Investigation on Anti-Inflammatory and Antiulcer Activities of *Anchusa azurea* Extracts and their Major Constituent Rosmarinic Acid

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This study investigated the anti-inflammatory and antiulcer activities of different extracts from the aerial parts and the roots of *Anchusa azurea* Miller var. *azurea* (Boraginaceae), as well as their major constituent, rosmarinic acid. The extracts were water (AWa, RWa) and methanol (AMe, RMe) extracts prepared from the aerial parts and the roots of *A. azurea*, respectively. The AMe extract was found to exert anti-inflammatory effects; so it was evaporated to dryness and the residue was dissolved in distilled water (AMeWa) and then further fractionated with *n*-hexane (AMeHe) and *n*-butanol (AMeBu). Anti-inflammatory activity was investigated in rats using carrageenan-induced acute inflammation, and antiulcer activity was investigated using indomethacin-induced gastric damage. The methanolic extract from the aerial parts, its *n*-butanol fraction, and rosmarinic acid, which was isolated from the *n*-butanol fraction of the AMe extract, showed significant dose-dependent anti-inflammatory activity. During the acute phase of inflammation, the anti-inflammatory activity of rosmarinic acid was comparable to that of ibuprofen. No antiulcer activity was observed. The experimental data demonstrate that *A. azurea* Miller var. *azurea* and rosmarinic acid display significant anti-inflammatory activity.

Key words: Anti-Inflammatory, *Anchusa azurea*, Rosmarinic Acid

Introduction

There are fifteen species of the genus *Anchusa* L. (Boraginaceae) found among the flora of Turkey (Chamberlain, 1978). Several species of *Anchusa* are used in folk medicine for treating open wounds and cuts (Dafni *et al.*, 1984; Lardos, 2006), rheumatism, arthritis, gout (Ali-Shtayeh *et al.*, 2000; El Beyrouthy *et al.*, 2009), stomach diseases, and weight loss (Al-Qura'n, 2009). In other instances, *Anchusa* is used as a diuretic agent (Baytop, 1984). Antibacterial and antifungal activity (Nader *et al.*, 1986) and the inhibition of aryl hydrocarbon hydroxylase activity (Alwan *et al.*, 1990) have also been reported in a study examining *A. strigosa*. An ointment prepared from a decoction of *A. azurea* Miller roots mixed with egg yolk and beeswax is used externally for wound healing (Honda *et al.*, 1996). The dried and powdered leaves are used externally as a desiccant for wounds (Yesilada *et al.*, 1995). Radical scavenging and iron-chelating activities of *A. azurea* have also been reported (El and Karakaya, 2004). In Jordan and Palestine, practitioners of folk medicine use the hot aqueous extracts of *A. strigosa* roots in the treatment of stomach ulcers. It was reported that these roots have both a protective and a treatment effect on ethanol-induced ulcers in rats and guinea pigs (Disi *et al.*, 1998). In another study, both the ethanol and aqueous extracts of *A. azurea* were found to be ineffective in anti-inflammatory and antinociceptive activity tests (Kupeli *et al.*, 2007). Pyrrolizidine alkaloids, flavonoids, triterpene saponins, fatty acids, and phenolic acids have been isolated from this species (Demirezer and Zeeck, 2000; Guil-Guerrero

et al., 2001; Erdemoglu et al., 2004; Kuruuzum-Uz et al., 2010).

Inflammatory diseases and ulcers are one of the world's major health problems. In inflammatory diseases, non-steroidal anti-inflammatory drugs are used as the first choice of treatment; however, their usage is limited because of the ulcerogenic side effects that they exert on the gastrointestinal system (Suleyman et al., 2008). In recent studies, investigators have sought to develop or discover new herbal drugs with anti-inflammatory effects and no ulcerogenic effects. Determining a compound that simultaneously demonstrates both anti-inflammatory and antiulcerogenic effects would represent significant development in ulcer and inflammation treatment (Albayrak et al., 2010). Recent studies have focused more attention on herbal drugs (Cadirci et al., 2007). Many experimental animal studies have demonstrated that plant extracts have lower toxicities than synthetic drugs (Suleyman et al., 1999; Odabasoglu et al., 2006). This is one of herbal drugs' most important advantages. Also, the wound-healing effect that the Anchusa species are reported to have is related to inflammation and ulcerogenic disorders. In our previous study on the aerial parts of A. azurea, phenolic acids, flavonoid glycosides, and triterpene glycosides were isolated, and the results of this study showed that A. azurea contains a very high concentration of rosmarinic acid (Kuruuzum-Uz et al., 2010). In order to evaluate the ethnobotanical information on Anchusa, water extracts used to form traditional medicines and various extracts, as well as rosmarinic acid, from A. azurea Miller var. azurea were investigated to determine their anti-inflammatory and antiulcer activities in experimental rat models.

Material and Methods

Plant material and preparation of extracts

Anchusa azurea Miller var. azurea was collected from Ankara-Beytepe on July 15, 2003 and identified by Dr. Hayri Duman of the Department of Biology Gazi University, Ankara, Turkey. Under the number HUEF 03012, a voucher specimen was deposited at the herbarium of Hacettepe University, Faculty of Pharmacy, Ankara, Turkey.

The extracts used in this study were water (AWa, RWa) and methanol extracts (AMe, RMe) prepared from the aerial parts and roots of A. azurea, respectively. Secondly, the methanol extract of the aerial parts, which exerted good anti-inflammatory activity, was dissolved in distilled water (AMeWa) and then further fractionated with n-hexane (AMeHe) and n-butanol (AMeBu) to give the respective extracts.

Air-dried and powdered aerial parts of the plant (600 g) were extracted three times with 3 L methanol under reflux for 3 h at 45 °C. The total filtrate was concentrated under reduced pressure to yield the methanol extract (AMe, 78 g, yield 13%). The methanol extract was dissolved in distilled water (150 mL) and partitioned with n-hexane (AMeHe, 10.4 g, yield 1.73%) and n-butanol (AMeBu, 7 g, yield 1.17%). The remaining aqueous layer was concentrated and then lyophilized (AMeWa, 15 g, yield 2.50%). Air-dried and powdered roots of the plant (480 g) were also extracted with methanol (5 x 1.5 L) under reflux for 3 h at 45 °C, and the filtrate was evaporated under reduced pressure (RMe, 22 g, yield 4.58%). In addition, aerial parts (100 g) and roots (100 g) were separately extracted with distilled water (2 x 1 L) at 45 °C during 2 h and filtered. Both of the filtrates were concentrated and then lyophilized (AWa, 11 g, yield 11%; RWa, 13 g, yield 13%).

Isolation of rosmarinic acid (RA)

The n-butanol extract prepared from the methanolic extract of the aerial parts of A. azurea was dissolved in methanol (MeOH) and then subjected to gel filtration over Sephadex LH 20 (Fluka, Steinheim, Germany) (200 g) with MeOH as eluant (100-mL fractions). Each fraction was monitored by TLC, and fractions with similar TLC patterns were collected to afford five main fractions (A – E): (A) fractions 1 – 10, 3.5 g; (B) fractions 11 – 14, 0.7 g; (C) fractions 15 – 25, 1.2 g; (D) fractions 26 – 28, 1.0 g; (E) fractions 29 – 32, 0.3 g. Fraction D was applied to a Sephadex LH 20 (40 g) column using MeOH as eluant. Fractions of 15 mL each were collected (D1 – D37). Fractions D1 – D26 were combined to give pure rosmarinic acid (150 mg), and fractions D27 – D37 (870 mg) were combined and applied to further column chromatography over silica gel eluting with CHCl$_3$/MeOH/H$_2$O (80:20:2 v/v/v, 150 mL and 70:30:3 v/v/v, 200 mL) and MeOH (100 mL). After this process, subfractions of D27 – D37 were subjected repeatedly to Sephadex LH-20 column chromatography for obtaining more pure rosmarinic acid (350 mg). Rosmarinic

acid was identified by comparing its NMR and mass data with those given in the literature (Kelley *et al.*, 1976). ^{1}H and ^{13}C NMR spectra were recorded on Varian (Palo Alto, CA, USA) Mercury plus 400 MHz (for proton) and 100 MHz (for carbon) instruments in CD_3OD using tetramethylsilane (TMS) as internal standard. EI mass spectra were recorded on a Finnigan (Waltham, MA, USA) MAT 95 spectrometer.

Animals

Male Wistar albino rats (185–200 g) were used. They were fed with standard laboratory chow and kept at 22 °C. All animals were obtained from the Experimental Animal Laboratory, Department of Pharmacology, Faculty of Medicine, Ataturk University, Ezurum, Turkey. The experiments were conducted according to the ethical norms approved by the Ethics Committee of Experimental Animal Teaching and Researcher Center (No. B.30.2.ATA.0.70/71). The animals were grouped before the experiments and kept under standard conditions. A minimum of six animals was used in each group.

Chemicals and drugs

Carrageenan was purchased from Sigma-Aldrich (Steinheim, Germany). The standard drugs that were used were indomethacin (Deva, Istanbul, Turkey), and ibuprofen and famotidine (Eczacibasi, Istanbul, Turkey). All other chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany).

Carrageenan-induced paw edema in rats

In the first series of our experiment, we investigated the anti-inflammatory activity of the primary extracts (water and methanol) of aerial parts and roots of *A. azurea* (AWa, RWa, AMe, RMe) using doses of 100 and 200 mg/kg body weight (bw) to treat carrageenan-induced paw edema in rats (Winter *et al.*, 1962). In this series, we used 25 mg/kg bw indomethacin as a reference drug. Initially, the rats were divided into 10 groups (*n* = 6), 9 of which received RWa, RMe, AWa, AMe (each at 100 and 200 mg/kg bw), and indomethacin (25 mg/kg bw), respectively, by oral gavage. The control group received an equal volume of distilled water as the vehicle. One h after administration of the extracts, 0.1 mL of 1%

carrageenan was injected into the hind paw of each animal. Before the carrageenan injection, the volume of the normal paw was measured up to the knee joint by plethysmometry. The carrageenan-induced increase in the paw volume (paw edema) was measured four times at 1-h intervals. The anti-inflammatory effects of the drugs were determined by comparing the results of the drug-treated groups with those of the control group. The percentages of the anti-inflammatory activities of the RWa, RMe, AWa, AMe extracts, and control drug (indomethacin) were calculated using the following formula: % inhibition = $(1 - D_t/D_0) \cdot 100$, where D_0 is the average inflammation of the control group of rats at a given time and D_t is the average inflammation of the drug-treated (*i.e.* extracts or reference indomethacin) rats at the same time.

In the second series of our experiments, we investigated the anti-inflammatory activity of different fractions of the AMe extract (AMeHe, AMeWa and AMeBu) and the reference drug indomethacin using the same model. The reason why we studied the fractions of AMe is that it was the most effective extract in the first experiment. The rats were divided into 8 groups. Each group consisted of six animals. There were 7 groups of rats that received RWa, RMe, AWa, AMe (each at 100 and 200 mg/kg bw), and indomethacin (25 mg/kg bw), respectively, by oral gavage. The control group received an equal volume of distilled water as vehicle. In the second series of experiments, the AMeBu extract exerted the highest anti-inflammatory effects; so, in the last series of the anti-inflammatory activity experiments, we investigated the anti-inflammatory effects of rosmarinic acid isolated as a major component from the AMeBu extract. For this series, we selected 7 groups of rats; each group consisted of six animals. The doses of rosmarinic acid, as well as of ibuprofen, both administered by oral gavage, were 10, 25, and 50 mg/kg bw. The last group received an equal volume of distilled water as vehicle. The anti-inflammatory effects of the extracts were determined as described above.

Indomethacin-induced gastric damage in rats

In this series of experiments, the effects of the prepared extracts on the indomethacin-induced gastric damage were analysed. Male Wistar rats fasted for 24 h had free access to water and were,

respectively, treated with the extracts and famotidine (20 mg/kg bw) by oral gavage. After 5 min of oral treatment, indomethacin (25 mg/kg bw) was administered to all groups of animals, and 6 h later the animals were sacrificed to evaluate their stomachs for ulcer areas.

Statistical analysis

Results are presented as mean ± S.D. Statistical calculations were done using SPSS 13.0 software. Data were analysed using one-way ANOVA with post-hoc LSD test. The level of statistical significance was defined to be $p < 0.05$.

Results and Discussion

In the present study, the anti-inflammatory and antiulcer activities related to the wound-healing activity of the extracts from the aerial parts and roots of *A. azurea* Miller var. *azurea* (Boraginaceae) have been investigated. In addition, rosmarinic acid, isolated as the major constituent from the methanolic extract of the aerial parts of *A. azurea* and its *n*-butanol fraction, was found to exhibit significant anti-inflammatory activity. The effects of the extracts and rosmarinic acid on acute carrageenan-induced inflammation and indomethacin-induced gastric damage in rats were investigated as well. As a result of the serial chromatographic separations, rosmarinic acid was isolated and its structure elucidated based on spectroscopic data (UV, IR, ^{1}H NMR, ^{13}C NMR, COSY, HMQC, HMBC, and EIMS) and compared with previously reported data (Kelley *et al.*, 1976) (Fig. 1)

The results of the initial anti-inflammatory activity assay indicated that the methanol extract of aerial parts (AMe) exhibited anti-inflammatory activity (30% at a dose of 200 mg/kg bw, Table I). So we fractionated the AMe extract to its water,

n-hexane, and *n*-butanol extracts. In the second experiment, only the AMeBu fraction, of all AMe fractions, exerted significant anti-inflammatory activity (42% at a dose of 200 mg/kg bw, Table II). The contents of the major compound rosmarinic acid in the AMe and AMeBu fractions were found to be 1.28 and 14.2%, respectively. So we isolated rosmarinic acid from the AMeBu extract and evaluated its anti-inflammatory effect as well.

The activity of rosmarinic acid at a dose of 50 mg/kg bw is comparable to that of indomethacin, a well-known prostaglandin synthesis inhibitor, at 25 mg/kg bw and ibuprofen at 50 mg/kg bw, (Tables I–III). The anti-inflammatory effects of the AMe and AMeBu extracts appear to be related to their rosmarinic acid content. However no antiulcer activity was observed (data not shown).

Acute anti-inflammatory activities of compounds or drugs are generally investigated using an acute aseptic model (paw edema) in which arthritis can be induced by histamine, formalin, dextran, or carrageenan. The carrageenan-induced paw edema is the most widely used primary test for the screening of new anti-inflammatory agents (Winter *et al.*, 1962). The carrageenan-induced inflammatory reaction has two phases: early and late. The sub-plantar injection of carrageenan produces a local edema that increases progressively and reaches its maximum within 3–5 h. It was shown that while the early phase is associated with the release of histamine, serotonin, and bradykinin, the late phase is usually associated with the release of prostaglandin (Ramprasath *et al.*, 2004). In our study, the anti-inflammatory effects of the drugs peaked 3 h after carrageenan injection. This period is accepted as the late phase of carrageenan-induced inflammation. Reaching the peak level of their anti-inflammatory effect after 3 h means that they probably inhibit prostaglandin synthesis. Cyclooxygenase (COX), responsible for prostaglandin synthesis, and lipoxygenase also have a role in carrageenan-induced inflammation. Non-selective COX inhibitors such as indomethacin, ibuprofen, and diclofenac sodium inhibit the COX-1 and COX-2 enzymes (Burke *et al.*, 2006). Indomethacin and ibuprofen are strong non-steroidal anti-inflammatory drugs (NSAIDs) that exert high anti-inflammatory effects (Burke *et al.*, 2006). In our study, the effects of these two NSAIDs used as reference anti-inflammatory agents and those of the AMe and, AMeBu extracts and rosmarinic acid were comparable.
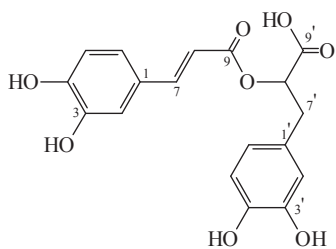

Fig. 1. Chemical structure of rosmarinic acid.

Table I. Effects of water and methanol extracts from roots (RWa, RMe) and aerial parts (AWa, AMe) of *A. azurea* and of indomethacin on carrageenan-induced rat paw edema.

Extract	Dose (mg/kg)	Paw volume [mL]		Increase in inflammatory paw volume [mL]	Anti-inflammatory effect (%)	*p*
		Before inflammation	After 3 h of inflammation			
RWa	100	0.83 ± 0.035	1.55 ± 0.087	0.72 ± 0.026	3	>0.05
RWa	200	0.87 ± 0.032	1.52 ± 0.098	0.65 ± 0.031	12	>0.05
RMe	100	0.75 ± 0.025	1.33 ± 0.056	0.58 ± 0.041	6	>0.05
RMe	200	0.73 ± 0.041	1.29 ± 0.12	0.56 ± 0.036	10	>0.05
AWa	100	0.79 ± 0.023	1.49 ± 0.078	0.70 ± 0.024	5	>0.05
AWa	200	0.79 ± 0.031	1.47 ± 0.068	0.68 ± 0.032	8	>0.05
AMe	100	0.78 ± 0.028	1.37 ± 0.074	0.63 ± 0.13	14	>0.05
AMe	200	0.80 ± 0.045	1.28 ± 0.055	0.52 ± 0.078	30	<0.05
Indomethacin	25	0.80 ± 0.03	1.18 ± 0.047	0.38 ± 0.015	49	<0.05
Control	-	0.84 ± 0.023	1.58 ± 0.099	0.74 ± 0.061	-	-

The results given are means ± S.D.

Table II. Effects of the *n*-hexane (AMeHe), water (AMeWa), and *n*-butanol (AMeBu) extracts, respectively, prepared from the methanol extract of aerial parts of *A. azurea* and of indomethacin on carrageenan-induced rat paw edema.

Extract	Dose (mg/kg)	Paw volume [mL]		Increase in inflammatory paw volume [mL]	Anti-inflammatory effect (%)	*p*
		Before inflammation	After 3 h of inflammation			
AMeHe	100	0.74 ± 0.041	1.53 ± 0.091	0.75 ± 0.052	-	>0.05
AMeHe	200	0.76 ± 0.024	1.48 ± 0.112	0.72 ± 0.046	-	>0.05
AMeWa	100	0.71 ± 0.019	1.46 ± 0.101	0.75 ± 0.034	-	>0.05
AMeWa	200	0.80 ± 0.023	1.48 ± 0.053	0.68 ± 0.063	4	>0.05
AMeBu	100	0.78 ± 0.037	1.32 ± 0.074	0.55 ± 0.073	22	>0.05
AMeBu	200	0.81 ± 0.018	1.21 ± 0.085	0.41 ± 0.075	42	<0.01
Indomethacin	25	0.78 ± 0.04	1.16 ± 0.091	0.38 ± 0.033	47	<0.05
Control	-	0.79 ± 0.025	1.50 ± 0.065	0.71 ± 0.048	-	-

The results given are means ± S.D.

Table III. Effects of rosmarinic acid (RA) and ibuprofen on carrageenan-induced rat paw edema.

Drug	Dose (mg/kg)	Paw volume [mL]		Increase in inflammatory paw volume [mL]	Anti-inflammatory effect (%)	*p*
		Before inflammation	After 3 h of inflammation			
RA	10	0.79 ± 0.03	1.32 ± 0.058	0.53 ± 0.024	13.2	>0.05
RA	25	0.85 ± 0.021	1.21 ± 0.051	0.36 ± 0.022	40	<0.05
RA	50	0.87 ± 0.012	1.20 ± 0.064	0.33 ± 0.019	45.9	<0.05
Ibuprofen	10	0.82 ± 0.025	1.36 ± 0.078	0.54 ± 0.018	11.5	>0.05
Ibuprofen	25	0.84 ± 0.031	1.32 ± 0.066	0.48 ± 0.041	21.4	>0.05
Ibuprofen	50	0.86 ± 0.021	1.27 ± 0.041	0.41 ± 0.032	32.8	<0.05
Control	-	0.79 ± 0.014	1.40 ± 0.087	0.61 ± 0.062	-	-

The results given are means ± S.D.

Rosmarinic acid is an important caffeoyl ester (phenolic depside) with proven medicinal properties and well-characterized physiological functions. The most recent studies on rosmarinic acid activity confirmed that caffeoyl esters, such as rosmarinic acid, exhibit the highest specific antioxidant activity, delay vitamin E depletion, and decrease the production of pro-inflammatory lysophosphatidylcholines (Cartron et al., 2001). rosmarinic acid prevents the oxidation of low-density lipoproteins and its constituents (vitamin E, phosphatidylcholine), which is compatible with its anti-inflammatory and antiatherosclerotic role in pathophysiological conditions (Cartron et al., 2001). Sanbongi et al. (2004) reported that the oral administration of rosmarinic acid is an effective intervention against allergic asthma, and Youn et al. (2003) demonstrated that rosmarinic acid suppressed synovitis in mice and suggested that it may be beneficial in the treatment of rheumatoid arthritis. Unlike antihistamines, rosmarinic acid prevents the activation of immune responder cells that cause swelling and fluid formation. All these data support our conclusion that rosmarinic acid is likely responsible for the anti-inflammatory effects of A. azurea extracts (AMe and its AMe-Bu fraction).

Nevertheless, the n-butanol extract of A. azurea and rosmarinic acid are good candidates for further investigation of their anti-inflammatory activity. In conclusion, our results support the traditional use of Anchusa species in treating inflammatory conditions and wounds.

Acknowledgements

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