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Original Article

Anti-inflammatory and Anti-diabetic Activity of *Ballota* L. species grown in Turkey

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ABSTRACT

Background and Aims: *Ballota* is represented by 12 species and 16 taxa in Turkey. Eleven of the 16 taxa are endemic to Turkey. *Ballota* have been used in different treatments in folk medicine. Anti-inflammatory drugs act by protecting lysosomal membrane integrity or inhibiting enzymes of prostaglandins and thromboxane inflammatory mediators synthesized by deactivating of cyclooxygenase (COX), COX-1 and COX-2 enzymes. Besides, degradation of lysosomal membrane brings on the release inflammatory mediators so human red blood cells (HRBC) can be used for evaluating of anti-inflammatory activity. One of the treatment approaches of diabetes mellitus is α-glucosidase enzyme inhibition. These enzymes play an important role in glucose releasing from carbohydrates. Due to the adverse effects of synthetic drugs, natural α-glucosidase inhibitors have been discussed as therapeutic agents to manage diabetes mellitus.
In the light of the traditional use of *Ballota* species, we aimed to investigate the anti-inflammatory and anti-diabetic activities of the ethanol and aqueous extracts from the aerial parts of 14 *Ballota* taxa.
Methods: HRBC membrane stabilizing and α-glucosidase inhibitory methods are used for determining anti-inflammatory and anti-diabetic activities of 14 *Ballota* taxa, respectively.
Results: According to our results, the ethanolic extracts showed higher membrane stabilization profile than aqueous extracts generally. And for anti-diabetic activity, it is concluded that aqueous and ethanol extracts of *Ballota glandulosissima* exhibited the maximum α-glucosidase inhibitory activity.
Conclusion: According to our results, IC_{50} values of anti-inflammatory effect of ethanolic and aqueous extracts of 14 *Ballota* samples ranged from 4,30-12,88 and 3,18-20,25 mg/ml, respectively. It is observed that aqueous extracts of *Ballota nigra* subsp. *anatolica* exhibited the maximum anti-inflammatory effect. Both aqueous and ethanol extracts of *Ballota glandulosissima* are found to exhibit the maximum α-glucosidase inhibitory activity with IC_{50} values of 2,18 and 2,30 µg/ml, respectively. Among the ethanolic extracts of *Ballota* species, the strongest α-glucosidase inhibitory activities are found as *B. glandulosissima*>*B. cristata*>*B. saxatilis subsp. brachyodonta* in descending order.

Keywords: α-glucosidase, anti-inflammatory activity, anti-diabetic activity, *Ballota* species

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INTRODUCTION

Ballota L. is represented by 12 species and 16 taxa in Turkey (Davis 1982). Eleven of the 16 taxa are endemic to Turkey. *Ballota* species belong to the Lamiaceae family, commonly distributed in the mild climate condition locations of the world. Morteza-Semnani and Ghanbarimasir reviewed traditional use of *Ballota* species and mentioned commercial products of *B. nigra* sold in Europe for sedative activity (Morteza-Semnani & Ghanbarimasir 2019). In Turkey, some species of *Ballota* have been used in folk medicine as antibacterial, antiulcer, antispasmotic, diuretic, choleretic, antihemorrhoidal and sedative agents (Çitoğlu et al., 1998; Baytop, 1999). Besides, *B. acetabulosa* was mentioned in the treatment of inflammation internally and to have external use for wounds and burns (Morteza-Semnani & Ghanbarimasir, 2019; Dulger & Sener, 2010). *Ballota hispanica*, endemic of Central Mediterranean region is reported to be used to treat skin problems and for its anti-diabetic activity (Riccobono, Ben Jemia, Senatore, & Bruno, 2016).

The main components of the *Ballota* species are flavonoids, labdane diterpenoids, and phenylpropanoids (Sever Yılmaz, Özbek & Saltan Çitoğlu, 2006).

Secondary metabolites, such as tannins, terpenoids, alkaloids and flavonoids, which have been found in plants have extensively different bioactivity properties. Antibiotics are commonly used in fighting bacterial infections and have widely beneficial effects for the health and quality of human life since their invention. However, occurrence of drug-resistant bacteria causes antibiotics to be less effective against certain illnesses. Anti-inflammatory agents that derived from natural sources play a significant role in the prevention and treatment of infection diseases (Bhalodia & Shukla, 2011).

Inflammation is a protective mechanism of living organisms against abnormal stimulation and covers complex series of biochemical activities performed by the body in response to injury or abnormal stimulation caused by a physical, chemical, or biological agent. In general, the generation of cytokines is accepted to play a major role inducing inflammatory process and free radicals can propagate inflammation by stimulating release of proinflammatory cytokines such as interleukin-1β, interleukin-6 and tumor necrosis factor-α (Libby, 2007). Drugs that are currently used for treatment of inflammatory conditions are non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids. Anti-inflammatory drugs act by protecting lysosomal membrane integrity or inhibiting enzymes of prostaglandins and thromboxane inflammatory mediators synthesized by deactivating of cyclooxygenase (COX), COX-1 and COX-2 enzymes. Besides, degradation of lysosomal membrane brings on the release inflammatory mediators so human red blood cells (HRBC) can be used for evaluating of anti-inflammatory activity. Some of these drugs such as aspirin, diclofenac, ketorolac, naproxen and piroxicam have toxic effects such as risk of gastrointestinal bleeding (Dinarello, 2010; Singh, Patil &Pal, 2012; Chowdhury, Azam & Jainul, 2014).

Diabetes mellitus is a major endocrine disorder, affecting approximately 5% of the world's population. According to WHO report, the global prevalence of diabetes has been rising from 4.7% to 8.5% since 1980 and direct medical costs, loss of works force both health services and national economies (WHO, 2016). Diabetes is characterized by abnormalities in carbohydrate, lipid and lipoprotein metabolisms, which not only lead to hyperglycemia but also cause many complications such as hyperlipidemia, hyperinsulinemia, hypertension and atherosclerosis (Ozkan, Kamiloğlu & Ozdal, 2016). One of the treatment approach of diabetes mellitus is α-glucosidase enzyme inhibition. These enzymes play an important role in glucose releasing from carbohydrates. A-glucosidase inhibitors lower the blood glucose level by delaying carbohydrate absorption in digestive tract. Recently, there are α-glucosidase inhibitory preparations used in handling hyperglycemia but these synthetic derivatives of α-glucosidase inhibitors have been reported as exhibiting adverse effects (Tundis, Loizzo & Menichini, 2010; Şöhretoğlu, Sari & Barut, 2018). So natural α-glucosidase inhibitors have been discussed as a therapeutic agents to manage diabetes mellitus.

Diseases associated with both inflammation and high blood glucose level have tended to increase and cause severe complications. In the light of traditional use of *Ballota* species, we aimed to examine 14 *Ballota* taxa from Turkey for their anti-inflammatory activities and anti-diabetic by using membrane stabilization method and *in-vitro* α-glucosidase inhibitory activity respectively as well as their therapeutic potentials. Two assays were carried out on both aqueous and alcoholic plant extracts in order to determine which could be a potential as a natural source for the treatment of diabetes and inflammation. We established *in-vivo* anti-inflammatory activity of two species of *Ballota* called *B. glandulosissima* and *B.inaequidens* in our earlier reports but to the best of our knowledge, the present study is the first report on comparison of anti-inflammatory and antidiabetic activity of 14 *Ballota* species (Özbek, Saltan Çitoğlu & Dülger 2004; Sever Yılmaz et al., 2006).

MATERIALS AND METHODS

Plant Material

Fourteen *Ballota* taxa were collected in different locations of Turkey. The species are listed in Table 1. Voucher specimens were deposited at the Herbarium belonging to the Ankara University Faculty of Pharmacy (AEF).

Preparation of extracts

From the air-dried and powdered materials of aerial parts of 14 *Ballota* taxa, 2 different extracts were prepared by using ethanol (%75) and water separately.

The air dried and powdered materials of the aerial parts of each *Ballota* taxa used in this study were weighed accurately and then, extracted with ethanol (75%). It was prepared by maceration 50 g of each plant powder in 300 ml of ethanol for 8 hours, in 3 days. The macerates obtained with ethanol were evaporated until dryness.

Additionally, the air dried and powdered materials of the aerial parts of the *Ballota* taxa used in this work were weighed accurately and then, extracted with water. It was prepared by

Table 1: Scientific names and collection places of Turkish *Ballota* species.

1.	*Ballota acetabulosa* (L.) Benth.	B1 İzmir: Yenifoça, 10 m, AEF 21602
2	*Ballota antalyense* F. Tezcan & H. Duman	C3 Antalya: Turunçova, 150 m, F. Tezcan & H.Duman 1701 (holo.: GAZI)
3	*Ballota cristata* P.H. Davis	C3 Isparta: Eğridir, 910 m, AEF19899
4	*Ballota glandulosissima* Hub.-Mor & Patzak	C3 Antalya: Kumluca, 500 m, AEF 19900
5	*Ballota inaequidens* Hub.-Mor & Patzak	C3 Antalya: Alanya, 200 m, AEF 19901
6	*Ballota larendana* Boiss. & Heldr.	A4 Ankara: Kızılcahamam, 830 m, AEF 21604
7	*Ballota latibracteolata* P.H. Davis & Doroszenko	C3 Antalya: Gazipaşa, 425 m, AEF 19902
8	*Ballota macrodonta* Boiss. & Bal.	B5 Kayseri: Yahyalı, 1150 m, AEF 19907
9	*Ballota nigra* L. subsp. *anatolica* P.H. Davis	B4 Ankara: Gölbaşı, 800 m, AEF 21601
10	*Ballota nigra* L. subsp. *uncinata* (Fiori & Beg.) Patzak	B1 Izmir: Gökçealan, 250 m, AEF 21607
11	*Ballota pseudodictamnus* (L.) Benth. subsp. *lycia* Hub.-Mor.	C2Muğla: Fethiye, 20m, AEF 21603
12	*Ballota rotundifolia* C. Koch	A8 Erzurum: Tortum Lake, 1200 m, AEF 21606
13	*Ballota saxatilis* Sieber ex. J & C. Presl subsp. *brachyodonta* (Boiss.) P.H. Davis & Doroszenko	C4 İçel: Silifke, 1400 m, AEF 21505
14	*Ballota saxatilis* Sieber ex. J & C.Presl subsp. *saxatilis*	C4 İçel: Anamur, 1530 m, AEF 19904

maceration 50 g of each plant powder in 300 ml of water for 8 hours, in 3 days. The macerates obtained with water were lyophilized.

Anti-inflammatory activity

The study protocol was approved by the ethics committees of the Faculty of Medicine of Ankara University, Ankara-Turkey (26.10.2015/16-695-15).

Preparation of Human Red Blood Cells (HRBC) Suspension

Fresh whole human blood was collected from healthy human volunteer who had not taken any anti-inflammatory or steroidal drug for 2 weeks prior the experiment and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 min. The packed cells were washed three times with equal volume of isosaline (0.85%, pH 7.2). The volume of the blood was measured and reconstituted as 10% *v/v* RBC's suspension with isosaline.

Heat Induced Hemolysis

As HRBC membrane is similar to lysosomal membrane structure, stabilization of membrane is taken as a measure of anti-inflammatory activity.

Membrane stabilizing activity of the extracts was assessed using heat-induced human erythrocyte hemolysis (Shinde et al., 1999; Yalçın, Yılmaz Sarıaltın & Çiçek Polat, 2020). Inhibition of hemolysis was implied as stabilization of the HRBC membrane. The reaction mixture consisted of extracts (0,5-20 mg/ml) or acetylsalicylic acid (0.05-0.5 mg/ml) and 10% HRBC suspension. Instead of test sample, solvent of the samples was used as a control and acetylsalicylic acid was a standard drug. All the centrifuge tubes containing reaction mixture were incubated at 56ºC for 30min. At the end of the incubation the tubes were cooled under running tap water. Another set of prepared samples were placed at 0°C in an ice bath. The reaction mixture was centrifuged at 2500 rpm for 5 min. Then the absorbance of the supernatant was measured on 96-well microplate spectrophotometer at 560 nm. The experiment was performed in triplicates for all the test samples. The percentage of inhibition of hemolysis was calculated then by the formula as given below. The concentration against the percentage of inhibition of hemolysis was plotted and the half maximal inhibitory concentration (IC_{50}) was calculated using this plot for each sample.

$$\% \text{ inhibition of hemolysis} = 100 \times \left[1 - \frac{(A_2 - A_1)}{(A_3 - A_1)}\right]$$

A_1: test sample unheated, A_2: test sample heated, A_3: control sample heated

Anti-diabetic Activity

***In-vitro* α-glucosidase inhibitory activity**

The α-glucosidase inhibitory activities of the ethanol and aqueous extracts were determined according to the method with slight modifications (Liu et al., 2014). The substrate solution pNPG was prepared with 0.2 M of Na-phosphate buffer (pH:6.8). The reaction mixture contained 10 µL of 0.02 U/µL α-glucosidase solution in 0.2 M Na-phosphate buffer (pH=6.8), 10 µL of sample (1-50µg/ml), 50 µL of Na-phosphate buffer, which were mixed and incubated at 37°C for 20 min. Then, 50 µL of 0.02 M PNPG was added, and the mixture was incubated at 37°C for another 30 min. Finally, the reaction was stopped by the addition of 100 µL 0.2 M Na_2CO_3 solutions. Acarbose (0.5-25 µg/ml) was used as a standard drug. The amount of the p-nitrophenol released from PNP-glycoside was quantified on a 96-well microplate spectrophotometer at 405 nm. The inhibitory activities of the samples on α-glucosidase were calculated by the following formula. The IC 50 value for each sample was determined graphically by plotting the percentage of inhibition and inhibitory concentration.

Inhibition (%) = [(Absorbance of control- Absorbance of test sample)/ Absorbance of control)] x 100

Statistical Analysis

All the tests were run in triplicate. SPSS 20.0 and 23.0 were used to examine the effect of different compounds and concentrations on anti-inflammatory and anti-diabetic activities. One-way analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by the Fisher's least significant difference (LSD) test. $p<0.05$ were reported as "statistically significant".

RESULTS

In this study, anti-inflammatory and anti-diabetic activities of ethanol and aqueous extracts from aerial parts of 14 *Ballota* taxa growing in Turkey were evaluated (Table 1).

Anti-inflammatory Activity

Anti-inflammatory activity was performed by evaluating the stabilization effects of extracts on hemolyzed erythrocyte membrane. HRBCs membrane stabilization activity of ethanolic and water extracts of *Ballota* sp. are given in Figure 1 and Figure 2.

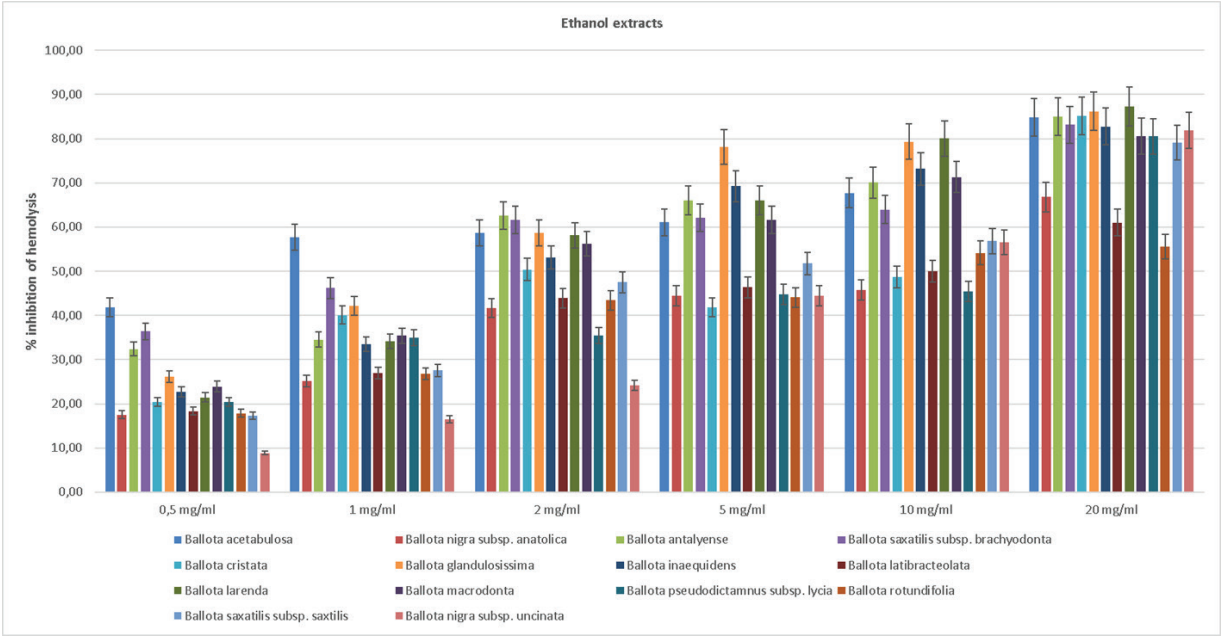

Figure 1: Percent inhibition of hemolysis of HRBC membrane in the presence of different concentrations of *Ballota* species

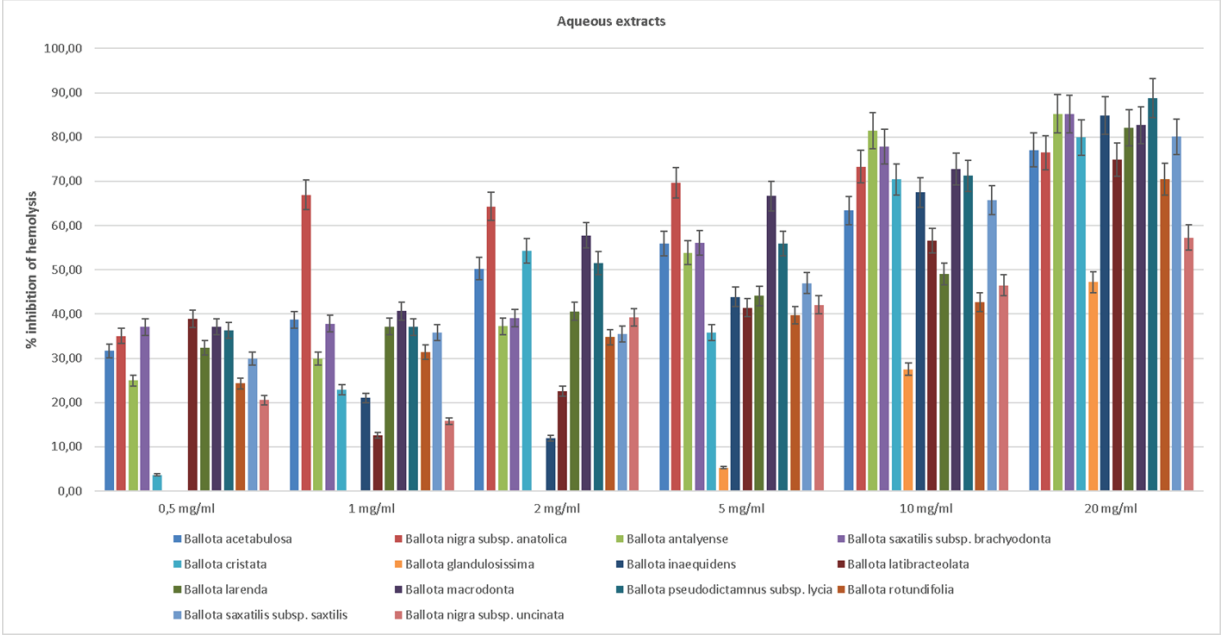

Figure 2. Percent inhibition of hemolysis of HRBC membrane in the presence of different concentrations of aqueous extracts of *Ballota* sp.

Table 2: *In-vitro* anti-inflammatory activities by using HRBC membrane stabilizing method

Plant name	IC_{50} (mg/ml) Mean ± SD	
	Ethanol extract	Aqueous extract
Control	-	-
Ballota acetabulosa	4,30±0,18*	7,24±0,01*
Ballota nigra subsp.*anatolica*	11,59±0,20*	3,18±0,10*
Ballota antalyense	5,46±0,02*	6,93±0,08*
Ballota saxatilis subsp. *brachyodonta*	5,31±0,02*	6,23±0,03*
Ballota cristata	8,45±0,06*	8,79±0,08*
Ballota glandulosissima	4,59±0,14*	20,25±0,04*
Ballota inaequidens	6,17±0,17*	9,69±0,17*
Ballota latibracteolata	12,06±0,44*	10,23±0,36*
Ballota larendana	5,61±0,06*	8,69±0,41*
Ballota macrodonta	6,47±0,15*	5,12±0,11*
Ballota pseudodictamnus subsp. *lycia*	9,65±0,05*	5,90±0,04*
Ballota rotundifolia	12,88±0,07*	11,49*[a]
Ballota saxatilis subsp *saxatilis*	8,67±0,07*	8,04±0,07*
Ballota nigra subsp. *uncinata*	9,89±0,03*	13,87±1,02*
Acetylsalicylic acid	0,28±0,01*	

*A p-value less than 0,05 was considered statistically significant compared to control. [a]SD<0,01 mg/ml

14 *Ballota* ethanolic extracts at all the doses (0.5–20 mg/ml) were found to protect the human erythrocyte membrane against lysis induced by heat. In the present study, the ethanolic extracts showed higher membrane stabilization profile than aqueous extracts except *B. nigra* subsp. *anatolica*, *B. latibracteolata*, *B. rotundifolia* and *B. saxatilis* subsp. *saxatilis*. As shown in Table 2, IC_{50} values of anti-inflammatory effect of ethanolic and aqueous extracts of 14 *Ballota* samples ranged from 4.30-12.88 and 3.18-20.25 mg/ml, respectively. Under the same experimental condition, inhibition of hemolysis of acetylsalicylic acid was 0.28 mg/ml.

On the basis of this study, it was concluded that aqueous extracts of *Ballota nigra* subsp. *anatolica* (IC_{50}=3.18 mg/ml) exhibited the maximum anti-inflammatory effect following by ethanolic extracts of *Ballota acetabulosa* and *Ballota glandulosissima* with a value of 4.30 and 4.59 mg/ml respectively.

Anti-diabetic activity

In-vitro anti-diabetic activity of the plant extracts was evaluated by measuring their inhibitory effect on α-glucosidase level and results were given in Table 3. Both aqueous and ethanol extracts of *Ballota glandulosissima* exhibited the maximum α-glucosidase inhibitory activity with IC_{50} values of 2.18 and 2.30 µg/ml, respectively. Generally, the aqueous extracts showed higher α-glucosidase inhibitory activity than the ethanol extracts. On the other hand, the ethanolic extract of *B. saxatilis* subsp. *brachyodonta* was found much stronger inhibition value (IC_{50}= 2.63 µg/ml) on α-glucosidase, compared with water extract (19.84 µg/ml). Similar results were also found for ethanol and water extracts of *B. cristata* with IC_{50} values of 2.60 and 17.82 µg/ml. The IC_{50} value of the reference compound, acarbose was 0.898 µg/ml.

DISCUSSION

Ballota species have been reported as being rich in phenolic compounds. They contain diterpenoids, flavonoids, phenylpropanoids, essential oils, tannins and saponins (Sever Yılmaz & Saltan Çitoğlu, 2003). In the present study, we applied membrane stabilization method and *in-vitro* α-glucosidase inhibitory activity to evaluate anti-inflammatory and anti-diabetic activities of 14 *Ballota* taxa growing in Turkey, respectively. According to our results; the ethanolic extracts showed higher membrane stabilization profile than aqueous extracts generally. Aqueous extracts of *Ballota nigra* subsp. *anatolica* exhibited the maximum anti-inflammatory effect following by ethanolic extracts of *Ballota acetabulosa* and *Ballota glandulosissima*. And for anti-diabetic activity, it was concluded that aqueous and ethanol extracts of *Ballota glandulosissima* (IC_{50}=2.18 and 2.30 µg/ml respectively) exhibited the maximum α-glucosidase inhibitory activity. In present study, among the *Ballota* species, the ethanolic extracts of B. *glandulosissima* showed both significant anti-diabetic and anti-inflammatory activity with 2.298 µg/ml and 4.59 mg/ml IC_{50} value, respectively.

According to our previous studies, *B. glandulosissima* and *B nigra* subsp. *anatolica* were found to have high antioxidant potential and *B. glandulosissima* was found as the richest spe-

Table 3: *In vitro* anti-diabetic activities of ethanol and aqueous extracts of *Ballota* sp. by α-glucosidase inhibitory method

Plant name	IC_{50} (µg/ml) Mean ± SD	
	Ethanol extract	Aqueous extract
Control	-	-
Ballota acetabulosa	26,93±0,03*	11,04±0,01*
Ballota nigra subsp.*anatolica*	19,21±0,01*	7,56±0,01*
Ballota antalyense	3,22±0,01*	3,68±0,02*
Ballota saxatilis subsp. *brachyodonta*	2,63± 0,01*	19,84±0,01*
Ballota cristata	2,60±0,01*	17,81±0,01*
Ballota glandulosissima	2,30± 0,01*	2,18±0,01*
Ballota inaequidens	15,67±0,03*	4,49±0,01*
Ballota latibracteolata	5,52± 0,01*	3,22±0,01*
Ballota larendana	14,83±0,01*	3,99±0,01*
Ballota macrodonta	4,56± 0,01*	4,52±0,01*
Ballota pseudodictamnus subsp. *lycia*	6,53± 0,01*	3,71±0,02*
Ballota rotundifolia	7,82± 0,01*	3,87±0,01*
Ballota saxatilis subsp *saxatilis*	9,16± 0,01*	4,50±0,02*
Ballota nigra subsp. *uncinata*	18,23±0,01*	6,07±0,01*
Acarbose	0,90± 0,01*	

cies with respect to flavonoid content We also studied the anti-inflammatory activities of *B. inaequidens* and *B. glandulosissima in-vivo* and aqueous extract of *B. inaequidens* was found to have better anti-inflammatory activity on carrageen induced hind paw edema in rats and the water extract *B. glandulosissima* showed anti-inflammatory activity, but in the present study, ethanolic extract of *B. glandulosissima* is found to have higher membrane stabilizing activity (Özbek, et al., 2004; Sever Yılmaz et al., 2006; Erdoğan Orhan, Sever Yılmaz & Altun, 2010; Sever Yılmaz, Ergene & Saltan Çitoğlu, 2015). Kumatakenin, pachypodol, 5-hydroxy-7,3',4'-trimetoxyflavone, velutin, salvigenin, retusin, corymbosin were found in *B. glandulosissima* (Çitoğlu, Sever & Antus, 2003). On the other hand, the opposite results were observed for *B.nigra subsp. anatolica* which aqueous extract showed the strongest membrane stabilizing activity. Considering our earlier studies on chemical profile of *Ballota* species, it could be assumed that the anti-inflammatory and anti-diabetic potential of *B. glandulosissima* and *B. nigra* subsp. *anatolica* were related to flavonoids and phenolic compounds and besides, the results of present study are in good agreement with our earlier reports. Earlier reports pointed out the strong correlation between phenolic compounds and antioxidant activity. Uysal et al. mentioned that the antioxidant activity of water extract of *B. macrodonta* was higher than methanolic extract connected with phenolic contents (Uysal et al., 2018). It is also well known that strong antioxidant activity could be linked to both anti-inflammatory and anti-diabetic effects. So, in conclusion, *B. acetabulosa, Ballota glandulosissima Ballota nigra* subsp. *anatolica* could be a good candidate for the treatment of inflammation and on the other hand, *Ballota saxatilis* subsp. *brachyodonta*, *Ballota cristata*, *Ballota glandulosissima* were found to significant anti-diabetic activities, directly related to the total amount of polyphenols and flavonoid content. The further step of this study was to isolate and identify the anti-inflammatory and anti-diabetic components of potential *Ballota* species.

CONCLUSION

Recently, the beneficial effects of natural products have become popular on the basis of low toxicity but it is clear that an evidence base approach is necessary for this issue. *Ballota* species have traditional use and are good candidates for treatment of inflammation and diabetes. Our results also support the medical profile of *Ballota* species but, of course, further studies are needed to be sure about the efficacy and safety.

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