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Flavonoids and essential oil composition of *Stachys lavandulifolia*, *Teucrium polium*, *Thymus daenensis* and *Ziziphora clinopodioides*

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Abstract

Iran has different environmental conditions due to its specific geographical location. Therefore, exploring essential oil and flavonoid components among medicinal plants in different areas may result in discoveries about valuable bioactive compounds. The present investigation aimed to study flavonoids profile and essential oils composition of four Lamiaceae species *i.e., Stachys lavandulifolia, Teucrium polium, Thymus daenensis* and *Ziziphora clinopodioides* from Oshtorankoh located on Zagros Mountains, Iran. A chemical study using two dimentional paper chromatography (2-DPC) and thin-layer chromatography (TLC) showed that the four studied species contained flavone *C* and *C-/O* glucosides and flavonoid sulphates. The present study revealed that *T. polium* had high levels of many flavonoids such as apigenin, genistein, isorhamnetin, kaempferol, quercetin, rutin, and vitexin compared to the other studied plants. The results of GC/MS analysis showed that *S. lavandulifolia* was rich in α-pinene (21.16 %) and 1,8-cineol (16.95 %), *T. polium* in α-pinene (12.21 %) and sabinene (9.55 %), *T. daenensis* in γ-Terpinene (36.98 %) and p-Cymene (26.87 %), and *Z. clinopodioides* in piperitone (16.84 %) and 1,8-cineol (16.11 %) components.

Key words: Flavonoids, essential oil, Iran, Lamiaceae, mint, Zagros

Introductıon

Iran has different environmental conditions due to specific geographical location. Therefore, exploring essential oil and flavonoids components among different medicinal plants in different areas of the country may result in discoveries about valuable components (Farajpour *et al.*, 2011; Farajpour *et al.*, 2017; Ebrahimi *et al.*, 2012). Mint is from Lamiaceae or Labiatae family with about 236 genera and 6900 to 7200 species worldwide, among which about 124 species and subspecies (30 %) are endemic to Iran (Akhmedov *et al.*, 2021). Many of these species are cultivated for their fragrant leaves and attractive flowers. Aerial parts and leaves are the most widely used plant parts. *Nepeta* (76 spp.), *Salvia.* (56 spp.), *Stachys* (34 spp.), *Scutellaria* (19 spp.), *Phlomis* (17 spp.), *Eremostachys* (16 spp.), *Thymus* (16 spp.) and *Teucrium* (12 spp.) are the largest genera in Iran (Naghibi *et al.*, 2005).

Mint plants are characteristically aromatic, and many of them are cultivated for their essential oils. They are widely used as spices and flavoring in food industries. Their oils are used in the perfumery, cosmetics, and pharmaceutical industries as bactericides, fungicides, virucides, antiparasitics, and pesticides (Bhatt *et al.*, 2021). More than 8000 flavonoids compounds have been identified in plants that afford protection against ultraviolet radiation, pathogens, and herbivores. They are considered health-promoting and disease-preventing dietary supplements. Epidemiological, clinical, and animal studies reveal that flavonoids may exert protective effects against various disease conditions including cardiovascular disease and cancer. Flavonoids also possess antibacterial, antiviral, and anti-inflammatory effects (Karak, 2019).

Lim *et al.* (2016) showed anti-inflammatory effects of *Stachys tibetica* polyphenols and flavonoids extracts. The leaves and aerial parts of *Thymus serpyllum* had antidiabetic effects (Mushtaq *et al.*, 2016). Also, myrcene, sabinene, β-phellandrene, and β-caryophylene were reported the main components in *S. lavandulifolia* essential oils (Shahnama *et al.*, 2015). Studies on different *S. lavandulifolia* populations indicated that the essential oil compounds can vary genetically (ecotype), environmentally, and geographically (Pirbalouti and Mohammadi, 2013). Twenty-four components were distinguished in *T. daenensis* essential oil from that thymol, α-terpinene, *p*-cymene, methyl carvacrol and α-thujene were the major components (Rustaiee *et al.*, 2010). The major essential oil compositions of *Teucrium polium* L. fruits were as follow α-pinene, β-pinene, limonene, elemol, and cubenol (Sabzeghabaie and Asgarpanah, 2016). The main chemical compositions of *Ziziphora clinopodiaides* aerial part essential oils were reported as carvacrol, α- & β-pinene, pulegone, cymene, iso-menthone, iso-menthol, menthyl acetate, p-menth-3-en-8-ol, neo iso- menthol, limonene, myrcene, piperitenone, 1,8-cineole, piperitone, spathulenol, β-pinene, borneol, germacrene D, ɣ-terpinene, and β-bourbonene (Mahboubi *et al.*, 2018). The antinociceptive effect of *Z. clinopodioides* essential oil and opioidergic system was studied in male rats (Mohammadifard and Alimohammadi, 2018). Due to the general tendency to use natural alternative medicines for the prevention and treatment of diseases, the consumption of plants containing flavonoids and essential oils as food and medicine has increased. .

The current study aimed to identify the flavonoids and essential oil compositions of four Lamiaceae species collected from the "Oshtorankoh" protected area in Lorestan Province, Iran, including *Stachys lavandulifolia, Teucrium polium, Thymus daenensis* and *Ziziphora clinopodioides.*

Materials and methods

Plant sample and preparation: The aerial parts of four Lamiaceae species including *Stachys lavandulifolia*, *Teucrium polium*, *Thymus daenensis*, and *Ziziphora clinopodioides* were collected from “Oshtorankoh” protected area, Lorestan, Iran (Table 1). Lorestan with a 28,294 km^2 area is located in western Iran in the Zagros Mountains (33°58´N, 48°39´E). Herbarium voucher samples of each species were prepared for reference and deposited at the Arak University Herbarium. The samples were air-dried for isolation and determination of their flavonoids and essential oils.

Flavonoids: The flavonoids of plants aerial parts were extracted using 70 % ethanol and the extracts were vacuum concentrated to dryness in a rotary evaporator. The flavonoids in each extract were isolated and detected using two-dimensional paper and thin-layer chromatography. Flavonoids extracts were prepared using the acid hydrolysis method adding chloride acid 2 M hydrochloric acid and placing them in a water bath at 100 °C for 30 min. After cooling, ethyl acetate was added to separate the flavonoid extract from the non-flavonoids extract. The flavonoid extract, soluble in ethyl acetate at 40 °C and 40 m/sec, was distilled off in a vacuum. Co-chromatography with standards was also performed where possible. All available flavonoid standards for comparison during the study were obtained commercially from Merck, Sigma, and Fluka. Cellulose sheet TLC plates were used and after running in three solvents separately, were viewed in UV245 nm. Each spot R_f-values and color comparing to standards helped flavonoids identification. R_f values were calculated and in comparison with used flavonoid standards identification and concentration of each spot were determined using Camag UV 254 and 366 nm, chrogramatographic map, and UV spectroscopy. Also Perkin-Elmer Lambda 15 UV/*Viz* Spectrophotometer was used for spots having similar hR_f.

Essential oil composition: Fifty g of dried aerial parts were hydro-distilled in a Clevenger type apparatus for 2.5 h in three replications. The EOs were kept at 4 °C in the dark before analysis. The essential oils were injected into a gas chromatograph and the most appropriate temperature planning of column was determined to complete the separation of essential oils. Then essential oils were injected into the Gas Chromatograph/Mass Spectrophotometer Aglient 7890 and compound mass spectra were obtained. Essential oils components of each sample were identified using Retention Index (RIs), studying mass spectra compared to Reference Spectra Library (Adams, 2007). In this study, GC including a mass detector Aglient 5975 C, an Electron Ionization source coupled with an Aglient 7890 GC apparatus was used. A 30-meter-long HP-5MS column, 0.25 mm internal diameter, and 0.25 µm internal layer thickness (film) were used. The GC injection site temperature (Inlet) were regulated to 280 °C, mass detector ionization source temperature was 150 °C, analyzer (Quadrupole) temperature was 230 °C, and the GC/MS intermediate section temperature was 280 °C. Each extract was inserted into the device through the injection site and the carrier gas (usually He or N) was entered into the device column. Then bypassing the sample and gas, extract components were separated and detected by the detector. Results, graphs were represented and each essential oil constituents’ percentages were recounted according to the FID chromatographic peak areas assuming that all the essential oil compounds comprise 100 %.

Results

Flavonoids: According to the results, all of the studied samples contained flavonoid sulphates and flavone *C*-& *C*-/*O*-glucosides (Table 1). *T. daenensis* had the most number of flavonoids and *Z. clinopodioides* had the lowest one. Aglycones were not found in any of them. Flavone *C*-& *C*-/*O*-glucosides and flavonoid sulphates were identified flavonoids series in 2-DPC and TLC. As Table 2 shows, rhamnetin, tricin, and morin were not found in the four studied species. However, all of them had isorhamnetin, kaempferol, luteolin, orientin, and quercetin. In addition, the samples had apigenin and vitexin (exception for *T. daenensis*). High levels of many flavonoids such as apigenin, genistein, isorhamnetin, kaempferol, quercetin, rutin, and vitexin were observed in *T. polium*.

Essential oils: Results of four Lamiaceae taxa aerial parts

Table 1. Sample information and aerial parts two-dimensional paper chromatographical data of four studied Lamiaceae

Voucher samples	Taxon	Altitude (m)	Total flavonoids (number)	Flavone *C*-& *C*-/*O* glucosides no.	Flavonoid sulphates (number)
CEF02	*Stachys lavandulifolia* vahl	2400	6	3	3
CEF55	*Teucrium polium* L.	1951	6	2	4
CEF70	*Thymus daenensis* Celak	2235	9	5	4
CEF73	*Ziziphora clinopodioides* Lam.	2223	3	2	1

Table 2. Thin Layer Chromatographical data of four studied Lamiaceae taxa aerial parts flavonoids from Iran.

Species	Flavonoids identification														
	Ap	Ch	Ge	Iso	Ka	Lu	Mo	My	Na	Or	Qu	Rh	Ru	Tr	Vi
S. lavandulifolia	+	-	-	+	+	±	-	++	-	++	+	-	++	-	++
T. polium	++	+	++	++	++	+	-	-	-	+	++	-	++	-	++
T. daenensis	-	-	-	+	+	+	-	+++	+	++	+	-	+	-	-
Z. clinopodioides	++	+++	++	+	+	+	-	+	-	+	+	-	-	-	+

Concentration of flavonoids: -(non flavonoid), ±(non or a few flavonoid), + (few flavonoid), ++ (middle concentration of flavonoid), +++ (high concentration of flavonoid). Ap: Apigenin; Ch: Chrysin; Ge: Genistein; Iso: Isorhamnetin; Ka: Kaempferol; Lu: Luteolin; Mo: Morin; My: Myricetin; Na: Naringenin; Or: Orientin; Qu: Quercetin; Rh: Rhamnetin; Ru: Rutin; Tr: Tricin; Vi: Vitexin.

essential oils studied from Iran using GC/MS showed, *S. lavandulifolia* was rich in α-Pinene (21.16 %) and 1,8-Cineol (16.95 %), *T. polium* in α-Pinene (12.21 %) and Sabinene (9.55 %), *T. daenensis* in p-Cymene (26.87 %) and γ-Terpinene (36.98 %), and *Z. clinopodioides* in 1,8-Cineol (16.11 %) and piperitone (16.84 %) (Table 3). Carvacrol and piperitone components were found only in *T. daenensis* and *Z. clinopodioides*, respectively. Based on the results, the maximum and minimum percentages of essential oil compositions detected by GC/MS belonged to *T. daenensis* (81.18 %) and *T. polium* (59.22), respectively. 1, 8-cineol, (E)-2-decenal, limonene, α-pinene, β-pinene, and (E)-2-undecenal compositions were found in the four studied species. According to the results, 14 components were detected in the essential oils of the four studied species, among which 9, 11, 10, and 9 components were observed in *S. lavandulifolia*, *T. polium*, *T. daenensis*, and *Z. clinopodioides*, respectively. All of the studied species had monoterpene, alcohol, aldehyde, and sesquiterpene while aromatic and fatty acid ester were found only in *S. lavandulifolia* and *T. polium*, respectively (Table 4).

Table 3. Essential oils compounds data of four Lamiaceae taxa aerial parts from Iran using GC/MS

Component	R.I.[a]	S.l.	T.p.	T.d.	Z.c.
α-Pinene	938	21.16[b]	12.21	2.03	3.8
(E)-2-Heptenal	965	4.73	5.85	0	0
Sabinene	979	3.58	9.55	0	4.52
β-Pinene	985	3.03	7.87	0.45	3.93
p-Cymene	1037	0	0.13	26.87	0
Limonene	1039	2.44	2.27	0.29	17.4
1,8-Cineol	1041	16.95	0.21	2.66	16.11
γ-Terpinene	1069	0	0	36.98	0.93
n-Nonanal	1110	4.88	6.73	0	0
Piperitone	1257	0	0	0	16.84
(E)-2-Decenal	1271	5.46	7.07	1.76	1.5
Thymol	1329	0	0.46	3.63	0
Carvacrol	1339	0	0	4.83	0
(E)-2-Undecenal	1374	5.32	6.87	1.68	1.04
Total (%)	-	67.55	59.22	81.18	66.07

[a] RI: Retention indices .[b] Values are the relative area percent (peak area relative to total peak area in total ion chromatogram). S.l: *S. lavandulifolia*; T.p.: *T. polium;* T.d.: *T. daenensis*; Z.c.: *Z. clinopodioides*.

Table 4. Essential oils compounds data of four Lamiaceae taxa aerial parts from Iran using GC/MS

Compounds	S.l*	T.p	T.d	Z.c
EO Compounds N.	9	8	6	4
EO Compounds %	54.76	41.64	91.55	90.4
Total EO N.	56	46	27	30
Total EO %	100	99.98	100	99.9
Monoterpene N.	14	10	14	21
Monoterpene %	53.90	37.92	90.95	89.4
Alcohol N.	5	4	2	0
Alcohol %	2.26	2.18	1.95	0.93
Aldehyde N.	23	21	4	7
Aldehyde %	35.61	47.18	9.38	8.63
Sesquiterpene N.	1	1	1	1
Sesquiterpene %	0.86	3.72	0.6	0.98
n-Alkane N.	3	1	0	0
n-Alkane %	1.43	0.47	0	0
Alkane N.	3	0	2	0
Alkane %	1.43	0	0.71	0
Alkene N.	3	7	0	0
Alkene %	5.09	7.19	0	0
Aromatic N.	1	0	0	0
Aromatic %	0.38	0	0	0
Fatty acid N.	1	1	0	0
Fatty acid %	0.47	0.74	0	0
Fatty acid ester N.	0	1	0	0
Fatty acid ester %	0	0.58	0	0
Ketone N.	0	0	1	0
Ketone %	0	0	0.31	0

*S.l: *S. lavandulifolia*; T.p: *T. polium;* T.d: *T. daenensis*; Z.c: *Z. clinopodioides*.

Discussion

Species of the *Teucrium* genus have been used for centuries and the plants are used for different purposes such as diabetes, gastrointestinal disorders, rheumatism, inflammations, tuberculosis, diuretic, antipyretic, tonic, diaphoretic, analgesic, and antihyperlipidemic (Jarić *et al.*, 2020). The present study shows high levels of many flavonoids such as apigenin, genistein, isorhamnetin, kaempferol, quercetin, rutin, and vitexin which were observed in *T. polium*. Therefore, different applications of these plants may be related to these components. The plant also had a low level of luteolin, this component was reported in *Teucrium* species (Macedonia, 2005). Different components such as luteolin-7-*O*-rutinoside and luteolin-7-*O*-glucoside, which have antioxidant activities, were identified in *Teucrium orientale* (Çakir *et al.*, 2006). Luteolin 3',4',5,7-tetrahydroxyflavone, is a common flavonoid used in Chinese traditional medicine for treating various diseases such as hypertension and inflammatory, it also has a high level of antioxidant activity (Lin *et al.*, 2008). Our results showed, from the isolated compounds of *Teucrium polium*, 47.18 % were aldehydes. Monoterpenes with 37.92 % consisted of α-pinene, β-pinene, and sabinene are a part of species essential oil (Amiri, 2010). Also, α-Muurolene, α-Cadinol, and δ-Cadinene were isolated from *T. Polium* (Guetata and Alghamdia, 2014). *T. polium* extract enhances the anti-angiogenesis effect of tranilast on human umbilical vein endothelial cells (Sheikhbahaei *et al.*, 2018). Based on the results, luteolin, isorhamnetin, kempfrol, myricetin, orientin, quercetin, and rutin were found in *Thymus daenensis*. Sonmezdag *et al.* (2016) showed that luteolin, luteolin 7-*O*-glucoside, and rosmarinic acid were the most evident phenolic compounds in the species. The results of GC/MS analysis revealed that the essential oil compounds of the plant contained monoterpenes (90.95 %), γ-Terpinene (36.98 %), and p-Cymene (26.87 %). Previous studies reported the following essential oil components in this species, γ-terpinene, α-pinene, camphene, myricetin, sabinene hydrate, terpenine, isovaleric acid, δ-3-carene, thymol, linalool, thymoquinone, and carvacrol (Sonmezdag *et al.*, 2016). The carvacrol component has therapeutic potential for preventing and treating colon cancer (Fan *et al.*, 2015). In addition, sesquiterpenes (60 %) consisting β- caryolhyllene, *α*-humulene and allo-aromadendrene, alkeholes (1.95 %), aldehydes (47.18 %), alkenes (9.38 %), alkanes (0.71 %), and ketones (0.31 %) were reported in *T. daenensis* (Tables 3 & 4). Growth inhibition of Gram-positive bacteria, *i.e., Staphylococcus aureus*, *Micrococcus luteus*, *Entrococcus faecalis*, *Streptococcus pyogenes* was observed using *T. daenensis* methanolic extract (Mojab *et al.*, 2008). This growth inhibition of different Gram-positive bacteria could be due to the presence of different components in the essential oil of aerial parts of the plant. The plants in *Stachys* species are consumed as herbal tea in the major parts of the world, and due to their antibacterial, antifungal, anti-inflammatory, and antioxidant capacity, they are widely used as a herbal remedy in alternative medicine (Goren, 2014). Flavonoid compounds are known for their antioxidant properties and are important for humans diet. Flavonoids make up one of the most pervasive groups of plant phenolics. In this study, high levels of some flavonoid components such as luteolin, myricetin, orientin, rutin, and vitexin were observed in aerial parts of *S. lavandulifolia*. Eghdami *et al.* (2011) showed a strong positive correlation between total phenolic content and antioxidant activity in this species. Due to different flavonoids contents the species can be used potentially as a readily accessible source of natural antioxidants (Eghdami *et al.*, 2011). The flavonoids were also identified in *S. lavandulifolia* methanolic extract (Rahimi Khoigani *et al.*, 2017). The essential oil extracted from *S. lavandulifolia* subsp. *lavandulifolia* had antimicrobial and antioxidant activities. The GC/MS analysis showed that the essential oil contained monoterpenes (53.9 %) and sesquiterpenes (0.86 %). In addition, sabinene, β-pinene, β-myrcene, 3-δ-carene, limonene, terpinolene, allo-Ocimene, verbenone, trans-carveol, nerol, geraniol, caryophyllene, and caryophyllene oxide components were detected in the essential oil of this plant. Karami *et al.* (2016) and Giuliani *et al.* (2017) reported two sesquiterpenes componentns including caryophyllene and caryophyllene oxide in *S. benthamiana* and *S. officinalis*, respectively. Arial parts including leaves, flowers, and stem of *Z. clinopodioides* are valuable parts of the plant, and used mostly for traditional food and medicine purposes. It is also used in the preparation of an aromatic tea for gastrointestinal disorders and as an aperitive, carminative, and antiseptic (Alp *et al.*, 2016). Our results revealed that the plant was rich in apigenin, chrysin, and genistein. The high level of chrysin component was reported earlier in this plant, however, some other components such as kaempferol, myricetin, orientin, and quercetin were also observed (Tian *et al.*, 2011). The chrysin flavonoid with different properties such as anti-oxidative and anti-inflammatory effects is a potential prophylactic agent in immunopathological and physicochemical injuries (Zeinali *et al.*, 2017). Therefore, it seems that the plant may have health-promoting and disease-preventing properties in modern lifestyles. The antioxidant activity of the plant could be due to the high level of 1,8-cineol in its essential oil (Al-musawi *et al.*, 2020). The component also has anti-inflammatory properties ans used for co-medication in inflammatory airway diseases (Juergens, 2014). Based on the results, the plant was rich in piperitone component, which was not observed in the three other studied plants. Shahverdi *et al.* (2004) reported that piperitone component (1 µL/ml) enhanced the antimicrobial activity of furazolidone and nitrofurantoin agains*t Salmonella* spp*., E. coli strains, Proteus* spp*., Enterobacter* spp*., Citrobacter spp., Klebsiella* spp. and *Serratia* spp*.* In the present study such valuable components including carvacrol, 1,8-cineol, p-cymene, (E)-2-decenal, (E)-2-heptenal, limonene, n-nonanal, α-pinene, β-pinene, piperitone, sabinene, γ-terpinene, thymol, and (E)-2-undecenal were detected in the essential oils of *S. lavandulifolia*, *T. polium, T. daenensis*, and *Z. clinopodioides*. The essential oil components can be affected by different factors including plant species, plant genetics, soil conditions, altitude, growth phase, plant part used, and environmental factors. Therefore, exploring phytochemical variations in different medicinal plant species could lead to discoveries about valuable components.

Iran has different environmental and climatic due to specific geography. Therefore, exploring essential oil components among different medicinal plants may lead to the discoveries about valuable components. There is little information about the essential oil components in medicinal plants from Oshtorankoh region. The present study revealed that *T. polium* had high levels of many flavonoids such as apigenin, genistein, isorhamnetin, kaempferol, quercetin, rutin, and vitexin compared to the other studied plants. According to the results of GC/MS analysis, *S. lavandulifolia* was rich in α-pinene and 1,8-cineol, *T. polium* in α-pinene, and sabinene, *T. daenensis* in p-Cymene and γ-Terpinene, and *Z. clinopodioides* in 1,8-cineol and piperitone components.

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