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EVALUATION OF ANTI-INFLAMMATORY AND ANTINOCICEPTIVE ACTIVITY OF FIVE ANATOLIAN *ACHILLEA* SPECIES

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Abstract

Achillea species have been used for their anti-inflammatory, analgesic, spasmolytic, hemostatic, digestive and cholagogue effects in Turkish folk medicine. In order to evaluate their folkloric utilization, both antinociceptive and anti-inflammatory activities of five Achillea species including Achillea wilhelmsii C.Koch, A. setacea Waldst&Kit, A. vermicularis Trin., A. phrygia Boiss.&Bal. and A. sipikorensis Hausskn. et Borm. were investigated. For the antinociceptive activity, p-benzoquinone-induced writhing test and for the anti-inflammatory activity, carrageenan-induced hind paw edema model in mice were employed. The ethanol extracts of A. wilhelmsii, A. setacea and A. vermicularis showed significant antinociceptive and anti-inflammatory activity at 500 mg/kg dose, per os, without inducing any apparent acute toxicity as well as gastric damage. A. phrygia was shown to possess only significant antinociceptive activity, on the other hand A. sipikorensis did not show any remarkable anti-inflammatory and antinociceptive activity.

Key words: *Anti-inflammatory activity, Antinociceptive activity, Achillea phrygia, Achillea wilhelmsii, Achillea setacea, Achillea sipikorensis, Achillea vermicularis, Asteraceae*

Anadolu'da Yetişen Beş *Achillea* Türünün Antinosiseptif ve Anti-Enflamatuvar Aktivitelerinin Değerlendirilmesi

Achillea türleri Türkiye'de halk arasında, anti-enflamatuvar, analjezik, spazmolitik, hemostatik, dijestif ve kolagog etkilerinden dolayı kullanılmaktadır. Folklorik kullanımı değerlendirmek amacıyla, beş Achillea türünün "Achillea wilhelmsii C.Koch, A. setacea Waldst&Kit, A. vermicularis Trin., A. phrygia Boiss.&Bal. and A. sipikorensis Hausskn. et Borm." antinonsiseptif ve anti-enflamatuvar aktiviteleri çalışıldı. Antinosiseptif aktivite için p-benzokinon nedenli kıvranma testi, anti-enflamatuvar aktivite için karragen-nedenli arka ayak ödemi testi uygulandı. A. wilhelmsii, A. setacea ve A. vermicularis'ten hazırlanan etanollü ekstre 500 mg/kg dozda herhangi bir akut toksisite ve gastrik hasar oluşturmaksızın kuvvetli antinosiseptif ve anti-enflamatuvar aktivite gösterdi. A. phrygia sadece kuvvetli antinosiseptif aktivite gösterirken A.sipikorensis herhangi bir anti-enflamatuvar ve antinosiseptif aktivite göstermedi.

Anahtar Kelimeler: *Antienflamatuvar aktivite, Antinosiseptif aktivite, Achillea phrygia, Achillea wilhelmsii, Achillea setacea, Achillea sipikorensis, Achillea vermicularis, Asteraceae*

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INTRODUCTION

The genus *Achillea* (Asteraceae), named after the mythological Greek warrior Achilles, comprises of approximately 85 species, most of which are endemic to Europe and the Middle East. Turkish flora possesses 40 *Achillea* species and 20 of them are endemic (1). On the other hand, some *Achillea* species have been known to be ethnopharmacologically used in folk remedies for various purposes such as haemorrhoid and wound healing (2). Especially, *A. millefolium* is frequently used against diarrhea, abdominal pain and stomachache in Turkish traditional medicine (3-5).

Several biological activity studies have been performed on various *Achillea* species, including antibacterial, antioxidant, anti-inflammatory and antispasmodic activities (6-10). Moreover, *Achillea* species are well-known to contain essential oil and their chemical compositions as well as antimicrobial activities have been well studied (7,9,11-15). According to the literature survey; there is no data carried out on the anti-inflammatory and antinociceptive activity of five *Achillea* species (*Achillea wilhelmsii*, *A. setacea*, *A. vermicularis*, *A. phrygia* and *A. sipikorensis*) growing in Turkey.

In our ongoing study on medicinal plants used in Turkish traditional medicine for the treatment of rheumatism and related inflammatory diseases, the objective of this study was to undertake the screening of five *Achillea* species in order to elucidate traditional use of these plants from the scientific point of view. The ethanolic and aqueous extracts prepared from the mentioned plants were tested in mice for anti-inflammatory activity using carrageenan-induced hind paw edema model and for antinociceptive activity using *p*-benzoquinone- induced abdominal contractions.

EXPERIMENTAL

Plant materials

Plant materials were collected from different localities in Turkey. Voucher specimens were authenticated by Prof. Dr. Hayri Duman of Department of Biology, Faculty of Science & Art, Gazi University, Ankara (Turkey) and were deposited in the Herbarium of Faculty of Pharmacy, Gazi University, Ankara (Turkey). Collection sites, parts used and herbarium numbers of the selected *Achillea* species as the subject of this study are listed in Table 1.

Preparation of plant extracts

Each plant material was dried under shade and powdered to a fine grade by using a laboratory scale mill. The plant parts and the extract yields (w/w) are given in Table 1. The extracts were prepared as given below:

Ethanolic (EtOH) extract: Dried plant material (10 g) was extracted with 90 % EtOH at room temperature for two times (x 200 ml). The combined ethanolic extracts were evaporated to dryness *in vacuo* to give crude extract.

Aqueous (H_2O) extract: Dried plant material (10 g) was extracted with distilled water at room temperature for two times (x 200 ml). The combined aqueous extracts were lyophilized to give the crude extract.

Table 1. The collection of plant parts, collection sites, herbarium numbers and percentage yields of EtOH and H_2O extracts of *Achillea* species

Plant name	Collected parts	Collection sites	Herbarium numbers	EtOH extract (w/w, %)	H_2O extract (w/w, %)
A.phrygia Boiss.&Bal.	Herb	Aksaray vicinity	GUE 2331	59.8	35.4
A.wilhelmsii C.Koch	Herb	Ankara-Kızılcahamam, Güvem village	GUE 2335	61.1	39.7
A.setacea Waldst&Kit	Herb	Ankara-Ahlatlıbel	GUE 2338	45.5	33.9
A.sipikorensis Hausskn. et Borm	Herb	Kayseri, Pınarbaşı, Eğrisöğüt village, A.Bey-Çayırköyü	AEF 22168	52.7	44.2
A.vermicularis Trin.	Herb	Van, Güzeldere passage	AEF 23170	63.9	46.1

Pharmacological procedures

Animals

Male Swiss albino mice (20-25 g) were purchased from the animal breeding laboratories of Refik Saydam Central Institute of Health (Ankara, Turkey). The animals left for two days for acclimatization to animal room conditions were maintained on standard pellet diet and water *ad libitum*. The food was withdrawn on the day before the experiment, but allowed free access of water. A minimum of six animals was used in each group. Throughout the experiments, animals were processed according to the suggested ethical guidelines for the care of laboratory animals (Gazi University Ethical Council Project Number: G.Ü.ET-05.004).

Preparation of test samples for bioassay

All the plant extracts were given orally to test animals in both 250 and 500 mg/kg doses after suspending in a mixture of distilled H_2O and 0.5% sodium carboxymethyl cellulose (CMC). The control group animals received the same experimental handling as those of the test groups except that the drug treatment was replaced with appropriate volumes of the dosing vehicle. Either indomethacin (10 mg/kg) or acetyl salicylic acid (ASA) (100 mg/kg and 200 mg/kg) in 0.5 % CMC was used as reference drug.

Antinociceptive activity

p-Benzoquinone-induced writhing test

p-Benzoquinone-induced abdominal constriction test was performed on mice for determination of antinociceptive activity (16). According to the method; 60 min after the oral administration of test samples, the mice were intraperitonally injected with 0.1 ml/10 g body weight of 2.5 % (w/v) *p*-benzoquinone (PBQ; Merck) solution in distilled H_2O. Control animals

received an appropriate volume of dosing vehicle. The mice were then kept individually for observation and the total number of abdominal contractions (writhing movements) was counted for the next 15 min, starting on the 5th min after the PBQ injection. The data represent average of the total number of writhes observed. The antinociceptive activity was expressed as percentage change from writhing controls. Aspirin (ASA) at 100 mg/kg and 200 mg/kg doses was used as the reference drug in this test.

Anti-inflammatory activity

Carrageenan-induced hind paw edema test

Carrageenan-induced hind paw edema model was used with modifications in measuring periods for determination of anti-inflammatory activity (17). The difference in footpad thickness between the right and left foot was measured with a pair of dial thickness gauge calipers (Ozaki Co., Tokyo, Japan). Mean values of treated groups were compared with mean values of a control group and analyzed using statistical methods. 60 min after the oral administration of test sample or dosing vehicle, each mice was injected with freshly prepared (0.5 mg/25 µl) suspension of carrageenan (Sigma, St.Louis, Missouri, USA) in physiological saline (154 nM NaCl) into subplantar tissue of the right hind paw. As the control, 25 µl saline solutions were injected into that of the left hind paw. Paw edema was measured in every 90 min during 6 h after induction of inflammation. The difference in footpad thickness was measured by a gauge calipers (Ozaki Co., Tokyo, Japan). Mean values of treated groups were compared with mean values of a control group and analyzed using statistical methods. Indomethacin (10 mg/kg) was used as the reference drug.

Acute toxicity

Animals employed in the carrageenan-induced paw edema experiment were observed during 48 h and morbidity or mortality was recorded, if happens, for each group at the end of observation period.

Gastric-ulcerogenic effect

After the antinociceptive activity experiment, mice were killed under deep ether anesthesia and stomachs were removed. Then the abdomen of each mouse was opened through the greater curvature and examined under dissecting microscope for lesions or bleedings.

Statistical analysis of data

Data obtained from animal experiments were expressed as mean standard error (±SEM). Statistical differences between the treatments and the control were evaluated by ANOVA and Students-Newman-Keuls post-hoc tests. $p<0.05$ was considered to be significant [* $p<0.05$; ** $p<0.01$; *** $p<0.001$].

RESULTS AND DISCUSSION

Five *Achillea* species have been evaluated for their *in vivo* anti-inflammatory and antinociceptive activities. The ethanolic and aqueous extracts were prepared from each species and their inhibitory effects on *p*-benzoquinone-induced writhing for the assessment of antinociceptive activity and carrageenan-induced hind paw edema model, a widely used screening protocol for anti-inflammatory activity to test the non-steroidal anti-inflammatory drugs, were examined in mice. Results of both assays are given in Tables 2 and 3, respectively.

The most widely used primary test for the screening of new anti-inflammatory agents is the carragenan-induced oedema in the rat hind paw (18). Establishment of oedema depends on the participation of kinins and polymorphonuclear leukocytes with their proinflammatory factors, including prostaglandins. The development of oedema in the paw of the mice, after the injection of carragenan, has been described by Vinegar et al. as a biphasic event. The initial phase, observed during the first hour, is attributed to the release of histamine and serotonin; the second one is due to the release of prostaglandin-like substances (19). It has been reported that the second phase of oedema is sensitive to both steroidal and non-steroidal anti-inflammatory agents (20). Based on this, it could be argued that the significant activity observed in the suppression of the first phase of carrageenan-induced inflammation may be due to inhibition of

the release of early mediators, such as histamine and serotonin, and the action on the second phase may be explained by an inhibition of cyclooxygenase.

As shown in Table 2, the ethanol extracts of A. wilhelmsii, A. setacea and A. vermicularis induced inhibition against carrageenan-induced inflammation which are dose-dependent over a range of 250-500 mg/kg, The result was quite comparable to indomethacin, reference sample, and were found to act in both phases of acute inflammation considerably.

In *p*-benzoquinone-induced writhing test exploited in the present study for the evaluation of antinociceptive activity on mice, which is known to lack of limitations such as that drugs other than analgesic will inhibit writhing; these include antihistamines, sympathomimetics and parasympathomimetics, central nervous system stimulants and adrenergic blockers. Such limitations are reported mainly for Koster test, which acetic acid solution is administered to induce writhings.

As shown in Table 3, the ethanol extracts of *A. wilhelmsii, A. setacea* and *A. vermicularis* displayed significant antinociceptive and anti-inflammatory activity at 500 mg/kg dose, *per os*, without inducing any apparent acute toxicity as well as gastric damage. *A. phrygia* was shown to possess only significant antinociceptive activity, on the other hand *A. sipikorensis* did not show any remarkable anti-inflammatory and antinociceptive activity.

The acute toxicity assessment has revealed that all extracts were safe in the administered doses.

Achillea species have been so far reported to contain sesquiterpenes, diterpenes, flavonoids, lignans, essential oil, and rarely triterpenes (13,21-29). There have been a few studies on anti-inflammatory and antinociceptive activities of some *Achillea* species (30-31), however, many studies were reported on the phytochemical contents of these species. *A. vermicularis* was shown to have guaianolide- and germacrene-type sesquiterpenes as well as flavonoids (24). *A. setacea* was found to contain sesquiterpenes, essential oil and flavonoids, while there are a few studies on *A. wilhemsii* (15,26,29,32,33).

On the other hand, sesquiterpene lactones, widely distributed in Asteraceae family, are known to possess anti-inflammatory activity by various mechanisms of action (34-38). In a previous study, it was shown that sesquiterpene lactones selectively inhibited DNA binding of transcription factors which control evolution of inflammation (35,39,40). Besides, in a number of studies, sesquiterpenes were reported to modulate many inflammatory processes including oxidative phosphorylation, platelet aggregation, the release of histamine from mast cells, serotonine from blood platelets as well as the rat paw and mouse-ear edema (34,41). It was also cleared that α-methylene-γ-lactone group was necessary for anti-inflammatory activity of sesquiterpene lactones in carrageenan-induced edema model (41).

Table 2. Effects of the extracts against carrageenan-induced paw edema in mice.

Material	Extract type	Dose mg/kg	Swelling thickness (x10^{-2}mm) ± SEM (% inhibition)			
			90 min	180 min	270 min	360 min
Control			45.0 ± 5.9	50.2 ± 5.7	57.7 ± 6.2	64.0 ± 5.7
Achillea phrygia Boiss.&Bal.	W	250	49.1 ± 4.4	52.7 ± 4.7	58.4 ± 5.2	63.4 ± 4.7
	W	500	38.5 ± 5.6 (14.4)	42.0 ± 5.5 (16.3)	44.8 ± 5.8 (22.4)	48.5 ± 5.9 (24.2)
	E	250	38.9 ± 4.0 (13.6)	41.7 ± 4.8 (16.9)	47.8 ± 5.0 (17.29	52.2 ± 4.5 (18.4)
	E	500	35.4 ± 4.7 (21.3)	39.3 ± 4.4 (21.7)	45.2 ± 4.6 (21.7)	49.0 ± 5.2 (23.4)
Achillea wilhelmsii C.Koch	W	250	39.4 ± 4.1 (12.4)	43.1 ± 4.3 (14.1)	48.6 ± 5.1 (15.8)	52.4 ± 4.4 (18.1)
	W	500	36.3 ± 5.2 (19.3)	39.7 ± 5.3 (20.9)	42.0 ± 3.1 **(27.2)***	45.3 ± 3.2 **(29.2)***
	E	250	37.3 ± 5.2 (17.1)	40.2 ± 5.4 (19.9)	45.7 ± 6.1 (20.8)	49.2 ± 5.39 (23.1)
	E	500	27.4 ± 3.2 **(39.1)***	31.7 ± 2.9 **(36.9)***	36.3 ± 3.3 **(37.1)***	40.6 ± 3.2 **(36.6)***
Achillea setacea Waldst&Kit	W	250	57.2 ± 4.7	59.1 ± 5.2	66.2 ± 5.8	71.8 ± 3.9
	W	500	49.3 ± 8.5	51.8 ± 8.7	55.2 ± 8.9 (4.3)	59.2 ± 8.7 (7.5)
	E	250	43.8 ± 5.9 (2.7)	45.4 ± 5.8 (9.6)	50.3 ± 7.1 (12.8)	53.4 ± 6.9 (16.6)
	E	500	33.7 ± 3.7 (25.1)	38.5 ± 3.6 (23.3)	38.8 ± 3.9 **(32.8)***	44.1 ± 4.2 **(31.1)***
Achillea sipikorensis Hausskn. et Borm	W	250	47.7 ± 5.1	53.3 ± 5.1	61.1 ± 5.4	67.7 ± 4.8
	W	500	40.9 ± 4.5 (9.1)	46.5 ± 3.6 (7.4)	51.8 ± 4.2 (10.2)	57.5 ± 4.1 (10.2)
	E	250	42.8 ± 6.9 (4.9)	45.7 ± 5.6 (8.9)	49.5 ± 5.7 (14.2)	54.0 ± 5.7 (15.6)
	E	500	36.7 ± 6.8 (18.4)	41.5 ± 5.8 (17.3)	47.9 ± 5.2 (16.9)	51.2 ± 5.3 (20.0)
Achillea vermicularis Trin.	W	250	43.3 ± 41 (3.8)	48.0 ± 3.8 (4.4)	54.6 ± 3.2 (5.4)	59.1 ± 2.7 (7.7)
	W	500	36.5 ± 6.6 (18.9)	40.1 ± 4.4 (20.1)	44.9 ± 4.7 (22.2)	48.3 ± 4.1 (24.5)
	E	250	33.9 ± 4.4 (24.7)	35.1 ± 2.9 **(30.1)***	40.4 ± 2.9 **(29.9)***	41.1 ± 3.3 **(35.8)****
	E	500	32.6 ± 4.4 (27.6)	32.6 ± 2.6 **(35.1)***	35.9 ± 3.7 **(37.8)****	40.1 ± 3.7 **(37.3)****
Indomethacin		10	29.5 ± 3.5 **(34.4)***	30.3 ± 3.3 **(39.6)****	35.0 ± 2.7 **(39.3)*****	38.3 ± 3.9 **(40.2)*****

*:p<0.05, **:p<0.01, ***:p<0.001 ; SEM: standard error mean
Abbreviations: W: water extract; E: ethanol extract

Table 3. Effect of the materials against *p*-benzoquinone-induced writhings in mice

Material	Extract type	Dose mg/kg	Number of writhings ± SEM	Inhibitory ratio (%)	Ratio of ulceration
Control			53.8 ± 5.9		0/6
Achillea phrygia Boiss.&Bal.	W	250	51.4 ± 6.2	4.5	0/6
	W	500	45.6 ± 4.5	15.2	0/6
	E	250	46.2 ± 5.1	14.1	0/6
	E	500	32.5 ± 3.6	**39.6****	0/6
Achillea wilhelmsii C.Koch	W	250	46.9 ± 8.3	12.8	0/6
	W	500	41.9 ± 5.4	22.1	0/6
	E	250	38.4 ± 4.5	28.6	0/6
	E	500	33.0 ± 2.0	**38.7****	0/6
Achillea setacea Waldst&Kit	W	250	52.6 ± 5.6	2.2	0/6
	W	500	50.8 ± 5.6	5.6	0/6
	E	250	44.1 ± 4.9	18.0	0/6
	E	500	36.3 ± 2.9	**32.5****	0/6
Achillea sipikorensis Hausskn. et Borm	W	250	47.9 ± 4.4	10.9	0/6
	W	500	44.8 ± 4.54	16.7	0/6
	E	250	44.3 ± 5.7	17.7	0/6
	E	500	40.8 ± 6.4	24.2	0/6
Achillea vermicularis Trin.	W	250	46.3 ± 5.8	13.9	0/6
	W	500	42.8 ± 4.4	20.4	0/6
	E	250	39.5 ± 4.7	26.7	0/6
	E	500	38.7 ± 4.2	**28.1***	0/6
ASA		100	28.7 ± 3.2	**46.6*****	3/6
		200	22.3 ± 3.0	**58.6*****	5/6

*:p<0.05, **:p<0.01, ***:p<0.001 ; SEM: standard error mean
Abbreviations: W: water extract; E: ethanol extract

Moreover, a wide range of essential oils have been reported to have anti-inflammatory and antinociceptive actions (42). Additionally, anti-inflammatory effect was also found in the components analyzed in the essential oils such as α-pinene, β-caryophyllene and 1,8-cineole. In another study, essential oil of *Lavandula hybrida* along with its main principles linalool and linalyl acetate was found to display potent analgesic activity after the oil inhalation (43). The results of another study carried out by Baylac and Racine on a number of essential oils against human leukocyte elastase (HLE), a protease which take place in the pathogenesis of inflammatory diseases, revealed that benzoin resinoid and turmeric oleoresin were potent inhibitors of HLE (44). Three *Eucalyptus* species were also reported to have anti-inflammatory and analgesic effects due to their essential oils (45). Iscan et al. found that *Achillea schisckinii* oil were not showed inhibitory effect, but *Achillea aleppica* subsp. *aleppica* oil was found to possess potent anti-inflammatory activity without inducing any apparent acute toxicity or gastric

damage in 200 mg/kg dose. The oil of *Achillea aleppica* subsp. *aleppica* contains 6.6% of α-bisabolol and its derivatives. These compounds may be partially responsible for anti-inflammatory activity of the oil. Their content in *Chamomile* oil is known to possess this activity exceeds 50% (46-48) and *A. aleppica* subsp. *aleppica* essential oil inhibited the writhes but was not as potent as ASA (49). Several GC-MS analysis studies on the essential oil composition on some *Achillea* species were reported. Essential oil of *A. clevannae* contains camphor, α-pinene, 1,8-cineole and linalool as main principles (9). *A. millefolium* oil consists of a number of monoterpenes such as α-pinene, β-pinene, 1,8-cineole, camphor, borneol as well as some sesquiterpene lactones of germacrene-derivative (28). 1,8-cineole was elucidated to be the major component in both *A. setacea* and *A. teretifolia* essential oils (15). In another study on essential oils of 10 *Achillea* species (*A. biserrata*, *A. clypeotala*, *A. crithmifolia*, *A. filipendula*, *A. macrophylla*, *A. pannonica*, *A. pyrenaica*, *A. sibirica*, *A. taygetea*, *A. tenuifolia*) were found to contain α-pinene, 1,8-cineole and camphor as well as germacrene D and bisabolene as the major constituents (25).

According to our results, all the ethanolic extracts of plants were shown to possess significant antinociceptive activity at 500 mg/kg dose, except that the aqueous and ethanol extracts from *A. sipikorensis* herbs. The ethanolic extracts from *A. wilhelmsii*, *A. setacea* and *A. vermicularis* exhibited potent anti-inflammatory activity against carrageenan-induced hind paw edema model in mice without inducing any gastric damage. The different behavior of the extracts might be possibly due to their phytochemical contents. To best of our knowledge, the present study is the first report on anti-inflammatory and antinociceptive activities of the mentioned *Achillea* species grown in Turkey. Therefore, we point to fact that *Achillea* species may reduce the risk of inflammation-related diseases that support their folkloric utilization. However, further studies must be conducted in order to clarify which constituent(s) of the extracts is responsible for these activities.

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