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Article

The *in vitro* and *in vivo* investigation of biological activities and phenolic analysis of *Helichrysum plicatum* subsp. *plicatum*

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In Turkey, *Helichrysum* genus is represented by 26 taxa belonging to 20 species in Turkish flora of which 14 ones are endemic to Turkey. The aerial parts of *Helichrysum plicatum* subsp. *plicatum* are used kidney stones, kidney and stomach ailments. The extraction procedures and solvents are important step in processing of bioactive constituents from the plant materials. Therefore, the aim of this study was to evaluate *in vitro* antioxidant, antimicrobial, anti-urease, anticholinesterase and *in vivo* anti-inflammatory activities of *Helichrysum plicatum* subsp. *plicatum* different extracts. In addition, the phenolic characterization of the Soxhlet and maceration methanol extracts which showed significant antioxidant, anti-urease, antimicrobial, anti-inflammatory and anticholinesterase activities were performed by HPLC-DAD and LC-MS/MS. In the present study, the Soxhlet methanol extract exhibited strong antioxidant, antimicrobial and anticholinesterase activities than other extracts. The maceration methanol extract showed the strongest anti-urease activity. The Soxhlet methanol and maceration methanol extracts showed *in vivo* anti-inflammatory activities very close to each other. As a result of this study, chlorogenic acid, dicaffeoylquinic acid, luteolin, luteolin-7-*O*-glucoside, naringenin-*O*-hexoside and isoquercitrin compounds were analysed in plant. Therefore, it is thought that methanol extracts can be used as a natural source in the future for food and pharmaceutical industries.

Keywords: *Helichrysum plicatum* subsp. *plicatum.* Anti-inflammatory. Anti-urease. Anticholinesterase. HPLC-DAD-MS.

INTRODUCTION

Natural antioxidants are more readily acceptable than synthetic antioxidants. Recently, much focus has been given to the involvement of active oxygen and free radicals in aging and disease processes like heart disease, inflammation, arthritis, immune system impairment and cancer. Oxidative stress is defined by an imbalance between increased levels of reactive oxygen species (ROS) and a low activity of antioxidant mechanisms. An increased oxidative stress can induce damage to the cellular structure and potentially destroy tissues. Antioxidants terminate ROS attacks and thus help prevent the diseases and health problems. There is a general trend to search safe and effective antioxidants from natural sources (Denys *et al*., 2013). Inflammation is the first response of body to infection or injury and is critical for both innate and adaptive immunity. It can be considered as part of the complex biological response of vascular tissues to harmful stimuli such as pathogens, damaged cells or irritans. The search for natural compounds and phytoconstituents that are able to interfere with these mechanisms by preventing a prolonged inflammation could be useful for human health (Parimelazhagan, 2016). The selection and extraction method of solvent for the extraction of plant material to determine the potential activity of the extract is one of the most important

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factors, since the solvent polarity and extraction method determines that compounds will be extracted or not. Thus, in many cases of newly studied plants, various extracts are prepared using different extraction methods and solvents (Azwanida, 2015).

The genus *Helichrysum* (Asteraceae) is represented by approximately 500 species in the World. In Turkey, *Helichrysum* genus is represented by 26 taxa belonging to 20 species in Turkish flora of which 14 ones are endemic for Turkey (Davis, 1975; Davis *et al.,* 1988; Kupicha, 1975; Erik, 2000). Various *Helichrysum* species have been widely used as folk remedy in Turkish folk medicine for diuretic and anti-asthmatic properties as well as againts kidney stones and stomache as decoction (Aslan *et al*., 2007; Suzgec *et al.,* 2005; Selcuk, Birteksoz, 2011). *Helichrysum plicatum* subsp. *plicatum* species are known as sarıçiçek and aerial parts of plant are used kidney stones, kidney and stomach ailments (infusion and decoction) and depreciatory (as tea) (Yeşil, Akalın, 2009). *Helichrysum plicatum* DC. subsp. *plicatum* is known with a rich flavonoids content, comprising 4.83% flavonoids such as helichrysins A and B, apigenin, naringenin, isoastragalin and isosalopurposide (Demir *et al*., 2009). The best of our knowledge, there is no reports on the effect of extraction methods on the biological activity of this plant. In addition to this, although the plant is used as tea, there is no study of the plants' anti- urease, anticholinesterase activity, and analysis of major phenolic compounds with responsible for the activity. Therefore, the aim of this study was to evaluate *in vitro* antioxidant, antimicrobial, anti-urease, anticholinesterase and *in vivo* anti-inflammatory activities of *Helichrysum plicatum* subsp. *plicatum* using a variety of extracts. And, the phenolic characterization of the Soxhlet and maceration methanol extracts, which showed the strongest biological activity were analyzed by HPLC-DAD and LC-MS/MS techniques.

MATERIAL AND METHODS

Chemicals

Folin Ciocalteu's phenol reagent, gallic acid, 2,4,6-tripyridyl-s-triazine, 2,2-diphenyl-1-picryl-hydrazyl (DPPH$^{\bullet}$) and ascorbic acid were obtained from Sigma Chemical Co.. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) and butylated hydroxytoluene was sourced from Fluka. All other reagents were of analytical grade.

Plant material and preparation of plant extracts

H. plicatum subsp. *plicatum* aerial parts were collected in 2016 from Uludağ-Bursa, Turkey and was authenticated by Asst. Prof. Dr. Ismail Senkardes. A voucher specimen (MARE-18080) was deposited at the herbarium of the Faculty of Pharmacy, Marmara University for future reference. The plant aerial parts was dried in shade (25 ^{o}C) and ground in a mechanic grinder (Renas, RBT1250) to fine powder. The powdered sample (50 g) was extracted with organic solvents (500 mL) such as petroleum ether (HPE), chloroform (HC) and methanol (HSM) using the Soxhlet method respectively until colorless. In addition, 15 gram of powdered sample was extracted with direct methanol (500 mL) (HMM) solvent using the maceration method until the colorless. The four different extracts were concentrated by rotary vacuum evaporator. All the extracts obtained were stored at -20 °C for future analysis.

Extract yield percentage and total flavonoid contents

The extraction yield was calculated to determine the effect of the solvents in order to extract the active compounds from the plant material (Murugan, Parimelazhagan, 2014).

The percentage yield was obtained using this formula:

$$\% \text{ extract yield} = A_2\text{-}A_1/A_0 \times 100$$

where A_2 is the weight of the extract and the container, A_1 is the weight of the container alone and A_0 is the weight of the initial dried sample.

The total flavonoid contents of the extract was determined using the aluminium chloride assay (Samatha *et al.,* 2012). 0.5 mL of extract solution was taken in different test tubes then 2 mL of distilled water was added. Next step was followed by the addition of 0.15 mL of sodium nitrite (5% $NaNO_2$, w/v) and allowed to stand for 6 min. Then 0.15 mL of aluminium trichloride (10% $AlCl_3$) was added and incubated for 6 min, followed by the addition of 2 mL of sodium hydroxide (NaOH, 4% w/v) and volume was equal to 5 mL with distilled water. The mixture was allowed to stand at ambient temperature for 15 minutes. Then, the absorbance was measured at 510 nm against a reagent blank. Total flavonoid contents were expressed as quercetin equivalents in milligram per milligram of extract (mg QuE /mg extract) ($R^2 = 0.9940$).

In vitro evaluation of biological activity

DPPH radical scavenging activity

The ability of free radical scavenging of four different extracts was determined using the DPPH method.

Briefly, 240 µL of DPPH solution (0.1 mM) was added to 10 µL of extracts prepared at different concentrations (5 mg/mL-0.5 mg/mL). Then the mixture was allowed to stand at room temperature for 30 min. The absorbance of the mixture was measured against the reference using a micro plate reader at 517 nm. The experiment was repeated three times and the results obtained in the experiment were given as IC_{50} = mg/mL (Wei *et al.,* 2010).

Trolox equivalent antioxidant activity

Briefly, 40 µL of four different extracts (5 mg/mL-0.5 mg/mL) prepared from plant material, 3960 µL of $ABTS^{•+}$ working solution were combined. The absorbance of the mixture was measured against the reference at 734 nm for 6 min. The standard curve was prepared using trolox and the data obtained in this study were expressed as mM trolox/mg extract (Re *et al.,* 1999).

Ferric reducing/antioxidant power (FRAP) assay

The ability of ferric reducing of different extracts (5 mg/mL-0.5 mg/mL) was evaluated using the FRAP method. Briefly, 3.8 mL of FRAP reagent was mixed with 0.2 mL of extract and the absorbance of the mixture was measured against the reference at 593 nm after 4 min. The standard curve was prepared using $FeSO_4.7H_2O$ and FRAP values of the extracts were expressed as a mM Fe^{2+}/mg extract (Benzie, Strain, 1996).

Anti-urease activity

Briefly, 500 µL of urease enzyme was added to 100 µL of the working solutions and incubated at 37 °C for 30 min. in the incubator. Then, 1100 µL of urea was placed in this mixture and the mixture was incubated for 30 min. at 37 °C in the incubator. R_1 (1% phenol, 0.005% sodium nitroprusside) and R_2 (0.5% NaOH, 0.1% sodium hypochlorite) reagents were added respectively to the mixture. The mixture was incubated for 2 h. in the incubator (37 °C) and then the absorbance of mixture was read at 635 nm against the reference (Ghous *et al.,* 2010).

Anticholinesterase activity of extracts

Inhibition of cholinesterases was evaluated using a 96-well microplate reader based on the method of Ellman *et al.* (1961) with some modifications. All reagent solutions (daily) were prepared using Tris-HCl buffer (50 mM, pH 8.0). Briefly, 20 µL of AChE solution were mixed with 20 µL of the sample and 40 µL of Tris-HCl buffer and the mixture was left at room temperature (25 °C) for 10 min. Then, 20 µL of ATChI (50 mM) was added the mixture and the mixture was incubated at 25 °C for 5 min. Then, 100 µL of 20 mM DTNB (containing 1 M NaCl and 0.2 M $MgCl_2.6H_2O$) was added in the mixture and the absorbance of mixture was measured at 412 nm against the reference. The experiments were performed in triplicate in each case. Galantamine was used as reference (Ellman *et al.,* 1961).

Antimicrobial activity

In this study, the *in vitro* antimicrobial activities of different extracts were studied using microdilution method as recommended by Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2006; CLSI, 2000; Perez, Pauli, Bazerque, 1990).

Test microorganisms

Staphylococcus aureus ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis ATCC 14153, Klebsiella pneumoniae* ATCC 4352, *Candida albicans* ATCC 10231, and MRSA.

***In vivo* evaluation of biological activity**

Anti-inflammatory activity

Paw edema was induced in Sprague-Dawley rats by sub-plantar injection of 0.1 mL of 1% (w/v) carrageenan in saline in the right paw. The different groups were treated orally with maceration methanol (HMM) and Soxhlet methanol extract (HSM) of *H. plicatum* subsp. *plicatum* (250 mg/kg, p.o.), indomethacin (5 mg/kg, p.o.) and saline (10 mL/kg, p.o.) 60 min. before the administration of carrageenan (Sezik *et al.,* 2010; Bayir *et al.,* 2011). The volume of the edema development and its duration were determined for 4 hours using plethysmometer 37140 (UgoBasile, Italy). The inhibitory activity was calculated according to the formula: % Inhibition = [(Vt-Vo) control - (Vt-Vo)]/[(Vt-Vo) control] x 100. Where Vt is the paw volume at time t, Vo is the paw volume before carrageenan injection, (Vt-Vo) is edema in paw after time t. The study was approved by Marmara University, Animal Experiments Local Ethics Committee (MÜHDEK-58.2017.mar).

Analysis of phenolic compounds

The active phenolic compounds in methanol extracts with strong biological activity were identified by HPLC-DAD based on comparison on-line ultraviolet absorption spectrum data and retention times acquired with authentic standards (Halpine, 1996). After that, they were confirmed by liquid chromatography with quadrupole time-of-flight mass spectrometry using targeted MS/MS

techniques with accurate mass measurement. Phenolic compounds were identified and characterized by comparing maximum UV absorptions, HPLC retention time and MS/MS of target peaks in the extracts with the standards. Chromatographic peaks for phenolic compounds were selected by using the theoretical $[M-H]^-$ as precursor ion. MS/MS analysis of phenolic compounds was carried out in negative mode using electrospray ionization.

Statistical analysis

The results of the antioxidant and anti-urease experiments were analyzed by the Graphpad Prism 5 program. The data obtained from anti-inflammatory activity were analyzed by PASW Statistics.

RESULTS AND DISCUSSION

In vitro evaluation of biological activity

Extract yield percentage and total flavonoid contents

The total flavonoid contents and yield percentage of different extracts were analysed and presented in Table I. The results from the present study showed that the total flavonoid contents in Soxhlet methanol extract higher than other extracts. The petroleum ether extract had the lowest total flavonoid contents. When the yields percentage of the different extracts were compared, maceration methanol extract found to have higher recovery over other extracts.

TABLE I - Extract yield percentage and total flavonoid contents of *H. plicatum* subsp. *plicatum*

	Total flavonoid (mgQUE/mg extract)	Extract yield (%)
HPE	0.784±0.018^a	3.613^a
HC	1.776±0.211^b	1.556^b
HSM	2.261±0.075^c	11.995^c
HMM	1.72±0.031^d	18.685^d

Values are mean of triplicate determination (n = 3) ± standard deviation. Means with different superscripts $^{(a-d)}$ are significantly different, $p<0.05$. QUE–Quercetin equivalents. HPE: Soxhlet petroleum ether extract; HC: Soxhlet chloroform extract HSM: Soxhlet methanol extract; HMM: Maceration methanol extract

Antioxidant activity of extracts

The antioxidant activities of different extracts were shown in Table II. The Soxhlet methanol (IC_{50}: 0.11±0.009 mg/mL) and maceration methanol (IC_{50}: 0.115±0.008 mg/mL) extracts exhibited the strongest free radical scavenging activity. The chloroform extract showed the lowest DPPH free radical scavenging activity. As shown in Table 2, the radical scavenging DPPH activities of all extracts showed lower than that of ascorbic acid (IC_{50}: 0.004±0.002 mg/mL).

The maceration methanol (46.19 mM trolox/mg extract), and Soxhlet methanol (44.26 mM trolox/mg extract) extracts showed stronger ABTS radical cation scavenging activity than other extracts. The chloroform extract had the lowest ABTS radical cation scavenging activity. In this study, when we compare the extraction techniques, it has been found that the extract obtained by Soxhlet and maceration from the sample showed strong DPPH and ABTS radical scavenging activity.

In this study, Soxhlet methanol (14.46 mM Fe^{2+}/mg extract) and maceration methanol (12.38 mM Fe^{2+}/mg extract) extracts were found to have stronger ferric reducing activity than the other extracts. The chloroform extract had the lowest ferric reducing activity. The results obtained from this study showed that both extraction techniques is the most suitable method to get the most powerful ferric reducing activity.

According to literature reviews, there are several studies on antioxidant activity of this plant. The antioxidant activity of the methanol extract obtained from maceration technical was examined using superoxide anion, hydrogen peroxide and DPPH methods. According to the results obtained study, methanol extract exhibited moderate superoxide anion (IC_{50}: 305.2 µg/mL), hydrogen peroxide (IC_{50}: 301.6 µg/mL), and DPPH (IC_{50}: 39 µg/mL) scavenging activity (Tatlı *et al.,* 2009). In another study, it was determined that plant's methanol extract from Soxhlet methods contained 87.36 mg GAE/g extract of phenolic content and showed moderate phosphomolybdenum activity (163.47 mg ascorbic acid equivalent/g extract) and DPPH radical scavenging activity (IC_{50}: 23.48 µg/mL) (Albayrak *et al.,* 2010).

In contrast to the above study, in our study, we examined the total flavonoids contents and antioxidant activity (DPPH, FRAP, ABTS) of four different extracts (Soxhlet petroleum ether, Soxhlet chloroform, Soxhlet methanol and maceration methanol extracts) obtained using different extraction methods. In addition, the effects of extraction methods/solvents on antioxidant activity were examined in this study. When the results obtained from the DPPH radical scavenging assay were compared with results of ours, it was found that Soxhlet methanol and maceration methanol extracts exhibited lower DPPH radical scavenging activity than above study. It was also found that maceration methanol and Soxhlet methanol extracts showed similar free radical activity in our study.

TABLE II - The antioxidant activity of *H. plicatum* subsp. *plicatum* extracts

Samples	DPPH (IC_{50}: mg/mL)	ABTS (mM trolox/mg extract)	FRAP assay (mM Fe^{2+}/mg extract)
HPE	0.12±0.03[a]	42.27±0.04[a]	9.63±0.004[a]
HC	0.24±0.02[b]	38.29±0.06[b]	8.63±0.25[b]
HSM	0.11±0.01[c,a]	44.26±0.04[c]	14.46±0.012[c]
HMM	0.115±0.008[d,a,c]	46.19±0.12[d]	12.38±0.056[d]
Ascorbic acid	0.004±**0.002**[e]		
BHA		52.63±0.01[e]	16.91±0.02[e]

Values are mean of triplicate determination (n = 3) ± standard deviation. Means with different superscripts [(a-e)] are significantly different, $p<0.05$. HPE: Soxhlet petroleum ether extract; HC: Soxhlet chloroform extract HSM: Soxhlet methanol extract; HMM: Maceration methanol extract.

The results obtained from our study showed that Soxhlet methanol extraction technique is the most suitable method/solvent to get the most powerful ferric reducing and DPPH radical scavenging activity. In addition, total flavonoid compounds analysis showed that the Soxhlet methanol extract exhibited the highest total flavonoid contents values. Flavonid compounds are known to have strong antioxidant effects. As a result, the strong antioxidant activity of this extract can be attributed to its contained higher amount of the flavonoid compounds.

Urease inhibitory activity

The results for the assessment of urease inhibitory activity of *H. plicatum* subsp. *plicatum* different extracts (25 µg/mL) were shown in Table III. The maceration methanol (13.13%) extract exhibited the strongest anti-urease activity. In the present study, maceration methanol methods/solvents were the most suitable solvents and methods to get the strongest anti-urease activity. According to this study, all the extracts had lower anti-urease activity than thiourea (97.04%). To the best of our knowledge, there have been no report in literature on the anti-urease activity of *H. plicatum* subsp. *plicatum* species. Therefore, the purpose of this study was to examine for the first time the anti-urease activity of this species's extracts obtained using different extraction methods and also to determine the most suitable extraction method and solvents. According to the results obtained from this study, the maceration method was found to have higher anti-urease activity over other methods.

Antimicrobial activity

In this study, the antimicrobial activity of the four different extracts from plant were investigated against *S. aureus*, *S. epidermidis*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *P. mirabilis,* MRSA and *C. albicans.*

TABLE III - The urease inhibitory activity of different extracts from *H. plicatum* subsp. *plicatum*

Samples	Urease inhibition (%) (25 µg/mL)
HPE	6.35±1.5[a]
HC	10.61±0.6[b]
HSM	8.08±1.24[c]
HMM	13.13±1.9[d]
Thiourea	97.04±0.14[e]

Values are mean of triplicate determination (n = 3) ± standard deviation. Means with different superscripts [(a-e)] are significantly different, $p<0.05$. HPE: Soxhlet petroleum ether extract; HC: Soxhlet chloroform extract HSM: Soxhlet methanol extract; HMM: Maceration methanol extract

According to the results obtained from the antimicrobial activity test, Soxhlet methanol extract from plant showed moderate antibacterial and antifungal activity against *S. aureus, S. epidermidis,* MRSA and the yeast *C. albicans* while, no activity was observed against *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *P. mirabilis* for any of the extracts. The petroleum ether extract obtained from Soxhlet method exhibited the significant antifungal activity against *C. albicans.* In addition, Soxhlet chloroform and maceration methanol extracts showed moderate activity against *C. albicans*, MRSA and *C. albicans*. According to this study, all the extracts showed lower antimicrobial activity than standard compounds such as cefuroxime-Na, ceftazidime, clotrimazole, oxacillin. When Soxhlet and maceration methods were compared between themselves, Soxhlet method was found to be more suitable for obtaining the significant antibacterial activity (Table IV).

To the best of our knowledge, there are some reports in literature on the antimicrobial activity of this

TABLE IV - The antimicrobial activity of *H. plicatum* subsp. *plicatum* extracts

Extracts / Standards	*Staphylococcus aureus* ATCC 6538	*Meticillin Resistant Staphylococcus aureus* ATCC 33591	*Staphylococcus epidermidis* ATCC 12228	*Escherichia coli* ATCC 8739	*Klebsiella pneumoniae* ATCC 4352	*Pseudomonas aeruginosa* ATCC 1539	*Proteus mirabilis* ATCC 14153	*Candida albicans* ATCC 10231
HPE	-	-	-	-	-	-	-	312
HC	-	1250		-	-	-	-	625
HSM	1250	1250	1250	-	-	-	-	625
HMM	-	-	-	-	-	-	-	625
Cefuroxime-Na	1.2	---	9.8	4.9	2.4	2.4	-	---
Ceftazidime	---	---	---	---	2.4	---	---	---
Clotrimazole	---	---	---	---	---	---	4.9	---
Oxacillin		≥4 µg/mL						

HPE: Soxhlet petroleum ether extract; HC: Soxhlet chloroform extract HSM: Soxhlet methanol extract; HMM: Maceration methanol extract. -: not active; --- : not tested; MIC: Mg/L.

species. The ethyl acetate, methanol, chloroform and acetone extracts from different parts (flowers, stem, leaves) of plant were tested *in vitro* against different bacterial species. According to the results obtained from the study, the ethyl acetate extracts from flowers, stem and leaves of plant have better antibacterial efficiency to *Bacillus brevis* FMC 3, *B.megaterium* DSM 32, *B. subtilis* IMG 22, *B. subtilis* var. *niger* ATCC 10, *Escherichia coli* DM, *Listeria monocytogenes* SCOTT A, *Micrococcus luteus* LA 2971, *Mycobacterium smegmatus* RUT, *Staphylococcus aureus* ATCC 25923, *Straptococcus thermophilus*, *Yersinia enterocolitica* than other extracts. All of the extracts from plant have shown no antibacterial efficiency to *Corynebacterium xerosis* (Erdogrul, Cakiroglu, Karaman, 2001). In another study, it was determined antimicrobial activity of that plant's methanol extract obtained from Soxhlet method and this extract was found to be active against *Aeromonas hydrophila* ATCC 7965, *Klebsiella pneumoniae* FMC 5, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus brevis* FMC 3, *B. cereus* RSKK 863, *B.subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 29213, *Candida albicans* ATCC 1223 and *S.aureus* ATCC 25923 (Albayrak *et al.,* 2010). The methanol extract of this species showed antifungal activity against *C. albicans* similar to the our study (Albayrak *et al.,* 2010). Unlike this study, methanol extracts did not show antibacterial activity against *K. pneumoniae* and *P. aeruginosa* in our study. However, when we compare the results of these studies, different results may be due to the use of different methods.

Anticholinesterase activity

The results for the assessment of cholinesterase inhibitory activity of *H. plicatum* subsp. *plicatum* different extracts (500 µg/mL) were shown in Table V. The Soxhlet methanol (58.51%) extract exhibited the strongest anticholinesterase activity. Soxhlet petroleum ether extract did not show cholinesterase inhibitory activity. In the present study, Soxhlet methanol method/solvent were the most suitable solvent and method to get the strongest anticholinesterase activity. According to this study, all the extracts had lower anticholinesterase activity than galantamine (88.14%). To the best of our knowledge, there have been no report in literature on the anticholinesterase activity of this species. Therefore, the purpose of this study was to examine for the first time the anticholinesterase activity of plant's extracts obtained using different extraction methods and also to determine the most suitable extraction method and solvents. According to the results obtained from this study, the Soxhlet method was found to have higher activity over other methods.

TABLE V - The anticholinesterase activity of different extracts from *H. plicatum* subsp. *plicatum*

Samples	Enzyme inhibition (%) (500 µg/mL)
HPE	-
HC	1.28 ± 0.24^{a}
HSM	58.51 ± 0.85^{b}
HMM	42.10 ± 0.79^{c}
Galantamine (200 µg/mL)	88.14 ± 0.14^{d}

Values are mean of triplicate determination (n = 3) ± standard deviation. **-:** not active. Means with different superscripts $^{(a\text{-}d)}$ are significantly different, $p<0.05$. HPE: Soxhlet petroleum ether extract; HC: Soxhlet chloroform extract HSM: Soxhlet methanol extract; HMM: Maceration methanol extract.

In vivo evaluation of anti-inflammatory activity

The injection of carrageenan showed a rapid increase in the paw volume, reaching its maximum 4 h. post-injection (Vazquez *et al.,* 2015). Indomethacin (5 mg/kg) indicated maximum anti-inflammatory effect at 4 hours after carrageenan injection by 89.73% (Table VI, $p<0.001$). HMM demonstrated a more prominent and intensive anti-inflammatory effect at first hour after carrageenan injection with 62.06% and at fourth hours with 66.58% of inhibitive capacity in the altered edema size compared to HSM. However, HSM's inhibitory effect reached maximum by 77.94% in second hour ($p<0.001$) and it was more intensive compared to HMM (Table VI, $p<0.01$). Carrageenan-induced edema is widely used as an experimental animal model in evaluation of acute inflammation and is believed to be biphasic, of which the first phase is mediated by the release of histamine and 5-hydroxytryptamine followed by kinin release and then prostaglandin in the later phase (Chaulya, Halda, Mukherjee, 2012; Alcaraz, Jimenez, 1988). Acute single-dose of HMM and HSM significantly reduced rat paw edema volume, especially at 2 hours after carrageenan injection. Some phenolic compounds have been reported to reveal inhibition of histamine release in mast cells, which is responsible for early phase of inflammation induced by carrageenan (Jabeur *et al.,* 2017). Thus, plant's acute anti-inflammatory effects may be based upon its phenolic composition. Consequently, phenolic compounds are possible to be the effective components, which trigger the anti-inflammatory action, but further investigations are needed to determine precise mechanisms. There have been no study on anti-inflammatory activities of maceration and Soxhlet methanol extracts from the aerial parts of plant. In this study, anti-inflammatory activity of extracts was examined for the first time and according to the obtained results, both extracts from plant showed strong anti-inflammatory activity ($p<0.001$).

Analysis of phenolic compounds

In this study, phenolic compounds in Soxhlet and maceration methanol extracts were analysed because these extracts showed strong antioxidant, anti-urease, antimicrobial, anti-inflammatory and anticholinesterase activity than other extracts. The phenolic compounds were identified as chlorogenic acid, dicaffeoylquinic acid, luteolin, luteolin-7-*O*-glucoside, naringenin-*O*-hexoside and isoquercitrin in methanol extracts by HPLC-DAD and ESI-Q-TOF LC/MS (Table VII). To the best of our knowledge, there are some reports in literature on the chemical contents of this species. In a study, apigenin, β-sitosterol 3-O-β-D-glikopiranozit, helichrysin A, helichrysin B, astragalin, isosalipurposit compounds were isolated from the plant's methanol extract (Demir *et al.,* 2009). In another study, it was analyzed that the plant methanol extract contained caffeic acid, chlorogenic

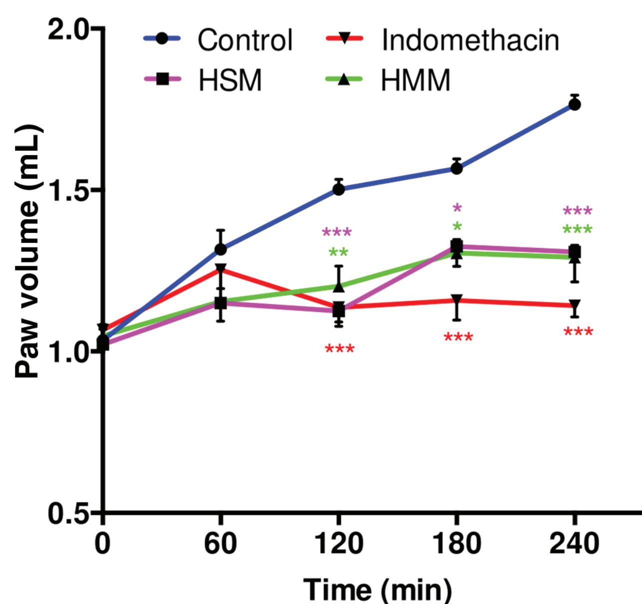

FIGURE 1 - Anti-inflammatory effect of Soxhlet methanol and maceration methanol extracts of plant on carrageenan-induced paw edema. Values are mean of triplicate determination and expresses as Mean ± SEM (Standard error mean); values are calculated using two-way ANOVA followed by Tukey posttests; ***$p<0.001$, **$p<0.01$ and *$p<0.05$ when compared to control group; n=6. HSM: Soxhlet methanol extract; HMM: maceration methanol extract.

TABLE VI - Results of % inhibition of paw edema in extracts from *H. plicatum* subsp. *plicatum* on carrageenan-induced paw edema (n=6)

Treatment	60 min	120 min	180 min	240 min
HSM (250 mg/kg)	54.61 %	77.94 %	43.05 %	60.82 %
HMM (250 mg/kg)	62.06 %	67.02 %	51.69 %	66.58 %
Indomethacin	34.04 %	85.01 %	82.89 %	89.73 %

HSM: Soxhlet methanol extract; HMM: Maceration methanol extract. Values are mean of triplicate determination.

TABLE VII - Identification of phenolic compounds in *H. plicatum* subsp. *plicatum*

Compounds	Rt (min)	[M-H]$^-$	*m/z* Fragment(s) (targeted MS/MS mode)
Chlorogenic acid	10.991	353.0913	191 (M-caffeoyl), 179 (M- quinic)
Dicaffeoylquinic acid	19.099	515.113	353, 284,173,135
Luteolin	23.594	285.0368	217, 199, 175, 151, 133
Luteolin-7-*O*-glucoside	17.019	447.0876	285
Naringenin-*O*-hexoside	13.447	433.1218	271, 151, 119, 65
Isoquercitrin	13.692	577.0977(M+TFA)	301

acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, apigenin, apigenin-7-glucoside, hesperidin, luteolin, and naringenin by using HPLC-DAD. In addition, according to obtained results, in methanol extract from flowers, leaves, and stem-barks identified the chlorogenic acid, quercetin and rutin compounds as the major compound (Albayrak *et al.,* 2010; Kolaylı *et al.,* 2010). Unlike the above study, in our study, the effects of extraction methods and solvents on biological activities were examined. Then, the phenolic compounds in Soxhlet and maceration methanol extracts were analysed by HPLC-DAD and LC-MS/MS. As a result of this study, dicaffeoylquinic acid, luteolin-7-*O*-glucoside, naringenin-*O*-hexoside and isoquercitrin were analysed for the first time in methanol extracts from plant's aerial parts.

It is important to select an extraction technique that is appropriate for the standardization of plant products and phytochemical analysis. When different extraction methods and different solvents are used, the different compounds are obtained and in this case the biological activity of the plants changes (Azwanida, 2015). Therefore, in this study, different extraction methods and solvents were selected. The effect of the methanol solvent on biological activities was found to be higher than that of other solvents. It was also observed that the extraction method had an important determining factor on activity results. In our investigation, we can get different results even if we use the same solvent since the amounts of bioactive compounds could be changed by the different extraction methods. However, in our study Soxhlet and maceration methods showed biological activity very close to each other. In addition, it was found that the methanol extracts obtained using both methods contained similar phenolic compounds.

Phenolic compounds (flavonoids, phenolic acids etc.) are known to exhibit strong biological activity (antioxidant, antimicrobial, anticancer, anti-inflammatory etc.) Also, the researchers use alcohols or aqueous alcohol mixtures for extracting of the polar flavonoids and glycosides (Li *et al*., 2014; Beer *et a*l., 2002; Anantharaju *et al*., 2016). In this study, parallel to the literatüre, it was determined that the plant exhibited strong biological activity due to its the phenolic compounds. Although both extracts contain similar phenolic compounds, there is a small difference between results and this difference is thought to be caused by change in the amount of the same or similar substances extracting into the solution.

CONCLUSION

In this study, we investigated the effect of extraction methods on biological activity and found that Soxhlet and maceration methanol extracts showed biological activity (*in vitro* and *in vivo*) close to each other. It was also found that antioxidant active compounds play an important role in anti-inflammatory activity. The phenolic compounds responsible for biological activity were analyzed by HPLC-DAD and LC-MS/MS and chlorogenic acid, dicaffeoylquinic acid, luteolin, luteolin-7-*O*- glucoside, naringenin-*O*-hexoside and isoquercitrin compounds were found to be in the Soxhlet and maceration methanol extracts. Therefore, these extracts can be used as natural antioxidants, antimicrobial, anticholinesterase, anti-inflammatory and anti-urease agents in the future for food and pharmaceutical industry.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication and dissemination of the information provided herein.

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