Available online on 30.08.2019 at http://jddtonline.info

Journal of Drug Delivery and Therapeutics

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Terpenoid composition of *Chenopodium ambrosioides* L. and its Antimicrobial activity from Uttarakhand Himalaya of India

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ABSTRACT

C. ambrosioides var. *ambrosioides* essential oil has been commercialized to control greenhouse insects and mites and the aerial parts of this plant have been used as condiment; traditional purgative for intestinal worms and acesodyne and in the Chinese traditional medicine, this herb can expel wind and treat rheumatism. Further the essential oil of *Chenopodium ambrosioides* showed the antimicrobial activity against several microbial species. Our investigations therefore, reveal presence of another chemotype in this region with *α*-terpinene and *p*-cymene as chemical markers of this chemotypes and its antimicrobial activity .

Keywords: *Chenopodium ambrosioides, α*-terpinene, *p*-cymene and antimicrobial activity.

Article Info: Received 25 June 2019; Review Completed 14 Aug 2019; Accepted 20 Aug 2019; Available online 30 Aug 2019

Cite this article as:

Bisht BS, Kumar A, Terpenoid composition of *Chenopodium ambrosioides* L. and its Antimicrobial activity from Uttarakhand Himalaya of India, Journal of Drug Delivery and Therapeutics. 2019; 9(4-A):612-617

http://dx.doi.org/10.22270/jddt.v9i4-A.3536

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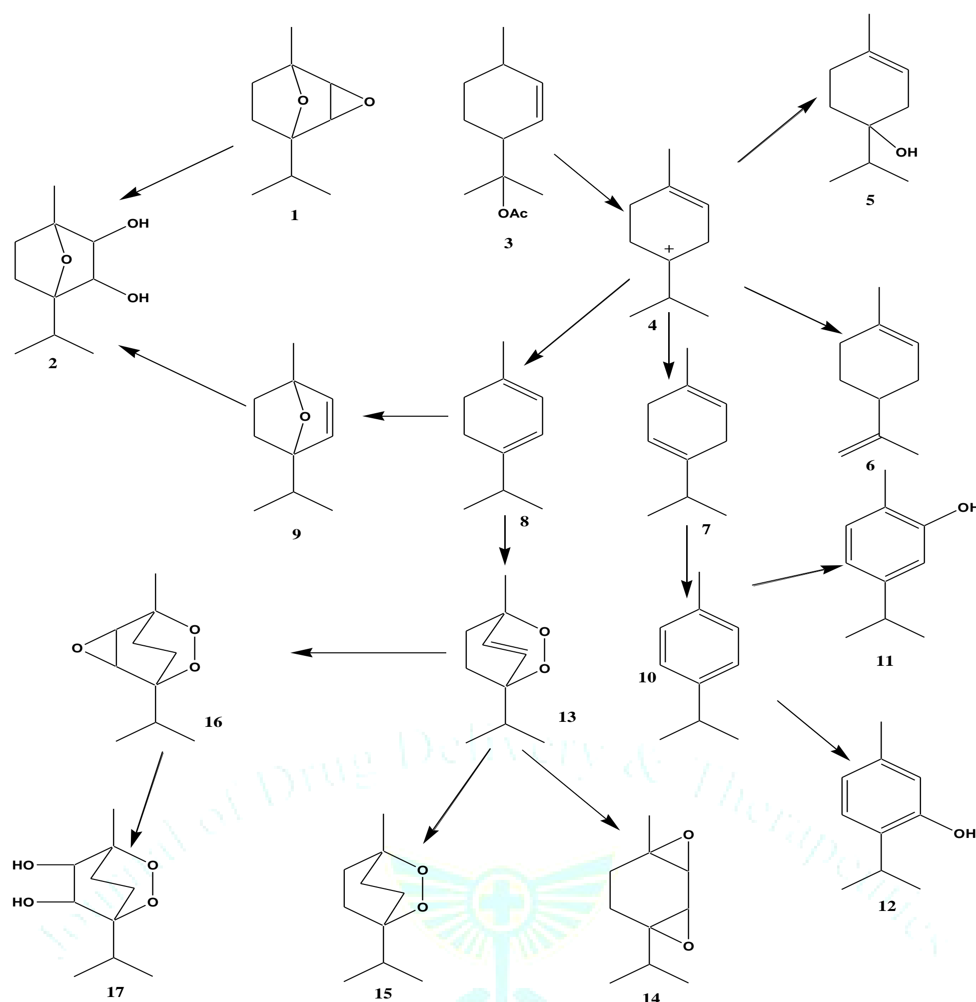
1. INTRODUCTION:

Plant-parasitic nematodes are responsible for substantial economic loss to agricultural crops. Nematode management is generally based upon chemical treatments, but environmental concerns and governmental regulations favour nematicides of natural origin [1,2]. One alternative is to screen naturally occurring compounds in plants which are known as plant secondary metabolites. Many plant constituents and metabolites including terpenoids have been investigated for activity against plant-parasitic nematodes [3-6]. A series of nematicidal substances of natural origin such as triglycerides, sesquiterpenes, alkaloids, steroids, diterpenes and flavonoids have been identified [2].

Chenopodium ambrosioides L. is an annual or perennial shrub, grows up to 1m high, leaves simple, alternate, occasionally opposite, lacking stipules, 2 - 12 cm long, 2.5. 9 cm wide, blade linear to broadly triangular in outline, margins entire serrated, serrate-dentate or lobed, with inflated glands, in senescence silvery shining. Inflorescences green, herbaceous, widely ramified, branches with flowers originate in the axilles of great leaves which are reduced in their upper parts. The bracts of the fruits green or brown, seeds one per flower, reddish brown. *C. ambrosioides* is mainly distributed in South America and throughout the tropical parts of the world. This species of plant is also distributed in the India. It is glandular erect herb, leaves oblong, or lanceolate, sinnuate-toothed, flowers yellowish-white. Flowering occurs in August–October [7].

Chemical composition of the essential oil of *C. ambrosioides* from different parts of the world has been widely reported such as from Brazil, Cuba, Mexico, Cameroon, Nigeria, Rwanda, China and India[8-17]. The essential oil of *C. ambrosioides* from China comprised bornylene, benzene, 1-methyl-4-(1- methylethyl), ascaridole and *α*-terpinene[14]. The oil of Indian and Brazilian *C. ambrosioides* was found to contain ascaridole as the main components (20-67%) besides hydrocarbons such as *p*-cymene, limonene and *β*-myrcene[8, 16, 17].

C. ambrosioides var. *ambrosioides* essential oil has been commercialized to control greenhouse insects and mites and the aerial parts of this plant have been used as condiment; traditional purgative for intestinal worms and acesodyne and in the Chinese traditional medicine, this herb can expel wind and treat rheumatism[18]. Further the essential oil of *Chenopodium ambrosioides* showed the strong antimicrobial activity against several microbial species like *Candida* species, *Aspergillus flavus, Aspergillus glaucus, Aspergillus niger, Aspergillus ochraceous* and *Collatotricum* species[11,8]. The biotransformation of *Chenopodium* constituents have been shown in **Fig 1**[20].



1. 1,4:2,3-diepoxy-*p*-menthane, **2.** ascaridole glycol, **3.** *α*-terpinyl acetate, **4.** *α*-terpinyl, **5.** terpin-1-ol, **6.** limonene, **7.** *γ*-terpinene, **8.** *α*-terpinene, **9.** 1,4-epopxy-*p*-enth-2-ene, **10.** *p*-cymene, **11.** carvacrol, **12.** thymol, **13.** ascaridole, **14.** isoascaridole, **15.** dihydroascaridole.

Fig 1. Formation of ascaridole and other terpenoid constituents present in *Chenopodium* species

The oil of *Chenopodium ambrosioides* was reported to possess strong fumigant activity against maize weevil[23], cytotoxic and antileishmanial properties of the essential oil of that species has also been reported[24]. The essential oil of *Chenopodium ambrosioides* showed the strong antifungal activity against several fungal species like *Candida* species, *Aspergillus flavus, Aspergillus glaucus, Aspergillus niger, Aspergillus ochraceous* and *Collatotricum* species[11, 8]. the oil of *Chenopodium ambriosoides* has been investigated only for its antimicrobial activity.

2. MATERIAL AND METHODS:

2.1. Plant material:

The fresh aerial parts of *Chenopodium ambrosioides* were collected from, Bhimtal (Nainital), Uttarakhand. Plant herbaria were identified from the Botanical Survey of India, Dehradun **(Acc. No. 113550)** and voucher specimen were deposited in the Phytochemistry Laboratory, Chemistry Department, Kumaun University, Nainital.

2.2. Extraction of the oil:

The fresh plant material (2 kg) was subjected to steam distillation. The distillates were extracted with *n*-hexane and dichloromethane. The organic phase was dried over anhydrous Na_2SO_4 and the solvent was distilled off.

2.3. GC and GC-MS analysis:

The oil was analyzed by using Nucon 5765 gas chromatograph equipped with Rtx-5 non-polar fused silica capillary column (30 m × 0.32 mm, 0.25 µm film coating). The oven temperature (60-210°C) was programmed at 3°C min^{-1} using N_2 as carrier gas at 4 Kg cm^{-2}. The injector temperature was 210°C, detector temperature 210°C and the injection volume 0.5 mL, using a 10% solution of the oil in *n*-hexane. GC-MS was conducted on a ThermoQuest Trace GC 2000 interfaced with a Finnigan MAT PolarisQ ion trap mass spectrometer equipped with Rtx-5 non-polar fused silica capillary column (30 m × 0.25 mm, 0.25 µm film coating). The oven temperature (60-210°C) was programmed at 3°C min^{-1} using helium as carrier gas at 1.0 min^{-1}. The injection, ion source and MS transfer line temperatures were 210°C, 220°C and 275°C, respectively, the injection volume was 0.1 mL, and the split ratio was 1:40. MS were taken at 70 eV with mass range of 40-450 amu.

2.4. Identification of constituents:

The 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, MS Library search (NIST and WILLEY) and by comparing with the MS literature data[19]. The relative

contents of individual components were calculated GC response on FID without using correction factor.

2.5. Growth and maintenance of test microorganism for antimicrobial Studies:

The *in vitro* antibacterial activities of the essential oils were evaluated against a total of five bacteria including two Gram positive *Micrococcus luteus* (MTCC-106) and *Bacillus subtilis* (MTCC-441) and three Gram negative bacteria *Pseudomonas aeruginosa* (MTCC-424), *Aeromonas hydrophila* subspecies *hydrophila* (MTCC-646) and *Escherichia coli* (MTCC-443). The antifungal activity of the oils was performed against *Streptomyces candidus* subspecies *azaticus* (MTCC-703) and *Candida albicans* (MTCC-227). The test strains were obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India. MTCC (Microbial Technology Culture Collection) numbers represents the standard strain numbers assigned to these microorganisms. The bacteria were maintained on nutrient broth (NB) at 37°C and fungi were maintained on malt yeast broth at 28°C.

Gram positive bacterial strain :

Name	**MTCC Number**
Micrococcus luteus	106
Bacillus subtilis	441

Gram negative bacterial strain :

Name	**MTCC Number**
Pseudomonas aeruginosa	424
Aeromonas hydrophila subspecies *hydrophila*	646
Escherichia coli	443

Fungal strains :

Name	**MTCC Number**
Streptomyces candidus subspecies *azaticus*	703
Candida albicans	227

2.6. Preparation of inoculums:

The Gram positive bacteria (*Micrococcus luteus, Bacillus subtilis*) and Gram negative bacteria (*Pseudomonas aeruginosa, Aeromonas hydrophila* subspecies *hydrophila, Escherichia coli*) were pre-cultured in nutrient broth overnight in a rotary shaker at 37°C, centrifuged at 10,00 rpm for 5 min, pellet was suspended in double distilled water. The fungal strains were pre-cultured in malt yeast broth at 35°C.

2.7. Determination of zone of inhibition:

The antimicrobial activity of the essential oils was investigated by the well diffusion method as adapted earlier was used [25-27]. The cultures were adjusted to $1×10^6$ CFU/mL with sterile water. 100 μL of the suspensions were spread over nutrient agar and malt yeast plates to obtain uniform microbial growth. Three –millimeter diameter wells were cut from the agar using a sterile cork-borer, and 30 μL of the essential oil/extract solution were delivered into the wells. The petri dishes were kept at 4 °C for 2 h. The plates were incubated at 37° C (24 h) and at 30° C (48 h) for bacterial and fungal strains, respectively. The diameter of the inhibition zones (mean values) were measured in millimeter and considered as the zone of inhibition (ZOI). DMSO was used as the negative control. Nalidixic acid and amikacin were used as positive controls for bacteria and fungi, respectively. All experiments were performed in triplicate.

2.8. Determination of the minimum inhibitory concentration (MIC):

The performed agar dilution susceptibility test was based on modified methods of NCCLS[25-27]. 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^{-1} 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 50 to 10 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 30 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 h., while fungal cultures were incubated at 30±2° C for 48 h. 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*. Each test was replicated three times.

3. RESULTS AND DISCUSSION:

The oil composition of the genus *Chenopodium* showed quantitative and qualitative variation in their terpenoid constituents and showed no correlation to the geographical distribution. Mostly ascaridole was found in the genus *Chenopodium* which was collected in different regions around the World.

The essential oil of Indian *C. ambrosioides* was found to contain *α*-terpinene, *p*-cymene and ascaridole as major constituents[16]. Ascaridole was first isolated in 1895 by a German pharmacist living in Brazil and it has been attributed with most of the vermifuge (worm-expelling) actions of the plant. In the early 1900's it was one of the major antihelmintics used to treat ascarids and hookworms in humans and pet animals[22]. Previously in an investigation in our laboratory, the *C. ambrosioides* collected from Almora (Uttarakhand) was found to contain ascaridole as a main constituent (57.89 %) along with 20.06 % limonene and 15.03 % *p*-cymene. A total of elevan compounds had been identified which constituted 96.92 % of the total oil[22]. But in contrast to these reports our sample collected from Bhimtal (Uttarakhand) was found to contain a total of 42 compounds out of which 35 compounds have been identified to constitute 99.2 % of the total oil. In this present investigation, unusually high content of *α*-terpinene (72.5 %) and *p*-cymene (20.6 %) were found as a main constituents alone constituting 93.1 % of the total oil content. The presence of ascaridole was limited to as low as 1.3 % while limonene was present in traces **(Table 1)**. Our investigations therefore, reveal presence of another chemotype in this region which *α*-terpinene and *p*-cymene as chemical markers of this chemotypes.

The essential oil of *C. ambrosioides* cultivated in China comprised bornylene (42.63%), benzene, 1-methyl-4-(1-methylethyl) (21.84%), ascaridole (18.36%) and *α*-terpinene (11.75%)[14]. The essential oils of *C. ambrosioides* were suggested to be split into three major categories[13] which includes oils rich in ascaridole (70-90%) (North AmerAican), oil in which ascaridole is the main components (20-67%) besides *p*-cymene, limonene or *β*-myrcene (India and Brazil), and finally the oil rich in *α*-pinene (30-32%) followed by pinocarveol (40-42%) and/or pinocarveol (62-65%) (Japan). The essential oils of the Indian *C. ambrosioides* belong to the first categories with ascaridole as a main component[21] except our chemotype. In some of the investigations it was also found to contain limonene and

trans-pinocarveol as a major constituent[10]. One more category of that plant species could be added in which α-terpinene and *p*-cymene were major constituents while ascaridole was noticed in a very low quantity[22].

Our investigation reveals that the sample collected from Bhimtal (Uttarakhand) belongs to that category of the plant *C. ambrosioides* in which α-terpinene (72.5 %) and *p*-cymene (20.6 %) were major components and ascaridole in a very low concentration.

For practical use, it is necessary to standardize the essential oil of *C. ambrosioides* found in different regions of Uttarakhand Himalayan because chemical composition of the essential oil varies greatly with geographical and environmental regions. In earlier reports the concentration of ascaridole, the main component of *Chenopodium* species was very high in the sample collected from Himalayan region[20] but present investigation shows almost absence of ascaridole as a constituent in a sample also collected from the Himalayan region of Uttarakhand.

The essential oil of the aerial parts of *C. ambriosoides* was found most active against *M. luteus* with zone of inhibition 14.67 ± 0.57 mm and also showed lowest MIC value 5 µL/mL against the same bacteria. The minimum activity of the oil of *C. ambriosoides* was found against *A. hydrophila.*

It is, therefore concluded that the *C. ambrosioides* collected from Bhimtal region of Uttarakhand shows chemotaxonomic dissimilarity with *C. ambrosioides* reported in an earlier study.

Table 1. Terpenoid Constituents of *Chenopodium ambrosioides* L.

S. NO.	COMPOUNDS	LRI	% FID (Present study)	CHEMOTYPE I [21]	IDENTIFICATION
1.	α-pinene	941	0.2	-	a,b
2.	β- pinene	982	0.1	-	a,b
3.	α- terpinene	1019	72.5	-	a,b
4.	*p*- cymene	1029	20.6	15.03	a,b
5.	limonene	1033	0.7	20.06	a,b
6.	*(Z)*- β-ocimene	1042	t	0.2	a,b
7.	*(E)*- β-ocimene	1053	t	-	a,b
8.	γ-terpinene	1062	t	-	a,b
9.	terpinolene	1085	2.5	-	a,b
10.	linalool	1100	t	-	a,b
11.	pinocarvone	1168	t	-	a,b
12.	*trans-p*-mentha-1(7),8-dien-2-ol	1190	0.3	-	a,b
13.	*cis-p*-mentha-1(7),8-dien-2-ol	1235	t	-	a,b
14.	ascaridole	1240	1.3	57.89	a,b
15.	thujyl alcohol		-	t	
16.	sabinol		-	t	
17.	thymol	1293	t	-	a,b
18.	carvacrol	1296	t	-	a,b
19.	*p*-cymen-7-ol	1298	t	-	a,b
20.	α-terpinyl acetate	1355	t	-	a,b
21.	citronyl formate		-	3.74	a,b
22.	diosphenol		-	t	
23.	piperitone oxide	1376	t	-	a,b
24.	β-maaliene	1384	t	-	a,b
25.	β-bourbonene	1386	0.1	-	a,b
26.	β-cubebene	1390	t	-	a,b
27.	β-cedrene	1410	0.2	-	a,b
28.	*(E)*-caryophyllene	1418	0.1	t	a,b
29.	aromadendrene	1445	t	-	a,b
30.	α-humulene	1454	0.1	-	a,b
31.	*(E)*- β-farnecene	1459	t	-	a,b
32.	β -chamigrene	1463	t	-	a,b
33.	ϒ-murrolene	1475	0.2	-	a,b
34.	ϒ- curcumene	1478	t	-	a,b
35.	germacrene D	1480	t	-	a,b
36.	ar-curcumene	1485	t	-	a,b
37.	δ-cadinene	1529	0.1	-	a,b
38.	caryophyllene oxide	1584	0.2	-	a,b
39.	α-bisabolol	1690	t	-	a,b
	Total identified		**99.2 %**		
	Monoterpene hydrocarbons		96.6 %		
	Oxygenated monoterpenes		1.6 %		
	Sesquiterpene hydrocarbons		0.7 %		
	Oxygenated sesquiterpene		0.3 %		

*a= Linear Retention Index (LRI), b=MS (GC-MS), c=NMR (^1H & ^{13}C), t = trace, less than 0.1%

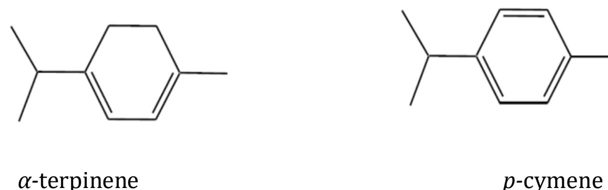


Fig. 2: Structure of the major constituents of *Chenopodium ambrosioides*

Table 2. Antibacterial and antifungal activity of the volatile constituents of *Chenopodium ambrosioides* showing zone of inhibition (ZOI) in mm ± standard deviation

Sample Code	Concentration (μL/mL)	*A. hydrophila*	*P. aeruginosa*	*E. coli*	*M. luteus*	*B. subtilis*	*C. albicans*	*S. candidus*
CSM-CA (A)	10	4.00 ± 0.00	NA	5.33 ± 1.15	8.00 ± 0.00	NA	5.67 ± 0.57	3.67 ± 0.57
	20	5.33 ± 1.15	4.00 ± 0.00	6.00 ± 0.00	9.33 ± 1.15	7.33 ± 1.15	6.67 ± 0.57	5.33 ± 1.15
	30	6.00 ± 0.00	5.33 ± 1.15	7.33 ± 1.15	10.67 ± 1.15	8.00 ± 0.00	9.00 ± 1.00	6.00 ± 0.00
	40	6.67 ± 1.15	6.00 ± 2.00	8.00 ± 0.00	13.33 ± 1.15	8.67 ± 1.15	10.67 ± 1.15	7.33 ± 1.15
	50	7.33 ± 1.15	8.00 ± 0.00	9.33 ± 1.15	14.67 ± 0.57	9.67 ± 0.57	11.67 ± 0.57	8.00 ± 0.00
Positive control	30 μg	14 [a]	16 [a]	14 [a]	20 [a]	22 [a]	10[b]	18[b]

CSM-CA (A) = Aerial part of *Chenopodium ambriosoides*, Control: a = Nalidixic acid, b = Amikacin, NA = Not active

Table 3. Antibacterial and antifungal activity of the volatile constituents of *Chenopodium ambrosioides* showing minimum inhibitory concentration (MIC) in μL/mL

Sample Code	*A. hydrophila*	*P. aeruginosa*	*E. coli*	*M. luteus*	*B. subtilis*	*C. albicans*	*S. candidus*
CSM-CA (A)	10	20	10	5	20	10	10

CSM-CA (A) = Aerial part of *Chenopodium ambrosioides*, Control: a = Nalidixic acid, b = Amikacin

ACKNOWLEDGEMENTS:

The authors are grateful to Prof. C.S. Mathela, Chemistry Department, of DSB campus, Kumaun University for providing laboratory and instrumentation facilities, Prof. Veena Pande, Head Biotechnology Department Bhimtal campus, Kumaun University for providing facility to conduct antimicrobial activity and one of us (BSB) is grateful to UCoST, Dehradun for Research fellowship.

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