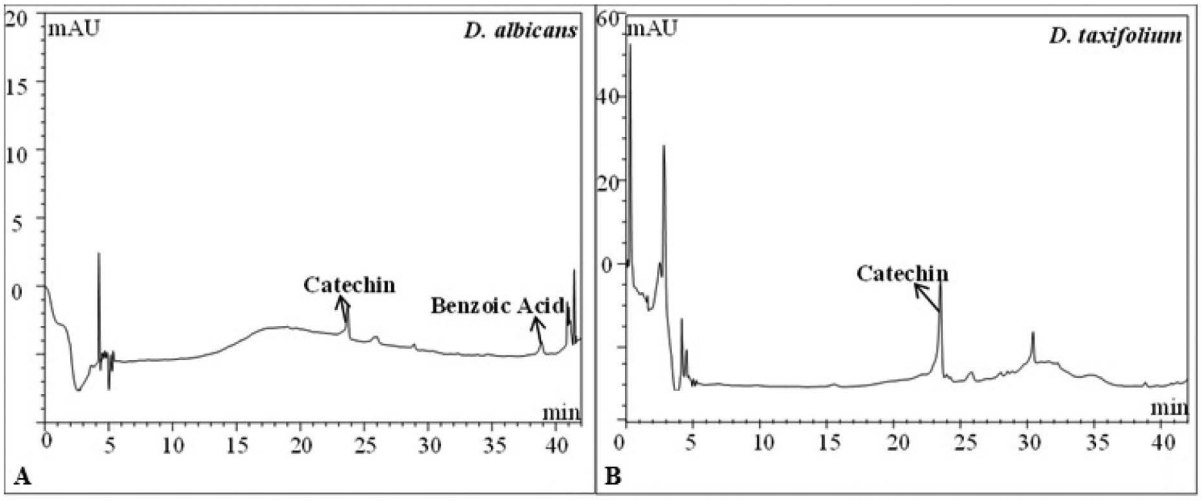

Fig. 2. HPLC chromatograms of the methanolic extract of *Diplophyllum albicans* (A) and *D. taxifolium* (B)

oxidant activity screening for all the methanol extracts are given in Table 3. All the extract samples ranged from 16.804 ± 0.356 to 23.423 ± 1.413 mg GAE/g pure extract using the TPC method, and from 95.042 ± 1.249 to 123.545 ± 1.732 µmol $FeSO_4.7H_2O$/g pure extract using FRAP. The DPPH power of the methanol extract of *D. taxifolium* (SC_{50} = 1.307 ± 0.016 mg/mL) was higher than that of the methanol extract of *D. albicans* (IC_{50} = 1.528 ± 0.017 mg/mL). The results of the antioxidant activity estimated by the TPC, FRAP, and DPPH methods on all the methanol extracts of *D. albicans* and *D. taxifolium* demonstrated similar general trends. All the examined *Diplophyllum* genus methanol extracts displayed good antioxidant activity, thus making our results consistent with earlier reports. A number of reports have shown that sesquiterpenes and oxygenated sesquiterpenes such as patchouli alcohol are also attributed to the high antioxidant potential of plants (Wei & Shibamoto 2007; Mansuri *et al.* 2020).

Urease and XO inhibitory activity of the methanol extracts of *D. albicans* and *D. taxifolium*. In the present study, we evaluated the enzyme inhibition effects of the methanol extracts of *D. albicans* and *D. taxifolium* on urease and XO using spectrophotometry (Baltaş *et al.* 2016; Baltaş 2017). The obtained results presented in Table 4 show the obtained IC_{50} values. When compared to the standard, the obtained results for inhibition of urease showed that the methanol extracts of *D. albicans* and *D. taxifolium* were more effective than XO. The methanol extracts of *D. albicans* and *D. taxifolium* exhibited extremely similar inhibitory effects on both urease and XO. Acetohydroxamic acid was used as the standard inhibitor for urease. In terms of the inhibition of urease activity, *D. taxifolium* was the most effective of the two liverwort species (6.304 ± 0.099 µg/mL) compared to acetohydroxamic acid (26.124 ± 0.077 µg/mL), with *D. albicans* showing weaker effects (9.711 ± 0.058 µg/mL). Furthermore, the inhibitory effects of the methanol extracts of *D. albicans* and *D. taxifolium* on XO revealed that *D. taxifolium* (10.355 ± 0.103 µg/mL) showed more potent inhibition than *D. albicans* (14.721 ± 0.135 µg/mL). However, all the methanol extracts demonstrated weaker effects compared with allopurinol, which was utilised as the positive control with an IC_{50} of 0.542 ± 0.009 µg/mL. Our results indicate that the methanol extracts of *D. albicans* and *D. taxifolium* show excellent potential for the inhibition of urease and such inhibitory effects could be directly connected to the secondary metabolites or phytochemicals which have led to the improvement of new pharmaceuticals (Saleem *et al.* 2020). This is the first report on urease and XO inhibition by methanol extracts of *D. albicans* and *D. taxifolium*, and it is clear that more needs to be learned about this genus.

Table 5. The determined phenolic compounds in the examined methanolic extracts of two liverwort species using the HPLC-UV method

	Sample	Phenolic Compounds (mg phenolic/ g extract)	
		Catechin	**Benzoic acid**
1	***D. albicans***	1.26	1.46
2	***D. taxifolium***	7.05	nd

n.d.: not detected

HPLC-UV screening of the phenolic content. Phenolic compounds have long been considered a source of natural antioxidants in herbal medicine, which have been used for different biological activities, thus promoting health benefits (Sönmezdag *et al.* 2017; Çelik *et al.* 2021; Rašeta *et al.* 2021). To the best of our knowledge,

no previous research has been carried out to define and quantify the phenolic contents of methanol extracts of *D. albicans* and *D. taxifolium*. Table 5 and Fig. 2 show the obtained results for the phenolic contents of the methanol extracts of both *Diplophyllum* species using HPLC-UV. The phenolic compounds of the methanol extracts of *D. albicans* and *D. taxifolium* were quite similar. According to the quantitative results of HPLC-UV, among the 18 phenolic standards, only catechin and benzoic acid were detected as phenolic compounds in both liverwort species (Fig. 2). Catechin was dominant in the methanol extracts of *D. taxifolium* (7.05 mg/g extract), and weaker in *D. albicans* (1.26 mg/g extract), while benzoic acid (1.46 mg/g extract) was only detected in the methanol extract of *D. albicans* (Fig. 2A). These findings suggest that the methanol extracts of *D. albicans* and *D. taxifolium* can be seen as a poor source in terms of the analysed phenolic standards, which is in line with previous reports. The phenolic composition of the liverwort species seems to be mostly identified by the presence of compounds in methanol extracts, as only luteolin was found in the *M. polymorpha* methanol extract using RP-HPLC (Gökbulut *et al.* 2012). In terms of the total phenolic compound detected by HPLC in our study, it was observed that while *D. albicans* was 2.72 mg per g extract, *D. taxifolium* was 7.05 mg per g extract since there was only one component present. In fact, the total phenolic contents of the samples discussed under the relevant sub-title were 16.804 and 23.423 mg GAE/g of pure extract for *D. albican* and *D. taxifolium*, respectively. It does not seem possible to make a mathematical comparison here because the determination of the total phenolic content is given in terms of the equivalence of a reference, as can be understood from the given gallic acid equivalent unit. However, in chromatographic analysis, the amount of phenolic substance in the sample is determined with high precision and accuracy, and there is no reference equivalence. The presence of one to two standards in the samples in the analyses does not necessarily indicate that these samples contain very small amounts of phenolic content since as many standards as are defined and determined can be analysed qualitatively and quantitatively in HPLC analysis. Aside from these standards, the samples are highly likely to have different amounts of phenolic content. The fact that the total phenolic content was measured in this study partly confirms this.

CONCLUSIONS

The current study examined the EOs of *D. albicans* and *D. taxifolium* using GC/FID/MS to identify their chemical composition as well as the antimicrobial, antioxidant, anti-urease, anti-XO, and phenolic compounds of all the analysed extracts (*n*-hexane and methanol). This is the first such record of these liverwort species. These liverwort species were found to be rich in sesquiterpene hydrocarbons and oxygenated sesquiterpenes. The major components of the investigated liverwort species are β-patchoulene, β-santalene, β-himachalene, cubebol, (*Z*)-β-elemonene, and cedrol. Additionally, the β-patchoulene ratio of the two liverwort species is also significant. The solvent extracts (*n*-hexane and methanol) of the investigated liverwort species can also be said to have medium antimicrobial activity. Furthermore, it was determined that the methanol extracts of the studied liverwort species had moderate to high antioxidant effects and displayed significant enzyme-inhibition activities against urease and XO. Catechin was seen to be the effective phenolic compound in the methanolic extract of both species. These findings suggest that *D. albicans* and *D. taxifolium* species require further more comprehensive studies considering their pharmacological effects.

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REZIME

Botanica SERBICA

Hemijska analiza i biološka aktivnost eteričnih ulja i ekstrakata dve vrste jetrenjača koje rastu u Turskoj

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Cilj ove studije bio je da se ispita hemijski sastav eteričnih ulja (EO) dve vrste roda *Diplophyllum* i proceni potencijal bioaktivnosti [antimikrobna, antioksidantna, antiureazna, antiksantin oksidaza (KSO)] i fenolna jedinjenja. Kod *Diplophyllum albicans* i *Diplophyllum taxifolium* identifikovane su 62 komponente, koje čine ≥ 99,6% ukupnog sastava EO. Glavne komponente nađene u ovim vrstama jetrenjača su β-pačulen, β-santalen, β-himahalen, i kubebol. Antimikrobni testovi su pokazali da ekstrakti rastvarača (n-heksan i metanol) iz ovih vrsta jetrenjača pokazuju slabu do umerenu antimikrobnu aktivnost. Pored toga, metanolni ekstrakti ovih vrsta pokazali su umeren do visok antioksidativni potencijal. Enzimski inhibitorni efekti vrsta su određeni korišćenjem ureaze i KSO za metanolne ekstrakte. Generalno, metanolni ekstrakti *D. albicans* i *D. taxifolium* su pokazali snažnu inhibiciju ureaze sa IC_{50} vrednostima 9,711 ± 0,058 i 6,304 ± 0,099 μg/mL, respektivno, gde je IC_{50} standarda (acetohidroksamične kiseline) 26,124 ± 0,077 μg/mL. Pored toga, HPLC-UV detekcija je pokazala da analizirani metanolni ekstrakti ovih vrsta jetrenjača sadrže samo katehin i benzoevu kiselinu. Ovi rezultati pokazuju da analizirane vrste jetrenjača poseduju antioksidantnu aktivnost i inhibiciju ureaze; te stoga poseduju potencijal za istraživanje novih bioaktivnih molekula.

Ključne reči: *Diplophyllum taxifolium*, *Diplophyllum albicans*, sastav ulja, GC/FID/MS, biološka procena, fenolni konstituenti, HPLC-UV