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Antinociceptive and Anti-inflammatory Activities of *Ballota inaequidens*

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Abstract

The water extract of *Ballota inaequidens* Hub.-Mor. & Patzak (Lamiaceae) was investigated for antinociceptive and anti-inflammatory activities in mice and rats. The tail-flick test, acetic acid–induced writhing test, and the carrageenan-induced rat paw edema test were used to determine these effects. Our findings show that *Ballota inaequidens* caused dose-related inhibition in the acetic acid–induced abdominal stretching in mice. The medium effective dose (ED_{50}) value of *Ballota inaequidens* was found to be 85.38 mg/kg. *Ballota inaequidens* showed no significant changes in the nociceptive threshold of the tail-flick test but did show an inhibition of paw edema induced by carrageenan. The ED_{50} value of *Ballota inaequidens* was found to be 99.42 mg/kg. The current study reveals that the water extract of *Ballota inaequidens* possesses promising antinociceptive and anti-inflammatory activities.

Keywords: Anti-inflammatory activity, antinociceptive activity, *Ballota inaequidens*, median lethal dose (LD_{50}).

Introduction

Ballota inaequidens Hub.-Mor & Patzak (Lamiaceae) is found in South Anatolia (Davis, 1982). *Ballota* species have been used in Turkish folk medicine as antiulcer, antispasmodic, diuretic, choleretic, anti-hemorrhoidal, and sedative agents (Meriçli et al., 1988; Vural et al., 1996; Çitoğlu et al., 1998; Baytop, 1999). *Ballota nigra* L. is used externally in the treatment of wounds and burns. It is orally taken to suppress coughs and upper respiratory inflammation (Yeşilada et al., 1993; 1995; Tuzlacı & Tolon, 2000). Vural et al. (1996) reported that *Ballota nigra* L. subsp. *anatolica* P.H. Davis and *Ballota larendana* Boiss. & Heldr. have antidepressant activity. *B. larendana* has also anxiolytic activity. The antimicrobial activities (Çitoğlu et al., 2003a) and the antioxidant activities (Çitoğlu et al., 2004a) of all *Ballota* species growing in Turkey was recently reported as well as the antifungal activities of some flavonoids isolated from *B. glandulosissima* Hub.-Mor. & Patzak (Çitoğlu et al., 2003b). The water extract of *B. glandulosissima* has been reported to have antinociceptive (Çitoğlu et al., 2004c), anti-inflammatory, and hepatoprotective activities (Özbek et al., 2004). Çitoğlu et al. (2004b) also reported antifungal activities of some diterpenoids and flavonoids from *B. inaequidens*.

The main components of the *Ballota* species are flavonoids, labdane diterpenoids, and phenylpropanoids (Sever, 2002). In our previous studies, three diterpenoids (hispanolone, ballonigrine, dehydrohispanolone) and ten flavonoids (kumatakenin, pachypodol, 5-hydroxy-7,3′, 4′-trimethoxyflavone, velutin, corymbosin, 5-hydroxy-3,7,4′-trimethoxyflavone, retusin, 5-hydroxy-7,4′-dimethoxyflavone, 5-hydroxy-3,6,7,4′-tetramethoxyflavone, ladanein) were isolated, chemically characterized and analyzed by HPLC in different of *Ballota* species (Çitoğlu et al., 1998, 1999, 2003b, 2004b; Sever, 2002).

This paper is a part of our ongoing studies on this genus (Çitoğlu et al., 1998, 1999, 2003a,b, 2004a,b,c; Sever, 2002; Özbek et al., 2004). *Ballota inaequidens* (BI) is claimed to be useful in respiratory disorders. Because our research group has conducted several studies on the biological activities of the crude extracts and isolates of *Ballota* species, the aim of this work was to assess the antinociceptive and anti-inflammatory activities of BI. To our knowledge, no data are available

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with respect to antinociceptive and anti-inflammatory activities of this plant.

Materials and Methods

Plant material

Ballota inaequidens Hub.-Mor & Patzak was collected in 1998 from flowering plants near Antalya (Turkey) by B. Sever and F. Tezcan. Taxonomic identy of the plant was confirmed by H. Duman, a plant taxonomist in the Department of Biological Sciences, Faculty of Art and Science, Gazi University (Ankara, Turkey). Voucher specimens were kept in the herbarium of Ankara University, Faculty of Pharmacy (AEF no. 19901).

Preparation of extract

The aqueous extract was prepared by macerating 20 g air-dried aerial part of plant powder in cold distilled water (300 mL) for 1 day. The macerate was evaporated and lyophilized (Kouadio et al., 2000). The extract yield was 20% (w/w).

Animals

The protocol for the study was approved by the Ethical Committee of Yüzüncü Yıl University Faculty of Medicine Animal Breeding and Research. Male Swiss albino mice (23–27 g) and Sprague-Dawley rats (160–210 g) were used in these experiments. The animals were housed in standard cages (48 cm × 35 cm × 22 cm) at room temperature ($20 \pm 2^{\circ}C$), with artificial light from 7:00 a.m. to 7:00 p.m. and provided with pelleted food (Van Animal Feed Factory) and water *ad libitum*.

Chemicals

Lambda-carrageenan type IV and indomethacin (Sigma, Steinheim, Germany), etodolac (Etol, Fako, Turkey), and aspirin (Bayer, Turkey) were used.

Acute toxicity test

Male mice were randomly assigned into six groups (T1–T6) with six animals in each group. Group T1 was treated with isotonic saline solution (0.1 mL, i.p.) as control and the other five groups received the aqueous extract by intraperitoneal (i.p.) administration at doses of 400, 800, 1600, 3200, and 6400 mg/kg body weight. The maximum volume injected was 0.2 mL. The groups were returned to their home cages and given free access to food and water. The mortality in each cage was assessed 24, 48, and 72 h after administration of the extract. The percent mortalities were converted to Probits and plotted against the $\log_{10}$ of the extract dose. Regression lines were fitted by the method of least squares, and confidence limits for the LD_{50} values were calculated by the method of Litchfield and Wilcoxon (1949) and Abdel-Barry et al. (1997).

Antinociceptive activity

Acetic acid–induced writhing test

The method of Koster et al. (1959) was used with slight modification. The animals were kept in a temperature-controlled environment ($22 \pm 2^{\circ}C$) with a 12 h light-dark cycle. Food and water were freely available. Abdominal writhing was introduced by i.p. injection of acetic acid (6%, 60 mg/kg). Animals were pretreated with the aqueous extract through i.p. administration, 30 min prior to acetic acid injection, and 5 min thereafter the test was started. The plant extract was tested at 30, 65, and 100 mg/kg i.p. BI dose (100 mg/kg) was selected according to LD_{50} value ($LD_{50} = 3.914$ g/kg), and after the experimental results, this dose was found effective. Thus, following doses were reduced to 65 and 30 mg/kg.

Control animals received the same volume of isotonic saline solution (ISS; 5 mL/kg). Acetylsalicylic acid at a dose of 300 mg/kg, which is a preferential dose in such studies, given orally was used as a standard for comparison (Hunskaar et al., 1985). After challenge, pairs of mice were placed in a glass cage measuring 44 cm × 44 cm × 25 cm. The number of stretchings occurring for 15 min after immediately the acetic acid injection was recorded. Six mice were used per group. Animals were sacrificed immediately after each 15-min experiment. The results were evaluated by calculating the mean number of stretchings per group, and they were represented as % inhibition of stretching movements with the control group (Tanker et al., 1996).

$$\%\ \text{analgesic activity} = ((n - n')/n) \times 100$$

where n is average number of "stretchings" of control group, and n' is average number of "stretchings" of test group.

Tail-flick test

Nociceptive response was assessed with a tail-flick apparatus (LSI Letica LE 7106, Spain) using a method initially described by D'Amour and Smith (1941). The animals were gently immobilized by using a glove, and the radiant heat was focused on a blackened spot 1–2 cm from the tip of the tail. Beam intensity was adjusted to give a tail-flick latency of 2–3 s in control animals. Measuring was terminated if the latency exceeded the end of time (20 s) to avoid tissue damage. In all the experiments, mice were tested three-times, 60 and 30 min before drug administration in the baseline latency determined and 30 min after drug administration. In addition, the mice were tested three-times after drug administration (30, 90, and 150 min). The BI extract was tested at 100, 200,

and 400 mg/kg i.p. Morphine at a dose of 10 mg/kg, given subcutaneously (s.c.), was used as a standard for comparison (Matsumoto et al., 2004).

Anti-inflammatory activity

The inhibitory activity of the extract on carrageenan-induced rat paw edema was determined according to the method of Winter et al. (1962) with slight modification. Forty-two rats of either sex were allotted into seven groups of six animals each. The rats were fasted for 12 h and deprived of water only during the experiment. Deprivation of water was to ensure uniform hydration and to minimize variability in edematous response. Inflammation of the right hind paw was induced by injecting 0.05 mL fresh lambda-carrageenan (phlogistic agent) into the subplantar surface of the right hind paw. Group I (control I) received 0.2 mL isotonic saline solution *per os* (p.o.). Group II, which served as control II, received 0.2 mL ethanol (p.o.). Group III (reference drug) received indomethacin (3 mg/kg, i.p.) (Rimbau et al., 1999). Group IV (reference drug) received etodolac (50 mg/kg, i.p.) (Inoue et al., 1991), while the remaining three groups received the extract at doses of 50, 100, and 200 mg/kg, i.p.

The measurement of foot volume was accomplished by displacement technique using the plethysmometer (Ugo Basile 7140 plethysmometer, Italy) immediately before and 3 h after the injection.

The inhibition percentage of the inflammatory reaction was determined for each animal by comparison with controls and calculated by the formula (Kouadio et al., 2000):

$$I\% = [(1 - (dt/dc)] \times 100$$

where *dt* is the difference in paw volume in the drug-treated group and *dc* the difference in paw volume in the control group.

Statistical analysis

All data expressed as mean ± standard error of the mean (SEM) were analyzed by the analysis of variance (one-way ANOVA), post hoc Tamhane's T2, and Tukey's HSD. Tukey's honestly significant difference test procedure, for multiple comparisons of p values of less than 0.5 were considered to be significant (Sümbüloğlu & Sümbüloğlu, 1998). Medium effective dose (ED_{50}) value was calculated by nonlinear regression analysis (SigmaPlot 2004 for Windows Version 9.01).

Results

Acute toxicity test

Mice have been used to determine the i.p. LD_{50} value of BI. Water extract of *B. inaequidens*, when administered i.p. in the dose range 400–6400 mg/kg to mice, did not produce any significant change in the autonomic or behavioral responses during the observation period. The LD_{50} value of the extract was found to be 3.914 g/kg in mice.

Antinociceptive activity

Acetic acid–induced writhing test

Water extract of *B. inaequidens* caused dose-related inhibition of the acetic acid–induced abdominal stretching response in mice (Table 1). When the abdominal stretching values of BI at all doses were compared with that of acetylsalicylic acid, no significant differences were observed at 30 and 65 mg/kg doses. Thus, BI is likely to have similar potency as acetylsalicylic acid at these doses. BI showed significant difference from acetylsalicylic acid group at 100 mg/kg dose. The medium effective dose (ED_{50}) value of *Ballota inaequidens* was found to be 85.38 mg/kg. Thus, BI was found more effective than acetylsalicylic acid at the same dose.

Tail-flick test

The results of the nociceptive threshold of tail-flick test of BI are shown in Table 2. At all time points examined, BI at three doses (100, 200, 400 mg/kg) produced no significant analgesic effects compared with that of control group.

Anti-inflammatory activity

Table 3 shows the results on antiedematous effect of intraperitoneally administered BI on carrageenan paw edema in rats. BI showed a significant anti-inflammatory effect in all doses studied, which peaked at doses of 200 mg/kg (85.85%) and 100 mg/kg (72.29%) with a lesser degree of inhibition at 50 mg/kg (58.54%). Compared with the controls, the greatest anti-inflammatory

Table 1. Effect of the water extract of *B. inaequidens* (BI) on acetic acid-induced writhing in mice (n = 6).

Treatment	Abdominal stretching (mean ± SEM)	% Inhibition of stretching
Control (ISS)	17.66 ± 1.66	—
Aspirin (300 mg/kg)	9.83 ± 0.60[a]	44.33
BI (30 mg/kg)	3.80 ± 3.80[a]	78.48
BI (65 mg/kg)	3.40 ± 2.71[a]	80.74
BI (100 mg/kg)	1.50 ± 1.02[ab]	91.50
F value	10.456	
p value	0.000	

ANOVA followed by Tukey's HSD test:
[a]$p < 0.05$ significantly different from control (isotonic saline solution; ISS) value.
[b]$p < 0.05$ significantly different from aspirin value.

Table 2. Effect of water extract of *B. inaequidens* (BI) on the latency of the tail-flick test in mice (n = 6).

Treatment	Measurements (mean ± SEM)			
	Before treatment	30 min	90 min	150 min
Morphine (10 mg/kg)	10.99 ± 0.64	15.70 ± 0.93	18.03 ± 0.68	10.88 ± 0.98
Aspirin (100 mg/kg)	8.95 ± 0.34	10.88 ± 0.58[a]	10.84 ± 0.51[a]	11.72 ± 0.54
Control (ISS)	9.71 ± 0.40	9.88 ± 0.35[a]	9.96 ± 0.39[a]	9.71 ± 0.28
BI (100 mg/kg)	10.06 ± 0.84	10.25 ± 0.32[a]	8.93 ± 0.79[a]	8.73 ± 0.58[b]
BI (200 mg/kg)	8.35 ± 0.37	10.31 ± 2.97	9.21 ± 2.14	9.60 ± 2.04
BI (400 mg/kg)	8.95 ± 0.39	7.93 ± 0.13[abcd]	7.01 ± 0.40[abc]	7.50 ± 0.31[bc]
F value		5.816	21.120	2.339
p value		0.000	0.000	0.061

ANOVA followed by Tamhane's T2 test:
[a] $p < 0.05$ significantly different from corresponding morphine value.
[b] $p < 0.05$ significantly different from corresponding aspirin value.
[c] $p < 0.05$ significantly different from corresponding control (isotonic saline solutions; ISS) value.
[d] $p < 0.05$ significantly different from corresponding BI (100 mg/kg) value.

activity was observed in the first reference group receiving indomethacin with a 95.7% regression of inflammation. Etodolac, the second reference agent, showed significant but weaker anti-inflammatory activity with 42.11% regression of edema. BI has significantly lower anti-inflammatory effect compared with indomethacin at 50 mg/kg dose and a similar effect at 100 and 200 mg/kg doses. When compared with etodolac, the extract had a statistically similar effect at 50 and 100 mg/kg, and higher activity at 200 mg/kg. BI showed no statistically meaningful difference with doses 50, 100, and 200 mg/kg. The ED_{50} value of *Ballota inaequidens* was found to be 99.42 mg/kg.

Table 3. Effect of intraperitoneal treatment with the water extract of *Ballota inaequidens* (BI) on the carrageenan-induced hind paw edema in rats (n = 6).

Groups	Paw edema at 3 h (mL ± SEM)	Percent inhibition (%)
Control-I (ISS)	1.043 ± 0.12	—
Control-II (ethyl alcohol)	0.988 ± 0.11	—
Etodolac (50 mg/kg)	0.572 ± 0.10[ab]	42.11
Indomethacin (3 mg/kg)	0.042 ± 0.02[abc]	95.70
BI (50 mg/kg)	0.431 ± 0.06[abd]	58.54
BI (100 mg/kg)	0.288 ± 0.08[ab]	72.29
BI (200 mg/kg)	0.147 ± 0.02[abc]	85.85
F value	21.711	
p value	0.000	

SEM, standard error of mean; ISS, isotonic saline solution.
ANOVA followed by Tukey's HSD test:
[a] $p < 0.05$ with respect to control-I group.
[b] $p < 0.05$ with respect to control-II group.
[c] $p < 0.05$ with respect to etodolac group.
[d] $p < 0.05$ with respect to indomethacin group.

Discussion

This plant is used as an infusion by the public (Baytop, 1999). Accordingly, as a preliminary study, we decided to prepare the water extract of the plant.

This is the first study to show antinociceptive and anti-inflammatory effects of BI on mice and rats, respectively. BI, at the doses tested, showed antinociceptive activity in writhing test, but did not show antinociceptive activity in tail-flick test in mice, relative to aspirin. That is, the BI extract shows a good protective effect on chemical (acetic acid injection) pain stimuli but not on thermal (tail-flick) pain stimuli. BI exhibited anti-inflammatory activity by reducing carrageenan-induced rat paw edema.

The antinociceptive and anti-inflammatory activities of a water extract of *Ballota glandulosissima* (BG) have also been reported (Çitoğlu et al., 2004c; Özbek et al., 2004). BG caused dose-related inhibition in the acetic acid–induced abdominal stretching in mice. The results obtained herein also showed the same finding. However, BI did not show any antinociceptive activity in the tail-flick test in mice relative to aspirin. However, BG showed significant changes in the nociceptive threshold of the tail-flick test (Çitoğlu et al., 2004c). In addition, anti-inflammatory activity of the water extract of *B. glandulosissima* has also been determined (Özbek et al., 2004). BG (100 mg/kg) caused a significant reduction (34.22%) in paw edema induced by carageenan. In this study, we found that BI caused more reduction (72.29%) at the same dose. Especially, the dose of 200 mg/kg BI has the highest activity (85.85%). We can say the water extract of *B. inaequidens* has better anti-inflammatory activity than the water extract of *B. glandulosissima*.

At this time, the exact mechanism responsible for these effects is not known. It could be due to the

flavonoid and phenylpropanoid content of the extract (Ferreres et al., 1986; Seidel et al., 1995; Çitoğlu et al., 1999, 2004c; Bertrand et al., 2000; Tuzlacı & Tolon, 2000; Sahpaz et al., 2002; Sever, 2002). These chemicals have been reported to have analgesic and anti-inflammatory properties (Di Carlo et al., 1999; Harborne & Williams, 2000; Miceli et al., 2005).

In conclusion, the current study revealed that the water extract of *Ballota inaequidens* possesses promising antinociceptive (centrally or peripherally) and anti-inflammatory activity and the median lethal dose (LD_{50}) of BI is 3.914 g/kg. Further investigations are necessary to elucidate the exact mechanism of antinociceptive (centrally or peripherally) and anti-inflammatory activity of this extract.

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