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Antimicrobial Activity and Phytochemical Analysis of Essential Oil of *Carum Carvi*, an Important Phytofood of Indian Folk Medicinal Plants

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Abstract: The present study was done to investigate the chemical constituents of *Carum carvi* Linn (Caraway) and to evaluate their antimicrobial activity. The fresh plants and seeds were collected from Himalayan hills of Uttarakhand state of India. Essential oil was obtained via steam distillation followed by chromatographic separation (TLC, GC-MS). Head space GC-MS was also done for quantification of highly volatile compounds. Biological activity of oil was evaluated by determination of minimum inhibitory concentration, which was found active against both bacteria and fungi. The MIC of the samples ranged from 50 µg/ml to 100 µg/ml. The antibacterial activity of *Carum carvi* essential oil is apparently due to carvone (23.3%), limonene (18.2%), carvacrol (6.7%), and linalool (0.3%), which inhibit the growth of fungi and bacteria.

Key Words: *Carum carvi* • essential oil • GC-MS • antimicrobial activity

Introduction

Caraway (*Carum carvi* L.) (Family-Apiaceae) is an aromatic plant used to flavour foods, added to fragrances, and for medical preparations. In particular, *Carum carvi* essential oil is used in liqueurs, mouthwashes, toothpastes, soaps, and perfumes. In addition, *C. cyminum* and *Carum carvi* are also used as antispasmodic, carminative, and appetite stimulant agents (Helander et al. 1976). Tewari and Mathela, 2003, confirmed the presence of carvone (60%) and limonene (35%) as the major chemical constituents of the essential oil of *Carum carvi* by GC and GC-MS studies showed (Tewari, et al. 2003). Chowdhury, 2002, investigated *Carum carvi* of Kumaun Himalaya that contain carvone as a major constituent (81.5%). The other constituents were citronellyl acetate, dihydrocarvone, eugenol, isolimonene and limonene oxide. Delta 3-carene, camphene, caryophyllene, carveol, p-cymene, dihydrocarveol, linalool, p-mentha-2, 8-dien-1-ol, myrcene, α–pinene, β-pinene, phellandrene, sabinene, α-terpinene and terpinelene were isolated in trace amounts (Chowdhury, 2002). Iacobellis, et al. 2005, analyzed *Carum carvi L.* by gas chromatography (GC) and GC-MS. The main components of *Carum carvi* oil were carvone (23.3%), limonene (18.2%), germacrene d (16.2%), and *trans*-dihydrocarvone(14.0%) (Iacobellis, et al. 2005). The antibacterial activity of *Carum carvi* essential oil is apparently due to carvone (23.3%), limonene (18.2%), carvacrol (6.7%), and linalool (0.3%), which inhibit the growth of fungi and bacteria (Farag et al. 1989; Helander, et al. 1998; Dorman and Deans, 2000; Kim, et al. 1995; Oosterhaven, et al. 1995; Naigre, et al. 1996). Antibacterial activity, determined with the agar diffusion method, was observed against Gram-positive and Gram-negative bacterial species in this study. The activity was

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particularly high against the genera *Clavibacter*, *Curtobacterium*, *Rhodococcus*, *Erwinia*, *Xanthomonas*, *Ralstonia*, and *Agrobacterium*, which are responsible for plant or cultivated mushroom diseases worldwide. In general, a lower activity was observed against bacteria belonging to the genus *Pseudomonas* (Iacobellis, et al. 2005).

Materials and Methods

Plant Collection & Identification: Plants & their seeds were collected from Dharchula District (about 3000 m amsl) of Uttarakhand, India, in the first week of October during flowering season. The plants were identified by Prof. Y.P.S. Pangtey, Botany Department, Kumaun University, Nainital, Uttarakhand, India and Dr. K.S. Negi, Principal Scientist, Regional Station, National Bureau of Plant Genetic Resource (NGPR), Niglat, Dist. Nainital, Uttarakhand, India.

Extraction of Essential Oil: The whole aerial part of the plant (1 to 4 inches tall) was used for the extraction of oil. The essential oil was obtained by steam distillation of fresh plant material (500 g) using a copper still fitted with spiral glass condensers. The distillate was saturated with NaCl and extracted with hexane. The hexane extract was dried using anhydrous sodium sulphate and the solvent was removed with a rotovap at reduced pressure at 25 °C to yield oil.

Thin layer chromatography: TLC was run on silica gel coated plates. Detection was done either by anisaldehyde followed by heating at 100 °C and observing under UV lamp.

GC and GC-MS analysis: GC-MS analysis was performed in Agilent 6890 Series equipped with MSD 19091S-433 system under following conditions.

GC Condition: Column: HP-5 MS, 5% Phenyl Methyl Siloxane, Max temperature:325 ^{0}C, Nominal length:30.0 m, Nominal diameter:250.00 um, Nominal film thickness:0.25 um, Mode: Constant flow, Inlet Temperature:250 ºC, Oven Temperature: 50 ºC hold 1 min, 250 ºC @4 ºC per min, Gas Type: Helium, Mode: Split less, Flow rate:1.2 ml/min (Constant Flow)

MS Parameters:MS System:Agilent 5975 inert mass selective detector,Ionisation Mode:EI (70ev),Acquisition Mode:Scan, Low Mass: 40.0, High Mass: 550.0, Threshold:150, MS Quadrupole:150 ºC, MS Source:230 ºC

Sample components were identified by matching their mass spectra with those in NIST 05 MS library search and by comparing with literature reports and gc retention indices (RI) [1].

Dynamic Headspace Gas Chromatography (DH/GC-MS): Dynamic Headspace Condition: 1.0 gm of sample is taken in 20 ml headspace vial. Headspace is created and these vapours are injected in GC equipped with MSD.

Zone temp: Vial Temperature: 120 ºC, Loop Temperature: 130 ºC, Transfer Line Temp:150 ºC

Event Time: Cycle Time: 80.0 min,Vial equilibration Time:15.0 min, Pressurizing Time:0.20 min, Loop Fill Time: 0.05 min, Loop Equilibration Time:0.05 min, Injection Time:10.0 min,Vial: Shake High: min

Headspace components were identified by matching their mass spectra with those in NIST 05 MS library search and by comparing with literature reports and GC retention indices (RI)[12].

Investigation of Antimicrobial Activity

Determination of Antimicrobial activity (minimum inhibitory concentration MIC) of oil extracts by two-fold serial dilution method:

Materials: Nutrient agar: Sisco Research Lab Pvt. Ltd., 26, Navketan Industrial Premises, Mumbai, 400099, India, Nutrient broth: Sisco Research Lab Pvt. Ltd., Mumbai, 400099, India, Sabouraud Dextrose agar: Sisco Research Lab Pvt. Ltd., Mumbai, 400099, India, Sabouraud Dextrose broth: Sisco Research Lab Pvt. Ltd., Mumbai, 400099, India, Glass tube 6" X ¾ " size: Borosil, Borosil Glassworks Ltd., Khanna Construction

House, 44, Dr. R.G. Thadani Marg, Worli, Mumbai-18, India, Glass tube (Microbiological glass pipettes of 1.0 ml and 2.0 ml capacity): Borosil, Borosil Glassworks Ltd., Khanna Construction House, 44, Dr. R.G. Thadani Marg, Worli, Mumbai-18,India, Chemical balance: Citizan Scale, (I)Pvt.Ltd.Citizan house, Govt.Industrial estate, Andhari (W) Mumbai-67, India, Autoclave:MAC, Macro Scientific Works (R), Jawahar Nagar Delhi-7, India, Hot air oven:MAC, Macro Scientific Works (R), Jawahar Nagar Delhi-7, India, Cell homogenizer: MAC, Macro Scientific Works (R), Jawahar Nagar Delhi-7, India, Bacterial incubator:MAC, Macro Scientific Works (R), Jawahar Nagar Delhi-7, India, Fungal incubator: MAC, Macro Scientific Works (R), Jawahar Nagar Delhi-7, India, Laminar Airflow: MAC, Macro Scientific Works (R), Jawahar Nagar, (Clean air work station) Delhi-7, India, Microscope: Olympus, Olympus (India) Pvt. Ltd., A-5, Mohan co-operative Industrial Area Mathura Road, New Delhi-110044, India, **Haemocitometer**:MAC, Macro Scientific Works (R), Jawahar Nagar Delhi-7,India, Distilled Water, Dimethyl sulphoxide: E Merck (India)Ltd. Worli, Mumbai-18, India

Test organisms: (a) Bacterial cultures: (i) *Staphylococcus aureus* (MTCC - 96) (ii) *Escherichia coli* (MTCC - 40) (b) Fungal cultures: (i) *Candida albicans* (MTCC – 227) (ii) *Cryptococcus neoformans* (ATCC-32045)

Methods: The test organisms; bacterial and fungal cultures (*Staphylococcus aureus* (S.a), *Escherichia coli* (E.c), *Cndida albicans* (C.a) and *Cryptococcus neoformans* (C.n.) were obtained from Central Drug Research Institute, Chattar Manzil Palace, Lucknow and maintained in the Microbiology laboratory, Department of Biotechnology, M.B. Govt. P.G.College, Haldwani, Nainital for further use on suitable growth promoting media.

Preparation of Nutrient agar slants for the maintenance of bacterial culture

Suitable quantity of nutrient agar medium, (peptone 5.0 g, NaCl 5.0g, yeast extract 2.0g, Beef extract 1.0 g/lit. pH 7.4±0.2 Agar 15.0 g) was accurately weighed and suspended in suitable amount of distilled water. The agar was dissolved by heating the medium in a water bath the melted agar was transferred of 6" x ¾" glass tubes containing approximately 10-12 ml in each tube. The medium containing tubes were plugged with non-absorbent cotton and sterilized at 15 lb/15 min in an autoclave. After sterilization the medium containing tubes were kept in slanting position with the help of a glass rod. The slants were incubated in a bacteriological incubator at 37 ºC for 24 h for the test of sterility of the nutrient medium.

Preparation of Sabouraud Dextrose agar slant for the maintenance of fungal strains

Suitable quantity of Sabouraud Dextrose agar medium (Glucose 20.0, Neopeptoone g/lit pH 5.6±0.2) was accurately weighed and suspended in suitable amount of distilled water. The SDA slants were also prepared and sterilized in the same manner as described earlier. The sterility of the SDA slants was also checked by incubating the tubes in a fungal incubator at 28 ºC for at least 72 h.These slants were stored in the refrigerator for periodical sub-culturing of bacterial and fungal strains.

Sub-culturing of bacterial and fungal strains in their maintenance media

A loopful culture from the stock of different strains was introduced into fresh agar slants to their respective media aseptically in the laminar air flow with the half of a sterile inoculating needle the inoculated agar slants were then incubated at the optimum temperature i.e 37º±1 ºC in case of bacteria and at 28º±1 ºC in case of fungal strains for 48 h. The purity of the strains was checked by staining and observation under microscope. Gram's staining procedure was adopted to stain the bacterial cultures and cotton blue stain was used to stain the fungal cultures. The

bacterial and fungal cultures were stored in refrigerator for further use.

Preparation of nutrient broth and Sabouraud's dextrose broth for the determination of minimum inhibitory concentration: 100 ml each of nutrient broth(Peptone 5.0, NaCl 5.0, yeast extract 2.0, Beef extract 1.0g/lit pH 7.4±0.2,) and Sabouraud's dextrose broth(Glucose 20.0, peptone 10g/Lit, pH 5.6±0.2) was prepared in 250 ml conical flask by dissolving suitable quantity of each broth in distilled water. The flasks containing the media were cotton plugged and sterilized as described earlier. Microbiological glass pipettes and assay tubes thoroughly washed and dried, cotton plugged with non absorbent cotton were sterilized in a hot air oven at 160 ºC for 2h.

Preparation of inoculums of test organisms: For the preparation of inoculums nutrient broth and sabourauds Dextrose broth were prepared in 6" x ¾" glass tubes. The tubes containing broth were sterilized. Bacterial cultures (*Staphylococcus aureus* (S.a) and *Escherichia coli* (E.c), yeast cultures *Cndida albicans* (C.a) and *Cryptococcus neoformans* (C.n.) were inoculated in the respective broth from the stock cultures stored in the refrigerator. The inoculated broth was incubated at optimum temperature for optimum period in the incubators.The fully grown cultures were diluted to such an extent with sterile medium prepared in 250 ml conical flask containing 100 ml nutrient broth/Sabouraud's dextrose broth that a cell concentration of 10^5 cells/ml (10^5/ml colony forming unit (CFU) was achieved. The cell counting was done by haemocitometer.

Preparation of stock solution of oil extracts

The oil extracts were accurately weighed and a stock solution of 1 mg/ml concentration was prepared in dimethyl sulphoxide (DMSO). DMSO was chosen as a carrier solvent because it is water miscible and does not inhibit the growth of the microbes under test a concentration of 200 µl/ml. Furthermore all the oil extracts were soluble in this organic solvent.

For the detection of minimum inhibitory concentration of a single oil extract against a single test organism at least six 4" x ½" assay tubes were arranged in a rack. The bulk medium containing 10^5 CFU/ml of test organism was dispensed in the assay tubes aseptically in such a way that the first tube contained 1.8 ml broth and rest tubes 1.0 ml each with the help of sterile microbiological glass pipettes 0.2 ml or 200 µl of sample from stock solution (1 mg/ml) of oil extract was added to the first tube in the row containing 1.8 ml inoculated broth and mixed thoroughly; 1.0 ml from this tube was taken out and added to the next tube. This process of dilution was repeated till the last or the sixth tube. In this way the concentration of the compound was reduced to its half in each tube. Culture control and solvent control (dimethyl sulphoxide) controls were also run simultaneously all the experimental tubes and control tubes were incubated at optimum temperature for optimum period of time i.e. at 37º±1ºC in case of detection of minimum inhibitory concentration against bacterial cultures for 24 h and at 28º±1ºC for fungal strains for 48 h. After the completion of incubation period the tubes were removed from the incubator and MIC was noted by judging the turbidity or comparing the growth with respective culture controls with unaided eye. In the same manner all the extracts were tested and biological activity was evaluated.

Results and discussion

GC-MS Analysis of Essential Oil: The seeds of *carum carvi* yielded 0.9% by weight of yellow oil with a pleasant aroma. The GC and GC-MS analysis of the essential oil from the seeds of *carum carvi* revealed the presence of eleven compounds and all of them were identified by comparing their mass spectra with ms library. Present study shows that the oil is rich in oxygenated monoterpenes. The

oxygenated monoterpenes, monoterpene hydrocarbons and aromatic aldehyde amounted to 90.63%, 8.92%, and 0.45% respectively. The major constituents are 87.59% (+)-(s)-carvone, 6.69% d-limonene, 1.33% o-cymene, 1.04% dihydrocarvone and 0.9% γ-terpinen. (Table 1, Fig 1)

Table 1: Chemical composition (%) of essential oil of *carum carvi* (seed).

Pk	RT	Area %	Common Name	Method of Identification
1	12.311	1.33	o-cymene	a,b
2	12.433	6.69	d-limonene	a,b
3	13.453	0.90	γ-terpinen	a,b
4	16.065	0.18	trans-p-mentha-2,8-dienol	a,b
5	17.924	1.04	dihydrocarvone	a,b
6	18.140	0.69	d-dihydrocarvone	a,b
7	18.856	0.55	neodihydrocarveol	a,b
8	19.060	0.38	cis-carveol	a,b
9	19.754	87.59	(+)-(s)-carvone	a,b
10	20.290	0.20	(-)-perillaldehyde;	a,b
11	20.384	0.45	cinnamaldehyde	a,b

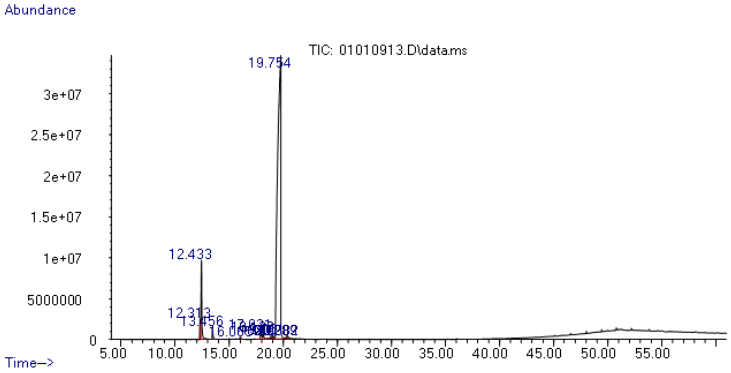

Fig 1. GC of essential oil of *carum carvi* (seed).

Dynamic Headspace GC-MS Analysis

The seeds of fresh *carum carvi* were shade dried and crushed to powder. One gram of powdered material was taken for dynamic headspace gc-ms analysis. Headspace gc-ms of *carum carvi* revealed the presence of seventeen volatile organic components and all of them were identified by comparing their mass spectra with ms library except one. The dominant component of the oil is a monoterpene hydrocarbon. The monoterpene hydrocarbons, oxygenated monoterpenes and sesquiterpenes amounted to 78.13%, 21.76% and 0.05% respectively. The major constituents are 73.64% d-limonene, 19.23% (+)-carvone, 2.26% β-pinene, 1.9% trans-dihydrocarvone and 0.41% 3-carene. (Table 2, Fig 2). In the present study, it is interesting to compare the results of the GC-MS of oil obtained by hydro distillation and dynamic head space GC-MS result of Carum carvi. The gc-ms of oil show 87.59% (+)-(s)-carvone and 6.69% d-limonene whereas dynamic headspace gc-ms show 73.64% d-limonene and 19.23% (+)-carvone as major components. It proves that d-limonene is precursor to carvone. It cracks to form isoprene at elevated temperature and gets oxidized easily in moist air to carveol and carvone.

Tewari and Mathela, 2003, has reported the presence of carvone (60%) and limonene (35%) as the major chemical constituents of the essential oil of Carum carvi (Raghunathan and Mittra, 1982). Chowdhury, 2002, investigated Carum carvi of Kumaun Himalayas and found that it contains carvone as a major constituent (81.5%) (Satyavati, et al. 1976).

Table 2: Chemical composition (%) of *carum carvi* by dynamic HD/GC-MS

Pk	RT	Area %	Common Name	Method of Identification
1	8.772	0.30	1R-α-pinene	a,b
2	10.253	0.39	β-terpinene	a,b
3	11.156	2.62	β-pinene	a,b
4	13.004	73.64	d-limonene	a,b
5	13.085	0.41	3-carene	a,b
6	13.307	0.39	γ-terpinene	a,b
7	14.030	0.18	limonene	a,b
8	15.533	0.07	limonene oxide	a,b
9	15.668	0.17	trans-limonene oxide,	a,b
10	16.513	0.06	1R-α-pinene	a,b
11	16.670	0.05	unidentifled	a,b
12	17.457	1.21	trans-dihydrocarvone	a,b
13	17.661	0.69	dihydrocarvone	a,b
14	17.812	0.39	neodihydrocarveol	a,b
15	19.275	19.23	(+)-carvone	a,b
16	23.420	0.05	caryophyllene	a,b

a = Retention Index; b = MS (GC-MS) Library

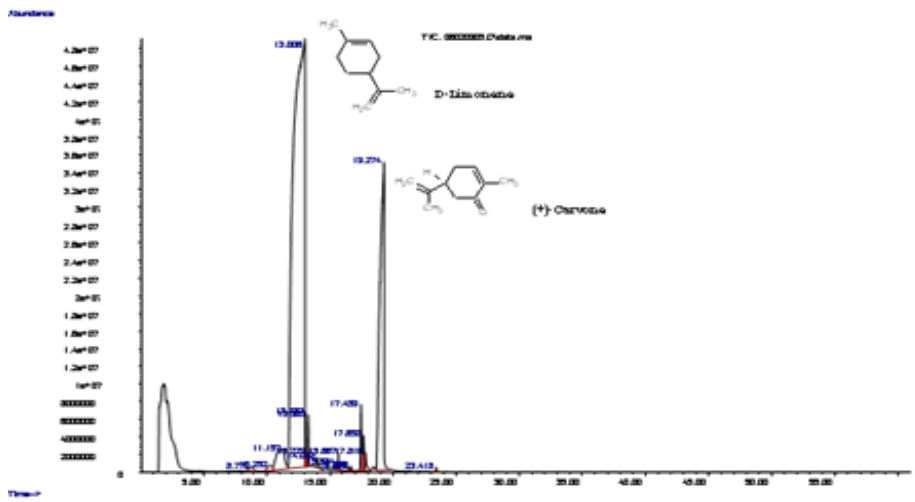

Fig 2. Dynamic headspace gc of *carum carvi* (seed).

In our studies also, the GC-MS of oil showed 87.59% (+)-(s)-carvone and 6.69% d-limonene. On the other hand, the percentage of these two components obtained by dynamic headspace GC-MS analysis was entirely different. It showed the presence of 73.64% d-limonene and 19.23% (+)-carvone in the plant. The reason behind lesser percentage of d-limonene obtained in GC-MS of oil extract obtained by hydro distillation is that d-limonene is the precursor to carvone. Fahlbusch et al. 2003; Simonsen, 1947, has reported that d-limonene is used to synthesize carvone via a three step reaction. In the studies of few reporters it has been proposed that d-limonene cracks to form isoprene at elevated temperatures and gets oxidized easily in moist air to carveol and carvone (Karlberg, et al. 1992; Mann, et al. 1994; Miyazawal, et al. 2002). From these findings it is clear that during extraction of oil by hydro distillation in clevenger type of apparatus, the compound d-limonene would have been converted to carvone due to which its GC-MS analysis showed higher percentage of carvone instead of d-limonene. However, in dynamic headspace isolation of compounds from the plant, the temperature is not so much elevated

for the conversion of d-limonene to carvone. So, the exact percentage of both the components was obtained.

Antimicrobial activity: The lowest concentration of a compound preventing appearance of turbidity or growth of test organism in the tube under test was considered to be the minimum inhibitory concentration of that compound. The MIC of oil extracts is given in the tabular form. The MIC of the samples ranged from 50 µg/ml to 100 µg/ml for bactericidal and fungicidal activity (table 3).

Table 3. Minimum Inhibitory concentration of oil extracts (MIC)

S.No.	Name of Oil extracts from	Test Organisms Used			
		Bacterial strains		Fungal strains	
		Staphylococcus aureus	***Escheridria coli***	***Candida albicans***	***Cryptococcus neoformans***
	Carum carvi (Family: Apiaceae)	Nil	50 µg/ml	100 µg/ml	100 µg/ml

Conclusion

From these findings it is clear that during extraction of oil by hydro distillation in clevenger type of apparatus, the compound d-limonene would have been converted to carvone due to which its GC-MS analysis showed higher percentage of carvone instead of d-limonene.It proves that d-limonene is precursor to carvone. It cracks to form isoprene at elevated temperature and gets oxidized easily in moist air to carveol and carvone. However, in dynamic headspace isolation of compounds from the plant, the temperature is not so much elevated for the conversion of d-limonene to carvone. So, the exact percentage of both the components was obtained. The antibacterial activity of *Carum carvi* essential oil is apparently due to carvone (23.3%), limonene (18.2%), carvacrol (6.7%), and linalool (0.3%), which inhibit the growth of fungi and bacteria.

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