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Comparative studies on anti-inflammatory and antidiabetic effects of some *Achillea* species from the Turkish flora

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ABSTRACT: This study aimed to determine antioxidant, antidiabetic and anti-inflammatory activities of methanol extracts of *Achillea crithmifolia* Waldst. & Kit. (AC), *A. millefolium* L. (AM), *A. nobilis* subsp. *neilreichii* (A.Kern.) Velen. (AN), *A. setacea* Waldst. & Kit. (AS) and *A. wilhelmsii K. Koch* (AW) species which grow naturally in Türkiye. DPPH/ABTS radicals scavenging, α-glucosidase inhibition and anti 5-lipoxygenase methods were used to determine for antioxidant, antidiabetic and anti-inflammatory activities, respectively. The total phenol content of the extracts was calculated using Folin-Ciocalteau solution and the highest phenol content was found in AC methanol extract (ACM) (56.71 mg GAE/g). In DPPH and ABTS radical scavenging activity assays, ACM showed the highest antioxidant activity according to IC_{50} values (54.85 and 79.77 µg/mL, respectively). According to the results of α-glucosidase inhibitory activity test, AN methanol extract (ANM) with an IC_{50} value of 245.50 µg/mL showed a better activity than acarbose (261.7 µg/mL) by inhibiting the enzyme. As a result of anti-inflammatory activity, it was observed that ACM had the highest activity (90.30 µg/mL). Therefore, ACM, which has the best antioxidant, anti 5-lipoxygenase activity and the second best value close to the standard in terms of α-glucosidase inhibitory activity, was fractionated with 3 different solvents (hexane-ACH, chloroform-ACC and ethyl acetate-ACEA). Biological activity studies were repeated on these fractions. The ACH fraction showed the best anti-inflammatory activity while the ACEA fraction showed the best antidiabetic activity. The biological activities of five *Achillea* species were not examined before by comparing with each other. In addition, antidiabetic and anti-inflammatory activity studies on *A. crithmifolia* were conducted for the first time.

KEYWORDS: *Achillea crithmifolia*; Anti 5-lipoxygenase; α-glucosidase inhibition; Anti-inflammatory; Antidiabetic.

1. INTRODUCTION

Health is a state of balance. This state of balance must be maintained in order to prevent the events that occur in our body, which may have negative consequences if they do not occur. Disturbances in this balance cause various diseases. Diabetes and chronic inflammation are progressive diseases in which this state of balance is disrupted. The incidence of diabetes is increasing every year around the world. It is known that oxidative stress and chronic inflammation cause disruption of glucose metabolism. In addition to this situation, it is thought that impaired fasting glucose may cause oxidative stress and chronic inflammation. Despite this, today there are not many alternative compounds that can be used for the treatment of these types of disorders, which are the cause and effect of each other. Additionally, the compounds used have the potential for side effects [1-4]. Asteraceae is the most diverse and largest family of the Angiosperm family. Members of this family can be found throughout the world, being common in arid and semiarid environments in subtropical and low temperate latitudes. Many species of the Asteraceae family are used in traditional medicine worldwide. The family Asteraceae includes mainly the genus *Achillea* and has 110-140 species worldwide. There are 61 taxa in the genus *Achillea*, 34 of which are endemic to Türkiye. The Achillea genus is one of the important medicinal plant groups and is frequently used in traditional folk medicine [1].

Natural resources have a special role in both the treatment and protection of diseases from the earliest periods of human history to the present day. Therefore, in parallel with the desire to live healthier and longer all over the world, the use of medicinal herbal products, food supplements and herbal medicines has become increasingly important. *Achillea* species are widely used in pharmaceutical and industrial fields due to their

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widespread use among the public, and their many pharmacological effects. *Achillea* species are generally used in traditional folk medicine in Turkey for rheumatic diseases, respiratory diseases, diabetes and high cholesterol, cancer and hemorrhoid treatment. Additionally, its uses in skin diseases, sexual disorders (menstrual problems, menopause, infertility, milk enhancer, prostate, sexual potency enhancer), gastrointestinal disorders and as nervous system sedative are also recorded [5]. Names such as "Ayvadana" and "Civanperçemi" have been attributed to *Achillea* species in different regions of Turkey [4]. The use of *A. millefolium* extracts as biological additives in concentrations ranging from 0.5% to 2% in the formulation of 65 cosmetic products has been approved by Commission E [6]. In this study, it was aimed to determine the status of *A. millefolium*, which is considered a medicinal species and is included in the composition of many cosmetic products, compared to other *Achillea* species growing in Türkiye in terms of biological activity and phenolic compounds content.

In this study, antioxidant, antidiabetic and anti-inflammatory activities of *Achillea crithmifolia* Waldst. & Kit. (AC), *A. millefolium* L. (AM), *A. nobilis* L. subsp. *neilreichii* (A.Kern.) Velen (AN), *A. setacea* Waldst. & Kit. (AS) and *A. wilhelmsii* K. Koch (AW) species were evaluated. Also, the total amounts of phenol and flavonoid compounds in these species were determined. The biological activities of five *Achillea* species were not examined before by comparing with each other. Additionally, studies of antidiabetic and anti-inflammatory activity on *A. crithmifolia* were carried out for the first time.

2. RESULTS

Low IC_{50} value (concentration that eliminates 50% of the radical or stops the activity of the enzyme by 50%) indicates high activity. When Table 1 is examined, ACM and ANM with IC_{50} values of 298.60 and 245.50 µg/mL are more effective than the other species against α-glucosidase enzyme. ACM showed the highest antioxidant activity against DPPH and ABTS radicals with IC_{50} values of 54.85 and 79.77 µg/mL, respectively. In the anti-inflammatory activity experiment, ACM showed the highest antilipoxygenase activity with an IC_{50} value of 90.30 µg/mL. ACM had the highest total phenol content with 56.71 mg/g, followed by ANM (49.88 mg/g), ASM (15.05 mg/g), AWM (10.92 mg/g) and AMM (7.40 mg/g) respectively. The highest total flavonoid content was 35.87(mg/g) in ACM, followed by ANM (32.93 mg/g), ASM (8.72 mg/g), AWM (6.28 mg/g) and AMM (5.96 mg/g), respectively. These results show that the reason why ACM has a good antioxidant activity can be explained by its high total phenol and flavonoid content. Anti-lipoxygenase activity values are listed as ACM> ANM> AWM> ASM> AMM. α-glucosidase inhibitor activity values are listed as ANM> ACM> ASM> AWM> AMM.

Table 1. Anti-inflammatory, antioxidant, antidiabetic activities and total compound contents of various extracts obtained from *Achillea* L. species

Assays	DPPH (IC_{50}, µg/mL)	ABTS (IC_{50}, µg/mL)	Anti 5-LOX (IC_{50}, µg/mL)	α-glucosidase inhibition (IC_{50}, µg/mL)	TPC (mg GAE/g extract)	TFC (mg QE/g extract)
ACM	54.85± 0.52^{b}	79.77± 0.04^{b}	90.30± 1.75^{b}	298.6± 2.76^{a}	56.71± 0.42^{a}	35.87± 0.31^{a}
AMM	532.50± 10.68^{f}	391.90± 2.05^{e}	471.60± 6.86^{d}	1201.0± 2.12^{d}	7.40± 0.05^{e}	5.96± 0.00^{b}
ANM	116.30± 3.04^{c}	116.80± 1.06^{c}	99.06± 1.31^{b}	245.50± 0.21^{a}	49.88± 2.23^{b}	32.93± 0.00^{a}
ASM	284.10± 8.91^{d}	340.00± 1.56^{e}	442.40± 1.13^{d}	494.70± 0.50^{b}	15.05± 1.86^{c}	8.72± 0.08^{b}
AWM	424.60± 0.00^{e}	280.60± 3.11^{d}	349.70± 0.57^{c}	724.20± 0.50^{c}	10.92± 2.17^{d}	6.28± 0.00^{b}
Ascorbic acid	40.23± 2.08^{a}					
Trolox		4.51± 0.07^{a}				
Indomethacin			21.4± 0.48^{a}			
Acarbose				261.70± 1.84^{a}		

* ACM, AMM, ANM, ASM, AWM: methanol extracts of *A. crithmifolia, Achillea millefolium, A. nobilis.* subsp. *neilreichii, A. setacea and A. wilhelmsii*, respectively.
** Total phenolic content was expressed as gallic acid equivalent (GAE).
*** Total flavonoid content was expressed as quercetin equivalent (QE).
**** Each value in the table is represented as mean ± SD (n=3). Different letter superscripts in the same line indicate significant differences ($p<0.05$).

This data showed that ACM has a remarkable antioxidant and anti-inflammatory activity, and ANM has a significant antidiabetic activity. Therefore, ACM, which has the best antioxidant, anti-lipoxygenase activity and the second best value close to the standard in terms of α-glucosidase activity, was fractionated with 3 different solvents (hexane, chloroform and ethyl acetate). Biological activity studies were repeated on these fractions.

Approximately 1.6 g of the most active ACM extract was weighed and suspended in water to obtain hexane, chloroform, ethyl acetate and water fractions respectively. The solvents in the resulting fractions were evaporated to dryness in a rotary evaporator at a temperature not exceeding 45-50 degrees. The yields of ACH, ACC, ACEA and ACW over ethanol extract were found to be 12.52, 18.58, 11.20 and 57.70%, respectively.

When Table 2 is examined; In the anti-inflammatory activity experiment, ACH showed the highest antilipoxygenase activity with an IC_{50} value of 35.02 µg/mL, while in the antidiabetic activity experiment, ACEA showed the best α-glucosidase inhibitor activity with an IC_{50} value of 222.10 µg/mL. At the same time, the activities of the extracts were below standards in all activity experiments except the α-glucosidase inhibitory activity value of ACEA. Anti-lipoxygenase activity values are listed as ACH> ACC> ACEA> ACW. α-glucosidase inhibitor activity values (IC_{50}, µg/mL) are listed as ACEA> ACH> ACC> ACW. These results show that it can be concluded that ACH and ACEA have good anti-inflammatory and antidiabetic activity, respectively.

Table 2. Anti-inflammatory, antidiabetic activities of various extracts obtained from *Achillea crithmifolia*

Assays	Anti 5-LOX (IC_{50}, µg/mL)	α-glucosidase inhibition (IC_{50}, µg/mL)	TPC (mg GAE/g extract)	TFC (mg QE/g extract)
ACH	35.02± 0.73^{a}	542.30± 2.76^{b}	20.76± 0.11^{c}	12.58± 0.07^{c}
ACC	89.51± 1.73^{b}	660.20± 4.10^{c}	65.29± 1.11^{c}	18.06± 0.33^{c}
ACEA	113.50± 1.56^{c}	222.10± 0.42^{a}	191.30± 0.42^{a}	97.97± 0.53^{a}
ACW	135.40± 0.92^{c}	853.20± 2.19^{d}	100.60± 1.1^{b}	40.59± 0.00^{b}
Indomethacin	21.4± 0.48^{a}			
Acarbose		261.70± 1.84^{a}		

* ACH, ACC, ACEA, ACW: Hexane, chloroform, ethyl acetate and water fractions of *A. crithmifolia*, respectively.
** Total phenolic content was expressed as gallic acid equivalent (GAE).
*** Total flavonoid content was expressed as quercetin equivalent (QE).
**** Each value in the table is represented as mean ± SD (n=3). Different letter superscripts in the same line indicate significant differences ($p<0.05$).

3. DISCUSSION

According to the detailed literature review, no activity studies on antilipoxygenase of *A. crithmifolia* species were found. However, there are studies on different *Achillea* species. In one of these studies, the hexane extract obtained from the above-ground parts of *A.sieheana* was found to have an IC_{50} value of 168 µg/mL against 5-lipoxygenase enzyme. It was found to be lower compared to the value of ACH (35.02 µg/mL) in current study [7]. At the same time, the α- glucosidase inhibitory activity of *A. crithmifolia* was not found in the literature. However, there are studies on different *Achillea* species. In one of these studies, it was reported that the ethyl acetate extract of *A. wilhelmsii* showed inhibitory activity against α-glucosidase enzyme with an IC_{50} value of 774 µg/mL. This result was found to be lower than the α-glucosidase activity of ACH (222.10 µg/mL) in the current study [7]. In conclusion, the different results in both antidiabetic and anti-inflammatory activity test may be due to the difference in species and extraction method.

When the literature was examined, polymethoxy flavone-derived compounds and phenolic compounds were identified in hexane and ethyl acetate extracts of different *Achillea* species, respectively [8,9]. Flavone and flavanol derivative compounds; sesquiterpene lactone derivatives (especially guaiane type); coumarins, lignans, amino acids, quinic acid and its derivatives; Essential oil components (terpenes: camphor, borneol, 1,8-cineole, sabinene, linalool) were isolated from *Achillea* species [10-23].

Phenolic compounds such as phenolic acids and flavonoids have been reported to have antidiabetic effects [24]. It has also been suggested that terpenic compounds are important anti-inflammatory agents [25]. Therefore, it can be thought that especially phenolic compounds are responsible for the antidiabetic effect of ACEA, and especially apolar compounds are responsible for the anti-inflammatory effect of ACH.

In traditional medicine, *Achillea* species are used in the treatment of diseases caused by inflammation. *Achillea millefolium* L. is approved by the EMA (European Medicines Agency) for healing various inflammations [26]. The findings of this study suggest that the activity results of the other four *Achillea* extracts examined were better than those of *A. millefolium*, which is considered a medicinal species.

4. CONCLUSION

The results of this study confirm the traditional use of *Achillea* species. In addition, antidiabetic and anti-inflammatory activity studies were conducted on *Achillea crithmifolia* for the first time, revealed the importance of the antidiabetic and anti-inflammatory activity of this plant compared to other *Achillea* species examined.

5. MATERIALS AND METHODS

A. crithmifolia Waldst. & Kit. (MARE-11895, Kırklareli), *A. setacea* Waldst. & Kit. (MARE-11893, Bolu), *A. wilhelmsii* K. Koch (MARE-11888, Ankara-Polatlı) and *A. nobilis* L. subsp. *neilreichii* (A. Kern.) Velen (MARE-11509, Bursa-Uludağ) were identified by Prof. Dr. Ertan Tuzlacı, also *Achillea millefolium* L. (MARE No-13212, İstanbul-Çatalca) were identified by Prof. Dr. Gizem Emre. Species samples were stored at Marmara University Faculty of Pharmacy Herbarium (MARE).

5.1. Preparation of Plant Extracts

The aerial parts of *Achillea* species collected during the flowering period were dried in the shade at room temperature. Powdered samples (50 g) were extracted by maceration with methanol three times (24 hours x 180 ml) at room temperature, filtered, and the solvent was removed at 50°C under vacuum. The dry extracts obtained were stored in the refrigerator for analysis.

5.2. Determination of the Total Phenolic and Flavonoid Content

The total phenolic content of the extracts was measured as described by Gao et al. with slight modifications described by Yıldırım et al. [27,28]. Ten µL of the extract in various concentrations (5000-9.77 µg/mL) was mixed with 20 µL of the Folin-Ciocalteu reagent (Sigma), 200 µL of H_2O, and 100 µL of 15% Na_2CO_3, and the absorbance was measured at 765 nm after 2 h of incubation at room temperature. Gallic acid was used as a standard, and the total phenolics were expressed as the mg gallic acid equivalent per g powder ethanol extract [27,28].

Total flavonoid content was determined following a method by Zhang et al. with slight modifications described by Yıldırım et al. [27,29] 25 µL extracts in various concentrations (5000-9.77 µg/mL) was mixed with 125 µL of ultra pure water and 7.5 µL of 5 % $NaNO_2$. After 6 min, 15 µL of 10% $AlCl_3.6H_2O$ was added. After 5 min, 50 µL NaOH (1 M) was added and this solution completed with 250 µL of ultra pure water. The absorbance was measured against the reagent blank at 510 nm. The standard curve for total flavonoids was made using quercetin standard solution (500-7.81µg/mL) under the same procedure as earlier described. The total flavonoids were expressed as milligrams of quercetin equivalents per g of dried extracts.

5.3. *In vitro* Antioxidant Activity

5.3.1. DPPH radical scavenging activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of the extract was measured according to Zou et al. [30]. Briefly, 10 µL of extracts in DMSO at different concentrations (5000-9.77 µg/mL) was added to a 190-µL methanol solution of DPPH (0.1 mM) in a well of 96-well plates. The mixture was shaken vigorously and allowed to stand in the dark at room temperature for 30 min. Absorbance readings were taken at 517 nm. The percentage of the radical scavenging activity of the extract and standard against DPPH was calculated according to the following formula:

DPPH radical-scavenging activity (%) = [(A0–A1)/A0]×100

where A0 is the absorbance of the control (containing all reagents except the test compounds), and A1 is the absorbance of the extracts/standard. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting the inhibition percentage against the extract concentration. Tests were carried out in triplicate. Ascorbic acid was used as the positive control.

5.3.2. ABTS radical scavenging activity

The ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity of the extract was measured according to Zou et al. [30]. Briefly, ABTS radical cations were prepared by mixing an equal volume of ABTS (7 mM in H_2O) and potassium persulfate (4.9 mM in H_2O), allowing them to react for 12-16 h at room temperature in the dark. Then, the ABTS radical solution was diluted with 96% ethanol to an absorbance of about 0.7 at 734 nm. Ten µL of the extracts in DMSO at different concentrations (5000-9.77 µg/mL) was added to 190 µL of the ABTS radical solution in a well of 96-well plates. The mixture was shaken vigorously and allowed to stand in the dark at room temperature for 30 min. Absorbance readings were taken at 734 nm. The percentage of the radical scavenging activity of the extract and standard against ABTS was calculated according to the following formula:

ABTS radical-scavenging activity (%) = [(A0–A1)/A0]×100

where A0 is the absorbance of the control (containing all reagents except the test compounds), and A1 is the absorbance of the extract/standard. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting the inhibition percentage against the extract concentration. Tests were carried out in triplicate. Trolox was used as the positive control.

5.4. Antidiabetic Activity

5.4.1. α-Glucosidase inhibitor activity

The anti-diabetic activity was evaluated as described by Ramakrishna et al. with slight modifications described by Sen et al. [31,32]. 10 µl of extracts was mixed with 40 µl of 0.1 M sodium phosphate buffer (pH 6.9), and 100 µl of α-glucosidase (obtained from *Saccharomyces cerevisiae*) prepared in buffer. The mixtures were incubated at 25°C for 10 minutes. Thereafter, 50 µl of 5 mM p-nitrophenyl-α-D-glucopyranoside (pNPG) prepared in buffer to the solutions was added. The mixtures were re-incubated at 25°C for 5 minutes, and their absorbance was recorded by reading in the microplate reader before and after incubation at 405 nm. Acarbose was used as standard. The percent inhibitory activity of extracts and standard against α-glucosidase enzyme were calculated according to the following:

α-glucosidase inhibitor activity (%) = [(A0-A1)/A0] ×100

where A0 is the absorbance of the control (containing all reagents except the test compounds), and A1 is the absorbance of the extracts/standard. Extracts or standard concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against extracts or standard concentration. Tests were carried out in triplicate.

5.5. *In vitro* Anti-Inflammatory Activity

5.5.1. Anti 5-lipoxygenase activity

The anti-inflammatory activity was evaluated as described by Phosrithong and Nuchtavorn with slight modifications described by Yıldırım et al. [27,32,33]. Ten µL at different concentrations of the extracts (5000-9.77 µg/mL) or standard indomethacine (250-0.49 µg/mL) was added to a 20 µL ethanol, 20 µL pure water, and 25-µL sodium borate buffer solution (0.1 M, pH 9) followed by the addition of 25 µL of a type V soybean lipoxygenase solution in a buffer (pH 9, 20.000 U/mL). After the mixture was incubated at 25 °C for 5 min, 100 µL of 0.6 mM linoleic acid solution was added and mixed well, and the change in absorbance at 234 nm was recorded for 6 min. Indomethacine was used as a reference standard. The percent inhibition was calculateæd using the following equation:

% inhibition: [(Acontrol−Asample)/Acontrol]×100

A dose-response curve was plotted to determine the IC_{50} values. IC_{50} is defined as the concentration sufficient to obtain 50% of the maximum anti-inflammatory activity. All tests and analyses were performed in triplicate.

5.6. Fractionation Process

Biological activity-guided fractionation was performed on the most active extract. Hexane, chloroform, ethyl acetate and water solutions were prepared respectively by liquid-liquid extraction using a separatory funnel. The solvents in the extracts were evaporated to dryness in a rotary evaporator at a temperature not exceeding 45-50 °C, and thus four extracts were obtained. These extracts were kept at +4 °C throughout the analysis.

5.7. Statistical Analysis

The data were given as means±standard deviations and analysed by one-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison tests using GraphPad Prism 5.

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