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Comparative study of the anti-inflammatory, antioxidant and urease inhibitory activities of *Eryngium kotschyi* Boiss. and *E. campestre* L. var. *virens* (Link) Weins extracts

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ABSTRACT: Despite widespread traditional usage of *Eryngium* species in Anatolia (Turkey), only a limited number of scientific studies exists on *E. kotschyi*, an endemic species. Previously, extracts from *E. campestre* and *E. kotschyi* were reported to have significant anti-inflammatory effects *in vivo*. This study aimed to investigate the *in vitro* anti-inflammatory, antioxidant, urease inhibitory activities of ethanol extracts of *E. kotschyi* and *E. campestre* var. *virens* roots as well as distilled water and ethanol extracts of *E. kotschyi* aerial parts. The NO and cytokine inhibitory effects were evaluated by Griess and ELISA assays. The antioxidant activities were tested on DPPH•, ABTS•+ and CUPRAC assays. The EtOH extract of *E. kotschyi* roots (EKr EtOH) and aerial parts (EKae EtOH) inhibited 50.08% and 41.52% of NO production at 100 µg/ml, respectively. The EtOH extract of the roots of *E. campestre* var. *virens* (ECr EtOH) and EKr EtOH provided 36.22% and 65.23% IL-6 inhibition and 44.24% and 56.84% IL-1α inhibition at 100 µg/ml. EKae EtOH exerted highest antioxidant activity on ABTS•+ (2.4±0.0005 µM trolox/mg extract) and CUPRAC (0.97±0.07 mM trolox/mg extract). This extract was also found the richest among all in terms of phenolics content (6.1±0.001 mg/GAE/g extract). EKr EtOH and ECr EtOH exhibited strongest DPPH• (IC_{50} = 1.132±0.057 mg/ml) radical scavenging and ferric reducing/antioxidant power (0.36±0.005 mM Fe2+/mg extract) activity respectively. The extracts exerted low urease inhibitory activity. Consequently, the results of this study might contribute to the elucidation of the mechanism of *in vivo* anti-inflammatory activity of the extracts.

KEYWORDS: *Eryngium kotschyi*; *Eryngium campestre* var. *virens*; anti-inflammatory; antioxidant; urease inhibition, phenolics.

1. INTRODUCTION

Eryngium (Apiaceae) species are known as 'boğadikeni' in Anatolia and represented by 25 species (1). *Eryngium kotschyi* Boiss., which is one of the endemic *Eryngium* species of Turkey, is a perennial plant which has amethyst colored, dense paniculate flowers [2, 3] . *Eryngium* species are traditionally used for a broad spectrum of diseases in Anatolia; notably roots are used for inflammatory diseases such as sinusitis, urinary infections, oedema or inflammations [4, 5].

Results of a literature survey showed that there are only a limited number of studies on *E. kotschyi* except of the studies that report cytotoxic, *in vivo* antinociceptive and anti-inflammatory activities of the plant [3, 6, 7]. On the other hand, *Eryngium campestre* L. var. *virens* (Link) Weins is a perennial plant which is usually about 30-60 cm in length. Cytotoxic, antibacterial, antitumor, anti-inflammatory and antinociceptive activities were previously reported for *E. campestre* [3, 8, 9, 10]. Triterpen saponins, flavonoids and coumarins were previously isolated from *E. campestre* while triterpene saponins were isolated from *E. kotschyi* [6, 11, 12, 13, 14, 15, 16]. In a previous work, the ethanol (EtOH) extract of the roots of *E. kotschyi* and *E. campestre* as well as dH_2O and EtOH extracts of the aerial parts of *E. kotschyi* exerted significant anti-inflammatory effects on carrageenan induced oedema model [3].

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Inflammation occurs as a defense mechanism of the body against various destructive stimuli (chemical, biological or physical), however when it becomes persistent as chronical inflammation, it contributes to the progression of many diseases. These diseases include inflammatory bowel diseases, neurodegenerative disorders, rheumatoid arthritis, heart attacks, Alzheimer's disease, and cancers [17, 18, 19]. Although inflammatory responses manifest itself in distinct ways among various inflammatory diseases, they can all be characterised by common mediators including prostaglandins, chemokines, inflammatory cytokines, and toxic molecules such as nitric oxide and free radicals [17, 18]. Among them nitric oxide (NO) is produced by many cell types in the body as a crucial mediator involving in critical physiological functions such as the regulation of vascular tone, neurotransmission and immune system, while in high concentrations NO and its reactive derivatives such as peroxynitrite involves in the pathogenesis of inflammation, sepsis and carcinogenesis [20]. Therefore, inhibition of NO production is endorsed to be an important measure to asses the activity of possible drug candidates against inflammation [21]. Likewise, cytokines are also of important value which are divided into two subgroups depending on their ability to induce or reduce inflammation; proinflammatory and anti-inflammatory cytokines, succesfully. Interleukin (IL)-1 and IL-6 which are classified as proinflammatory cytokines, involve in processes such as inflammation, tissue destruction and shock, and induce the transcription of proinflammatory genes [22, 23]. It was shown that IL-6 levels are raised in many inflammatory diseases such as systemic lupus erythematosus, psoriasis, rheumatoid arthritis, Crohn's disease, systemic juvenile idiopathic arthritis and ankylosing spondylitis [24].

Oxidative stress occurs when the antioxidant defence mechanisms of the body failes to balance the effects of reactive oxygen species (ROS). The progression of various diseases including inflammatory diseases, cancer and diabetes is found to be associated with oxidative stress [25]. Researches indicate that oxydative stress is induced by inflammatory process or vice versa and reduces cellular antioxidant capacity [26].

Helicobacter pylori is a major cause of chronic inflammation of stomach. Peptic ulcer and neoplastic processes on gastric mucosa have been shown to be related with the activity of the pathogens in stomach. Urease activity of *H. pylori* allows it to survive in the acidic condition of stomach. The pathogen's urease pathway have been widely targeted by drug discovery studies [27].

In order to further elucidate the previously reported mechanism on the *in vivo* anti-inflammatory activity of the extracts from the roots and aerial parts of *E. kotschyi*, the present study was designed to investigate the *in vitro* anti-inflammatory and antioxidant potentials of these extracts. The *in vitro* anti-inflammatory and antioxidant activities of the root extract of *E. campestre* var. *virens* was also studied. Moreover, to obtain an opinion on the potential benefits of these extracts against *Helicobacter pylori* infection their urease inhibitory activities were studied. The antioxidant activities and the phenolic contents of the plant extracts were repeatedly shown to be correlated. The phenolic compounds are known to be strong antioxidants [28]. For that reason, the chemical compositions of the extracts were also compared based on their total phenolic contents.

2. RESULTS

2.1. Total phenolic contents

The highest total phenolic content was determined in EKae EtOH (6.1±0.001 mgGAE/g extract). The total phenolic contents of EKae dH_2O (3.4±0.002 mg GAE/g extract) and EKr EtOH (3.1±0.004 mgGAE/g extract) were found to be close. On the other hand, ECr EtOH (0.2±0.05 mg GAE/g extract) was found to contain the lowest levels of phenolics.

2.2. Effects of the extracts on cell viability

The non-toxic concentrations of the extracts on Raw 264.7 macrophages were determined by WST-1 assay (Figure 1). The indicated concentrations (12.5, 25, 50 and 100 µg/ml) of extracts with lipopolysachharides (LPS, 1 µg/ml) were applied to cells, and incubated at 37 ^{o}C for 24 h. The viability of the cells were not significantly decreased by the treatment with the EtOH extract of the aerial parts of *E. kotschyi* [EKae EtOH] (Figure 1A). On the other hand, the dH_2O extract of the aerial parts of the same plant [EKae dH_2O] applied at the same concentration slightly reduced the cellular viability of the cells at the highest two concentrations (Figure 1C). The cell viabilities were 85.70±4.21% and 80.65±7.42% at 50 and 100 µg/ml concentrations, respectively. The EtOH extract of the roots of *E. kotschyi* [EKr EtOH] only slightly decreased the cell viability (80.96±3.60%) at 100 µg/ml concentration (Figure 1B). The EtOH extract of the roots of *E. campestre* var. *virens* [ECr EtOH] did not significantly decreased cellular viability (Figure 1D). L-N6-(1-iminoethyl) lysine (L-NIL) at 10 µM did not significantly reduce the viability of Raw 264.7 cells.

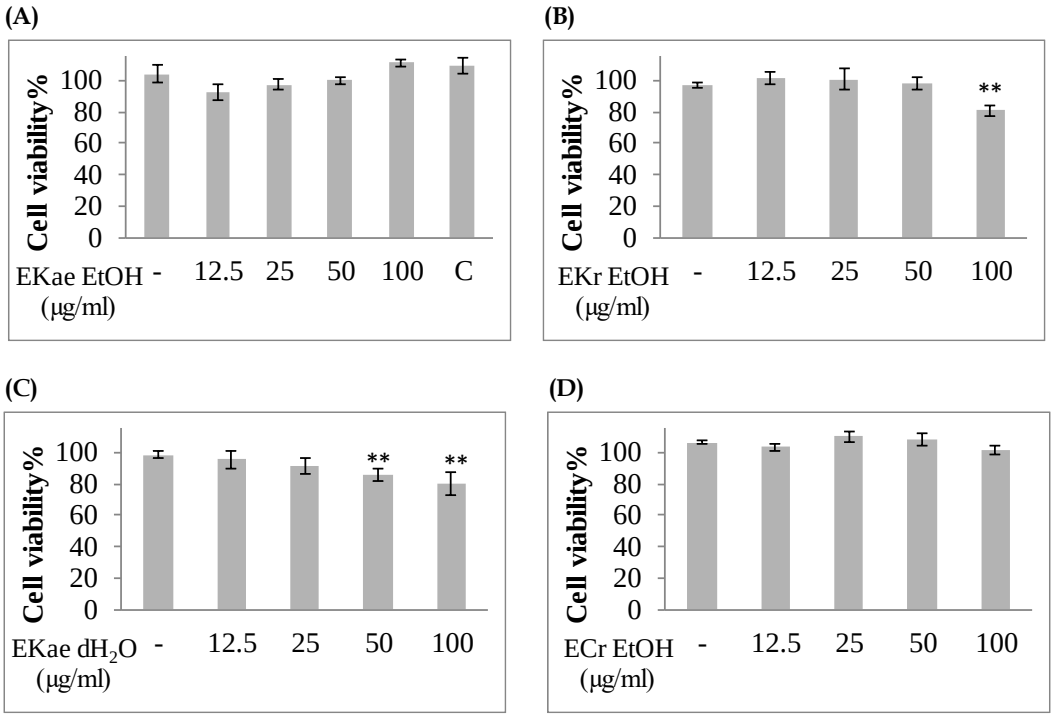

Figure 1. Effects of *E. kotschyi* extracts [aerial EtOH (A), root EtOH (B), aerial dH_2O (C)] and *E. campestre* var. *virens* root EtOH extract (D) on the viability of Raw 264.7 macrophages as determined by WST-1 assay. Indicated concentrations of extracts were applied to cells with LPS (1 µg/ml) for 24 h. C: L-NIL applied at 10 µM concentration. (-): Only media treated control group. Error bars represents the mean ± SEM value for three experiments. Values of * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ were considered statistically significant compared to only media treated control group.

2.3. Effects of the extracts on NO production

Raw 264.7 cells were co-incubated with the indicated concentrations (12.5, 25, 50 and 100 µg/ml) of the extracts and LPS (1 µg/ml) for 24 h to determine the NO inhibitory activities. As shown in Figure 2, the treatment of cells with LPS resulted in a significant increasement of NO levels compared to only media treated control group. Although EKae dH_2O (Figure 1C) and ECr EtOH (Figure 1D) did not exert remarkable inhibition at these concentrations, the other two extracts exerted significant inhibitions. EKr EtOH inhibited 4.31%, 12.47%, 19.62 %and 50.08% of NO production at 12.5, 25, 50 and 100 µg/ml concentrations respectively. On the other hand, EKae EtOH inhibited 13.85%, 24.61%, 33.44%, 41.52% of NO production at the same concentrations. It is noteworthy that despite the fact that EKr EtOH was found to be more active at high concentration (100 µg/ml), the activities of EKae EtOH was found to be higher at lower concentrations. L-NIL which is a known inhibitor of NO, exerted 86.94% inihibition at 10 µM concentration.

2.4. Effects of the extracts on the production of IL-6 and IL-1α on LPS-induced Raw 264.7 macrophages

Raw 264.7 macrophages were co-incubated with both LPS (1 µg/ml) and the indicated concentrations (12.5, 50 and 100 µg/ml) of the extracts and for 24 h. After that the media was collected and IL-6 levels were measured. As shown in Figure 3 the treatment of cells with LPS significantly increased IL-6 levels compared to only media treated control group. ECr EtOH inhibited 11.05%, 18.10%, 36.22% of IL-6 production at these concentrations (Figure 3D). EKr EtOH exerted remarkable inhibition (65.23%) only at the highest concentration (Figure 3B). On the other hand, the activities of EKae dH_2O and EKae EtOH were found weak. Dexamethasone which is a known inhibitor of IL-6 production, exerted 80.14% inihibition at 5 µM concentration.

All extracts were tested for their IL-1α inhibitory potentials at 100 µg/ml concentrations. EKae dH_2O and EKae EtOH did not significantly inhibit IL-1α productions. EKr EtOH significantly inhibited IL-1α in a concentration dependent manner. This extract provided 38.94% and 56.84% decrease in IL-1α levels at 50 and 100 µg/ml concentraions compared to only LPS treated control group (Figure 4 B). ECr EtOH inhibited 50.92%, 44.24% of IL-1α production at these concentrations (Figure 4C).

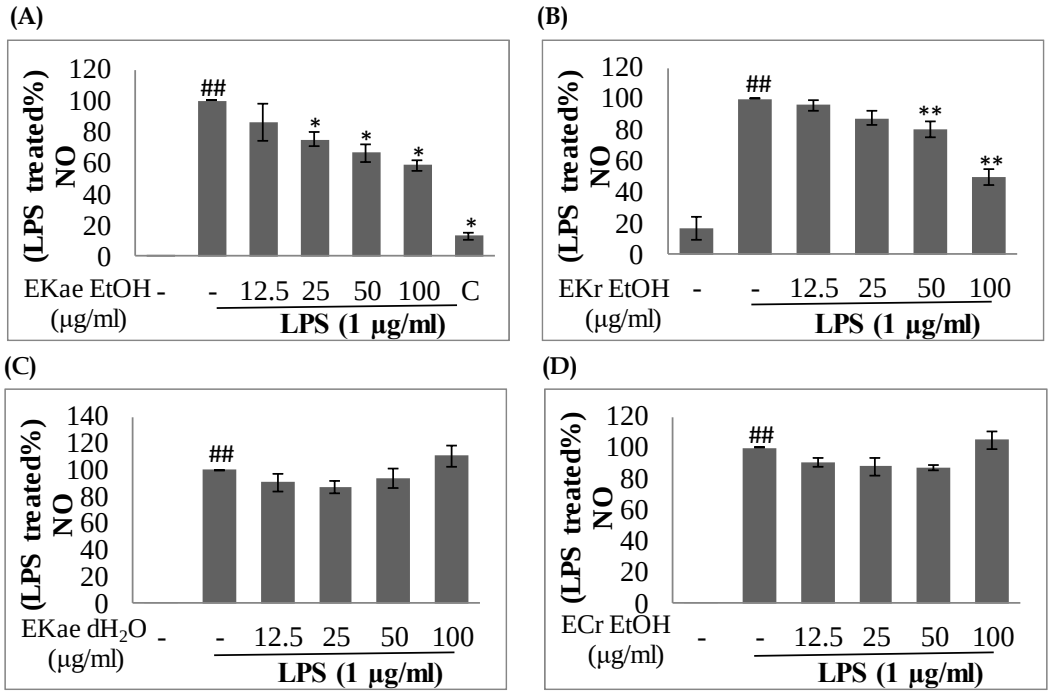

Figure 2. Effect of *E. kotschyi* extracts [aerial EtOH (A), root EtOH (B), aerial dH_2O (C)] and *E. campestre* var. *virens* root EtOH extract (D) on the NO productions of LPS induced Raw 264.7 macrophages as determined by Griess Assay. The indicated concentrations of extracts was co-incubated with LPS (1 µg/ml) for 24 h. C: L-NIL (10 µM) was applied. (-): Only media treated control group. Error bars represents the mean ±SEM values for three experiments. Values of * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ vs. LPS treated control group and # $p \leq 0.05$, ## $p \leq 0.01$ and ### $p \leq 0.001$ vs. only media treated group were considered statistically significant.

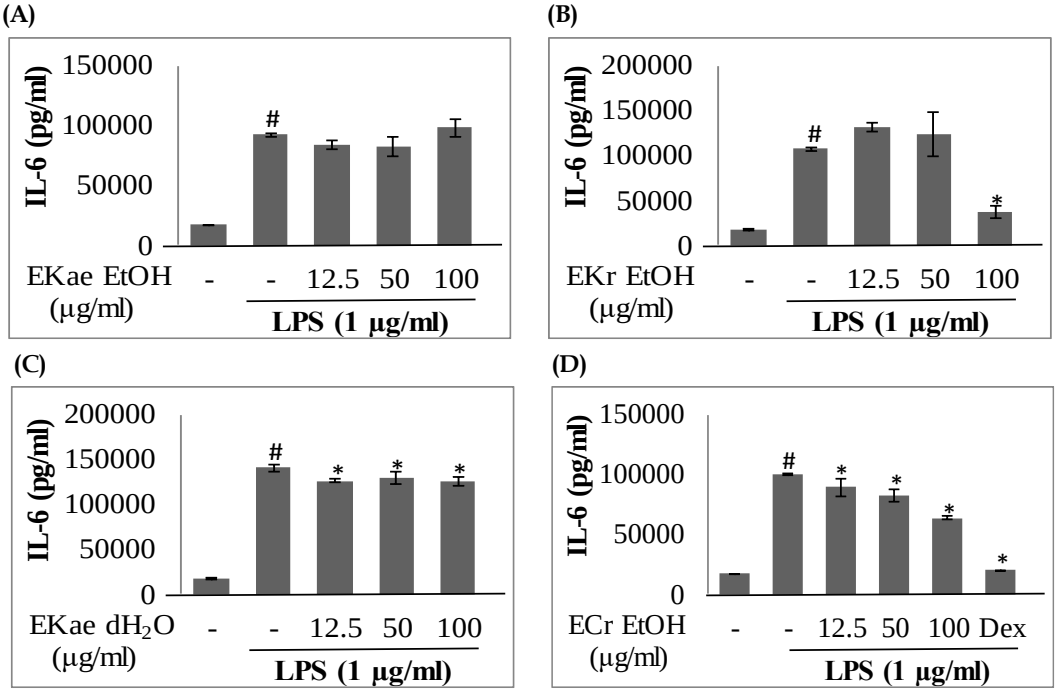

Figure 3. Effect of *E. kotschyi* extracts [aerial EtOH (A), root EtOH (B), aerial dH_2O (C)] and *E. campestre* var. *virens* root EtOH extract (D) on the IL-6 productions of LPS induced Raw 264.7 macrophages. IL-6 concentrations were determined by ELISA. Cells were co-incubated with the indicated concentrations of extracts and LPS (1 µg/ml) for 24 h. Dexamethasone (Dex) was applied at 10 µM concentration. (-): Only media treated control group. Error bars represent the mean ± SEM. Values of * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ vs. LPS treated control group and # $p \leq 0.05$, # # $p \leq 0.01$ and # # # $p \leq 0.001$ vs. only media treated group were considered statistically significant.

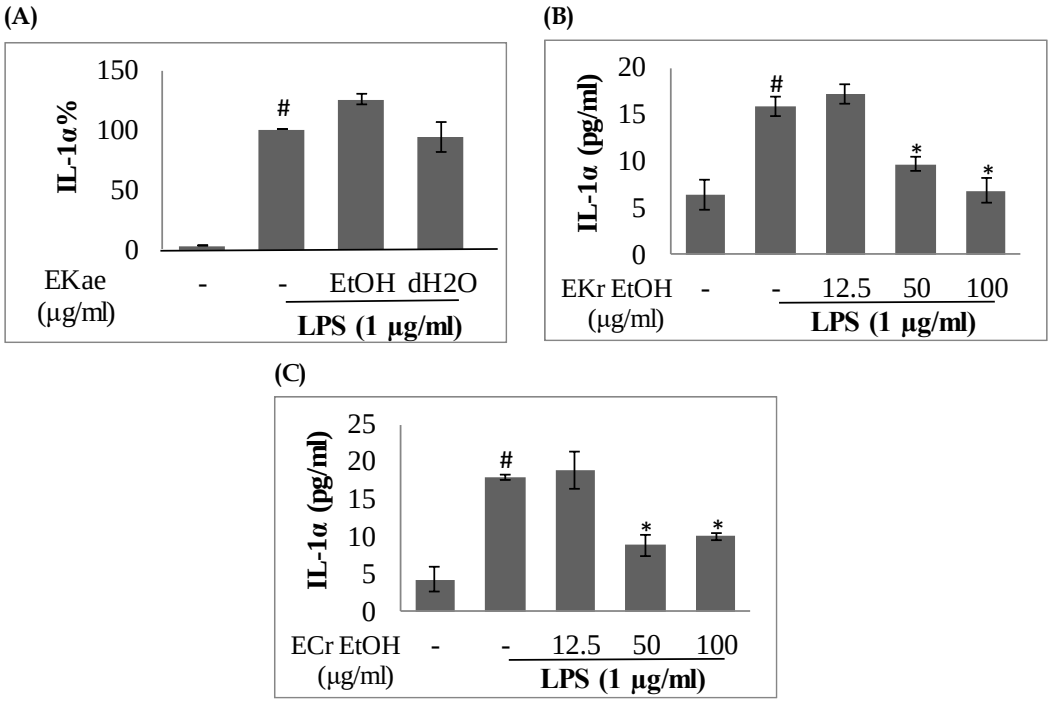

Figure 4. Effect of *E. kotschyi* extracts [aerial EtOH (A), root EtOH (B), aerial dH_2O (A)] and *E. campestre* var. *virens* root EtOH extract (C) on the IL-1α productions of LPS induced Raw 264.7 macrophages. IL-1α levels were determined by ELISA. Cells were treated with the indicated concentrations of extracts and LPS (1 µg/ml) for 24 h. (-): Only media treated control group. Error bars represents the mean ±SEM values. Values of * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ vs. LPS treated control group and # $p \leq 0.05$, # # $p \leq 0.01$ and # # # $p \leq 0.001$ vs. only media treated group were considered statistically significant.

2.5. DPPH• radical scavenging activity

The highest DPPH• radical scavenging activity is detected for EKr EtOH, followed by EKae dH_2O, EKae EtOH and ECr EtOH (Table 1). The IC_{50} values are 1.132±0.057, 1.222±0.05, 2.327±0.075, 2.84±0.19 mg/ml respectively. The free radical scavenging activities of the extracts were found to be lower than BHT (0.15±0.026 mg/ml). A linear relationship was not observed between DPPH• radical scavenging activity and the amount of total phenolic contents.

Table 1. The antioxidant and urease inhibitory activities of plant extracts.

Samples	Extract Yield (%)	DPPH• (IC_{50}, mg/ml)	$ABTS^{•+}$ (µM trolox/mg extract)	FRAP (mM Fe^{2+}/ mg extract)	CUPRAC (mM trolox/mg extract)	Total phenolics (mg GAE/ g extract)	Urease inhibition (%) (12.5 µg/ml)
EKae dH_2O	12.00	1.222±0.05[a]	2.3±0.0001[a]	0.22±0.003[a]	0.66±0.01[a]	3.4±0.002[a]	-
EKae EtOH	5.60	2.327±0.075[b]	2.4±0.0005[a,b]	0.31±0.003[b]	0.97±0.07[b]	6.1±0.001[b]	-
ECr EtOH	6.80	2.84±0.19[c]	1.2±0.0002[c]	0.36±0.005[c]	0.51±0.009[c]	0.2±0.05[c]	3.06±0.07[b]
EKr EtOH	13.80	1.132±0.057[d]	2.3±0.002[a,b,d]	0.24±0.006[a,d]	0.80±0.06[d]	3.1±0.004[d]	10.04±0.1[a]
BHT		0.15±0.026[e]	13±0.001[e]	1.1±0.12[e]	5.78±0.07[e]		
Thiourea							86.17±1.2[c]

Values are mean of triplicate determination (n = 3) ± standard deviation; means with different superscripts [(a-e)] are significantly different, $p<0.05$, GAE–Gallic acid equivalents.

2.6. $ABTS^{•+}$ radical cation scavenging activity

As shown in Table 1, $ABTS^{•+}$ radical scavenging activities of the extracts were as follows the EKae EtOH, EKae dH_2O, EKr EtOH, ECr EtOH. The values were 2.4±0.0005, 2.3±0.0001, 2.3±0.002 and 1.2±0.0002 µM trolox/mg extract, respectively. The free radical scavenging activities of both extracts was found to be less

than BHT (13±0.0001 µM trolox/mg). A linear relationship was observed between $ABTS^{\bullet+}$ radical scavenging activity and the amount of total phenolic contents.

2.7. Ferric reducing antioxidant/power capacity

As shown in Table 1, ECr EtOH (0.36±0.005 mM Fe2+/mg extract) exhibited the highest ferric reducing antioxidant capacity followed by EKae EtOH (0.31±0.003 mM Fe2+/mg extract), EKr EtOH (0.24±0.006 mM Fe2+/mg extract) and EKae dH_2O (0.22±0.003 mM Fe2+/mg extract). The activities of the extracts were less than BHT. A linear relationship was not observed between ferric reducing antioxidant capacity and the amount of total phenolic contents.

2.8. Cupric ion reducing antioxidant capacity

The cupric reducing antioxidant capacity of the extracts were as follows EKae EtOH (0.97±0.07 mM trolox/mg extract), EKr EtOH (0.80±0.06 mM trolox/mg extract), EKae dH_2O (0.66±0.01 mM trolox/mg extract) and ECr EtOH (0.51±0.009 mM trolox/mg extract). A linear relationship was observed between cupric reducing antioxidant capacity and the amount of total phenolic contents.

2.9. Urease inhibitory activity

The urease inhibitory activities of the extracts were tested at 12.5 µg/ml concentration. EKr EtOH exerted 10.04±0.1% inhibition of urease activity. On the other hand, ECr EtOH showed 3.06±0.07% inhibition. Both extracts exerted lower activity than thiourea, a reference compound, which provided 86.17±1.2% inhibition at the same concentration. While the dH_2O and EtOH extracts of the aerial parts of *E. kotschyi* did not exert significant urease inhibition (Table 1).

3. DISCUSSION

Despite the common traditional usage of *Eryngium* species for a wide spectrum of diseases in Anatolia, a literature survey revealed that, only a limited number of scientific studies have been carried out so far particularly on *E. kotschyi*, which is one of the endemic species to Turkish flora [2, 4, 5]. The *in vivo* anti-inflammatory and anti-nociceptive activities of *E. kotschyi* and *E. campestre* were previosly reported [2; 29]. From the MeOH extract of the roots of *E. kotschyi*, bioactivity guided fractionation procedures led to the identification several active fractions and a moderate effective triterpen saponin [29]. In a previous study Kupeli et al. evaluated the *in vivo* anti-inflammatory activities of the aerial and root parts of various *Eryngium* species and reported that dH_2O and EtOH extracts of the aerial parts of *E. kotschyi* as well as the EtOH extract of the root parts of *E. kotschyi* exerted significant *in vivo* anti-inflammatory activities [3]. In order to cast light onto the anti-inflammatory activity mechanism of these extracts, the present study investigated the *in vitro* effects of the extracts on NO, IL-6 and IL-1α production on LPS induced Raw 264.7 macrophages. EKae EtOH which was determined to be the richest extracts of phenolics, significantly inhibited NO production of LPS induced Raw 264.7 cells, but did not exert significant inhibition on IL-6 and IL-1α levels. On the other hand, EKae dH_2O which is the second richest extract in phenolics, failed to inhibit NO production, but slightly reduced IL-6 levels. It's noteworthy that the aerial parts of of *E. kotschyi* did not exert high anti-inflammatory activity on the tested parameters except that the EtOH extract of the aerial parts exerted signifacant NO inhibitory activity. In the present study the roots of *E. kotschyi* were also investigated along with the roots of *E. campestre* var. *virens*. EtOH extracts of both plants exerted significant inhibitory activity on IL-1α productions. Although the ECr EtOH did not effect NO production, EKr EtOH significantly inhibited NO productions. ECr EtOH which contains the lowest levels of phenolics, significantly inhibited IL-6 production. The EtOH extract of the roots of *E. campestre* var. *virens* only exerted inhibitory activities on cytokine secrection. The EtOH extract of the roots of *E. kotschyi* exerted inhibitory activity on all parameters and it was the most active one against NO. Compared to aerial parts the roots parts of both plants exerted higher activities on all parameters except the EtOH extract of the aerial parts of *E. kotschyi* which exerted significant NO inhibitory activity. Literature survey revealed there is only a limited number of studies on the *in vitro* anti-inflammatory effects of the plants particularly against the activated macrophages. In a previous study, the EtOH extract from the aerial parts of *E. campestre* significantly inhibited NO secretion of cytokine stimulated murine brain endothelial cells and LPS induced murine monocyte/macrophage-like cell line P388D1 [10].

The extracts prepared from the aerial and root parts of *E. kotschyi* and the roots of *E. campestre* var. *virens* were also compared by means of their antioxidant activities and total phenolic contents. EKae EtOH having the richest phenolics content exerted the highest antioxidant activity by $ABTS^{\bullet+}$ and CUPRAC assays. On the

other hand, EKr EtOH and EKae dH_2O exerted the highest antioxidant potential on DPPH• assay. ECr EtOH showed weak antioxidant potential on all assays except FRAP assay, although possessing the least phenolic content among all. In a recent study, the MeOH extract and subextracts obtained from the MeOH extract (ethyl acetate, *n*-butanol and water) of the flowering aerial parts of *E. kotschyi* was investigated for their total phenolic and total flavonoid compounds as well as their antioxidant capacity (DPPH•, $ABTS^{+\bullet}$ and FRAP methods). In this study, the highest total phenolic and total flavonoid content was found in ethyl acetate subextract which also showed the highest antioxidant capacity [30]. In a previous study, the boiling water extracts of the aerial parts and roots of *E. kotschyi* were investigated for their antioxidant potentials by DPPH• radical scavenging assay, phosphomolybdenum assay and reducing power assays. The boiling water extract of *E. kotschyi* aerial and root parts exerted high antioxidant capacity with IC_{50} values 39.38 µg/ml and 39.42 µg/ml by DPPH• radical scavenging assay, and 50.90% and 49.62% as equivalent to α-tocopherol (mg/g) by phosphomolybdenum assay. Reducing power analysis verified the antioxidant capacity [31]. In the present study, the dH_2O extract prepared at room temperature from the aerial parts of *E. kotschyi* was also found to exert DPPH• activity (IC_{50} = 1.222±0.05 mg/ml). Difference with the results might be due to the differences between the extraction techniques. The DPPH• method better responds to polar compounds in the extract. The increasing temperature for the extraction procedure seems to increase the DPPH• activity of the extract. Our results suggest that *E. kotschyi* aerial parts is a potential candidate for further investigations in anti-inflammatory activity as well as its promising antioxidant activity.

The antioxidant activity of the aerial parts of *E. campestre* was reported in a number of studies [32, 33, 34, 35, 36, 37]. However only a few studies reported the antioxidant activity of its roots. Different solvent extracts (ethyl acetate, *n*-butanol and aqueous extracts) prepared from roots of *E. campstre* was reported to exert good antioxidant activities due to its polyphenolic compounds [33]. The MeOH extract of the roots was also evaluated by an antioxidant activity assay based on the Briggs–Rauscher (BR) reaction as well as TEAC and DPPH• assays and IC_{50} value for DPPH• assay was estimated as 216.0 ± 4.0 µg/ml [38].

The urease inhibitory activites of the extracts were also evaluated as a measure to investigate anti-*Helicobacter pylori* activities of the extracts. Only EKr EtOH exerted 10.04% urease inhibition. The remaining extracts were not active at 12.5 µg/ml concentration. All extracts exerted lower activity than Thiourea.

4. CONCLUSION

It's revealed that the EtOH extracts of the root parts of *E. kotschyi* and *E. campestre* var. *virens* exerted the highest inhibitory activities on NO, IL-6 and IL-1α productions. Containing relatively lower levels of phenolic compounds, these extracts also exerted the highest antioxidant activities on DPPH• and FRAP assays, respectively. Further investigations are needed to explain the chemical composition of these extracts to explain the observed activity. The extracts prepared from the aerial parts of *E. kotschyi* (EtOH and dH_2O) exerted high antioxidant activities on $ABTS^{\bullet+}$, FRAP and CUPRAC assays, and on DPPH• and $ABTS^{\bullet+}$ assays, respectively. These extracts were also determined to contain the highest levels of total phenolics that may contribute to explain the antioxidant activity of these extracts. On the other hand, the EtOH extracts of the aerial part of *E. kotschyi* exerted significant inhibitory activity on NO production.

Consequently, the results of this paper might shed light on the effects of the extracts on inflammatory mediators which might account for the previously reported *in vivo* anti-inflammatory activity of the *E. kotschyi* extracts. Besides the inhibitory activities of the extracts on NO and cytokines, the antioxidant activity of the extracts might contribute to the anti-inflammatory activity of the extracts. The total phenolic contents of the extracts were also reported. As far as we know this is the first report on the *in vitro* anti-inflammatory and urease inhibitory activities of *E. kotschyi.*

5. MATERIALS AND METHODS

5.1. Reagents and Chemicals

LPS, dexamethasone, dimethylsulfoxide (DMSO), Griess reagent, Folin Ciocalteu's phenol reagent, 2,4,6-tripyridyl-s-triazine, gallic acid, and 2,2-diphenyl-1-picryl-hydrazyl (DPPH•) were obtained from SigmaAldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycine solution and Fetal bovine serum (FBS) was purchased from Invitrogen/Gibco (Grans Island, NY, USA). Enzyme linked immunosorbent assay (ELISA) kits for TNFα and IL-1α were from R&D Systems (MN, USA) and eBiosciences respectively. L-NIL was sourced from Cayman. Butylated hydroxytoluene and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) were obtained from Fluka. WST-1 reagent was purchased from Roche

Applied Science (Mannheim, Germany).Urease (lyophilized 5 U/MG EC 3.5.1.5) were sourced from Merck KGaA. All other reagents were of analytical grade.

5.2. Plant material

The aerial parts and roots of *E. kotschyi* Boiss., Akaydın 13365 were collected from Sarıveliler and the roots of *E. campestre* L. var. *virens* (Link) Weins, Akaydın 13338 were collected from Kazancı (Karaman-Turkey) in July, 2010. The material was identified by G.A. (one of the authors). Voucher specimen are deposited at the Herbarium of the Faculty of Education, Hacettepe University, Ankara, Turkey.

5.3. Extraction

Dried and powdered roots of *E. kotschyi* and roots of *E. campestre* var. *virens* (10 g, each) as well as aerial parts of *E. kotschyi* were separately extracted two times with 90% EtOH (200 ml) at room temperature. The extracts were filtered through a filter paper. Evaporation of the solvents to dryness under reduced pressure yielded crude *E. kotschyi* root EtOH extract [EKr EtOH], *E. campestre* var. *virens* root EtOH extract [ECr EtOH] and *E. kotschyi* aerial parts EtOH extract [EKae EtOH] (Table 1). The extracts were filtered and the organic solvent was evaporated to dryness under reduced pressure (40°C). On the other hand, the dried and powdered aerial parts of *E. kotschyi* (10 g) was extracted two times with dH_2O (200 ml). The extract was filtered and lyophilized [EKae dH_2O] (Table 1) [3].

5.4. Determination of total phenolic contents

FCR method was used to measure the total phenolic contents. Extract (5 µL) and water (225 µL) was mixed in the plate. Afterwards, Folin-Ciocalteu reagent (diluted 1/3 with distilled water, 5 µL) and 2% sodium carbonate solution (15 µL) were added to the mixture. It was then lefted at room temperature (RT) for 2 hours. Finally the absorbance was read at 760 nm against the reference using a micro plate reader. The results were calculated and expressed as mg gallic acid equivalents/g extract [39].

5.5. Cells and cell culture

Dulbecco's modified Eagle's medium (DMEM) was supplemented with 10% Fetal Bovine Serum (FBS), 4 mM L-glutamin, 100 IU/ml penicillin and 100 µg/ml streptomycin to prepare cell culture media. The Raw 264.7 macrophages (ATCC TIB-71) were grown in the cell culture media at 37°C in a humidified atmosphere containing 5% CO_2.

5.6. WST-1 assay for cell viability

The viability of Raw 264.7 macrophages were tested by using a WST-1 assay kit (Roche Applied Sciences). This assay depends on the reducement of a tetrazolium-based salt dye to a purple colored formazan salt by metabolically active cells. Raw 264.7 cells (22.500 cells/well) were seeded into 96-well plates in 10% FBS-DMEM. The cells were treated with the indicated concentrations of the extracts ranging from 12.5 µg/ml to 100 µg/ml with 1 µg/ml LPS, and incubated for 24 hrs (37°C). The supernatant was then discarded and WST-1 reagent was added directly to the wells [final concentration of 5% (v/v)]. The cells were incubated with the reagent at 37°C for an additional 60 mins. Finally, absorbance was measured between 420-480 nm (λ_{max} 450 nm). All extracts and standards were dissolved in DMSO. They were then diluted with DMEM. The concentration of DMSO in the cell culture medium was not more than 0.1% (v/v).

5.7. Griess assay

The NO inhibitory activities were evaluated by Griess assay on LPS induced Raw 264.7 macrophages. The 96-well plates were used to seed Raw 264.7 cells at a density of 22.500 cells/well, in 10% FBS-DMEM. The indicated (12.5 µg/ml-100 µg/ml) concentrations of the extracts with 1 µg/ml LPS was applied to cells for 24 hrs at 37°C. Accumulated nitrite in the culture medium was determined by Griess Assay. For this purpose, 50 µl of cell culture medium and 50 µl of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid was mixed and incubated for 10 mins. After that, 50 µl of 0.1% (w/v) naphtylethylenediamine-HCl (Promega) was added. The mixture was then incubated at room temperature for an additional 10 mins. The absorbance was measured at 550 nm by a microplate reader. The amount of nitrite was calculated from the serial dilution standard curve prepared from $NaNO_2$.

5.8. Enzyme immunoassay for quantification of cytokines (IL-1α, IL-6)

The cytokine inhibitory activities were evaluated by ELISA on LPS induced Raw 264.7 macrophages. The 96-well plates were used to seed Raw 264.7 cells at a density of 22.500 cells/well, in 10% FBS-DMEM. The extracts and 1 µg/ml LPS were together applied to cells for 24 hrs (37°C). The inhibitory effects of extracts on the production of cytokines were determined by Sandwich ELISA method. The supernatants were harvested and tested in agreement with the manufacturer's instructions for the relevant ELISA kit.

5.9. DPPH· radical scavenging activity

The free radical scavenging capacity of extracts were determined by DPPH· test. The DPPH· solution (0.1 mM, 240 µL) was added to extracts (10 µL) prepared at varying concentrations ranging from 5 mg/ml to 0.5 mg/ml. The mixture was then lefted at RT for 30 min. The absorbance was measured against the reference at 517 nm. The results obtained from three experiments were given as IC_{50} = mg/ml [40].

5.10. Trolox equivalent antioxidant activity

Briefly, 50 µL of each extract was taken into the plate and 50 µL of water was added. Afterwards, 150 µL of $ABTS^{\bullet+}$ working solution were added to the mixture. The absorbance of the mixture was measured against the reference using a microplate reader at 734 nm for 6 min. The standard curve was prepared using trolox. The results were expressed as µM trolox/mg extract [41].

5.11. Ferric reducing/antioxidant power (FRAP) assay

The ferric reducing ability of extracts were evaluated using the FRAP method. The FRAP reagent (10 µL) and extracts (190 µL) were mixed. The absorbance of the mixture was measured after 4 mins against the reference at 593 nm. The $FeSO_4.7H_2O$ standard cureve was prepared and FRAP values were expressed as a mM Fe^{2+}/mg extract [42].

5.12. Cupric reducing antioxidant capacity (CUPRAC) assay

CUPRAC method was used to asses the antioxidant capacity. The neocuproine ethanolic solution ($7.3.10^{-3}$ M, 60 µL), Cu (II) (1.10^{-2} M, 60 µL), and 1 M NH_4Ac buffer solution (60 µL) were mixed in plate. Extracts (each in 60 µL) and EtOH (pure, 10 µL) were added to the initial mixture to make the total volume of 250 µL and it was vortexed for 10 s. The absorbance was measured exactly after 60 mins against a reagent blank at 450 nm. The results were reported as trolox equivalents (mM trolox/mg extract) [43].

5.13. Urease inhibitory activity

Urease enzyme (500 µL) was mixed with the working solution (100 µL). The mixture was incubated in the incubator at 37°C for 30 min. After that, 1100 µL of urea was added and the mixture was incubated for an additional 30 min. in the incubator (37°C). The reagents; R1 (1% phenol, 0.005% sodium nitroprusside) and R2 (0.5% NaOH, 0.1% sodium hypochlorite) were added to the mixture respectively. It was then incubated for 2 hrs in the incubator at 37°C. The absorbance was read against the reference at 635 nm [44].

5.14. Statistical analysis

The data obtained from triplicate experiments were reported as mean ± standard deviation (SD) or mean ± standard error ofthe mean (SEM). Graphpad Prism 5 Demo or PASW Statistics were used to analyse data. The determination of the significance of the differences between means were achieved by Tukey's Multiple Comparison Test or Mann Whitney U test. The p-values less than 0.05 were considered statistically significant.

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