World Journal of Pharmaceutical and Life Sciences
WJPLS
www.wjpls.org
SJIF Impact Factor: 7.409

IN VITRO ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITY OF VARIOUS EXTRACTS OF *LEUCUS ASPERA* (WILLD.) LINN. AERIAL PARTS

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Article Received on 29/12/2023 **Article Revised on 19/01/2024** **Article Accepted on 09/2/2024**

ABSTRACT
Various Extracts of *Leucus aspera* (Willd.) Linn. Aerial Parts were investigated for the *in vitro* antioxidant effects. Ethanolic extract (EA), Ethyl acetate extract (Eac), Chloroform extract (CH), Diethyl ether erxtract (DE), Petroleum Ether extract (PE) and n-butanol extract (NB) were prepared by successive soxhlation method and estimation of total phenolics and total flavonoids were carried out. Further, the antioxidant activity of the extracts were studied using five *in vitro* models. The amount of total phenolics was estimated to be highest in EA (56.3 ≧of pyrocatechol equivalent per mg of extract) while minimum in NB (7.9 ≧of pyrocatechol equivalent per mg of extract), whereas total flavonoids were found to be maximum in PE and lowest in EA. The reducing power of the extracts, the antioxidant activity, the DPPH radical scavenging activity, nitric oxide scavenging activity, superoxide radical scavenging activity were also concentration dependant. The order of antioxidant activity was found to be EA>PE>NB>Eac>DE>CH. This could be attributed to more amount of total phenolics in the polar solvents and more amount of total flavonoids in non polar solvents. From these results, it was suggested that EA and PE could effectively act as antioxidative agents. Further studies may be done with these extracts on diseases which are associated with the accumulation of free radicals.

KEYWORDS: *Leucus aspera*, Free radical scavenger, Antioxidants.

INTRODUCTION

The role of free radical reactions in disease pathology is well established. It suggests that these reactions are necessary for normal metabolism but can be detrimental to health as well including outcome of various diseases like diabetes, immunosupression, neurodegenerative diseases and others [Harman, 1998]. Free radicals lead to cellular necrosis, which is implicated in some membrane pathophysiological conditions, including atherosclerosis, rheumatoid arthritis as well as toxicity of many xenobiotics [Kappus, 1985].

Leucas aspera (Willd.) Linn. (Family: Lamiaceae) commonly known as 'Thumbai' [Rai *et al*, 2005] is distributed throughout India from the Himalayas down to Ceylon. [Nadkarni, 1976] The plant is used traditionally as an antipyretic and insecticide. Flowers are valued as stimulant, expectorant, aperient, diaphoretic, insecticide and emmenagogue. Leaves are considered useful in chronic rheumatism, psoriasis and other chronic skin eruptions. Bruised leaves are applied locally in snake bites. [Rai *et al*, 2005; Shirazi, 1947].

Further, studies reveal the presence of various phytochemical constituents mainly triterpenoids, oleanolic acid, ursolic acid and b-sitosterol, nicotine, sterols, flavonoids, glucoside, diterpenes, phenolic compounds (4-(24-hydroxy-1-oxo-5-n-propyltetracosanyl)-phenol). These studies reveal that *L. aspera* is a source of medicinally active compounds and have various pharmacological effects; hence, this drug encourage finding its new therapeutic uses.

Phenols and flavonoids are natural products, which have been shown to possess antioxidant property [Baumann *et al*, 1979]. As *Leucas aspera* contains large amounts of phenols and flavonoids it is thought worthwhile to investigate the antioxidant activity of the aerial parts of *Leucas aspera* in a scientific manner.

MATERIALS AND METHODS

Plant material. The plant was identified by the taxonomists of the Botanical Survey of India, Govt. of India, Howrah. A voucher specimen is kept in our department (JP/PH/TG/03/02) for further reference. Fresh aerial parts of the young and matured plants were collected in bulk from the rural belt of Salipur, Orissa, India during early summer, washed, shade dried and then milled in to coarse powder by a mechanical grinder.

Extraction and isolation. The powdered plant material (2 kg) was extracted successively with n-hexane, petroleum ether (60-80°C), diethylether, chloroform, ethylacetate and ethanol (95%) in a soxhlet apparatus in increasing order of polarity. The respective solvents were removed under reduced pressure, which produced different extracts. All the extracts were evaluated for antioxidation activity.

Chemicals Used

All the chemicals and reagents used in the study were of analytical grade.

Estimation of Total Phenolic Compounds

The method of Naczk *et al*, 1989 was followed. 0.1ml of 10mg/ml aqueous solution of the extract was diluted with 46 ml of distilled water in an Erlen meyer flask. Afterwards, 1 ml of Folin – Ciocalteu Reactive (FCR) was added into this mixture followed by addition of 3 ml of Na_2 CO_3 (2%) after 3 min. Subsequently, mixture was shaken on a shaker for 2 h at room temperature and then absorbance was measured at 760 nm. The concentration of total phenolic compounds was determined as micrograms of pyrocatechol equivalent by using the equation that was obtained from the standard pyrocatechol graph.

Determination of total flavonoids

Total flavonoids were estimated using aluminum chloride colorimetric assay described by Zhisen et al. [Zhishen *et al*, 1999]. To 0.5 mL of samples/standard, 150 µL of 5 % sodium nitrate and 2.5 mL of distilled water were added. After 5 min, 0.3 mL of 10% $AlCl_3$ was added. At 6 min, 1 mL of 0.001 M NaOH and 0.55 mL distilled water was added to the mixture and left at RT for 15 min. Absorbance of the mixtures was measured at 510 nm. Samples of extract were evaluated at a final concentration of 0.1 and 0.15 mg/mL. Total flavonoid contents were expressed in terms of catechin equivalent, CAE (standard curve equation: y = 0.000× + 0.001, R^2 = 0.998), mg of CA/g of dry extract. The experiment was repeated three times at each concentration.

In Vitro Antioxidant Activity Reducing Power

The reducing power of the different extracts of *Leucas aspera* was determined according to the method of Oyaizu, 1986. Accurately weighed 10 mg of the extracts in 1 ml of distilled water was mixed in to the mixture of 2.5 ml of 0.2M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixtures were then incubated at 50°C for 20 min. Following incubation, 2.5 ml of 10% trichloroacetic acid was added to the mixtures, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1 %) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated the increased reducing power.

Antioxidant Activity

The antioxidant activity was determined according to the thiocyanate method of Gulchin et al, 2002.[20] 1 ml of different concentrations of various extracts of *Leucas aspera* was added to linoleic acid in potassium phosphate buffer (2.5 ml, 0.04 M, pH 7.0). The mixed solution was incubated at 37°C. The peroxide value was determined by reading the absorbance at 500 nm, after reaction with ferrous chloride and thiocyanate after two hours of incubation. The solutions without added extracts were used as blank samples. Percent inhibition was calculated from the formula:

$$\% \text{ inhibition} = (A_c - A_t)/ A_c \times 100$$

Where A_c and A_t are absorbance of control and test samples respectively.

DPPH radical scavenging assay

Free radical scavenging ability of the extracts was tested by DPPH radical scavenging assay as described by Blois, 1958 and Desmarchelier *et al*, 1997. The hydrogen atom donating ability of the plant extractives was determined by the decolorization of methanol solution of 2,2-diphenyl- 1-picrylhydrazyl (DPPH). DPPH produces violet/purple color in methanol solution and fades to shades of yellow color in the presence of antioxidants. A solution of 0.1 mM DPPH in methanol was prepared, and 2.4 mL of this solution was mixed with 1.6 mL of extracts in methanol at different concentrations (12.5–150 µg/mL). The reaction mixture was vortexed thoroughly and left in the dark at RT for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. BHT was used as reference. Percentage DPPH radical scavenging activity was calculated by the following equation:

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

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the extractives/standard. Then % of inhibition was plotted against concentration, and from the graph IC_{50} was calculated. The experiment was repeated three times at each concentration.

Assay for Nitric Oxide Scavenging Activity

The method of Sreejayan *et al*, 1997 was followed. For the experiment, sodium nitroprusside (10 mM) in

phosphate buffered saline was mixed with different concentrations of the extracts dissolved in methanol and incubated at room temperature for 2½ h. The same reaction without the sample but equivalent amount of methanol served as control. After incubation period, 0.5 ml of Griess reagent was added. Absorbance of the chromophore formed was measured at 546 nm. Ascorbic acid was used as positive control.

The procedure is based on the principle that, Sodium nitroprusside solution spontaneously generates nitric oxides, which reacts with oxygen to produce nitrite ions that can be estimated using Greiss reagent. Scavengers of nitric oxides compete with oxygen leading to reduce production of nitrite ions

Determination of total antioxidant capacity

Total antioxidant capacity (TAC) of samples was determined by the method reported by Prieto *et al*, 1999. The assay is based on the reduction of Mo(VI) to Mo(V) by samples and formation of green colored phosphate/Mo(V) complex at acidic pH. 0.5 mL of samples/standard at different concentrations (12.5–150 µg/mL) was mixed with 3 mL of reaction mixture containing 0.6 M sulphuric acid, 28 mM sodium phosphate and 1 % ammonium molybdate into the test tubes. The test tubes were incubated at 95 °C for 10 min to complete the reaction. After cooling at RT, sample absorbance was measured at 695 nm using a spectrophotometer against a blank solution. CA was used as standard. A typical blank solution contained 3 mL of reaction mixture and the appropriate volume of the same solvent used for the samples/standard. The blank was incubated at 95°C for 10 min and the absorbance was measured at 695 nm. Increased absorbance of the reaction mixture indicates increased total antioxidant capacity. We used standard/samples at five different concentrations ranges from 12.5 to 150 µg/mL for each antioxidant assay. Concentrations were selected on the basis of trial and error to fit the range of concentration that can fully represent the rational change of antioxidant activity with the increasing concentration of the samples. Also, we assumed that such range of concentrations is useful for smooth calculation of IC_{50}. The experiment was repeated three times at each concentration.

Assay for Superoxide Radical Scavenging Activity

The assay for superoxide radical scavenging activity was performed as per standard procedure [Beuchamp and Fridovich, 1971]. The reaction mixture contained 50 mM phosphate buffer (pH 7.6), 20 µg riboflavin, 12 mM EDTA and 0.1 mg/ml of NBT (nitro blue tetrazolium), all added in chronological sequence. Reaction was started by illuminating the reaction mixture containing different concentrations of the sample extracts for 90 sec and then measuring the absorbance at 590nm. Ascorbic acid was taken as the positive standard.

Statistical Analysis

Data for the in vitro antioxidant activity was expressed as Mean ± SD from three separate observations.

RESULTS

Determination of Total Phenolics

The study reveals that 1g of EA, Eac, CH, DE, PE, NB of *Leucas aspera* contains 56.3, 42.7, 35.5, 20.1, 12.3 and 7.9 mg of pyrocatechol equivalent respectively (Table 1).

Determination of Total Flavonoids

The study reveals that 1g of EA, Eac, CH, DE, PE, NB of *Leucas aspera* contains 0.19, 0.17, 0.21, 0.33, 0.59 and 0.42 mg of catechin equivalent respectively (Table 1).

Reducing Power of *Leucas aspera*

Fig. 1 shows the reductive capacity of various extracts of *Leucas aspera* as compared with ascorbic acid. The reducing power of the extracts were found to increase with increasing concentration of the extracts, which is comparable with the standard drug ascorbic acid. The reducing power was maximum in EA and comparable in PE and minimum in CH.

Antioxidant Activity

Fig. 2 reveals that the extracts possesses significant antioxidant activity with IC_{50} value for EA, Eac, CH, DE, PE, NB of *Leucas aspera* being 126.22, 300, 551.48, 400, 166.76 and 207.51 µg/ml respectively.

DPPH Radical Scavenging Activity

We have observed that the % Inhibition of the extracts increased in a dose dependent manner, and the IC_{50} value was calculated to be 7.05, 23.73, 69.59, 41.71, 9.75 and 17.64 µg/ml for EA, Eac, CH, DE, PE, NB of *Leucas aspera* which was calculated from the regressional line. (Fig 3).

NO Scavenging Activity

Extracts of *Leucas aspera* possess significant NO Scavenging activity with IC_{50} value being 16.17, 45.23, 115.64, 76.24, 22.56, 37.18 µg/ml for EA, Eac, CH, DE, PE, NB of *Leucas aspera* respectively, indicating that the extracts may contain compounds that are able to scavenge nitric oxide (Fig. 4).

Superoxide Radical Scavenging Activity

We have observed that the extracts reduced the absorbance in a dose dependent manner, and the IC_{50} value was calculated to be 7.49, 10.35, 28.29, 20.89, 9.18 and 11.87 µg/ml for EA, Eac, CH, DE, PE, NB of *Leucas aspera* which was calculated from the regressional line. (Fig 5).

DISCUSSION

Although oxygen is essential for life, its transformation to reactive oxygen species (ROS) may

provoke uncontrolled reactions. Such challenges may arise due to exposure to radiation, chemicals or by other means. Antioxidants may offer resistance against the oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation and some other mechanism [Kakkar *et al*, 1984]. Phenols are very important plant constituents because of their free radical scavenging ability due to their hydroxyl groups [Maehly and Chance, 1954]. The phenolic compounds may contribute directly to antioxidant action. It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in human beings [Lowry *et al*, 1951]. Extracts of *L. aspera* is found to contain phenolic compound in significant amount, which attributes to its rationality of possessing antioxidant activity.

For measurements of the reductive ability, we investigate Fe^{+3} to Fe^{+2} transformation in the presence of ethanolic extract using the method discussed earlier. The reducing capacity of the extract may serve as a significant indicator of its potential antioxidant activity [Gillman *et al*, 1997].

Nitric oxide (NO) exhibits numerous physiological properties and it is also implicated in several pathological states [Hatano *et al*, 1989]. However, the specificity of this assay has been questioned since nitrite is one final product of the reaction of nitric oxide with oxygen, through intermediate such as NO_3, N_2O_4, N_2O_3 [Tsao and Akhtar, 2005]. Therefore the decrease in the nitrite production could also be due to interaction of the extract with other nitrogen oxides [Heir *et al*, 1995].

The in vitro superoxide radical scavenging activity is measured by riboflavin/ light/ NBT (Nitroblue tetrazoline) system reduction. The method is based on generation of superoxide radicals by auto oxidation of riboflavin in the presence of light. The superoxide radical reduces NBT to a blue colored formazone that can be measured at 560 nm [Beuchamp and Fridovich, 1971]. The capacity of the various extracts of *Leucas aspera* to inhibit the colour to 50% is measured in terms of IC_{50}. Superoxide radical is known to be very harmful to the cellular components as a precursor of more ROS [Meir *et al*, 1995]. It has been found to have significant superoxide radical scavenging activity, which ultimately adds to its antioxidant potential.

Leucas aspera is reported to be rich in polyphenol and flavonoids. The amount of total phenolics was estimated to be highest in EA (56.3 µg of pyrocatechol equivalent per mg of extract) while minimum in NB (7.9 µg of pyrocatechol equivalent per mg of extract), whereas total flavonoids were found to be maximum in PE and lowest in EA. The order of antioxidant activity was found to be EA>PE>NB>Eac>DE>CH. This could be attributed to more amount of total phenolics in the polar solvents and more amount of total flavonoids in non polar solvents. It can be postulated that the cumulative amount of these substances in various extracts led to observed antioxidant effect. From these results, it was suggested that EA and PE could effectively act as antioxidative agents. Further studies may be done with these extracts on diseases which are associated with the accumulation of free radicals.

ACKNOWLEDGEMENTS

The authors are also thankful to the taxonomists of Botanical Survey of India, Shibpur, Howrah, India for proper identification of the plant.

Table 1: Determination of Total Phenolics and Total Flavonoids of various extracts of Leucas aspera.

	EA	Eac	CH	DE	PE	NB
Total Phenolics[#]	56.3 ± 2.7	42.7 ± 2.5	35.5 ± 2.6	20.1 ± 1.8	12.3 ± 0.8	7.9 ± 0.5
Total Flavonoids[*]	0.19 ± 0.008	0.17 ± 0.012	0.21 ± 0.017	0.33 ± 0.024	0.59 ± 0.035	0.42 ± 0.031

#Pyrocatechol equivalent in mg/g of extract

*Catechin equivalent in mg/g of extract. n=3, Values are Mean ± S.D.

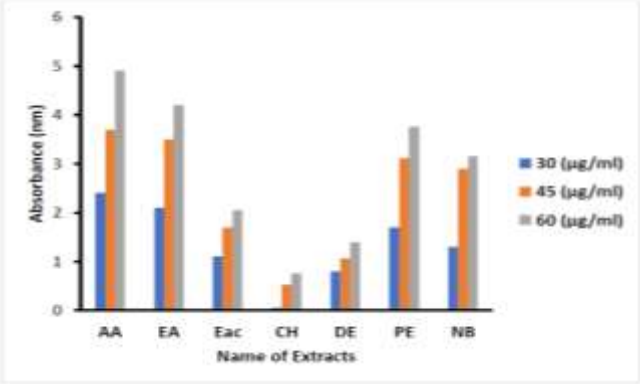

Fig. 1: Reducing property of various extracts of *Leucas aspera*; Values are Mean ± SD of n=3 observations.

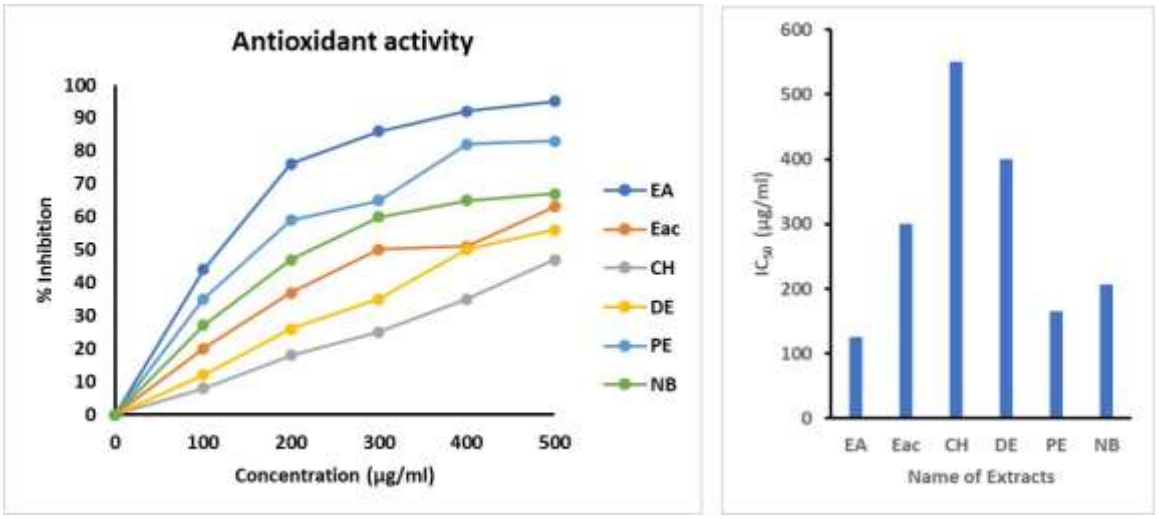

Fig. 2: Antioxidant activity of various extracts of *Leucas aspera.*

n=3, Values are Mean ± S.D.

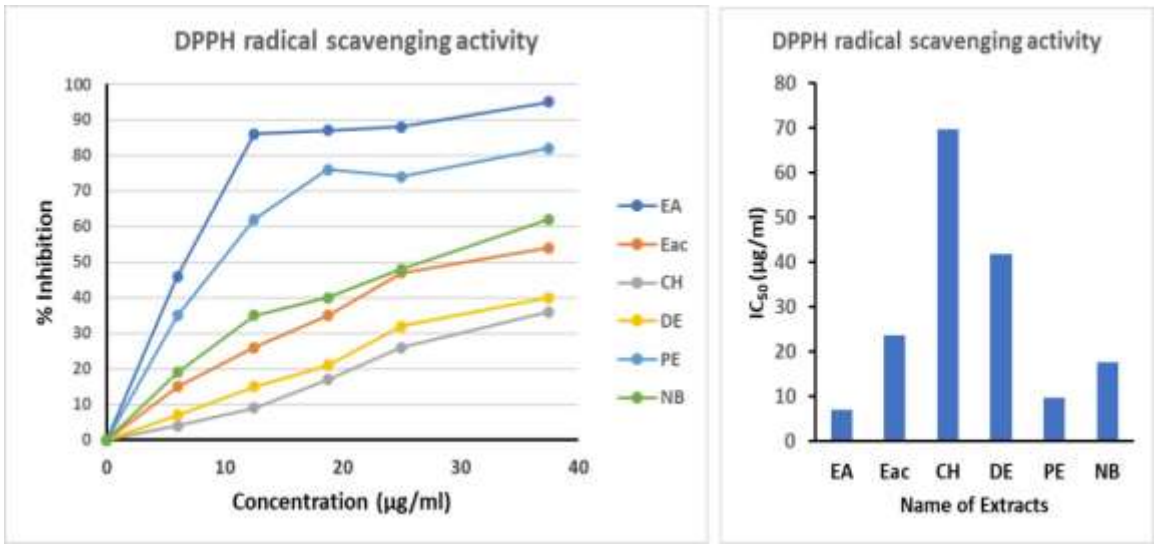

Fig. 3: DPPH radical scavenging activity of various extracts of *Leucas aspera.*

n=3, Values are Mean ± S.D.

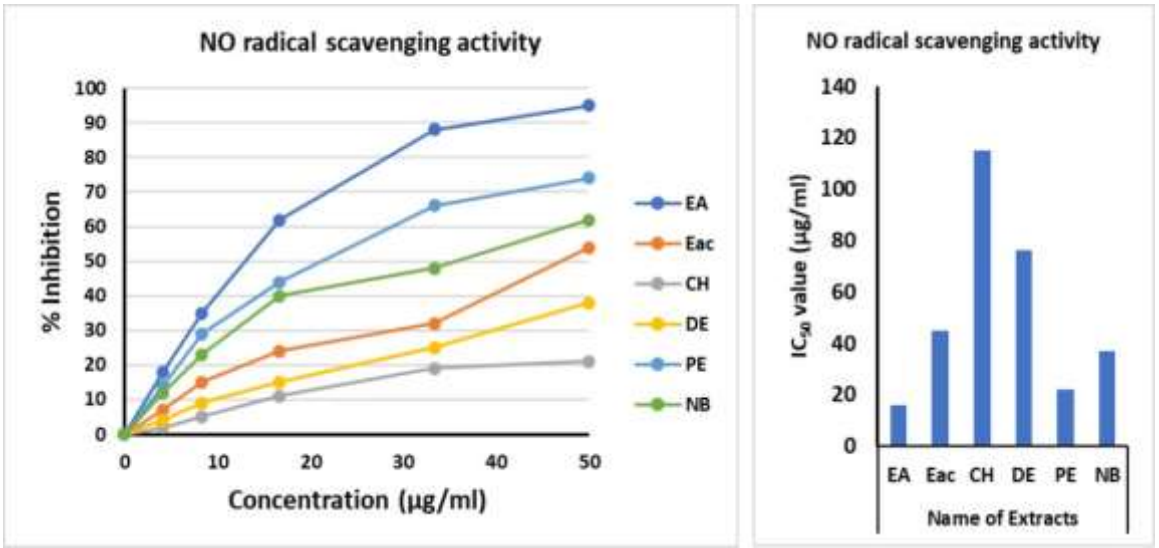

Fig. 4: Nitric oxide radical scavenging activity of various extracts of *Leucas aspera.*

n=3, Values are Mean ± S.D.

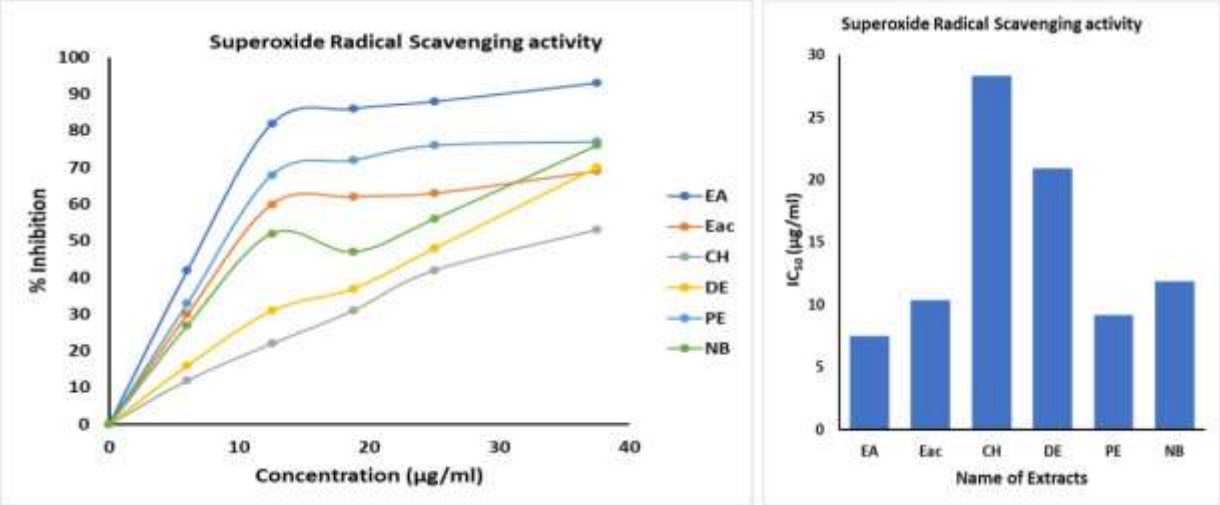

Fig. 5: Super oxide radical scavenging activity of various extracts of *Leucas aspera.*

n=3, Values are Mean ± S.D.

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