Journal of Oleo Science
Copyright ©2024 by Japan Oil Chemists' Society
J-STAGE Advance Publication date : January 16, 2024
doi : 10.5650/jos.ess23055
J. Oleo Sci.

Haplophyllum tuberculatum (Forssk.) A. Juss Essential Oils: Seasonal Contents Variation, Bioactivity of the Traditionally-favored, High-yield and Frequent-use Summer Season Oil

Fatma Elshibani[1,2], Abdullah Alamami[3], Riaz Khan[4,5], Ghassan M. Sulaiman[6], and Hamdoon A. Mohammed[4,7]*

[1] *Department of Pharmacognosy, Faculty of Pharmacy, University of Benghazi, Benghazi, LIBYA*
[2] *Department of Pharmacognosy, Faculty of Pharmacy, Assalam International University, Benghazi, LIBYA*
[3] *Department of Basic Medical Sciences, Faculty of Pharmacy, University of Benghazi, Benghazi, LIBYA*
[4] *Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, Qassim University, Qassim 51452, SAUDI ARABIA*
[5] *Manav Rachna International Institute of Research and Study (MRIIRS, formerly Manav Rachna International University, MRIU), Faridabad, HR 121 001, INDIA*
[6] *Division of Biotechnology, Department of Applied Sciences, University of Technology, Baghdad, 10066, IRAQ*
[7] *Department of Pharmacognosy and Medicinal Plants, Faculty of Pharmacy, Al-Azhar University, Cairo 11371, EGYPT*

Abstract: ***Haplophyllum tuberculatum*** **(Forssk.) A.Juss. volatile oils were obtained by distillation of the aerial parts of the plant growing in Libya during the summer and spring seasons. A yield and componential analysis revealed that the summer season oil, which is frequently used in traditional medicaments by North African communities, was high in yield (0.858%) compared to the spring season oil (0.47%), and distinguished by the presence of major and various diverse constituents, some of which are considered chemical markers. Owing to the traditional and high incidence of use of the summer-produced essential oil for the treatment of several disorders, including hepatic diseases, and fatigue, the oil was pharmacologically investigated for its varied bioactivities of anti-microbial, *in vivo* anti-oxidant, and *in vitro* anti-cancer properties. Thirty-three compounds were identified and represented 96.2% of the peaks in the GC-chromatogram of the summer oil, in which the major volatile constituents were δ-3-carene (21.5%), bornyl acetate (16.9%), and limonene aldehyde (15.2%). The summer-based essential oil of the plant demonstrated moderate anti-bacterial activity against Gram-positive bacteria and a relatively strong antibacterial effect against Gram-negative bacteria as compared to the positive antibacterial controls, ampicillin and gentamicin, respectively. Also, antifungal activity against *Aspergillus* sp. was observed. The summer-produced oil also exhibited *in vivo* antioxidant and *in vitro* anti-cancer activities.**

Key words: cytotoxicity, antimicrobial activity, antioxidant activity, essential oil, GC-MS, glutathione level, *Haplophyllum tuberculatum*, seasonal oil

1 Introduction

Haplophyllum, a large plant genus that falls into the family Rutaceae, comprises 161 genera and over 1900 species and is distributed worldwide. Its representatives are found in tropical and subtropical regions, particularly in Asia, the Far East, the Middle East, and the African and Australian continents[1]. The family consists of economically significant plants that are used for several purposes, including medicine, food, vegetable production, and seminal agricultural products. The plants have ornamental uses, together with medicinal purposes, particularly essential oil from some species[2]. There are about 70 major plant species in the family that are encountered in the MENA (Middle East and North Africa) regions, China, and Siberia[3]. The family's plants primary distinguishing trait is the existence of secretory cavities, which are found mostly in the reproductive organs and the leaves[4]. In MENA countries, the commonly encountered plant species, *H. tu-*

*Correspondence to: Hamdoon A. Mohammed, Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, Qassim University, SAUDI ARABIA
E-mail: ham.mohammed@qu.edu.sa ORCID ID: https://orcid.org/0000-0003-2896-6790
Accepted December 4, 2023 (received for review March 28, 2023)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/ http://mc.manusriptcentral.com/jjocs

berculatum, is often used for treatment of headaches, arthritis, abdominal flatulence, and muscle spasms[5]. It is also used as a rectal suppository preparation by new mothers after childbirth[6]. In addition, *H. tuberculatum* is traditionally used in Libya as an antiseptic against injuries, ulcers, symptomatic liver disorders, and as a calming and refreshing agent. The plant is also a good sleep inducer[7]. It is also used for hyperglycemia, fever, and inflammation[7]. It has also found use as an anthelmintic, for reducing body weight, and relieving gastrointestinal disorders such as constipation, flatulence and gas release[7]. The plant is frequently used as a hypotensive medicament and to relive menstrual pain and common cold symptoms, including flu, throat inflammation, tonsillitis, and cough[7].

As part of the chemical investigations, the analysis of the plant's constituents showed several classes of secondary metabolites, representing various chemical constituents, including phenolics and alkaloids, as well as essential oils, resins, and other chemical ingredients from the stem, leaves, flowers, and fruits of the plant[8]. The *Haplophyllum* species, *i.e.*, *H. tuberculatum*, also termed as, *Ruta tuberculata* Forssk. is mostly endemic to the regions of Libya[9], and is found scattered in the Mediterranean region[10]. Among the alkaloids, the presence of flindersine, γ-fagarine, (+)-tuberine, haplotubinone, haplotubine, and quinolone-type compounds was established[11]. Among non-volatile constituents, *e.g.*, lignan, furocoumarin, coumarin, and flavonoids were identified from the plant[8].

An extensive literature survey revealed that the plant, *H. tuberculatum*, contained high concentrations of essential oils as well. The GC-MS examination of the volatile oil of *H. tuberculatum* exhibited β-phellandrene as the predominant constituent[12]. The same plant from other parts of the world showed considerable variations in the oils' constituents, oils' concentrations, and oil yields from the plant, which varied for various reasons, including seasonal, environmental, biotic, and abiotic factors[13].

With the plant being held in high esteem by the locals for being extremely beneficial to human health, it was pertinent to investigate the oil's pharmacological activities in conjunction with its traditional uses, as the oil is frequently used by the public and traditional healers. The study focused on investigating the impact of seasonal variations in the oils for the summer and spring seasons through plant material collection at these times and observing the yields and quality of the seasonally produced oil together with its biological activity. Hence, the current investigation planned to ascertain the differences in the oils' yields and oils' quality through the variability of the constituents obtained in different seasons, *i.e.*, the summer and spring. Owing to the folklore prestige of the plant for the use of its different parts and the essential oil obtained from it, an extensive biological activity examination of the plant oil was also undertaken. The traditionally preferred as well as high-yield and distinct fragrance note of the summer oil implied that it be examined for its broad chemical diversity in terms of the oil's constituents and suggested its bioactivity evaluation coinciding with its broad ethno-medicinal use. The cutting-edge component of the current study is the evaluation of the extracted summer oil's biological activity as an *in vivo* antioxidant, antibacterial, and anticancer agent.

2 Materials and Methods

2.1 Plant materials

Two separate batches of the aerial portions of *Haplophylum tuberculatum* (Forssk.) A.Juss. fresh plants were collected from the western region of Libya during late March (spring) and July (summer). Dr. Houssein Eltaguri, faculty member, Department of Botany, Faculty of Science, University of Benghazi, identified the plant material, and a sample was deposited in the departmental herbarium (# 1-10604).

2.2 Procurement of essential oil from *H. tuberculatum*

Hydro-distillation was performed on fresh plant samples of *H. tuberculatum* aerial parts. The collected plant samples (1.2 kg) in both seasons were separately subjected to the distillation process through the Clevenger apparatus for 5 continuous hours[14]. Oil yields were estimated according to the initial dry weight of the plant material (v/w). The obtained oil, after drying with anhydrous sodium sulphate, was kept in a refrigerator for subsequent examinations.

2.3 Gas chromatography-Mass spectroscopy study (GC-MS)

The volatile oils obtained from both batches (summer and spring) were subjected to GC analysis using an Agilent GC-MS instrument (6890 model). The separations of the oil constituents were run on the fused-silica column (30 m, 0.25 mm, film thickness 0.25 μm) composed of cross-linked 5% phenyl polysiloxane (HP-5MS, USA, Hewlett Packard) as the stationary phase material. The temperature was risen from 80℃ to 260℃ with an average of 8℃/min. The ultimate temperature was sustained for 15 minutes. The ionization energy was 70 eV, the quadrupole was at 150℃, the ion source at 230℃, and the flow rate of the carrier gas was maintained at 0.1 mL/min. The scan range was 40–500 m/z at 3.62/scan. For identification, the fragmentation and retention indices of oil components were analyzed using a standard with a sequence of alkanes (C8-C40). The retention indexes reported for *H. tuberculatum* volatile constituents besides the reported retention indexes obtained for the analysis of different essential oils under similar GC experimental conditions, were also used for the identification of the plant oil constituents. The constituents were also

identified by comparing their mass spectral fragmentation pattern with the NIST database (John Wiley Library, # 229119).

2.4 *In vivo* anti-oxidant activity

Adult male Sprague-Dawley rats weighing 150–180 g were utilized in the test. All the animals were housed according to the recommended hygienic standards and fed a typical laboratory diet. The antioxidant activity of *H. tuberculatum* essential oil was measured in alloxan-induced diabetic rats by measuring blood glutathione levels, with vitamin E serving as the standard control. The rats were randomly distributed among 4 groups (G1-G4, n = 6). The G1 served as a negative control group (non-diabatic normal group). Diabetes mellitus was induced in other groups (G2 to G4) by using the literature method[15]. The second group of animals (G2, untreated) served as the diabetic reference group. The G3 and G4 groups served as treatment groups with the standard, vitamin E, and plant essential oil. The serving dose of the plant's essential oil was 50 mg per kg of the animals' body weight as a daily dose for one week. Following one week of treatment, blood samples were collected in accordance with the known procedure to determine glutathione levels[16].

2.5 Antimicrobial activity

The summer-produced essential oil was evaluated for its antimicrobial activity against *Proteus vulgaris* RCMB010085, *Escherichia coli* RCMB010056, and *Pseudomonas aeruginosa* RCMB010043 (Gram-negative); *Streptococcus pyrogens* RCBM010015, *Staphylococcus aureus* RCMB010027, and *Bacillus subtilis* RCBM010067 (Gram-positive) bacteria; and the fungal strains *Aspergillus niger* RCMB02542, together with *Candida albicans* RCMB05035, using a modified well-diffusion method[17]. For bacterial strains, the bacteria were sub-cultured on brain heart infusion agar. The agar was streaked with the organisms' respective broth cultures (0.5 McFarland solution which corresponded to the microbial concentration of 10^8 CFU/mL[18]), and left for 15 min for the absorption to occur. The end of a sterile Pasteur pipette (5 mm in diameter) was used to make wells in agar, and 10 μL of the oil was poured into each hole. For fungi, wells were made on sabouraud dextrose agar. Gentamicin (100 μg/mL) and ampicillin (100 μg/mL) were employed as antibacterial standard agents, while amphotericin B (250 μg/mL) was used as a standard for antifungal activity. DMSO (at a concentration of 1% v/v) was used as a negative control. After 24 h of incubation at 37℃ in case of bacteria and 48 h at 25℃ for the fungi. The diameters of the inhibition zones were measured in millimeters by measuring the diameter of circular inhibition zones around the well using a pachymeter, and data were expressed as mean ± SD of three independent experiments[19].

2.6 Cytotoxicity assay using sulforhodamine B staining

The cytotoxic properties of the summer-produced essential oil obtained from the aerial parts of the plant was *in vitro* investigated according to the reported method[20]. Four human carcinoma cell lines, *i.e.*, liver cell line (HEPG2), breast cell line (MCF7), larynx cell line (HEP2), and colon cell line (HCT116), were investigated for ascertaining the cytotoxicity. The cells were cultured in DMEM medium supplemented with fetal bovine serum (10%) and streptomycin/penicillin (1%) under the conditions of 5% CO_2 and 95% air at 37℃ in T 25 cm^2 tissue culture flasks. For cytotoxicity, and cells growth assays, cells were seeded in a 96-well plate at a density of 1×10^4 cells/well and incubated for 24 h before being treated with the oils to allow the cell attachment to the surface of plate wall. Different concentrations of each essential oil (0, 1, 2.5, 5, and 10 mg/mL) were added to the cells. Each concentration was performed in triplicate. Cells were incubated for 48 h under the above-mentioned conditions. After 48 h of incubation, cells were fixed with ethanol, washed with phosphate buffer saline, and stained with sulfo-Rhodamine B stain. The exogenous stain was removed by washing with acetic acid, and the attached stain was recovered by Tris-EDTA buffer. Color intensity was measured using a Biotex microplate reader. The survival curve of each cell line after the specified oil was obtained by plotting the relationship between the surviving fraction and the drug concentration. By fitting the curves to the straight-line equation, IC_{50} (the concentration that causes 50% of the cells death) was calculated. The viability percentages were calculated according to the following equation:

$$\text{Viability of cells (\%)} = (\text{Absorbance of sample} / \text{Absorbance of the control}) \times 100$$

2.7 Statistical analysis

The results of each group were stated as the mean along with their standard deviations. A one-way ANOVA was carried out to assess statistically significant changes.

3 Results and Discussion

3.1 Analyses of the essential oils: GC and GC-MS investigations

The GC-MS study of the essential oils of the aerial parts of *H. tuberculatum* revealed significant alterations in the number and nature of the constituents. It also exhibited quantitative variations in the constituents' concentrations depending on the plant material collection times (Figs. 1 and 2). The summer vegetative season was shown to have a much greater essential oil yield (0.858 percent of the weight of the fresh plant material), which dropped by the spring flowering season to 0.47 percent of the fresh plant

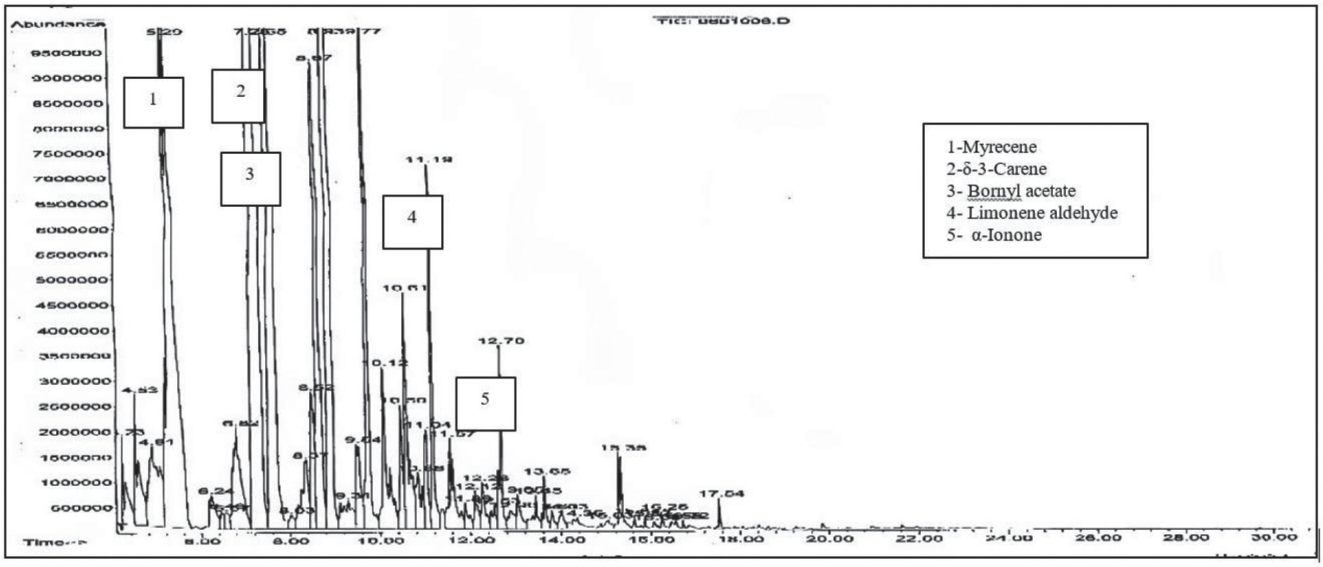

Fig. 1 GC chromatogram of the essential oil of the aerial parts of *H. tuberculatum* obtained in the summer.

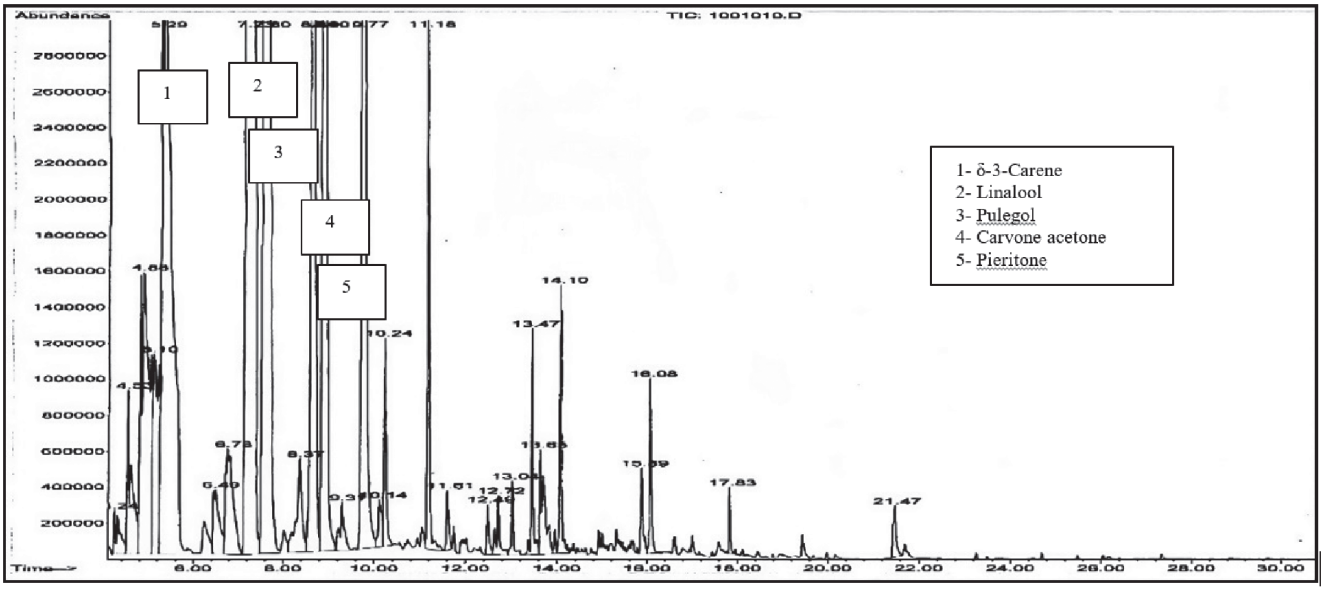

Fig. 2 GC chromatogram of the spring season's essential oil of the aerial parts of *H. tuberculatum*.

material, which dropped to 54% at the gross level for the spring-sourced produced oil.

The variability in environmental parameters, *i.e.*, climatic temperature, duration of sun exposure, and precipitation, seemed to have a direct impact on the oil yields during the two seasons[21]. Table 1 shows the average percentage of the total identified compounds in the essential oils obtained across the two seasons. The GC-MS analysis facilitated the identification of 33 compounds from the summer, and 22 compounds from the spring seasons' oils, representing 96.2%, and 92.5%, respectively, of the total constituents represented by their peaks in their respective GC-chromatograms. The oxygenated compounds were the most prevalent in the spring season oil at 72.1% composition, as compared to the non-oxygenated compounds at 20.4% for the same season. The summer season-sourced oil had different levels of oxygenated (53.1%) and non-oxygenated constituents (43.1%).

Furthermore, the summer oil sample primarily contained oxygenated monoterpenes at 46.2%, with bornyl acetate at 16.9%, and limonene aldehyde at 15.2%. On the other hand, linalool was present at 25.0% and piperitone was found to be present at 12.1% as primary components of the spring-season oil, whereas monoterpene oxygenated contents were present at 71.5% of the analyzed constituents. The oxygenated sesquiterpenes were in a lower pres-

Table 1 Identified seasonal components in the essential oils of *H. tuberculatum*.

Serial	Identified Constituents	Spring (Yield, %)	Summer (Yield, %)	KI cal.	KI rep.
1.	α-Pinene	0.3	0.9	939	939
2.	Myrecene	1.1	13.2	991	991
3.	α-Phellandrene	3.7		1003	1004
4.	β-Phellandrene	1.9	2.5	1030	1031
5.	δ-3-Carene	10.1	21.5	1031	1015
6.	Linalool oxide		0.6	1073	1081
7.	Dehydro-linalool	1.3		1091	1092
8.	Linalool	25.0	2.4	1097	1091
9.	*Neo*-dihydro carveol		2.3	1198	1193
10.	Pulegol	17.7	0.2	1215	1214
11.	Carveol	1.4	1.4	1229	1230
12.	Carvotane acetone	11.6		1247	1245
13.	Piperitone	12.1		1253	1260
14.	Carvenone	0.3		1258	1258
15.	*trans*-Carvyl acetate		1.5	1289	1288
16.	Bornyl acetate	1.7	16.9	1311	1293
17.	Limonene aldehyde		15.2	1329	1310
18.	*trans*-Piperitol acetate		2.8	1343	1343
19.	β-Isocomene		2.2	1407	1407
20.	Davana furan		0.9	1416	1414
21.	Carvone hydrate		1.0	1424	1424
22.	α-Ionone	0.4	4.3	1430	1427
23.	Neryl acetone	0.20	1.7	1436	1431
24.	β-Humulene	0.5		1436	1439
25.	Cabreuva oxide A	0.2	0.4	1447	1448
26.	bis-Ether Davana		0.5	1452	1452
27.	α-Humulene		0.3	1455	1454
28.	Cabreuva oxide D		0.4	1480	1483
29.	γ-Curcumene		0.4	1483	1483
30.	α-Amorphene		0.4	1485	1484
31.	Germacrene D	0.6	0.4	1485	1489
32.	α-Zingiberene	0.8		1494	1495
33.	γ-Amorphene	0.8		1495	1498
34.	Bicyclo-germacrene	0.6	0.3	1500	1500
35.	(*Z*)-γ-Bisabolene		0.9	1515	1529
36.	Geranyl butanoate	0.2	0.1	1564	1562
37.	*n*-Tridecanol		0.1	1572	1572
38.	Neryl isovalerate		0.1	1584	1586
39.	1-Hexadecene		0.1	1590	1600
40.	β-Capaen-4-α-ol		0.2	1591	1590
41.	*E*-β-Elemenone		0.1	1601	1594
	Oxygenated Constituents				
	Non-terpenoids	0.4	5.8		
	Monoterpenoids	71.5	46.2		
	Sesquiterpenoids	0.2	1.1		
	Non-Oxygenated Constituents				
	Non-terpenoids	0.0	0.1		
	Monoterpenoids	17.1	38.1		
	Sesquiterpenoids	3.3	4.9		
	Total	92.5	96.2		

ence in the summer and spring samples, precisely at 1.1% and 0.2%, of the identified constituents, respectively. The non-oxygenated monoterpenes were found to be in 38.1% abundance in the summer oil sample, with δ-3-carene as the predominant component at 21.5%, which was also detected in the spring-based oil sample at a relative concentration of 10.1%, nearly half of the contents. Furthermore, during the summer and spring seasons, the essential oils of *H. tuberculatum* contained minor levels of non-oxygenated sesquiterpenes ranging from 4.97% to 3.3%, respectively. In the summer, β-isocomene was the predominant non-oxygenated sesquiterpene component at 2.2%, while γ-amorphene was the major compound in the spring season oil at 0.8%. The percentage of oxygenated non-terpenoidal constituents in the summer season oil was 5.8%, and 0.4% in the spring season produced oil, which was down by 5.4% in abundance. The non-oxygenated non-terpenoidal components were at 0.1% in the summer season oil, while they were absent in the spring season oil. The environmental and climate-based factors and the seasonal metabolic changes towards adaptation by the plants in the two extreme seasons seemed to have produced an impact on the contents and quantities of the oils, thereby contributing to distinct odor and composition of the oils. Under the seasonal variations of the biotic and abiotic factors, including environmental pressor; the oils may also have used by the plants for different purposes, including pollination support, pest control, insect repellents, and physico-mechanical adaptation and responses to the climate and surroundings[22]. To the best of our knowledge, this is the first report on the effects of seasons on the chemical composition of the oil of *H. tuberculatum* growing in the MENA region, particularly in the Libyan wild.

3.2 Comparisons of the oils: Summer and spring oils from different geographic locations

The essential oils of *H. tuberculatum* revealed distinct variations in chemical constituents from two different seasons. However, the volatile oil of *H. tuberculatum* obtained in the United Arab Emirates has been reported to contain α-phellandrene (10.7–32.9%) as major constituent component, followed by limonene (9.6%), α-pinene (8.0%), δ-3-carene (6.0%), and β-caryophyllene (2.8%)[13]. The essential oil sample from Egypt was found to have δ-3-carene as the prominent component (23.3%), which coincided with our findings[13]. The volatile oil of *H. tuberculatum* grown in Iran included the primary ingredients as limonene (27.3%), α-pinene (21.9%), myrcene (6.9%), and camphene (5.2%), while δ-3-carene accounted for only 3.3% of the total oil constituents[13].

The oil obtained from the plant species growing in Oman showed α-phellandrene (23.3%) as the major constituent, followed by limonene (12.6%), (*Z*)-ocimene (12.3%), β-caryophyllene (11.6%), myrcene (11.3%), and β -phellandrene (10.9%)[12]. The essential oils of the plant growing in Algeria were collected at three different stages of the plant's development, revealing qualitative and quantitative changes between the samples' essential oil constituents. This variation included differences in the plant's main components, such as limonene, -phellandrene, -3-carene, myrcene, and piperitone[23]. Differences in the essential oil constituents of the plant were attributed to harvesting time, local climate, phenological cycle and several other variable seasonal factors. It could also be that these samples belonged to different chemotypes. However, more investigation is needed to confirm this proposition. The Table 2 demonstrated the major constituents of the oil of *H. tuberculatum* from different locations and at different seasons. Nonetheless, the *H. tuberculatum* growing in Libya achieved a high percentage of oil yields[24]. Furthermore, the reported and currently detected major constituents of *H. tuberculatum* like myrecene, α-phellandrene, β-phellandrene, δ-3-carene, linalool, piperitone, bornyl acetate, limonene, and linalyl acetate have been detected at more or less variable concentrations in other species of the plant, *e.g.*, *H. perforatum*, *H. robustum*, and *H. villosum*[25–27].

3.3 Biological activity

3.3.1 Anti-microbial activity

Based on the antimicrobial activity under the experimental conditions used and the data obtained (Table 3), it was concluded that the *H. tuberculatum* oil exhibited moderate biological activity against Gram-positive bacteria, and significant antimicrobial activity against the Gram-negative bacteria, although at a lower level than the positive control. A good level of antifungal bioactivity was also demonstrated against *C. albicans*, the effect that is consistent with the reported data for the bioactivity of the essential oil of the plant obtained from different plant sections, *i.e.*, stems and leaves, against different Candida species, *C. albicans*, *C. glabrata*, and *C. parapsilosis*[28]. Due to the seasonally variable chemical constituents nature of the essential oils, the antimicrobial activity was not correlated to any specific compound, but the presence of major phenolics, terpenes, alcohols, aldehydes, and ester based compounds were contemplated to exhibit the obtained antimicrobial activity[29]. Previous studies on the antibacterial activity of *H. tuberculatum* oil obtained from Oman showed partial growth inhibition of *E. coli* and *Bacillus subtilis*, which supported the current findings on this Libyan *H. tuberculatum* oil[12]. Another study on the *H. tuberculatum* Libyan species having flowers-based essential oils showed lowered inhibition zones in the antimicrobial activity assays than the essential oils currently obtained from the aerial parts of this plant[24]. The antifungal activity of *H. tuberculatum* may be attributed to R-(+)-limonene, S-(−)-limonene and octanol[28]. Previous work demon-

Table 2 *H. tuberculatum* essential oil constituents from different geographic locations.

Location	Yield, %	Season	Major Constituents	Refer
Oman	0.21	October	β-Phellandrene (23.3%), limonene (12.6%), (*Z*)-β-ocimene (12.3%), β-caryophyllene (11.6%), myrcene (11.3%), α-phellandrene (10.9%)	12)
Libya	0.4-1.5	April	β-Phellandrene, α-Terpinene, β-terpinene, and γ-terpinene	35)
Algeria	0.91	April	α-Phellandrene (2.1%), β-phellandrene (3.0%), terpinene-4-ol (3.2%), *p*-cymene-8-ol (2.9%), piperitone (17.8%), 2,4-bis(1,1-dimethylethyl)-phenol (28.3%), 1E,4E-germacrene-B (2.1%), hexadec-1-ene (3.2%), octadec-1-ene (2.1%)	44)
United Arab Emirates		May	α-Phellandrene (10.7–32.9%), β-caryophyllene (6.3-12.8%), β-pinene (7.6-8.0%), limonene (4.0-9.6%), δ-3-carene (5.5-6.0%), linalool (15%), linalyl acetate (10.6%), β-caryophyllene (9.7%), α-terpineol (6.7%)	13)
Saudi Arabia		May	*Trans-p-menth*-2-en-1-ol (19.2%), *cis-p*-menth-2-en-1-ol (13.2%), myrcene (10.1%), δ-3-carene (8.8%), β-phellandrene (6.9%), limonene (6.6%), *cis*-piperitol (6.4%)	45)
Egypt	0.65-0.31	June	δ-3-Carene (23.8% and 48.2%), *Cis-p*-Menth-2-en-1-ol (7-12.3%), *trans*-p-Menth-2-ene-1-ol (9.7-14.1%), and *cis*-piperitol (5.9 -10.4%)	46)
Tunisia	0.24	June	cis-p-menth-2-en-1-ol (8.7-16.8), *Trans*-p-menth-2-ene-1-ol (8.2-18.2%), β-phellandrene (14.5%), trans-piperitol (5.5-12.1), 1-octyl acetate (5.4-8.8), piperitone (6.7-14.30%), α-phellandrene (4.4%), β-myrcene (3.5%), *n*-octyl acetate (3.5%)	47,48)
Iran	0.02	June	Linalool (15.5%), α-pinene (7.9%), and limonene (5.3%)	49)

Table 3 The results of the antimicrobial testing of the essential oil of aerial parts *H. tuberculatum*.

Tested Microorganisms	Zone of inhibition (milli meter, mean ± S.D.)	
	H. tuberculatum (10 μL of the essential oil)	Standard
Fungi		Amphotericin B (250 μg/mL)
Aspergillus niger, RCMB02542	12 ± 1.2	21.9 ± 0.6
Candida albicans, RCMB05035	22 ± 1.2	26.4 ± 0.7
Gram-Positive Bacteria		Ampicillin (100 μg/mL)
Staphylococcus aureus, RCMB010027	15 ± 1.2	28.9 ± 1.2
Streptococcus pyrogens, RCBM010015	14 ± 1.2	26.4 ± 0.3
Bacilus subtilis, RCBM010067	17 ± 1.2	32.4 ± 1.2
Gram-Negative Bacteria		Gentamicin (100 μg/mL)
Proteus vulgaris, RCMB 010085	34 ± 1.2	38 ± 0.6
Pseudomonas aeruginosa, RCMB 010043	20 ± 2.1	29 ± 0.6
Escherichia coli, RCMB010056	25 ± 1.5	35 ± 0.218

Significance different from control, $p < 0.05$ n=3; summer essential oil was chosen for the antimicrobial screening because it was the most productive yield.

strated that oil purified from *H. tuberculatum* has weak fungicidal activity against *Alternaria alternata*, *Stemphylium solani*, *Curvularia lunata*, *Fusarium oxysporium*, and *Bipolaris* sp., but *Curvularia lunata* and *Bipolaris* sp. were more susceptible to the poisoning effects of the oil at higher doses. The presence of monoterpene hydrocarbons in agar medium have been demonstrated to inhibit the mycelia growths of *Curvularia pallescens* and *Fusarium oxysporium*[30]. The oil apparently has no effects on the germination of spores of the pathogenic fungi tested[12]. The variations in the antimicrobial effects of the plant essential oils obtained at three different phenological stages

Table 4 Antioxidant activity of the essential oil and vitamin E in male Sprague Dawley rats.

Animal Groups	Blood, Glutathione (mg)	% of change = Control - Result/ Control × 100
Control	36.2 ± 1.3	
Diabetic	21.3 ± 0.4	41.1
Diabetic +Vitamin E (7.6 mg/kg)	35.7 ± 0.8	1.4
Diabetic and *H. tuberculatum* (50 mg/kg)	33.6 ± 0.9	8.4

Table 5 Cytotoxic activity of the essential oil of *H. tuberculatum* (IC_{50} μg/mL).

Sample	Cell lines			
	HEPG 2	MCF 7	HEP2	HCT116
H. tuberculatum essential oil	5.91	8.91	7.9	6.3
Doxorubicin	7.0	2.4		1.9

have been reported and attributed to the changes in the minor as well as major constituents of these oil samples[23].

3.3.2 *In vivo* antioxidant activity

According to the method used, the oil sample showed considerable antioxidant potential (Table 4). The antioxidant activities exhibited by the oil is manifested by the chemical constituents present in the oil sample. The higher contents of oxygenated monoterpenes and hydrocarbons also seemed to play the part[31]. Previous results have also confirmed the terpenes antioxidant activity[32]. The activity of *H. tuberculatum* essential oils against oxidative stress was also evaluated, employing H_2O_2 as the oxidant inductor on the astrocytes which were used as the cellular model. *H. tuberculatum* essential oils also showed high scavenging activity and protected the human astrocytoma U373-MG cells against the H_2O_2 damage. The essential oils also prevented cells death, and inhibited the ROS production caused by the H_2O_2[33]. Previous study also substantiated the *in vitro* antioxidant capacity of *H. tuberculatum*. A direct relationship can be drawn between the phenolic contents of the sample and the *in vitro* antioxidant activity[34]. The current results indicated the ability of *H. tuberculatum* essential oil to restore the glutathione levels in the alloxan induced diabetic animals, and the glutathione depletion in the rats wherein the *H. tuberculatum* essential oil increased the glutathione level from 21.3 ± 0.4 mg in the untreated diabetic rats to 33.6 ± 0.9 mg after treatment with the oil sample (Table 4). However, the antioxidant restoring activity of the *H. tuberculatum* essential was found lower as compared to the activity of vitamin E (35.7 ± 0.8 mg), and also compared to the negative control group of animals (36.2 ± 1.3 mg) (Table 4).

3.3.3 Cytotoxic effects

The summer-sourced essential oil exhibited remarkable cytotoxic properties against HEPG2 cell-lines as represented by the lower IC_{50} (5.91 μg/mL) value in comparison to the standard drug, doxorubicin (IC_{50} equaled to 7.0 μg/mL) (Table 5). The findings also indicated remarkable cytotoxic effects of the oil against the MCF 7 (breast cancer, IC_{50} value of 8.91 μg/mL as compared to the 2.4 μg/mL for the doxorubicin), HEP2, IC_{50} value of 7.9 μg/mL), and HCT116 (IC_{50} value of 6.3 μg/mL compared to 1.9 μg/mL for the doxorubicin) cell lines. These finding also corroborated the previous study, which exhibited the cytotoxic potential of the *H. tuberculatum* oil against the HEPG2 and H1299[35] cells.

3.3.4 The chemobiological relationship

Current findings on the chemo-biological properties of the *H. tuberculatum* essential oils can be co-related to the oils' nature and contents which at the primary levels can be defined by the presence and compositional concentrations of several constituents present in the oil. However, attributing individual compound (s) exhibiting any specific activity is difficult. The biological effects of the oil might also be related to the synergistic effects of the specific constituents in the oil and they could also be possibly generated by one component of the major or minor presence in the oil. Therefore, the antimicrobial and cytotoxic effects of the essential oil may be attributed to the presence of δ-3-carene which is present in high concentration (21.50%), which has also been reported in another study as having cytotoxic and antimicrobial activities[36]. The cytotoxic effect of the oil might also attributed to the presence of myrecene (13.18%), which had been reported for its activity against A549 lung cancer and HEPG 2 liver cancer cell lines and represented in high concentration among cytotoxic essential oils[14, 37–39]. Furthermore, myrecene had also exhibited potential antimicrobial effects against several microbial strains including *Staph. aureus* and *A. tumefaciens*[40], and could possibly be playing

primary role in the antimicrobial activity of the currently tested oil. Several components present in the *H. tuberculatum* essential oil have been reported for their antioxidant activity. For example, linalool and bornyl acetate, which were found at 2.38% and 16.93% in the summer-produced oil of *H. tuberculatum*, have been reported for their potential antioxidant activity, and were represented as major constituent of the rosemary essential oil[41]. In addition, carveol, α-ionone and neryl acetone have been reported for their antioxidant effects[42), 43]. The antioxidant behavior of the *H. tuberculatum* oil, used as a traditional remedy for liver disorders, seemingly also contributed to liver protection, when tested against the CCl_4 generated oxidative stress.

4 Conclusion

Haplophyllum tuberculatum Forssk, an important medicinal plant also found in northern Libya, showed seasonally variable oil quality and contents. The plant has been utilized as part of local traditional medicament to treat various ailments and several symptomatic disorders of common health problems. The plant's juice has been used to treat warts, infections, skin discoloration, and parasitic infections, especially stomach worms. Since the plant's summer oil is remarkably beneficial and finds folklore-based uses, its seasonal oil contents and yields were analyzed, together with its antioxidant, antimicrobial, and cytotoxic activities. The novelty of the current work lies in the assessment of the *in vivo* antioxidant, antimicrobial, and anticancer biological activities of the produced summer oil. The summer-sourced essential oil was twice the yield of the spring season oil's yield, as well as the summer-sourced oil, which was tested owing to its high yield and preferred traditional uses, as recommended by the locals, was found biologically active as an anti-microbial, anti-oxidant, and cytotoxic agent. The yield and biological activity substantiated the traditional wisdom of using the summer-produced oil. The study also confirmed the health benefits and associated biological effects of the summer season's frequent oil as part of the medicinal chest of the local tribes. The bioactivity evaluations and chemical diversity of the summer season's oil also suggested potent applications of the oil in further developing its medicinal uses on modern pharmacological evaluations and chemical investigations lines, which have the potential of the oil constituents to be developed as future drugs. The cytotoxicity experiment that has to be performed against normal cells in order to determine the selectivity indices of essential oils from *H. tuberculatum* against various cancer cell types is one limitation of the current information. Future goals in this regard includes employing the MTT assay to determine the selectivity and anticancer activity of plant's essential oils, as well as conducting apoptotic assays and observing morphological changes in the cells under a fluorescence microscope.

Acknowledgements

Researchers would like to thank the Deanship of Scientific Research, Qassim University for funding publication of this project.

Conflict of Interest Statement

The authors declare there are no conflicts of interest.

References

1) Kubitzki, K.; Kallunki, J.A.; Duretto, M.; Wilson, P.G. *Flowering Plants: Eudicots*. Springer, pp. 276-356 (2010). doi: 10.1007/978-3-642-14397-7_16
2) ur Rehman, F. *Citrus leprosis* and its impacts on citrus food industry: A review. *EC Agric.* **6**, 34-39(2020).
3) Willis, J.C. *A dictionary of flowering plants and ferns*. Cambridge Press(1980).
4) Tekin, M.; Eruygur, N. The structural studies on the medicinal plant *Haplophyllum telephioides*. *Rev. Bras. Farmacogn.* **26**, 544-552(2016).
5) Hamdi, A.; Viane, J.; Mahjoub, M.A.; Majouli, K.; Gad, M.H.H. *et al.* Polyphenolic contents, antioxidant activities and UPLC–ESI–MS analysis of *Haplophyllum tuberculatum* A. Juss leaves extracts. *Int. J. Biol. Macromol.* **106**, 1071-1079(2018). doi.org/10.1016/j.ijbiomac.2017.08.107
6) Foster, S.; Duke, J.A. *Eastern/central medicinal plants and herbs*, in Pet, pp.18-19(2000).
7) Soumia, H.; Zahia, B.; Aminata, O.E.H.-K.; Hanane, B.; Zohra, B. *et al.* Ethnobotanical study and phytochemical screening of six medicinal plants used in traditional medicine in the Northeastern Sahara of Algeria(area of Ouargla). *J. Med. Plants Res.* **9**, 1049-1059(2015).
8) Hamdi, A.; Majouli, K.; Abdelhamid, A.; Marzouk, B.; Belghith, H. *et al.* Pharmacological activities of the organic extracts and fatty acid composition of the petroleum ether extract from *Haplophyllum tuberculatum* leaves. *J. Ethnopharmacol.* **216**, 97-103(2018). doi.org/10.1016/j.jep.2018.01.012
9) Abu-Aziza, E.B.; El-Barasi, Y.M.; Rahil, R.O. Flora, vegetation and human activities of Wadi Der-na-El-Jabal. *ContROL* **1**, 6(2017).
10) Saidi, A.; Hambaba, L.; Bensaad, M.S.; Melakhessou, M.A.; Bensouici, C. *et al.* Phenolic characterization using cLC-DAD analysis and evaluation of *in vitro* and

in vivo pharmacological activities of *Ruta tuberculata* Forssk. *Antioxidants* **11**, 1351(2022). doi.org/10.3390/antiox11071351
11) Mohammadhosseini, M.; Venditti, A.; Frezza, C.; Serafini, M.; Bianco, A.; Mahdavi, B. The genus Haplophyllum Juss.: Phytochemistry and bioactivities—A review. *Molecules* **26**, 4664(2021). doi.org/10.3390/molecules26154664
12) Al-Burtamani, S.K.S.; Fatope, M.O.; Marwah, R.G.; Onifade, A.K.; Al-Saidi, S.H. Chemical composition, antibacterial and antifungal activities of the essential oil of *Haplophyllum tuberculatum* from Oman. *J. Ethnopharmacol.* **96**, 107-112(2005). doi.org/10.1016/j.jep.2004.08.039
13) Al Yousuf, M.H.; Bashir, A.K.; Veres, K.; Dobos, Á.; Nagy, G. *et al.* Essential oil of *Haplophyllum tuberculatum*(Forssk.)A. Juss. from the United Arab Emirates. *J. Essent. Oil Res.* **17**, 519-521(2005). doi.org/10.1080/10412905.2005.9698981
14) Mohammed, H.A. Phytochemical analysis, antioxidant potential, and cytotoxicity evaluation of traditionally used *Artemisia absinthium* L.(Wormwood)growing in the central region of Saudi Arabia. *Plants* **11**, 1028(2022). doi.org/10.3390/plants11081028
15) Eliasson, S.G.; Samet, J.M. Alloxan induced neuropathies: Lipid changes in nerve and root fragments. *Life Sci.* **8**, 493-98(1969). doi.org/10.1016/0024-3205(69)90442-1
16) Beutler, E. Improved method for the determination of blood glutathione. *J. Lab. Clin. Med.* **61**, 882(1963).
17) Ezzat, S.M.S.; El-Hawary, S.; El Shabrawy, A.E.R.; El-Shibani, F. Evaluation of the phenolic and flavonoid contents, antimicrobial and cytotoxic activities of some plants growing in Al Jabal Al-Akhdar in Libya. I. *J. Pharmacogn. Phytochem. Res.* **8**, 1083-1087(2016).
18) Jasim, A.J.; Sulaiman, G.M.; Ay, H.; Mohammed, S.A.A.; Mohammed, H.A. *et al.* Preliminary trials of the gold nanoparticles conjugated chrysin: An assessment of anti-oxidant, anti-microbial, and *in vitro* cytotoxic activities of a nanoformulated flavonoid. *Nanotechnol. Rev.* **11**, 2726-2741(2022). doi/10.1515/ntrev-2022-0153/html
19) Hawar, S.N.; Al-Shmgani, H.S.; Al-Kubaisi, Z.A.; Sulaiman, G.M.; Dewir, Y.H.; Rikisahedew, J.J. Green synthesis of silver nanoparticles from *Alhagi graecorum* leaf extract and evaluation of their cytotoxicity and antifungal activity. *J. Nanomater.* **2022**, 1-8(2022). doi.org/10.1155/2022/1058119
20) Mohammed, H.A.; Abd-Elraouf, M.; Sulaiman, G M.; Almahmoud, S.A.; Hamada, F.A. *et al.* Variability in the volatile constituents and biological activities of *Achillea millefolium* L. essential oils obtained from different plant parts and by different solvents. *Arab. J. Chem.* 105103(2023). doi.org/10.1016/j.arabjc.2023.105103
21) Elshibani, F.; Alshalmani, S.; Mohammed, H.A. *Pituranthos tortuosus* essential oil from Libya: Season effect on the composition and antioxidant activity. *J. Essent. Oil Bear. Plants* **23**, 1095-1104(2020). doi.org/10.1080/0972060X.2020.1843550
22) Mohammed, H.A.; Emwas, A.-H.; Khan, R.A. Salt-tolerant plants, Halophytes, as renewable natural resources for cancer prevention and treatment: Roles of phenolics and flavonoids in immunomodulation and suppression of oxidative stress towards cancer management. *Int. J. Mol. Sci.* **24**, 5171(2023). doi: 10.3390/ijms24065171
23) Aberrane, S.; Djouahri, A.; Djerrad, Z.; Saka, B.; Benseradj, F. *et al.* Changes in essential oil composition of *Haplophyllum tuberculatum*(Forssk.)A. Juss. aerial parts according to the developmental stage of growth and incidence on the biological activities. *J. Essent. Oil Res.* **31**, 69-89(2019). doi.org/10.1080/10412905.2018.1511483
24) Sabry, O.M.; El Sayed, A.M.; Slee, A.A. Potential antimicrobial, anti-inflammatory and anti-oxidant activities of *Haplophyllum tuberculatum* growing in Libya. *J. Pharmacogn. Nat. Prod.* **2**, 1-5(2016).
25) Gholivand, M.B.; Rahimi-Nasrabadi, M.; Batooli, H.; Samimi, H. Chemical composition and antioxidant activity of the essential oil and various extracts of *Haplophyllum robustum* Bge. *Nat. Prod. Res.* **26**, 883-891(2012). doi.org/10.1080/14786410903374462
26) Mohammadhosseini, M.; Nekoei, M.; Mashayekhi, H.A.; Aboli, J. Chemical composition of the essential oil from flowers, leaves and stems of *Haplophyllum perforatum* by using head space solid phase microextraction. *J. Essent. Oil Bear. Plants* **15**, 506-515(2012). doi.org/10.1080/0972060X.2012.10644080
27) Azadi, B.; Khaef, S.; Ziarati, P. Chemical composition of *Haplophyllum villosum*(M. B.)G. Don essential oil. *J. Essent. Oil Bear. Plants* **17**, 1161-1164(2014). doi.org/10.1080/0972060X.2014.958556
28) Hamdi, A.; Majouli, K.; Flamini, G.; Marzouk, B.; Marzouk, Z.; Heyden, Y.V. Antioxidant and anticandidal activities of the Tunisian *Haplophyllum tuberculatum*(Forssk.)A. Juss. essential oils. *South African J. Bot.* **112**, 210-214(2017). doi.org/10.1016/j.sajb.2017.05.026
29) Knobloch, K.; Pauli, A.; Iberl, B.; Weigand, H.; Weis, N. Antibacterial and antifungal properties of essential oil components. *J. Essent. Oil Res.* **1**, 119-128(1989).
30) Singh, G.; Singh, O.P.; Maurya, S. Chemical and biocidal investigations on essential oils of some Indian Curcuma species. *Prog. Cryst. Growth Charact. Mater.* **45**, 75-81(2002).
31) Bhavaniramya, S.; Vishnupriya, S.; Al-Aboody, M.S.;

Vijayakumar, R.; Baskaran, D. Role of essential oils in food safety: Antimicrobial and antioxidant applications. *Grain Oil Sci. Technol.* **2**, 49-55 (2019). doi.org/10.1016/j.gaost.2019.03.001
32) Baccouri, B.; Rajhi, I. Potential antioxidant activity of terpenes. *Terpenes Terpenoids-Recent Adv*. 53-62 (2021).
33) Eissa, T.F.; González-Burgos, E.; Carretero, M.E.; Gómez-Serranillos, M.P. Compositional analysis and *in vitro* protective activity against oxidative stress of essential oils from Egyptian plants used in traditional medicine. *Nat. Prod. Commun.* **9**, 1934578X1400900939 (2014). doi.org/10.1177/1934578X140090093
34) Al-Brashdi, A.S.; Al-Ariymi, H.; Al Hashmi, M.; Khan, S.A. Evaluation of antioxidant potential, total phenolic content and phytochemical screening of aerial parts of a folkloric medicine, *Haplophyllum tuberculatum* (Forssk) A. Juss. *J. Coast. Life Med.* **4**, 315-319 (2016).
35) Sabry, O.M.M.; El Sayed, A.M.; Alshalmani, S.K. GC/MS analysis and potential cytotoxic activity of *Haplophyllum tuberculatum* essential oils against lung and liver cancer cells. *Pharmacogn. J.* **8**, 66-69 (2016).
36) Ben Salem, S.; Znati, M.; Jabrane, A.; Casanova, J.; Ben Jannet, H. Chemical composition, antimicrobial, anti-acetylcholinesterase and cytotoxic activities of the root essential oil from the Tunisian *Ferula lutea* (Poir.) Maire (Apiaceae). *J. Essent. Oil Bear. Plants* **19**, 897-906 (2016).
37) Orlando, J.B.; Silva, B.O.; Pires-Cunha, C.L.; Hiruma-Lima, C.A.; Gaivão, I.O.; de M., Maistro, E.L. Genotoxic effects induced by beta-myrcene following metabolism by liver HepG2/C3A human cells. *J. Toxicol. Environ. Heal. Part A* **82**, 176-185 (2019). doi.org/10.1080/15287394.2019.1577195
38) Bai, X.; Tang, J. Myrcene exhibits antitumor activity against lung cancer cells by inducing oxidative stress and apoptosis mechanisms. *Nat. Prod. Commun.* **15**, 1934578X20961189 (2020).
39) Mohammed, H.A.; Eldeeb, H.M.; Khan, R.A.; Al-Omar, M.S.; Mohammed, S.A.A. *et al.* Sage, *Salvia officinalis* L., constituents, hepatoprotective activity, and cytotoxicity evaluations of the essential oils obtained from fresh and differently timed dried herbs: A comparative analysis. *Molecules* **26**, 5757 (2021). doi.org/10.3390/molecules26195757
40) Rasoul, M.A.A.; Marei, G.I.K.; Abdelgaleil, S.A.M. Evaluation of antibacterial properties and biochemical effects of monoterpenes on plant pathogenic bacteria. *Afr. J. Microbiol. Res.* **6**, 3667-3672 (2012).
41) Mohammed, H.A.; Al-Omar, M.S.; Mohammed, S.A.A.; Aly, M.S.A.; Alsuqub, A.N.A.; Khan, R.A. Drying induced impact on composition and oil quality of rosemary herb, *Rosmarinus officinalis* Linn. *Molecules* **25**, 2830 (2020). doi.org/10.3390/molecules25122830
42) Serafim, C.A.D.L.; Araruna, M.E.C.; Alves Júnior, E.B.; Silva, L.M.O.; Silva, A.O. *et al.* (-)-Carveol prevents gastric ulcers via cytoprotective, antioxidant, antisecretory and immunoregulatory mechanisms in animal models. *Front. Pharmacol.* **12**, 736829 (2021). doi.org/10.3389/fphar.2021.736829
43) Marumoto, S.; Okuno, Y.; Miyazawa, M. Inhibition of β-secretase activity by monoterpenes, sesquiterpenes, and C13 norisoprenoids. *J. Oleo Sci.* **66**, 851-855 (2017). doi.org/10.5650/jos.ess16188
44) Mechehoud, Y.; Chalard, P.; Figuérédo, G.; Marchioni, E.; Benayache, F.; Benayache, S. Chemical composition of the essential oil of *Haplophyllum tuberculatum* (Forssk.) LA Juss. from Algeria. *Res. J. Pharm. Biol. Chem. Sci.* **5**, 1416-1419 (2014).
45) Al-Rehaily, A.J.; Alqasoumi, S.I.; Yusufoglu, H.S.; Al-Yahya, M.A.; Demirci, B. *et al.* Chemical composition and biological activity of *Haplophyllum tuberculatum* Juss. essential oil. *J. Essent. Oil Bear. Plants* **17**, 452-459 (2014).
46) El-Naggar, E.B.; El-Darier, S.M.; Abdalla, A.; El-Mekanen, S.; Švajdlenka, E.; Zemlièka, M. Chemical composition of essential oil of *Haplophyllum tuberculatum* (Rutaceae) grow wild in different habitats of Egypt. *Glob. J. Pharmacol.* **8**, 385-393 (2014).
47) Sriti, J.; Mejri, H.; Bachrouch, O.; Hammami, M.; Liman, F. Antioxidant activity and chemical constituents of essential oil and extracts of *Haplophyllum tuberculatum* from Tunisia. *J. New Sci. Agric. Biotechnol.* **43**, 2373-2381 (2017).
48) Hamdi, A.; Majouli, K.; Vander Heyden, Y.; Flamini, G.; Marzouk, Z. Phytotoxic activities of essential oils and hydrosols of *Haplophyllum tuberculatum*. *Ind. Crops Prod.* **97**, 440-447 (2017). doi.org/10.1016/j.indcrop.2016.12.053
49) Javidnia, K.; Miri, R.; Banani, A. Volatile oil constituents of *Haplophyllum tuberculatum* (Forssk.) A. Juss. (Rutaceae) from Iran. *J. Essent. Oil Res.* **18**, 355-356 (2006).