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Original paper

Researches on the Epilobium angustifolium L. ethanol extract, Turkey

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Abstract

In this paper, *Epilobium angustifolium* L. in Turkey was investigated for total phenolics, flavonoids, and anthocyanin amounts. Furthermore, the ethanol extract of the leaves was evaluated for antioxidant activities as ABTS, hydroxyl radical scavenging, superoxide radical scavenging, power of reduction, and bleaching of beta-carotene. The total phenolic and flavonoid contents of the ethanolic extract were found to be $468,11 \pm 1,53$ micrograms of pyrocatechol per milligram of the extract and $103,05 \pm 2,36$ μg catechin per milligram of the extract, respectively. As the extract concentration increased, the amount of anthocyanins showed a decrease (17.92 ± 8.08 and 13.56 ± 8.22 for the 1 mg/mL and 2 mg/mL concentrations, respectively). In the β -carotene bleaching test, higher activity was observed than BHA (1.540 ± 0.053 and 1, respectively). The results of the reduction power, hydroxyl radical scavenging, and ABTS tests gave either very close values or higher than the standards chosen. Superoxide radical scavenging activity gave a lower value than the Trolox selected as standard. Antioxidant activity of ethanol extract was found to be 94.22 ± 0.05958 (ABTS), 78.77 ± 0.84099 (hydroxyl radical scavenging) and 47.86 ± 0.03915 (superoxide radical scavenging). The experimental data indicate that Turkey *Epilobium angustifolium* L. ethanol extract can be used pharmacologically.

Keywords *Epilobium angustifolium* L., ethanol extract, antioxidant activities.

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Introduction

Reactive oxygen species (ROS) are widely accepted to be associated with diseases like Alzheimer's, Parkinson's, aging, and cancer (KIM & al., 2016 [9]; LIOCHEV, 2013 [14]; WEN & al., 2013 [33]). Antioxidants are considered to prevent oxidative damage by eliminating reactive oxygen species. In particular, natural antioxidants are considered to have more effective and have safety in reducing ROS in excessive amounts (NDHLALA & al., 2010 [17]). Therefore in recent years the number of studies on plants, which have been used for folk medicine for centuries, is increased. The genus *Epilobium* is one of the widely used medicinal plants in the world. *Epilobium angustifolium* L. (another name *Chamaenerion angustifolium*), belonging to the Onagraceae family, is a showy, perennial, commonly self-growing 3-5 ft. tall wild plant. *Epilobium* plants can be divided into two groups according to their flower's size: the small ones (*E. roseum*, *E. parviflorum*, *E. montanum*) and the large ones (*E. angustifolium*, *E. hirsutum*) (STOLARCZYK & al., 2013 [29]). There are more than 200 species of *Epilobium* plants in the world, and 21 species have been identified in Turkey, especially in the Aegean, Black Sea, and Marmara regions.

As a folk medicine, *Epilobium angustifolium* is widely used to treat various gastrointestinal diseases such as mouth ulcers, swelling, stomach ulcers, duodenal ulcers, gastritis, colitis, dysentery, diarrhea, prostate or urinary problems (LEBEDA & al., 2004 [13]; KUJAWSKI & al., 2011 [12]; FESHCHENKO & al., 2019 [7]). It has also been used to treat voiding disorders, prostatic adenoma and benign prostatic hyperplasia (BPH) (STOLARCZYK & al., 2013 [29]; DENG & al., 2019 [5]). It has been reported that *Epilobium* is used as a tea in the treatment of migraine headaches, insomnia, anemia and colds (BUSHUEVA & al., 2016 [3]). There are many studies on the antitumor, anti-inflammatory, analgesic, antiviral and antioxidant activities of *Epilobium angustifolium* extract in the literature (TÓTH & al., 2009 [31]; KOSALEC & al., 2013 [11]; TITA & al., 2001 [30]; WEBSTER & al., 2008 [32]). The reason for these activities is that *Epilobium* species are rich in polyphenols, steroids, triterpenoids, and fatty acids, especially Oenothin B (a dimeric macrocyclic ellagitannin) (SCHEPETKIN & al., 2009 [24]; RAMSTEAD & al., 2012 [21]; KISS & al., 2011 [10]).

The plant has been studied for antioxidant activities in China, Poland, Croatia, Serbia, Russia, and many countries (SHIKOV & al., 2006 [25]; STAJNER & al., 2007 [28]; STOLARCZYK & al., 2013 [29]; MONSCHEIN & al., 2015 [16]; DENG & al., 2018 [6]). There are only two studies about the antioxidant activity of Turkish *Epilobium*. One of the studies is our previous study which contains the antioxidant activities of the aqueous extract of the plant (ONAR & al., 2012 [18]). The other is related to some biological activities of the ethanol and methanol extracts (SAYIK & al., 2017 [23]) DPPH and metal chelating activity were investigated for antioxidant activity.

Total phenolics, flavonoids, and anthocyanin amounts were also examined in *Epilobium angustifolium* L. in Turkey. Furthermore, its ethanolic extract was evaluated

for antioxidant activities like ABTS, radical scavenging experiments with hydroxyl and superoxide, power of reduction, and test of beta-carotene bleaching. This study aimed to complete the missing data of *Epilobium angustifolium* L. plant grown in Turkey.

Materials and methods

Plant collection and extraction

We collected the leaves of *E. angustifolium* in September, from Canakkale district, Turkey, and identification was performed by Prof. Dr. Kerim Alpınar, a faculty member of Istanbul University, Faculty of Pharmacy. Voucher specimens are being kept in the Herbarium of the Faculty of Pharmacy, Istanbul University with the code (ISTE 83909). Deionized water was used to wash the leaves and they were dried for five days to a week left in the shade, and the medium was at room temperature. After the leaves were dried, manual grinding of them yielded a fine powder.

The powder was extracted in the soxhlet apparatus with absolute ethanol at 75-79°C for 22 h. The extract obtained was evaporated at 45°C, and the remainder was dried and stored in a container that is sufficiently air-tight. The extract's yield was 11.84 % w/w.

Determination of total phenolic content

With Folin-Ciocalteu reagent, it was possible to measure the total phenolics in the extract, and minor modifications were applied to the method by SLINKARD and SINGLETON (1977) [26]. In brief, plant extract (0.1 mL; 1000 to 1500 µg/mL) was taken to tube and the final volume was made, with distilled water, to 4.6 mL. The Folin-Ciocalteu reagent was diluted three times with distilled water, and 0.1 mL of it along with 0.3 mL of 2% Na₂CO₃ solution were added and, after vortexing the tube, it was allowed to settle 2 h with shaking intermittently. With a spectrophotometer set at 760 nm, the measurement of absorbance was performed. In the extract, the total phenolic compounds were calculated as milligrams of pyrocatechol (with the help of calibration curve) and as milligrams of pyrocatechol equivalents (expressed as milligrams of the extract).

Determination of total flavonoid content

SAKANAKA's method (2005) [22] was employed for total flavonoid content, and catechin was used as a standard flavonoid. Briefly, in a test tube, 1.25 mL of distilled water was added to the extract (0.25 mL, 1000 to 3000 micrograms per milliliter) or a standard solution of (+)-catechin (20 to 100 microgram per milliliter), and then 75 µL of a sodium nitrite solution (5%) was introduced. 6 min later, 150 µL of an aluminum chloride solution (10%) was mixed with the others and the resulting mixture was rested for another 5 minutes and finally, x M NaOH (0.5 mL) was added. The final volume was made to 2.5 mL with distilled water and mixed well. Using a spectrophotometer at 510 nm, the absorbance was immediately measured. From the

calibration curve, the results were calculated as milligram of (+)-catechin equivalents per milligram of the extract.

Determination of total anthocyanin

The determination of the content of total anthocyanins for *Epilobium angustifolium* L. was performed according to GIUSTI and WROLSTAD (2001) [8]. The samples are prepared by dissolving the dry plant in methanol and acidifying it with 1% HCl. With a spectrophotometer, absorbances of 1 and 2 mg/mL samples (pH values were at 1.0 and 4.5) were measured at two different wavelengths, namely at 510 and 700 nm. The total absorbance of anthocyanin depended on two different pH values and two different absorption wavelengths and was calculated with the formula as seen below:

$$A = [(A_{510} - A_{700})_{\text{pH } 1.0} - (A_{510} - A_{700})_{\text{pH } 4.5}]$$

Bleaching test of β -carotene

To a boiling flask which contains linoleic acid (20 mg) and Tween-40 (200 mg) (Sigma) was added 0.2 mL solution of approximately of trans- β -carotene (10 mg) (type 1, synthetic, Sigma, St. Louis, MO, USA), and they were dissolved in chloroform (10 mL). After all chloroform was evaporated, distilled water (50 mL) was introduced to the flask and vigorously shaken. A 5-mL aliquot was taken and mixed with 0.2 mL of the plant extract (20-1000 (μg / mL)). The contents of the test tubes were heated at 50 °C, on a water bath. Absorbances at 470 nm were recorded after incubation periods of 60 and 120 minutes. Butylated-hydroxy-anisole (BHA) was the positive and the emulsion of linoleic acid and β -carotene was the negative control. The following formula calculates the relative antioxidant activities (RAA):

$$\text{RAA} = \text{Abs of sample} / \text{Abs of BHA} \quad (\text{BRUNI \& al., 2004 [2]})$$

Activity of ABTS radical scavenging

ABTS^{•+} scavenging activity was described by ARNAO & al. (2001) [1]. This procedure was followed to measure the activity of our plant extract. 7.4 millimolar ABTS^{•+} solution and 2.6 millimolar K₂S₂O₈ (potassium persulfate) solution were used. These two stock solutions in equal amounts was mixed and kept in reaction for twelve hours at room temperature in the dark, and 60 mL of methanol was used to dilute 1 mL of the ABTS^{•+} solution. Every time of essay measurement, a fresh solution of ABTS^{•+} was prepared.

The plant extracts (in a volume of 150 μL) were reacted with 2.850 mL of the ABTS^{•+} solution for two hours at dark. The spectrophotometer was set to record absorbances at 734 nm. We used the following equation for calculating the ABTS^{•+} scavenging activity:

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

A₀= without ethanolic extract sample (with methanol)
A₁= ethanol extract sample

Reducing power

A 100-1000 μg sample of extracts in 1 mL of distilled water was mixed with phosphate buffer (pH 6.6, 0.2 M, 2.5

mL) along with 2.5 mL of 1% [K₃Fe(CN)₆], and incubation of the mixture was performed for 30 min, at 50 °C. Afterward, trichloroacetic acid 10% (2.5 mL), was added to the mixture, followed by centrifugation at 3000 rpm (10 minutes). Finally, a 2.5 mL-supernatant was mixed with distilled water (2.5 mL) and 0.5 mL of FeCl₃ (0.1%), and the spectrophotometer was set at 700 nm wavelength, then the absorption was read (OYAIZU, 1986 [19]). Increased absorbance is indicative of increased reducing power.

Hydroxyl radical scavenging activity

According to CHUNG & al., 1997 [4], deoxyribose method was used for the affection of hydroxyl radicals by antioxidative effect of the plant extract. In the reaction mixture, there were sodium phosphate buffer (0.2 M, 0.45 mL, pH 7.4), 2-deoxyribose (10 mM, 0.15 mL), FeSO₄-EDTA (10 mM, 0.15 mL), hydrogen peroxide (10 mM, 0.15 mL), distilled water (0.525 mL) and the extract (0.075 mL) were added in a test tube. Hydrogen peroxide initiated the reaction. After incubation for 4 h at 37 °C, by adding 2.8% TCA (0.75 mL) and 1.0% of thiobarbituric acid (0.75 mL), the reaction was stopped. After boiling the mixture for 10 min, the reaction contents were cooled on an ice bath, and the absorbance was measured with a spectrophotometer set at 520 nm. The following equation calculates hydroxyl radical scavenging activity:

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

A₀ is control sample's absorbance and A₁ is sample's absorbance.

Superoxide radical scavenging activity (SOD)

Superoxide anion scavenging activity of the aqueous extract of the plant was determined by LIU & al.'s method (1997) [15]. A phenazine methosulfate (PMS)-NADH system that is non-enzymatic generates superoxide radicals by the oxidation of NADH (nicotinamide adenine dinucleotide) and quantified by NBT's reduction (nitroblue tetrazolium). The mixture contained Tris-HCl (16 mM, 3 mL, pH 8), NBT (50 mM, 1 mL), and NADH (78 mM, 1 mL) and diluted samples (1 mL). By adding phenazine methosulfate (PMS, 10 mM, 1 mL), the reaction was initiated. The tubes were incubated at 25 °C for 5 minutes and the absorbances were recorded with a spectrophotometer set at 560 nm against blank. If the samples' absorbances are low, it means that the superoxide scavenging activity is higher. The formula below calculates the percentage inhibition of superoxide anion:

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

A₀ is the control's absorbance. A₁ is the samples' absorbance of the sample.

Results and discussions

In this work, *Epilobium angustifolium* L. plant collected from Çanakkale, Turkey was used. The plant was identified by University of Istanbul (Herbarium code ISTE 83909). The yield of the ethanol extract is 11.84 % (w/w) and was kept at -20 °C.

Total phenolic and flavonoid content

The total phenolic group in the compound and the number of potentially oxidizable groups (total phenolic content) were determined by the Folin-Ciocalteu reagent using the Slinkard and Singleton method (SLINKARD and SINGLETON, 1977 [26]). This method includes an electron transfer between the antioxidants and molybdenum ion, and the formed blue-colored products from the reaction can be measured in a spectrophotometric manner. This reaction is valid in an alkaline environment and does not work in any other medium (pH ≈ 10). The amount of total phenolics in the plant extract was found from the drawn calibration curve as milligram equivalent of pyrocatechol. Total flavonoid

content was determined as catechin equivalent as a standard flavonoid compound (SAKANAKA & al., 2005 [22]). Reporting of the results were as the milligram amount of (+)-catechin equivalents per milligram amount of the extract from the drawn calibration curve. Flavonoidal and phenolic compounds are responsible for the biological activities of the plant extracts (SOBRATTEE & al., 2005 [27]). In the ethanolic extract, the total phenolic and flavonoidal contents were 468.11± 1.53 micrograms per milligram of extract (in 1500 micrograms per milliliter plant concentration) and 103,05 ± 2,36 micrograms of catechin per milligram of extract (in 3000 micrograms per milliliter plant concentration), respectively, which was presented in Table 1.

Table 1. Total phenolic compounds and total flavonoids in ethanol extract from *Epilobium angustifolium* L. leaves

Concentration (µg/ml)	Total phenolic compound (µg pyrocatechol/mg extract)*	Total flavonoid (µg catechin/mg extract)*
1000	174,68±19,97	24,16 ± 0,78
1250	360,52± 6,14	-----
1500	468,11± 1,53	-----
2000	-----	57,96 ± 1,06
3000	-----	103,05 ± 2,36

* Values are means of independent analyses of the extract±SD in triplicate, SD: Standard deviation

Anthocyanins

Anthocyanins (Ac), which responsible for colorization (blue, purple, and red) in many plants, are water-soluble flavonoids. Not only as food colorants but also as

antioxidants. anthocyanins are becoming densely important. In our study, when the concentration of sample increased, the number of anthocyanins showed a decrease. The total anthocyanin amount is represented in Table 2 (GIUSTI and WROLSTAD, 2001 [8]).

Table 2. Total anthocyanins in *Epilobium angustifolium* L. dry leaves

Sample	2h	24h
1 mg/mL	17,92 ± 8,08	22,64 ± 14,29
2 mg/mL	13,56 ± 8,22	16,55 ± 8,22

Values are means of independent analyses of the extract±SD in triplicate, SD: Standard deviation

β-carotene bleaching test

β-carotene bleaching test yielded that ethanolic plant extracts gave the best results, with RAA (relative antioxidant

activity) values. It was observed to have higher activity than butylated-hydroxy-anisole (BHA), which was taken as a positive control, especially at concentrations of 500 and 1000 µg / mL (Table 3).

Table 3. β-carotene bleaching test

Sample	Concentration (µg/mL)	RAA (60 min)	RAA (120 min)
Ethanolic extract	20	0.738 ± 0.053	0.686 ± 0.019
	100	0.966 ± 0.053	0.853 ± 0.074
	250	0.983 ± 0.021	0.903 ± 0.027
	500	1.050 ± 0.054	0.964 ± 0.092
	1000	1.540 ± 0.053	1.030 ± 0.005
BHA		1	1
Negative control ^a	20	0.673 ± 0.033	0.406 ± 0.014
	100	0.676 ± 0.005	0.435 ± 0.010
	250	0.677 ± 0.015	0.444 ± 0.017
	500	0.687 ± 0.013	0.451 ± 0.004
	1000	0.718 ± 0.024	0.456 ± 0.019

^aNegative control: Linoleic acid and β-carotene emulsion

Values are means of independent analyses of the extract±SD in triplicate, SD: Standard deviation

ABTS activity

The ABTS^{•+} (ABTS cation radical) is formed with a loss of an electron from the nitrogen atom of ABTS the chemical name of which is 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid, absorbing at 743 nm (giving a color of bluish-green). When Trolox or another hydrogen-donating antioxidant is present, the nitrogen atom quenches the hydrogen atom, yielding decolorization of the solution (PISOSCHI and NEGULESCU, 2011 [20]).

Potassium persulfate or manganese dioxide can be used to oxidize ABTS, and ABTS^{•+} has a loss of absorbance at 743 nm in the presence of Trolox, which is the standard antioxidant. The method was applied, as based on the reduction of absorbance of the cation radical, to the content determination of antioxidants in plant extracts. Hydrogen-donating antioxidants and chain-breaking antioxidants are determined by the ABTS method, which could be applied to both lipophilic and hydrophilic compounds (ARNAO & al., 2001 [1]).

As a result of the trial, it was found that the ethanolic plant extract showed an activity equivalent to Trolox, which was chosen as the witness antioxidant. Higher levels of anthocyanins, flavonoids, and total phenolic compounds are attributed to the highest ABTS scavenging activity.

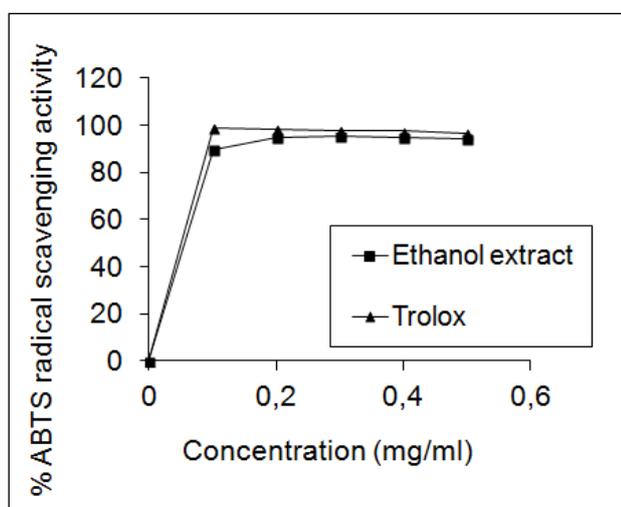


Figure 1. ABTS radical scavenging activity of *Epilobium angustifolium* L. ethanol extract

Reducing power activity

Electron-donating activity is often attributed to Fe³⁺ reduction, and this is an important phenolic antioxidant mechanism. With the other antioxidant properties, there is a strong correlation. In this assay, the presence of reductants, referred to as the presence of antioxidants in the samples, leads to a reduction of the Fe³⁺/ ferricyanide complex to the Fe²⁺ form. At 700 nm, the concentration of Fe²⁺ complex could be measured from the formation of the Perl's Prussian Blue complex. If a compound has a good reducing capacity, it implies that its antioxidant potential has been significant (OYAZU, 1986 [19]).

In Figure 2., it can be seen that *E. angustifolium* L. ethanolic extract and standards behave as reducing entities

in the dose-response curves. The reduction power of the ethanolic extract is almost equal BHT's performance and better than of tocopherol.

The reductive order of the power of the ethanolic extract and standards showed this increase: Tocopherol < BHT < Ethanolic extract < BHA. It means that the ethanolic extract of *E. angustifolium* did behave as an electron donor, having the capacity to react with free radicals and convert them into products that are more stable. The outcome of reduction terminates the radical chain reactions, so very damaging results are prevented. One could attribute this activity to the presence of phenolic compounds like *E. angustifolium*, having natural antioxidants.

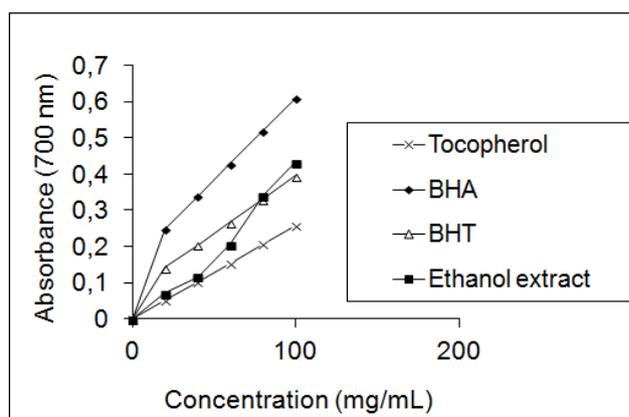


Figure 2. Reducing power activity of *Epilobium angustifolium* L. ethanol extract

Hydroxyl radical scavenging activity

There is a multitude of ways to be sure about the ability of the formation of hydroxyl radicals, and deoxyribose test is one of them. This method uses iron(III) chloride (FeCl₃) and ethylenediaminetetraacetic acid (EDTA) complex, and in the presence of ascorbic acid, the complex is reduced to Fe²⁺-EDTA, and ascorbic acid is oxidized. Upon adding hydrogen peroxide (H₂O₂), Fe²⁺-EDTA complex is oxidized to Fe³⁺-EDTA complex again and HO[•] is produced. This is referred to as the Fenton reaction, generating the highly reactive hydroxyl radical (Fe²⁺ + H₂O₂ → Fe³⁺ + HO[•] + HO[•]) (CHUNG & al., 1997 [4]).

Hydroxyl radicals, which are not terminated by one of the components of the mixture, attack deoxyribose and cause the degradation of it into many small fragments. Some of these could react with thiobarbituric acid after heating (ensuring that the pH is acidic), which yields a pink-colored product the quantification of which can be performed by spectrophotometry. Chromagen formation is inhibited by the compounds which could scavenge hydroxyl radicals. Without ascorbic acid, the deoxyribose method can be performed. If the samples have pro-oxidant ability or not could be determined, because the compounds that are substituted for ascorbic acid may react in Fenton's reaction or not at all. Some of them cause inhibition of chromagen, and hydroxyl radicals are not involved, but after stable metal chelates are formed, which block the formation of hydroxyl radicals. When identifying chelates

with iron compounds, EDTA is not recommended for use. If a chelate is not present, iron ions are found to bind to deoxyribose and cause damage to hydroxyl radicals specific to the site. If there is a species which is capable of forming iron complexes, there will be less hydroxyl radical present, and the characteristic pink color is reduced.

Hydroxyl radical scavenging activity of *Epilobium angustifolium* L. ethanolic extract is higher than that of ascorbic acid and of gallic acid (Figure 3).

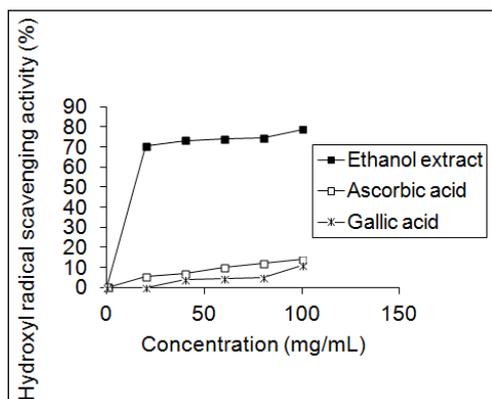


Figure 3. Hydroxyl radical scavenging activity of *Epilobium angustifolium* L. ethanol extract

Superoxide radical scavenging activity (SOD)

Superoxide anion by itself is a weak oxidant, but it produces, in the end, hydroxyl radicals and singlet oxygen, both of which are known to contribute to oxidative stress. The scavenging activity of the superoxide anion could be determined by following the procedure by LIU & al. (1997) [15].

Superoxide radicals are generated from dissolved oxygen with the effect of PMS-NADH coupling and they could be detected with the fact that they are able to reduce NBT. A decrease in absorbance at 560 nm, the plant extract, and the reference compound (Trolox) are indicative of the quenching of the superoxide radicals in the reaction mixture.

The generation of superoxide anion in terms of inhibition tells us about the activity of scavenging superoxide anion, and therefore, the production of formazan is reduced. Our ethanolic extract of *E. Angustifolium* has a scavenging activity of radicals that is lower than that of Trolox, but it is not zero (Figure 4).

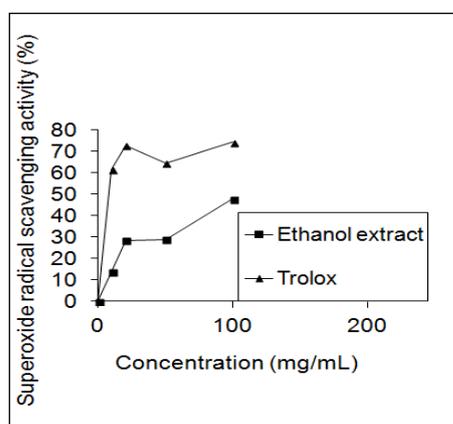


Figure 4. Superoxide radical scavenging activity of *Epilobium angustifolium* L. ethanol extract

Statistical analysis

Results were expressed in triplicate as mean \pm standard deviation (SD) analyses.

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Conflict of Interest

The authors have no conflict of interest to declare.

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