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Chemical Composition, Antimicrobial and Antioxidant Activities of *Crupina crupinastrum* as a Medicinal Plant Growing Wild in West of Iran

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In this study, the hydro-distilled volatile oil from the aerial parts of *Crupina crupinastrum* was investigated by GC-MS and GC-FID. A total of 25 compounds representing 86.4% of the volatile oil were identified. The main constituents were linoleic acid (19.1%), n-decane (12.4%) and ethyl hexadecanote (7.8%). The antioxidant activity of essential oil and methanolic extract was evaluated with DPPH radical scavenging activity. The total phenolic and flavonoids contents were also determined spectrophotometerically. The antimicrobial activity of essential oil of *C. crupinastrum* was examined against four gram-negative and five gram-positive bacteria. The preliminary phytochemical analysis of the methanolic extract carried out using standard procedures. The data of this study suggests that *C. crupinastrum* has potential for application as an antioxidant and antimicrobial agent in pharmaceutical and food industries.

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Introduction

Nowadays multi-drug resistant (MDR) human pathogens are considered among the most important health threatening problems worldwide. Application of natural antibacterial such as plants essential oil and extracts has been recently gained increasing attention [1]. Oxidative stress in body by free radicals or reactive oxygen species leads to the development of diseases like cancer, cardiovascular, diabetes, and cirrhosis. Endogenous antioxidant defense as well as natural and dietary antioxidants are required to reduce the effect of oxidative stress in our system [2].
Crupina crupinastrum (Moris) Vis. is herbaceous annual plant belonging to the compositae family. The genus *Crupina* (pers.) DC. have three species herbaceous annual include *C. crupinastrum*, *C. intermedia* and *C. vulgaris* in Iran [3]. *C. crupinastrum* in folk medicine in the decoction form was used for treatment of the infections and wounds [4]. Antimicrobial activity of a group of herbal medicinal plants grown in Jordan studied against two bacterial species, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and one fungi, *Candida albicans* that antimicrobial activity was highest in *C. crupinastrum* extract which gave the largest inhibition zone (DIZ 24 mm) at 60 ppm [5]. Inhibitory effect of ethanolic extract from C. *crupinastrum* and four Jordanian medicinal plant were studied against phytopathogenic fungi that highest inhibition effect of extract was recorded at 1000 ppm *C. crupinastrum* showed 26% of inhibition on *Rhizoctonia solani* [6]. In this study, we reported on chemical constituents and bioactivities of essential oil and methanolic extract of the aerial part of *C. crupinastrum*, aiming at the assessment of its potential medicinal uses.

Material and Method

Plant Material

The aerial part of *C. crupinastrum* at the beginning of flowering stage was collected from Bankul area, Ilam province, Iran, at an altitude of 1550m in July 2016. A voucher specimen (no70) has been deposited with the herbarium of the school of pharmacy, Hamadan, Hamadan province, Iran. Plant material was taken immediately to the laboratory to be dried at ambient temp. (Min: 12 °C and max: 25 °C) and shade condition.

Isolation of Essential Oil

Samples of aerial parts (100 g) were cut into segments of approximately 1 cm and subjected to hydrodistillation in a Clevenger-type apparatus for 3.5 h as described in British Pharmacopoeia (1993). The distilled essential oil was dried (Na_2SO_4) and keep in closed dark vials at 4 °C until the analysis.

GC and GC/MS Analyses

GC analysis was performed using a Thermoquest gas chromatograph with a flame-ionization detector (FID). The injector and detector temps were 250 and 300 °C, resp. The analysis was carried out using fused silica capillary DB-5 column (60 m 9 0.25 mm; film thickness 0.25 lm). Oven temp. was programmed from 60 to 250 °C at the rate of 5 °C/min, and finally held isothermally for 10 min. N_2 was used as carrier gas at a flow rate of 1 ml/min. GC/MS analysis was performed using a Thermoquest-Finnigan gas chromatograph equipped with the above-mentioned column, used under the same conditions coupled to a TRACE mass spectrometer. He was used as the carrier gas. Ionization voltage was kept at 70 eV. Ion source and interface temps. were kept 200 and 250 °C, resp. Mass range was scanned from 43 to 456 m/z.

Identification of Compounds

The essential oil constituents were identified by comparison of the relative retention indices calculated with respect to homologous of n-alkanes (C_6–C_{25}) [7], MS library search (Wiley and Adams) and comparison of mass spectrum reported in the literature [8]. The relative amounts of individual components (%) were determined using area percentage method relative to total peak area from the GC-FID analysis, without using correction factor.

Preparation of the Extract

The extract was prepared from aerial parts by maceration method. 200 g of the powdered plant bodies were extracted using 2L of methanol. The extraction was carried out during 72 h at room temperature. The extract was filtered using a Whatman filter paper and then concentrated in vacuum at 40 °C using a rotary evaporator. The residue obtained was stored in a freezer until further tests.

Phytochemical Screening

The preliminary phytochemical analysis of the methanolic extract carried out using standard procedures to identify the various constituents as described by Ugochukwu [9] and Bargah [10].

Test for Saponins

5 ml of extract was shaken vigorously with 5 ml of distilled water in a test tube and warmed. The formation of stable foam was taken as an indication of the presence of saponins.

Test for Alkaloids

3 ml of extract was stirred with 3 ml of 1% HCl on steam bath. 1 ml of mixture was taken separately in two test tubes. Few drops of Dragendorff's reagent were added in one tube and occurrence of orange red precipitated was taken as positive.

Test for flavonoids

To 1 ml of extract, 1ml of 10% lead acetate solution was added. The formation of a yellow precipitate was taken as a positive test for presence of flavonoids.

Test for Terpenoids

2 ml of the extract was dissolved in 2ml of $CHCl_3$ and evaporated to dryness. 2ml of conc. H_2SO_4 was then added and heated for about 2 minutes. Development of a grayish color indicates the presence of terpenoids.

Test for tannins

About 2 ml of the extract was stirred with 2ml of distilled water and few drops of ferric chloride ($FeCl_3$) solution were added. Formation of green precipitate was indication of presence of tannins.

Test for Phlobatannins

Deposition of a red precipitate when 2mls of extract was boiled with 1ml of 1% aqueous hydrochloric acid was taken as evidence for the presence of phlobatannins.

Test for Steroids

A red color produced in the lower chloroform layer when 2 ml of extract was dissolved in 2 ml of chloroform and 2 ml concentrated sulphuric acid was added in it, indicates the presence of steroids.

Test for phenols

The extract (500mg) was dissolved in 5ml of distilled water. To this, few drops of neutral 5% ferric chloride solution were added. A dark green color indicated the presence of phenolic compounds.

Tests for proteins

2 ml of 0.2% Ninhydrin solution with the extract was boiled, appeared violet color indicate the presence of proteins and amino acids.

Tests for carbohydrates

A mixture of Fehling solutions A and B with equal volumes was boiled and added to crude plant extract. A red color precipitate indicated the presence of reducing sugars.

Measurement of Free Radical-Scavenging Activities (DPPH Assay)

The capacity of *C. crupinastrum* essential oil and methanolic extract to scavenge DPPH was determined according to the technique reported by [11]. The absorbance of the reaction mixture at 517 nm was measured and BHT was used for comparison. The percent of DPPH• discoloration of the samples was calculated according to the formula:

Percent inhibition = (A_o - As/ A_o) x 100

Where A_o is the absorbance of the control (containing all reagents except the test compound), and As is the absorbance of the mixture containing the test compound. Sample concentration providing 50% inhibition (IC_{50}) was obtained by plotting the inhibition percentage against sample concentrations.

Determination of total phenolics content

The total phenolics content (TPC) of the plant extract was determined according to the Folin–Ciocalteu procedure. Total phenols content was expressed as milligram gallic acid equivalents per gram of plant extract (mg (GAE)/g) [12].

Determination of total flavonoid

Determination of total flavonoid content (TFCs) was done using the colorimetric method [13]. 20 µl of sample solution (1 to 200 µg/ml) was diluted in 60 µl of methanol and 10 µl of 5% $AlCl_3$. Then, 10 µl of 0.5 M potassium acetate was added into the mixture and the total volume was made up to 200 µl using distilled water. The solution was shaken and kept for 30 min. The absorbance was measured at 415 nm. TFCs were expressed as mg of quercetin equivalents per gram of dry weight of extracts (mg QE/g) using the calibration curve of quercetin as standard reagent. All tests were carried out in triplicates.

Antimicrobial Activities

The essential oil of *C. crupinastrum* was tested individually against a range of 9 bacteria, including *Escherichia coli* ATCC 25922 (American Type Culture Collection number), *Klebsiella pneumoniae* ATCC 10031, *Bacillus cereus* PTCC 1015 (Persian Type Culture Collection number), *Bacillus pumilus* PTCC 1274, *Bacillus subtilis* ATCC 465, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29737 and *Pseudomonas aeruginosa* ATCC 85327. The antimicrobial activity of essential oil was determined by the disk diffusion method using Mueller–Hinton Agar plates with determination of inhibition zones. Also the MIC values were determined by the broth microdilution assay as described by Eftekhar [14].

Results and discussion

Hydrodistillation of dried aerial parts of *C. crupinastrum* afforded a light yellow color essential oil in 0.1% yield (w/w %) relative to dry weight of plant. Twenty five compounds representing 86.4% of the essential oil were identified. The compounds were identified by GC–MS and quantified by GC–FID. The compounds are listed according to their elution from the DB-5 column (Table 1). The major compounds were linoleic acid (19.1%), n-decane (12.4%), ethyl hexadecanote (7.8%), octadecanoic acid (7.0%) (Fig. 1). The analysis of the essential oil showed that non terpenoides in *C. crupinastrum* essential oil were found as the main class of compounds.

According to literature the essential oil of different plants such as *Morus alba, Morus nigra, Retama monosperma*, *Euphorbia hirta*, and *Ballota nigra Subsp. Kurdica* were rich in non terpenoides including n-alkanes and fatty acid [15-17].

The major non-hydrocarbon compound of *C. crupinastrum* is linalool as well as in different compositae plants the linalool is the major compound of essential oil such as *Lavandula angustifolia, Chrysanthemum*, *Francoeuria undulata*, *Achillea aucheri* and *Petasites albus* [18-23].

Table 1. Percentage composition of the essential oils of *C. crupinastrum*

	Compounds	RI*	Percentage
1	(3Z)-Octen-2-ol	972	0.5
2	Decene	986	0.6
3	n-Decane	1000	12.4
4	Linalool	1095	0.9
5	cis-Pinocamphone	1172	1.0
6	Dodecane	1200	4.9
7	Carvacrol	1298	0.6
8	n-Tetradecane	1400	0.7
9	Liguloxide	1534	0.5
10	Spathulenol	1577	0.3
11	Hexadecanoic acid	1921	2.4
12	Phytol	1942	2.1
13	Isophytol	1946	2.8
14	(E,Z)-Geranyl linalool	1987	3.7
15	Ethyl hexadecanote	1992	7.8
16	Isopropyl hexadecanoate	2024	0.6
17	(6Z,10E)-Pseudo phytol	2030	3.5
18	(Z)-Falcarinol	2035	4.5
19	Methyl linoleate	2095	2.0
20	Heneicosane	2100	3.2
21	Linoleic acid	2132	19.1
22	Oleic acid	2141	0.4
23	(E)-Phytol acetate	2218	1.9
24	Octadecanoic acid		7.0
25	3α-Acetoxy-Manool	2359	2.9
	Total		**86.4**

***RI: retention index**

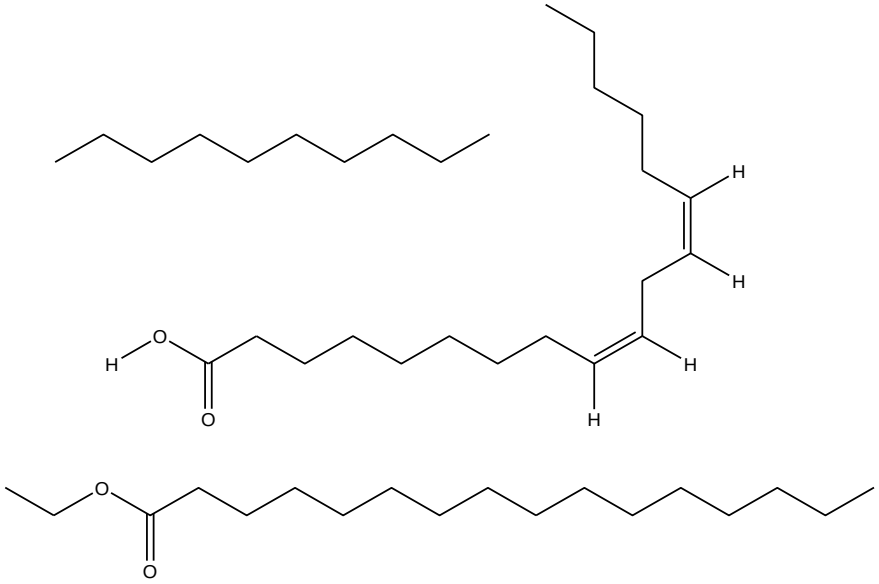

Fig. 1. The structure of major compounds from *C. crupinastrum* essential oil (n-Decane, Linoleic acid and Ethyl hexadecanote).

Preliminary phytochemical tests for extract was studied by standard method. Result showed extract contain bioactive constituents include: phlobatannins, steroids, phenolics, flavonoids, tannins and saponins (Table 2). The preliminary phytochemical screening tests are useful in the detection of bioactive principles and subsequently may lead to the drug discovery and development. Further, these tests facilitate their quantitative estimation and qualitative separation of pharmacologically active chemical compounds. Further the presence of different phytoconstituents in the methanolic extract may be responsible for the therapeutic properties of *C. crupinastrum*.

Table 2. Preliminary phytochemical screening of *C. crupinastrum* extract

phytochemical constituents	Test methods	result
Carbohydrates	Fehling's solutions	-
Glycosides	Keller-kilani	-
Pheolics	Ferric chloride	+
Tannins	Ferric chloride	3+
Alkaloids	Dragendorff's	–
Proteins & amino acids	Ninhydrin test	2+
Saponins	Foam test	+
Flavonoids	Alkaline reagent	2+
Phlobatannins	Precipitate test	-
Terpenoids	-	+
Steroids	Salkowski,s test	2+

+ Presence; - Absence

Free radical scavenging capacities of essential oil and methanolic extract of *C. crupinastrum* measured by DPPH assay are shown in Table 3. According to the results the highest scavenging activity was found for methanol extract ($IC_{50=}$ 58.1 µg/ml), followed by essential oil ($IC_{50=}$ 59.6 µg/ml). The total phenolics of methanolic extract of the plant were measured using Folin–Ciocalteu's assay. The total phenolic and flavonoid content of the methanolic extract were (110.8 mg GAE/g sample) and (66.6 mg QE /g Sample), respectively (Table3). Flavonoids and tannins are phenolic compounds and plant phenolics are a major group of compounds that act as primary antioxidants or free radical scavengers [24]. Since these compounds were found to be present in the extracts, it might be responsible for the potent antioxidant capacity of *C. crupinastrum*.

Table 3. Antioxidant activity, total phenolic content and total flavonoid content of the essential oil and methanolic extract from *C. crupinastrum*

Sample	DPPH assay IC_{50}(µg/ml)	TPC mg gallic acid /g Sample	TFC mg quercetin /g Sample
Essential Oil	59.6±0.2	-	-
Methanol extract	58.1±0.2	110.8±0.4	66.6±0.1
BHT	24±0.2	-	-

Values were the means of three replicates ± standard deviation.

The essential oil of *C. crupinastrum* was tested against four Gram-negative and five Gram-positive bacteria. The results indicated that the essential oil had moderate to high inhibitory activity against the Bacillus subtilis, and Bacillus cereus (Table 4). In the study by Khalil et al. including evaluation of antimicrobial activity against pathogenic microorganisms by extracts from herbal Jordanian plants the antimicrobial activity was highest in *C. crupinastrum* extract (5, 10, 15, 20, 40 and 60 ppm) which gave the largest inhibition zone (DIZ 24 mm) at 60 ppm [5].

Conclusion

The main constituents of *C. crupinastrum* volatile oils were linoleic acid (19.1%), n-decane (12.4%) and ethyl hexadecanote (7.8%) and the volatile oil was rich in non terpeneoids. The secondary metabolites of methanolic extract were pheolics, tannins, saponins, flavonoids, terpenoids, steroids, proteins and amino acids. Also the high antioxidant activity and medium antimicrobial activity effect of the *C. crupinastrum* supports its potential as a prospective source of antimicrobial and antioxidant agent in pharmaceutical and food industries.

Table 4. *In vitro* antibacterial activities of *C. crupinastrum* essential oil.

Sample	Microorganism								
	Bacillus pumilus	***Bacillus subtilis***	***Staphylococcus aureus***	***Bacillus cereus***	***Klebsiella pneumoniae***	***Enterococcus faecalis***	***Escherichia coli***	***Staphylococcus epidermidis***	***Pseudomonas aeruginosa***
Essential Oil	14 [a] (15) [b]	18 (7.5)	10 (15)	18 (7.5)	10 (15)	11 (15)	13 (15)	11 (>15)	-
Tetracycline[c]	nt	21 (3.2)	20 (3.2)	nt	nt	nt	- (nt)	34 (1.6)	nt
Gentamicin[d]	nt	- (nt)	- (nt)	nt	nt	nt	23 (3.2)	- (nt)	nt
Ampicilline[e]	15 (15)	14 (15)	13 (15)	nt	nt	nt	12 (15)	19 (15)	nt

a: Zone of inhibition (in mm) includes diameter of the disc (6 mm), b: Minimum inhibitory concentration values as mg ml^{-1}, (–): Inactive, (7 – 13): moderately active, (> 14): highly active, nt: not tested, c: Tested at 30 µg/disc, d: Tested at 10 µg/disc, e: Tested at 10 µg/disc.

Conflict of interest

Authors certify that there is no actual or potential conflict of interest in relation to this article.

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