# Antioxidant Properties of Extracts from Leaves of *Evolvulus alsinoides* Linn.

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**ABSTRACT**

**Introduction:** In the present study, hexane, chloroform, ethyl acetate, butanol and methanol extracts of fruits of *Evolvulus alsinoides* Linn (Convolvulaceae) were examined total phenolics (TP) and *in vitro* antioxidative capacity. **Methods**: For the determination of total phenolics (TP) and *in vitro* antioxidative capacity, established assay methods such as 1, 1 - diphenyl – 2- picryl hydroxyl (DPPH) radical assay, reducing power, ferric ion chelating assay, superoxide anion, and nitric oxide scavenging activity assays were used with reference to synthetic antioxidant butyl hydroxyl toluene (BHT). One way analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) were carried out. **Results:** The TP ranged from 1686 ± 1.527 mg GAE /100 of Dry weight (DW) to 1255 ± 0.020 mg GAE/100 of Dry weight (DW). The data obtained from the study showed high levels of antioxidant activity of the fruit extracts. **Conclusion:** From the findings, it was observed that there was a well correlation between antioxidant activity and total phenolic/flavonoid contents. These results may be useful to further analyze wild edible fruits that contain most antioxidant activity in order to identify the active constituents.

**Keywords:** Antioxidant activity, butyl hydroxyl toluene, Convolvulaceae, *Evolvulus alsinoides* Linn., phenolic content

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## INTRODUCTION

Free radicals including reactive oxygen species (ROS) induce oxidative damage to biomolecules and have been implicated with variety of chronic diseases including cancer, diabetes, atherosclerosis, neurodegenerative disorders and arthritis.[1] Natural antioxidant mechanisms can be inefficient, hence dietary intake of antioxidant compounds becomes important.[2] Epidemiological studies have indicated the relationship between the plant antioxidants and reduction of chronic diseases.[3] These benefits are thought to result from the antioxidant components of plant origin, vitamins, flavonoids, and carotenoids.[4,5] The studies in recent years have shown that polyphenols in plants scavenge active oxygen species and effectively prevent oxidative cell damage.[6]

*Evolvulus alsinoides* L., belonging to Convolvulaceae family, is known as morning glory and *Sankhapushpi*, grows wildly in open grassy places throughout India. The plant is used in dementia.[7] *E. alsinoides* contains alkaloids betaine, sankhapushpine and evolvine, scopoletis, scopolin, umbelliferone, 6-methory-7-0-b-glucophyranoside coumarin queretine-3-o-b-glucophyrenoside are reported. [8] It was reported to possess antibacterial and anthelmintic, anti ulcer and anti catatonic activity, and immunomodulatory activity.[9,10,11] The leaves exhibited significant scavenging activity against DPPH and superoxide radicals and inhibited acetylcholinesterase enzyme.[12] The ethanol extract of *E. alsinoides* showed potent antioxidant activity.[13] Antioxidant compounds scopoletin, umbelliferone, scopolin and 2-methyl-1,2,3,4-butanetetrol were isolated from *E. alsinoides*.[14]

In the present investigation, we determined the total phenolics content and antioxidant activity of leaves of different of *E. alsinoides* were carried out. The antioxidant and free radical scavenging activity were evaluated by means of DPPH· radical quenching test, reducing power, $O_2^{\cdot-}$ scavenging assay, ·OH scavenging assay, NO scavenging activity, ferrous ion chelation and inhibition of LPO.

## MATERIALS AND METHODS

### *Chemicals*

The solvents used in the present work were purchased from Qualigenes. Folin-Ciocalteu reagent, DPPH, Gallic acid and quercetin were procured from Sigma, USA. Ammonium molybdate tetrahydrate, aluminium chloride,

TCA were acquired from E. Merck (INDIA) Limited. Mumbai, India. nitro blue tetrazolium (NBT), ferrozine were purchased from HI-MEDIA, Pvt Ltd, India. All other reagents were of analytical grade.

### Plant material and extraction

The plant material was collected from Kerala and Tamilnadu states, India during 2008-09. The plant was identified and authenticated by Botanical Survey of India (Southern Circle), Coimbatore. A voucher specimen has been deposited at the herbarium of Karpagam University. Fifty grams of each powder was successively extracted in soxhlet apparatus with petroleum ether, chloroform, ethyl acetate, *n*-butanol and methanol (250 ml). The extracts, thus collected, were evaporated to dryness using rotary flash evaporator (Buchi type, Switzerland) under reduced pressure at less than $40^0$ C. The crude extracts were used for assessing the antioxidant capacity.

### Determination of total phenolics

Total phenolic contents in the extracts were determined by Folin-Ciocalteu method.[15] In brief, an aliquot of the extracts was mixed with 5 ml of Folin-Ciocalteu reagent previously diluted with water (1:10 v/v) and 4 ml (7.5 g/l) of saturated sodium carbonate ($Na_2CO_3$). The tubes were allowed to stand for 15 min and the total phenols were measured using UV/VIS spectrophotometer (ELICO SL-164, Elico Ltd, Hyderabad) at 765 nm. Total phenol content was expressed in terms of gallic acid equivalent in mg/100 g of DM.

### DPPH· scavenging activity

The effect of extracts on DPPH· was determined using the method of Blois.[16] To 1 ml of different concentrations (500, 400, 300, 200, 100 μg/ml) of extracts, 5 ml of methanol solution of DPPH· (0.1 mM) was added, vortexed, followed by incubation at 27ºC for 20 min. The control was prepared without any extract and absorbance of the sample was measured at 517 nm using UV/VIS spectrophotometer. The ability to scavenge DPPH· radical was calculated by the following equation: DPPH· radical scavenging activity (%) = $[(Abs_{control} - Abs_{sample})]/(Abs_{control}) \times 100$.

### Reductive ability

Total reducing power was determined as described by Oyaizu.[17] One ml of sample solution at different concentrations (500, 400, 300, 200, 100 μg/ml) was mixed with 2.5 ml of phosphate buffer (0.2 mol/l, pH 6.6) and 2.5 ml of potassium ferricyanide (1%). The mixture was incubated at 50ºC for 20 min. To this, 2.5 ml of trichloroacetic acid (TCA, 10%) was added and centrifuged at 3000 *g* for 10 min. The supernatant (5 ml) was mixed with 1 ml of ferric chloride (0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

### Superoxide anion scavenging activity

Measurement of superoxide anion scavenging activity was based on the method described previously.[18] Superoxide radicals were generated in PMS-NADH systems by oxidation of NADH and assayed by the reduction of nitro blue tetrazolium (NBT). Three ml of sample solutions at different concentrations (500, 400, 300, 200, 100 μg/ml) were mixed with 1 ml of NBT (156μM) and 1 ml of NADH (468 μM). The reaction started by adding 0.1 ml of phenazine metho sulphate (PMS) solution (60 μM) to the mixture. The reaction mixture was incubated at 25ºC for 5 min, and the absorbance at 560 nm was measured against blank samples. The percentage inhibition of $O_2^{\bullet -}$ generation was calculated using the following formula: Inhibition of $O_2^{\bullet -}$ generation (%) = $[(A_{control} - A_{sample}) / A_{control}] \times 100$.

### Scavenging capacity towards hydroxyl ion (˙OH) radicals

The ˙OH scavenging activity of the extracts was determined according to the method described previously.[19] Different concentrations (500, 400, 300, 200, 100 μg/ml) of extracts were added with 1.0 ml of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 ml of EDTA solution (0.018%), and 1.0 mL of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4). The reaction was started by adding 0.5 ml of ascorbic acid (0.22%) and incubated at 80-90° C for 15 min in a water bath. The reaction was then terminated by the addition of 1.0 ml of ice-cold TCA (17.5% w/v). Three millilitres of Nash reagent (75.0 g of ammonium acetate, 3.0 ml of glacial acetic acid, and 2 ml of acetyl acetone were mixed and raised to 1 L with distilled water) was added and left at room temperature for 15 min. The intensity of the color formed was measured at 412 nm against reagent blank. BHT was considered as the reference standard. The ˙OH scavenging activity is calculated by the following formula: HRSA (%) = 1-(difference in absorbance of sample/difference in absorbance of blank) × 100.

### Scavenging activity against nitric oxide

Nitric oxide interacts with oxygen to produce stable products, nitrite and nitrate. Scavengers of nitric oxide compete with oxygen, leading to a reduced production of nitrite. The concentration of nitrite in aqueous solution was assayed spectrophotometrically by using the Greiss reagent, with which nitrite reacts to give a stable product absorbing at 546 nm.[20] Nitric oxide radicals were generated from sodium nitroprusside (SNP) solution at physiological pH. Sodium nitroprusside (1 ml of 10 mM) was mixed with 1 ml various concentrations (500, 400, 300, 200, 100 μg/ml) of extracts in phosphate buffer (pH 7.4). The mixture was incubated at 25ºC for 150 min. To 1 ml of the incubated solution, 1 ml of greiss reagent (1% sulphanilamide, 2% ortho phosphoric acid and 0.1% napthyl ethylene diamine dihydrochloride) was added. Absorbance was read at 546 nm and percentage inhibition was calculated using the formula: inhibition (%) = $[(A_{control} - A_{sample}) / A_{control}] \times 100$.

### Iron chelating activity

The iron chelating activity was measured by the decrease in absorbance at 562 nm of the iron (II) –ferrozine complex.[21] The reaction mixture contained 0.5 ml of various concentrations (500, 400, 300, 200, 100 μg/ml) of the extracts, 0.1 ml of ferric chloride (0.6 mM) and 900 μl methanol. The mixture was shaken and left at room temperature for 10 min. To this, 0.1 ml of ferrozine (5 mM) in methanol was added, mixed and left for 5 min to complex the residual $Fe^{2+}$. The absorbance of the resulting solution was measured at 562 nm. The ability of the extracts to chelate ferrous ion was calculated relative to the control (consisting of iron and ferrozine only) using the formula: Chelating effect (%) = $[(Abs_{control} - Abs_{sample})] / (Abs_{control}) \times 100$.

### Reduction of lipid peroxidation

Inhibition of lipid peroxidation (LPO) in rat lever homogenate was determined in terms of formation of thiobarbituric acid reactive substances (TBARS) with minor changes.[22] In brief, different concentrations (500, 400, 300, 200, 100 μg/ml) of extracts of *E. alsinoides* and standard (10-80 μg $mL^{-1}$) were individually added to 0.2 mL of liver homogenate (10%) extracted with KCl (15%). To the above mixture, 0.1 mL of $FeSO_4$ (10 mM) solution was added to initiate LPO. The volume of the mixtures was finally made up to 2 mL with phosphate buffer (0.1 mM, pH 7) and incubated at 37º C for 30 min. At the end of the incubation period, reaction mixture (0.3 mL) was added with 1 mL of TBA (0.8%, w/v) and 0.1 mL of TCA (20%) solution. The mixture was then heated on a water bath at 100º C for 60 min. After cooling, *n* – butanol (4 mL) was added in each tube and centrifuged at 3000 x *g* for 10 min. The absorbance of the organic upper layer was read at 532 nm. Butyl hydroxyl toluene (BHT) was used for comparison. The percentage reduction of LPO was calculated as follows: Reduction of TBARS (%) = $1\text{-}Sample_{532nm}/Control_{532nm} \times 100$ where, $Sample_{532nm}$ was absorbance of the sample and $Control_{532nm}$ was absorbance of control.

### Statistical analysis

The experimental data were mean ± standard deviation of three measurements (n = 3). Linear regression analysis was used to calculate the efficient concentration ($IC_{50}$) values. One way analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) were carried out. The *P* values of less than 0.05 were adopted as statistically significant.

## RESULTS AND DISCUSSION

### Total phenolic content

In our study, the content of the total phenolics in different extracts of MeOH was determined using Folin–Ciocalteau method expressed as gallic acid equivalents is shown in Table 1. Total phenolic content of different *E. alsinoides* extracts were solvent dependent. The estimation of phenolic content amongst different solvent extracts of *E. alsinoides* revealed that the MeOH extract contained higher phenol content of 1686 ± 0.029 mg/100g GAE DW. The content in the total phenolics in the extracts decreased in the order of EA extract > BuOH extract > PE extract > $CHCl_3$ extract. As different *E. alsinoides* extracts exerted different

**Table 1.** Total phenolics content of different extracts of *E. alsinoides*

| Extracts | TP (mg/100g) [a] |
|---|---|
| PE | 1347 ± 0.025 |
| $CHCl_3$ | 1255 ± 0.020 |
| EA | 1560 ± 0.022 |
| BuOH | 1540 ± 0.030 |
| MeOH | 1686 ± 1.527 |

Each value is presented as Mean ± Standard Deviation (n = 3). [a]mg GAE/100g DW

reactive oxygen species scavenging activities, there may be different kinds of total phenolic compounds in different *E. alsinoides* extracts.

## DPPH· scavenging activity

The dose-response DPPH· scavenging activity of various extracts studied in this study is shown in Figure 1. Among the extracts from *E. alsinoides*, MeOH extract displayed significantly higher DPPH· scavenging activity ($IC_{50}$ = 0.095 mg/mL) as compared to other four extracts ($p < 0.05$). The BuOH extract showed the weakest quenching capacity with an $IC_{50}$ value of 0.445 mg/mL. The effectiveness of antioxidants as DPPH· scavengers ranged in the following order: EA extract (0.1 mg/mL) > PE (0.140 mg/mL) > $CHCl_3$ (0.245 mg/mL) > BuOH (0.44 mg/mL). The standard antioxidant, BHT, displayed DPPH· quenching capacity with $IC_{50}$ value of 0.026 mg/mL. When considering the organic solvent extracts of *E. alsinoides*, the DPPH· scavenging capacities increased towards the MeOH extract with increasing the polarity of the solvent. Also, DPPH radical scavenging activities were increased with an increased content of total phenolics in the extracts.

## Reducing power

The reducing power of the crude extracts was examined as a function of their concentration. The reducing capacity for investigated extracts of *E. alsinoides* is illustrated in Figure 2. Out of five extracts, the EA displayed the highest reductive capacity with an absorbance of 0.732 at 1 mg/mL followed by BuOH, MeOH, PE and $CHCl_3$ extracts. Also the reducing ability of each extract was concentration dependent and comparable to that of the control, BHT with an absorbance of 1.634 at 1 mg/mL. For the determination of reducing capacity, "$Fe^{3+}$-$Fe^{2+}$ transportation" in the presence of extract was observed. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom.[23] The reducing power of the extracts might be due to the di and mono hydroxyl substitutions in the aromatic ring which possess potent hydrogen donating abilities.

## Superoxide scavenging activity

The $O_2^{\bullet -}$ radical is one of the most dangerous free radicals in humans and also the source of hydroxyl radical ($OH^{\cdot}$).[24] In our study, the extracts of *E. alsinoides* were screened for their $O_2^{\bullet -}$ scavenging activity using PMS-NADH-NBT assay. In the PMS/NADH-NBT system, $O_2^{\bullet -}$ derived from dissolved oxygen by PMS-NADH coupling reaction reduces NBT. The effect of extracts (PE, $CHCl_3$, EA, BuOH and MeOH) on $O_2^{\bullet -}$ in PMS-NADH/NBT system is shown in Figure 3. The effect of MeOH and $CHCl_3$ extracts was the highest with $IC_{50}$ value of 0.1 and 0.27 mg/mL respectively [Table 2] which was significantly ($p < 0.05$) lesser than that of BHT ($IC_{50}$ 0.016 mg/mL). The EA extract ($IC_{50}$ 0.29 mg/mL) caused a concentration dependent elevation in $O_2^{\bullet -}$ scavenging activity and the BuOH ($IC_{50}$ 0.395 mg/mL) and PE ($IC_{50}$ 0.41 mg/mL) extracts showed least activity. It is also noteworthy to state that higher scavenging activity was detected in high polar solvent extracts.

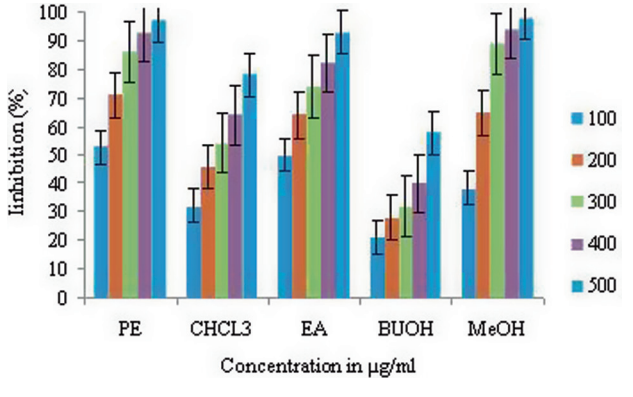


**Figure 1.** DPPH· scavenging activity of extracts of *e. alsinoides*.

The data are presented as mean value ± standard deviation SD (n = 3). PE – Petroleum ether, $CHCL_3$ – Chloroform, EA – Ethyl acetate, BuOH – Butanol, MeOH – Methanol, BHT – Butyl Hydroxyl Toluene.

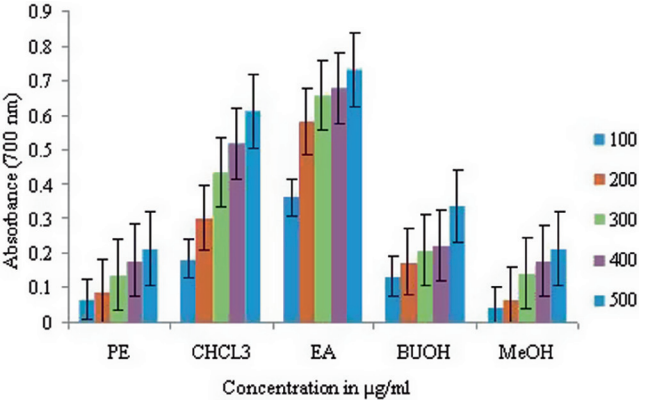


**Figure 2.** Reductive ability of extracts of *e. alsinoides*.

The data are presented as mean value ± standard deviation SD (n = 3). PE – Petroleum ether, $CHCL_3$ – Chloroform, EA – Ethyl acetate, BuOH – Butanol, MeOH – Methanol, BHT – Butyl Hydroxyl Toluene.

**Table 2.** Antioxidant capacities of different extracts of *E. alsinoides*

| | $IC_{50}$ (mg/mL) | | | | | |
|---|---|---|---|---|---|---|
| **Extracts** | **DPPH·** | **$O_2^{\cdot-}$** | **OH·** | **NO** | **Metal chelating** | **LPO** |
| PE | 0.140 ± 0.42 | 0.41 ± 0.64 | 0.485 ± 0.06 | 0.5 ± 0.62 | 0.5 ± 0.22 | 0.29 ± 0.48 |
| $CHCl_3$ | 0.245 ± 0.22 | 0.27 ± 0.32 | 0.46 ± 0.82 | 0.49 ± 0.48 | 0.475 ± 0.06 | 0.35 ± 0.84 |
| EA | 0.1 ± 0.06 | 0.29 ± 0.64 | 0.40 ± 0.34 | 0.48 ± 0.32 | 0.5 ± 0.08 | 0.33 ± 0.34 |
| BuOH | 0.44 ± 0.44 | 0.395 ± 0.22 | 0.38 ± 0.52 | 0.48 ± 0.02 | 0.480 ± 0.04 | 0.44 ± 0.62 |
| MeOH | 0.095 ± 0.42 | 0.1 ± 0.04 | 0.29 ± 0.08 | 0.44 ± 0.22 | 0.410 ± 0.04 | 0.25 ± 0.12 |
| BHT | 0.02 ± 0.02 | 0.016.4 ± 0.04 | 0.04.34 ± 0.12 | 0.046 ± 0.22 | ND | ND |

The data are presented as mean value ± standard deviation SD (n = 3). ND – Not Determined. PE – Petroleum ether, $CHCL_3$ – Chloroform, EA – Ethyl acetate, BuOH – Butanol, MEOH – Methanol, BHT – Butyl Hydroxyl Toluene.

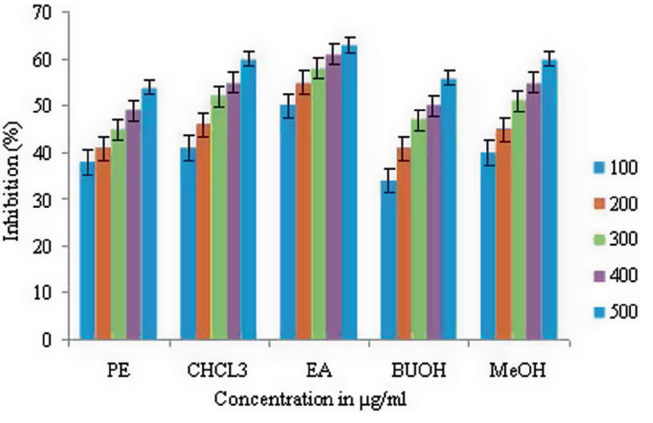


**Figure 3.** $O2^{\cdot-}$ scavenging capacity of extracts of *e. alsinoides*.

The data are presented as mean value ± standard deviation SD (n = 3). PE – Petroleum ether, $CHCL_3$ – Chloroform, EA – Ethyl acetate, BuOH – Butanol, MeOH – Methanol, BHT – Butyl Hydroxyl Toluene.

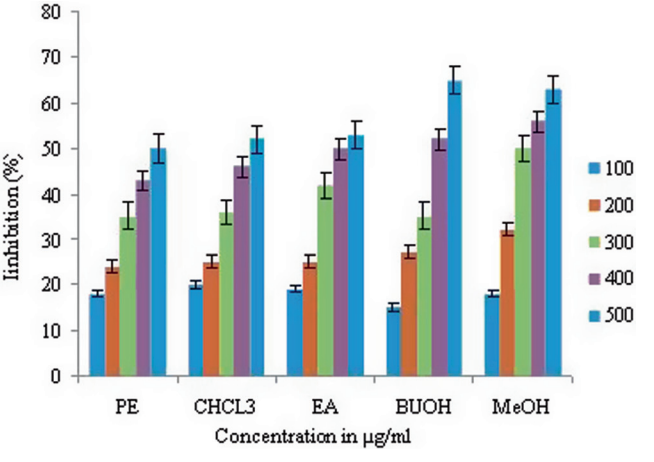


**Figure 4.** ·OH scavenging capacity of extracts of *e. alsinoides*.

The data are presented as mean value ± standard deviation SD (n = 3). PE – Petroleum ether, $CHCL_3$ – Chloroform, EA – Ethyl acetate, BuOH – Butanol, MeOH – Methanol, BHT – Butyl Hydroxyl Toluene.

## ·OH scavenging activity

Hydroxyl radical (·OH) which is the most reactive free radical, has the capacity to conjugate with nucleotides in DNA, cause strand breakage, and lead to carcinogenesis, mutagenesis and cytotoxicity.[25] The scavenging activity of the fruit extracts on ·OH is presented in the figure 4. The extracts from the leaves displayed dose dependent scavenging activity against the ·OH species, of which, MeOH and BuOH extracts were the most effective ($IC_{50}$ 0.29 mg/mL and 0.38 mg/mL respectively ). The PE ($IC_{50}$ 0.485 mg/mL), $CHCl_3$ ($IC_{50}$ 0.46 mg/mL), and EA ($IC_{50}$ 0.40 mg/mL) extracts displayed moderate scavenging capacity. The synthetic antioxidant, BHT, exhibited ·OH scavenging capacity with $IC_{50}$ value of 0.016 mg/mL.

## Nitric oxide radical scavenging activity

In addition to reactive oxygen species, nitric oxide (NO) is also implicated in inflammation, cancer and other pathological conditions. The NO generated from sodium nitroprusside reacts with oxygen to form nitrite. From the results obtained, the extracts dose-dependently inhibit nitrite formation by directly competing with oxygen in the reaction with nitric oxide [Figure 5]. All the extracts exhibited strong to moderate NO scavenging activity *in vitro* in the following order: MeOH ($IC_{50}$ 0.44 mg/mL) > BuOH ($IC_{50}$ 0.48 mg/mL) EA ($IC_{50}$ 0.48 mg/mL) $CHCl_3$ ($IC_{50}$ 0.49 mg/mL) and PE ($IC_{50}$ 0.5 mg/mL). Standard compound, BHT, displayed NO scavenging activity with $IC_{50}$ value of 0.046 mg/mL [Table 2].

## Ferrous ion chelating ability

Metal chelating activity is significant since it reduces the concentration of the catalyzing transition metal in lipid peroxidation.[26] Figure 6 shows concentration dependent chelating effects of the extracts from *E. alsinoides* on the $Fe^{2+}$ - ferrozine complex. The MeOH extract displayed

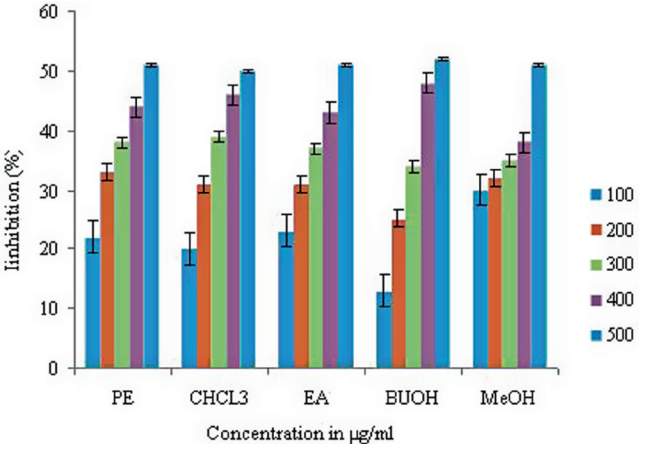


**Figure 5.** NO radical scavenging capacity of extracts of *e. alsinoides.*

The data are presented as mean value ± standard deviation SD (n = 3). PE – Petroleum ether, $CHCL_3$ – Chloroform, EA – Ethyl acetate, BuOH – Butanol, MeOH – Methanol, BHT – Butyl Hydroxyl Toluene.

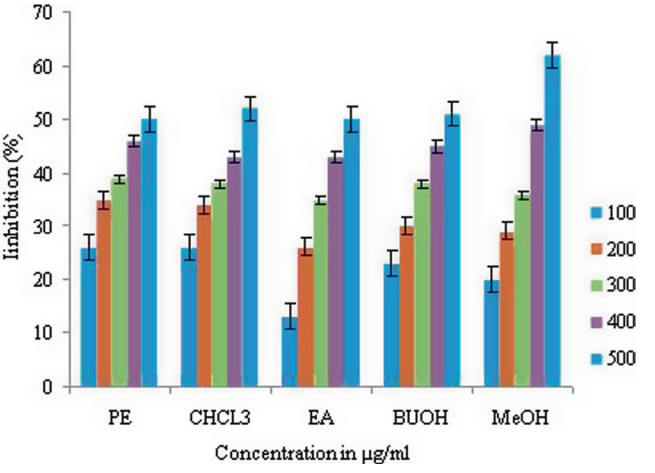


**Figure 6.** Metal chelating capacity of extracts of ***e. alsinoides***.

The data are presented as mean value ± standard deviation SD (n = 3). PE – Petroleum ether, $CHCL_3$ – Chloroform, EA – Ethyl acetate, BuOH – Butanol, MeOH – Methanol, BHT – Butyl Hydroxyl Toluene.

the highest chelating activity ($IC_{50}$ 0.410 mg/mL) followed by $CHCl_3$ extract ($IC_{50}$ 0.475 mg/mL), BuOH ($IC_{50}$ 0.480 mg/mL), EA extract ($IC_{50}$ 0. 5 mg/mL) and PE extract ($IC_{50}$ 0. 5 mg/mL) [Table 2]. Chelating agents may serve as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of metal ion. Accordingly, it is suggested that the low to moderate ferrous ions chelating effects of these extracts would be somewhat beneficial to protect against oxidation damage.

### *Inhibition of lipid peroxidation*

Lipid peroxidation plays an important role in causing oxidative damage to biological systems and its by-product malondialdehyde (MDA) induces damage to other biomolecules.[27] The dose dependent inhibitory effect of extracts from *E. alsinoides* on lipid peroxidation (LPO) is shown in Figure 7. In the present study, the MeOH extract was able to inhibit the generation of LPO efficiently with an $IC_{50}$ of 0.25 mg/mL). The EA, BuOH, $CHCl_3$ and PE extracts exhibited moderate LPO inhibitory activity with the $IC_{50}$ values of 0.29 mg/mL, 0.33 mg/mL, 0.44 mg/mL and 0.35 mg/mL respectively [Table 2].

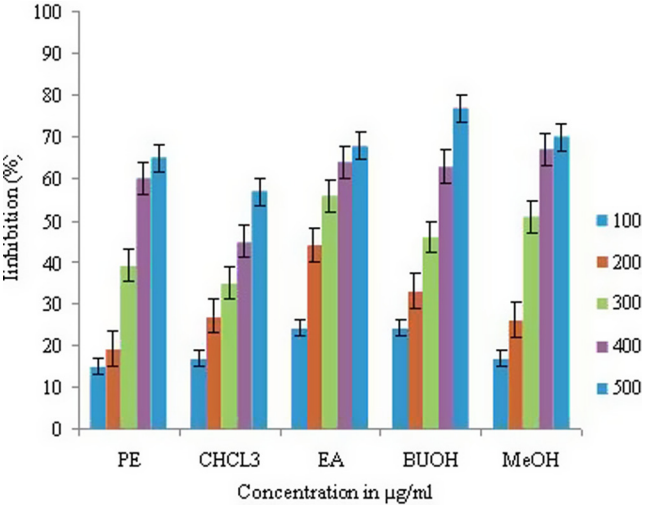


**Figure 7.** Lipid peroxidation inhibitory activity of extracts of *e. alsinoides.*

The data are presented as mean value ± standard deviation SD (n = 3). PE – Petroleum ether, $CHCL_3$ – Chloroform, EA – Ethyl acetate, BuOH – Butanol, MeOH – Methanol, BHT – Butyl Hydroxyl Toluene.

## CONCLUSION

Recent years have seen an exponential increase in research antioxidant properties of fruits and vegetables. If it is accepted that higher intakes of natural antioxidants containing phenolics are associated with long-term health benefits, then the results presented in this paper offer possible avenues toward health promotion by identifying those compounds. The health promoting properties of fruits of *E. alsinoides* may be due to its antioxidant properties and is also attributed to its multitherapeutic characteristics. Thus, *E. alsinoides* might be useful in the development of raw materials of medicine.

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