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Research Article

Chemical composition of volatile extract of *Gnaphaliumluteo-album* l. Its and its antibacterial activity

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ABSTRACT

The quantitative and qualitative analysis of the oil by GC and GC-MS, led to the identification of 14 constituents, constituting 96.13 % of the volatile extract.It was mainly characterized by the dominant presence of hydrocarbons (41.6 %) followed by oxygenated sesquiterpene (25.1 %). Dodecane (31.10 %) was found as major compound with other major constituents6,10,14-trimethyl-2-pentadecanone (18.23 %), indol (8.12 %), 5-methyl undecane (7.99 %), *γ*-cuprene (7.03 %) and isoledene (6.05 %). The antimicrobial activity of the volatile extract was determined by well diffusion method. The extract was found to be significantly active for *Klebsiellapneumoniae, Salmonella enterica*and*Escherichia coli* and can be used as antimicrobial agent for these strains. High antibacterial activity of the oil against all microorganisms indicates good bioactive potential of the oil.

INTRODUCTION

The genus *Gnaphalium*, a variable annual or perennial herb distributed worldwide, comprises approximately 200 species of the Compositae (Asteraceae) family that belongs to the tribe Gnaphalieae. Among them, 19 species are widespread in the Yangtze and Pearl river basins of China[1]. Itis an annual herbaceous plant that grows widely in East Asia, including the temperate regions of China, Korea, and Japan as well as some high altitude tropical regions of India, Nepal, and Thailand. The species is traditionally used as a wild vegetable in the Guangdong and Fujian provinces of China[2,3]. Every year after the traditional Ching Ming festival, it is extensively harvested nationally as a wild vegetable and then processed into a variety of foods, such as drinks, canned products, and frozen vegetables. *G. affine* is believed to be of high nutritional value since it has a reasonable proportion of the eight essential amino acids for human body, a high content of minerals, trace elements, and vitamins, and is thus considered suitable to be developed into a functional food[4]. This plant is also known as *Helichrysumluteoalbum*. Its common names are Jersey Cudweed[5] and Weedy Cudweed, is a cosmopolitan weed. It grows as an erect herb up to 70 centimetres high, branching from the base. Flowers are cream, yellow, white, or pink[6]. This species is so widely distributed that it is unclear where it is native and where naturalised. In general it is considered naturalised in North and South America, and native to every other continent except Antarctica[7].The leaves are used as an astringent and vulnerary. The tomentum is applied as counter irritant for gout.

More than 125 chemical constituents have been isolated from the genus Gnaphalium, including flavonoids, sesquiterpenes, diterpenes, triterpenes, phytosterols, anthraquinones, caffeoylquinic acid derivatives, and other compounds. The extracts of this genus, as well as compounds isolated from it, have been demonstrated to possess multiple pharmacological activities such as antioxidant, antibacterial and antifungal, anti-complement, antitussive and expectorant, insect antifeedant, cytotoxic, anti-inflammatory, antidiabetic and antihypouricemic properties. Leaves contain luteolinglucoside. Flowers contain luteolin, its glucosides, a chalconeglucoside, dehydro-p-asebotin and other flavonoids. The plant also contains resin, tannin, essential oil, bitter principles and phytosterin[8]. The essential oil composition of this plant from Turkey was found to contain 44 compounds and major compounds were hexanal, undecane, and heptanal[9].

Materials and Methods

Plant collection and extraction

The plant material was collected from Ramgarh (district Nainital) and identified from Botanical survey of India, Dehradun and Department of Botany, DSB Campus, Kumaun University, Nainital. The specimen herbarium has been deposited in Botanical survey of India, Dehradun and Department of Botany, DSB Campus, Kumaun University, Nainital. Fresh aerial parts (2 kg) were subjected to steam distillation. The distillates were

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saturated with NaCl and extracted with *n*-hexane and dichloromethane. The organic phase was dried over anhydrous Na_2SO_4 and the solvent was distilled off in rotary vacuum evaporator at 30°C.

Gas chromatography

The oil was analyzed by using *Shimadzu QP-2010* gas chromatograph equipped with Omega wax SP-2560 column. Details of the column are given below

Column Name : SP-2560
Serial Number : 24056
Film Thickness : 0.20 um
Column Length : 100.0 m
Inner Diameter : 0.25 mm ID
Column Max Temp : 250 C

The oven temperature (100-240˚C) was programmed at 3˚C min^{-1} using N_2 as carrier gas at 4 Kg cm^{-2}. The injector temperature was 260˚C, detector temperature 270˚C and the injection volume 0.5 µL, using a 10% solution of the oil in *n*-hexane.

Gas chromatography / Mass spectroscopy

GC-MS was conducted on a Gas Chromatograph Mass Spectrometer (GCMS) - *Shimadzu QP-2010 Plus with Thermal Desorption System TD 20* ion trap mass spectrometer equipped with Omega wax SP-2560 column. Details of the column are given below

Column Name : SP-2560
Serial Number : 24056
Film Thickness : 0.20 um
Column Length : 100.0 m
Inner Diameter : 0.25 mm ID
Column Max Temp : 250 C

The oven temperature (100-240˚C) was programmed at 3˚C min^{-1} using helium as carrier gas at 1.21 mL min^{-1}. The injection, ion source and Interface temperatures were 260˚C, 230˚C and 270˚C, respectively, the injection volume was 0.1µL, and the split ratio was 10. MS were taken at 70 eV with mass range of 40-450 amu.

Retention Indices

Besides the spectral methods, the identification of the essential oil constituents was done by calculation of their retention indices and compared with those of the literature reports. Retention indices were experimentally determined by the following formula:

$$\mathbf{RI = 100 \times N_0 + 100\,[RT_{un} - RT_{No}] / [RT_{N1} - RT_{No}]}$$

RT_{un} = RI value of the compound to be identified.
RT_{No} = n-alkane eluted before the unknown peak.
RT_{N1} = n-alkane eluted after the unknown peak.
N_0 = Carbon number from which the standardization is done.

Identification of constituents

Characterization of constituents was done on the basis of Linear Retention Index (LRI, determined with reference to homologous series of *n*-alkanes C_9-C_{24}) under identical experimental condition, co-injection with available compounds, mass spectral fragmentation pattern and library search (NIST and WILLEY) and by comparing with the MS literature data[10] and by ^{1}H-NMR and ^{13}C-NMR spectral data. The percentage contents of the constituents were determined by FID response on GC. The known compounds were further confirmed by comparing with the authentic samples.

Antimicrobial activity

Growth and maintenance of test microorganism for antimicrobial studies

The *in vitro* antibacterial activities of the samples was evaluated against a total of five bacteria including one gram positive *Stephylococcusaureus* (MTCC-3160) and four gram negative bacteria *Klebsiellapneumoniae* (MTCC-3384), *Pseudomonas aeruginosa*(MTCC-424), *Salmonella enterica* (MTCC-98) and*Escherichia coli* (MTCC-443). The test strains were procured from the Institute of Microbial Technology (IMTECH), Chandigarh, India. MTCC (Microbial Technology Culture Collection) numbers represent the standard strain numbers assigned to these microorganisms. The bacteria were maintained on nutrient broth (NB) at 37°C and fungus was maintained on malt yeast broth at 28°C.

Determination of zone of inhibition

The antimicrobial activity of the oils and extracts was investigated by the well diffusion method using 24–48 hours grown strains reseeded on nutrient broth (bacterial strains) and Malt yeast agar (fungal strains)[11]. The cultures were adjusted to 1×10^6 CFU/mL with sterile water. Then 100 µL of the suspensions were spread over nutrient agar and malt yeast plates to obtain uniform microbial growth. In petri plates well prepared with the help of borer (bore 2 mm diameter). The wells were filled with 20 µL of essential oil solutions in the inoculated nutrient/malt yeast extract agar plates. The petri dishes were kept at 4 °C for 2 hours. The plates were incubated at 37 (24 hours) and at 30 °C (48 hours) for bacterial and fungal strains, respectively. The diameter of the inhibition zones were measured and considered as the zone of inhibition (ZOI). All experiments were performed in triplicate.

Determination of the minimum inhibitory concentration (MIC)

The performed agar dilution susceptibility test was based on modified methods of NCCLS and CLSI[12, 13]. To determine the minimum inhibitory concentration (MIC) of the potent oils a series of dilutions of each potent oil

ranging from 10-50 µL/mL were prepared In the agar-well diffusion technique, serial dilutions of the essential oils were prepared by diluting oil with DMSO to achieve a decreasing concentration range from 60 to 5 µL/mL using 100 µL of a suspension containing 1×10^6 CFU/mL of bacteria spread on nutrient agar plates, whereas the fungal strains were reseeded on Potato dextrose agar (PDA) plates. The wells were filled with 20 µL of essential oil solutions in the inoculated nutrient/malt yeast extract agar plates. The bacterial plates were incubated at 37±2 °C for 24–72 hours, while fungal cultures were incubated at 30 ± 2°C for 48 hours. The MIC was defined as the lowest concentration of the oil inhibiting the visible growth of each bacterium on the agar plate so the least concentration of each essential oil showing a clear zone of inhibition was taken as the MIC. DMSO was used as the negative control. Gentamicin and kanamycin were used as positive control for bacteria andnystatin for fungi.

Results and discussion

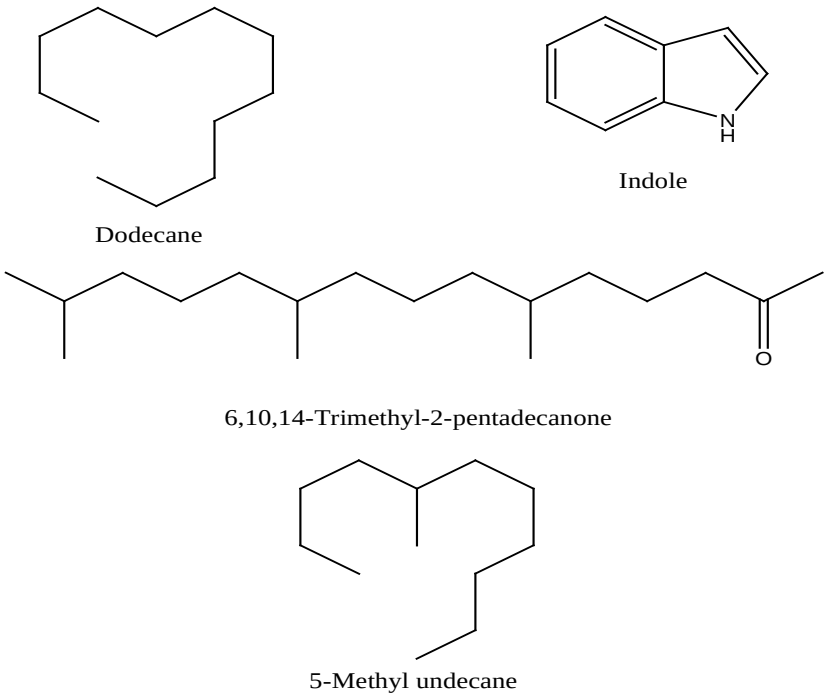
Terpenoid composition

The quantitative and qualitative analysis of the oil by GC and GC-MS, led to the identification of 14 constituents, constituting 96.13 % of the oil. The identified compounds are listed in Table 1 in order of their elution in Omega wax SP-2560 column. The oil was dominated by hydrocarbon dodecane (31.10 %). The other major constituents were 6,10,14-trimethyl-2-pentadecanone (18.23 %), indol (8.12 %), 5-methyl undecane (7.99 %), *γ*-cuprene (7.03 %) and isoledene (6.05 %). The structure of major compounds has been given in Figure 1.

Table 1: Essential oil composition of *Gnaphaliumluteo-Album L.*

S. No.	Compounds	RI	% Composition (FID)	Mode of Identification*
1.	undecane	1100	2.51	a, b
2.	indole	1190	**8.12**	a, b
3.	5-methyl undecane	1195	**7.99**	a, b
4.	dodecane	1200	**31.10**	a, b, c
5.	α-copane	1250	2.36	a, b
6.	limonene aldehyde	1327	t	a, b
7.	isoledene	1374	**6.04**	a, b
8.	*β*-caryophyllene	1417	4.12	a, b
9.	*E-β*-fernecene	1505	1.76	a, b
10.	sesquisabinene	1525	t	a, b
11.	*γ*-cuprene	1532	**7.03**	a, b, c
12.	caryophyllene oxide	1582	1.89	a, b, c
13.	veridiflorol	1592	4.98	a, b
14.	6,10,14-trimethyl-2-pentadecanone	1610	**18.23**	a, b
	Total		96.13	

*Mode of identification: Retention Index (LRI, Based on homologous series of n-alkanes; C_8-C_{24}), coinjection with Standards/Peak enrichment with known oil constituents, t= trace (<0.1%); (-) = not detected, a = Retention Index (RI) on Rtx-5 capillary column; b = MS (GC/MS) comparison with NIST and WILLEY and literature (Adams, 2007); c= co-injection with the standard compound.



Dodecane

Indole

6,10,14-Trimethyl-2-pentadecanone

5-Methyl undecane

Figure1: Structures major compounds of *Gnaphaliumluteo-Album* L. essential oil.

Antimicrobial activity

In this study, five microbial species were used to determine the possible antimicrobial activity of the oil and shown in Table 2. Of the species used, *Klebsiellapneumoniae* is the microbe responsible for destructive changes to human lungs via inflammation and hemorrhage with cell death (necrosis) that sometimes produces a thick, bloody, mucoid sputum (currant jelly sputum).The most common condition caused by Klebsiella bacteria outside the hospital is pneumonia, typically in the form of bronchopneumonia and also bronchitis. These patients have an increased tendency to develop lung abscess, cavitation, empyema, and pleural adhesions. It has a high death rate of about 50%, even with antimicrobial therapy. The mortality rate can be nearly 100% for people with alcoholism and bacteremia.Interestingly, the oil showed good activity against *Klebsiellapneumoniae* with ZOI 15.00 ± 0.00 mm and 50 µL/ mL MIC value. As shown inTables 2.2 the generation of most microbial species was inhibited by the oil. Activity of the oil has been compared with standard antimicrobial compounds in Fig 2. *Escherichia coli*, belonging to the normal flora of humans, is an enterohemmoragic bacteriumcauses serious cases of food poisoning and preservatives to eliminate its growth are needed. Oil was found to be active against *E. coli* with 13.00 ± 0.00 mm ZOI and 50 µL/ mL MIC value. The oil has also shown significant activity against *Pseudomonas aeruginosa* and *Salmonella enterica* (ZOI = 12.00 ± 0.00 and 11.00 ± 0.00 mm, respectively) with MIC value of 50 µL/ mL for each while least effective against *Stephylococcusaureus*(ZOI = 10.00 ± 0.58 mm and MIC = 100 µL/mL). Gentamicin (30 µg/disc) and kanamycin (30µg/disc) were used as positive controls.

Table 2: Antimicrobial activity of essential oil of *Gnaphaliumluteo-Album* L.

Bacterial strain	Oil		Standard antimicrobial			
			Gentamycin		**Kanamycin**	
	ZOI* (mm)	**MIC** (µL/mL)	**ZOI** (mm)	**MIC** (µL/mL)	**ZOI** (mm)	**MIC** (µL/mL)
KlebsiellaPneumoniae	**15.0±0.00**	**50**	10.67±0.58	50	10.67±0.58	50
Stephylococcusaureus	**10.00±0.58**	**100**	17.33±0.58	50	16.33±0.58	50
Escherichia coli	**13.0±0.00**	**50**	14.67±0.58	50	13.33±0.58	75
Salmonella enterica	**11.0±0.00**	**50**	10.67±0.58	50	9.67±0.58	100
Pseudomonas aeruginosa	**12.00±0.00**	**50**	13.33±0.58	50	12.67±0.58	50

*Zone of inhibition (ZOI) is given at 200 µl/ml. MIC= Minimum inhibitory concentration.

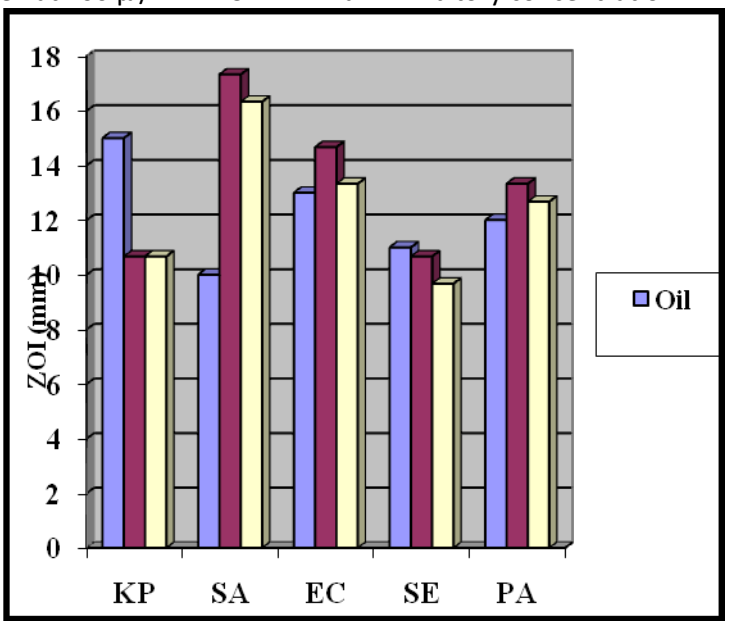


Figure 2.Comparative antimicrobial activity of the essential oil.

KP = *KlebsiellaPneumoniae*
SA = *Stephylococcusaureus*
EC = *Escherichia coli*
SE = *Salmonella enterica*
PA = *Pseudomonas aeruginosa*

The antimicrobial activity of the oil has been compared with the standard antimicrobial compounds in Figure 2.

Conclusion

The oil was mainly characterized by the presence of hydrocarbons (41.6 %) followed by oxygenated sesquiterpene (25.1 %). Dodecane (31.10 %) was found as major compound with other major constituents6,10,14-trimethyl-2-pentadecanone (18.23 %), indol (8.12 %), 5-methyl undecane (7.99 %), *γ*-cuprene (7.03 %) and isoledene (6.05 %). This report is in contrast to the previous report which shows hexanal and undecane as major constituentsbut presence of the hydrocarbons is significant in both the reports[9].

High antibacterial activity of the oil against all microorganisms indicates good bioactive potential of the oil. The oil was found to be significantly active for *KlebsiellaPneumoniae, Salmonella enterica*and*E. coli* and can be used as antimicrobial agent for these strains. Antimicrobial activity of essential oils is difficult to correlate to a specific compound due to their complexity and variability. It has been mainly explained through C_{10} and C_{15}terpenes with aromatic rings and phenolic hydroxyl groups able to form hydrogen bonds with active site of target enzymes, although other active terpenes, as well as alcohols, aldehydes and esters and their synergic effects can contribute to the overall antimicrobial effect of essential oils[14].Therefore, the antibacterial results observed in this investigation might be related to the presence of Dodecane, 6,10,14-trimethyl-2-pentadecanone, indol, 5-methyl undecane, *γ*-cuprene and isoledene. Since the major compound of the oil is hydrocarbon, the synergistic effects of the diversity of major and minor constituents in the essential oils should have more accountability for their overall biological activity.

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