JOTCS
Journal of the Turkish Chemical Society
Section: A
Chemistry
TURKISH CHEMICAL SOCIETY

Phytochemical Profiling and Evaluation of *Marrubium sivasense* Aytaç, Akgül & Ekici for Antioxidant Activity and Inhibition Effects on α-Amylase, Lipoxygenase, Xanthine Oxidase and Tyrosinase Enzymes

Fatih Göger[1,2,*], Gülmira Özek[1], Mehmet Tekin[4], Süleyman Yur[1,2], Temel Özek[1,2]

[1]Anadolu University, Faculty of Pharmacy, Department of Pharmacognosy, 26470, Eskişehir, Turkey.

2Anadolu University, Medicinal Plant, Drug and Scientific Research Center (AUBIBAM), 26470, Eskisehir, Turkey.

3Anadolu University, Yunus Emre Vocational School, Department of Pharmacy, Program in Pharmacy Services, 26470, Eskişehir Turkey.

4Trakya University, Faculty of Pharmacy, Department of Pharmaceutical Botany, 58140 Edirne, Turkey.

Abstract: In the present study, the extract of an endemic plant ourkey Marrubium sivasense Aytaç, Akgül & Ekici was investigated for phytochemical profile, antioxidant properties and inhibition of several enzymes. The chemical composition of the extract was investigated with LC-MS/MS technique. Forsythoside B, verbascoside and leucoseptoside were identified as the main compounds. The free radical scavenging activity of the extract was determined against DPPH• radicals (IC50 0.34 mg/mL) and ABTS•+ cation radicals (TEAC 2.3 mM). In the xanthine-xanthine oxidase (XO) system and in β-carotene bleaching assay the extract demonstrated moderate activity (Inh% 16.54 and Inh% 43±1.26, respectively). The extract demonstrated hypoglycemic activity (Inh% 49.67±1.09) via inhibition of porcine pancreatic α-amylase. The antiinflammatory effect tested via inhibition of 5-LOX was found as Inh% 18.71±0.74. However, no antityrosinase activity was found.

Keywords: *Marrubium sivasense*, extract, LC-MS/MS; antioxidant, xanthine oxidase, α-amylase, tyrosinase.

Submitted: January 23, 2019. Accepted: May 29, 2019.

Cite this: Göger F, Özek G, Tekin M, Yur S, Özek T. Phytochemical Profiling and Evaluation of Marrubium sivasense Aytaç, Akgül & Ekici for Antioxidant Activity and Inhibition Effects on α-Amylase, Lipoxygenase, Xanthine Oxidase and Tyrosinase Enzymes. JOTCSA. 2009;6(3):272–82.

DOI: https://doi.org/10.18596/jotcsa.516888.

*Corresponding author. E-mail: fatihgoger@gmail.com, Tel: +90-2223350580 Extn.:3663-3707, Fax: +90-2223306809

INTRODUCTION

The genus Marrubium L. (Lamiaceae) consists of 40 species indigenous in Europe, the Mediterranean and Asia. In the Flora of Turkey, the genus is represented by 21 taxa, of which 12 are endemic (1-3). The endemism rate (57%) shows that Turkey is an important center of diversity for the genus (4, 5). Several aspects on chemical and pharmacological potent of the genus Marrubium were reported by Meyre-Silva et al (6). A previous phytochemical studies on Marrubium species resulted with polyphenols (7), essential oils (8), labdane diterpenoids and sterols (9, 10). Biological activity investigations of Marrubium species encompasses antibacterial (11), antioxidant, antiproliferative (12), antinociceptive (13), antidiabetic (14), antihypertensive (15), hepatoprotective (16), antiinflammatory (17), cardioprotective (18) and cytotoxic/cytostatic (19) potentials. Some Marrubium species are traditionally used to treat various diseases, including asthma, pulmonary infections, inflammation and hypotension, as cholagogues and sedative agents, and for pain relief. M. vulgare (horehound) is cultivated in Lithuania (20) and serves as raw material and source for food flavoring and for medicinal purposes (herbal extracts) and beverage industries (21). The plant has also been used as a substitute for hop in beer-breweries and as an ingredient of pastilles, as a flavoring in stick candy, and candy drops (22, 23). In Turkey, Marrubium species are known as "Agor çalbasi" (in Sivas province), "şalba" (in Antalya province) and "kayişkiran otu" (in Kirklareli province) (24).

Marrubium sivasense Aytaç, Akgül & Ekici is the local endemic species in Sivas province of Turkey (1). The local name of this plant is "kukasotu". Taking into consideration the previous literature data on the chemistry and biological activities of Marrubium species as well as lacking information about M. sivasense, we aimed to investigate chemical composition and biological potential of this species. Therefore, the extract of M. sivasense was screened for antioxidant activity using different in vitro methods like 2,2-diphenyl-1-picrylhydrazyl (DPPH˙) radical scavenging test, Trolox equivalent antioxidant capacity test and β-carotene bleaching test. The antioxidant effect of extract on oxidative damage was also evaluated with enzymatic method using XO system that generated superoxide anion radical (O2-). The test samples which interact with XO can affect the kinetics of xanthine oxidation to uric acid which causes hyperuricacidemia associated with gout (25). XO has a role in the generation of reactive oxygen species in various pathologies such as viral infection, inflammation, brain tumors or the process of ischemia/reperfusion. Thus, inhibitors of XO are expected to be therapeutically useful for the treatment or prophylaxis of these diseases. Earlier, it has been supposed that the essential oil of M. peregrinum L. may contain compounds that would be more effective in the treatment of gout from the widely used allopurinol because of they can inhibit XO as well as neutralize O2-• (26). In literature there is no any information about effect of Marrubium sivasense on XO enzyme. Therefore, it was aimed to investigate inhibitory effect of M. sivasense extract on XO enzyme.

In the literature there is information about antiinflammatory properties of M. vulgare and M. allyson (27, 28). The phenylpropanoid glycosides from M. vulgare have been reported as the strong inhibitors of enzymes associated with inflammation. Therefore, it was interesting to investigate the extract of M. sivasense against 5-lipoxygenase (5-LOX) enzyme, which linked to inflammation process.

Recently, the perspectives of M. vulgare as antidiabetic natural product have been discussed in several papers (14, 29, 30). The investigation of M. sivasense inhibitory activity against pancreatic α-amylase, which is known as key enzyme in digestion of dietary carbohydrate in organism, may give information on antihyperglycemic potential of the plant. The inhibitors of □-amylase enzyme may be effective in retarding carbohydrate digestion and glucose absorption to suppress postprandial hyperglycemia.

A literature search revealed information about tyrosinase inhibition potent of flavonoids and phenylethanoid glycosides isolated from M. velutinum and M. cylleneum. (7). In the scope of the present work, we attempted to investigate in vitro potential of M. sivasense against tyrosinase enzyme which is the key

enzyme in production of melanin (31). The central role of tyrosinase in dopamine neurotoxicity as well as contribution to the neurodegenerative Parkinson's disease has earlier been well documented (32). The inhibitors of tyrosinase found application in cosmetic products for whitening and depigmentation after sunburn as well as for the treatment of hyperpigmentation. Nowadays, there is increasing demand for naturally derived inhibitors of tyrosinase due to diverse side effects of synthetic products. The tyrosinase inhibitory activity of M. velutinum and M. cylleneum was reported to be due to flavonoid and phenylethanoid glycosides (7). It was supposed, that flavonoids and cinnamic acid derivatives may contribute to antityrosinase activity of M. vulgare extract (33). The above mentioned reports encouraged us to investigate M. sivasense for antityrosinase activity. The present research work is the first contribution into the chemistry and biological activities of M. sivasense, endemic species from Turkey.

MATERIALS AND METHODS

Chemicals and Enzymes

3,4-Dihydroxyl-L-phenilalanine, β-carotene, linoleic acid, Tween-20, butylated hydroxytoluene (BHT), gallic acid, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH˙), amonium acetate, kojic acid, acarbose, 3,4-dihydroxy-L-phenylalanine (L-DOPA), nordihydroguaiaretic acid (NDGA), allopurinol, α-amylase from porcine pancreas (Type VI-B, EC 3.2.1.1), tyrosinase from mushroom (EC 1.14.18.1), xanthine oxidase from bovine milk (Grade IV) and lipoxidase from Glycine max (Type I-B) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Soluble starch extra pure, iodine and potassium iodide were purchased from Merck (Darmstadt, Germany). Sodium phosphate, disodium phosphate, aluminum chloride, water and methanol were extra pure analytical grade. A C9–C40 n-alkane standard solution was purchased from Fluka (Buchs, Switzerland). All solvents were purchased from Sigma Aldrich (Germany) and were of analytical grade.

Equipment

Shimadzu 20A HPLC system equipped with Applied Biosystem 3200 Q trap MS/MS detector was used for chromatographic analysis of the extract. Microtiter plate assay was performed with Biotek Powerwave XS microplate reader. Ultrapure water was obtained from a Direct-Q Water Purification System (Germany). Spectrophotometric measurements were carried out with a UV-Pharma Spec 1700 (Shimadzu) spectrophotometer.

Plant Material

The aerial parts of M. sivasense were collected in Sivas province, vicinity of Mancilik village of Kangal district, on June, 2015 and dried under the shade. Botanical identification was performed by Dr. Mehmet Tekin (Trakya University, Faculty of Pharmacy, Department of Pharmaceutical Botany). The voucher specimen is stored in the Herbarium of Cumhuriyet University, Faculty of Science (CUFH) under herbarium code Tekin 1686.

Preparation of the Extract

Aerial parts of M. sivasense (10.0 g) were powdered and subjected to maceration in methanol (200 mL) by shaking (3000 per min) at room temperature for 24 h. The obtained liquid extract was filtered and dried under vacuum. The dried methanol extract was kept at 4°C until phytochemical analysis and biological activity tests.

Liquid-Chromatography - Mass Spectrometry (MS/MS)

LC-MS/MS analysis was carried out using an Absciex 3200 Q trap MS/MS detector. The experiments were performed with a Shimadzu 20A HPLC system coupled to an Applied Biosystems 3200 Q-Trap LC-MS/MS instrument equipped with an ESI source operating in negative ion mode. For the chromatographic separation, a GL Science Intersil ODS 250 × 4.6 mm, i.d., 5 µm particle size, analytical column operating at 40ºC was used. The solvent flow rate was maintained at 0.5 mL/min. The detection was carried out with PDA and MS detectors. 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. LC-ESI-MS/MS data were collected and processed by Analyst 1.6 software.

Identification of Compounds

Identification of phenolic constituents was based on matching of mass-spectral patterns obtained with LC-MS/MS system.

Free Radical Scavenging Activity (DPPH˙ assay)

The hydrogen atoms or electrons donation ability of M. sivasense extract was evaluated according to bleaching of purple colored DPPH° stable radicals by using of the method of Brand-Williams (34) with slight modifications. The solution of DPPH° (0.08 mg/mL, in methanol) was freshly prepared daily, kept in the dark at 4 oC between the measurements. The solutions of the extract (10 mg/mL) and gallic acid (0.1 mg/mL) were prepared in methanol. In the experiment, 100 µL of the sample (extract/ standard) solution and 100 µL of DPPH solution were pipetted by multichannel automatic pipette (Eppendorf Research® plus, Germany) into 96-flat bottom well plate cells and allowed to stand in the dark for 30 min. The control well contained 100 µL methanol (instead of the sample) mixed with 100 µL of DPPH. The decrease in the absorbance was recorded at 517 nm. Gallic acid (standard) was used as the positive control. The experiments were performed in triplicate. The free radical scavenging activity of the samples was expressed as percentage of inhibition calculated according to Equation 1:

$$Inh\% = \left(\frac{Abs_{control} - Abs_{sample}}{Abs_{control}}\right) \times 100 \quad \text{(Eq. 1)}$$

where, Abscontrol is the absorbance of the control (containing all reagents except the test compound), Abssample is the absorbance of the sample with added DPPH reagent. The IC50 values were obtained by plotting the DPPH˙ scavenging percentage of each sample against the sample concentration. Data were analyzed using the SigmaPlot software (Version 12.0).

Trolox Equivalent Antioxidant Capacity (TEAC Assay)

The free radical scavenging activity of the extract was tested against 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+) cation radicals according to the procedure described by Re et al. (35) with slight modifications. 7 mM ABTS and 2.5 mM K2S2O8 dissolved in 10 mL ultrapure water were allowed to stand in the dark for 16 h at room temperature to create ABTS•+ cation radicals. Prior to the assay, ABTS•+ solution was diluted with absolute ethanol to get an absorbance between 0.7-0.8 at 734 nm. The solutions of the extract (5 mg/mL) and Trolox (standard, 3.0; 2.0; 1.0; 0.5; 0.25; 0.125 mM) were prepared in methanol. In the experiment, the sample solution (10 uL) was mixed with 990 µL ABTS•+ solution. Ten microliter of methanol instead of the sample mixed with ABTS•+ solution was used as the control. Gallic acid solution (0.1 mg/mL) was used as the positive control. Decrease in the absorbance after 30 minutes of incubation was recorded at 734 nm to get linear Trolox equation. ABTS•+ scavenging activity of the sample was expressed as Trolox equivalent antioxidant capacity and calculated using linear equation obtained for Trolox ($y=33.644\times x+ 2.6523$, $r^2=0.9942$).

β-Carotene/Linoleic Acid Peroxidation Inhibition Assay

β-Carotene/linoleic acid peroxidation inhibition assay used linoleic acid as the model lipid substrate in an emulsified form (with Tween-20) lies in between methods employing only model substrates (e.g. DPPH˙) and those using real lipids (36). Inhibition of lipid peroxidation by M. sivasense extract was measured according to method of Marco (37) with slight modifications. Briefly, β-carotene (5 mg) dissolved in chloroform (5 mL) was added to flask containing linoleic acid (120 mg) and Tween-20 (1200 mg). The content of flask was vigorously shaken and chloroform was evaporated under the vacuum. After evaporation, pure water (300 mL) was added and shaken vigorously. BHT (1 mg/mL) was used as the standard inhibitor. The sample (extract/ standard) solution (10 uL) and β-carotene emulsion (2 mL) were mixed in the deep well plate. After that, 300 µL of the mixture was placed by multichannel automatic pipette (Eppendorf, Germany) into a 96-well microplate cells and incubated at 50°C for 2 h.

Control was prepared without sample or standards according to the same procedure. The rate of β-carotene bleaching was monitored by measuring the absorbance at 15 min periods at 470 nm in an ELISA microplate reader (Biotek Powerwave XS). Analyses were run in triplicate and the results were expressed as average of inhibition percentage values with SEM calculated according to Equation 2:

$$AA\% = \left[1 - \frac{(Abs0_{sample} - Abs120_{sample})}{Abs0_{control} - Abs120_{control}}\right] \times 100 \quad \text{Equation (2)}$$

where, AA is an antioxidant activity, Abs0sample and Abs120sample are the absorbance values of the sample at 0 min and 120 min, Abs0control and Abs120control are the absorbance values of the control at 0 min and 120 min.

Xanthine Oxidase (XO) Inhibition Assay

The XO inhibition assay was carried out according to procedure reported by Chen (38) with slight modifications. Namely, the stock solution of the extract (5 mg/mL) prepared in methanol was diluted with phosphate buffer (0.1 M, pH 7.5) up to 1 mg/mL. In the experiment, the sample solution (100 µL), buffer (1.75 mL) and 40 µL of XO (0.33 U/mL in sodium phosphate buffer) were pipetted in quartz cuvette and pre-incubated for 10 min at 25 °C. The reaction was initiated by addition of the substrate solution (100 µL 0.5 mM xanthine in buffer). Then, the mixture was subjected to the second incubation for 12 min at 25 °C. The sample blanks contained all reaction reagents and 40 µL buffer instead of enzyme. The control cuvette contained all the reagents without the sample (plus equivalent amount of the sample solvent was added). Control incubations, representing 100% enzyme activity were conducted in the same manner replacing the plant extract with the solvent used for the solution of the extract. The standard inhibitor of XO, allopurinol (1 mM in DMSO) was used as the positive control. Absorbance at 295 nm was recorded with a spectrophotometer. The percentage inhibition was calculated according to Equation 3:

$$Inh\% = \left[\frac{(Abs_{control} - Abs_{control\ blank}) - (Abs_{sample} - Abs_{sample\ blank})}{Abs_{control} - Abs_{control\ blank}}\right] \times 100 \quad \text{(Eq. 3)}$$

where Abscontrol and Abscontrol blank are the absorbance values of the control and its blank, Abssample and Abssample blank are the absorbance values of the sample and its blank.

Tyrosinase Inhibition Assay

An inhibitory activity of M. sivasense extract on tyrosinase was assessed using the modified microplate method reported by Masuda (39). The solution of the extract (1 mg/mL) was prepared in phosphate buffer (pH 6.8) with adding DMSO. The experiment was carried out as follow: eight wells were used, A (three wells, control), B (one well, blank), C (three wells, sample), and D (one well, blank), which contained the following reaction mixtures: A, 120 µL of phosphate buffer (0.1 M, pH 6.8) and 40 µL of tyrosinase (33.3 U/mL) in the buffer; B, 160 µL of the buffer; C, 80 µL of the buffer, 40 µL of tyrosinase (33.3 U/mL) in the buffer, 40 µL of the sample-buffer solution containing DMSO; D, 120 µL of the buffer and 40 µL of the sample solution containing DMSO. Pipetting was performed with multichannel automatic pipette (Eppendorf Research® plus, Germany). Kojic acid (0.01-0.1 mg/mL in buffer) was used as the positive control. The contents of each well were mixed and then preincubated at 23°C for 10 min, before 40 µl L-DOPA (2.5 mM) in the buffer was added. After incubation at 23°C for 15 min, the absorbance at 475 nm was measured using an ELISA microplate reader (Biotek Powerwave XS). The percentage inhibition of the tyrosinase activity (Inh %) was calculated according to Equation 4:

$$Inh\% = \frac{(Abs_A - Abs_B) - (Abs_C - Abs_D)}{(Abs_A - Abs_B)} \times 100 \quad \text{(Eq. 4)}$$

α-Amylase Inhibition Assay

The inhibitory potential of the extract on activity of α-amylase was measured using Caraway-Somogyi iodine/potassium iodide (I/KI) method (40) with slight modifications. The substrate solution (0.05 %) was prepared by dissolving soluble potato starch (10 mg) in 20 mL ultrapure water then boiling for 10 min and cooling to room temperature before use. As a positive control experiment, acarbose (0.01-0.1 mg/mL in buffer) was used. The

experiment was carried out as follows: 20 mM sodium phosphate buffer (pH 6.9) was pipetted in the 96-well flat bottom plates with multichannel automatic pipette (Eppendorf, Germany), then 25 µL sample solution and 50 µL α-amylase (0.8 U/mL in buffer) were added and incubated for 10 min at 37ºC. After incubation, 50 µL substrate solution was added to the mixture. The mixture was subjected to the second incubation for 10 min at 37ºC. The reaction was stopped by addition of 25 µL HCl (1 M). Finally, 100 µL of I/KI reagent was added to the wells. The sample blanks contained all reaction reagents and 50 µL buffer instead of enzyme. The control wells contained all reaction reagents and 25 µL solvent (instead of the sample solution). The control incubations, representing 100% enzyme activity were conducted in the same manner replacing the plant extract with the solvent used for solution of the extract. The absorbance values were recorded for the sample and blank at 630 nm. The percentage inhibition of the α-amylase activity (Inh %) was calculated according to Equation 3.

Lipoxygenase Inhibition Assay

Inhibition of 5-LOX activity was measured with spectrophotometric method described by Albano et al. (41). The solutions of the extract (1 mg/mL) and NDGA (0.1 mg/mL) were prepared in methanol. The substrate solution (4 mM) was prepared as follows: 50 mg linoleic acid, 50 mg Tween-20, 500 µL NaOH (1 M) were mixed and the total volume (40 mL) was adjusted with 0.1 M phosphate buffer (pH=8). The enzyme solution was prepared in phosphate buffer (273000 U/mL). In the experiment, 50 µL of the sample solution (extract/standard), 2.5 mL phosphate buffer and 50 µL of 5-LOX were mixed in quartz cuvette and incubated for 10 min at 25 ºC. Then, the reaction was initiated by adding linoleic acid (25 µL). Increase in the absorbance at 234 nm was recorded for 6 min. The percentage inhibition was calculated according to equation 3.

Statistical Analysis of Data

Data obtained from antioxidant and enzyme inhibition experiments were expressed as mean standard error (±SEM). IC50 values were estimated using a nonlinear regression algorithm.

RESULTS AND DISCUSSION

In the literature, there are a number of papers promising about phytochemical properties and biological activities of diverse Marrubium species (7,11-19). However, there is no information about phytochemistry and biological potent of endemic species M. sivasense. The main objective of the present work was to evaluate chemical composition and confirm the biological potent of non-volatile metabolites of M. sivasense. The extract was subjected to evaluations for free radical scavenging, lipid peroxidation inhibition and Trolox equivalent antioxidant capacity. Another goal was to determine inhibitory potential of the extract on enzymes involved in oxidation metabolism (XO), digestion of carbohydrates (α-amylase), process of inflammation (5-LOX), and linked to melanin formation and Parkinson's disease (tyrosinase).

Composition of Extract

In scope of the research, M. sivasense methanol extract phenolics were determined with LC-MS/MS technique. Phenylethanoid glycosides: forsythoside B, verbascoside, alyssonoside, martynoside were identified as the main compounds. Chromatographic profile of the extract is presented on Figure 1.

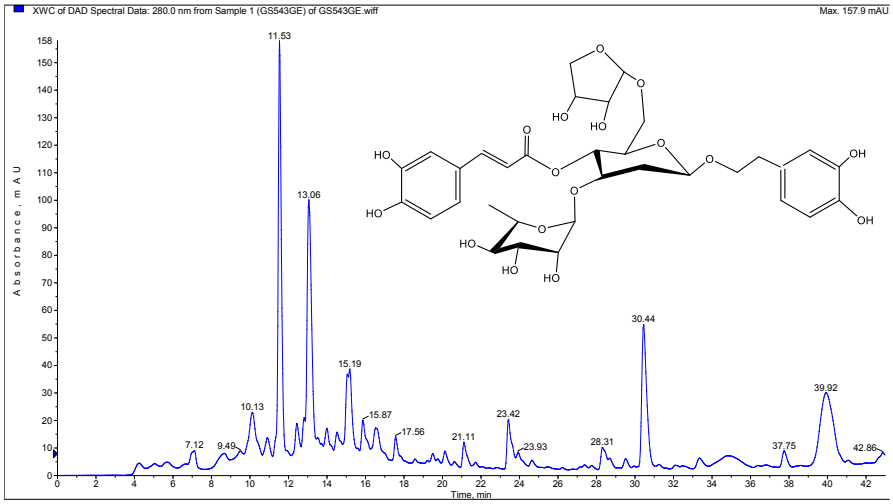

Figure 1: Liquid-chromatographic profile of the methanol extract of *Marrubium sivasense*.

The list of the constituents detected with MS detector is summarized in Table 1. The composition of the extract was constituted by phenylethanoid glucosides as well as cumaroyl glycosides of apigenin. Forsythoside B, verbascoside and leucoseptoside were found as the main phenolic constituents in M. sivasense. Several of these constituents have earlier been reported for M. alysson, M. anisodon and M. cylleneum (42-44).

Table 1: Characterization of chromatographic peaks detected in the methanolic extract of *Marrubium sivasense.*

Rt	$[M-H]^-$ *m/z*	Fragments	Compound	Reference
11.5	755	593,461, 315, 297, 179,	Forsythoside B	(45)
13.0	623	461, 315, 179	Verbascoside	(43, 46)
13.2	769	593, 575	Alyssonoside	(43, 46)
14.0	463	300, 271	Quercetin glucoside	(7)
15.2	637	461, 315, 297, 175	Leucoseptoside A	(43,45, 46)
15.9	431	311, 269	Apigenin glucoside	(45, 47)
16.3	461	445, 297, 283, 255	Chrysoeriol glucoside	(42)
17.6	651	193, 175, 160	Martynoside	(7)
20.1	377	249, 205	Unknown	
21.1	577	431, 311, 269	Apigenin coumaroylglucoside	(7, 44, 48)
23.4	577	431, 413, 269, 145,	Apigenin coumaroylglucoside	(7, 44, 48)
28.3	723	576, 269	Similar to Apigenin di-coumaroylglucoside	(44,48)
30.5	267	223	Unknown	

Antioxidant Activity

Antioxidant activity assessments of M. sivasense extract were performed in vitro by using non-enzymatic and enzymatic systems employing different model substrates: stable free radical DPPH• and cation radical ABTS+• as well as real substrates: linoleic acid peroxides and superoxide anion radicals (O2-•). Such approach allowed obtaining rather realistic results about the antioxidant potent of the sample. The extract demonstrated antioxidant activity in all antioxidant activity assays. Namely, the extract scavenged DPPH˙ free radicals with IC50 0.34 mg/mL. It was more effective than BHT (IC50 0.50 mg/mL.), but less effective than ascorbic acid (IC50=0.03 mg/mL.) and gallic acid (IC50 0.003 mg/mL.). The extract prevented bleaching of β-carotene by inhibition of linoleic

acid peroxidation with Inh% 43. In the TEAC assay, the extract demonstrated noteworthy ABTS●+ bleaching activity equivalent to 2.3 mM of Trolox. Such significant activity may be observed due to phenolic compounds detected in the extract as reported in Table 2. In the xanthine/xanthine oxidase system, the extract demonstrated moderate inhibitory effect (16.54 %). As can be seen from the antioxidant activity determination results (Table 2), the extract of M. sivasense may be considered as potential source of antioxidant agents in prevention of oxidative damage. LC-MS/MS analysis revealed that extract of M. sivasense contained phenylethanoid glucosides: forsythoside, verbascoside and leucoseptoside. It has earlier been reported that these compounds displayed notable antioxidant capacity (49). In the literature, there is increasing evidence that polyphenols have multiple beneficial effects on preventing of oxidative damage. The phenol content is well known to correlate with higher antioxidant activity in plant extracts (50). The mechanism by which polyphenols exert their effects is not fully clarified, but a consensus was reached that polyphenols protect membranes, proteins, and DNA against damage by scavenging free radicals generated through oxidative metabolism.

Enzyme Inhibition Assays

Inhibitory Effect of the Extract on Mushroom Tyrosinase Activity

In scope of the research work, the inhibitory effect of M. sivasense extract on mushroom tyrosinase activity was evaluated. The extract was found to be inactive when tested at a concentration of 1 mg/mL (Table 2).

Inhibitory Effect of the Extract on Porcine Pancreatic α-Amylase Activity

M. sivasense extract was evaluated in vitro for hypoglycemic activity via inhibition of the porcine pancreatic α-amylase. As can be seen in Table 2, the extract demonstrated inhibitory activity (Inh% 49.67). It seems to be that M. sivasense can be considered as perspective potential source of natural phytochemicals with hypoglycemic effect. Earlier, antidiabetic phytochemicals from different Marrubium species have been proved to be effective agents. The hypoglycemic effect of M. vulgare extract was clinically documented in patients with type II non-controlled diabetes mellitus (51). It was reported, that M. radiatum extract exerted the strongest activity against α-amylase and α-glucosidase (52). The evidenced-based therapeutic usage of many plants is scarce. Nowadays, there is demand for new effective and safe natural products with hypoglycemic property. The efficacy of known synthetic hypoglycemic products is debatable. So, the plants reputed for their antidiabetic effect should be verified either experimentally or clinically.

Inhibitory Effect of the Extract on Lipoxygenase Activity

Antiinflammatory effect of M. sivasense was tested with 5-LOX enzyme inhibition system. Actually, 5-LOX catalyzes the oxidation of arachidonic acid, produces 5(S)-hydroxyperoxyeicosatetraenoic acid (5-HETE) which undergoes dehydration, resulting in the formation of leukotriene. Enzymatic hydrolysis of leukotriene, as well as conjugation with other substances, leads to the formation of inflammatory mediators.

In the present study, the extract of M. sivasense was found to be as moderate inhibitor of 5-LOX with Inh% 18.71 when tested at 5 mg/mL concentration (Table 2).

Table 2. The biological activity of *M. sivasense* extract*.

	DPPH° - IC_{50} (mg/mL)	β-Carotene[a], Inh%	TEAC[a], mM	XOD[b], Inh%	α-AML[c], Inh%	LOX[a], Inh%	TYR[b], Inh%
Extract	0.34	43.04	2.3	16.54	49.67	18.71	N/A
Gallic acid	0.003	-	-	-	-	-	-
Ascorbic acid	0.03	-	-	-	-	-	-
BHT[b]	0.50	85.0	-	-	-	-	-
Acarbose[d]	-	-	-	-	98.0	-	-
NDGA[e]	-	-	-	-	-	86.38	-
Allopurinol[f]	-	-	-	81.02	-	-	-
Kojic acid[d]	-	-	-	-	-	-	84.0

*Activity values are presented as results of triplicate experiments n=3, p<0.01; BHT: butylated hydroxytoluene; N/A: not active; α-AML: α-amylase; LOX: lipoxygenase; TYR: tyrosinase; NDGA: nordihydroguaiaretic acid; §the deviation from the mean is <0.01 of the mean value; Concentrations of the tested sample: [a)]5 mg/mL;[b)]1 mg/mL; [c)]0.5 mg/mL; [d)]0.3 mg/mL; [e)]0.1 mg/mL; [f)]1.0 mM.

CONCLUSION

The liquid chromatographic analysis of Marrubium sivasense methanol extract resulted with identification of phenolic constituents, namely forsythoside B, leucoseptoside A, verbascoside, alyssonoside, martynoside, as well as glucosides of quercetin, apigenin and chrysoeriol, and coumaroylglucoside of apigenin. Evaluation of the extract in different model systems revealed that M. sivasense methanol extract is good inhibitor of α-amylase enzyme. However, the extract did not demonstrate noteworthy effect on 5-LOX, XOD and tyrosinase enzymes. The data gathered from the current study support that M. sivasense is a good source of flavonoid constituents and other bioactive compounds that might be responsible for the observed antioxidant and some enzyme inhibitory activities. These interesting results encourage further investigation geared towards the isolation of potent phytochemicals of different polarity from M. sivasense.

ACKNOWLEDGEMENTS

Authors are grateful to Anadolu University Scientific Research Department for supporting this research project (Grant Number 1705S171).

REFERENCES

1. Aytaç Z, Akgul G, Ekici M. A New Species of Marrubium (Lamiaceae) from Central Anatolia, Turkey. Turk J Bot. 2012;36:443-9.

2. Akgül G, Ketenoglu O. The new subspecies for the Flora of Turkey: Marrubium cephalanthum Boiss. & Noë subsp. akdağhicum (Lamiaceae). In: Ürgüp, Turkey: IXth National Conference on Ecology; 7-10 October;, Nevşehir: Nevşehir University Faculty of Science; 2009.

3. Tuylu M, Büyükkartal HN, Akgül G, Kalyoncu H. Marrubium lutescens Boiss. ve M. cephalanthum Boiss. & Noë subsp. akdaghicum (Lamiaceae)'un gövde ve yaprak özelliklerinin anatomik olarak karşılaştırılması. Süleyman Demirel Univ. J Nat Appl Sci. 2017;21(1):113-7.

4. Firat M. Marrubium eriocephalum (Lamiaceae); a Species New to the Flora of Turkey, with Contributions to its Taxonomy. PhytoKeys. 2016(58):9-20.

5. Davis PH, Mill RR, Kit T. Flora of Turkey and the East Aegean Islands (Suppl. 1). Edinburgh: Edinburgh University Press; 1988. p. 202-3.

6. Meyre-Silva C, Cechinel-Filho V. A Review Of The Chemical And Pharmacological Aspects of the Genus Marrubium. Curr Pharm Des. 2010;16(31):3503-18.

7. Karioti A, Protopappa A, Megoulas N, Skaltsa H. Identification of Tyrosinase Inhibitors from Marrubium velutinum and Marrubium cylleneum. Bioorg Med Chem. 2007;15(7):2708-14.

8. Golmakani H, Rabbani Nasab H, Sharifan M, Kamali H, Yadollahi A. The Essential Oil Composition and Antibacterial Activity of Marrubium duabense Murata from North Khorassan Province, Iran. J Essent Oil Bear Plants. 2016;19(4):963-71.

9. Masoodi M, Ali Z, Liang S, Yin H, Wang W, Khan IA. Labdane Diterpenoids from Marrubium vulgare. Phytochem Lett. 2015;13:275-9.

10. Piozzi F, Bruno M, Rosselli S, Maggio A. The Diterpenoids of The Genus Marrubium (Lamiaceae). Nat Prod Commun. 2006;7:585-92.

11. Dehbashi Z, Mazaheri M, Saeedi S, Sabbagh SK. Antibacterial Activity of Marrubium vulgare L. Against Antibiotic Resistance Klebsiella pneumoniae Strains. Adv Herb Med. 2015;1(3):9-14.

12. Hamedeyazdan S, Sharifi S, Nazemiyeh H, Fathiazad F. Evaluating Antiproliferative And Antioxidant Activity of Marrubium crassidens. Adv Pharm Bull. 2014;4(Supp1):459-64.

13. De Jesus R, Cechinel-Filho V, Oliveira A, Schlemper V. Analysis of the Antinociceptive Properties of Marrubiin Isolated From Marrubium vulgare. Phytomed. 2000;7(2):111-5.

14. Boudjelal A, Henchiri C, Siracusa L, Sari M, Ruberto G. Compositional Analysis And In Vivo Anti-Diabetic Activity Of Wild Algerian Marrubium vulgare L. Infusion. Fitoterapia. 2012;83(2):286-92.

15. Namjoyan F, Azemi ME, Abdollahi E, Goudarzi N, Nikan K. Angiotensin I Converting Enzyme Inhibitory Activities Of Hydroalcoholic Extract of Nardostachys jatamansi, Prangos ferulacea and Marrubium vulgare. Jundishapur J Nat Pharm Prod. 2015;10(2):e17255.

16. Ettaya A, Dhibi S, Samout N, Elfeki A, Hfaiedh N. Hepatoprotective Activity Of White Horehound (Marrubium Vulgare) Extract Against Cyclophosphamide Toxicity In Male Rats. Can J Physiol Pharmacol. 2015;94(4):441-7.

17. Saad S, Ouafi S, Chabane D. Anti-Inflammatory And Acute Toxicity Evaluation Of Aqueous Infusion Extract Obtained From Aerial Parts of Marrubium deserti de Noé growing in Algeria. Afr J Tradit, Complement Altern Med. 2016;13(1):71-5.

18. Yousefi K, Soraya H, Fathiazad F, Khorrami A, Hamedeyazdan S, Maleki-Dizaji N, et al. Cardioprotective Effect Of Methanolic Extract Of Marrubium vulgare L. on Isoproterenol-Induced Acute Myocardial Infarction In Rats. Ind J Exp Biol. 2013;51(8):653-60.

19. Argyropoulou A, Samara P, Tsitsilonis O, Skaltsa H. Polar Constituents Of Marrubium Thessalum Boiss. & Heldr.(Lamiaceae) And Their Cytotoxic/Cytostatic Activity. Phytother Res. 2012;26(12):1800-6.

20. Pukalskas A, Venskutonis PR, Salido S, de Waard P, van Beek TA. Isolation, Identification And Activity Of Natural Antioxidants From Horehound (Marrubium Vulgare L.) Cultivated in Lithuania. Food Chem. 2012;130(3):695-701.

21. Letchamo W, Mukhopadhyay S. Variability in Chromosomes, Herb Yield, Essential Oil

Content And Potentials Of Horehound For North American Commercial Production. J Horticult Sci. 1997;72(5):741-8.

22. Weel KG, Venskutonis PR, Pukalskas A, Gruzdiene D, Linssen JP. Antioxidant Activity Of Horehound (Marrubium vulgare L) Grown in Lithuania. Eur J Lipid Sci Tech. 1999;101(10):395-9.

23. Orhan IE, Belhattab R, Şenol F, Gülpinar A, Hoşbaş S, Kartal M. Profiling of cholinesterase inhibitory and antioxidant activities of Artemisia absinthium, A. herba-alba, A. fragrans, Marrubium vulgare, M. astranicum, Origanum vulgare subsp. glandulossum and Essential Oil Analysis Of Two Artemisia Species. Ind Crops Prod. 2010;32(3):566-71.

24. Tuzlaci E. A Dictionary of Turkish Plants. Melisa Matbaacilik: ALFA Press; 2006.

25. Song H-P, Zhang H, Fu Y, Mo H-y, Zhang M, Chen J, et al. Screening for Selective Inhibitors Of Xanthine Oxidase From Flos Chrysanthemum Using Ultrafiltration LC–MS Combined With Enzyme Channel Blocking. J Chromatogr B. 2014;961:56-61.

26. Kaurinovic B, Vlaisavljevic S, Popovic M, Vastag D, Djurendic-Brenesel M. Antioxidant Properties Of Marrubium Peregrinum L.(Lamiaceae) Essential Oil. Molecules. 2010;15(9):5943-55.

27. Sahpaz S, Garbacki N, Tits M, Bailleul F. Isolation and Pharmacological Activity Of Phenylpropanoid Esters From Marrubium vulgare. J Ethnopharmacol. 2002;79(3):389-92.

28. Essawy SS, Abo-Elmatty DM, Ghazy NM, Badr JM, Sterner O. Antioxidant and Anti-Inflammatory Effects Of Marrubium Alysson Extracts In High Cholesterol-Fed Rabbits. Saudi Pharm J. 2014;22(5):472-82.

29. Benkhnigue O, Ben Akka F, Salhi S, Fadli M, Douira A, Zidane L. Catalogue Des Plantes Médicinales Utilisées Dans Le Traitement Du Diabète Dans La Région d'Al Haouz-Rhamna (Maroc). J Anim Plant Sci. 2014;23(1):3539-68.

30. Orch H, Douira A, Zidane L. Étude Ethnobotanique Des Plantes Médicinales Utilisées Dans Le Traitement Du Diabète, Et Des Maladies Cardiaques Dans La Région d'Izarène (Nord du Maroc). J Appl Biosci. 2015;86(1):7940-56.

31. Tsukamoto K, Jackson IJ, Urabe K, Montague PM, Hearing V. A Second Tyrosinase-Related Protein, TRP-2, Is A Melanogenic Enzyme Termed Dopachrome Tautomerase. EMBO J. 1992;11(2):519-26.

32. Xu Y, Stokes AH, Freeman WM, Kumer SC, Vogt BA, Vrana KE. Tyrosine mRNA is Expressed In Human Substantia Nigra. Mol Brain Res. 1997;45(1):159-62.

33. Namjoyan F, Jahangiri A, Azemi ME, Arkian E, Mousavi H. Inhibitory Effects of Physalis alkekengi L., Alcea rosea L., Bunium persicum B. Fedtsch. and Marrubium vulgare L. on Mushroom Tyrosinase. Jundishapur J Nat Pharm Prod. 2015;10(1):1-6.

34. Brand-Williams W, Cuvelier M-E, Berset C. Use Of A Free Radical Method To Evaluate Antioxidant Activity. LWT-Food Sci Technol. 1995;28(1):25-30.

35. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant Activity Applying An Improved ABTS Radical Cation Decolorization Assay. Free Rad Biol Med. 1999;26(9-10):1231-7.

36. Prior RL, Wu X, Schaich K. Standardized Methods For The Determination Of Antioxidant Capacity And Phenolics In Foods And Dietary Supplements. J Agric Food Chem. 2005;53(10):4290-302.

37. Marco GJ. A Rapid Method For Evaluation Of Antioxidants. J Am Oil Chem Soc. 1968;45(9):594-8.

38. Chen C-H, Chan H-C, Chu Y-T, Ho H-Y, Chen P-Y, Lee T-H, et al. Antioxidant Activity Of Some Plant Extracts Towards Xanthine Oxidase, Lipoxygenase And Tyrosinase. Molecules. 2009;14(8):2947-58.

39. Masuda T, Yamashita D, Takeda Y, Yonemori S. Screening For Tyrosinase Inhibitors Among Extracts Of Seashore Plants And Identification Of Potent Inhibitors From Garcinia subelliptica. Biosci Biotech Biochem. 2005;69(1):197-201.

40. Yang X-W, Huang M-Z, Jin Y-S, Sun L-N, Song Y, Chen H-S. Phenolics from Bidens bipinnata And Their Amylase Inhibitory Properties. Fitoterapia. 2012;83(7):1169-75.

41. Albano SM, Lima AS, Pedro MGMLG, Barroso JG, Figueiredo AC. Antioxidant, Anti-5-Lipoxygenase And Antiacetylcholinesterase Activities Of Essential Oils And Decoction Waters Of Some Aromatic Plants. Rec Nat Prod. 2012;6(1):35.

42. Michelis F, Tiligada E, Skaltsa H, Lazari D, Skaltsounis A-L, Delitheos A. Effects of the Flavonoid Pilloin Isolated From Marrubium Cylleneum On Mitogen-Induced Lymphocyte Transformation. Pharm Biol. 2002;40(4):245-8.

43. Çalis I, Hosny M, Khalifa T, Rüedi P. Phenylpropanoid Glycosides From Marrubium alysson. Phytochem. 1992;31(10):3624-6.

44. Hussain A, Perveen S, Malik A, Afza N, Iqbal L, Tareen RB. Urease Inhibitiory Flavone Glucosides from Marrubium anisodon. Pol J Chem. 2009;83(7):1329-35.

45. Mitreski I, Stanoeva JP, Stefova M, Stefkov G, Kulevanova S. Polyphenols in Representative Teucrium Species in the Flora of R. Macedonia: LC/DAD/ESI-MS (n) profile and content. Nat Prod Commun. 2014;9(2):175-80.

46. Kırmızıbekmez H, Montoro P, Piacente S, Pizza C, Dönmez A, Çalış İ. Identification by HPLC-PAD-MS and Quantification by HPLC-PAD of Phenylethanoid Glycosides of Five Phlomis Species. Phytochem Anal. 2005;16(1):1-6.

47. Ulubelen A, Topcu G, Kolak U. Labiatae flavonoids and their bioactivity. In: Atta ur R, editor. Studies in Natural Products Chemistry. Vol. 30: Elsevier; 2005. p. 233-302.

48. Buckingham J, Munasinghe VRN. Dictionary of Flavonoids with CD-ROM: CRC Press; 2015.

49. Tsvetkov D, Dmitrenok A, Tsvetkov YE, Menshov V, Yashunsky D, Yashin AY, et al. Phenylethanoid Glycosides From Teak Wood Knots And Their Antioxidant Activity. J Biol Active Prod Nat. 2016;6(4):272-81.

50. Zhang H, Tsao R. Dietary Polyphenols, Oxidative Stress And Antioxidant And Anti-Inflammatory Effects. Curr Opin Food Sci. 2016;8:33-42.

51. Herrera-Arellano A, Aguilar-Santamaria L, Garcia-Hernandez B, Nicasio-Torres P, Tortoriello J. Clinical trial of Cecropia obtusifolia and Marrubium vulgare Leaf Extracts On Blood Glucose And Serum Lipids In Type 2 Diabetics. Phytomed. 2004;11(7-8):561-6.

52. Loizzo MR, Saab AM, Tundis R, Menichini F, Bonesi M, Piccolo V, et al. In vitro Inhibitory Activities Of Plants Used In Lebanon Traditional Medicine Against Angiotensin Converting Enzyme (ACE) And Digestive Enzymes Related To Diabetes. J Ethnopharmacol. 2008;119(1):109-16.