Phenolic constituents, antioxidant and anti-inflammatory effects of *Heliotropium europaeum* (Boraginaceae)

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Heliotropium europaeum L. (Boraginaceae) has been traditionally used by Mediterranean peoples as folk medicine against several types of disease to treat diverse illnesses. In this study, the antioxidant, and anti-inflammatory activities of *H. europaeum* extracts were investigated for the first time. Additionally, the methanol extract was analysed for phenolic acids and flavonoids by HPLC. The antioxidant potencies of the extracts (ethanol, methanol, chloroform, water) were carried out via six different antioxidant assays (radical quenching (ABTS and DPPH), metal chelating, reducing power (FRAP), phosphomolybdenum and β-carotene/linoleic acid assay) and the methanol extract showed the highest antioxidant activity. To determine the anti-inflammatory effects of *H. europaeum* extract in LPS-stimulated RAW 264.7 macrophage cells, basically two methods were used. While Griess assay was applied to detect the nitrite level as an index of nitric oxide (NO) production, enzyme-linked immunosorbent assay (ELISA) was used for determining the levels of pro-inflammatory cytokines (TNF-α, IL-6 and IL-12) generated in the culture. *H. europaeum* inhibited not only NO production but also the levels of proinflammatory cytokines, without any cytotoxicity. HPLC analysis showed that the major phenolic compounds in the extract were caffeic acid (15971.41 µg/g), 2,5-dihydroxybenzoic acid (11469.11 µg/g) and epicatechin (7243.33 µg/g). These results indicate that *H. europaeum* possesses potent antioxidant and inflammatory effects associated with its bioactive phenolic constituents.

Keywords: *Heliotropium europaeum*, pro-inflammatory cytokines, antioxidant, phenolic compounds

INTRODUCTION

A great number of medicinal plants in Turkey have widely been used in many fields such as food, tea, spices, ornaments, smell, taste industry, perfumes and cosmetics by the people for many years [1]. Plants contain thousands of constituents and are a valuable source of new and biologically active molecules. Identifying plants with therapeutic properties is a great purpose of novel research. In recent years, the requisition for medicinal plants and their individual bioactive components have dramatically enhanced due to the comprehensive growth of nutraceutical, pharmaceutical, cosmeceutical, or food industries [2].

Heliotropium is a genus of flowering plants in the Boraginaceae family, which is distributed through temperate regions but more abundantly in the Mediterranean region [3]. There are 270 to 330 species in this genus, which are commonly known as heliotropes. The word “heliotrope” in Greek, has been remarked for plants that have the characteristic of turning their leaves in the orientation of the sun [4, 5]. In Turkey, this genus is represented by 16 species, 4 of which are endemic [6]. *Heliotropium europaeum* L. (called ‘akrep otu’ in Turkish) possesses a wide variety of biological activities, including antitumor, anti-inflammatory, antimicrobial, insecticidal and antispas-

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modic effects and is used as antipyretic, cholagogue, emmenagogue, cardiotonic and anthelmintic, in the treatment of gastrointestinal disorders, headache and gout. Moreover, it is applied topically for snake bites and scorpion stings and for promoting wounds [7-9]. Besides recent studies have revealed the antibacterial and antifungal activities of *H. europaeum* essential oil [7]. Twenty-six compounds were identified in the essential oil of *H. europaeum* represented 91.4% of the total essential oil [10]. The essential oils of some *Heliotropium* species can be used in cosmetics, aromatherapy, massage, perfumery, soap and candle making.

Inflammation is a body's natural defense system triggered by a variety of harmful stimuli such as damaged cells, pathogens, lipopolysaccharides, or irritants. Immune cells, especially macrophages, release biochemical mediators, such as pro-inflammatory cytokines (e.g., tumor necrosis factor-alpha (TNF-α) interleukin-6 (IL-6) and IL-12), nitric oxide (NO) and chemokines to coordinate the immune reaction that will eliminate the inflammatory triggers and promote tissue repair and recovery [11]. A prolonged inflammatory response is often associated with chronic diseases such as cancer, arthritis and autoimmune disorders. In ancient times, inflammation and its related disorders treated with some plants or plant-based treatments including herbs, weeds, fruits and vegetable parts. It has been demonstrated by experimental studies that different plant-based extracts and their chemical constituents possess the anti-inflammatory activity [12, 13]. Macrophages are essential for host immunity and by detecting pathogenic substances, they initiate and regulate inflammatory responses. Lipopolysaccharide (LPS), an endotoxin derived from Gram-negative bacteria, is a powerful activator of macrophage cells, and activated macrophages are known to produce inflammatory mediators [14].

Plant extracts have been used as natural therapeutic agents against inflammation, characterised by an overproduction of inflammatory mediators such as reactive oxygen species (ROS) and pro-inflammatory cytokines [15]. Recently, there has been considerable interest in finding natural antioxidants from plant materials as alternatives to synthetic ones. Antioxidants are substances that neutralise both free radicals and their negative effects. The data from both scientific reports and laboratory studies show that plants contain a wide range of substances that possess antioxidant activities [16]. Phytochemical analysis of *H. europaeum* indicated the presence of alkaloids, terpenoids, steroids, saponins, flavonoids, phenols, and tannins [7]. Also, the fatty acid composition of the seed oils of *H. europaeum* was reported in a previous study [17]. Most of the previous reports were focused on the alkaloid content and the associated toxic effects, while other active metabolites and their beneficial biological activities were mostly neglected. In this respect, the present study for the first time aimed to investigate the effects of *H. europaeum* on LPS-induced inflammatory responses of murine RAW 264.7 macrophages. Additionally, the antioxidant activities and phenolic and flavonoid contents of *H. europaeum* were also determined.

MATERIALS AND METHODS

CHEMICALS

The chemicals and reagents for the HPLC, antioxidant and anti-inflammatory assays were purchased from Sigma-Aldrich (Germany), Merck (USA) and Boster Biologicals (USA).

PLANT MATERIAL AND EXTRACTION

The individuals of *H. europaeum* were collected in July 2014 from Denizli-Turkey (Pamukkale University Kınıklı Campus, maquis shrubland, 430 m) and identified (Voucher No: C. Ozay 3002). The aerial parts of the plant were air-dried and coarsely ground. The extracts were prepared using ethanol, methanol, chloroform, and water in a shaker water bath for 12h. Subsequently, the extracts were concentrated at reduced temperature and pressure and lyophilised. The crude extracts were kept at -20°C until needed. The dried extracts were weighed to determine the percent of yield [18]. The percentage yield was obtained using this formula W1/W0 × 100. Where W1 is the final weight of the extract and W0 is the initial weight of sample.

DETERMINATION OF TOTAL SECONDARY METABOLITES AMOUNT

To obtain total levels of phenolic, flavonoid and saponin contents in the extracts, colorimetric assays were used as described in the previous paper [19]. These contents were expressed as gallic acid (mg GAEs/g), quercetin (mg QEs/g) and quillaja (mg QAEs/g) equivalents, respectively.

PHENOLIC COMPOUND CHARACTERİZATION BY HPLC

Phenolic compounds of *H. europaeum* were analysed by RP-HPLC (Shimadzu, Japan) as described by Caponio et al. [20] with some modifications. Separation was performed at 30°C by using a reversed phase column (Agilent Eclipse XDB C-18, 250 mm × 4.6 mm, 5 µm) using the mixture of two solvents (A: the acetic acid solution 3% and B: methanol) as a mobile phase. Gradient conditions were particularised at a flow rate of 0.8 mL/min as follows: 93% A + 7% B for 0-20 min, 72% A + 28% B in 20-28 min, 75% A + 25% B in 28-35 min, 70% A + 30% B in 35-60 min, 67% A + 33% B in 60-62 min, 58% A + 42% B in 62-70 min, 50% A + 50% B in 70-73 min, 30% A + 70% B in 73-75 min, 20% A + 80% B in 75-80 min, 0% A + 100% B in 80-81 min, 93% A + 7% B in 81-90 min. Phenolic compounds in the methanolic extract of *H. europaeum* were expressed as µg/g ex-

tract, which were analysed with diode array dedector (DAD) at 280 nm (for the phenolic acids) and 320 and 360 nm (for the flavones, flavonoles). The identification of each target compound was based on a combination of retention time and spectral matching.

ANTIOXIDANT ACTIVITY ASSAYS

- DPPH radical scavenging activity

When the 1,1- diphenyl-2-picrylhydrazyl (DPPH) radical gets an electron from the antioxidant compound, the color changes from violet to yellow which is detected colorimetrically. The impact of the extracts on DPPH radical was determined with the method described by Ozay and Mammadov [18] with slight modifications. Various concentrations of the extracts were added to DPPH metanolic solution, and the mixture was incubated for 30 min in the dark at room temperature. After incubation, the absorbances were measured at 517 nm. The synthetic antioxidant, butylated hydroxytoluene (BHT) was used as positive control. The results were indicated as the half-maximal inhibitory concentration (IC_{50}) values.

- ABTS radical cation activity

The ABTS assay is based on the generation of a blue/green 2,2' azino-bis (3-ethylbenzothiazloine-6 sulfonic acid) (ABTS) radical cation that can be reduced by antioxidants. ABTS radical removal activity was analyzed as described by Re et al. [21] with some modifications. Freshly prepared and diluted ABTS solution were mixed with the various solvent extracts of *H. europaeum* and the absorbances were read after 30 min at 734 nm. BHT was used as positive control. The results were indicated as IC_{50}.

- β-Carotene/linoleic acid assay

The ability of the extracts regarding the inhibition of linoleic acid oxidation was investigated using β-carotene test system according to Ozay and Mammadov [18]. This method is based on the monitoring of the color opening of β-carotene by alkyl peroxides formed by free radical chain reaction by heat and air oxidation of linoleic acid. The results were calculated with the formula using the initial and final absorbances of samples and control group.

$[1 - (A_C - A_S / A_C° - A_S°)] \times 100$

where A_C and $A_C°$ were absorbance values initial and final measurement (120 min) of contol group; A_S and $A_S°$ were absorbance values of samples or standard, respectively. BHT was used as standard antioxidant.

- Phosphomolybdenum (PM) assay

This assay is based on the reduction of Mo (IV) to Mo (V) with antioxidant agents. The green color resulting from the reduction is measured at 695 nm. PM assay of *H. europaeum* extracts carried out Prieto et al. [22]. Results are given as ascorbic acid equivalents (mg AEs/g extract).

- Ferric reducing antioxidant power (FRAP) assay

The principle of this method is based on the reduction of a Fe(III)-tripyridyltriazine (TPTZ) complex to Fe(II)-TPTZ in the presence of antioxidants. The results measured at 593 nm are given as equivalent to trolox (mg TEs/g extract). This assay was carried out according to Apak et al. [23].

- Metal chelating activity

The ferrous chealating capacity of *H. europaeum* extracts was determined with Dinis et al. [24]'s method with slight modification. According to this method, extracts inhibit ferrozine complexing with Fe^{2+} and color expansion is determined spectrophotometrically (562 nm). The results were provided as ethylenediaminetetraacetic acid (EDTA) equivalents (mg EDTAEs/g extract).

- Cell viability assay

The extract used in all cell culture assays was diluted in the growth media of the RAW 264.7 cell line. The vehicle for the initial stock of the extract was 0.1% dimethyl sulfoxide (DMSO). The effect of *H. europaeum* on cell viability was determined by MTT assay [41]. RAW 264.7 cells were mechanically scraped and plated at 5×10^3 cells/well in 96-well plates containing 100 µl of DMEM medium with 10% heat-inactivated FBS and incubated for 24 h (37°C and 5% CO_2). After incubation, the cells were treated with different concentrations of the extract (50-400 mg/mL) for 24 h. Then, MTT solution was added to the wells and incubated for 4h at 37°C. Finally, DMSO was added to dissolve the formazan crystals and absorbance was measured at 570 nm. Cell viability (%) was calculated as follows:

Cell Viability (%) = $(Abs_{sample} / Abs_{control}) \times 100$

- Griess nitrite assay

The NO production was measured as described by Han et al. [25]. RAW 264.7 cells at 5×10^3 cells/well were cultured in 96-well plates in triplicate for 24 h and preincubated with different concentrations of the extract (50-200 µg/mL) for 1 h and were then stimulated with LPS (1 µg/mL). The culture supernatants were collected 24 h after the LPS stimulation and the concentrations of NO were measured according to the Griess reaction. 50 µL of supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid) and incubated for 10 min at room temperature, then the absorbance was measured at 550 nm. Finally, the concentration of nitrite was calculated from a standard curve ($y = 0.0123x + 0.0185$, $R^2 = 0.9995$) drawn with known concentrations (1-100 µM) of sodium nitrite dissolved in DMEM.

- Pro-inflammatory cytokine determination

RAW 264.7 cells were pretreated with different concentrations of the extract (50-200 µg/mL) for 1 h and

Table I - Extract yield and total secondary metabolites amount of *H. europaeum* according to different solvents (mean ± SD)

	Chloroform	Ethanol	Methanol	Water
Extraction yield (%)	5.13±0.11[a]	11.48±0.17[a]	16.51±0.20[b]	20.12±0.14[b]
TPA (mg GAEs/g)	13.04±0.20[a]	27.35±0.28[b]	42.01±0.43[c]	33.11±0.34[b]
TFA (mg QEs/g)	5.01±0.09[a]	20.64±0.27[b]	38. 14±0.45[c]	27.45±0.32[b]
TSA (mg QAEs/g)	nd	11.09±0.15[a]	18.22±0.22[a]	30.35±0.46[b]

TPA: total phenolic amount; *TFA:* total flavonoid amount; *TSA:* total saponin amount; *GAEs:* gallic acid equivalents; *QEs:* quercetin equivalents; *QAEs:* quillaja equivalents, *nd:* not detected. In each row, different letters indicate significant difference ($P \leq 0.05$)

then LPS (1 µg/mL) was added to the treatment cells to activate the macrophages. After 24 h of cell incubation, the supernatants of cell cultures were collected and used to measure the levels of TNF-α, IL-6, and IL-12 proteins using ELISA kit (Boster, USA) according to the manufacturer's instructions. ELISA results were recorded using a microplate spectrophotometer at 450 nm and the corrected absorbance was noted as per manufacturer's instruction. Results were expressed in pg/mL. Each sample was measured in triplicate and values were derived from the standard curve.

- Statistical analysis

The results obtained in this study are expressed as mean ± standard deviation (SD). Statistical analysis and data processing were performed by using SPSS 22.0. Comparisons of the treatments among groups were analysed by one-way ANOVA with post-hoc Tukey's test. Significance was accepted as $P \leq 0.05$.

RESULTS AND DISCUSSION

This contemporary age is extensively involved in herbal medicine investigation as witnessed by plenty of peer-reviewed research on herbs published by the research groups. A vast number of novel insights have been asserted, and many conventional beliefs have been confirmed, refused, or adopted again. However, even by conceding the abundance of research being performed, the search for novel drugs from plants stand before us as an ongoing necessity [26, 27].

Heliotropium is one of the most significant plant genera to have conventional folklore significance, hence is a potential source of bioactive compounds [28]. In this study, total phenolic content of *H. europaeum* extracts was calculated equivalent to gallic acid and the highest content was observed in methanol extract (42.01 ± 0.43 mgGAEs/g). Our results showed that methanol extract have the highest total flavonoid amount (38.14 ± 0.45 mgQEs/g), while water extract have the highest total saponin amount (30.35 ± 0.46 mgQAEs/g). Total saponin content could not be determined in the chloroform extract, because when the extract was mixed with the highly polar vanillin-sulfuric acid solution, a cloudy emulsion was formed. Therefore, the saponin content could not be measured spectrophotometrically in the chloroform extract. The yield of extracts from *H. europaeum* is listed in Table I. The efficiency of extracts prepared with four solvents with different polarities was calculated. The highest extract amount obtained from water extract (% 20.12). This result can be related to that water have the highest polarity. However, methanol, ethanol and chloroform present the lower extraction yield with a percentage of 16.51%, 11.48% and 5.13%, respectively.

Although there are many studies on alkaloids of the genus *Heliotropium*, few studies have described its phenolic compounds [29, 30]. Because methanol extract has a higher antioxidant capacity and total secondary metabolites amount than others, methanol extract was used in HPLC analysis and anti-inflammatory activity assays. To identify the phenolic compounds in the methanolic extracts of the aerial parts of *H. europaeum*, 15 standard compounds (gallic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, 2,5-dihydroxybenzoic acid, chlorogenic acid, vanillic acid, epicatechin, caffeic acid, *p*-coumaric acid, ferulic acid, rutin, ellagic acid, naringin, cinnamic acid, quercetin) were used in the HPLC analysis. The phenolic compounds were detected in the extract with varying amounts and were listed in Table II. According to these results, caffeic acid (15971.41 µg/g), 2,5-dihydroxybenzoic acid (11469.11 µg/g), epicatechin (7243.33 µg/g) and quercetin (4465.59 µg/g) are most common phenolic compounds of methanol extract of *H. europaeum.* It has been reported that these phenolic acids possess anti-inflammatory and anti-oxidative activities [31-33]. The results of the present study are in accordance with the results of Jasim and Hamad [30]. Six phenolic compounds (syringic acid, silybin, kaempferol, apigenin, caffeic acid and genistein) were isolated and identified from Iraqi *H. europaeum* plant. According to their result, one of the most abundant phenolic compounds in *H. europaeum* extract was caffeic acid. Al-Saleem et al. [28] reported that eight phenolic compounds were isolated from the aerial parts of *H. europaeum,* which was collected from Saudi Arabia. The isolated compounds were identified as kaempferol (1), luteolin (2), quercetin (3), kaempferol-3-O-glucoside (4), and luteolin-7-O-glucoside (5), in addition to caffeic acid (6), rosmarinic acid (7), and methyl rosmarinate (8). These phenolic metabolites from *H. europaeum* exhibited antioxidant, angiotensin-converting enzyme

(ACE) and lipoxygenase (LOX) inhibitory activities.
It is known that phenolic compounds are molecules with antioxidant activity due to their hydroxyl groups and phenolic rings [34]. To determine the antioxidant activity of *H. europaeum* extracts with different solvents, we preferred six diverse methods to compare the results with each other and provide more reliable data. The antioxidant activity of the extracts is presented in Table III. β-carotene/linoleic acid and phosphomolybdenum assay were used to evaluate total antioxidant capacity and the water extracts (%75.06 ± 1.46) have the highest antioxidant activity according to β-carotene/linoleic acid assay, but methanol extracts (85.24 ± 0.62 mgAEs/g) possess most effective activity in phosphomolybdenum assay. DPPH and ABTS radical scavenging assays are commonly carried out for fast evaluation of antioxidant activity because of their stability in the radical form and simplicity of the assay. In both DPPH (IC_{50}: 28.02 ± 0.37 µg/mL) and ABTS (IC_{50}: 37.55 ± 0.06 µg/mL) tests, methanol extracts showed the highest radical scavenging activity. In a previous study, the highest DPPH radical scavenging activity of *Heliotropium strigosum* was observed in ethyl acetate (94.5%) and aqueous (94.7%) fractions [3]. When the DPPH assay was applied to the *Heliotropium samolifolium* subsp. *erzurumicum*, the highest activity was found in above-ground ethanol+aqueous, chloroform and below-ground ethanol extracts [1]. FRAP assay was carried out to determine the reduction power of the extracts. Results which are equivalent to trolox standard, show that aqueous extracts have high reducing power activity (97.08 ± 1.67 mgTEs/g). And also, ferrious metal chelating capacity of the extracts were evaluated, and results were given as the standard equivalent of EDTA. According to this test, methanolic extract had highest chelating capacity (32.07 ± 0.65 mgTEs/g) ($P \leq 0.05$). Due to the different antioxidant potentials of compounds with different polarity antioxidant activity of the extracts are strongly dependent on the solvent type [19].
The total phenolic and flavonoid amount of *H. crispum* methanol extract were reported earlier as 24.84 mg GAE/g and 19.73 mg REs/g, respectively [35]. According to these results, *H. europaeum* methanolic extract used in the current study had more abundant (42.01 mg GAEs/g) total phenolic amounts than *H. crispum.* Savran et al. [36] have demonstrated that methanol extracts from different plant species remarkable bioactive components, which is in agreement with the present results. In present study, some phytochemical analysis and biological activity of *H.*

Table II - Phenolic compounds characterization of methanolic *H. europaeum* extract by HPLC (mean ± SD).

No	Identified phenolic compounds	RT (min)	UV_{max} (nm)	LOD (µg/mL)	µg/g extract (mean ± SD)
1	Gallic acid	6.8	280	0.015	47.83 ± 0.42
2	3,4-dihydroxybenzoic acid	10.7	280	0.031	68.95 ± 0.61
3	4-hydroxybenzoic acid	15.7	280	0.014	70.64 ± 0.65
4	2,5-dihydroxybenzoic acid	17.2	320	0.753	11469.11 ± 228.2
5	Chlorogenic acid	18.2	320	0.011	307.34 ± 3.11
6	Vanillic acid	19.2	320	0.112	847.95 ± 6.47
7	Epicatechin	21.3	260	0.433	7243.33 ± 120.1
8	Caffeic acid	22.7	280	0.018	15971.41 ± 250.3
9	*p*-coumaric acid	26.1	320	0.020	468.76 ± 4.24
10	Ferulic acid	30.1	320	0.012	241.47 ± 2.54
11	Rutin	45.6	360	0.576	217.76 ± 2.15
12	Ellagic acid	47.7	240	0.455	1345.09 ± 15.10
13	Naringin	49.7	280	0.404	109.95 ± 1.17
14	Cinnamic acid	67.8	280	0.016	265.02 ± 2.71
15	Quercetin	71.1	360	0.578	4465.59 ± 74.20

RT: retention time, LOD: limit of detection

Table III - Antioxidant activity of *H. europaeum* extracts (mean ± SD).

	Chloroform	Ethanol	Methanol	Water	BHT
β-carotene/linoleic acid assay (% inhibition)	50.01±1.10^{a}	62.11±1.35^{a}	70.13±1.40^{b}	75.06±1.46^{b}	95.02±1.66^{c}
Phosphomolybdenum assay (mg AEs/g)	61.76±1.33^{a}	73.08±1.46^{a}	85.24±0.62^{b}	78.44±0.48^{a}	nd
DPPH assay (IC_{50} value, µg/mL)	125.06±1.84^{d}	35.82±0.68^{b}	28.02±0.37^{b}	84.15±0.51^{c}	9.40±0.05^{a}
ABTS assay (IC_{50} value, µg/mL)	120.54±1.80^{d}	45.16±1.13^{b}	37.55±0.06^{b}	71.26±1.42^{c}	20.15±0.30^{a}
FRAP assay (mg TEs/g)	32.27±0.01^{a}	86.03±0.67^{b}	92.00±1.72^{b}	97.08±1.67^{b}	nd
Metal chelating activity (mg EDTAEs/g)	11.04±1.07^{a}	15.71±0.27^{a}	32.07±0.65^{b}	26.48±0.30^{b}	nd

AEs: ascorbic acid equivalents, *TEs:* trolox equivalents, *EDTAEs:* EDTA equivalents, *nd:* not detected. In each row, different letters indicate significant difference ($P \leq 0.05$)

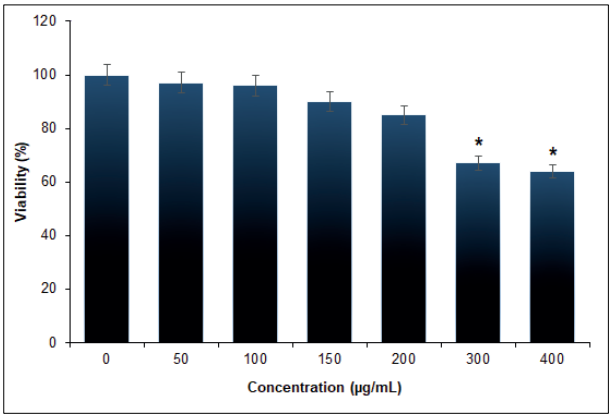

Figure 1 - Effect of *H. europaeum* extract on cell viability of RAW 264.7 cell line. Data are presented as mean ± SD. *$P \leq 0.05$

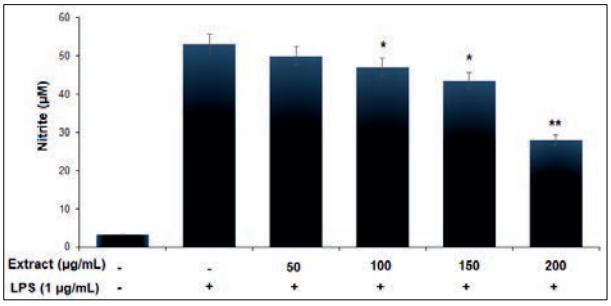

Figure 2 - Effect of *H. europaeum* extract on nitric oxide production by LPS-induced macrophage RAW 264.7 cells. Data are presented as mean ± SD. *$P \leq 0.05$, **$P \leq 0.01$; compared to the LPS-treated group.

europaeum extracts was determined. Our studies were revealed that *H. europaeum* methanolic extracts possess the high total phenolic, flavonoid amount and also total antioxidant capacity, radical scavenging activity and chealating power. The results on the total phenolic content in this study displayed a similar propensity to those of the antioxidant abilities. Accordingly, the high content of total phenolics in the extracts might explain the antioxidant properties of the extracts. These results were consistent with other results in literature which demonstrated a strong relationship between antioxidant activities and total phenolic contents [18, 37].

Drugs for inflammation treatment are effective but have serious side effects when used for a prolonged time. For this reason, it is crucial to search for new and safe anti-inflammatory agents. Medicinal plants are a valuable source of novel molecules and efficient alternative strategy for newer therapeutics development [38]. The potential anti-inflammatory effects of *H. europaeum* were examined and RAW 264.7 murine macrophage cell line, which is frequently used as an in vitro model in studies of inflammation, was selected in this study. Detection of suitable concentration ranges, which are not toxic, can be used for further *in vitro* anti-inflammatory screening assays of the extracts. Cytotoxicity assay was performed for methanol extracts at different concentrations (50-400 µg/mL) and the results were shown in Figure 1. The viability of RAW 264.7 cells decreased below 70% after 24 hours exposure to extracts at 300 and 400 µg/mL. Other concentrations of the extracts, that resulted in a cell viability > 70%, were used for the nitrite assay. Therefore, the possibility that the observed NO production inhibitory activities were due to the cytotoxicity was excluded.

Because NO is considered as an important parameter of inflammation and NO production is related with many inflammatory diseases, we investigated the effect of *H. europaeum* on LPS-induced NO production in RAW 264.7 macrophages by using Griess assay. The quantity of nitrite in the culture medium was measured as an indicator of NO production. Amount of nitrite (µM), a stable metabolite of NO, significantly decreased due to the rising extract concentration (Fig. 2). A 200 µg/mL *H. europaeum* methanol extract demonstrated the highest nitrite inhibitory activity. As for the pro-inflammatory cytokines (TNF-α,

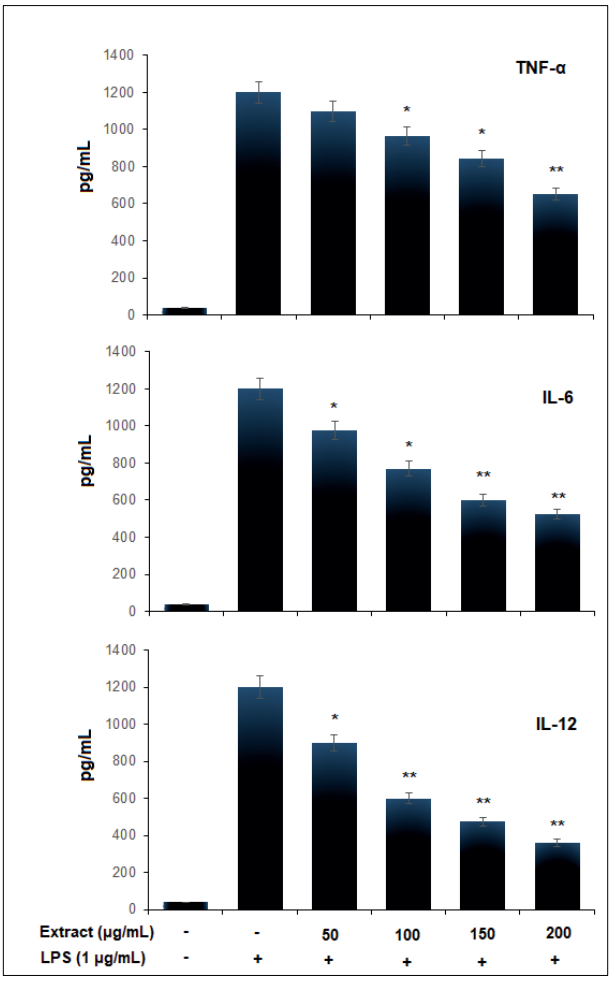

Figure 3 - Effects of *H. europaeum* extract on TNF-α, (a) and IL-6 (b) and IL-12 (c) generation by LPS-induced RAW 264.7 macrophage cells. Data are presented as mean ± SD. *$P \leq 0.05$, **$P \leq 0.01$; compared to the LPS-treated group.

IL-6 and IL-12) levels, *H. europaeum* has a greater effect on decreasing IL-6 and IL-12 levels than TNF-α level. TNF-α, IL-6 and IL-12 are three major macrophage-derived mediators of inflammatory responses in mammals. In this respect, it is important to investigate their quantities. As shown in Figure 3, there was a 3-fold reduction in LPS induced IL-12 production in RAW 264.7 cells pre-treated with 200 µg/mL of extract. Two major cytokines; TNF-a and IL-6 are implicated in the pathogenesis of rheumatoid arthritis, ulcerative colitis and several other inflammatory disorders [39]. A previous study showed that isolating two compounds (4,7,8-trimethoxy-naphthalene-2-carboxylic acid and 6-hydroxy-5,7-dimethoxy-naphthalene-2-carbaldehyde) from *Heliotropium ovalifolium* inhibited the release of IL-6 and TNF-a by LPS-stimulated THP-1 (human monocytic cell line) cells [40]. In another study, the whole-plant butanol fraction of *Heliotropium europaeum* showed anti-inflammatory activity on ROS [41].

CONCLUSIONS

Phenolic compounds possess a remarkable anti-inflammatory capacity due to their multiple inhibitory activities of pro-inflammatory mediators. In this study, we first demonstrated the anti-inflammatory effect of *H. europaeum* using RAW 264.7 macrophage cells. *H. europaeum* inhibited not only NO production but also the levels of proinflammatory cytokines. The antioxidant and anti-inflammatory activities observed could be related to the polyphenolic compounds in the extract. However, the isolation of bioactive compounds and analysis of the molecular mechanisms responsible for the potential anti-inflammatory effect should be performed.

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