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Original Article

GC/MS Analysis and Potential Cytotoxic Activity of *Haplophyllum tuberculatum* Essential Oils Against Lung and Liver Cancer Cells

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ABSTRACT

Background: *Haplophyllum tuberculatum* is a plant belongs to family rutacee. It is rich in volatile oils, fixed oils, alkaloids, and furanocoumarins. It is well known for the huge number of folkloric uses in middle east. **Objective:** The aim of this study is to establish the chemical composition of the essential oils of Libyan *H. tuberculatum* and to investigate their cytotoxic potentialities.**Materials and Methods:** The essential oils of the aerial parts and flowers of *H. tuberculatum* growing in Libya were prepared by hydrodistillation. GC/MS analyses were performed on a Shimaduz capillary gas chromatograph (GC 17A ver.3) instrument directly coupled to mass spectrometer-MS QP5050A. Oil A and F of *H. tuberculatum* at different concentrations (0-50 µg/ml) in DMSO were tested for cytotoxicity against human tumor cell lines. **Results:** Oil yield was found 0.4 and 1.5 (v/w %) on dry weight basis respectively. GC/MS analysis resulted in identification of total 35 compounds. 15 compounds were common to both oils. oil A of *H. tuberculatum* exhibited antitumor activities against. liver carcinoma cell line (HEPG2) and lung carcinoma cell line (H1299) 4.7 µg/ml and 4.1 µg/ml. **Conclusion:** Essential oil of the aerial parts of *H. tuberculatum* is potentially active against lung (H-1299), and liver (HEPG2) carcinoma cell lines. The observed cytotoxic, activities can be attributed to the dominance of α and γ-terpinene in this oil.

Key words: *Haplophyllum tuberculatum*, Essential oils, Cytotoxicity, Lung cancer, Liver cancer.

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INTRODUCTION

In Egypt the flowering aerial parts of *H. tuberculatum* are used as a drink to relieve fever, for abdominal upset, anemia, gastric pains, intestinal worms, malaria, as aphrodisiac, and the decoction is used for rheumatic pains.[1] In Oman, the leaves are used as a remedy for headaches and arthritis and also used for treatment of skin infections, discoloration and parasitic diseases.[2] In Saudi Arabia, *H. tuberculatum* is used to treat malaria, rheumatoid arthritis and gynecological disorders,[3] while in Sudan the herb is used as an antispasmodic, to treat allergic rhinitis and breathing difficulties.[4] The plant is rich in alkaloids, fixed oils, volatile oils and furanocoumarins.[5-8] No published report was recorded concerning the analysis of the volatile oil of the aerial parts and the flower of Libyan *H. tuberculatum* and their biological potentialities.

MATERIALS AND METHODS

Plant material

Samples of the aerial parts and flowers of *Haplophyllum tuberculatum* (Forssk) A. Juss (*Rutaceae*) were obtained from Benghazi, Libya, identified by Dr. Reem Samir Hamdy, Lecturer of Plant Taxonomy, Botany Department, Faculty of Science, Cairo University, Giza, Egypt. A voucher specimen of the aerial parts and the flowers of *H. tuberculatum*, were kept in the herbarium of Department of Pharamacognosy, Faculty of Pharmacy, Cairo University as a reference material specimen No. 2015224.

Preparation, characterization and analysis of the essential oil

Fresh samples of aerial parts and flowers of *H. tuberculatum* (500 g) were subjected separately to hydro-distillation. The percentage yield was calculated on dry weight (v/w) basis of the plant materials. The essential oil was dried over anhydrous sodium sulfate and kept refrigerated until analysis.

Sample preparation for GC/FID and GC/MS analysis

Samples prepared by mixing 5µl of dehydrated essential oil with approximately one ml of dichloromethane in auto sampler vials. Injection volume was 1 µl injected directly to GC-FID and GC-MS.

Gas chromatography-mass spectrometry analysis (GC/MS)

GC/MS analyses were performed on a Shimaduz capillary gas chromatograph (GC 17A ver.3) instrument directly coupled to mass spectrometer-MS QP5050A. Capillary column SLB-5ms (30 m x 0.25 mm, film thickness 0.25 µm) was used under the following condition: oven temperature programmed from 40°C (3 min), then temperature increased at rate 12°C/min to 180°C where it is hold for 5 min, then temperature increased at rate 40°C/min to reach 240°C, where it is finally hold for 5 min. The injector temperature was 240°C and was set at split ratio 1:54. Carrier gas was He at flow rate 0.9 ml/min. The mass spectrometer operated on electronic (EI) ionization mode at 70 eV with scan range 40-500. The transfer line temperature was 230°C.

Gas chromatography analysis (GC-FID)

The GC analysis was carried out using Shimaduz GC gas chromatograph (GC-17 ver.3) system. FID detector temperature was 240°C. To obtain the same elution order with GC/MS simultaneous auto injection was done on a duplicate of the same operational conditions. Identification of the essential oil components were carried out by comparing their relative retention times with those of authentic samples or by comparing their relative retention indices (RRI). The later were computed using a mixture of a continuous series of *n*-alkane hydrocarbons (C4-C28) run on SLB-5ms (non-polar) column using the same conditions as described above. The components of the oils were fully unambiguously identified by their mass spectral fragmentation patterns with those reported in computerized MS-data bank spectral libraries (NIST and WILEY)[10] or reported in the literature.[11-12] The compounds are arranged in order to GC elution on SLB-5ms capillary column. Relative percentages were calculated based on the GC-FID peak areas without the use of correction factors and are compiled.

In vitro screening for cytotoxic activity

Human tumor cell lines: lung carcinoma cell line (H-1299) and liver carcinoma cell line (HEPG2), maintained in the laboratory of the

Cancer Biology Department of National Cancer Institute, Cairo, Egypt, were used. Oil A and F of *H. tuberculatum* at different concentrations (0-50.000 µg/ml) in DMSO were tested for cytotoxicity against the forementioned human tumor cell line adopting sulforhodamine B stain (SRB) assay.[13] The relation between surviving fractions and oil concentration was plotted to get the survival curve of each tumor cell line after the application of the specific concentration. The results were compared to those of the standard cytotoxic drug, Doxorubicin (10 mg Adriamycin hydrochloride, in 5 ml IV injection, Pharmacia, Italy) at the same concentrations. The dose of the test solutions which reduces survival to 50% (IC_{50}) was calculated.

Determination of median lethal dose LD_{50}

The LD_{50} of the volatile oils was determined according to the procedures developed by Karber (1941).[14]

Drugs and chemicals

Doxorubicin (10 mg Adriamycin hydrochloride, in 5 ml IV injection, Pharmacia, Italy).

Statistical analysis

All data were expressed as mean ± SE and the statistical significance was evaluated using the ANOVA test followed by Duncan's multiple range tests.

RESULTS

GC/MS analysis of essential oils

Hydro distillation of the aerial parts and flowers of *H. tuberculatum* yielded 0.4 and 1.5 % v/w of clear yellow colored oil exhibiting a characteristic agreeable odor. The specific gravity and refractive index were 0.975, 0.968 and 1.487, 1.495, respectively. GC-MS analysis of different oil samples of *H. tuberculatum* revealed relevant quantitative and qualitative variability. The components were categorized according to their chemical nature and listed in Table 1. The oils of the aerial parts (A) and flowers (F) of *H. tuberculatum* showed approximately similar amounts of hydrocarbons and oxygenated constituents The lower percentage of hydrocarbons was recorded for the oil F (78.28%). Meanwhile, the highest percentage of hydrocarbon constituents was found in oil A (89.82%).

Terpene hydrocarbons were detected in appreciable amounts in oil A (82.34%). Monoterpenes prevailed in the sample A (75.78%) while, in sample F (72.81%) with the major α-terpinene ranged from (26.40-24.45%) and β-terpinene ranged from (17.13-14.40%), respectively. Sesquiterpenes e.g. Zingiberine and β-sesquiphellandrene were detected in oil A in traces only (0.36%) and (0.03%). Other monoterpene hydrocarbons constituents detected in high amounts were: α-pinene (1.43-1.33%), β-myrcene (5.69-6.05%) and 3-carene (3.87-5.43%), respectively. The profile of both samples were characterized by the presence of β-phellandrene (10.0-10.40%), 3,4-dimethyl-1,5-cyclooctadiene (6.03-6.67%) and β-myrecene (5.69-6.04%). In oil A Oxygenated monoterpenes were represented by 1,8 cineole (1.67%) and piperitone (5.55%).

Evaluation of cytotoxic activity

On assessing the cytotoxic activity under the experimental condition adopted and from data displayed in Table 2 it could be concluded that the oil A of *H. tuberculatum* exhibited variable antitumor activities against the two tested cell line *viz.* liver carcinoma cell line (HEPG2) and lung carcinoma cell line (H1299). In this respect, the oil A of *H. tuberculatum* demonstrated the lowest IC50% when tested against H-1299 and HEPG2 cells (4.7 µg/ml and 4.1 µg/ml) respectively.

On the other hand, the oil F showed relatively higher IC50% against H-1299 and HEPG2 cells (42.3 µg/ml and 19.7 µg/ml) respectively, which is comparable to the standard cytotoxic drug, doxorubicin (Table 3-4). Results obtained during assessment of the cytotoxic potential of

Table 1: Percentage composition of essential oil of the aerial parts and flowers of *H. tuberculatum*

No.	Compound	* RI	A (%)	F (%)
1	1-Butanol-3-methylacetate	880	0.52	0.77
2	α-Thujene	929	0.32	-
3	α-Pinene	937	1.43	1.33
4	Pentanol	972	-	0.02
5	Cyclohexen,1-methy-4-(1-ethylethyldiene	977	1.12	-
6	β-Myrcene	990	5.69	6.05
7	Pentane-2,2-dimethyl	1003	0.08	-
8	Octanal	1005	0.36	-
9	Isovaleric acid isobutyl ester	1010	0.33	-
10	α –Phellandrene	1010	-	2.33
11	3-Carene	1013	3.87	5.43
12	Cineol 1,4	1020	3.82	0.08
13	Iso-terpinolene	1021	-	0.20
14	2-Pentanone ethylacetone	1025	0.54	0.28
15	Acetophenone-4`-methyl	1029	-	0.27
16	Cyclooctadiene-3,7-dimethyl	1034	6.03	6.66
17	β-phellandrene	1036	10.40	10.00
18	Eucalyptol	1039	1.67	0.70
19	Cis B-Ocimene	1049	0.39	0.42
20	1-Cyclo propyl pentane	1074	0.42	0.25
21	Non-2-en-1-ol	1077	-	0.49
22	n-amyl iso valerate	1099	0.27	0.43
23	Linaloal	1101	-	1.38
24	Butyric acid-2-methyl	1103	-	0.52
25	Isovaleric acid iso pentyl ester	1105	-	2.32
26	n-amyl isovalerate	1108	-	3.23
27	Octanol (n-octan-1-ol)	1130	-	0.50
28	α-Terpinen	1133	26.40	24.45
29	β-Terpinen	1151	17.13	14.40
30	Ocimenol	1175	0.03	0.05
31	Trans piperitol	1207	-	4.66
32	γ-Terpinen	1219	9.07	7.76
33	Piperitone	1267	5.55	2.07
34	(-)-Zingiberene	1505	0.36	-
35	β-Sesquiphellandrene	1540	0.04	-
	Identified components		95.83	97.05
	Monterpenes hydrocarbons		82.34	72.81
	Oxygenated monoterpenes		13.09	7.51
	Sesquiterpens hydrocarbons		0.40	0.00
	Oxygenated sesquiterpenes		0.00	0.00
	Aliphatic hydrocarbons		6.58	6.47
	Aliphatic oxygenated compounds		1.51	9.97
	Oxygenated components		13.09	17.77
	Non-oxygenated components		82.74	79.28

Components are listed in order of their elution. RI*: Relative retention index on SLB-5ms column.

Table 2: Results of cytotoxic activity of the essential oil of *H. tuberculatum*

Tested Solutions	IC_{50} µg/ml	
	Lung carcinoma H1299	Liver carcinoma HEPG2
Essential oil A	4.7	4.1
Essential oil F	42.3	19.7
Standard drug (Doxorubicin)	3.70	7.00

Table 3: Drug cytotoxicity of *H.tubrtculatum* on Liver carcinoma cell line HEPG-2

Conc µg/ml	Essential oil A	Essential oil F	Doxorubicin
	Mean ± SD		
0.000	1.000 ± 0.047	1.000 ± 0.047	1.000 ± 0.009
5.000	0.407 ± 0.050	0.857 ± 0.055	0.536 ± 0.017
12.000	0.311 ± 0.065	0.602 ± 0.060	0.441 ± 0.017
25.000	0.323 ± 0.050	0.423 ± 0.029	0.377 ± 0.017
50.000	0.355 ± 0.036	0.380 ± 0.055	0.338 ± 0.011

N=6

Table 4: Drug cytotoxicity of *H.tubrtculatum* on Lung carcinoma cell line H-1299

Conc µg/ml	Essential oil A	Essential oil F	Doxorubicin
	Mean ± SD		
0.000	1.000 ± 0.050	1.000 ± 0.050	1.000 ± 0.057
5.000	0.465 ± 0.033	0.593 ± 0.041	0.353 ± 0.014
12.000	0.224 ± 0.041	0.597 ± 0.044	0.339 ± 0.007
25.000	0.181 ± 0.012	0.639 ± 0.075	0.303 ± 0.023
50.000	0.230 ± 0.032	0.552 ± 0.067	0.369 ± 0.011

N=6

the oil *H. tuberculatum* are in accordance with formerly reported data related to γ-terpinene.[15]

Determination of median lethal dose LD_{50}

The 24-hours LD_{50} was approximately more than 0.05 ml/kg b. wt. for the essential oils. These results showed that the essential oils are safe and non-toxic.

To the best of our knowledge, this is the first report on the chemical composition of the essential oil of Libyan *H. tuberculatum*. Nevertheless, the composition of the essential oil from *H. tuberculatum* grown in different regions of Egypt was previously studied.[16] Monoterpenes are non-nutritive dietary components found in the essential oils of herbs. γ-terpinen, the major component of the oil in the present work (26-38%) was detected only in trace amounts in the essential oil obtained from *H. tuberculatum* in other countries.[17-19] On the contrary, limonene (absent in the Libyan sample), linalool, and beta caryophyllene, were present in major quantities (12.8 %, 15.5% and 12.77%) in the essential oil of the *H. tuberculatum* of Iran, United Arab Emirates, Oman and Iran respectively.[17-19] Moreover, the hydrocarbons, β-phellandrene, α-pinene, and 3-carene were present in Libyan and Egyptian samples in comparable amounts. The difference in oil yield obtained and the composition of essential oil in plants were affected by genetical and environmental conditions, which is determined by growth region and harvesting time in terms of onto genetical and diurnal variability.

On assessing the cytotoxic activity under the experimental condition adopted and from data displayed in Table 3 it could be concluded that the oil A of *H. tuberculatum* exhibited variable antitumor activities against the two tested cell line *viz,* liver carcinoma cell line (HEPG2) and lung carcinoma cell line (H1299). In this respect, the oil A of *H. tuberculatum* demonstrated the lowest IC50% when tested against H-1299 and HEPG2 cells (4.7 µg/ml and 4.1 µg/ml) respectively. On the other hand, the oil F showed relatively higher IC50% against H-1299 and HEPG2 cells (42.3 µg/ml and 19.7 µg/ml) respectively, which is comparable to the standard cytotoxic drug, doxorubicin (Table 3-4). Results obtained during assessment of the cytotoxic potential of the oil *H. tuberculatum* are in accordance with formerly reported data related to γ-terpinen.[15] The study was the first concerning the cytotoxic activity of the essential oils of *H. tuberculatumon* tumor cell lines.

CONCULSION

In conclusion, this report was the first paper indicating the chemical composition and investigating the cytotoxic potentialities of the essential oils of Libyan *H. tuberculatum*. It is found that the essential oils of the aerial parts of *Halpophyllum tuberculatum* are potentially active against lung (H-1299), and liver (HEPG2) carcinoma cell lines. The observed cytotoxic, activities can be attributed to the dominance of α and γ-terpinene.

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CONFLICT OF INTEREST

The authors report no declarations of interest.

ABBREVIATION USED

A: The essential oils of the aerial parts, **DMSO:** Dimethyl sulfoxide, **F:** The essential oil of the flower, **GC/FID:** Gas chromatography/Flame ionization detector, **GC/MS:** Gas chromatography/Mass spectrometry analysis, **IC_{50}%:** The dose of the test solutions which reduces survival to 50%, **RI:** Retention indices, **R_t:** Retention times, **SD:** Standard deviation, **SRB:** Sulforhodamine B stain assay.

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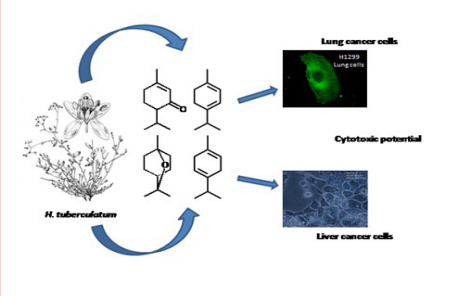
PICTORIAL ABSTRACT



SUMMARY

- GC/MS analyses of the aerial parts and flowers of *H. tuberculatum* growing in Libya were performed.
- Analyses resulted in identification of total 35 compounds. 15 compounds were common to both oils.
- Oils at different concentrations (0-50 µg/ml) in DMSO were tested for cytotoxicity against human tumor cell lines.
- Essential oil of the aerial parts of *H. tuberculatum* is potentially active against lung (H-1299), and liver (HEPG2) carcinoma cell lines.
- The observed cytotoxic activities can be attributed to the dominance of α and γ-terpinene in this oil.

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Research Interests of Dr. Salmin are:

1. Isolation and Structure Elucidation of Secondary Metabolites From Natural Sources "Terrestrial Plants And Marine Organisms" With Important BioPharmaceutical Properties Such As Anticancer, Antimicrobial, Anti- inflammatory and Antiviral Activities Using Different Advanced Chromatographic and Spectroscopic Techniques.

2. Running biological and pharmacological screening for chemical, natural and semi-synthetic compounds.

3. Formulation of natural products as complementry and alternative medicine.