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Changes in composition and antioxidant activities of essential oils in *Phlomis anisodonta* (Lamiaceae) at different stages of maturity

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ABSTRACT

The composition of hydro-distilled essential oils of *Phlomis anisodonta* Boiss. subsp. *occidentalis* Jamzad in vegetative, flowering and post flowering stages were investigated using GC and GC-MS, leading to identification of 41, 26 and 23 compounds, respectively. In all three samples, sesquiterpenes were the main components. In vegetative stage the main components of the oil were germacrene-D (14.3%), bicyclogermacrene (12.4%) and α-pinene (6.8%); in flowering stage, germacrene-D (52.6%) and β-caryophyllene (15.9%); and in fruiting stage, germacrene-D (27.9%), bicyclogermacrene (17.6%), caryophyllene oxide (14.7%) and β-caryophyllene (11.3%). The samples were also subjected to screening for their possible antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and β-carotene-linoleic acid assays. In the first experiment, the free radical scavenging activity of polar subfraction of methanol extract in fruiting stage was superior to all other extracts (IC50= 41±0.4 µg/ml). When using linoleic acid system, oxidation of the linoleic acid was effectively inhibited by the non-polar subfraction of methanol extract in different stages. The results of this study show that the composition of essential oils varies considerably both in different parts of the plants and at different stages of development. When using the plants for medicinal purposes, it is important to be aware of the effective developmental phase, i.e. when the most effective components reach their peak volume, as well as the most useful parts.

Keywords: Phenology; Volatile oils; Terpenoids; Germacrene-D; Bicyclogermcrene

Introduction

Oxidation processes are intrinsic in the energy management of all living organisms and are therefore kept under strict control by several cellular mechanisms (1). However, the excessive production of free radicals and the unbalanced mechanisms of anti-oxidant protection result in the onset of numerous diseases and accelerate ageing. The antioxidants with small molecular weight are considered as possible protection agents reducing oxidative damage to the human body, when the internal enzymatic mechanisms fail or are inadequately efficient (2). The plant

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kingdom produces a wide range of natural antioxidants. However, there is still not enough knowledge about the practical usefulness of most of them. In the group of secondary plant metabolites, antioxidant phenolics are commonly found in various fruits, vegetables and herbs and they have been shown to provide a defense against oxidative stress from oxidizing agents and free radicals (3).

Phlomis L. is a large genus in the plant family Lamiaceae with over 100 species distributed throughout Eurasia and North Africa. In Iran, this genus is represented by 19 species (9 species are endemic) (4). These plants have various uses that differ from one country to another. Some species of *Phlomis* are used in folk medicine as stimulants, tonics, diuretics and for the treatment of ulcers and haemorrhids (5). There are reports indicating various activities such as antiinflammatory, immunosuppressive, antimutagenic, antinociceptive, antifibriel, free radical scavenging, anti-allergic, anti-malarial and antimicrobial effects for some of these species. Their flowers are generally used as herbal tea to treat gastrointestinal troubles and to promote good health by protecting the liver, kidney, bone and cardiovascular system. In addition, some *Phlomis* species have culinary uses (5).

The aim of this research was to study the essential oil composition of *Phlomis anisodonta* Boiss. subsp. *occidentalis* Jamzad growing wild in W Iran (4) at different developmental stages as well as antioxidant activity of the plant extracts. These results can be used to investigate the optimal harvesting time of this plant for relevant industries.

Materials and Methods

Plant material

Phlomis anisodonta Boiss. subsp. *occidentalis* Jamzad was collected at three different times representing different phases of plant growth (vegatative, flowering and fruiting stage) on April 10, May 15 and August 2, 2012, respectively from Lorestan province in W Iran. The samples were identified by Dr. Mohammad Mehrnia and the voucher specimen has been deposited in Lorestan Research Center of Agriculture and Natural Resources Khoramabad, Iran (voucher no. 5711). The fresh aerial parts were shade dried (10 days) at room temperature.

Isolation of the essential oils

The essential oils were extracted by hydro-distillation of dried plant materials for 2.5 h (100 g of sample in 500 mL of distilled water) using a Clevenger-type apparatus as recommended by European Pharmacopia. The resulted essential oils were dried over anhydrous sodium sulphate and stored at +4˚C until tested and analyzed.

Preparation of methanol extracts

Dry aerial parts of *P. anisodonta* subsp. *occidentalis* were grinded into powder. The dried powder (50 g) was extracted with 70% methanol (MeOH) by Soxhlet apparatus at 60ºC for 6 h (6). The extracts were then filtered and concentrated *in vacuo* at 45ºC yielding a waxy material. The resulting extracts were suspended in water and partitioned with chloroform ($CHCl_3$) to obtain water-soluble (polar) and water-insoluble (non-polar, chloroformic) sub-fractions. Extracts were concentrated, dried and kept in the dark at +4ºC until tested.

Analysis of the oils

FID-GC was carried out using a Hewlett-Packard 6890 apparatus equipped with HP-5 capillary column (phenyl methyl diloxane, 25 m×0.25 mm i.d., 0.25 µm film thickness); carrier gas, He; split ratio, 1:25, and flame ionization detector. Temperature progamme: 60ºC (2 min) rising to 240ºC at 4ºC/min, injector temperature, 250ºC, detector temperature, 260ºC. GC-MS was performed using Hewlett-Packard 6859 apparatus equipped with quadrupole detector, on a HP-5 column (see GC), operating at 70 eV ionization energy, using the same temperature program and carrier gas as above. Retention indices were calculated by using retention times of *n*-alkanes that were injected after the oils at the same chromatographic conditions according to Van Den Dool method (7).

Identification of the components was based on comparison of their mass spectra with those of internal Wiley GC-MS spectral library, or with published mass spectra and those described by Adams and others (8).

Antioxidant activity - DPPH assay

The antioxidant activity of each oil or extract was evaluated on the basis of its activity in scavenging the stable DPPH radical, using a slight modification of the method described by Sarker et al. (9). Briefly, stock solutions (10 mg/ml each) of the essential oils, extracts and the synthetic standard antioxidant BHT were prepared in methanol. Dilutions are made to obtain concentrations ranging from 1 to 5×10^{-10} mg/ml. Diluted solutions (2 ml each) were mixed with 2 ml of freshly prepared 80 µg/ml DPPH methanol solution and allowed to stand for 30 min in the dark at room temperature for any reaction to take place. Ultraviolet (UV) absorbance of these solutions was recorded on a spectrophotometer at 517 nm using a blank containing the same concentration of oils or extracts or BHT without DPPH. Inhibition of free radical DPPH in percent (I%) was calculated as follow:

$$I\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. The sample concentration providing 50% inhibition (IC_{50}) was calculated by plotting inhibition percentages against concentrations of the sample. All tests were carried out in triplicates and IC_{50} values were reported as means±SD of triplicates.

β-Carotene/linoleic acid bleaching assay

In this test, the method of Miraliakbari and Shahidi was used with slight modification (10). A stock solution of *β*-carotene and linoleic acid was prepared with 0.5 mg of *β*-carotene in 1 ml chloroform, 25 µl of linoleic, acid and 200 mg Tween 40. The chloroform was evaporated under vacuum and 100 ml of aerated distilled water was then added to the residue. The samples (2 g/l) were dissolved in DMSO and 350 µl of each sample solution was added to 2.5 ml of the above mixture in test tubes. The test tubes were incubated in a hot water bath at 50ºC for 2 h, together with two blanks, one contained the antioxidant BHT as a positive control and the other contained the same volume of DMSO instead of the extracts. The absorbance was measured at 470 nm on an ultraviolet spectrophotometer. Antioxidant activities (inhibition percentage, I%) of the samples were calculated using the following equation:

$$I\% = (A_{\beta\text{-carotene after 2h assay}} / A_{\text{initial } \beta\text{-carotene}}) \times 100$$

where $A_{\beta\text{carotene after 2 h assay}}$ is the absorbance of *β*-carotene after 2 h assay remaining in the samples and $A_{\text{initial } \beta\text{-carotene}}$ is the absorbance of *β*-carotene at the beginning of the experiments. All tests were carried out in triplicates and inhibition percentages were reported as means±SD of triplicates.

Assay for total phenolics

The level of total phenols in the oils and extracts of *Phlomis anisodonta* subsp. *occidentalis* in different developmental stages were determined using Folin-Ciocalteu reagent and external calibration with gallic acid (11). Solutions of the extracts (0.1 ml each) containing 1000 µg of the extracts were taken individually in volumetric flasks, 46 ml of distilled water and 1 ml Folin-Ciocalteu reagent were added, and the flasks were thoroughly shaken. After 3 min, 3 ml of 2% Na_2CO_3 solution were added and the mixtures were allowed to stand for 2 h with intermittent shaking. Absorbance was measured at 760 nm. The same procedure was repeated for all the standard Gallic acid solutions (0–1000 mg/0.1 ml) and a standard curve obtained with the following equation:

$$\text{Absorbance} = 0.0012 \times \text{gallic acid (µg)} + 0.0033$$

Total phenols of the extract, as Gallic acid equivalents, was determined by using the absorbance of the extract measured at 760 nm as input to the standard curve and the equation. All tests were carried out in triplicates and phenolic contents as Gallic acid equivalents were reported as means±SD of triplicate determinations.

Total flavonoid content (TFC)

Total flavonoid contents of methanol extracts of *Phlomis anisodonta* subsp. *occidentalis* in maturity stage was determined utilizing the aluminum chloride colorimetric method as described byArvouet-Grand *et al*. (12). Briefly, 1 ml of 2% aluminum trichloride ($AlCl_3$) in methanol was mixed with the same volume

Table 1. Composition of essential oil of *Phlomis anisodonta* subsp. *occidentalis* at different growth stages

No.	Compound	RI	Vegetative stage	Flowering stage	Fruiting stage
1	α−pinene	935	6.8	0.8	0.5
2	camphene	947	2.2	0.3	−
3	β-pinene	974	0.4	−	0.7
4	β-myrcene	985	0.4	0.1	−
5	limonene	1025	1.1	0.5	−
6	linalool	1099	0.5	1.1	0.3
7	nonanal	1103	0.2	−	0.6
8	α-terpineol	1192	0.2	0.6	0.5
9	fenchyl acetate	1222	1.6	−	0.3
10	bornyl acetate	1285	1.2	0.2	−
11	bicycloelemene	1333	1.5	0.1	−
12	α-copaene	1372	0.3	1.4	2.2
13	β-bourbonene	1380	0.8	0.9	−
14	β-cubebene	1386	0.2	−	0.5
15	β-elemene	1388	0.7	2.8	5.9
16	β-caryophyllene	1418	1.2	15.9	11.3
17	α- amorphene	1470	1.2	−	0.1
18	germacrene-D	1480	14.3	52.6	27.9
19	β-ionone	1483	0.6	−	−
20	β-selinene	1484	−	0.2	0.3
21	bicyclogermacrene	1494	12.4	3.4	17.6
22	germacrene-B	1495	3.3	0.7	−
23	α-muurolene	1500	0.3	4.1	2.4
24	β-bisabolene	1511	1.3	−	−
25	δ-cadinene	1526	1.5	0.1	−
26	trans-β-farnesene	1527	0.5	1.9	3.1
27	γ-elemene	1564	1.5	0.3	−
28	nerolidol	1565	1.4	−	−
29	1,5-epoxysaliva-4(14)-ene	1582	−	−	0.8
30	spathulenol	1589	5.1	0.5	0.2
31	caryophyllene oxide	1596	1.1	4.2	14.7
32	globulol	1598	2.0	−	−
33	veridiflorol	1608	2.6	0.3	0.1
34	carotol	1614	1.0	−	−
35	α-cadinol	1656	3.5	0.2	−
36	T-muurolol	1676	0.86	−	−
37	α-hexylcinnamic-aldehyde	1740	0.3	−	−
38	2-pentadecanone-6,10,14-trimethyl	1845	5.5	3.1	3.8
39	1,2-benzendicarboxylic acid	1872	2.2	−	−
40	hexadecanoic acid	1972	1.5	−	−
41	dibutyl phthalate	2085	2.4	−	0.3
42	phytol	2240	1.0	0.1	0.2
43	eicosane	2740	2.1	−	−
	oxygenated monoterpene hydrocarbons		10.9	1.7	1.2
	monoterpenes		3.7	1.9	1.7
	sesquiterpene hydrocarbons		41.6	84.4	71.3
	oxygenated sesquiterpenes		17.6	5.2	15.8
	Total		88.7	96.4	94.3

of the extracts (2000 µg). Absorption readings at 415 nm were taken after 10 min against a blank sample consisting of a 1 ml extract solution with 1 ml methanol without $AlCl_3$. The concentrations of flavonoid compounds were calculated according to the following equation that was obtained from the standard quercetin graph:

Absorbance – 0.026 quercetin (µg) – 0.0060 (R^2: 0.9887).

Results

Essential oil components

The hydro-distillation of the dried aerial parts of *Phlomis anisodonta* susbsp. *occidentalis* gave yellow oil in 0.06, 0.25 and 0.11% (w/w) yields from the vegetative, flowering and fruiting stages, respectively.

The chemical composition of essential oil during three different maturation stages is presented in Table 1. As can be seen, the studied oils were resolved into 41, 26 and 23 components at the vegetative, flowering and fruiting stages, respectively. This compositional trend which is characterized by a decrease of the oil complexity during the plant development suggests that numerous metabolic pathways were elicited in the secondary metabolism of *P. anisodonta* subsp. *occidentalis*. The major constituents of the essential oil in vegetative stage were germacrene-D (14.3%), bicyclogermacrene (12.4%) and α-pinene (6.8%). In the volatile oil of flowering stage, germacrene-D (52.6%), β-caryophyllene (15.9%), caryophyllene oxide (4.2%) and bicyclogermacrene (3.4%) were the main components. In the oil obtained from fruiting stage, germacrene-D (27.9%), bicyclogermacrene (17.6%), caryophyllene oxide (14.7%) and β-caryophyllene (11.3%) were the principal components.

Antioxidant activity

The antioxidative capacity of the samples was evaluated using DPPH and β -carotene/linoleic acid assays. In both assays, butylated hydroxyl toluene (BHT) was used as the positive control.

According to the results given in Table 2, the higher DPPH-radical-scavenging activity belonged to polar subfraction of methanol extract in fruiting stage which had the lowest IC_{50} value (41±0.4 µg/ml).

The effect of the oil and methanolic extracts on inhibition of lipid peroxidation was assayed using β-carotene bleaching induced linoleic acid peroxidation methods. The results of β-carotene bleaching test showed that the inhibition capacity percentage of the non-polar subfraction of methanol extracts in fruiting stage (91.4±0.6) was higher than other fractions (Table 2). In β-carotene/linoleic acid assay, fruiting stage was the best time for antioxidant activity.

Assays for total phenolics and flavonoids

The results of the colorimetric analysis of total phenolics and flavonoids are given in Table 3. As shown the polar sub-fraction of methanol extracts have higher total phenolic and flavonoid compounds. On other hand, in fruiting stage highest phenolic and flavonoid compounds were found. It is evident that there is a relationship between the antioxidant ability and total phenol and flavonoid contents.

Table 2. Antioxidative capacities of the essential oils and methanol extracts of *Phlomis anisodonta* subsp. *occidentalis* at different developmental stages. Results are means of three different experiments. Means followed by same letter in each column are not significantly different at the 5% level.

Plant oils, methanol extracts and controls	Test system					
	DPPH IC_{50} (µg/ml)			β-Carotene/linoleic acid (inhibition rate %)		
	Vegetative stage	Flowering stage	Fruiting stage	Vegetative stage	Flowering stage	Fruiting stage
Essential oil	177±1.2[b]	208±1.9[b]	150±1.2[b]	68.3± 0.4[b]	79.2± 0.5[c]	75.7± 0.5[b]
Polar sub-fraction	130±0.9[c]	88±0.6[c]	41±0.4[c]	59.1±0.4[c]	81.2±0.6[b]	71.1±0.5[c]
Non polar-sub-fraction	310±2.7[a]	431±3.3[a]	288±2.1[a]	81.8± 0.6[a]	90.1± 0.6[a]	91.4± 0.6[a]
BHT		18.9 ± 0.3			96.4 ± 0.9	

Table 3. The results of the colorimetric analysis of total phenolics and flavonoids in *Phlomis anisodonta* subsp. *occidentalis* at different developmental stages. Results are means of three different experiments. Means followed by same letter in each column are not significantly different at the 5% level.

Sample	Phenolic content (µg GEs/mg extract)[y]			Flavonoid content (µg QEs/mg extract)[z]		
	Vegatative stage	Flowering stage	Fruiting stage	Vegetative stage	Flowering stage	Fruiting stage
Polar sub-fraction	423±3.2[a]	113±0.9[b]	552±3.9[a]	69.2±0.4[a]	99.6±0.8[a]	134.3±1.2[a]
Non polar sub-fraction	136±0.8[b]	226±3.1[a]	198±1.1[b]	31.4± 0.3[b]	58.9± 0.5[b]	19.7± 02[b]

[y] GEs, Gallic acid equivalents. [z] Not studied

Discussion

The observed increase in the amount of the essential oil during different growth stages reflects a developmental regulation of the essential oil biosynthesis. Therefore, the flowering stage is characterized by an intensive biosynthesis of essential oil presumably caused by the increase of the number of secretary structures following the expansion of flowers and/or as a response to pressure implied by herbivores, pollinators and phytophages. In contrast, at the fruiting stage, the decrease of essential oil content seems to be linked with the activation of catabolic pathways concomitant leading to a decrease in the densities of secretary structures following the senescence of leaves. These dynamics of essential oil accumulation during plant development have been already observed in *Hypricum perforatum*, *Artemisia pallens*, *Salvia officinalis*, *Salvia fructicosa* cv. Newe Ya'ar No. 4, *Balsamita major* and *Artemisia molinieri* (13). The essential oils' yields varied considerably from month-to-month and is also influenced by the micro-environment (sun or shade) in which the plant is growing. Pala-Paul et al. (2001) reported month-to-month variations in the essential oil composition and yield of *Santolina rosmarinifolia*, which could be attributed to precipitation and temperature (14). Results obtained by Badi et al. (2004) for *Thymus vulgaris* (thyme) also indicated that the timing of harvest is critical to both yield and oil composition (15).

In all three samples, the total amount of the sesquiterpene hydrocarbon fractions (61.6%, 84.4% and 71.3% in vegetative, flowering and fruiting stages, respectively) were higher than the monoterpene fractions (14.6%, 3.6% and 2.9% in vegetative, flowering and fruiting stages, respectively) and germacrene-D was the most representative sesquiterpene which has been identified in other species of *Phlomis* as the main compound too. Among the three growth stages, the oil of vegetative stage had the highest monoterpene fraction (14.6%) with α-pinene (6.8%) being the main component. This compound has been identified in *P. lanata* (25.4%), *P. olivieri* (11.7%) and *P. fruticosa* flowers (8.9%) and leaves (6.6%), as the main component; whereas in other reported oil analyses of *Phlomis* spp., the amount of α-pinene was either low or nil (16). Our results showed that, flowering stage of *P. anisodonta* subsp. *occidentalis* is the best time for obtaining the essential oil and germacrene-D.

The essential oils from many *Phlomis* spp. have been studied by means of gas chromatography coupled with mass spectrometry (GC-MS) techniques. Based on these reports researchers often divide *Phlomis* species into four chemotypes: 1- rich in sesquiterpene: in this group, two main components, i.e. germacrene-D and β-caryophyllene are redundant; 2- rich in monoterpene and sesquiterpene: the main components of this class are α-pinene , limonene, linalool, germacrene-D and β-caryophyllene; 3- fatty acids, aliphatic compounds and alcohol (diterpenoic alcohol, fatty acid alcohol) constituting the main components: this group contains a high percentage of hexadecanoic acid, trans-phytol and 9,12,15-octadecatrien-1-ol; 4- rich in terpene, fatty acids, aliphatic compounds and alcohol (diterpenoic alcohol, fatty acid alcohol) as main constituents: this group contains hexadecanoic acid, α-pinene and germacrene-D as major fatty acids, as well as monoterpene and sesquiterpene, respectively (17). Regarding the above mentioned classification, our results showed that *P. anisodonta* subsp. *occidentalis* is placed in the first group.

The DPPH-radical-scavenging activity of the oils

and extracts increased during the progress of developmental stages. Therefore, fruiting stage was the best time for harvesting the plant for obtaining most antioxidant activity. The higher amount of phenolic and flavonoid compounds in polar sub-fraction of methanol extract in fruiting stage can result in the dominance of free radical scavenging activity in the same treatment (18).

Previous studies indicated that the methanol extracts of *Phlomis fruticosa* L. and *Phlomis lanata* Willd. have antioxidant activity. They prevent bleomycin-Fe (II) catalyzed arachidonic acid super oxidation. Also, forsythoside B, acteoside (the two major phenylethylalcohol glycoside in *Phlomis*) purified from *Phlomis caucasica* Rech.f. and samioside obtained from *Phlomis samia* L. were found to be potent scavengers of 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical (17).

Antioxidants function either by inhibiting the formation of free alkyl radicals in the initiation step or by interrupting the propagation of the free radical chain. In truncating the propagation step, the antioxidants function as hydrogen donors (19).

Phenolic and flavonoid antioxidants are products of secondary metabolism in plants, and the antioxidant activity is mainly due to their redox properties and chemical structure, which can play an important role in chelating transitional metals, inhibiting lipoxygenase and scavenging free radicals (20).

The antioxidant activity of phenolic compounds is reported to be mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. Organic extracts may be more beneficial than isolated constituents, because other compounds present in the extracts can change the chemical or biological properties of bioactive individual components (21).

Conclusion

The results obtained from this study showed that the amount and composition of essential oil is strongly dependent on the developmental stage of the plant (phenology), and therefore harvesting time is one of the most important factors influencing the oil quality of *Phlomis anisodonta* subsp. *occidentalis*. Also, evaluation of antioxidant activity of *P. anisodonta* subsp. *occidentalis* demonstrated that the essential oils and extracts in this taxon have moderated antioxidant activity which is influenced by maturity and high activity during fruiting stage. Further studies are needed to understand the impact of harvesting time on the pharmaceutical values of *Phlomis anisodonta* subsp. *occidentalis*.

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