

Full Length Research Paper

Elastase, tyrosinase and lipoxigenase inhibition and antioxidant activity of an aqueous extract from *Epilobium angustifolium* L. leaves

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Herbs have been utilized to treat acute and chronic disorders since thousand years. *Epilobium angustifolium* L. (Onagraceae) is used as herbal and digestive plant all over the world. The *Epilobium* extracts are reported to have analgesic, anti-microbial, antimotility, antiproliferative, antiinflammatory, antitumor and antiandrogenic activities. In the present study, the antioxidant activity and elastase, tyrosinase, lipoxigenase inhibitor capacity of *E. angustifolium* L. were examined. Total phenolic and total flavonoid content, reducing power, superoxide anion radical scavenging, hydroxyl radical scavenging, 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activities were used to evaluate the antioxidant activities. The results were compared with natural and synthetic antioxidants. The aqueous extract of *E. angustifolium* exhibited strong elastase ($EC_{50} = 42.72 \pm 2.38 \mu\text{g/ml}$), tyrosinase ($EC_{50} = 33.03 \pm 3.71 \mu\text{g/ml}$) and lipoxigenase inhibitory activities ($EC_{50} = 0.57 \pm 0.06 \mu\text{g/ml}$). The extract also showed good antioxidant activity in all antioxidant tests. According to these results, *E. angustifolium* may be considered as an important source for pharmaceutical, cosmetic and food manufactures due to its elastase, tyrosinase, lipoxigenase inhibitory activities and antioxidant activities.

Key words: *Epilobium angustifolium* L., antielastase, antityrosinase, antioxidant, activity, lipoxigenase, inhibition.

INTRODUCTION

Skin is a fundamentally important organ of the body, protecting it from damage caused by direct contact with outside environment. Ultraviolet light (UV) leads to alterations in the composition of the skin, including the accumulation of elastic fibres, collagen reduction and degeneration and deposition of glycosaminoglycans (Moon et al., 2010). Oxidative stress derived from UV in sunlight induces different hazardous effects in the skin, including sunburn, photo-aging and DNA mutagenesis.

Elastin an important structural protein of extracellular

matrix, is the major component of the elastic fibres, which provide resilience and elasticity to many tissues including skin, lungs, ligaments and arterial walls (Wiedow et al., 1990). Elastase is the only enzyme capable of degrading elastin, an insoluble elastic fibrous protein of animal connective tissue (Antonicevich et al., 2007). Elastase is known to cause rheumatoid arthritis, pulmonary emphysema, and other chronic inflammatory diseases by the protein degradation of human tissues. It also degrades elastin, which is closely related to the elasticity and restoration of skin, and induces wrinkles and the lack of elasticity. Elastase activity increased significantly with age and results in reduced skin elastic properties, aging and sagging (Robert, 2001). Inhibition of the elastase activity could be employed as a useful method

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to protect against skin aging (Wiedow et al., 1990). Tyrosinase is an enzyme of the undesirable browning of fruits and vegetables, and is involved in the natural development of skin, hair and eye coloring (Roh et al., 2004) and in the initial step of melanin synthesis. Over-activity of this enzyme leads to overproduction of melanin leading to hyper-pigmentation of the skin. Hyper-pigmentation can also be caused by excessive exposure to UV light, drug reaction and also occurs during ageing. The accumulation of an excessive level of epidermal pigmentation can cause some dermatological disorders associated with freckles, melasma, ephelide and senile lentiginos (Khan et al., 2006). Tyrosinase is linked to Parkinson disease and other neurodegenerative diseases in mammals (Xu et al., 1998; Asanuma et al., 2003). In addition, tyrosinase has been reported to be involved in the molting process of insects (Shiino et al., 2001) and it may be characterized as a potential target site for the control of insects pests. Therefore, the control of tyrosinase is important in relation to browning control of vegetables and fruits, and potent tyrosinase inhibitors have also gained increasing importance in medicinal and cosmetic products in relation to hyperpigmentation. Some commercially available tyrosinase inhibitors used as chemical and fungal derived skin-lightening agents have been proven to have chronic, cytotoxic and mutagenic effects on humans (Wang et al., 2006). Therefore, it is still necessary to search tyrosinase inhibitors with potent activities and lower side effects.

Lipoxygenases (LOX) are a family of iron-containing enzymes that catalyse the dioxygenation of polyunsaturated fatty acids in lipids. Lipoxygenases have recently become of interest, as they are considered as the key enzymes in the biosynthesis of leukotrienes that have been postulated to play an important role in the pathophysiology of several inflammatory and allergic diseases. They also play a significant role in cancer, cell growth, metastasis, cell survival and induction of tumor necrosis factor (TNF) (Khan et al., 2007). Increased expression of cyclooxygenase (2) and specific LOX enzymes have been observed in several epithelial cancers such as breast, renal, pancreatic and prostate cancers. Inhibitors of lipoxygenases have attracted attention initially as potential agents for the treatment of inflammatory and allergic diseases, but their therapeutic potential has been expanded to certain types of cancer and cardiovascular disease (Werz and Steinhilber, 2006). Free radical species and reactive oxygen species (ROS) may cause oxidative damage. Antioxidants can react with ROS and quench free radicals giving rise to restriction of radical chain propagation, eventually preventing tissue damage. Many antioxidants based on drug formulations are used for the prevention and treatment of complex diseases like stroke, cancer, atherosclerosis, and Alzheimer's disease. Oxidation of lipids, which is the main cause of quality deterioration in many food systems, may lead to off-flavors and formation of toxic compounds, and may lower the quality and nutritional value of foods.

Lipid oxidation is also associated with aging, membrane damage, heart disease and cancer (Ramarathnam et al., 1995). Natural antioxidant properties from plant species have medicinal functions. Natural antioxidant constituents are the most important compounds in cosmetic and food industries because of their capacities to decrease the free radical mediated degradations of tissues and cells in human organism (Jin et al., 2004). Consequently, there is a requirement to explore new natural sources of antioxidants to replace synthetic antioxidants.

The genus *Epilobium* is widely distributed all over the world and consist of over 200 species, among which the most known are *E. angustifolium*, *E. hirsutum* and *E. parviflorum*, perennial herbs generally named Willow herb, with reference to the willow-like nature of their leaves (Battinelli et al., 2001). Various members of the genus *Epilobium* have been used in folk medicine internally for prostate disease (Melchior, 1972), menstrual and gastrointestinal disorders (Hiermann et al., 1986), rectal bleeding and externally as antiphlogistic (Štajner et al., 2007). In Russia, due to a sweet and pleasant taste, it is usually consumed as tea for the treatment of stomach ulceration, gastritis and sleeping disorders (Štajner et al., 2006). The leaves and young shoot tips can be used in salads, soups or cooked as a vegetable (Štajner et al., 2007).

Phytochemical screenings of *Epilobium* species have revealed the presence of steroids, triterpenes, fatty acids, phenolic acids, macrocyclic tannins, and flavonoids in the aerial parts (Barakat et al., 1997). The antioxidant activity and phenolic compounds, including other *Epilobium* subspecies have been studied, but only limited studies have been reported on the antioxidant activity of *E. angustifolium* L. aqueous extracts (Štajner et al., 2007).

In this study, we have investigated the elastase, tyrosinase, lipoxygenase inhibitory activities of the aqueous extract from *E. angustifolium* L. for the first time as well as its antioxidant activity. The total phenolic and flavonoid contents were also determined to find out the relationship between free radical scavenging assays.

MATERIALS AND METHODS

Plant material

E. angustifolium leaves were collected in September from Canakkale in Turkey and identified by Prof. Dr. Kerim Alpinar from the Faculty of Pharmacy, Istanbul University. Voucher specimens are deposited in the Herbarium of the Faculty of Pharmacy, Istanbul University (Herbarium code number, ISTE 83909). The leaves were washed with deionized water and dried for 5 to 7 days in the shade at room temperature. The dried leaves were manually ground to a fine powder.

Preparation of extract

Ground dried leaves powder (8 g) was extracted with boiled distilled water (200 ml) for 15 min while stirring, in order to prepare a 4%

(w/v) infusion. The extract was filtered and evaporated to dryness under reduced pressure at 40°C in a rotary evaporator, then weighed to determine the total extractable compounds (EC). The crude extract was transferred to vials and kept at -20°C and dissolved in water for the assessment of antioxidant activity.

Elastase inhibitory activity

Elastase activity was examined by using N-succinyl-Ala-Ala-Ala-p-nitroanilide (STANA) as a substrate and by the measuring the release of p-nitroaniline at 410 nm (James et al., 1996). The reaction was carried out in a 200 mM Tris-HCl buffer (pH 8.0) containing 5 mM N-suc-(Ala)₃ p-nitroanilide and 20 µg/ml elastase. 50 µl of elastase inhibitor solution was mixed with the elastase and incubated for 15 min at 37°C prior to the addition of the STANA substrate. Then 0.9 ml of 50 mM Tris-HCl buffer (pH 7.8) and 20 µl 50 mM STANA were added and incubated for 40 min at 37°C. The change in absorbance was measured at 410 nm. The percent inhibition of elastase was calculated as follows:

$$\text{Inhibition (\%)} = (1 - B/A) \times 100$$

Where A is the enzyme activity without inhibitor and B is the activity in the presence of inhibitor. The EC₅₀ defined as the concentration of plant extract required to inhibit elastase activity by 50% was determined.

Tyrosinase inhibitor activity

The tyrosinase inhibitor activity of the sample was estimated according to the method of Vanni et al. (1990). Briefly, test reaction mixtures were prepared by adding 10 µl tyrosinase to 10 µl plant extract and then adding 20 µl 1.5 mM L-tyrosine and 110 µl of 0.1 M sodium phosphate buffer (pH 6.5). The resulting mixture (150 µl) was incubated for 10 min at 37°C and absorption at 490 nm was measured. The percent inhibition of tyrosinase activity was calculated as follows:

$$\text{Inhibition (\%)} = (1 - B/A) \times 100$$

Where A is the enzyme activity without inhibitor and B is the activity in presence of inhibitor. The EC₅₀ defined as the concentration of plant extract required to inhibit tyrosinase activity by 50%, was determined.

Lipoxygenase inhibitor activity

Lipoxygenase inhibitor activity was determined by slightly modifying the spectrophotometric method developed by Tappel (1962). A mixture of 0.8 ml of 100 mM phosphate buffer (pH 8), 0.1 ml (U/ml) lipoxygenase and 50 µl plant extract was incubated for 10 min at 25°C.

The reaction was then initiated by addition of 50 µl 20 µM linoleic acid. This resulting solution mixed well and incubated and room temperature. After 6 min, the change of absorbance at 234 nm was read to measure conjugated diene produced. The percent inhibition of lipoxygenase activity was calculated as:

$$\text{Inhibition (\%)} = (1 - B/A) \times 100$$

Where A is the enzyme activity without inhibitor and B is the activity in presence of inhibitor.

The EC₅₀ defined as the concentration of plant extract required to inhibit lipoxygenase activity by 50%, was determined.

Determination of total phenolic contents

Total phenolics in *E. angustifolium* extract were determined with Folin-Ciocalteu reagent, according to the method of Slinkard and Singleton (1977) with some modifications. Briefly, 0.1 ml of the *E. angustifolium* extract (1000 to 1500 µg/ml) was transferred into a test tube and the volume made up to 4.6 ml with distilled water. After addition of 0.1 ml Folin-Ciocalteu (previously diluted 3-fold with distilled water) and 0.3 ml 2% Na₂CO₃ solution, the tube was vortexed and then allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm in a spectrophotometer. The amount of total phenolic compounds in the *E. angustifolium* extract was calculated as mg of pyrocatechol equivalent from the calibration curve and as mg pyrocatechol equivalents per mg of extract.

Determination of total flavonoid contents

Total flavonoid content was determined by using a method described by Sakanaka et al. (2005) using catechin as a standard flavonoid compound. Briefly, 0.25 ml of the extract (1000 to 3000 µg/ml) or (+)-catechin standard solution (20 to 100 µg/ml) was mixed with 1.25 ml of distilled water in a test tube, followed by the addition of 75 µL of a 5% sodium nitrite solution. After 6 min, 150 µL of a 10% aluminium chloride solution was added and the mixture was allowed to stand for a further 5 min before 0.5 ml of M NaOH was added. The mixture was brought to 2.5 ml with distilled water and mixed well. The absorbance was measured immediately at 510 nm using a spectrophotometer. The results were expressed as mg of (+)-catechin equivalents per mg of extract.

Reducing power

The reducing power of the *E. angustifolium* extract was measured according to the method of Oyaizu (1986). Various concentrations of extracts (20 to 100 µg) in 1 ml of distilled water were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide (1%, w/v), and the mixture was incubated at 50°C for 30 min.

Afterwards, 2.5 ml of trichloroacetic acid (10%, w/v) was added and the mixture was centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of upper-layer solution was mixed with 2.5 ml distilled water and 0.5 ml FeCl₃ (0.1 %, w/v), and the absorbance was measured at 700 nm. α-Tocopherol, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were used as standard antioxidants.

Superoxide anion radical scavenging activity

Superoxide anion scavenging activity of water extract of *E. angustifolium* was determined according to the method of Liu et al., (1997). Superoxide radicals were generated in a non-enzymatic phenazine methosulphate (PMS)-NADH system by oxidation of NADH and assayed by the reduction of nitroblue tetrazolium (NBT). The reaction mixture consisted of 3 ml of Tris-HCl (16 mM, pH 8), 1 ml of NADH (78 µM) and 1 ml of NBT (50 µM) and 1 ml of diluted samples.

The reaction was initiated by adding of 1 ml of phenazine methosulphate (PMS) (10 µM) to the mixture. The tubes were incubated at 25°C for 5 min and the absorbance at 560 nm was recorded against blank samples in a spectrophotometer. A lower absorbance of the reaction mixture indicated a higher superoxide anion scavenging activity.

The percentage inhibition of superoxide anion generation was calculated using the following formula:

Superoxide anion radical scavenging activity (%) = $(A_0 - A_1 / A_0) \times 100$

A_0 is the absorbance of the control. A_1 is the absorbance of the sample.

Hydroxyl radical scavenging activity

The effect of *E. angustifolium* extract on hydroxyl radicals was assayed by using the deoxyribose method (Chung et al., 1997). The reaction mixture contained 0.45 ml of 0.2 M sodium phosphate buffer (pH 7.4), 0.15 ml of 10 mM 2-deoxyribose, 0.15 ml of 10 mM $\text{FeSO}_4\text{-EDTA}$, 0.15 ml of 10 mM hydrogen peroxide, 0.525 ml of distilled water and 0.075 ml of extract in a tube. The reaction was started by the addition of hydrogen peroxide. After incubation at 37°C for 4 h, the reaction was stopped by adding 0.75 ml of 2.8% TCA and 0.75 ml 1.0% of thiobarbituric acid. The mixture was boiled for 10 min, cooled in an ice bath and then measured at 520 nm. Hydroxyl radical scavenging activity was calculated in the following equation:

Hydroxyl radical scavenging activity (%) = $(A_0 - A_1 / A_0) \times 100$

A_0 is the absorbance of the control and A_1 is the absorbance of sample.

DPPH radical scavenging activity

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of the *E. angustifolium* extract was measured according to the procedure described by Brand-Williams et al. (1995). Dilution series (0.25 to 1 mg/ml) were prepared for each aqueous extract in methanol, 0.1 ml of each dilution was added to 3.9 ml of a 6×10^{-5} M methanolic solution of DPPH followed by vortexing. The mixture was shaken vigorously and allowed to stand in the dark at room temperature for 30 min. The decrease in absorbance of the resulting solution was then measured spectrophotometrically at 517 nm against methanol. The DPPH radical scavenging activity was calculated using the following equation:

DPPH radical scavenging activity (%) = $(A_0 - A_1 / A_0) \times 100$

A_0 is the absorbance of the control. A_1 is the absorbance of the sample

ABTS radical scavenging activity

The ABTS^{•+} scavenging activity of the *E. angustifolium* extract was measured according to the procedure described by Arnao et al. (2001). The stock solutions included 7.4 mM ABTS^{•+} solution and 2.6 mM potassium persulfate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS^{•+} solution with 60 ml methanol to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using the spectrophotometer. Fresh ABTS^{•+} solution was prepared for each assay.

E. angustifolium extracts (150 μL) were allowed to react with 2850 μL of the ABTS^{•+} solution for 2 h in a dark condition. Then the absorbance was taken at 734 nm using a spectrophotometer. The ABTS^{•+} scavenging activity was calculated using the following equation:

ABTS radical scavenging activity (%) = $(A_0 - A_1 / A_0) \times 100$

A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

Statistical analysis

Results were expressed as mean \pm standard deviation (SD) of triplicate analyses. The correlation coefficient (r^2) between the parameters tested was established by regression analysis.

RESULTS AND DISCUSSION

Elastase inhibitory activity

Elastase is known to cause rheumatoid arthritis, pulmonary emphysema, and other chronic inflammatory diseases by the protein degradation of human tissues. It also degrades elastin, which is closely related to the elasticity and restoration of skin, and induces wrinkles (An et al., 2005). Recently, there is an increased demand for natural substances such as green plant products have been in increased demand in the global market as new agents for cosmeceutical purposes (Aburjai and Natsheh, 2003). In the present study, *E. angustifolium* was investigated for potential effectiveness as antiaging agent for the ingredients of cosmetic preparations. The inhibition effect of elastase activity is shown in Table 1. We have found that all concentrations exerted inhibitory effects on elastase in a dose-dependent manner. The inhibition was increased with increasing extract concentration. The inhibition percentage of *E. angustifolium* extract at 100 $\mu\text{g/ml}$ concentration was $82.19 \pm 2.38\%$. EC_{50} values were found 42.72 ± 2.38 $\mu\text{g/ml}$. A high correlation was observed between antielastase activity and phenolic compounds ($r^2 = 0.9982$). Kang et al. (1994) reported that the inhibition of the elastase activity of ginseng was about 90% at 0.14 mg/ml. The present results indicate that the *E. angustifolium* has a better activity than the other sources reported (An et al., 2005). Terpenoids, flavonoids, phenolic compounds have been shown to possess antielastase activity (Lee et al., 2001). This high antielastase activity is attributed to the presence of natural antioxidants such as flavonoids, terpenoids and phenolic compounds in *E. angustifolium*. Therefore, it can be concluded that *E. angustifolium* may have a potential role on skin care in the cosmetics for its enzyme inhibition activity.

Tyrosinase inhibitor activity

Tyrosinase inhibitors are chemical agents capable of reducing enzymatic reactions, such as food browning and melanisation of human skin. Therefore, these inhibitors have good commercial potential in food and cosmetic industries (Lim et al., 2009). Tyrosinase inhibitor activity of *E. Angustifolium* is reported the first time in this study

Table 1. Elastase, tyrosinase, and lipoxygenase inhibitor activities of *Epilobium angustifolium* L. extracts.

Elastase activity			Tyrosinase activity			Lipoxygenase activity		
Concentration ($\mu\text{g/ml}$)	Inhibition (%)	EC ₅₀ ($\mu\text{g/ml}$)	Concentration ($\mu\text{g/ml}$)	Inhibition (%)	EC ₅₀ ($\mu\text{g/ml}$)	Concentration ($\mu\text{g/ml}$)	Inhibition (%)	EC ₅₀ ($\mu\text{g/ml}$)
10	11.03 \pm 1.03		0.1	4.9 \pm 0.70		0.1	21.81 \pm 15.12	
25	42.72 \pm 2.38	42.72 \pm 2.38	1	12.07 \pm 1.53	33.03 \pm 3.71	0.2	31.25 \pm 1.76	0.57 \pm 0.06
50	72.16 \pm 2.88		5	13.89 \pm 0.13		0.5	46.58 \pm 0.46	
100	82.19 \pm 2.38		10	15.67 \pm 5.19				

Values are means \pm SD.

and the results are shown in Table 1. It was found that the *E. angustifolium* inhibited the tyrosinase enzyme activity. The inhibition was increased with increasing extract concentration (Table 1). A high antityrosinase (15.67 \pm 5.19%) was seen in 10 $\mu\text{g/ml}$ (Table 1). EC₅₀ values were found 33.03 \pm 3.71 $\mu\text{g/ml}$. The tyrosinase activity was correlated with phenolic compounds ($r^2 = 0.9942$). It was evident that *E. angustifolium* extract had a stronger effect on tyrosinase inhibition compared to various plant extracts in literature (Moon et al., 2010).

Jo et al. (2011) have reported that *Magnolia* species showed low tyrosinase inhibition with an average 8.63% inhibition of 1000 $\mu\text{g/ml}$ (IC₅₀ = 10555.55 $\mu\text{g/ml}$). Various naturally occurring anti melanogenesis reagents contain a phenolic structure (Kim, 2007). Many tyrosinase inhibitors are polyphenol derivatives of flavonoids (Briganti et al., 2003; Seo et al., 2003). Structural differences in phenolic compounds found in *E. Angustifolium* may also play a critical role in occurrence of tyrosinase inhibition. Nerya et al. (2004) stated that the most important factor in efficacy of the chalcones against tyrosinase is the location of the hydroxyl groups on aromatic ring. The mechanism of tyrosinase inhibition activity is an important factor for skin whitening in cosmetic industry and for food browning inhibition in food

industry (An et al., 2005; Vamos–Vigyazo, 1981).

Lipoxygenase inhibitory activity

The effect of the aqueous extract of *E. angustifolium* on lipoxygenase activity is shown in Table 1. Lipoxygenase inhibitory activity of *E. angustifolium* was found to be increased dose dependently. The results were expressed as EC₅₀ values, calculated from the regression equations prepared from the concentration of the samples.

A high lipoxygenase inhibition (46.58 \pm 0.46%) was seen in 0.5 $\mu\text{g/ml}$ extract concentration (Table 1). EC₅₀ values were found 0.57 \pm 0.06 $\mu\text{g/ml}$. Kiss et al. (2011) have demonstrated that *Epilobium* species inhibited the activity of lipoxygenase with IC₅₀ = 16 to 28 $\mu\text{g/ml}$. Our result (0.57 $\mu\text{g/ml}$) showed that *E. angustifolium* water extract had higher lipoxygenase inhibitor activity.

A good relationship between polyphenol contents and lipoxygenase inhibitor activity has been reported by Aquila et al. (2009). This good lipoxygenase inhibitor activity is attributed to the presence of natural antioxidants such as phenolic compounds in *E. angustifolium*. This plant is used by people against prostate cancer in Turkey. In this study, it is found that the water extract of

E. angustifolium has a good lipoxygenase inhibitor activity, therefore the usage of this plant by people against prostate cancer is scientifically confirmed.

Total phenolic and flavonoid contents

Phenols are very important plant constituents as they are good scavengers due to their hydroxyl groups. Phenolic compounds and flavonoids have been shown to be responsible for the antioxidant activity of many plants (Geetha et al., 2005). These antioxidants also possess diverse biological activities such as antiinflammatory, antiatherosclerotic and anticarcinogenic which may be related to their antioxidant activities (Chung et al., 1997). It was determined that there was 42.10 \pm 10.76 μg pyrocatechol equivalent of phenolic compounds in 1 mg of the *E. angustifolium* extract. A high correlation was observed between the total phenolic content and hydroxyl radical scavenging activity ($r^2 = 0.8577$), DPPH radical scavenging activity ($r^2 = 0.9285$), and superoxide radical scavenging activity ($r^2 = 0.9999$) of the extract. These data are in accordance with others, which have shown that a high total phenolic content increases antioxidant activity, and that there is a linear correlation between phenolic content and antioxidant activity

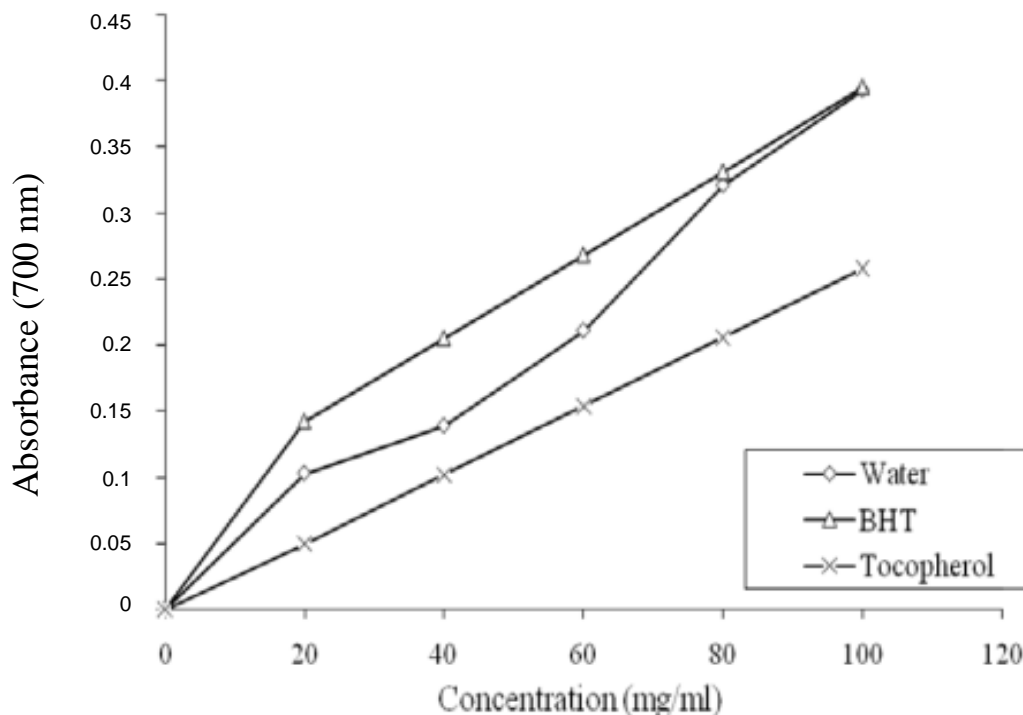


Figure 1. Reducing power of the water extract from *Epilobium angustifolium* L. BHT and tocopherol were used as reference antioxidants. Values are means \pm SD ($n=3$).

(Holasoava et al., 2002). These results indicate that the high antioxidant activity of *E. angustifolium* extract may be associated with its total phenolic content. Polyphenolic compounds like flavonoids have been labelled as "high level" natural antioxidants based on their abilities to scavenge free radicals and active oxygen species (Birt et al., 2001). They contain conjugated ring structures and hydroxyl groups having the potential function as antioxidants *in vitro* or cell free systems by scavenging superoxide anion, singlet oxygen, lipid peroxyradicals, and stabilizing free radicals involved in oxidative processes through hydrogenation or complexing with oxidizing species (Klahorst, 2002).

The concentration of flavonoids was 22.76 ± 1.34 μ g catechin equivalent of phenolic compounds in 1 mg of the water extract. Phytochemical screening of *Epilobium* species have revealed the presence of phenolic acid, macrocyclic tannins, and flavonoids (in particular myricitrin, isomyricitrin, quercitrin, and quercetin-3-O- β -D-glucuronide) in the aerial parts of the plant (Barakat et al., 1997).

Reducing power

Fe^{3+} reduction is often used as an indicator of electron-donating activity, which is an important mechanism of

phenolic antioxidant action, and can be strongly correlated with other antioxidant properties (Dorman et al., 2003). In the reducing power assay the presence of reductants (antioxidants) in the samples could result in the reduction of the Fe^{3+} /ferricyanide complex to its ferrous form. Amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's Prussian blue at 700 nm. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity.

Figure 1 shows the dose-response curves for the reducing powers of the extract from *E. angustifolium* and standards. The reducing power of water extract was nearly equal to that of BHT. Results of the reducing power indicate that the reducing capacity of *E. angustifolium* was higher when compared to standard tocopherol, indicating that it is an excellent natural antioxidant.

Reducing power of extract and standards decreased in order of water extract \geq BHT > tocopherol. These results revealed that the extract of *E. angustifolium* was electron donor and also could react with free radicals, converting them to more stable products. The outcome of the reducing reaction is to terminate the radical chain reactions that may otherwise be very damaging. This good antioxidant activity is attributed to the presence of natural antioxidants such as phenolic compounds in

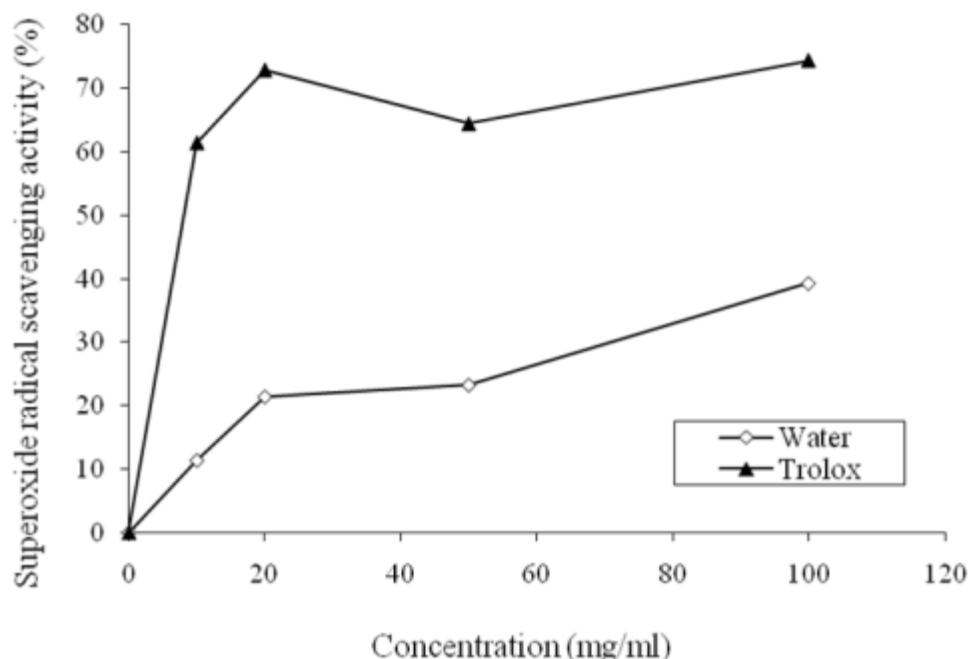


Figure 2. Superoxide radical scavenging activity of the water extract from *Epilobium angustifolium* L. Trolox was used as reference antioxidant. Values are means \pm SD (n=3).

E. angustifolium.

Superoxide anion radical scavenging activity

Superoxide anion is a free radical created from the normal process of energy generation in the human body. Superoxide anion is toxic to cells and tissues and can act as precursor to other ROS (Korychka-Dahl and Richardson, 1978). These species are produced by a number of enzyme systems. The radicals may also play an important role during the peroxidation of unsaturated fatty acids and other potential susceptible substances (Sakanaka et al., 2005; Halliwell and Gutteridge, 1984). It has also been reported that antioxidant properties of some flavonoids are effective mainly via scavenging of superoxide anion radicals (Yen and Duh, 1994).

In a biological system, its toxic role can be eliminated by superoxide dismutase (SOD) (Chung et al., 2005). Figure 2 shows the dose response curves of superoxide-scavenging activities of the extracts from *E. angustifolium* by the PMS-NAOH superoxide-generating system. Water extract at 100 mg/ml exhibited $39.32 \pm 0.02\%$ superoxide radical scavenging activity. On the other hand at the same concentration, Trolox exhibited ($74.36 \pm 0.02\%$) activity. These values were lower than that of the same dose of Trolox ($74.36 \pm 0.02\%$). The superoxide radical scavenging activity of those samples were as follows: Trolox > water extract. EC_{50} values of *E. angustifolium* extract and Trolox were found to be 127.15 ± 0.08 and

7.41 ± 0.008 mg/ml, respectively. When compared to Trolox, the superoxide scavenging activity of the extract was found to be low. This could be due to the presence of reactive concentration of bioactive constituents and mixture of other nutrients in the extract. The superoxide radical scavenging activity in the water extract was correlated with phenolic compounds ($r^2 = 0.9999$). These results indicated that the radical scavenging capacity of the water extract might be mostly related to its concentration of phenolic hydroxyl group.

Hydroxyl radical scavenging activity

The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecules found in living cells (Hochstein and Atallah, 1988). This radical has the capacity to join nucleotides in DNA and cause strand breakage which contributes to carcinogenesis, mutagenesis and cytotoxicity. This reactive hydroxyl radical can cause oxidative damage to DNA, lipids and proteins (Shukla et al., 2009). Hydroxyl radical scavenging capacity of an extract is directly related to its antioxidant activity (Babu et al., 2001). Figure 3 shows the dose response curves of radical scavenging activities of the extracts and reference antioxidant (ascorbic acid) on the hydroxy radicals. Water extract and ascorbic acid scavenging hydroxyl radicals by 8.89 ± 0.83 and $5.56 \pm$

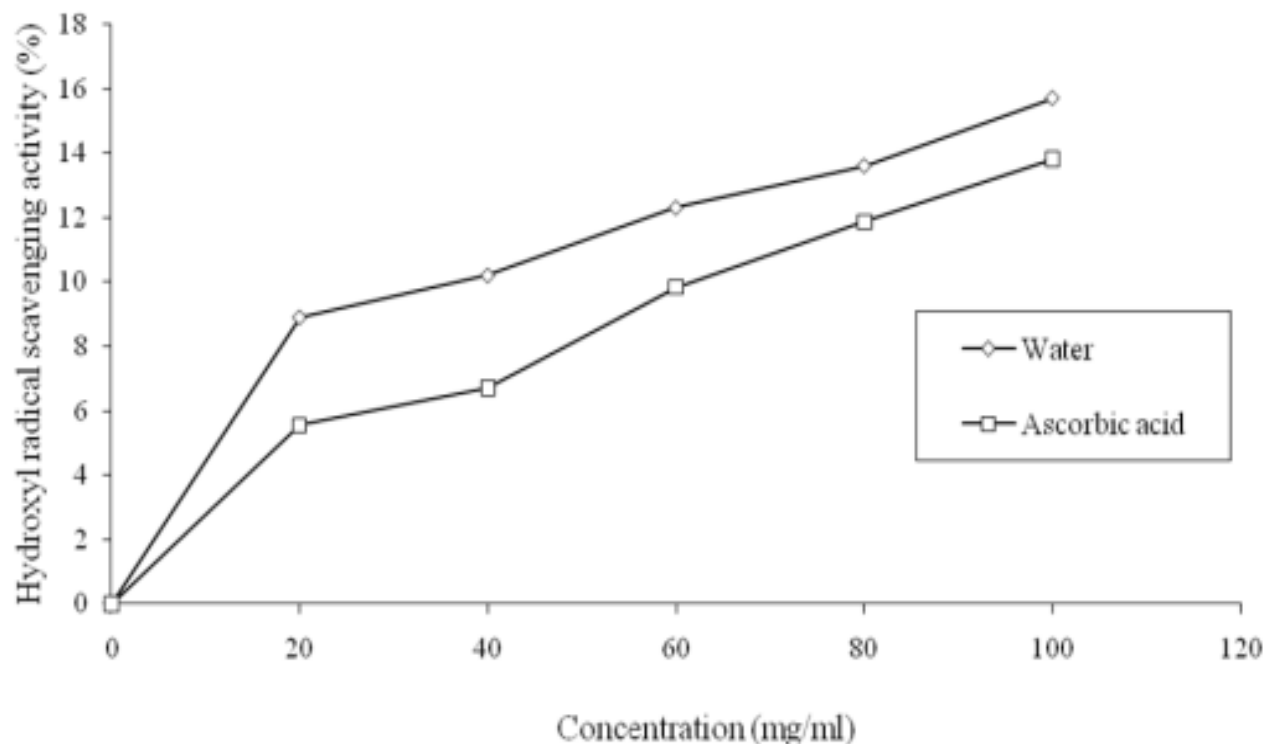


Figure 3. Hydroxyl radical scavenging activity of the water extract from *Epilobium angustifolium* L. Ascorbic acid was used as reference antioxidant. Values are means \pm SD (n=3).

0.18% at 20 mg/ml, and 15.60 ± 1.32 and $13.84 \pm 0.12\%$ at 100 mg/ml, respectively. These values were significantly higher than the value of the positive control ascorbic acid. The scavenging abilities on hydroxyl radicals were in descending order: water>ascorbic acid. A higher hydroxyl radical scavenging activity is associated with a lower EC_{50} value. EC_{50} values for extracts, and ascorbic acid on hydroxyl radical scavenging activity were found as 2.64 ± 0.22 , and 2.34 ± 0.02 mg/ml, respectively. A high correlation was observed between hydroxyl radical scavenging activity and phenolic compounds ($r^2 = 0.8577$). *E. angustifolium* extract was also capable of reducing DNA damage at all concentrations used. All results showed antioxidant activity in dose dependent manner. These results indicated that water extracts from *E. angustifolium* are effective scavengers for hydroxyl free radicals. The high value of hydroxyl radical scavenging activity of this plant might be the reason why it is widely used in traditional medicine for prostate cancer.

DPPH radical scavenging activity

DPPH has been widely used to evaluate the free radical scavenging effectiveness of various antioxidant substance (Ozcelik et al., 2006). A chain in lipophilic radicals was initiated by the lipid autoxidation. DPPH is a

stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. Antioxidants react with DPPH, reducing a number of DPPH molecules equal to the number of their available hydroxyl groups (Xu et al., 2005). Figure 4 shows, the dose response curves of DPPH radical scavenging activity of the extracts from *E. angustifolium*. The extract was capable of scavenging DPPH radicals in a concentration-dependent manner.

The scavenging activity of *E. angustifolium* extract, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and ascorbic acid on DPPH radicals increased between 5 to 10 mg/ml and were 90.68 ± 0.45 , 94.19 ± 0.29 , 85.84 ± 0.61 and $93.50 \pm 0.23\%$ at a concentration of 10 mg/ml, respectively. Water extract, BHA and BHT showed similar DPPH radical scavenging activities, while ascorbic acid was a considerably less effective DPPH radical scavenger. The DPPH radical scavenging activity was correlated with phenolic compounds ($r^2 = 0.9286$). These results indicated that the radical scavenging capacity of the water extract might be mostly related to concentration of phenolic hydroxyl groups. EC_{50} values for ascorbic acid, water extract, BHA and BHT on DPPH radical scavenging activity were found as 2.59 ± 0.01 , 2.70 ± 0.09 , 2.94 ± 0.03 and 3.47 ± 0.07 mg/ml. Štajner et al. (2007) has reported that Willow herb's showed a relatively high DPPH radical scavenging effect

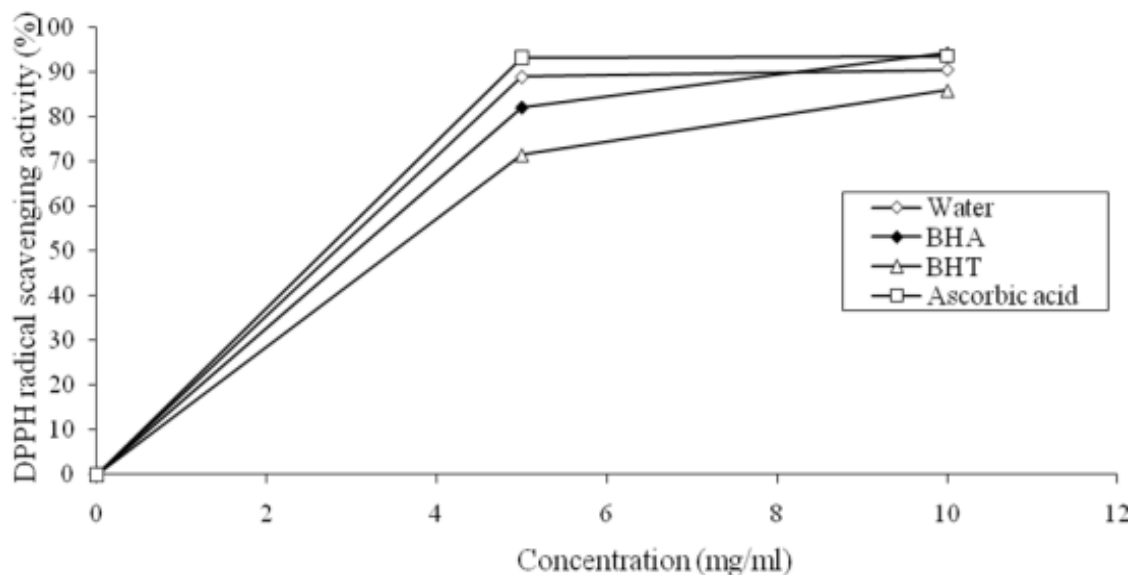


Figure 4. DPPH radical scavenging activity of the water extract from *Epilobium angustifolium* L. BHA, BHT and ascorbic acid were used as reference antioxidants. Values are means \pm SD (n=3).

(95.57 \pm 6.57%). This value is similar to the our findings..

ABTS radical scavenging activity

The ABTS^{•+} scavenging activity is also one of the most commonly used methods to evaluate the antioxidant activity (Zhang et al., 2009). The ABTS^{•+} scavenging activity of water extract compared to Trolox are shown in Figure 5. They increased with increasing concentration reaching 96.10 \pm 0.12% and these values were comparable to those of the positive control Trolox 96.49 \pm 0.03% at a concentration of 0.5 mg/ml. EC₅₀ values for *E. angustifolium* extract and Trolox on ABTS^{•+} scavenging activity were found as 0.052 \pm 0.003 and 0.051 \pm 0.004 mg/ml, respectively. A high correlation was observed between ABTS^{•+} scavenging activity and phenolic compounds ($r^2 = 0.6546$).

Conclusions

The results of the present study are indicating that water extract of *E. angustifolium* L. exhibits good antioxidant, antielastase, antityrosinase and lipoxigenase inhibitor activities. We have demonstrated that the aqueous extract of *E. angustifolium* L. contained high level of flavonoid and phenolic compounds. Phenolic compounds and flavonoids present in the plant kingdom are mainly responsible for the antioxidant and enzyme inhibitor activity. Therefore, *E. angustifolium* L. could be used as a source of natural antioxidant and antielastase, antityrosinase, and lipoxigenase inhibitor in

pharmaceutical, cosmetic and food industries. The high value of antioxidant and lipoxigenase inhibitor activity of this plant obtained in this study may partly support and explain the use of its extracts in folk medicine for prostate disease treatment.

The water extract of *E. angustifolium* L. could be beneficial for conventional herbalism and used as a natural remedy in the future. In conclusion, *E. angustifolium* L. extract may be considered as a main lipoxigenase, tyrosinase and elastase inhibitor and free radical scavenger. Therefore, this plant may be appreciated as a medicinal food mainly at skin, prostate, Parkinson and other diseases.

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Abbreviations: **ABTS**, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; **BHA**, butylated hydroxyanisole; **BHT**, butylated hydroxytoluene; **DPPH**, 2,2-diphenyl-1-picryl-hydrazyl; **DTNB**, 5,5'-dithiobis(2-nitrobenzoic acid); **EC₅₀**, efficiency concentration; **EC**, extractable compounds; ferrozine, 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine; **LOX**, lipoxigenase; **NADH**, reduced nicotinamide adenine dinucleotide; **NADPH₂**, reduced nicotinamide adenine dinucleotide phosphate; **NBT**, nitroblue tetrazolium; **PMS**, phenazine methosulphate;

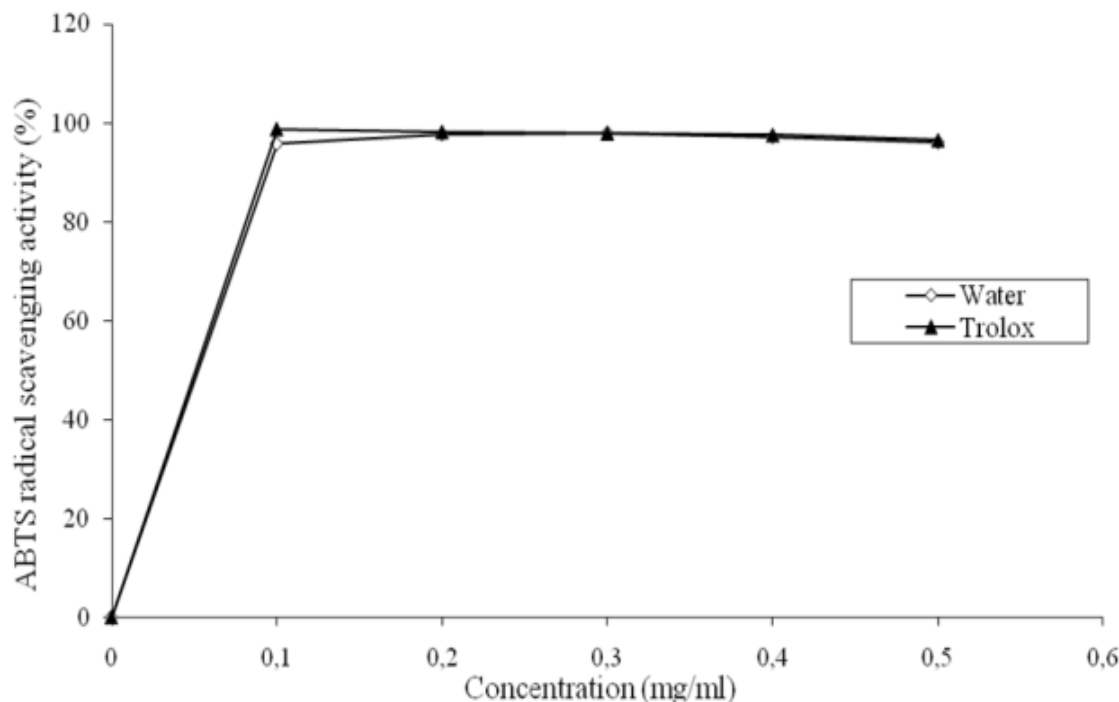


Figure 5. ABTS radical scavenging activity of the water extract from *Epilobium angustifolium* L. Trolox was used as reference antioxidant. Values are means \pm SD (n=3).

ROS, reactive oxygen species; **STANA**, N-succinyl-Ala-Ala-Ala-p-nitroanilide; **TBA**, tiobarbituric acid; **TCA**, trichloroacetic acid; **Trolox**, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; **TNF**, tumor necrosis factor; **UV**, ultraviolet light.

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