Journal of Plant Physiology and Breeding

2022, 12(1): 39-49 ISSN: 2008-5168

Research paper

Essential oil composition and metabolites changes of *Artemisia melanolepis* Boiss at different phenological stages

Seyed Mehdi Razavi* and Maryam Enferadi

Received: October 7, 2021 Accepted: December 9, 2021
Department of Biology, Faculty of Sciences, University of Mohaghegh Ardabili, Ardabil, Iran
*Corresponding author; Email: razavi694@gmail.com

Abstract
Artemisia melanolepis Boiss is an endemic species in Iran distributed in north and north west of the country. In the present work, metabolites' content of the aerial parts of *A. melanolepis* was analyzed at three growth stages. The non-volatile primary and volatile secondary metabolites of the plants were obtained by extraction and hydro distillated methods, respectively, and then were analyzed using a GC-MS. Our results showed that the non-volatile primary metabolites from amino acids, organic acids, sugars, and sugar alcohols declined considerably from the vegetative state to fruiting period of the plants. The results also indicated that thymol was the main constituent of the volatile oils at all phonological stages with various percentages in each stage. The major volatile secondary metabolite was alpha-terpineol at the vegetative and flowering stages (6.09 and 6.92%, respectively), followed by 4-carene (4.96%) at the fruiting stage. It was concluded that the metabolites profile of *A. melanolepis* was considerably altered at different stages of the life cycle.

Keywords: *Artemisia melanolepis*; essential oil; phenological stage; thymol

How to cite: Razavi SM and Enferadi M, 2022. Essential oil composition and metabolites changes of *Artemisia melanolepis* Boiss at different phenological stages. Journal of Plant Physiology and Breeding 12(1): 39-49.

Introduction

The *Artemisia* genus comprised of 64 species in the flora of Iran and some species are endemic. Many species of the genus are used in Iran for spice, food flavoring agent, or medicinal herb due to their pleasing scents, and have been named as Darmeneh or Afsantin in different parts of the country (Podlech *et al.* 1986).

Artemisia melanolepis Boiss is an endemic species in Iran distributed in north and north-west of the country. It is a perennial herb with small stem as tall as 4-10 cm with ovate leaves. The plant forms small to medium clones in slopes and hills at the altitude of 3500 to 4200 m (Podlech *et al.* 1986). The essential oils *of Artemisia* contain a variety of volatile secondary metabolites such as terpenoids, phenylpropanoids, and aliphatic compounds. Different species of *Artemisia* genus have been used in Iran as folk medicine for digestive disorders (Mahboubi 2014). There are also a number of reports on biological activities of *Artemisia* species in the literature. They may exhibit antifungal, insecticidal, antiviral, antitumor, antipyretic, antioxidant, anticoagulant, antihemrrhagic, and antihepatitic activity (Burits *et al*. 2001; Juteau *et al.* 2002; Shafi *et al.* 2004; Vajs *et al.* 2004; Zhang *et al.* 2015; Azimian and Roshandel 2016; Sharifivash and Shokrpour 2017).

Plant metabolism consist of two distinct process called primary and secondary metabolism. Whereas the former involves in the production of primary metabolites with certain roles in the plant growth and development, the later produces some products named secondary metabolites. These

compounds only play some roles in the plant survival by producing attractants for pollinators or providing chemical defense against herbivores and pathogens (Nigam *et al.* 2019).

Phenology is regarded as the periodic events in the life cycle of a plant and how these events are impacted by the seasonal alterations in climate. Phenological stages alter the plant metabolic pathways and thus the plant metabolite reserves including volatile and non-volatile metabolites (Daghbouche *et al.* 2020). It is also well documented that some biological and pharmacological properties of the *Artemisia* species are attributed to the volatile compounds consisting of the essential oils (Abad *et al.* 2012). In a previous work, we described the essential oils profile of *A. austriaca* from north-west of Iran (Razavi *et al.* 2014). As a part of our continuous investigations on the phytochemistry of *Artemisia* genus in north-west of Iran, in this paper, we are reporting the volatile metabolites and the composition of the essential oils of *A. melanolepis* Boiss at different phonological stages. We also describe the changes in the non-volatile primary metabolites of the plant at different periods of the life cycle.

Materials and Methods

Plant material

The aerial parts of *A. melanolepis* Boiss were collected at the vegetative (5-7 leaves stage), flowering (early blooming) and fruiting (early fruiting) stages from Alvars region in Sabalan mountains, Ardabil, north-west of Iran (38° 10′ 5″ N/ 47°54′ 56″E) at an altitude of 3031 m, in May-August 2019. A total of 30 fresh samples were collected at each phenological stage. All collected samples were dried at room temperature. A voucher specimen (No. 1395-1) of this collection was deposited in the Department of Biology, University of Mohaghegh Ardabili, Ardabil, Iran.

Extraction procedure

The nonvolatile metabolites were extracted from 300 mg of the plant samples at different phenological stages using methanol (70%). After homogenization with the liquid nitrogen, the extracts were sonicated at 70 °C for 60 min. Samples were centrifuged at 12,000 g for 10 min. Then, 2 ml of supernatant was lyophilized. For the silylation reaction, 90 µL BSTFA reagent (with TMCS 1%) and 100 µL hexane were added to the lyophilized samples. Then, the vials were tightened by caps and heated for 45 min at 75 °C. Ten microliters of samples were injected to GC Ms. For quantification, decanoic acid (capric acid) with a concentration of 0.30 µg/mL was added to the samples as the internal standard.

Hydrodistillation

To obtain volatile metabolites, the dried aerial parts of the plants at three phonological stages (100 g) were also subjected to hydro-distillation for 4h using a Clevenger-type apparatus. The resulting oils were subsequently dried over anhydrous sodium sulphate and dissolved in *n*-hexane (1 mL) for analysis.

GC-MS analysis

The silylated extracts and hydrodistillated oils were separately analyzed using an Agilent 7890B series GC with a fused methyl silicon HP-5 MS column (30 m × .25 mm i.d., .25 µm film thickness) and fitted with Agilent 5977 A Series MS system. Helium was used as carrier gas at a flow rate of 1 mL/min. The programmed temperature was increased from 50 to 320 °C at a rate of 4 °C/min and finally held for 10 min. The injector

temperature was set at 320 °C with the splitless condition. The mass spectral (MS) data were obtained at the following conditions: ionization potential 70 eV; ion source temperature 200 °C; quadrupole temperature 100 °C; solvent delay 3 min; scanning rate of .4 s; mass range of 40-460 amu. EM voltage of 3000 volts. The compounds were identified based on direct comparison of the MS data with those for the standard compounds, and via computer matching with the NIST NBS54 K Library and Wiley Library.

For quantitation (% area), the GC analysis was also performed on an Agilent 7890 B series apparatus fitted with a FID detector. The FID detector temperature was 320 ºC. To obtain the same elution order as with GC-MS, simultaneous auto-injection was performed on a duplicate of the same column applying the same operational conditions. Relative percentage of the separated compounds were calculated from FID chromatograms.

Statistical analysis

Statistical analysis was done using SPSS 21 software. Means were compared using Duncan's multiple range test at $p \leq 0.05$.

Results

The GC-MS analysis revealed a number of nonvolatile primary metabolites that change in different phenological stages of *A. melanolepis*. The identified metabolites belong to various groups such as amino acides (alanine, glycine, valine, tryptophan, methyl cysteine, N-methyl-L-glutamic acid, methyl glycine), sugars/sugar alchols (ribitol, editol, glucose), organic acids (oxalic acid, succinic acid, 4-hydroxyphenylpyruvic acid, malonic acid, phenyl pyruvic acid, isocitric acid, urocanic acid), and hydrocarbons (heptadecane, octadecan) (Table 1). Table 2 presents the amount of the metabolites at three stages of the plant's life cycle. As it shown, all of identified nonvolatile metabolites declined from the vegetative to fruiting period. The largest reduction was seen in the content of sugars and alchol sugars that reached to 350% from the vegetative state to fruiting. Also, the glucose reserves were 2.8 mg/ml in the vegetative stage and declined to 1.5 and 0.6 µg/mL at the flowering and fruiting stages, respectivly. The amino acids like alanin, glycine, valin, glutamic acid, serin, and triptophan diminished up to 30% from the vegetative stage to fruiting. Similiar results were recorded for the organic acids such as succinia acid and fatty acids like palmitic acid with a reduction of 25 to 35% from the vegetative to fruiting stage.

Aerial parts of *A. melanolepis* yielded 0.3, 0.1, and 0.1% (V/W) of yellowish oils in hydrodistillation at vegetative, flowering, and fruiting stages, respectively. Table 3 lists the constituents in the essential oils at three phenological stages in order of elution from the HP-5MS column, their percentages and their retention indices on the column. The oil at the vegetative stage comprised of 27 components representing 94.7% of the total oil. Also, 26 and 21 components were detected from the flowering and fruiting stages representing 98 and 95.3 % of the oil, respectively.

Table 1. List of identified metabolites using GC-MS

Name of metabolites	Retention time
L-alanine	4.39
L-glycine	4.61
L-valine	5.60
Sarcosine (methylglycine)	6.41
L-serin	6.72
Oxalic acid	6.78
Ethylmalonic acid	7.15
L-glutamic acid	7.23
D-glucose	7.43
L-pipecolic acid	8.10
Malonic acid	9.48
Decanoic acid (cappric acid)	10.75
Succinic acid	10.75
L-iditol	11.14
4-Hydroxyphenilpyrovic acid	12.30
Oleic acid	12.28
L-triptophan	13.61
Phenyl pyrovic acid	14.24
Isocitric acid	14.58
Urocanic acid	17.02
Nonacosanoic acid	17.90
Indoleacetaldehyde	21.76
Ribitol	21.98
Palmitic acid	23.10
N-methyl-L-glutamic acid	26.01
Methylcysteine	33.23
Heptadecane	36.76
Octadecane	40.45
Nonadecane	43.97
Eicosane	46.98

Table 2. Variation of the metabolites involved in the important metabolic pathways of *A. melanolepis* at different phenological stages

Name of metabolites	Concentration (µg/mL) at three phenological stages		
	Vegetative	Flowering	Fruiting
L-Alanine	4.1±0.0.05^{a}	3.8±0.0.04^{b}	3.1±0.0.02^{c}
L-Glycine	9.5±0.0.06^{a}	8.8±0.0.05^{b}	7.2±0.0.05^{c}
L-Valine	3.8±0.0.02^{a}	2.8±0.0.02^{b}	2.7±0.0.01^{c}
Oxalic acid	150±2.6^{a}	110±2.1^{b}	108±0.8^{c}
D-Glucose	2.8±0.0.1^{a}	1.5±0.0.06^{b}	0.6±0.0.02^{c}
Malonic acid	0.6±0.0.01^{a}	0.5±0.0.01^{b}	0.4±0.0.01^{c}
Cappric acid	0.3±0.0.02^{a}	0.3±0.0.01^{a}	0.3±0.0.01^{a}
Succinic acid	9.3±0.0.4^{a}	7.6±0.0.3^{b}	6.7±0.0.2^{c}
L-Iditol	5.3±0.0.3^{a}	4.1±0.0.2^{b}	1.7±0.0.07^{c}
L-Tryptophan	8.6±0.0.03^{a}	7.7±0.0.02^{b}	6.1±0.0.02^{b}
4-Hydroxyphenilpyrovic acid	0.5±0. 02^{a}	0.4±0. 01^{b}	0.3±0. 01^{b}
Isocitric acid	0.07±0. 003^{a}	0.06±0. 004^{a}	0.07±0. 003^{a}
Ribitol	1.6±0. 05^{a}	0.9±0. 03^{b}	0.5±0. 01^{c}
palmitic acid	3.4±0. 2^{a}	2.8±0. 2^{b}	2.2±0. 1^{c}
Methylcysteine	0.33±0. 07^{a}	0.29±0. 05^{b}	0.27±0. 04^{c}

In each row, different letters represent significant difference between means at $p \leq 0.05$ (± SE)

Table 3. Components of the essential oils in the aerial parts of *Artemisia melanolepis* at different phenological stages based on GC-MS analysis

Component	KI	Vegetative		Flowering		Fruiting	
		RT	%	RT	%	RT	%
Octane	801	3.16	3.22	3.17	3.04	3.15	1.80
(E)-2-hexenal	803	3.97	0.11	-	-	3.97	0.17
(-)-α-pinene	940	5.40	0.26	-	-	5.40	0.24
2,3-dehydro-1,8-cineole	993	-	-	-	-	6.46	0.32
Decane	1002	6.59	0.96	6.59	1.06	6.59	0.96
(+)-2-carene	1004	-	-	-	-	6.95	0.21
α-terpinene	1018	6.95	0.36	6.95	1.61	-	-
P-cymene	1027	7.10	0.24	7.10	0.86	-	-
O-cymene	1028	-	-	-	-	7.10	0.27
R(+)-limonen	1030	7.18	0.30	-	-	7.18	0.27
β-phellandrene	1033	-	-	7.19	0.54	-	-
1,8-cineole	1034	7.24	1.32	7.24	1.39	7.25	1.19
γ-terpinene	1061	7.78	0.74	7.78	1.81	7.77	2.34
α-terpinolene	1092	8.42	0.28	8.65	0.53	8.42	0.24
Δ3-carene	1101	8.64	0.39	-	-	8.65	0.47
Nonanal	1103	-	-	8.76	2.18	-	-
Camphor	1148	9.84	3.82	9.85	4.64	9.83	3.24
Borneol	1172	10.42	2.61	10.43	2.74	10.42	2.54
Terpinene-4-ol	1180	10.73	3.24	10.76	5.46	-	-
(+)-4-carene	-	-	-	-	-	11.11	4.96
α-terpineol	1192	11.13	6.09	11.16	6.92	-	-
Dodecane	1202	11.31	0.35	11.32	0.53	11.31	0.37
Thymol	1293	14.88	60.65	14.99	45.08	14.82	70.36
Carvacrol	1302	15.18	2.95	15.25	3.31	15.16	2.52
Crvacrol acetate	1375	17.04	3.35	17.08	5.32	17.02	2.36
Caryophyllene (Z)	1412	-	-	-	-	24.15	0.39
β-selinene	1493	21.48	0.75	21.49	3.13	25.84	0.20
Zingiberene	1497	-	-	21.75	0.59	-	-
α-selinene	1501	-	-	25.35	0.79	-	-
β-himachalene	1508	-	-	25.45	1.19	-	-
β-bisabolene	1508	22.13	0.25	25.94	1.43	-	-
Germacrene A	1512	24.16	0.41	-	-	-	-
δ-cadinene	1515	25.44	0.24	25.40	0.68	-	-
α-bisabolene	1515	25.63	1.02	-	-	-	-
δ-bisabolene	1518	25.92	0.39	-	-	-	-
α-bisabolol	1689	-	-	26.66	3.37	-	-
Eicosene	2004	30.80	0.19	-	-	-	-
Henicosane	2103	31.95	0.21				
(Z,E)-α-farnesene	1509	-	-	30.80	0.51	-	-
Docosane	2204	-	-	31.95	0.36	-	-

KI: Kovats index; RT: Retention time

The results indicated that thymol was the main constituent of the oil at all phonological stages. This followed by alpha-terpineol (6.09 and 6.92%) at the vegetative and flowering stages, respectively, and 4-carene (4.96%) at the fruiting stage.

Oxygenated monoterpenes were recognized as the main components of the distillated oils at all of the plant's phenological stages (Table 4).

Table 4. Proportion of various components of essential oil in the aerial parts of *Artemisia melanolepis* at different phenological stages

Component class	Real area (%)		
	Vegetative stage	Flowering	Fruiting
Oxyganated monoterpenes	84.03	74.86	82.53
Monoterpenes	2.18	5.35	8.53
Sesquiterpenes	2.04	8.32	0.59
Oxygenated sesquiterpenes	1.02	3.379	0
Hydrocarbones	1.28	7.17	1.50
Others	4.15	0	2.17
Total identified	94.70	98.07	95.32

Discussion

The results demonstrated that the content of primary metabolites of *A. melanolepis* such as amino acids, sugars, and organic acids declined with the progression of phenological stages to the fruiting period. This can be attributed to the translocation of nonvolatile primary metabolites from leaves toward developing seeds to reserve metabolites and nutrient for embryos, which supports them in the growth and development process. Similar results were obtained from other plants like sunflower and *Vitis labrusca* (Köse and Çelik 2017). The translocated amino acids not only play a vital role in the synthesis of seed-storage proteins but also serve as precursors for the biosynthesis of secondary metabolites and as a source of energy for embryos. The flow of organic acids and sugars from leaves and stems to seeds may be a reason for intensifying the respiration to supply energy for the embryos.

Our results revealed that thymol is the characteristic volatile metabolite in *A. melanolepis* at all of the phenological stages. Thymol is an oxygenated monoterpene with various bioactivities. It has been shown that thymol has antibacterial and fungicidal activities on plant pathogens. This compound has also herbicidal properties on some common weeds (Nesrollahi and Razavi 2017). It can be assumed that thymol could play an allelopathic role for the plant to combat surrounding competing plants, pathogens or herbivores. Allelopathic potential has been previously described for some *Artemisia* species that is attributed to presence of volatile terpenoids in the genus (Barney *et al*. 2005; Kegode and Ciernia 2005). In arid and semi-arid climates, plants inhibit the growth of surrounding plants by producing volatile compounds such as thymol and therefore overcome them in competing on the space, water, and nutrients. In the last decades, thymol has also been used as a novel botanical pesticide in the tropical agriculture (Liu *et al.* 2017).

The results demonstrated that there is a variation in quantity and quality of chemical constituents of the essential oils of *A. melanolepis* in the aerial parts at various phonological stages. Although thymol as the dominant component, characterized 60.6 and 70.3% of the oil at the vegetative and fruiting stages, respectively, it declined to 45% at the flowering stage. On the other hand, sesquiterpenes and hydrocarbons raised approximately to 20% at the flowering stage. This implies a certain shift in the metabolism of

terpenoids during the phenological stages. It is well known that metabolism of secondary compounds is highly changed by the plant age and phonological stage (Karousou *et al.* 2000). We previously described the increase in the sesquiterpene and reduction in the monoterpene content of the oil from the leaves of *Prangos uloptera* (Apiaceae) at the flowering stage as well as (Razavi *et al.* 2009) that shows the metabolic shift of monoterpene biosynthesis from leaves to flower organs. It might be responsible for the attraction of pollinators at the anthesis period.

The essential oil composition of many *Artemisia* species has been previously investigated. According to the major components of essential oils, it is possible to divide *Artemisia* species into two groups and a few subgroups:

1) Species producing oxygenated monoterpenes as major constituents of the oil

i) Species with camphor and 1,8-cineole as characteristic compounds, such as *A. oilveriana* (Rustaiyan *et al.* 2000), *A. spicigera* (Guvenalp *et al.* 1998), *A. deserti* (Rustaiyan *et al.* 2000), *A. frigida* (Atuzhanova *et al.* 1999), *A. radicus* (Atuzhanova *et al.* 1999), *A. incana* (Çetin *et al.* 2009), *A. austriaca* (Razavi *et al.* 2014), *A. abrotanum* (Tabanca *et al.* 2011), *A. distans* (Konatchiev *et al.* 2011), *A. longifolia* (Lopez-Lutz *et al.* 2008), *A. pontica* (Tabanca *et al.* 2011), *A. armeniaca* (Kazemi *et al.* 2010), and *A. splendens* (Kazemi *et al.* 2010).

ii) Species with thujone as the major component, such as *A. scoparia* (Mirjalili *et al.* 2007), *A. arborescens* (Beyrouthy *et al.* 2011), *A. sieberi* (Farzaneh *et al.* 2006; Ghorbani *et al.* 2008), *A. fukudo* (Yoon *et al.* 2010), and *A. absinthifolia* (Rezaeinodehl and Khangholi 2008).

iii) Species with camphor as the main constituent, such as *A. afra* (Nibret and Wink 2010), *A. gorgonum* (Ortet *et al.* 2010) and *A. santanicum* (Kordali *et al.* 2005).

iv) Species dominated by thymol as the characteristic component, such as *A. melanolepis (*Present work*).*

2) Species containing mainly sesquiterpene hydrocarbons

i) Species with germacrene D as the main constituent, such as *A. vulgaris* (Judžentienė and Buzelytė 2006) and *A. parviflora* (Rana *et al.* 2003).

ii) Species that produce artemisin as the major compound, such as *A. annua* (Viuda-Martos *et al.* 2010; Bilia *et al.* 2014) and *A. douglasiana* (Setzer *et al.* 2004).

It is interesting to note that thymol as the characteristic compound of the essential oils in *A. melanolepis* is not present in other *Atremisia* species or may be found in a trace amount. This can indicate a chemotaxonomic significance. In the Flora Iranica book (Podlech *et al.* 1986), *Artemisia* genus divides into 3 subgenera according to morphological features. *A. melanolepis* along with some other species of the genus such as *A. armeniaca, A. austriaca, A. incana, A. splendens, and A. annua* belongs to subgenus 1 named *Atremisiae* subgenus. However, it differs from other species of the subgenus in the essential oil composition. Although the essential oils of the majority of species in this subgenus are enriched with 1, 8- cineole and camphor, the oil of *A. melanolepis* is characterized with thymol as the

main constituent. Differences in phytochemicals of *A. melanolepis* from the related species (in subgen. *Artenisiae*) suggest that this species might be transferred to a new distinct subgenus or section of the genus. The differences can also be used as a diagnostic criterion for the distinction of *A. melanolepis* from allied species. It was previously documented that *A. splendens* is very close to *A. melanolepis* and the two species commonly distribute in the mountains of northern Iran from 3550 to 4200 m (Podlech *et al.* 1986). The oil profile can be used to distingush the doubtful populations.

Conclusion

The metabolites profile of *A. melanolepis* was considerably altered at different stages of its life cycle. Although the quantity of nonvolatile metabolites like sugars and sugar alcohols declined from the vegetative period to the fruiting stage, the volatile metabolites differed in quality (types of compounds) at different phenological stages.

Acknowledgment

Authors are grateful for the support of this research project provided by the University of Mohaghegh Ardabili. We would like to thank Mr. Kamali for his cooperation in the GC-MS analyses.

Conflict of interest

The authors declare that they have no conflict of interest with any organization concerning the subject of the manuscript.

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تغییرات ترکیب اسانس و متابولیت‌های گیاه درمنه در مراحل متفاوت فنولوژیک

سید مهدی رضوی* و مریم انفرادی

گروه زیست شناسی، دانشکده علوم، دانشگاه محقق اردبیلی، اردبیل
*مسئول مکاتبه؛ Email: razavi694@gmail.com

چکیده

گیاه درمنه (*Artemisia melanolepis*) بومی ایران است و در شمال غرب و شمال ایران انتشار دارد. در این تحقیق، محتوی متابولیت‌های اندام هوایی گیاه در سه مرحله رشد مورد واکاوی قرار گرفت. متابولیت‌های غیرفرار و فرار به ترتیب با روش‌های عصاره گیری و تقطیر آبی به دست آمدند و سپس هر کدام با روش خاص با دستگاه کروماتوگرافی گازی متصل به طیف سنج جرمی مورد تجزیه قرار گرفتند. نتایج نشان داد که متابولیت‌های غیرفرار از گروه اسیدهای آمینه، اسیدهای آلی، قندها و الکل قندها به طور قابل توجهی از مرحله رویشی به میوه دهی کاهش پیدا کردند. همچنین نتایج نشان داد که تیمول به عنوان ترکیب شاخص اسانس این گیاه در تمام مراحل زیستگردی گیاه به شمار می‌آید. بعد از این ترکیب، آلفا ترپینول به ترتیب با ۶ و ۶/۹ درصد در مراحل رویشی و گلدهی و ۴-کارن با ۴/۹ درصد در دوره میوه دهی ترکیبات اصلی به حساب می‌آیند. می‌توان نتیجه گرفت که نیمرخ متابولیت‌های گیاه به طور کامل در مراحل مختلف فنولوژیک متفاوت است.

واژه‌های کلیدی: اسانس؛ تیمول؛ درمنه؛ مرحله فنولوژیک