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Original Research Article

Chemical composition of the essential oil, total phenolic contents and antioxidant activity of *Vitex pseudo-negundo* seeds collected from northeastern Iran

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ABSTRACT

In this study, the essential oils obtained by hydrodistillation of the seeds of *Vitex pseudo-negundo* (Verbenaceae), growing wild in Sabzevar, Khorasan Razavi Province (Iran), were analyzed by GC and GC/MS. The mean yield of total volatile oils was 0.8% (w/w). Thirty seven compounds representing 92.4% of the seed oil were identified. The main components of the oil were hexadecanoic acid (8.0%), elemol (7.0%) and α-bisabolol (6.1%). The oil was rich in sesquiterpene hydrocarbons (51.2%). The total flavonoids of different extracts of the plant, were found the range 56-195 mg/g, with the maximum amount being in the methanol extract. In addition, the antioxidant activities of the extracts were measured by radical scavenging activity of antioxidants against the free radical 2,2'-diphenyl-1-picrylhydrazyl (DPPH). It was found that the antioxidant activities of the extracts of *Vitex pseudo-negundo* seed are higher than those of the standard synthetic antioxidants, BHT, ascorbic acid and gallic acid.

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1. Introduction

Vitex pseudo-negundo (Verbenaceae) grows naturally in the vicinity of seasonal rivers in Iran (Rechinger, 1986; Mozaffarian, 1996). *V. pseudo-negundo*, commonly referred as chaste tree, chaste lamb, tree of chastity and Abraham's balm (English names). The biological activities, e.g., antibacterial activity (Moghadam et al., 2010; Seyyednejad and Motamedi, 2010), anti-brucella (Motamedi et al., 2010), antifungal (Svecova et al., 2013), antioxidant (Mozdianfard et al., 2012; Sridevi et al., 2012), anti-inflammatory (Chouhan et al., 2012), fumigant toxicity (Sahaf and Moharramipour, 2008; Sahaf et al., 2008), and antimicrobial (Stojkovic et al., 2011) characteristics of some species of *Vitex* have been reported.

Antioxidants have been found in many plant materials and supplements. Because of their natural origin, the antioxidants obtained from plants are claimed to be of greater benefit than those of synthetic ones (Rohman et al., 2010). It is suggested that natural antioxidants from plants have fewer side effects than do synthetic antioxidants, some of which have been found to be genotoxic (Chen et al., 1992). Therefore, investigation of the biological activity and chemical composition of medicinal plants as potential sources of natural antioxidants seems meritorious. The basic aim of this research was to characterize the essential oil of seeds of *V. pseudo-negundo* and to determine the total phenolic content and radical scavenging activity in various extracts of the seeds.

In this study, the volatile oil prepared by hydrodistillation of *V. pseudo-negundo* seeds from Sabzevar, Khorasan Province (Iran), was studied by GC and GC/MS. Total phenolic content and radical scavenging capacity were used to determination of antioxidant activity of different extracts of the plant's seed.

2. Experimental

2.1. Chemicals

Solvents (methanol, chloroform, ethyl acetate) were purchased from Merck (Darmstadt-Germany). Gallic acid and 2,2'-diphenyl-1-picrylhydrazyl (DPPH)

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were obtained from Sigma Chemical Co., St Louis, MO, USA. The Folin-Ciocalteu phenol reagent and butylated hydroxytoluene (BHT) were purchased from Fluka Chemie AG, Buchs, Switzerland. All other solvents and chemicals were of analytical grade.

2.2. Plant material

The plant material (Fig. 1) was collected in June 2013 from Sabzevar in Khorasan Province, Iran (Fig. 2). A voucher specimen with an assigned number of IAUS325 has been deposited in the herbarium of the Research Center of Natural Resources, Sabzevar, Iran. The collected plant material was air-dried in darkness at room temperature (20 ºC). Afterwards, the seeds of dried plant were separated from the plant and stored in dark, tightly sealed containers until needed.

Fig. 1. Representation of *Vitex pseudo-negundo* in the sampling area.

Fig. 2. Google map of Sabzevar, northeastern Iran.

2.3. Essential oil isolation

Air-dried seeds of *V. pseudo-negundo* (100 g) were subjected to hydrodistillation in a Clevenger-type apparatus for three hours to produce oils. The yield of total essential oil was found to be 0.8% (w/w). The oils were dried over anhydrous sodium sulfate and stored in dark and sealed vials at 4 ºC before analysis.

2.4. GC analysis

GC analysis was performed using a Shimadzu GC-9A gas chromatograph, equipped with an HP-5MS fused silica column (30 m×0.25 mm i.d., film thickness 0.25 µm). The oven temperature was held at 50 ºC for five minutes and then programmed to 250 ºC at a rate of 3 ºC/min. The injector and detector (FID) temperatures were 290 ºC. Helium was used as carrier gas at a flow-rate of 1 mL/min.

2.5. GC/MS analysis

GC/MS analysis was carried out on a Hewlett-Packard 6890 gas chromatograph fitted with a fused silica HP-5MS capillary column (30 m×0.25 mm i.d., film thickness 0.32 µm). The oven temperature was programmed from 60 to 220 ºC at 6 ºC/min. Helium was used as carrier gas at a flow-rate of 1 mL/min. The chromatograph was coupled to a Hewlett-Packard 5973 mass selective detector with an ionization voltage of 70 eV.

2.5.1 Qualitative and quantitative analyses

Constituents of the volatile oils were identified by the comparison of their retention indices relative to C_9-C_{24} *n*-alkanes and of their mass spectral fragmentation patterns with those reported in the literature (Adams, 2007) and stored in a MS library (Wiley 275). The quantification of the components was performed on the basis of GC peak area data obtained from separation on the HP-5MS column.

2.6. Preparation of the extracts

The conventional maceration method was used for preparing the extracts. In this regard, dried and powdered seed of *V. pseudo-negundo* (50 g) were extracted by 400 mL of some organic solvents. This process replicated trice with the same volume of fresh solvent. Three solvents having different polarities (methanol, ethyl acetate, chloroform) were used. An overhead stirrer mixed the materials for 24 h. All the mixtures were filtered through Whatman paper No. 41. The solvents were removed below 40 ºC using a rotary evaporator (Heidolph, Germany) and finally stored at 4 ºC for further use.

2.7. Determination of total phenolic contents

The total phenolic contents of the prepared extracts were determined spectrophotometrically by Folin-Ciocalteu method according to the procedure reported previously (Singleton et al., 1999) with some modifications. Briefly, 500 µL of extract solution (in methanol, ethyl acetate, chloroform), 1500 µL distilled water and 500 µL of 1:10 Folin-Ciocalteu reagent were mixed for 1 minute. Then 5 minutes later, 1000 µL of sodium carbonate (5.0%) was added and the mixture shaken. After two hours in the dark at room temperature, the absorbance was measured at 760 nm using a UV-Visible spectrophotometer (Unico UV-2100, China). The total phenolic concentration was

calculated from gallic acid (GA) calibration curve (5-100 mg/L). The total phenolic content of each extract was expressed as gallic acid equivalents (GAE)/g of extracts averaged from three replicates.

2.8. DPPH radical scavenging activity assay

The ability of the plant extract to scavenge DPPH free radicals was assessed by a standard method (Mensor et al., 2001). Briefly, certain volumes of different extracts were added to a prepared 0.004% solution of DPPH in methanol. Stock solutions of extracts were prepared to have a concentration of 1 mg/mL. Dilutions were made to obtain concentrations of 20, 40, 60 and 80 µg/mL. Diluted solutions (1 mL each) were mixed with 1 mL of a methanol solution of DPPH having a concentration of 0.004%. After 30 min. in the dark at room temperature (23 ºC), the absorbance was recorded at 517 nm. Ascorbic acid, gallic acid and BHT (20, 40, 60, 80 µg/mL) were used as reference compounds. The control contained all the reagents except the extract. The percentage of scavenged DPPH was calculated using equation 1. The data reported here are mean values±standard deviation (n=3).

$$\text{DDPH scavenging activity} = 100 \times (A_c - A_s)/A_c \quad \text{(eqn. 1)}$$

Where A_c is the absorbance of the control and A_s is the absorbance of the sample. IC_{50} values calculated denote the concentration of sample required to decrease the absorbance at 517 nm by 50%.

3. Results and Discussion

3.1. Essential oil profile

As a part of on-going work on the chemical analysis of oils obtained from the wild plants of Iran, it was decided to re-investigate the oils of this specific plant (*V. pseudo-negundo*). Accordingly, hydrodistilled volatile oils from the crushed dry seeds of *V. pseudo-negundo* (Verbenaceae) from Sabzevar (Iran), were analyzed by means of GC and GC/MS instruments. The air-dried seed of the plant yielded 0.8% (w/w) oil. The oil was clear and yellowish. The forty four components that were identified in the oil of the seed accounted for 92.4% of the total detected compounds. Table 1 lists formulas, percentages, and retention indices of identified compounds in the oil. As can be seen, the main components are hexadecanoic acid (8.0%), elemol (7.0%) and α-bisabolol (6.1%).

In this study, GC and GC/MS analyses of the oil revealed the occurrence of several monoterpene hydrocarbons (MH), oxygenated monoterpenes (OM), sesquiterpene hydrocarbons (SH), oxygenated sesquiterpenes (OS) and non-terpenoid hydrocarbons (NH). Six monoterpene hydrocarbons (12.0%), seven oxygenated monoterpenes (13.1%), nine sesquiterpene hydrocarbons (22.3%), eleven oxygenated sesquiterpenes (28.9%) and four non-terpene hydrocarbons (16.1%) were characterized in this oil. The data lead to an overall rank order of constituent groups: OS>SH>NH>OM>MH for the seed oil.

3.2. Chemical compositions of the essential oils of *Vitex pseudo-negundo* in similar reports

In the report of Azar et al. (2011), using the microwave assisted-based methods, namely solvent-free microwave extraction (SFME) and microwave assisted headspace solid phase microextraction (MA-HS-SPME), headspace solid phase microextraction (HS-SPME), characterized. As shown in detail in this study, using the hydrodistillation approach, twelve compounds were totally characterized of which the major constituents were α-pinene (33.2%), α-terpinyl acetate (23.9%), limonene (15.0%) and β-caryophyllene (7.3%). Moreover, as being reported in this attempt, using the SFME technique ten compounds were screened among which the most abundant compounds were respectively α-terpinyl acetate (36.3%), β-caryophyllene (34.8%), bicyclogermacrene (9.5%) and α-pinene (8.1%). Using the headspace-based techniques involving HS-SPME and MA-HS-SPME, 18 and 19 compounds were respectively characterized. The main compounds of the former technique (HS-SPME), were α-pinene (30.0%), α-terpinyl acetate (12.1%), β-caryophyllene (11.0%) and limonene (10.3%), while using the latter approach (MA-HS-SPME), α-pinene (24.5%), limonene (17.1%) and spathulenol (16.2%) were found as the most occurring natural compounds in the corresponding profile.

Tayebee et al. (2007) have reported limonene (28.8%), myrcene (17.06%) and caryophyllene oxide (27.5%) as the major constituents of essential oils obtained from leaf, flower and gramineous stipes of *Vitex pseudo-negundo*, from Sabzevar province, northeastern Iran in August 2004.

In a related study, Safaei-Ghomi and Meshkatalsadat (2010), have studied the chemical profiles of the essential oils and volatiles of *V. pseudo-negundo* by using three different methods involving classical hydrodistillation (HD), microwave-assisted hydrodistillation (MAHD) as well as ultrasonic assisted head space solid phase microextraction (UA-HS-SPME). All the extracted essential oils and volatiles were subsequently analyzed using nano scale injections onto an injection port of a GC/MS apparatus. This strategy led to identification of a total of 32 constituents, representing 95.67 to 96.65% of the oil. As shown in this study, the most abundant natural compounds occurring in the chemical profiles of the oils and volatiles using the three aforementioned

Table 1

Constituents of essential oils from seed of *Vitex pseudo-negundo* obtained by hydrodistillation.[a]

No.	Compound	Formula	Percentage	RRI[b]	Class
1	α-Pinene	$C_{10}H_{16}$	3.3	939	MH[c]
2	β-Pinene	$C_{10}H_{16}$	1.7	979	MH
3	β-Myrcene	$C_{10}H_{16}$	1.5	990	MH
4	*p*-Cymene	$C_{10}H_{14}$	2.4	1024	MH
5	Limonene	$C_{10}H_{16}$	2.2	1029	MH
6	1,8-Cineole	$C_{10}H_{18}O$	3.2	1031	OM[d]
7	Terpinolene	$C_{10}H_{16}$	0.9	1088	MH
8	Linalool	$C_{10}H_{18}O$	2.7	1096	OM
9	Pinocarveol	$C_{10}H_{16}O$	1.6	1139	OM
10	Pinocarvone	$C_{10}H_{14}O$	0.4	1164	OM
11	Terpinen-4-ol	$C_{10}H_{18}O$	3.1	1177	OM
12	*p*-Cymen-8-ol	$C_{10}H_{14}O$	0.6	1182	OM
13	Bornyl acetate	$C_{12}H_{20}O_2$	1.5	1288	OM
14	α-Copaene	$C_{15}H_{24}$	4.1	1376	SH[e]
15	β-Bourbonene	$C_{15}H_{24}$	0.9	1387	SH
16	β-Elemene	$C_{15}H_{24}$	2.7	1390	SH
17	α-Cedrene	$C_{15}H_{24}$	1.1	1410	SH
18	β-Caryophyllene	$C_{15}H_{24}$	2.5	1417	SH
19	γ-Muurolene	$C_{15}H_{24}$	3.9	1478	SH
20	α-Curcumene	$C_{15}H_{24}$	2.0	1489	SH
21	γ-Cadinene	$C_{15}H_{24}$	0.7	1520	SH
22	δ-Cadinene	$C_{15}H_{24}$	4.4	1524	SH
23	Elemol	$C_{15}H_{26}O$	7.0	1548	OS[f]
24	(*E*)-Nerolidol	$C_{15}H_{26}O$	1.1	1561	OS
25	Spathulenol	$C_{15}H_{24}O$	4.2	1577	OS
26	Guaiol	$C_{15}H_{26}O$	0.9	1600	OS
27	*epi*-Cedrol	$C_{15}H_{26}O$	0.7	1619	OS
28	α-Muurolol	$C_{15}H_{26}O$	2.2	1646	OS
29	Selin-11-en-4-α-ol	$C_{15}H_{26}O$	0.9	1658	OS
30	Bulnesol	$C_{15}H_{26}O$	1.1	1671	OS
31	α-Bisabolol	$C_{15}H_{26}O$	6.1	1685	OS
32	Acorenone-B	$C_{15}H_{24}O$	0.9	1690	OS
33	(*E*)-Nerolidyl acetate	$C_{17}H_{28}O_2$	3.8	1717	OS
34	Tetradecanoic acid	$C_{14}H_{28}O_2$	1.8	1777	NH[g]
35	Hexadecanoic acid	$C_{16}H_{32}O_2$	8.0	1992	NH
	Total identified		**92.4**		

[a]The compounds have been arranged according to their retention indices on an HP-5 MS capillary column
[b]Kovats retention indices given in the literature
[c]Monoterpene hydrocarbons
[d]Oxygenated monoterpenes
[e]Sesquiterpene hydrocarbons
[f]Oxygenated sesquiterpenes
[g]Non-terpene hydrocarbons

methods (HD, MAHD and UA-HS-SPME) were α-terpinyl acetate (23.2, 27.0, 29.3%), *trans*-β-farnesene (1.6, 21.4, 1.8%), α-pinene (20.2, 12.3, 6.2%), limonene (13.5, 10.9, 11.7%), β-caryophyllene (12.4, 0.0, 28.4%) and bicyclogermacrene (5.6, 7.8, 0.0%), respectively.

In another report focusing on characterization of chemical profiles relating to the essential oils from leaf, flower and fruit of *V. pseudo-negundo*, the most prevailing compounds in the profile of the leaf oil were α-pinene (35.9%), limonene (12.2%) and bicyclogermacrene (9.5%). On the other hand, the fruit oil of the plant was mainly composed of α-pinene (31.7%), bicyclogermacrene (14.5%) and limonene (11.5%). However, the flower oil of *V. pseudo-negundo* contained high quantities of monoterpene hydrocarbons such as α-pinene, and limonene as well as a sesquiterpene hydrocarbon like bicyclogermacrene (Hadj Mohammadi et al., 2006).

3.3. Content of phenolic compounds

Methanol, ethyl acetate and chloroform extracts were prepared to examine the total phenolic contents and antioxidant activities of *V. pseudo-negundo* seeds. The total phenolic contents in the examined plant extracts using the Folin-Ciocalteu's reagent were expressed in terms of gallic acid equivalents (the standard curve equation: $Y=0.0086x+0.0175$; $r^2=0.999$). Regarding the obtained results, the total phenolic compounds in the extracts examined ranged from 55.8 to 194.5 mg GA/g of dry extract (see Table 2). The total phenolics in the organic extracts of *V. pseudo-negundo* depends on the solvent nature used for extraction, with the most polar solvent being most effective, which is consistent with the high polarity of

most phenols (Mohsen and Ammar, 2008).

Table 2
Total content of phenolic compounds in the organic extracts from the seeds of *V. pseudo-negundo* expressed in terms of gallic acid equivalents (mg of GA/g of extract).

Extract	Absorbance	mg of GA/g of extract[1]
Chloroform	1.37	55.8±3.7
Ethyl acetate	1.75	148.7±1.9
Methanol	1.51	194.5±4.2

[1]Each value is the average of three analyses±standard deviation.

3.4. Antioxidant activity

The antioxidant activities of different extracts of the seeds of *V. pseudo-negundo* were determined using a methanolic solution of DPPH. DPPH is a very stable free radical. Unlike *in vitro* generated free radicals, such as the hydroxyl radical and superoxide anion, DPPH has the advantage of being unaffected by side reactions such as metal ion chelation and enzyme inhibition. A freshly prepared DPPH solution exhibits a deep purple color with an absorption maximum at 517 nm. This purple color generally fades when antioxidant molecules quench DPPH free radicals (Amarowicz et al., 2003). The antioxidant activity of three different extracts from the species *V. pseudo-negundo* is expressed in terms of percentage of inhibition (%) and IC_{50} values (µg/mL) (Table 3). In addition to the plant extracts, the antioxidant activities of three standard compounds --BHT, gallic acid and ascorbic acid-- were obtained for comparison.

The antioxidant activities of *V. pseudo-negundo* extracts showed values ranging from 8.6% to 72.4%. The finding the current report showed that the most active extract was the methanol extract, which neutralized 50% of free radicals at a concentration of 61.3 µg/mL. However, a rather moderate activity was found for ethyl acetate, while the chloroform extract had the least activity. In comparison to IC_{50} values of BHT, ascorbic acid and gallic acid, methanol extract from *V. pseudo-negundo* showed a moderate capacity for neutralization of DPPH radicals.

The extraction of antioxidant substances of different chemical structures, was achieved using solvents of different polarity. Numerous investigations of plant extracts have revealed the presence of high concentrations of phenols in the extracts obtained using polar solvents. There is a correlation between the concentration of phenolic compounds found in the extracts of *V. pseudo-negundo* and their antioxidant activity, with the methanol extract having the highest concentration of total phenols (Table 2) and DPPH scavenging capacity (Table 3).

4. Concluding remarks

The chemical composition of the essential oil of seeds from *V. pseudo-negundo* (Verbenaceae) growing in Sabzevar, northeast of Iran, was investigated. The results demonstrated that the chemical profile of the essential oil of the same species can change depending on a variety of conditions, including climate, time of collection, and the ground composition of the sampling area besides of growth stages of plant. Also, results of our study suggest the great value of the species *V. pseudo-negundo* for use in pharmacy and phytotherapy. Based on this information, it could be concluded that this plant is a natural source of antioxidant substances of high importance. It was found that the highest concentration of phenolic compounds in the extracts was obtained using the solvent of highest polarity, namely methanol. Further studies of this plant species should be aimed at determining whether the oils and extracts have medicinal value.

Conflict of interest

The author declares that there is no conflict of interest.

Table 3
Antioxidant (DPPH scavenging) activities of investigated seed extracts and standard antioxidants presented as percentage of DPPH radicals inhibition and IC_{50} values (µg/mL).

	DPPH assay (percentage of inhibition)				
Solvent	**Extract concentration**				**IC_{50} (µg/mL)**
	20 ppm	**40 ppm**	**60 ppm**	**80 ppm**	
Chloroform	9.8±0.7[a]	18.0±0.8	31.8±1.9	42.4±1.4	94.6±6.9
Ethyl acetate	8.6±1.6	26.7±2.2	42.7±2.5	56.1±4.3	75.2±3.3
Methanol	14.0±0.6	23.6±1.0	48.0±1.8	72.4±4.1	61.3±2.7
	Standard antioxidant concentration				
Standard antioxidant	**20 ppm**	**40 ppm**	**60 ppm**	**80 ppm**	**IC_{50} (µg/mL)**
BHT	76.0±4.8	93.8±0.8	94.6±1.1	96.7±0.9	14.9±0.9
Ascorbic acid	34.2±5.2	51.8±2.4	67.2±0.6	85.5±1.7	40.3±1.1
Gallic acid	82.8±1.6	91.0±1.9	91.5±2.6	92.6±0.9	7.9±1.2

[a]Standard deviations based on three replicate analyses.

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