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ASCARIDOLE AND RELATED PEROXIDES FROM THE GENUS *CHENOPODIUM*

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Aim: Detection of monoterpenoid ascaridole and other terpenoids in the genus *Chenopodium* from the East Mediterranean.

Method: Distribution of ascaridole in leaves of 13 species medicinal plant belonging to the genus *Chenopodium* was examined with the help of the GC-MS method.

Results: *cis*-Ascaridole was found as a major peroxy monoterpenoid (up to 46.9 %) in the oil. Three minor isomers: *cis*-isoascaridole (1.1-6.4 %), *trans*-ascaridole (1-2 %), and *trans*-isoascaridole (1-2 %) were also detected. The biological activities and biosynthesis of ascaridole are further discussed.

Conclusions: The results on Ascaridol and the major volatiles and semi-volatiles of wild species belonging to the genus *Chenopodium* provide important information on biologically active monoterpenoid compounds and volatile metabolites biosynthesized in wild medicinal plants growing in the East Mediterranean.

INTRODUCTION

In the past several decades, natural peroxides have been isolated from a wide variety of plants, and marine organisms[1, 2]. Extensive pharmacological screening performed on microorganisms and other species resulted in discovery of novel peroxides with antitumor, antibacterial, antimalarial, and antiviral agents[1, 3]. Many natural and synthetic peroxides were used in the past as therapeutic agents[1, 3].

Species of the family Chenopodiaceae are widely distributed in the East Mediterranean area, where they are often used commercially as spices or drugs because of the presence of useful secondary metabolites. The most characteristic constituents are flavonols, essential oils and terpenes[4-7]. The genus *Chenopodium* includes varieties of weedy herbs (more than 200 species) native to much of Europe, Asia, India, China and both North and South America[8]. Goosefoot is common name for the genus *Chenopodium,* as well as for the goosefoot family Chenopodiaceae. Various plant parts of different species of *Chenopodium* have been traditionally used in the treatment of several disorders[9]. *C. ambrosioides* (also known as American wormseed oil, chenopodium oil, or Baltimore oil) is rich of monoterpenes[10]. The seed and fruit contain a large amount of essential oil which has a main active compound in it called ascaridole. Herb *C. ambrosioides* is a plant widely known in popular medicine as anti-helminthic, vermifuge, emmenagogue and abortifacient[11, 12]. It is used for the treatment of digestive, respiratory, uro-genital, vascular and nervous disorders, for metabolic disturbances such as diabetes and hypercholesterolemia, and as sedative, antipyretic and antirrheumatic[13]. Kishore et al.[14] described the fungitoxicity of chenopodium oil against dermatophytes such as *Aspergillus fumigatus* and *Cladosporium trichoides*.

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, cats, dogs, horses, and pigs[15-18]. Essential oil from the fresh *Chenopodium ambrosioides* contains the ascaridol (40-70 %), α-perpinene, *p*-cymeme, glycol, and isoascaridol[19]. Ascaridole (also known as ascarisin; 1,4-epidioxy-*p*-menth-2-ene) is a bicyclic monoterpene that has an unusual bridging peroxide functional group. Ascaridole has been documented with sedative and pain-relieving properties as well as antifungal effects[20]. Ascaridole was found to be a potent inhibitor *in vitro* development of *Plasmodium falciparum*[21], *Trypanosoma cruzi*[22], and *Leishmania amazonensis*[23]. Ascaridole also showed activity against different tumor cell lines *in vitro* (CCRF-CEM, HL60, MDA-MB-231). The findings are the first hint that ascaridole may be an interesting novel candidate drug for cancer treatment[24]. A few review articles devoted to pharmaceutical application of ascaridole have been published[25, 26].

In this paper, we report the detection of monoterpenoid ascaridole and other terpenoids in the genus *Chenopodium* from the East Mediterranean.

EXPERIMENTAL

Plant material

All species of the genus *Chenopodium* were collected from January to May during 2003-2006. Two species *C.*

Table 1. Ascaridole and other terpenoids in essential oils of the genus *Chenopodium* from the East Mediterranean

Compounds*	KI#	A⊥	B	C	D	E	F	G	H	I	J	K	L	M
1.α-Pinene	939	3.5	1.0			4.4		9.5	1.4	1.6			2.6	1.2
2.Camphene	953		2.4			8.1						4.8	1.0	2.2
3.β-Pinene	980	2.9	1.0			3.7		4.2	2.2			2.1		1.8
4.β-Myrcene	991	3.2				1.3	1.2			1.9			1.9	
5.p-Mentha-1(7),8-diene	1004		1.4									2.3		2.2
6.Δ^3-Carene	1011			1.8			2.1			1.8	3.4			3.1
7.α-Terpinene	1018	4.5	7.2	23.5	4.4	2.1	3.6	2.3	12.3	6.4	27.2	2.2	21.4	9.6
8.*cis-p*-Menth-2-en-1-ol	1121		1.5		2.3						2.4			3.6
9.*p*-Cymene	1026	2.2	11.3		28.5	11.2	3.6		3.6	3.5	9.8	13.2	15.2	2.1
10.Limonene	1031	9.4	9.7	1.7	3.6		24.2		23.2	1.7			6.1	10.9
11.*trans-p*-Menth-2-en-1-ol	1140		3.6				4.5					3.9		2.6
12.γ-Terpinene	1062	1.6	3.6	14.2		1.1	6.8	2.1	3.2	2.3	16.6	1.6	2.9	1.0
13.*p*-Menta-2,8-diene	1086						4.7				3.5			2.3
14.Camphor	1143		3.3			24.8			2.2			8.7		
15.Pinocarvone	1162	1.8								1.4		3.2		11.2
16.Terpin-1-ol	1177	1.6							1.7			2.7		
17.*trans*-Carveol	1217	1.3										4.1	2.1	
18. Dihydroascaridole	1230	3.1			4.5			6.8		2.6	1.4			1.8
19.*cis*-Ascaridole	1237	27.5	14.8	22.1	31.4	19.1	13.3	38.9	12.2	46.9	8.4	5.2	4.2	5.7
20. *cis*-Isoascaridole	1282	6.4	2.2	4.3	6.2	2.1	4.8	5.1	3.2	3.4	2.2	1.6	2.5	1.1
21.Tymol	1290		1.3							1.2			2.6	
22.Carvacrol	1298		1.7								1.6		3.2	
23.*trans*-Ascaridole	1301	2.4	1.5		1.1			2.5		6.5				
24.*trans*-Isoascaridole	1337	1.0	1.6		1.3		1.1	3.1		2.2				
25.α-Terpinyl Acetate	1350	8.9	2.5	2.5	2.2			7.9	13.7	1.1	2.8	2.3	3.6	
26.β-Elemene	1391			3.3									4.1	1.8
27. E-Caryophyllene	1418		1.6	2.4								3.3	6.5	2.5
28.β-Selinene	1485			3.5									2.3	
Minor and non-identified		18.7	11.5	20.7	14.5	30.3	30.1	17.6	21.1	15.5	20.7	38.8	17.8	33.3

* Are shown compounds over 1.0% from total

Kovatz Index, column DB-5;

NIST compounds were identified based on NIST mass spectra library search (NIST 2005).

Most of these compounds were further confirmed by comparing their mass spectra and retention times with standard compounds.

⊥ Plant species: **A**, *C. murale;* **B**, *C.foliosum*; **C**, *C. rubrum*; **D**, *C. missouriense*; **E**, *C. opulifolium*; **F**, *C. polyspermum*; **G**, *C. vulvaria*; **H**, *C. album*, **I**, *C. ambrosioides*; **J**, *C. ficifolium*, **K**, *C. quinoa*; **L**, *C. botrays*; **M**, *C. urbicum*

murale and *C. foliosum* were collected in Hermon mountain area; two species *C. rubrum* and *C. missouriense*, in Kinnererth Lake area; *C. opulifolium*, and *C. polyspermum*, in Beit Shemesh Forest (Jerusalem area). Four species were collected in the Dead Sea area: *C. vulvaria* (Newe Zahar), *C. album* and *C. ambrosioides* (Jordan Ghour), and *C. ficifolium* (Almog). Two species were collected in the Negev Desert area: *C. quinoa* (Ramat Negev), and *Chenopodium botrays* (Arad), and one species *C. urbicum* was collected in Kinnroth Valley.

Preparation of the essential oil

Extraction of volatiles and semi volatiles was performed. Air-dried leaves of each species (6-12 g) were mixed with 250 ml of double distilled water (DDW) and 0.03 g 4-isopropyl phenol as internal standard and subjected to steam distillation for 3 hrs at atmospheric pressure. The water distillate containing oil was distributed two times with hexane (50 ml each) with the help of a separatory funnel. A 50-ml of 10 % sodium hydroxide solution was added to the hexane layer and stirred at room temperature for 5 minutes and the two phases were separated. The aqueous fraction was washed with 50 ml of hexane, and the hexane fractions were combined, dried with anhydrous sodium sulfate and were labeled as "non-phenolic" fraction. To the basic water layer, 1 N HCl was added with stirring until pH = 3. The solution was then extracted three times with hexane (50 ml each). The hexane fractions were combined and dried over anhydrous sodium sulfate and labeled as "phenolic" fraction. The oil was dried over anhydrous sodium sulphate and studied by GC–MS as described previously[27].

GC-MS analysis

Essential oils were analyzed using Shimadzu GC-17A connected to MS-QP5050A. The GC-MS was operated

Table 2. Production of ascaridole (AL, % from total compounds) in essential oils of the genus *Chenopodium* collected in different world regions

Species	Collected place	AL	Ref.
European Region			
C. ambrosioides	Cagliari, Italy	76.0	43
C. ambrosioides	Karlsruhe, Germany	69.3	44
C. ambrosioides	Germany	60.0	45
C. ambrosioides	Montpellier, France	62.1	46
C. ambrosioides	Spain, Salamanca	41.1	47
C. botrys	Manoteras, Spain	7.5	48
C. botrys	Bratislava, Slovakia	40.0	49
C.anthelminticum	Olomouc, CSSR	44.0	50
C. ambrosioides	Hungary	37.1	51
Former USSR Region			
C. anthelminticum	Moldavia, USSR	39.1	52
C. ambrosioides	Tomsk, USSR	23.3	53
C. ambrosioides	Kharkov, USSR	66.8	54
C. ambrosioides	Donetsk, USSR	71.8	55
C. ambrosioides	Feodosia, USSR	75.3	55
C. ambrosioides	Odessa, USSR	86.0	55
C. ambrosioides	Kemerovo, USSR	45.0	56
C. ambrosioides	Omsk, USSR	70.0	56
C. ambrosioides	Kharkov, USSR	50.0	57
South American Region			
C. pumilio	Tucuman, Argentina,	7.9	58
C. multifidum	Santa Fe, Argentina	51.0	59
C. ambrosioides	Santa Fe, Argentina	20.0	60
C. ambrosioides	Argentina	33.2	61
C. ambrosioides	Argentina	55.0	62
C. ambrosioides	Brazil	68.7	61
C. ambrosioides	Rio Grande Sul, Brazil	17.1	63
C. hircinum	Brazil	41.5	64
C. multifidium	Brazil	58.5	64
North American Region			
C. ambrosioides	USA	70.0	65
C. ambrosioides	USA	61.5	66
C. ambrosioides	USA	65.0	67
C. ambrosioides	Maryland, USA	44.0	68
C. ambrosioides	USA	60.0	69
C. ambrosioides	Cuba	3.9	70
C. ambrosioides	Cuba	22.5	71
African Region			
C. ambrosioides	Rwanda	1.7	72
C. ambrosioides	Oyo State, Nigeria	0.7	73
C. ambrosioides	Nigeria	2.5	74
C. ambrosioides	Madagascar	41.8	19
C. ambrosioides	Uganda	40.7	75
Asian Region			
C. ambrosioides	India	46.0	76
C. ambrosioides	India	17.7	77
C. ambrosioides	Peshawar, Pakistan	46.0	78
C. ambrosioides	Tehran, Iran	*cis*- 43.4	79
C. ambrosioides	Tehran, Iran	*trans*- 6.4	79
C. ambrosioides	Nanjing, China	23.5	80
C. ambrosioides	Japan	60.0	81
C. ambrosioides	Japan	45.0	82

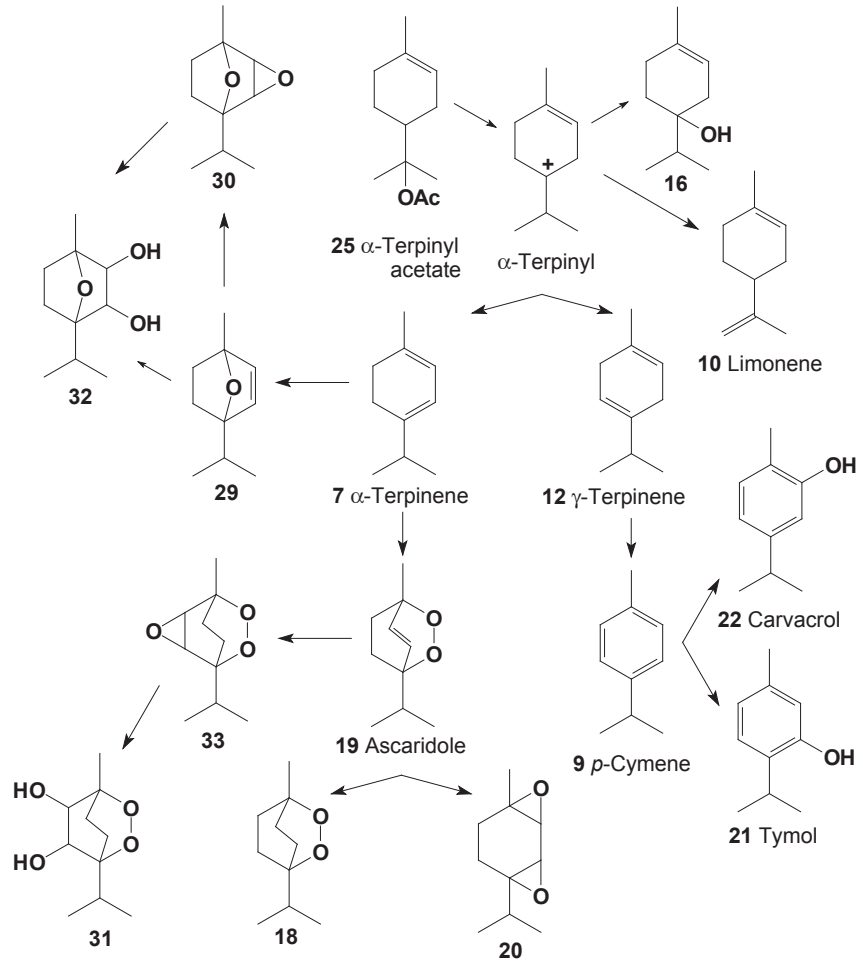

Fig. 1. The proposed mechanism involves the formation of ascaridole and other monoterpenoid constituents in the genus *Chenopodium* (number under structure see in Table 1, and explanation in the text)

in the electron impact ionization mode (EI) at 70 eV. Sampling the hexane extract was carried out by taking out 1 µl sample from 2 ml vials using an AOC-20i autosampler while sampling of the headspace of the dry leaves was performed by taking 0.2 ml from 27 ml HS vials using a Shimadzu HSS-4A autosampler. The HS vials were sealed with silicon rubber septa and aluminum caps after introduction of the sample while the AOC vials were sealed with 8mm double-faced rubber septa and screw cap with 12 mm hole. The GC was equipped with a fused silica capillary column; DB-5, 30m x 0.25 mm i.d., coating thickness is 0.25 µm, Supelco (Sigma-Aldrich Inc., USA). The GC-MS operating conditions were as follows: the carrier gas flow rate was 1.6 ml He/min. Injector and detector temperatures were 230 °C and 250 °C respectively. Split ratio was 1:30. The column temperature was held at 60 °C for 2 minutes, then raised from 60 °C to 100 °C at 3 °C/min and from 100 to 280 °C at 30 °C/min and held there for 2 min. Solvent cut time was 4 minutes and the starting time of the chromatogram was 5 minutes. Mass range was from 30 to 350 Daltons, and scan interval was 0.5 seconds. Detector voltage was set to 1.50 kV. For HS samples, vial temperature was set to 100 °C and the syringe temperature was set to 110 °C. The identification of the compounds was based mainly on their retention times in comparison with those from authentic standards. The standards were injected separately in addition to adding them to the leaves extracts (spiking) to enhance the relevant peaks of interest. Identification of some peaks was based on matching of their MS spectra with NIST/EPA/NIH Mass Spectral Library (NIST 2005).

All the essential oil standards (ascaridole, thymol, carvacrol, α-pinene, β-myrecene *o*-cymene, *p*-cymene, γ-cymene, limonene, γ-terpenene, linalool), internal standard (4-isopropyl phenol), GC grade n-hexane and anhydrous sodium sulfate were purchased from Sigma–Aldrich Inc. (USA).

RESULTS AND DISCUSSION

The essential oils of leaves of 13 species medicinal plant belonging to the genus *Chenopodium* were isolated by normal SD. Our results are in accord with previous investigations indicating that the two major constituents of the oil of *Chenopodium* were for: *C. murale*, ascaridole –

27.5 %, limonene – 9.4 %; *C.foliosum*, ascaridole – 14.8 %, *p*-cymene – 11.3 %; *C. rubrum,* α-terpinene – 23.5 %, ascaridole – 22.1 %; *C. missouriense,* ascaridole – 31.4 %; α-terpinene – 28.5 %; *C. opulifolium,* camphor – 24.8 %, ascaridole – 19.1 %; *C. polyspermum,* limonene – 24.2 %, ascaridole – 13.3 %; *C. vulvaria,* ascaridole – 38.9 %, α-terpinyl acetate – 7.9 %; *C. album,* limonene – 23.2 %, ascaridole – 12.2 %; *C. ambrosioides, cis*-ascaridole – 46.9 %, *trans*-ascaridole – 6.5 %; *C. ficifolium,* α-terpinene – 27.2 %, γ-terpinene – 16.6 %; *C. quinoa, p*-cymene – 13.2 %, camphor – 8.7 %; *C. betrays,* α-terpinene – 21.4 %, *p*-cymene – 15.2 %, and *C. urbicum,* pinocarvone – 11.2 %, limonene – 10.9 %. Distribution of ascaridole and other compounds of *Chenopodium* essential oils are summarized in Table 1. According to the obtained data *cis*-ascoridole varied from 4.2 % in *C. betrays* to 46.9 % in *C. ambrosioides,* and *cis*-isoascaridole was also present in all studied species, and varied from 1.1 % in *C. urbicum* to 6.4 % in *C. murale*. Other derivatives of ascaridole such as dihydroascaridole, *trans*-ascaridole, and *trans*-isoascaridole are present in some plant species[28, 29].

The oil composition of the genus *Chenopodium* varied quantitively and qualitively within and between natural populations and showed no correlation to the geographical distribution. Distribution of ascaridole in the genus *Chenopodium* which was collected in different regions around the World is shown in Table 2. It is from these results clear that a more accurate quantitative determination of dihydroascaridole (**18**), ascaridole (**19**), and isoascaridole (**20**) in essential oils can be obtained by combination of GC-MS. Thus, the total content of the three compounds is available from GC-MS analysis and was found in the samples employed in the present studies using a non-polar column.

Compounds (**7**, **9**, **10**, **16**) and (**18-22**) detected during this study in the essential oils of leaves from 13 species medicinal plant belong to the genus *Chenopodium*. Other compounds have been isolated by different authors. *cis*-Ascaridole (**19**) is the dominant isomer in nature (see Tables 1 and 2), while *trans*-ascaridole is present usually in trace amounts. Recently, *trans*-ascaridole (6.38 %) was isolated from *Chenopodium ambrosioides* cultivated in Iran[30]. Dihydroascaridole was isolated for the first time by Paget in 1938 from *Chenopodium* oil[31]. *cis*-Isoascaridole (**20**) was found in the essential oil of all analyzed species from Mediterranean area (Table 1), and it was also found in: 13 pepper and peppercorn samples of different species, colloquially also referred to as pepper (*Capsicum frutescens*, *Coriandrum sativum*, *Pepper* (spice), *Pimenta dioica*, *Piper* sp., *P.cubeba*, *P.nigrum*, *P. retrofractum*, *Schinus terebinthifolius*, *Tasmannia lanceolata*, *Zanthoxylum piperitum*, *Zanthoxylum simulans piperitum*[32]; in *Rhododendron* spp.[33]; in several species of Brazilian Euphorbiaceae: *Croton sponderianus*, *C. essequiboenis, C. argyophylloides, C. affinis mucronifolius, C. rhamnifolius, C. nepetaefolius, C. jacobinenis, C. micans*, *C. zehntneri anethole*, and *C. zehntneri eugenol*[34].

1,4-Epoxy-*p*-enth-2-ene (**29**) was found in *Chenopodium* oil by Halpern in 1951 (ref.[35]) 1,4:2,3-Diepoxy-*p*-menthane (**30**) and (**31**,**33**) were isolated from the essential oil from the leaves of *Curcuma longa* (Bangladesh)[36]. Ascaridole glycol (**32**) was present in oil of *Tanacetum* (syn. *Chrysanthemum*) *fruticulosum* from Iran[37]; Japanese *Ledum palustre* var. *nipponicum et yesoense*[38]; and Indian *Zanthoxylum rhetsa*[39].

The biosynthesis of ascaridole from the conjugated symmetric diene α-terpinene (a major component of the oil from *C. rubrum* and *C. ficifolium*) was shown to be catalyzed by a soluble iodide peroxidase isolated from homogenates of *C. ambrosioides* fruit and leaves[40]. The enzymatic synthesis of ascaridole was confirmed by GC-MS of the product, which was also shown to be racemic. Optimal enzymatic activity occurred at pH 4.0 in the presence of 2.5 mM H_2O_2 and 1 mM NaI. Peroxidase activity was susceptible to proteolytic destruction only after periodate treatment, suggesting an association of the enzyme(s) with polysaccharide material. Ascaridole biosynthesis from α-terpinene was inhibited by cyanide, catalase, and reducing agents, but not by compounds that trap superoxide or quench singlet oxygen. A peroxide transfer reaction initiated by peroxidase-generated I^+ is proposed for the conversion of α-terpinene to ascaridole. Photolysis of ascaridole in hydrocarbon solvents at 185 nm gave isoascaridole as the major product and *p*-mentha-1,3-diene as the minor product, whereas at 354 nm additional[41]. *iso*-Ascaridole and *p*-$MeC_6H_4SO_3H$ in Et_2O mixed with ice cooling gave an oil from which on standing was deposited 1,4:2,3-diepoxy-*p*-menthane (**30**) (ref.[42]). Based on our and other authors' experimental data, we have proposed biosynthetic pathway of ascaridole and related products in plants (see Fig. 1).

CONCLUSIONS

In this study, we have described the separation and identification of ascaridol and the major volatiles and semi-volatiles of wild species belonging to the genus *Chenopodium*. Comparable results of the percentages of semi-volatile phenols were obtained using conventional SD-GCMS. This report provides important information on biologically active monoterpenoid compounds and volatile metabolites biosynthesized by medicinal plant growing wild in the East Mediterranean.

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