RESEARCH ARTICLE

In vitro antioxidant potential of leaf extracts of the endemic plant *Becolepsis nervosa* Decne. ex. Moq. (Periplocaceae)

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ABSTRACT

The aim of the present study was to investigate the antioxidant activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *Becolepsis nervosa* leaf. Antioxidant activity was evaluated by using *in vitro* antoxidant assay models like, DPPH radical scavenging activity, superoxide radical scavenging activity, hydroxyl radical scavenging activity, ABTS radical cation scavenging activity and reducing power assay. All the antioxidant activities were compared with standard antioxidant such as ascorbic acid. Ethanol extract of the plant showed effective DPPH radical scavenging activity superoxide radical scavenging activity and reducing power assay. Ethyl acetate extract showed effective hydroxyl radical scavenging activity and ABTS assay. IC_{50} values of all the extracts of *B. nervosa* were found to be low than ascorbic acid in DPPH radical scavenging activity model and ABTS assay. In addition, total phenolic content (TPC) and total flavonoid content (TFC) of the extract were evaluated. It was observed that the ethyl acetate extract contained high level of phenolic content that might have accounted for the strong activity observed against the free radicals. The results obtained in this study showed that the leaves of *B. nervosa* have antioxidant properties which provde a basis for the traditional use of the plant.

Keywords: *Becolepsis nervosa*, *in vitro* antioxidant activity, phenolics and flavonoids.

INTRODUCTION

Free radical is a chemical species which contains an unpaired electron spinning on the peripheral layer around the nucleus. Free radicals generated from the oxygen are called Reactive oxygen species (ROS). In biological systems, during the cellular respiration, reactive oxygen species (ROS) like hydroxyl radical (.OH), superoxide anion (.O2−) and hydrogen peroxide (H_2O_2) are generated, as the natural consequence of oxidation reactions. [1] Reactive oxygen species (ROS) damage living cells causing lipid, protein, and DNA oxidation.[2] They are involved in the development of various diseases such as diabetes, rheumatic disorders,[3] aging, cancer, cardiovascular or neurodegenerative disorders [4,1], malaria and gastric ulcer.[5]

Antioxidants neutralize free radicals by donating one of their own electrons. They act as scavengers and play the housekeeper's role by mopping up free radicals. They can greatly reduce the damage by neutralizing the free radicals before they can attack the cells and prevent damage to lipids, proteins, enzymes, carbohydrates and DNA.[6] Antioxidants can be classified into two major classes i.e., enzymatic and non-enzymatic. The enzymatic antioxidants are produced endogenously which include superoxide dismutase, catalase, and glutathione peroxidase. The non-enzymatic antioxidants include tocopherols, carotenoids, ascorbic acid, flavonoids and tannins which are obtained from natural plant sources.[7] Although the human body produces antioxidant enzymes to neutralize free radicals,[8] a diet rich in edible antioxidants is recommended to assist the human body to protect itself from free radicals.

Many plants from nature possess antioxidant and antimicrobial activities. The interest in searching natural antioxidants has recently increased. These natural products could be used in food or in medicinal materials to replace synthetic antioxidants which are about to be restricted owing to their side effects such

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as carcinogenesis.[5] Many medicinal plants contain large amounts of antioxidants, such as polyphenols, which can play an important role in adsorbing and neutralizing free radicals, in quenching singlet and triplet oxygen, or in decomposing peroxides.

The compounds that are responsible of antioxidant activity could be used for the prevention and treatment of free radical-related disorders.[9] Indeed, the consumption of antioxidants prevents different diseases such as neurological degeneration, inflammatory disorders, coronary diseases, aging and cancers.[10] Hence, the studies on natural antioxidants have gained increasingly greater importance. A large number of different plants have been studied as new sources of natural antioxidants.[11-14]

Becolepsis nervosa is one of the medicinally important plants belonging to the family Periplocaceae. This plant is endemic to Nilgiri Biosphere Reserve, Western Ghats, Tamil Nadu. For the first time, we report here the antioxidant properties of leaf extracts of *Becolepsis nervosa*. To our knowledge, there are no previous reports concerning *in vitro* antioxidant activities of this plant part extracts. The purposes of this study were to determine the total phenolic and the total flavonoid contents and to evaluate a positive relationship between the antioxidant activity and the totoal phenolic and flavonoid content of the various solvent of *Becolepsis nervosa* leaf extracts.

MATERIALS AND METHODS

The leaves of *Becolepsis nervosa* Decne. ex. Moq. were freshly collected from Kothagiri, Nilgiri Biosphere Reserve, Western Ghats, Tamil Nadu. The plant specimen was identified and authenticated in Botanical Survey of India, Southern Circle, Coimbatore, Tamil Nadu, India. A voucher specimen was deposited in Ethnopharmacology unit, Research department of Botany, V. O. Chidambaram College, Tuticorin, Tamil Nadu.

Preparation of Plant extract

The leaves were shade dried, powdered and extracted with petroleum ether (BNPE), benzene (BNB), ethyl acetate (BNEA), methanol (BNM) and ethanol (BNE) respectively using cold extraction in shaker for 48h at room temperature. The extracts were concentrated in a rotary evaporator to obtain concentrated extracts which were then used for the estimation of total phenolic, flavonoid and the assessment of antioxidant activity.

Estimation of Total Phenolics

Total phenolic contents was estimated using Folin-Ciocalteau reagent based assay as previously described[15] with little modification. To 1mL of each extract (100µg/mL) in methanol, 5mL of Folin-Ciocalteau reagent (dluted ten-fold) and 4mL (75g/L) of Na_2CO_3 were added. The mixture was allowed to stand at 20ºC for 30min and the absorbance of the developed colour was recorded at 765nm using UV-VIS spectrophotometer. 1mL aliquots of 20, 40, 60, 80, 100 µg/mL methanolic gallic acid solutions were used as standard for calibration curve. The absorbance of solution was compared with gallic acid calibration curve. The total phenolic content was expressed as gallic acid equivalents (GAE g/100g dry weight of extract).

Estimation of Flavonoids

The total flavonoid content was determined according to Eom *et al*. [16] An aliquot of 0.5 mL of sample (1mg/mL) was mixed with 0.1mL of 10% aluminium chloride and 0.1mL of potassium acetate (1M). In this mixture, 4.3mL of 80% methanol was added to make 5mL volume. The mixture was vortexted and the absorbance was measured spectrophotometrically at 415nm. The value of optical density was used to calculate the total flavonoid content present in the sample.

DPPH radical scavenging activity

The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant component. This method is based on the reduction of DPPH in methanol solution in the

presence of a hydrogen donating antioxidant due to the formation of the non radical form DPPH-H.[17]

The free radical scavenging activity of all the extracts was evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) according to the previously reported method.[17] Briefly, an 0.1mM solution of DPPH in methanol was prepared, and 1mL of this solution was added to 3 ml of the solution of all extracts in methanol at different concentration (50,100,200,400 & 800µg/mL).The mixtures were shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance was measured at 517 nm using a UV-VIS spectrophotometer (Genesys 10S UV: Thermo electron corporation). Ascorbic acid was used as the reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability to scavenging the DPPH radical was calculated by using the following formula.

DPPH scavenging effect (% inhibition) = $\{(A_0 - A_1)/A_0)*100\}$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged

Hydroxyl radical scavenging activity

The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell.[18] Stock solutions of EDTA (1mM), FeCl3 (10mM), Ascorbic Acid (1mM), H_2O_2 (10mM) and Deoxyribose (10 mM), were prepared in distilled deionized water.

The assay was performed by adding 0.1mL EDTA, 0.01mL of $FeCl_3$,0.1mL H_2O_2, 0.36mL of deoxyribose, 1.0mL of the extract of different concentration (50,100,200,400 &800µg/mL)dissolved in distilled water,0.33mL of phosphate buffer (50mM , pH 7.9), 0.1mL of ascorbic acid in sequence . The mixture was then incubated at 37^{0}C for 1 hour. 1.0mL portion of the incubated mixture was mixed with 1.0mL of 10%TCA and 1.0mL of 0.5% TBA (in 0.025M NaOH containing 0.025% BHA) to develop the pink chromogen measured at 532nm. The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula.

Superoxide radical scavenging activity

The superoxide anion scavenging activity was measured as described by Srinivasan *et al.*[19] The superoxide anion radicals were generated in 3.0 ml of Tris – HCL buffer (16 mM, P^H 8.0), containing 0.5 mL of NBT (0.3mM), 0.5 ml NADH (0.936mM) solution, 1.0 mL extract of different concentration (50,100,200,400 & 800µg/mL), and 0.5 mL Tris – HCl buffer (16mM, P^H 8.0). The reaction was started by adding 0.5 mL PMS solution (0.12mM) to the mixture, incubated at 25^{o}C for 5 min and the absorbance was measured at 560 nm against a blank sample, ascorbic acid. The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula

Antioxidant activity by radical cation (ABTS. +)

ABTS assay was based on the slightly modified method of Huang *et al.*[20] ABTS radical cation (ABTS+) was produced by reacting 7mM ABTS solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS + Solution were diluted with ethanol to an absorbance of 0.70+0.02 at 734 nm. After addition of 100µL of sample or trolox standard to 3.9 mL of diluted ABTS+ solution, absorbance was measured at 734 nm by Genesys 10S UV-VIS (Thermo scientific) exactly after 6 minutes. Results were expressed as trolox equivalent antioxidant capacity (TEAC). The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula.

Reducing power

The reducing power of the extract was determined by the method of Kumar and Hemalatha.[21] 1.0 mL of solution containing 50,100,200,400 &800µg/mL of extract was mixed with sodium phosphate buffer (5.0 mL, 0.2 M, pH6.6)

and potassium ferricyanide (5.0 mL, 1.0%): The mixture was incubated at 50°C for 20 minutes. Then 5mL of 10% trichloroacetic acid was added and centrifuged at 980 g (10 minutes at 5°C) in a refrigerator centrifuge. The upper layer of the solution (5.0 mL) was diluted with 5.0 mL of distilled water and ferric chloride and absorbance read at 700 nm. The experiment was performed thrice and results were averaged.

Statistical analysis

Antioxidant activities like DPPH radical scavenging activity, hydroxyl radical scavenging activity, superoxide radical activity, ABTS radical cation scavenging activity and reducing powers were estimated in triplicate determinations. Data were analyzed using the statistical analysis system SPSS (SPSS software for windows release 17.5; SPSS Inc., Chicago IL, USA) Estimates of mean, standard error for aforesaid parameters were calculated.

RESULT AND DISCUSSION

Table 1 shows the presence of total phenolic and total flavonoid contents in various extracts of *B. nervosa* leaf. Phenolic contents expressed as gallic acid equivalens varied from 0.26 to o.55 g 100 g^{-1}. The phenolic contents of the extracts decreased in the following order BNEA > BNP > BNM > BNB > BNE. The BNEA possessed the highest total phenolic contents (0.5458 g GAE/100g) while BNE comprised of lowest total phenolics content (0.2611 g GAE/100g) extract. The total flavonoid content in the extracts was expressed as g/100g quercetin equivalent. The methanol extract has more flavonoid content (0.5588 g 100 g^{-1}) than ethanol extract (0.2588 g 100 g^{-1}). In the present study, the phenolic content was very less in ethanol extrac when compared to other solvents. Our result was supported by.[22] They reported that ethanol and methanol showed lowest phenolic conent.

Polyphenolic flavonoids are occurring ubiquitously in food and medicinal plants. They occur as glycosides and contain several phenolics hydroxyl groups. Many flavonoids are found to be strong antioxidants effectively scavenging the reactive oxygen species because of their phenolics hydroxyl groups. Phenolic compounds possess a wide specrum of biological effects including antioxidant and free radical scavenging.[23] Results obtained from experimental data revealed that there might be correlation between total phenolic and antoxidant capacity of different extracts of *B. nervosa.* However, some literature demonstrated that antioxidant was not soley dependent on phenolic content but it may be due to other phytoconstituents as tannins, triterpenoid or combine effect of them.

Free radicals are known to play a definite role in a wide variety of pathological manifestations. Antioxidants fight against free radicals and protect us from various diseases. They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defence mechanisms.[24] In the present study, antioxidant capacity of various solvent of *B. nervosa* leaf extracts were examined using five different assays.

DPPH radical scavenging activity

1, 1 Diphenyl-2-picrylhydrazyl (DPPH) is a kind of stable organic free radical.[25] Compared with other methods, the DPPH assay has multiple advantages such as good stability, credible sensitivity, simplicity and feasibility (Ozcelik *et al.,* 2003).[26] In this assay, the radical scavenger present in the sample extract will decolorized the purple coloured DPPH solution to yellow due to the reduction of the stable DPPH radicals to diphenyl picrylhydrazine in the presence of hydrogen donating antioxidant.[27]

The results of the DPPH radical scavenging activity of leaf extracts of *B. nervosa* is shown in Fig 1. IC_{50} is often used to express the amount or concentration of extracts needed to scavenge 50% of the free radicals. IC_{50} value is inversely proportional to the scavenging activity of leaf extracts. Smaller IC_{50} corresponds to a higher antioxidant activity of the plant extract.[28] The present study revealed that the ethanol extract of leaf had the highest scavenging activity with low IC_{50} value of 17.24 µg/mL while benzene leaf extract had the

lowest scavenging activity with high IC_{50} value of 23.18 µg/mL. The IC_{50} value of all the extracts was found to be lower than those of ascorbic acid (26.16 µg/mL). It is because of the presence of phenolic compounds in these fractions. This showed that the *B. nervosa* leaf extracts contained high amount of radical scavenging compounds with proton donating ability. The different concentrations of various extracts of *B. nervosa* showed antioxidant activities in a dose dependent manner.

Superoxide radical scavenging activity

Superoxide radical is considered a major biological source of reactive oxygen species.[29] Although superoxide anion is a weak oxidant, it gives rise to generation of powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress.[30] Superoxide anions can be generated either form a membrane-associated NADPH oxidase [31] or as a result of electron leakage from mitochondrial electron transport.[32]

In the present study, the superoxide radical scavenging effect of different extracts was compared with the same dose of ascorbic acid ranging from 50 to 800µg/ml. The IC_{50} values in superoxide scavenging activities were in the order of BNM > BNE > BNB > BNPE > BNEA. Lesser the IC_{50} value for an extract is considered to be associated with higher ability to donate hydrogen radical (i.e) antioxidant activity. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound under test.[33] When compared to ascorbic acid, the superoxide scavenging activity of the alcohol extracts (both methanol and ethanol) were found to be high (Fig. 2)

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 molecule found in living cells.[34] Hydroxyl radical scavenging capacity of an extract is directly related to its antioxidant activity. Among the reactive oxygen species, the hydroxyl radical is the most reactive and induces severe damage to the adjacent biomolecules. [35]

The hydroxyl radical scavenging activity of various solvent extracts of *B. nervosa* was in the order of BNEA > BNE > BNM > BNPE > BNB respectively (Fig. 3). The IC_{50} values of various solvent leaf extracts were 18.27 µg/mL, 21.41 µg/mL, 22.67 µg/mL, 23.41 µg/mL 24.22 µg/mL and ascorbic acid with IC_{50} value of 20.16 µg/mL (Fig.6). Though the antioxidant potential of various solvent extracts was found to be lower than those of ascorbic acid, the present study revealed that ethyl acetate extract has prominent hydroxyl radical scavenging activity than the other solvent extract.

ABTS radical assay

ABTS radical scavenging assay involves a method that generates a blue/ green $ABTS^{+}$ chromophore via the reaction of ABTS and potassium persulfate. The ABTS radical cation is generated by the oxidation of ABTS with potassium persulfate, its reduction in the presence of hydrogen donating antioxidants is measured.

Differences for the ABTS radical cation scavenging capacities of various solvents and reference compound (ascorbic acid) was recorded in this study (Fig.4). Among various solvent extracts of *B. nervosa* leaf, BNEA possessed the highest ABTS radical scavenging activity (19.33µg/mL), while BNB showed the lowest ABTS radical scavenging activity (27.13 µg/mL). When compared to ascorbic acid, all the extracts possessed strong ABTS scavenging activity. Our observation is supported by other researchers.[36]

Reducing power assay

The reducing capacity of the extract is another significant indicator of antioxidant activity. In the reducing power assay, the presence of antioxidant in the reduction of Fe^{3+} to Fe^{2+} by donating an electron. Increasing absorbance at 700nm indicates an increase in reducing ability.[37] The antioxidant present in the extracts of *B. nervosa* caused their reduction of Fe^{3+}/

ferricyanide complex to the ferrous form and thus proved the reducing power. Figure 5 shows the dose response for the reducing power of various solvent extracts (50-800µg/ml) from *B. nervosa.* It was found that the reducing power of plant extract increases with increasing concentration of each solvent. The ranking order for reducing power was BNM > BNE > BNPE > BNB > BNEA respectively. Significantly higher reducing power was (0.436±0.61 at 800µg/ml) evident in BNM extract. Similar result was recorded by Jagadeeswari *et al.* [38] Previous reports suggested that the reducing properties have been shown to exert antioxidant action by donating of a hydrogen atom to break the free radical chain.[39]

CONCLUSION

In the present investigation, leaf extract of *B. nervosa* exhibited satisfactory scavenging effects on DPPH, ABTS, hydroxyl radicals, superoxide radicals and reducing power. It was observed that methanol extract of *B. nervosa* leaf showed concentration dependent free radical scavenging activity and this antioxidant effect may be due to higher content of phenolic and flavonoid compounds. However the exact compounds responsible for the antioxidant activity of *B. nervosa* are not clear. Therefore further work is necessary to isolate and characterize those constituents. From this study, it is concluded that, *B. nervosa* leaf extract as promising natural sources of antioxidants can be used in pharmaceutical fields for the prevention of free radical mediated diseases. However, pharmacological studies are suggested to confirm the antioxidant ability before going for commercialization. Further, detailed studies on isolation of phytoconstituents of the plant extracts are essential to characterize them as biological antioxidants.

Table 1: Total phenolic and flavonoid content of different extracts of *Baeolepsis nervosa* leaf.

S. No	Extracts	Total Phenolic Content ($g/100g^{-1}$)	Total Flavonoid Content ($g/100g^{-1}$)
1	Petroleum ether	0.4736	-
2	Benzene	0.5458	-
3	Ethyl acetate	0.3278	-
4	Methanol	0.4	0.5588
5	Ethanol	0.2611	0.2588

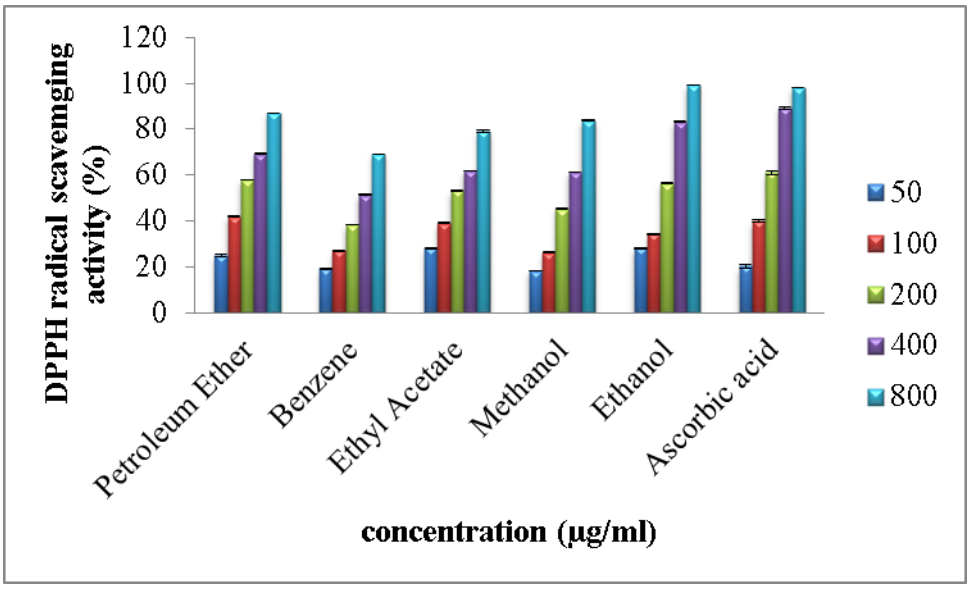

Fig. 1: DPPH radical scavenging activity of different extracts of *Baeolepsis nervosa* leaf.

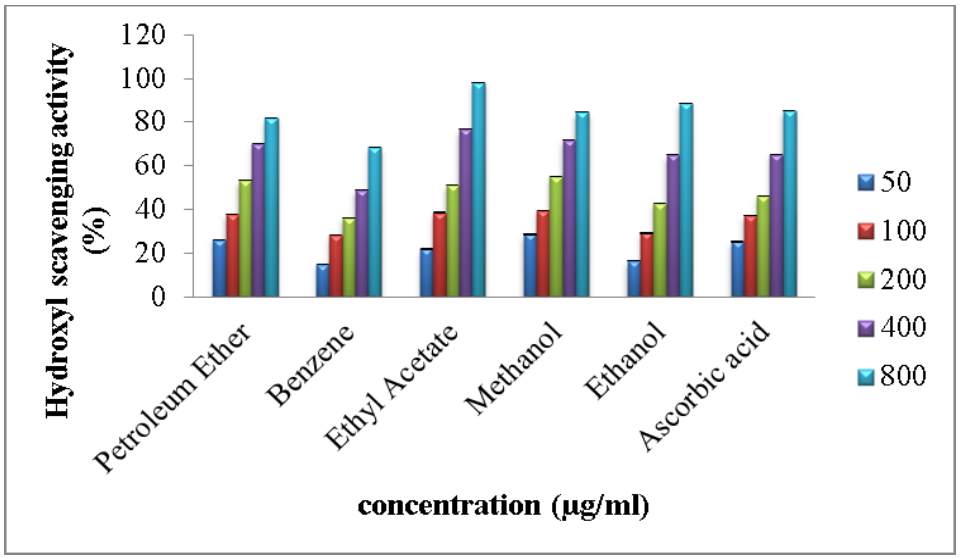

Fig. 2: Hydroxyl radical scavenging activity of different extracts of *Baeolepsis nervosa* leaf.

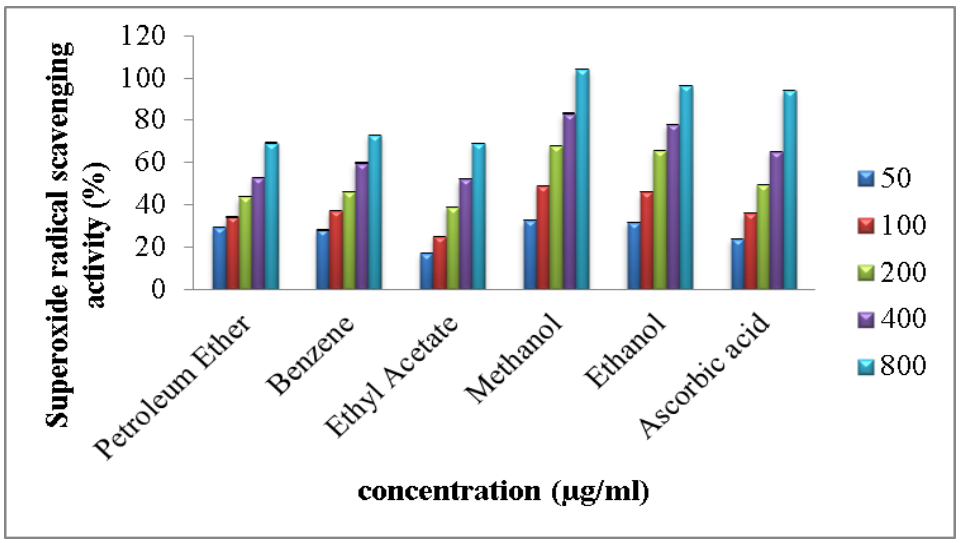

Fig. 3: Superoxide radical scavenging activity of different extracts of *Baeolepsis nervosa* leaf.

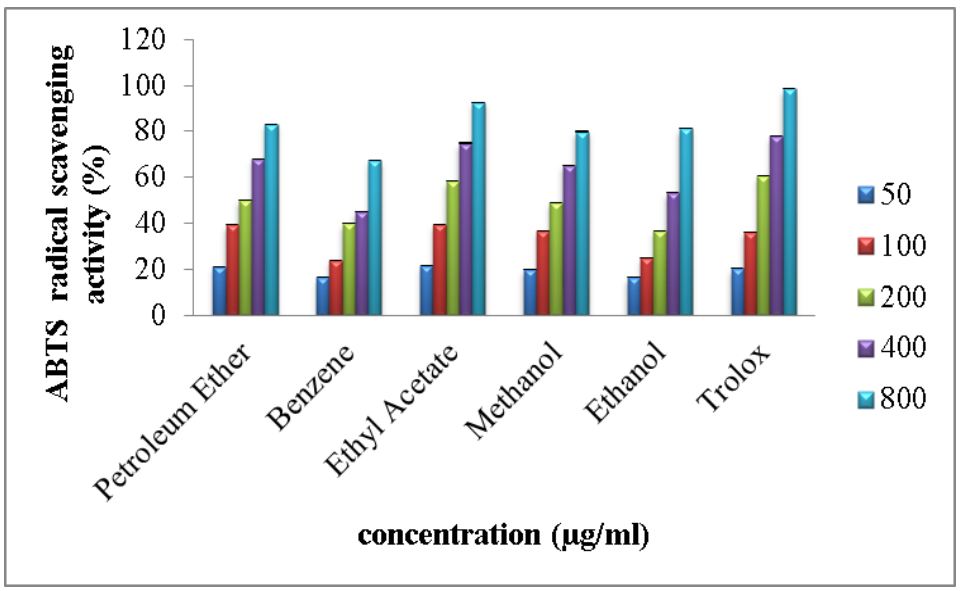

Fig. 4: ABTS radical cation scavenging activity of different extracts of *Baeolepsis nervosa* leaf.

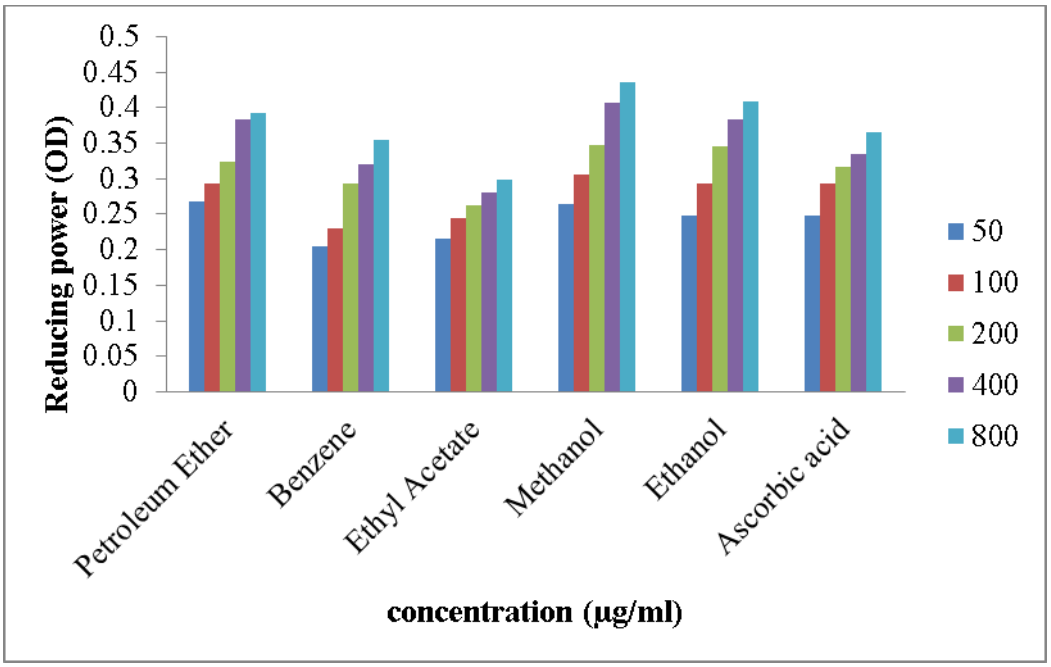

Fig. 5: Reducing power ability of different extracts of *Baeolepsis nervosa* leaf.

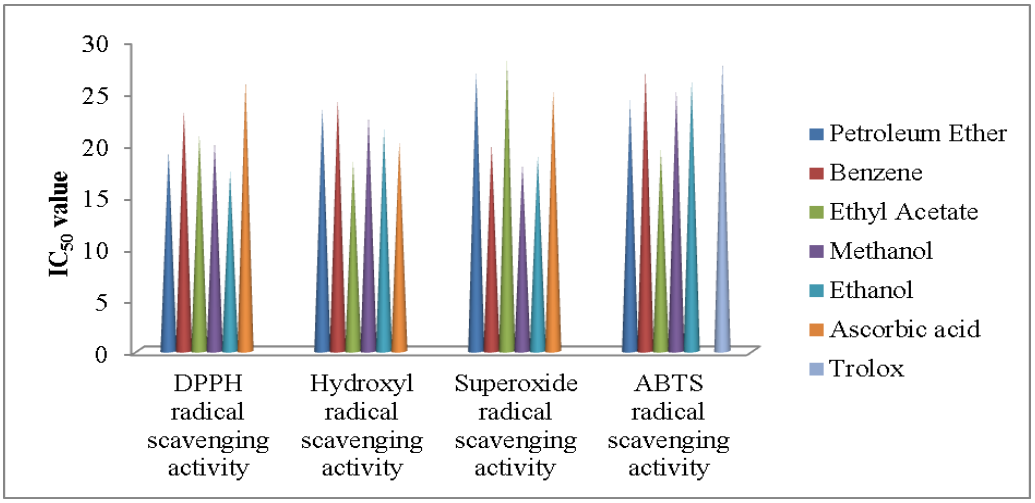

Fig. 6: IC_{50} values of different extracts of *Baeolepsis nervosa* leaf.

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