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Latin American Journal of Pharmacy
(formerly *Acta Farmacéutica Bonaerense)*
Lat. Am. J. Pharm. **39** (1): 109-15 (2020)

Received: September 2, 2019
Revised version: October 14, 2019
Accepted: October 15, 2019

Evaluation of Antioxidant Activities, Phenolic Constituents and Essential Oil Composition of *Marrubium heterodon* (Benth.) Boiss. & Balansa from Turkey

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***SUMMARY*. This study comprises the antioxidant activities, phenolic constituents and essential oil composition of *Marrubium heterodon* (Benth.) Boiss. & Balansa, an endemic species distributed in Turkey. Essential oil was obtained by hydrodistillation from the aerial parts, and analysed by gas chromatography (GC) and GC/mass spectrometry (MS). In total, 38 components representing 86.8% of the oil were determined. The major components of the essential oil were α-pinene (17.3%), myrtenal (11.9%) and α-terpineol (5%). The phenolic composition of the aqueous, methanolic and ethyl acetate extracts were evaluated by liquid chromatography (LC)-MS/MS. The antioxidant activity of each extract was determined by using the *in vitro* 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, 2,2'-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical cation decolorization and Copper (II) reducing antioxidant capacity (CUPRAC) assays. To the best of our knowledge, this is the first report on the antioxidant activities, phenolic constituents and essential oil composition of *M. heterodon*.**

RESUMEN. Este estudio comprende las actividades antioxidantes, los componentes fenólicos y la composición de aceites esenciales de *Marrubium heterodon* (Benth.) Boiss. & Balansa, una especie endémica distribuida en Turquía. El aceite esencial se obtuvo por hidrodestilación de las partes aéreas, y se analizó por cromatografía de gases (GC) y GC/espectrometría de masas (MS). En total, se determinaron 38 componentes que representan el 86.8% del aceite. Los componentes principales del aceite esencial fueron α-pineno (17.3%), myrtenal (11.9%) y α-terpineol (5%). La composición fenólica de los extractos acuosos, metanólicos y de acetato de etilo se evaluó por cromatografía líquida (LC)-MS/MS. La actividad antioxidante de cada extracto se determinó usando la eliminación *in vitro* de radicales 2,2-difenil-2-picrilhidrazilo (DPPH), catión radical de ácido 2,2'-azino-bis-3-etilbenzotiazolina-6-sulfónico (ABTS), decoloración y ensayos de cobre (II) que reducen la capacidad antioxidante (CUPRAC). Hasta donde sabemos, este es el primer informe sobre las actividades antioxidantes, los componentes fenólicos y la composición de aceites esenciales de *M. heterodon*.

INTRODUCTION

Marrubium L. is a herbaceous genus of Lamiaceae, comprising about 40 species in the Irano-Turanian and Mediterranean phytogeographic regions [1]. The genus is represented by 20 species (24 taxa) in Turkey, of which 11 are endemic [2].

The genus *Marrubium* has a widespread traditional usage on treatment of various diseases, such as, asthma, pulmonary infections, inflammation and hypotension, as a cholagogue and a sedative agent, additionally it contains several diterpenoids (*e.g.* marrubiin) and phytochemical constituents comprise mainly diterpenes, polyphenols, steroids, phenylpropanoids and flavonoids with important antimicrobial and antioxidant properties [3]. In addition, the pharmacological activities of the genus such as cytotoxicity [4], immunomodulating [4], vasorelaxant [5,6], antispasmodic [7], hypolipidemic [8], hypoglycemic [9] and analgesic properties [10] were reported.

Previous reports on the essential oils of *Marrubium* species showed antioxidant, antimicrobial activities and cytotoxic properties [11-13]. In the related studies, γ-eudesmol, germacrene D, and β-caryophyllene were determined as major

KEY WORDS: endemic, essential oil, GC-MS, Lamiaceae, LC-MS/MS, *Marrubium heterodon*.

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ISSN 0326 2383 (printed ed.)
ISSN 2362-3853 (on line ed.)

components. The studies conducted on the Marrubium species from Turkey showed that spathulenol [14], α-pinene [15], (Z)-β-farnesene [16] and hexadecanoic acid [17,18], β-caryophyllene [19], and (Z,Z)-farnesyl acetone [20] were the main components of Marrubium essential oils.

The epidermis of *M. heteredon* had glandular hairs. Multicellular branched eglandular and glandular hairs were distributed throughout the entire stem [21].

In the present study, it was aimed to determine the major constituents such as the essential oil and phenolics of *Marrubium heterodon* (Benth.) Boiss. & Balansa, an endemic species from Turkey. In addition, to reveal the potential bioactivity, antioxidant properties of the aqueous, methanolic and ethyl acetate extracts of the plant were determined by DPPH radical scavenging assay, ABTS radical cation decolorization assay and copper (II) reducing antioxidant capacity (CUPRAC) assay.

MATERIALS AND METHODS

Plant material and isolation of the oils

Aerial parts of *Marrubium heterodon* (local name: Kınalı Kekik) were collected at flowering stage from Bolkar mountain, Niğde-Turkey. The essential oil was obtained from the air-dried aerial parts by hydrodistillation for 3 h with Clevenger-type apparatus. A voucher specimen (No: Arabacı 2976) is deposited in the herbarium of the Faculty of Pharmacy, İnönü University, Malatya, Turkey.

Essential oil analysis

Agilent Technologies 6890N Network system gas chromatograph equipped with a FID and an Innowax column (60 m x 0.25 mm i.d., 0.25 µm film thickness) were used for the GC analysis of the essential oil. Injector and detector temperatures were at 250 °C. The oven temperature was linearly raised from 60 to 250 °C at a rate of 5 °C/min, then kept constant at 250 °C for 20 min. Helium was used as the carrier gas at a flow rate of 1.7 mL/min. GC/MS analyses were carried out under the same conditions (column, oven, temperature, flow rate of the carrier gas) with GC by Agilent Technologies 6890N Network system gas chromatograph equipped with an Agilent Technologies 5973 inert Mass Selective Detector (Agilent G3180B Two-Ways Splitters with Makeup gas) in the electron impact mode (70eV). The mass range m/z was between 10 and 550. The column, temperature program and injection temperature were performed as described above. Injection was carried out at automatic mode. FLAVOR2, NIST05a, NIST08 and WILEY8 were used for library search. Relative retention indices were calculated according to series of linear alkanes C8-C23 used as a reference. For quantification purposes area percent reports obtained by FID were used. Relative retention indices (RRI) and relative percentages (%) of the essential oil components are listed in Table 1.

Preparation of the extracts

The air-dried aerial parts were grinded. The aqueous, methanolic and ethyl acetate extracts were obtained by macerating plant materials (5 g) for 24 h with 150 mL of each solvent. The maceration was repeated 3 times. The extracts were filtered and solvents were evaporated to dryness under vacuum using a rotary evaporator. The yield of aqueous, methanolic and ethyl acetate extracts were obtained as 1.26, 0.83, and 0.22 g, respectively. The extracts were stored at 4 °C.

LC-MS/MS analysis of the extracts

Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis was carried out using an Absciex 3200 Q trap MS/MS detector. Experiments were performed by using LC-MS/MS (20A HPLC system, Shimadzu) coupled to an Applied Biosystems 3200 Q-Trap instrument equipped with an ESI source operating in negative ion mode. GL Science Intersil ODS 150 × 4.6 mm, i.d., 3 µm particle size, and octadecyl silica gel analytical column operating at 40 °C have been used for the chromatographic separation. The solvent flow rate was maintained at 0.5 mL/min. Detection was carried out with PDA (photodiode array) detector. The elution gradient consisted of mobile phases (A) acetonitrile:water: formic acid (10:89:1, v/v/v) and (B) acetonitrile:water:formic acid (89:10:1, v/v/v). The composition of B was increased from 10% to 100% in 40 min. Liquid Chromatography-Electrospray Ionization-tandem Mass Spectrometry (LC-ESI-MS/MS) data were collected and processed by Analyst 1.6 software.

DPPH radical scavenging assay

DPPH radical scavenging activity was carried out by using Brand-Williams *et al.* [22] method, adopted with some modifications. 1000-31.25 µg/mL concentration serial dilutions were prepared. 150 µL of each diluted extracts were added to 50 µL freshly prepared solution of

Compound	RRI [a]	RRI [b]	Methods of Identification [c]	Composition %
α-Pinene	1012	1012	RRI, MS	17.3
Myrcene	1167	1166	RRI, MS	1.2
Limonene	1213	1217	RRI, MS	4.3
p-Cymene	1295	1287	RRI, MS	0.3
(Z)-3-Hexenol	1397	1394	RRI, MS	1.0
Nonanal	1424	1422	RRI, MS	0.5
1-Octen-3-ol	1464	1460	RRI, MS	0.6
Pentadecane	1496	1500	RRI, MS	0.6
Copaene	1527	1535	RRI, MS	3.4
Linalool	1544	1537	RRI, MS	2.4
α-Bergamotene	1593	1594	RRI, MS	0.9
Hexyl tiglate	1608	1602	RRI, MS	0.9
Verbenol	1625	1665	RRI, MS	0.4
Myrtenal	1647	1648	RRI, MS	11.9
trans-Piperitol	1676	1675	RRI, MS	0.4
Myrtenyl acetate	1701	1704	RRI, MS	1.1
α-Terpineol	1713	1710	RRI, MS	5.0
Carvone	1731	1737	RRI, MS	1.5
α-Muurolene	1741	1738	RRI, MS	0.6
β-Damascenone	1783	1790	RRI, MS	0.7
Geranylacetone	1824	1825	RRI, MS	0.3
Calamenene	1877	1859	RRI, MS	0.6
Piperitenone	1948	1949	RRI, MS	1.2
Neric acid	2294	2294	RRI, MS	0.8
Tricosane	2303	2300	RRI, MS	3.5
Farnesyl acetone	2382	2384	RRI, MS	1.8
Tetracosane	2400	2400	RRI, MS	0.6
Kaurene	2418	2425	RRI, MS	2.3
Indole	2451	2451	RRI, MS	1.2
Pentacosane	2495	2500	RRI, MS	0.6
5-Hydroxymethyl furfural	2543	2537	RRI, MS	2.0
cis-Phytol	2556	2555	RRI, MS	0.4
Benzyl benzoate	2652	2655	RRI, MS	0.5
Tetradecanoic acid	2672	2670	RRI, MS	2.8
Heptacosane	2698	2700	RRI, MS	4.8
Octacosane	2776	2800	RRI, MS	3.8
Hexadecanoic acid	2843	2890	RRI, MS	2.9
Nonacosane	2897	2900	RRI, MS	1.7
	Total identified			86.8
	Monoterpene hydrocarbons			23.1
	Oxygenated monoterpenes			23.6
	Sesquiterpene hydrocarbons			6.1
	Oxygenated sesquiterpenes			0
	Diterpene hydrocarbons			2.3
	Oxygenated diterpenes			0.4
	Others			31.3

Table 1. Essential oil composition of Marrubium heterodon. [a] Relative retention indices calculated against n-alkanes, [b] Relative retention indices from literature [27-29], [c] Methods used for identification of the compound.

DPPH in a 96-well microplate with 3 repetitions and left in a dark for 30 min. After incubation, the absorbance was calculated at 517 nm for each concentration relative to a blank absorbance. The IC_{50} was calculated from the graphical plot of the percent inhibition versus extract concentration. Butylated hydroxytoluene (BHT) was used as reference.

ABTS radical cation decolorization assay

The antioxidant capacity was determined by following Re *et al.* [23] with suitable modifications. The ABTS radical cation was produced by mixing 7 mM ABTS in H_2O and 2.45mM potassium persulfate in the dark at room temperature for 12 h. Dilutions were made to obtain concentrations of 1000-15.625 µg/mL; 150 µL of each diluted extracts were added to 50 µL ABTS solution in a 96-well microplate with 3 repetitions. After incubation 30 min in the dark, the absorbance was calculated at 734 nm for each concentration relative to a blank absorbance. The IC_{50} was calculated from the graphical plot of the percent inhibition versus extract concentration. Gallic acid was used as reference.

Copper (II) reducing antioxidant capacity assay

The copper (II) reducing activity was carried out as described by Apak *et al.* [24] with slight modifications. The copper (II) chloride solution, 7.5 mM alcoholic neocuproine solution, an ammonium acetate aqueous buffer at pH 7.00 and distilled water added in a 96-well microplate and incubated for 30 min in the dark during shaking. After incubation the absorbance was calculated at 450 nm. CUPRAC activity was expressed as Gallic acid equivalent (mg GA/g DW).

Statistical analysis

All samples were replicated three times for each antioxidant methods. Data obtained from antioxidant experiments were expressed as mean standard error (±SEM). Data were compared by one-way analysis of variance (ANOVA) with Tukey's test for the findings of significant differences.

RESULTS AND DISCUSSION

Essential oil composition

The essential oil yield from the aerial parts was relatively very low and calculated as 0.04% (v/w). Overall, 38 compounds were characterized representing 86.8% of the oil. The major components of the essential oil were determined as α-pinene (17.3%), myrtenal (11.9%), and α-terpineol (5%), respectively. *M. heterodon* was rich in oxygenated monoterpenes with the ratio 23.6 %. α-Pinene, a monoterpene hydrocarbon, was identified as one of the main components in the report on the essential oil obtained from *Marrubium vulgare* L. [15] and *M. persicum* [25]. α-Terpineol was observed in low amount in the essential oil of *M. velutinum* Sm. and *M. peregrinum* L. [26]. The relative percentages and the chemical composition of *M. heterodon* essential oil is reported in detail as shown in Table 1.

Phenolic composition of the extracts

A total of 18 compounds were determined in methanol extract most of which were flavonoid and phenyl propanoid derivative. Previous reports on the phenolic compounds were identified in the methanol extract were reported in several *Marrubium* species [30-34] except for *M. heteredon*. Phenolic profile of the ethyl acetate extract was not entirely identified, anisofolin is the only compound determined in the ethyl acetate extract, which was previously identified in *M. vulgare* [34].

Kaempferol-3-O-glucoside and rutin were determined in the aqueous extract. It was not possible to determine other constituent of the aqueous extract. The LC chromatograms of the methanolic, aqueous, and ethyl acetate extracts were given in Figs. 1-3, respectively and the identified compounds was given in Table 2. The peaks of the determined compounds were marked in the chromatograms.

Antioxidant activity

Antioxidant properties of the plants could be correlated with the radical scavenging and reducing power capacity assays [35,36]. In this study, aqueous, methanol and ethyl acetate extracts of *M. heterodon* were used to determine DPPH scavenging, ABTS radical cation decolorization and copper (II) reducing power properties. Radical scavenging activities of extracts were expressed as percentage of DPPH and ABTS radicals inhibition and IC_{50} values (µg/mL). The results are given in Table 3. Reducing copper (II) ion power activity was expressed as gallic acid equivalent and the results are presented in Table 4.

In the DPPH assay, the highest inhibitory activity was measured as 186.23 µg/mL (IC_{50}) in

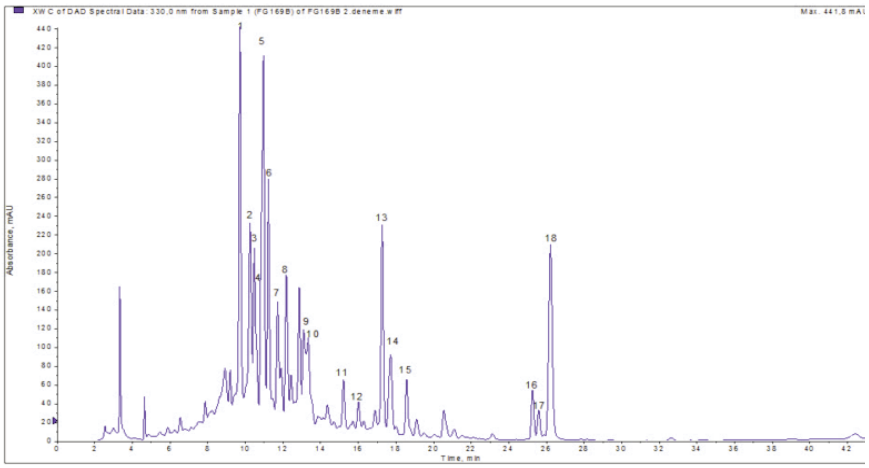

Figure 1. LC chromatogram of the methanol extract of *M. heteredon* (at delta 330 nm).

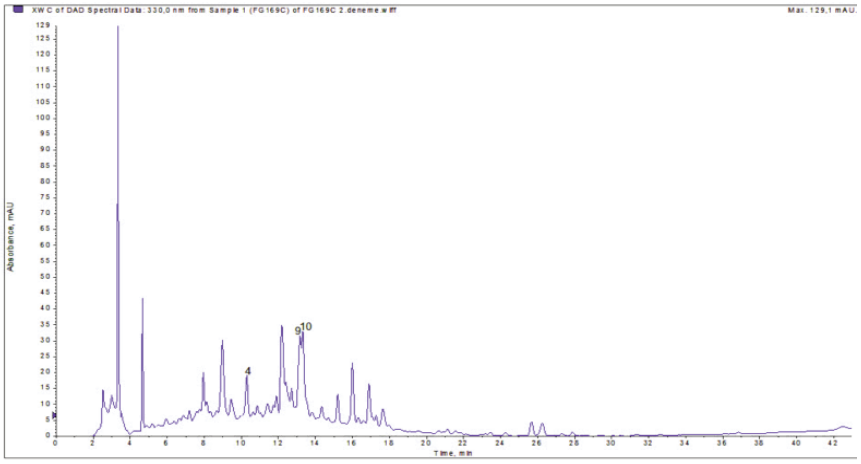

Figure 2. LC Chromatogram of the aqueous extract of *M. heteredon* (at delta 330 nm).

No	Rt	M-H	MS2	Identified as	Ext	Ref
1	9.6	755	623, 593,461, 447, 179, 161	Forsythoside B	M	30
2	10.3	179	135	Caffeic acid	M	30
3	10.5	755	593, 461, 161	Samioside	M	31
4	10.8	609	301, 271	Rutin	M, A	32
5	10.9	623	461, 315, 305, 179, 161	Verbascoside	M	30
6	11.3	769	593, 575, 461, 193, 175, 160	Alyssonoside	M	30
7	11.7	463	301, 271, 255	Quercetin glucoside	M	32
8	12.1	593	357, 285, 255, 241	Kaempferol rutinoside	M	32
9	13.2	447	327, 284, 255, 227	Kaempferol-3-O glucoside	M,A	33
10	13.4	477	357, 314/315, 299/300, 285, 271	Unknown	M,A	
11	15.2	651	505, 475, 193, 175, 160	Martynoside	M	30
12	16.3	193	161, 134	Smilar to dihydroxycinnamic acid	M	
13	17.3	593	447, 285	Kaempferol rutinoside	M	32
14	17.6	623	477, 315, 307, 299, 287, 271	Isorhamnetin rutinoside	M	32
15	18.5	577	431,413, 269, 145, 117	Apigenin coumaroylglucoside	M	32
16	24.7	446	402, 358, 210, 166, 150	Unknown	E,M	
17	25.3	723	577, 559, 453, 289, 269	Anisofolin	E,M	34
18	26.2	487	Poor ionised (301, 175)	Unknown like quercetin derivative	E,M	

Table 2. Phenolic constituents of *Marrubium heterodon* extracts determined by LC-MS/MS E: Ethyl acetate, M: Methanol, A: Aqueous.

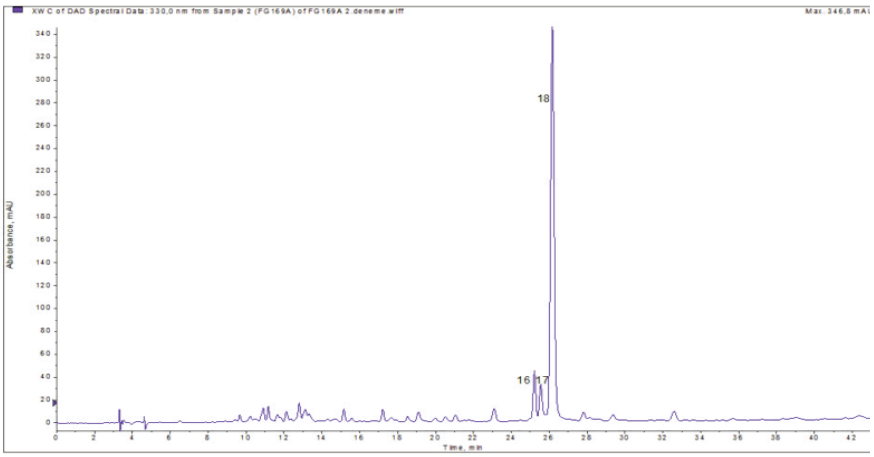

Figure 3. LC Chromatogram of the ethyl acetate extract of *M. beteredon* (at delta 330 nm).

Extract	DPPH (IC_{50} µg/mL)	ABTS (IC50 µg/mL)
Aqueous	NA	326.84±2.82
Methanolic	186.23±1.33	153.28±2.47
Ethyl acetate	281.28±3.68	204.86±2.72
BHT*	24.92±0.94	-
Gallic acid*	-	9.45±0.39

Table 3. DPPH radical scavenging and ABTS radical cation decolorization capacities of the extracts *: references. Results are mean ± standard deviation of three independent analyses, NA: no activity. ($p < 0.05$).

Extract	CUPRAC (mg GA/g DW)
Aqueous	19.36±2.74
Methanolic	50.53±3.17
Ethyl acetate	30.12±1.68

Table 4. Copper (II) reducing antioxidant capacities of the extracts. Results are mean ± standard deviation of three independent analyses. GA: gallic acid, DW: dry weight ($p < 0.05$).

methanolic extract and 281.28 µg/mL (IC_{50}) in ethyl acetate extract. The aqueous extract was not active in the DPPH assay. BHT was used as reference in the assay and the IC_{50} value was determined as 24.92 µg/ µg/mL. In the ABTS radical cation decolorization assay, the inhibitory activities of the methanolic, ethyl acetate and aqueous extracts were measured as 153.28, 204.86 and 326.84 µg/mL (IC_{50}), respectively. Gallic acid was used as reference and the IC_{50} value was determined as 9.45 µg/mL. In the CUPRAC assay, the strongest reducing activity was observed from the methanolic extract with 50.53 mg GA/g DW. This was followed by the ethyl acetate and aqueous extract at 30.12 and 19.36 mg GA/g DW, respectively. The highest antioxidant activities were observed in methanolic extract at all the assays.

Radical scavenging capacity and reducing oxidant power assays is a feasible method to determine antioxidant properties of plant extracts. Our results revealed that, the methanolic extract of *M. heterodon* exhibited a remarkably high antioxidant activity according to the ABTS, DPPH and CUPRAC assays. This activity may be due to flavonoids and phenylpropanoids detected in methanol extract. In addition, some of the compounds detected in LC-MS/MS such as forsythoside B [37], caffeic acid [38], samioside [39], rutin [40], verbascoside [37], and alyssonoside [41] were reported to exhibit significant antioxidant activity. The structure-antioxidant activity relationships of the flavonoids and phenylpropanoids are clearly identified [42].

CONCLUSIONS

Antioxidant results revealed that methanol extract provides the highest activity. With the LC-MS/MS analysis, the rich phenolic content of the methanol extract compared to the aqueous and ethyl acetate extracts was also illuminated. It was determined that the compounds detected in the extracts were also identified before in other *Marrubium* species. For the first time, myrtenal and α-terpineol were characterized as main compounds of an essential oil of a *Marrubium* species. Monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, diterpene hydrocarbons and oxygenated diterpenes comprised the volatile profile of the essential oil. To the best of our knowledge, this is the first report on the antioxidant activities, phenolic and essential oil composition of *M. heterodon.*

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