Asian Journal of Chemistry; Vol. 23, No. 10 (2011), 4271-4275

ASIAN JOURNAL OF CHEMISTRY

www.asianjournalofchemistry.co.in

Composition of Essential Oil of Leaves Stems and Roots by Different Extraction Methods of *Thymus kotschyanus* Boiss. & Hohen var. *pseuderiophorus* Rech. f.p.p. Grown Wild in Iran

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(*Received*: 17 August 2010; *Accepted*: 3 June 2011) AJC-10025

The essential oils of leaves, stems and roots of *Thymus kotschyanus* Boiss. and Hohen var. *pseuderiophorus* Rech. f. p.p. (Lamiaceae) that obtained by hydro distillation (HD), solvent free microwave extraction (SFME), microwave assisted extraction (MAE) with hexane and mixture of water and hexane, solid phase micro extraction (SPME) were analyzed by means of GC and GC/MS. Identification of components was done by comparison of the relative retention indices and mass spectra with authentic reference. In all of the above methods, carvacrol was the major compound in the oils of different organs. The identified compounds in the oil of leaves, stems and roots represent (98.4, 99.6, 97.7, 99.6 and 98.4 %), (99.3, 92.5, 99.1, 99.1 and 99.8 %) and (98.3, 99.9, 98.4, 97.1 and 100 %), respectively of the essential oils.

Key Words: *Thymus kotschyanus*, Lamiaceae, Essential oil composition, Carvacrol, Hydro distillation.

INTRODUCTION

Essential oils are complex mixtures of volatile substances generally present at low concentration that are widely used in the perfume industries, in the pharmaceutical sector and in the food and human nutrition field. Before such substances can be analyzed, they have to be extracted from the matrix. In this study we extracted essential oil from leaves, stems and roots of *Thymus kotschyanus* var. *pseuderiophorus* Rech. f. p. p from wild populations in north of Iran by different methods and then analyzed by GC-MS.

The genus *Thymus* includes about 350 species word wide and is distributed mainly in temperate Eurasia. In Iran 14 species are present, four being endemic[1,2]. The chemical composition of essential oils of various *Thymus* species has been extensively studied[3-15]. Water distilled oil obtained from the aerial parts of *T. kotschyanus*, growing wild in north of Iran has been reported. The major compounds were thymol (31.8 %), carvacrol (24.3 %), *p*-cymene (12.3 %) and 1,8-cineole (5.8 %)[16]. In previous study, we work on water distilled oil of leaves, stems and roots of *Thymus kotschyanus* var. *pseuderiophorus* Rech. f. p. p from wild populations in north of Iran. The major compound of these organs was carvacrol (69.5, 71.6 and 71.3 %), respectively[16].

However, to the best of our knowledge no report on the oils from the leaves, stems and roots of this plant exists, leading us to the present work.

EXPERIMENTAL

Leaves, stems and roots of *T. kotschyanus* var. *pseuderiophorus* Rech. f.p.p. were collected in May 2008 from Firoozkooh, Province of Tehran. Voucher specimens have been deposited at the herbarium of the Research Institute of Forest and Rangelands (TARI), Tehran, Iran.

Essential oil extraction

Hydro distillation: The oil of dry organs (100 g) of *T. kotschyanus* var. *pseuderiophorus* Rech. f.p.p. were obtained by hydro distillation using a Clevenger type apparatus for 4 h. The yields were (0.82, 0.41 and 0.21 %).

Solvent free microwave extraction: For Solvent free microwave extraction, a Milestone srl operating at 2450 MHz was used. The maximum power of the oven was 1000 w which was measured using ATC-EO sensor. Solvent free microwave extraction was performed at atmospheric pressure, 100 g of fresh organs of plant material was heated using an optimize fixed power of 800 w for optimize time 25 min without added any solvent or water. A Clevenger system outside the microwave cavity condensed the distillate continuously. Condensed water was refluxed to the extraction vessel in order to provide uniform condition of temperature and humidity for extraction. The essential oil was collected, dried on anhydrous sodium sulphate and stored at until analyzed. The yields of this method were (0.85, 0.35 and 0.35 %) (w/w), respectively.

Microwave-assisted extraction (MAE): Microwave-assisted extraction was performed at atmospheric pressure, using the above microwave oven. In MAE procedure with hexane (Romil, England), 20 g of different parts of plant material was inserted into an extraction vessel and 100 mL of hexane was added. The extraction time was 2 min and the extraction temperature, setting the microwave extractor at maximum power. After cooling the vessel, it was opened and the supernatant was filtered. Then the filtrate was deposited in the freezer during 12 h in order to precipitate fixed waxes and oils, after the second filtration, the extract was filtered through a column containing activated carbon, in order to eliminate the pigments[17].

All stages of the MAE methods, being a mixture of water and hexane (1:1) is similar to MAE method with hexane, however after cooling the vessel and before filtration, water phase was separated from hexane phase. Other steps like MAE (H) were done on the hexane phase. Finally, the filtrate was reduced by rotary evaporation. The extract was collected, dried under anhydrous sodium sulphate, stored at ºC until used. The extraction yield of MAE with hexane and mixture of water and hexane were (0.71 %, 0.5 %), (0.3 %, 0.25 %) and (0.21 %, 0.15 %)(w/w), respectively which collected in amber coloured vials dehydrated with anhydrous sodium sulfate and kept at ºC until being analyzed.

Head space solid phase micro extraction apparatus and procedure: This method is proposed for rapid qualitative analysis of plant damageable volatile organic compound (VOC) emission. Sampling of the plant emission is carried out by the solvent free preparation technique head space-solid phase micro extraction (HS-SPME). The method incorporates sampling, extraction and concentration of the sample component[18].

A manual SPME holder and 65 µm PDMS-DVB fiber from Supelco (Bellefonte, USA) were used for the SPME procedure. The fiber was condition at 250 ºC for 0.5 h in GC injector. 0.5 g of powdered samples were placed in 20 mL sample vials sealed with septum-type caps from supelco (Bellefonte, USA) and heated for 15 min at 70 ºC. After this time the SPME needle was pierced the septum, the PDMS

TABLE-1
PERCENTAGE COMPOSITION OF LEAF OF *Thymus kotschyanus* Var. *pseuderiophorus* Rech. f.p.p. BY DIFFERENT METHODS

Compounds	KI	HD (%)	SFME (%)	MAE (%)(H)	MAE (%) (H + W)	SPME (%)	SPME* (%)
α-Thujene	930	2.2	–	–	–	–	0.6
α-Pinene	939	–	0.2	–	–	–	5.3
Camphene	954	1.6	0.4	0.4	0.2	0.3	4.7
β-Pinene	979	0.3	–	–	–	–	0.8
3-Octanone	984	–	–	–	–	–	0.5
Myrcene	991	1.4	0.4	–	–	–	3.5
α-Phellandrene	1003	0.3	–	–	–	–	0.5
α-Terpinene	1017	–	0.4	0.4	–	–	3.3
p-Cymene	1025	7.3	3.5	2.0	0.8	1.4	20.8
Limonene	1029	–	–	–	–	–	–
β-Phellandrene	1030	–	–	–	–	–	2.4
1,8-Cineol	1031	–	1.1	–	–	0.3	2.6
γ-Terpinene	1060	4.1	1.5	1.3	0.6	0.7	10.8
Z-Sabinene hydrate	1070	0.8	4.2	0.8	0.8	0.9	3.5
α-Terpinolene	1089	0.4	–	–	–	–	0.5
E-Sabinene hydrate	1098	–	1.4	–	0.3	0.4	0.9
Camphor	1146	–	1.1	–	0.3	0.4	1.3
Borneol	1169	8.9	12.5	4.8	3.9	5.1	8.6
1,4-Terpineol	1177	–	1.3	–	0.4	0.4	0.6
Thymoquinone	1252	–	5.5	0.9	2.1	1.1	–
Bornyl acetate	1289	–	–	–	–	–	–
Thymol	1290	–	1.8	2.7	2.0	2.4	–
Carvacrol	1299	69.5	62.3	81.6	86.9	82.8	28.2
β-Caryophyllene	1419	–	0.7	–	0.2	0.8	0.6
γ-Muurolene	1480	–	–	–	–	0.3	–
β-Bisabolene	1506	–	0.3	0.2	–	0.4	–
α-Bisabolene	1507	0.9	1.0	1.8	0.6	–	–
γ-Cadinene	1514	–	–	0.4	–	0.4	–
Caryophyllene oxide	1583	–	–	–	–	–	–
Carotol	1595	–	–	–	–	–	–
τ-Cadinol	1640	0.6	–	0.4	0.5	0.3	–
Total (%)		98.4	99.6	97.7	99.6	98.4	100
Monoterpene (%)		96.8	97.6	94.9	98.3	96.2	99.4
Sesquiterpene (%)		1.6	2.0	2.8	1.3	2.2	0.6
Oxygenated compound (%)		79.9	91.2	91.2	97.2	94.1	46.2
Number of compound		13	18	13	14	17	20
Total peak area ($\times 10^8$)		32.4	8.6	19.3	6.1	6.2	0.7
Yield (% w/w)		0.82	1.4	1.1	0.9	–	–

TABLE-2
PERCENTAGE COMPOSITION OF STEM OF *Thymus kotschyanus* Var. *pseuderiophorus* Rech. f.p.p. BY DIFFERENT METHODS

Compounds	KI	HD (%)	SFME (%)	MAE (%) (H)	MAE (%) (H + W)	SPME (%)
α-Thujene	930	1.3	–	–	–	–
Camphene	954	1.4	–	0.1	0.1	0.2
Sabinene	975	0.2	–	–	–	–
Myrcene	991	0.8	–	–	–	–
α-Phellandrene	1003	0.2	–	–	–	–
α-Terpinene	1017	0.8	–	–	–	–
p-Cymene	1025	4.2	7.6	0.2	0.3	0.5
1,8-Cineole	1031	–	0.4	–	0.1	0.2
γ-Terpinene	1060	2.2	–	0.2	0.1	0.2
Z-Sabinene hydrate	1070	0.6	–	0.4	0.5	0.5
α-Terpinolene	1089	0.2	–	–	–	–
Linalool	1097	0.2	3.7	0.2	–	–
E-Sabinene hydrate	1098	–	–	–	0.2	0.3
Camphor	1146	0.4	–	0.2	0.3	0.4
Borneol	1169	11.8	5.4	3.2	3.8	4.5
4-Terpineol	1177	–	3.0	0.2	0.3	0.3
Thymoquinone	1252	–	–	0.7	2.3	1.5
Thymol	1290	–	–	1.5	2.0	2.1
Carvacrol	1299	71.6	65	90.4	87.5	87.0
β-Caryophyllene	1419	0.4	5.9	0.2	0.1	0.4
E-Cadina-1(6),4-diene	1477	0.1	–	–	–	–
β-Bisaboolene	1506	0.8	1.5	0.2	0.2	0.4
α-Bisaboolene	1507	1.0	–	0.7	0.6	0.4
γ-Cadinene	1514	–	–	0.2	0.2	0.6
δ-Cadinene	1523	–	–	0.2	0.1	–
Caryophyllene oxide	1583	0.3	–	–	–	–
τ-Cadinol	1640	0.8	–	0.3	0.4	0.3
Total (%)		99.3	92.5	99.1	99.1	99.8
Monoterpene (%)		95.9	85.1	97.3	97.5	97.7
Sesquiterpene (%)		3.4	7.4	1.8	1.6	2.1
Oxygenated compound (%)		85.7	78.6	97.1	97.4	97.1
Number of compound		20	8	17	18	17
Total peak area ($\times 10^8$)		35.9	1.5	2.4	6.1	4.1
Yield (%w/w)		0.41	0.88	0.65	0.61	–

fiber was extended through the needle and exposed to the head-space above the sample for 5 min. After an optimize extraction time (5 min), the fiber was drown into the needle and then the needle was removed from the septum and inserted directly on to the injection port of the GC. The desorption of analytes from the fiber coating was performed by heating the fiber in the split less (250 ºC) injection port at for 3 min.

Yield: Essential oil yield was expressed in terms of the weight of the oil collected per gram of dry plant material.

Analysis: The essential oils obtained by several methods under different condition were analyzed by gas chromatography and gas chromatography coupled to mass spectrometry.

GC analysis of the oils was performed on a Shimadzu 15A gas chromatograph equipped with a split/split less injector (250 ºC). Nitrogen was used as carrier gas (1 mL/min) and the capillary column used was DB-5 (30 m × 0.25 mm, film thickness 0.32 µm). The column temperature was kept at 60 ºC for 3 min and then heated to 220 ºC with 5 ºC/min rates and kept constant at 220 ºCC for 5 min.

Relative percentage amount were calculated from peak area using a shimadzu C-R4A chromatopac without the use of correction factors.

GC-MS analysis was performed using a Hewlett-Packard 6890/5973 GC-MS instrument with a HP-5MS column (30 m × 0.25 mm, film thickness 0.32 µm. The column temperature was as like as GC condition. Helium was used as carrier gas (1 mL/min). Mass spectra were taken at 70 eV.

Identification of the constituents of each oil was made by comparison of their mass spectra and retention indices (RI) with those given in the literature and those authentic samples[19].

RESULTS AND DISCUSSION

The percentage composition of the oils is given in Table-1 in order of their elution from the DB-5 column. As can be seen from the Table-1, especially in MAE and SFME methods, the oils from leaves, stems and roots of *T. kotschyanus* var. pseuderiophorus Rech. f.p.p. are rich in regard to oxygenated monoterpenes small amount of monoterpene hydrocarbons and very few sesquiterpenoids (Fig. 1). Comparing these results with pervious investigation on oils of the genus *Thymus* showed that they were also dominated by monoterpenes.

Carvacrol was the major compound in the oils of leaves, stems and roots. The highest percentage of carvacrol was (86.9, 90.4 and 94.2 %) in MAE: (H + W), (H) and (H) methods respectively.

It can therefore, be concluded that leaves and stems offers higher essential oil yield from *T. kotschyanus* var. *pseuderiophorus* Rech. f.p. p. in comparison with roots.

TABLE-3
Percentage composition of root of *Thymus kotschyanus* Var. *pseuderiophorus* Rech. f.p.p. BY DIFFERENT METHODS

Compounds	KI (%)	HD (%)	SFME (%)	MAE (%) (H)	MAE (%) (H + W)	SPME (%)
α–Thujene	930	0.1	–	–	–	–
α-Pinene	939	5.3	–	–	–	–
Camphene	954	3.3	–	–	–	–
Sabinene	975	0.2	–	–	–	–
Myrcene	991	1.1	–	–	–	–
α-Phellandrene	1003	0.3	–	–	–	–
α-Terpinene	1017	0.3	–	–	–	–
p-Cymene	1025	2.2	0.5	0.2	–	–
Limonene	1029	0.7	–	–	–	–
1,8-Cineole	1031	0.1	–	–	–	–
γ-Terpinene	1060	1.2	–	–	–	–
z-Sabinene hydrate	1070	–	1.5	–	–	–
E-Sabinene hydrate	1098	–	0.7	–	–	–
Camphor	1146	0.3	0.9	–	–	–
Borneol	1169	4.8	8.2	1.8	2.1	2.3
4-Terpineol	1177	0.4	0.8	–	–	–
Endow-fancy acetate	1220	1.6	–	–	–	–
Thymoquinone	1252	–	4.2	–	1.2	0.6
Bornyl acetate	1289	0.4	–	–	–	–
Thymol	1290	2.0	1.9	1.7	1.6	2.1
Carvacrol	1299	71.3	80.1	94.2	91.1	93.9
Diocese	1382	0.5	–	–	–	–
β-caryophyllene	1419	0.8	–	–	–	–
β-bisabolene	1506	0.2	–	–	–	0.3
α-bisabolene	1507	0.4	1.1	0.5	0.6	0.8
Carotol	1595	0.4	–	–	–	–
τ-Cadinol	1640	0.4	–	–	0.5	–
Total (%)		98.3	99.9	98.4	97.1	100
Monoterpene (%)		95.6	98.8	97.9	96	98.9
Sesquiterpene (%)		2.7	1.1	0.5	1.1	1.1
Oxygenated compound (%)		81.7	98.3	97.7	96.5	98.9
Number of compound		24	10	5	6	6
Total peak area (× 10^8)		14.1	5.0	1.6	2.6	2.9
Yield (% w/w)		0.27	0.57	0.45	0.4	–

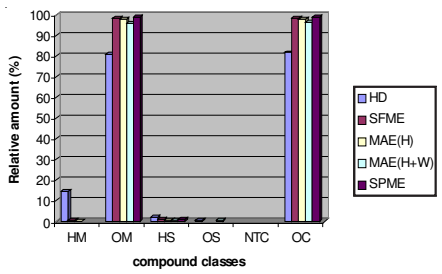

Fig. 1

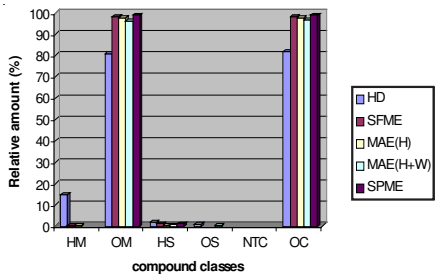

Fig. 2

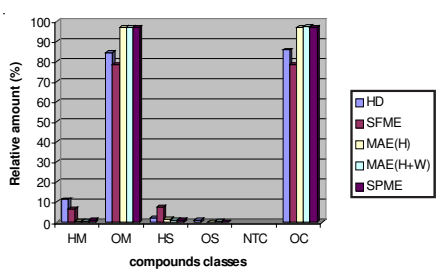

Fig. 3

Concerning the comparison of five techniques in terms of isolation times MAE, SPME and SFME were clearly fast (2, 5, 25 min), While about 3h were required for hydro distillation.

The reason for reduction in extraction time in SFME and MAE methods may be due to the high pressure gradient formed inside the plant material. Microwave absorption results in significant internal heating thus creating significantly higher internal pressure which enhances oil extraction in a shorter time from the parts of the plant.

The higher abundance of oxygenated compounds in SFME and MAE oils than in HD oil is related to the rapid heating of polar substances by microwave and to the smaller amount of water used, which prevented the decomposition of principal oxygenated constituents by thermal and hydrolytic reaction[20].

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