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Research Article

Antimicrobial activity and volatiles profiling of *Pulicaria crispa* and *P.incisa* growing wild in Egypt as determined *via* headspace SPME and steam distillation

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ABSTRACT:
Volatile profile in *Pulicaria crispa*(Forssk.) Olivand *Pulicaria incisa*(Lam.) DC aerial parts was determined*via*headspace solid phase microextraction (HS-SPME) and steam distillation. A total of 59 volatiles were identified in *P. crispa* and *P. incisa*volatile blends. Carvotanacetone predominated the essential oil (76%)and HS (99%) blendsof *P. incise*.Incontrast, P. *crispa* essential oil was enriched in carvotanacetone (48%) and hexanal (11%) versus abundance of β-caryophyllene (98%) inits HS sample.Both oils were assessed for their antimicrobial activity and were active against *Staphylococcusaureus, Bacillus subtilis, Escherichia coli* and *Candida albicans* with *P. incisa* being more active with minimum inhibitory concentration of 4.9, 5.2, 2.8 and 5.7 µg/ml, respectively.

KEYWARDS: *Pulicaria incise, Pulicaria crispa, Headspace, Essential oil, GC/MS, Antimicrobial activity.*

1. INTRODUCTION:

The genus *Pulicaria* belongs to the tribe Inuleae, subrtibe Inulinae of the Astreaceae family, comprising*ca*. 100 species distributed worldwide[1].Plants within thatgenus are used as traditional remedies in folk medicine,*for examplePulicaria*. *crispa* (Forssk.) Oliv. is used by the people of Southern Egypt and Saudi Arabia to treat inflammation and as an insect

repellent [2]. Also *Pulicaria incisa* (Lam.) DC is used in Upper Egypt and by Bedouins to treat tachycardia and other heart diseases [3].

Phytochemical studies on *Pulicaria* revealed for the presence of flavonoids, sesquiterpenoids and diterpenoidsas major constituents[4-6]. Isolates from *Pulicaria*exhibit a myriad of biological activities [7-9]. For example,triacetoxy-pulicanon isolated from *P. canariensis* demonstrates cytotoxic effect against human myeloid leukemia cell line HL-60 [10]. Meanwhile, "pulichalconoid B"a chalcone isolated from *P. incisa* exhibits astroprotective effect [11], whereas 2.3-Dihydro-5.10-epiaromatin (pseudoguaianes) from *P. crispa* exhibits anticancer activity *in vitro* against human bladder carcinoma cell line, EJ-138 [1].

The presence of a characteristic aroma for *P. crispa* and *P. incisa*infers the presence of volatile constituents that contribute to their odour. Nevertheless, few studies were reported on their volatiles composition. Fifty six volatiles were identified in *P. crispa*from Iran which was enriched in 1,8-cineol(21%), alloaromadendrene epoxide (17%) and α-terpineol (8%)[12]. With regards to *P. crispa*collected from southeastern coast of Egypt, major volatile componentsincluded carvotanacetone (93%) and linalool (3.5%) [13],suggestive for a geographical origin impact on volatiles composition withinthat genus. Volatile constituents of *P. incisa* from Algeria included high percentage of chrysanthenone and dimethyl phenols [14], while carvotanacetone (66%) and chrysanthenone (13.26%) were the major constituents in the essential oil obtained for *P. incisa* sub. Candolleana from Egypt [15]. Additionally, the essential oil of *P. undulate* from Sudan was characterized by high oxygenated monoterpenes levels (68%)with (+) carvotanacetone (55.8%) as the major constituent [16].

To provide insight on *P. crispa* and *P. incisa* volatiles profile, two extraction methodsfor volatiles collection were employed including headspace solid phase microextraction (SPME) and steam distillation**.** Headspace SPME is a relatively superior technique for volatiles profiling being solvent free and involves no heating step in oil preparation, and can be conveniently used to collect volatile fractions as a time-saving technique [17].Additionally, SPME enables the enrichment of volatiles from gas or liquid samples, over a fused-silica fiber coated with an appropriate sorbent layer, then subsequent desorption of these analytes leading to detection of less abundant volatiles. While steam distillation considered as one of the cheapest way to extract essential oils with high yield, SPME provides a more realistic and clean picture of plants volatile profile. HS-SPME has been successfully applied for screening complex volatile mixture and for complementing information obtained from other volatiles analysis techniques [17,18]. Taking advantages of the complementarily of both volatiles collection methods, our aim was to apply both hot distillation and cold SPME extraction methods for volatile profiling in two *Pulicaria* species i.e., *P. crispa* and *P.incisa*.

2. EXPERIMENTAL:

2.1. Plant material

Aerial parts including stem, leaf, flower etc. from *P. incisa* and *P. crispa* were collected in May 2013 from waddi Hagoul-Suez road, Egypt. The plants were identified by Prof. Dr. Ibrahim Elgarf, Professor of taxonomy, Faculty of science, Cairo university, Egypt. Voucher specimens for *P. incisa* (13.2.17.2) and *P. crispa* (13.2.17.1) have been deposited in the Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Egypt.

2.2.SPME material and chemicals

SPME holder and fiber coated with 100 μm polydimethyl siloxan (PDMS) were supplied by Supelco (OaKville, ON, Canada). Before use,

fibers were conditioned according to the supplier's instructions. All other chemicals and terpene standards were provided from Sigma Alderich (St. Louis. Mo., U.S.A.)[18].

2.3. Essential oil extraction and analysis

Extraction and analysis of the essential oil from aerial parts of *P. incisa* and *P. crispa* followed the procedure described by Farag [18]. Briefly fresh ground aerial parts (100 g) were distilled for 3 h in a modified Clevenger apparatus with distilled water. The distillate was extracted with chloroform (GC grade) and concentrated under nitrogen gas to give brownish yellow oil. The oil was dried over Na_2SO_4 and stored at -20 °C until further analysis. The fresh aerial parts yielded 0.18% and 0.043% (v/w) respectively.

2.4. SPME headspace volatiles isolation

Headspace volatiles were collected and analyzed according to22. Briefly, *Pulicaria* species aerial parts were ground, and 1 g was placed inside clear glass vials 20 ml and spiked with 10 µg hexenyl acetate added as an internal standard. Vials were then immediately capped and placed on a temperature controlled tray for 1 h at 50 °C with the SPME fiber inserted into the headspace above the sample. Adsorption was timed for 30 min.

2.5. GC-MS data processing and volatiles analysis

SPME fibers were desorbed at 210 °C for 1 min in the injection port of a Shimadzu Model GC-17A gas chromatograph interfaced with a Shimadzu model QP-5000 mass spectrometer (Japan). Volatiles were separated on a DB5-MS column (30 m length, 0.25 mm inner diameter, and 0.25 µm film (J&W Scientific, Santa Clara, CA, USA). Injections were made in the splitless mode for 30 s. The gas chromatograph was operated under the following conditions: injector 220 °C, column oven 38 °C for 3 min, then programmed at a rate of 12 °C/min to 180 °C, kept at 180 °C for 5 min, and finally ramped at a rate of 40 °C min^{-1} to 220 °C and kept for 2 min, He carrier gas at 1 mL min^{-1}. The transfer line and ion–source temperatures were adjusted at 230 and 180 °C, respectively. The HP quadrupole mass spectrometer was operated in the electron ionization mode at 70 eV. The scan range was set at m/z 40–500 [19]. Volatile components were identified using the procedure described previously [17], by comparing their retention indices (RI) relative to n-alkanes (C6-C20), mass matching to NIST, WILEY library database and with authentic standards whenever available. Peaks were first deconvoluted using AMDIS software (www.amdis.net) prior to mass spectral matching.

2.6. Antimicrobial activity assay

The antimicrobial activity of essential oil samples was investigated against the following micro-organisms available in stock culture at The Medical Mycology Lab, Regional Center for Mycology and Biotechnology, Al-Azhar University, Gram-positivebacteria: **[***Bacillus subtilis* (ATCC 5230), *Staphylococcus aureus*(ATTC 3556)]. Gram-negative bacteria: *Echerichia coli* (ATCC 35218). Fungi:*Candida albicans*(ATCC 90028). The minimal inhibitory concentration (MIC) values, which represent the lowest tested compounds and standard drugs (ciprofloxacin against bacterial strains and Amphotericin B against fungal strains) concentrations that completely inhibit the bacterial growth were determined using a micro-well dilution method. The inoculums were prepared and the suspensions were adjusted to 10^6 CFU/ml. Essential oils under investigation and the standards drugs were performed in a 96-well plate. Each well of the micro plate include 40 µl of the growth medium Brain Heart Infusion (BHI) plus 10% fetal serum bovine (FBS), 10 µl of inoculums and 50 µl of the diluted compounds. The clarithromycin and DMSO are used as positive and negative

controls, respectively. The plates were incubated at 37 °C for 24 h. After that, 40 µl of tetrazolium salt {2,3-bis[2-methyl-4- nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide} (XTT) was added. The plates were incubated in the dark for 1 h at 37 °C, after which colorimetric change in the XTT reduction assay was measured using a microtiter plate reader (Tecan Sunrise absorbance reader; Tecan UK, Reading United Kingdom) at 492 nm. The percentage of inhibition was calculated as 1- (mean of test well / mean of control wells) x 100 [20].

3. RESULTS and DISCUSSION:

3.1. Volatiles analysis

GC/MS analysisof *P. crispa* and *P.incisa* aerial parts resulted in the identifications of 54 volatiles constituting 91.5% of the total volatiles make up.To assess for biological variance, 3 biological replicates for each plant specimen were extracted and analyzed in parallel under identical conditions. The complete list of identified volatiles in *P. incisa* and *P.crispa* is presented in **Table 1**.*P. incisa* constituted 30 of the identified volatiles compared to 31 in the *P. crispa*and with 7 constituents found common in both species. GC/MS analysis revealed for the presence of 19 and 12 compounds in the essential oil and HS samples of *P. incisa,* respectively corresponding to 99.9% and 99.8% of the total volatiles make up. Identified volatiles belonged to different classes *viz,* terpenoids, aldehydes, alcohols, esters, ketones, aliphatic hydrocarbons, phenols/aromatics. Among volatilesin *P. incisa,* oxygenated monoterpenes amounted for themajor volatile classin both hydrodistilled oil and HS sample accounting for 89.7% and 99.6%, respectively of its volatile oil blend. Carvotanacetone(*p*-Menthenone) was found as the main component in both samples at 76% and99% followed by *p*-menthadiene-dione,though thelatter was present at only 8%. It should be noted that this is the first report of essential oil chemical compositionof *P. incise* (LAM.) from Egypt.

In *P. crispa* essential oil and HS samples,17 compounds were identified and with oxygenatedmonoterpenes amounting for (52%) of its volatile blend. Similar to *P. incisa,* p-menthenone (carvotanacetone) was the main component (48.5%). Next to oxygenated monoterpenes, oxygenated hydrocarbons amounted for 27% of thevolatile blend with hexanal as the main component (11.4%). In contrast, sesquiterpene hydrocarbons appeared as the major components in *p. crispa*HS sample (98.5%) with β-caryophyllene (98.4%) as the main constituent. These results confirm previous report of *P. crispa*essential oil from southern coast of Egyptrevealing carvotanacetone as the main component (93%) [13]. However,*P. crispa* from Iran showed different composition with1,8-cineoleas the main constituent[12].

3.2. Antimicrobial Assay

Essential oils enriched in carvotanacetone has been reported to possessstrong bactericidal activity [21], which warranted assessing *P. crispa* and *P. incisa* considering their abundance in such terpene. Minimum inhibitory concentration (MIC), lowest concentration to inhibit visible growth, was used herein for assessment of oils antimicrobial efficacy. The MIC value for *P. incisa* oil varied between2.8± 0.1to 5.7 ± 0.1 µg/ml, while in *P. crispa* oil ranged from 3.3 ± 0.1 to 7.1 ± 0.1µg/ml according to the organism tested, **Table 2**.Examined microorganisms included *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Candida albicans*.These results reveal that *P. incisa*essential oil exhibit in general comparable antimicrobial activity to that of *P. crispa*as exemplified by their similar MIC values, **Table 2**. The tested essential oils revealed a good spectrum activity against the tested organisms in comparison with the antibiotic standards (ciprofloxacin against

bacterial strains and amphotericin B against fungal strains). MIC for ciprofloxacin ranged from 0.8± 0.033 to 1.8 ± 0.05 µg/ml and for amphotericin B was 0.9 ± 0.036 µg/ml.

Both essential oils were found more potent against Gram-negative bacteria more than Gram-positive and in agreement with results obtained previously by Ahmed et al. who investigated the antimicrobial effect of *P. crispa* extract [22]. MIC for *P. incisa* against *Escherichia coli*was 2.8 µg/ml while against *S. aureus* and *B. subtilis* were 4.9 and 5.2 µg/ml respectively. In *P. crispa* MIC against *E.coli*was 3.3µg/ml versus 6.1 µg/ml against *S. aureus* and *B. subtilis*.Abundance of carvotanacetone in both oils is likely to mediate for their antimicrobial effects. Nevertheless, other volatiles i.e., *p*-mentha-diene-dione and (*E*)-2-hexenal present at moderate levels might act synergistically to enhance oil overall antibacterial effect.

4. CONCLUSION:

In conclusion, HS-SPME technique combined withhydrodistillation provided the first comprehensive volatiles profile in *P.crispa* and *P. incisa* aerial parts. The present work focused on ability of two different extraction techniques to trap the volatile fractions from *P. crispa* and *P. incisa* prior to their subsequent analysis using GC/MS. The most notable difference in chemical composition was the abundance of oxygenated monoterpenes in all volatile blends, except in *P. crispa* HS in which sesquiterpene hydrocarbons was the major form. The presence of carvotanacetone at such high levels in both species could justify for their notable antimicrobial effects. Whether carvotanacetone could also serve as chemotaxonomic marker for*Pulicaria* species need to be confirmed by investigating other species oil composition.The tested essential oils showed a relatively high antimicrobial activity against the microorganisms used. Both essential oils are effective against Gram-negative bacteria more than Gram-positive, The low susceptibility of Gram-positive bacteria could be attributed to the presence of hydrophobic lipopolysaccharide in their outer membrane which provide protection against antimicrobials [23]. Other effects yet to be pursued for these oils include cytotoxic and antioxidant activitiespreviously reported in the genus [10,11,15].

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Tables and Figures:

Table 1. Volatile composition (%) of *P. crispa* and *P. incisa* as determined *via* SPME and steam distillation

Peak No.	Rt.(min)	RI	Compound	***P. incisa***		***P. crispa***	
				E. oil	**SPME**	**E. oil**	**SPME**
1	4.45	770	4-Hydroxy-3-methylbutanal	---	---	8.61	---
2	5.03	800	Octane	1.54	---	3.84	---
3	5.08	802	Hexanal	0.99	---	11.36	---
4	5.61	829	2,6-Dimethylheptane	0.56	---	---	---
5	6.17	857	(*E*)-2-Hexenal	---	---	4.04	---
6	6.56	877	1-Hexanol	---	---	1.34	---
7	7.02	900	Nonane	2.02	---	4.91	---
8	7.1	904	Heptanal	---	---	1.64	---
9	8.68	993	3,5-Heptadien-2-ol, 2,6-dimethyl	1.28	---	---	---
10	8.81	1000	Decane	1.36	---	3.4	---
11	9.27	1029	p-Cymene	1.07	---	---	---
12	9.6	1049	Benzeneacetaldehyde	---	---	1.17	---
13	10.23	1082	β-Linalool	---	0.077	---	---
14	10.26	1093	3-Carene, 2-acetyl	---	0.061	---	---
15	10.39	1100	Undecane	0.57	---	1.56	---
16	10.42	1102	β-Linalool	1.27	---	2.78	0.035
17	10.5	1107	Chrysanthenone	1.05	---	---	---
18	10.61	1114	Camphenone	0.47	---	---	---
19	11.69	1192	Unknown hydrocarbon	---	0.016	---	---
20	11.77	1195	Isothujol	0.75	---	---	---
21	11.83	1200	Nonane	---	---	1.23	---
22	11.89	1204	*p*-menthenol	---	---	1.12	---
23	11.95	1209	Menthone	0.46	---	---	---
24	12.62	1260	*p*-Mentha-diene-dione	8.02	---	---	---
25	12.66	1263	Carvotanacetone (*p*-Menthen-one)	76.17	99.4	48.54	0.18
26	13.05	1172	*p*-Allyl-anisole (Estragole)	---	---	---	0.017
27	13.07	1294	*p*-Cymen-2-ol (Isothymol)	0.53	---	---	---
28	13.13	1298	Docosene	0.82	---	---	---
29	13.2	1304	Thymol	0.55	---	---	---
30	13.23	1221	Cyclopentylcyclohexane	---	0.018	---	---
31	13.39	1322	Unknown	---	0.011	---	---
32	13.75	1352	Isopropyl phenylacetate	---	0.012	---	---
33	13.94	1348	6,10-Dimethyl-4-undecanol	---	---	---	0.0076
34	13.98	1371	Unknown sesquiterpene hydrocarbon	---	0.018	---	---
35	14.27	1394	(*Z*)-Jasmone	---	0.13	---	---
36	14.35	1384	Isomyrcenyl acetate	---	---	---	0.18
37	14.36	1393	Patchoulane	---	0.011	---	---
38	14.42	1404	δ-Selinene	---	---	1.07	---
39	14.44	1405	(Z)-Jasmone isomer	0.46	---	---	---
40	14.49	1396	Geranyl isobutyrate	---	---	---	0.22
41	14.54	1440	Unknown hydrocarbon	---	0.022	---	---
42	14.59	1272	Linalyl acetate	---	---	---	0.22
43	14.76	1440	*β*-Farnesene	---	---	---	0.011
44	14.78	1419	*β*-Caryophyllene	---	---	1.12	98.43
45	14.91	1292	Myrcenyl acetate	---	---	---	0.03
46	15.08	1458	*α*-Farnesene	---	0.02	---	---
47	15.13	1465	*α*-Curcumene	---	0.037	---	---
48	15.13	1448	*α*-Farnesene	---	---	---	0.012
49	15.15	1518	*α*-Bisabolene	---	---	---	0.026
50	15.36	1467	*β*-Selinene	---	---	---	0.08

Table 1. Continued.

Peak No.	Rt.(min)	RI	Compound	***P. incisa***		***P. crispa***	
				E. oil	***SPME***	***E. oil***	***SPME***
51	15.38	1486	Aromadendrene	---	0.042	---	---
52	15.47	1493	(*E*)-α-Bergamotene	---	0.025	---	---
53	15.5	1479	Linalyl iso-valerate	---	---	---	0.25
54	15.52	1480	Trimethyl-1,5-heptadien-4-ol	---	---	---	0.17
55	15.66	1507	δ-Cadinene	---	0.012	---	---
56	15.66	1492	*α*-Guaiene	---	---	---	0.02
57	15.72	1496	*β*-Himachalene	---	---	---	0.014
58	16.55	1564	Unknown sesquiterpene	---	---	1.12	---
59	17.25	1608	Caryophyllene oxide	---	---	1.1	---
Identified Compounds							
Monoterpene hydrocarbons				3	---	4.96	---
Oxygenated monoterpenes				89.73	99.668	52.44	0.8396
Sesquiterpene hydrocarbons				---	0.128	3.31	98.593
Oxygenated sesquiterpenes				---	---	1.1	0.47
Aromatics				---	0.049	1.17	---
Hydrocarbons				4.94	0.056	9.98	---
Oxygenated hydrocarbons				2.27	---	26.99	---

Notes: Alloilconstituents wereidentified by(i) comparison of massspectral data with NIST library database , (ii)comparison of massspectrum withliterature data, (iii)RRI, relativerefractive indextoahydrocarbonseriesC_6–C_{20}.Relativeconcentrationas%basedontriplicate measurements. ---,notdetected.

Table 2. Antimicrobial activity of *Pulicaria* species eesential oils using XTT assay (n=3). Results are expressed as average ± Std. deviation

Organism	*P. incisa* V.O. (µg/ml)	*P. crispa* V.O. (µg/ml)	Ciprofloxacin (ug/ml)	Amphotericin B (ug/ml)
Staphylococcus aureus ATCC 3556	4.984 ± 0.083	6.085 ± 0.059	1.807 ± 0.055	NT
Bacillus subtilis ATCC 5230	5.173 ± 0.061	6.085 ± 0.059	0.872 ± 0.033	NT
Escherichia coli ATCC 35218	2.802 ± 0.113	3.298 ± 0.139	1.542 ± 0.016	NT
Candida albicans ATCC 90028	5.730 ± 0.077	7.117 ± 0.052	NT	0.911 ± 0.036

Figure 1:GC-MS chromatograms of volatiles collected from *P. crispa* and *P. incisa* as determined via headspace SPME and steam distillation.The corresponding compound names for peaks are shown in Table 1