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SCAVENGING EFFECT OF STEM EXTRACTS OF *NIEBUHRIA APETALA* DUNN ON FREE RADICAL

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ABSTRACT

Extracts of stem of Niebuhria apetala Dunn was assessed for its antioxidant activity by in vitro methods. Antioxidant activity was studied using 1, 1- Diphenyl-2-Picrylhydrazyl (DPPH), Superoxide scavenging activity, Hydroxyl scavenging activity, ABTS radical scavenging and reducing power assay. Antioxidant activities compared with ascorbic acid as standard antioxidant. Results showed that the crude extracts exhibited significant DPPH, superoxide, hydroxyl, ABTS and reducing power assay. From results, it is concluded that the ethanol extract is the most potent antioxidant. This holds great promise for the use as a source of strong antioxidant compounds.

KEY WORDS

Free radicals, ROS, Oxidative stress, Antioxidant activity, Medicinal plant

INTRODUCTION

Generation of large amounts of free radicals, particularly ROS, and their high activity plays a significant role in the progression of a large number of diseases like inflammation, atherosclerosis, stroke, heart disease, diabetes mellitus, multiple sclerosis, cancer, Parkinson's disease, Alzheimer's disease, etc., [1, 2]. Therefore, immense interest has been newly focused on the normal foods, medicinal plants and phytochemicals due to their recognized abilities to scavenge free radicals [3, 4]. Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) and free radicals such as hydroxyl radical (-OH) and superoxide anion (O_2-), are produced as normal products of cellular metabolism. Fast production of free radicals can guide to oxidative damage to biomolecules and may result in disorders such as cancer, diabetes, asthma, cardiovascular diseases, inflammatory disease, neurodegenerative diseases and premature aging. Many medicinal plants contain large amounts of antioxidants, such as polyphenols, vitamin C, vitamin E, selenium, β-carotene, lycopene, lutein, and other carotenoids, which play important roles in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides.

Capparis species is also known as Caper plants. These are familiar as a potential cause of valuable nutrients and biochemical compounds with physiological function. The various biological activities include antibacterial, hepatoprotective, anthelmintic, antifungal, antidiabetic, anti-inflammatory, anti-cancer, and anti-hyperlipidemic. These actions as well as folk medicinal uses of Caper plants have been featured to the presence of functional bioactives, for example phenolic acids, flavonoids, alkaloids, natural sugars, vitamins, phytosterols, and organic acids [5]. Hence in the current study, the extracts of leaf and bark of *N. apetals* was investigated for their antioxidant activity

using DPPH radical scavenging activity, hydroxyl radical scavenging activity, superoxide scavenging activity, ABTS radical cation scavenging activity and reducing power assay.

MATERIALS AND METHODS

Collection of plants

The fresh plant parts of *Niebuhria apetala* (stem) were collected from Petchiparai, Western Ghats, Kanyakumari District, Tamil Nadu, India. The gathered samples were cut into small pieces and shade dried until the fracture is identical and even. The dried plant material was crushed or grinded by using a blender and separated to get uniform particles by using sieve No. 60. The final uniform powder was used for the extraction of active constituents of the plant material.

Preparation of extract

100 g of the coarse powder of *N. apetala* stem was extracted successively with 250 ml of alcoholic and organic solvents (Peroleum ether, Benzene, Ethyl acetate, Methanol and Ethanol) in a Soxhlet apparatus for 24 hrs. All the extracts were filtered through Whatman No. 41 filter paper discretely and all the extracts were contemplated in a rotary evaporator. All the concentrated extracts were subjected for *in vitro* antioxidant activity.

Antioxidant activity

DPPH radical scavenging activity

The DPPH is a constant free radical and is extensively used to measure the radical scavenging activity of antioxidant component. This process is based on the reduction of DPPH in methanol solution in the company of a hydrogen donating antioxidant due to the arrangement of the non-radical form DPPH-H [6]. Using 1, 1- diphenyl-2-picryl-hydrazyl (DPPH) the free radical scavenging action of all the extracts was assessed as per the previously reported process [6]. DPPH of 0.1 mM solution in methanol was prepared. 1 ml of this solution was poured into 3 ml of the solution at different concentrations (50, 100, 200, 400 and 800 µg/ml). The mixtures were shaken dynamically and allowed to stand at room temperature for 30 minutes. After that the absorbance was measured at 517 nm using a UV-VIS spectrophotometer (Genesys 10s UV: Thermo electron corporation). Ascorbic acid was employed as the reference. The lesser absorbance values of reaction mixture identify higher free radical scavenging action. Using the subsequent formula, the ability to scavenge the DPPH radical was computed.

DPPH scavenging effect (% inhibition) = (A0 - A1) / A0 X 100

Where, A0 is the absorbance of the control and A1 is the absorbance of the test samples and reference. All the tests were repeated thrice, and the outcomes were averaged.

Hydroxyl radical scavenging activity

According to the adapted method of Halliwell *et al.* [7] the scavenging ability for hydroxyl radical was projected. Stock answers of $FeCl_3$ (10 mM), Ascorbic Acid (1 mM), EDTA (1 mM), H2O2 (10 mM) and Deoxyribose (10 mM) were put in distilled deionized water. The assay was executed by adding 0.1 ml EDTA, 0.1 ml H_2O_2, 0.01 ml of FeCl3, 0.36 ml of deoxyribose, 1.0 ml of the extract of diverse concentration (50, 100, 200, 400 & 800 µg/ml) dissolved in distilled water, 0.33 ml of phosphate buffer (50 mM, pH 7.9), 0.1 ml of ascorbic acid in sequence. The mixture was then incubated at 37°C for 1 hour. 1.0 ml of the incubated mixture was mixed with 1.0 ml of 10% TCA and 1.0 ml of 0.5% TBA (in 0.025 M NaOH containing 0.025% BHA) to develop the pink chromogen measured at 532 nm. The hydroxyl radical scavenging accomplishment of the extract is calculated as % inhibition of deoxyribose. By using the successive equation, the degradation is calculated

Hydroxyl radical scavenging activity = (A0 - A1) / A0 X 100

here, A0 is the absorbance of the control, A1 is the absorbance of the test samples and reference. All the tests were carried out in triplicates and the results were averaged.

Superoxide radical scavenging activity

The superoxide anion scavenging action was calculated as elucidated by Srinivasan *et al.* [8]. The superoxide anion radicals were made in 3.0 ml of Tris - HCl buffer (16 mM, pH 8.0) containing 0.5 ml of NBT (0.3 mM), 0.5 ml NADH (0.936 mM) solution, 1.0 ml extract of different concentrations (50, 100, 200, 400 & 800 µg/ml) and 0.5 ml Tris - HCl buffer (16 mM, pH 8.0). The reaction was started by adding 0.5 ml PMS solution (0.12 mM) to the mixture, incubated at 25°C for 5 min and the absorbance was estimated at 560nm against a blank sample, ascorbic acid. The percentage inhibition was determined by using the following equation

Superoxide radical scavenging activity = (A0 - A1) / A0 X 100

where, A0 is the absorbance of the control and A1 is the absorbance of the test samples and reference. All the tests were achieved in triplicates and the results were averaged.

Antioxidant Activity by Radical Cation (ABTS+)

ABTS assay was supported on the slightly modified technique of Huang *et al.* [9]. By reacting 7 mM ABTS solution with 2.45 mM potassium persulphate, ABTS radical cation (ABTS+) was prepared. This mixture is permitted to be in the dark at room temperature for 12-16 hrs previous to use. With ethanol to an absorbance of 0.70 + 0.02 at 734 nm the ABTS+ solution was added. Following this trolox standard to 3.9 ml of diluted ABTS+ solution or addition of 100 µL of sample, absorbance was calculated at 734 nm by Genesys 10S UV-VIS (Thermo scientific) accurately after 6 minutes. Results were expressed as trolox equivalent antioxidant capacity (TEAC).

ABTS radical cation activity = (A0 - A1) / A0 X 100

where, A0 is the absorbance of the control and A1 is the absorbance of the test samples and reference. All the tests were repeated thrice, and the end results were averaged.

Reducing Power

The reducing power of the extract was established by the method of Kumar and Hemalatha [10]. 1.0 ml of solution containing 50, 100, 200, 400 & 800 µg/ml of extract was mixed up with sodium phosphate buffer (5.0 ml, 0.2 M, pH 6.6) 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 refrigerated centrifuge. Using 5.0 ml of distilled water the upper layer of the solution (5.0 ml) was diluted and ferric chloride and absorbance is read at 700 nm. The experiment was performed thrice, and results were averaged.

RESULTS AND DISCUSSION

Plants produce a diverse array of bioactive molecules, making them rich sources of diverse type of medicines. Most of the bioactive molecules have probably evolved as chemical defence against predation or infection. Out of the 265000 species of flowering plants identified on planet earth so far, only 0.5% of them have been studied in detail for chemical composition and medicinal principles. The radical scavenging activities of stem extracts of *N. apetala* were tested using the 'stable' free radical, DPPH. DPPH has the benefit of not affected by certain side reactions, for example metal ion chelating and enzyme inhibition. This is in contrast to the laboratory generated free radicals such as the hydroxyl radical and superoxide anion [11, 12]. DPPH radical scavenging is regarded to be a good *in vitro* model generally used to assess antioxidant efficacy within a very short time. DPPH disappears, in its radical form, on reduction by an antioxidant compound or a radical species, to become a stable diamagnetic molecule follow-on in the colour change from purple to yellow, due to the formation of diphenyl picryl hydrazine (DPPH), which could be taken as an indication of the hydrogen donating ability of the tested samples [13, 14]. Figure 1 shows free radical scavenging activity of the crude extracts at different concentrations. The radical scavenging activity of methanol extract increased with increasing concentrations, with 29.16%, 43.16%, 67.97%, 84.84% and 116.56% scavenging activity for 50, 100,200,400 & 800 mg/ml extract, respectively (Figure 1). The IC_{50} value was found to be 28.22 mg/ml (Table 1). These results indicated that the ethanol extract exhibited the ability to quench the DPPH radical, which indicated that extract was good antioxidant with radical scavenging activity.

Figure 1: Effect of different solvent extract of *N. apetala* stem on DPPH assay

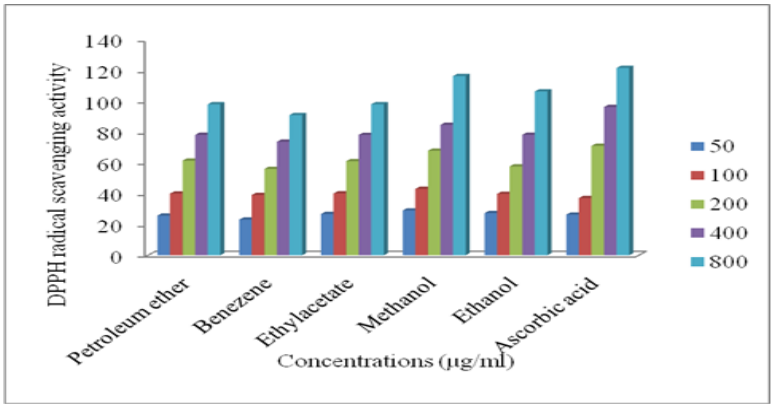
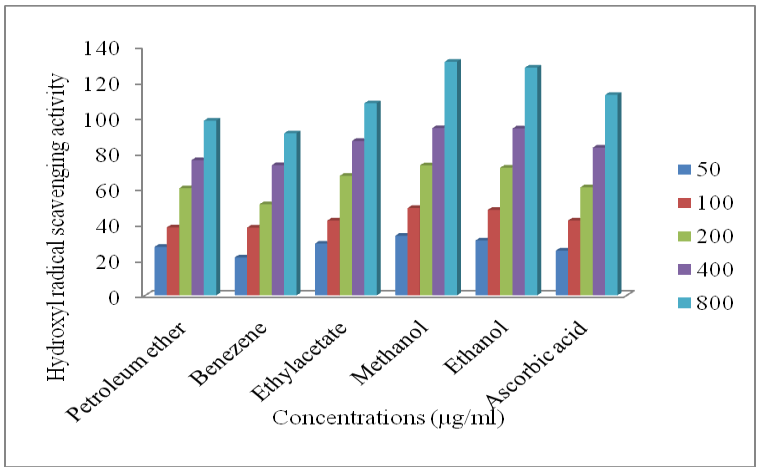


Table 1: IC_{50} values of different solvent extracts of the stem extracts of *N. apetala*

Solvents	IC_{50} (µg/ml)			
	DPPH	Hydroxyl	ABTS	Superoxide anion
P.ether	22.94	23.84	27.33	26.13
Benzene	21.73	20.16	29.08	28.16
Ethyl acetate	23.16	26.22	29.16	30.88
Methanol	28.22	31.16	28.16	38.27
Ethanol	26.84	30.26	28.93	36.37
Ascorbic acid	29.16	28.17	-	30.16
Trolox	-	-	30.22	-

Hydroxyl radical scavenging revealed that the methanol extract of *N. apetala* (IC_{50}: 31.16 mg/ml) scavenged free radicals compared to other extracts. Ascorbic acid (IC_{50}: 28.17 mg/ml) (Table 1) however exhibited strong antioxidant activity compared to the extracts (figure 2). Hydroxyl radicals are one of the quick indicators of the lipid peroxidation process by abstracting hydrogen atom from unsaturated fatty acids or simply auto-oxidation of polyunsaturated fatty acids found primarily in membranes [15]. Scavenging of OH- is an essential antioxidant activity, for the reason that it has very high reactivity, which can easily cross the cell membranes at specific sites and reacts with the most part of the biomolecules and furthermore cause tissue damage and cell death. Thus, removing OH-is very important task for the protection of living systems [16].

Figure 2: Effect of different solvent extract of *N. apetala* stem on Hydroxyl assay



The ABTS assay is supported on the inhibition of the absorbance of radical cation ABTS, which has a typical long wave length absorption spectrum. The ABTS chemistry engages the direct generation of ABTS+ radical mono cation with no association of any intermediary radical. In view of the fact that it is a decolourization assay, the radical cation is performed previously to addition of antioxidant test system, more willingly than the generation of the radical taking place commonly in the presence of antioxidant. The activity was found to be increased in a dose-dependent manner from 21.84% to 106.24 % at a concentration of 50-800 mg/ml. The ethanol extract exhibited an IC_{50} value of 28.93 mg/ml (Table 1). Therefore, the ABTS radical scavenging activity of ethanol extract indicates its ability to scavenge free radicals, thereby preventing lipid oxidation via a chain-breaking reaction.

The result revealed for superoxide radical scavenging assay is figure 4. Superoxide scavenging activity of *N. apetala* stem was increased markedly with increased concentrations of the extract. Thus, higher inhibitory effects of the extract on superoxide anion formation noted here in possibly renered its promising antioxidant potential. The half inhibition concentration IC_{50} value of stem methanol extract of *N. apetala* was 38.27 mg/ml (Table 1). Superoxide anions are the most common free radicals formed *in vivo* and are generated in a variety of biological systems and the concentration of superoxide anions increases under conditions of oxidative stress [17] (Lee *et al.*, 2002). It was therefore proposed to

measure the comparative interceptive ability of the methanol extracts to scavenge the superoxide radical.

Reducing power assay measures the electron-donating capacity of an antioxidant [18, 19]. In this assay, depending on the reducing power of each compound, the yellow 152 colours of the test solution changes to various shades of green and blue. Presence of reducers causes the conversion of the Fe3+/ferricyanide complex to the ferrous form which may serve as a significant indicator of its antioxidant capacity [12]. Figure 5 presents the reductive capabilities of the methanol extract of *N. apetala*. In the concentration range investigated, all the extracts demonstrated reducing power that increased linearly with concentration. At 50, 100,200,400 & 800 mg/ml, reducing power of ethanol extract were found to be 0.388, 0.421, 0.459, 0.516 & 0.567 respectively. The reducing power of the extract might be due to their hydrogen-donating ability. Possibly, ethanol extract contains high amounts of reductone, which could react with radicals to stabilize and terminate radical chain reactions.

Figure 3: Effect of different solvent extract of *N. apetala* stem on Superoxide anion assay

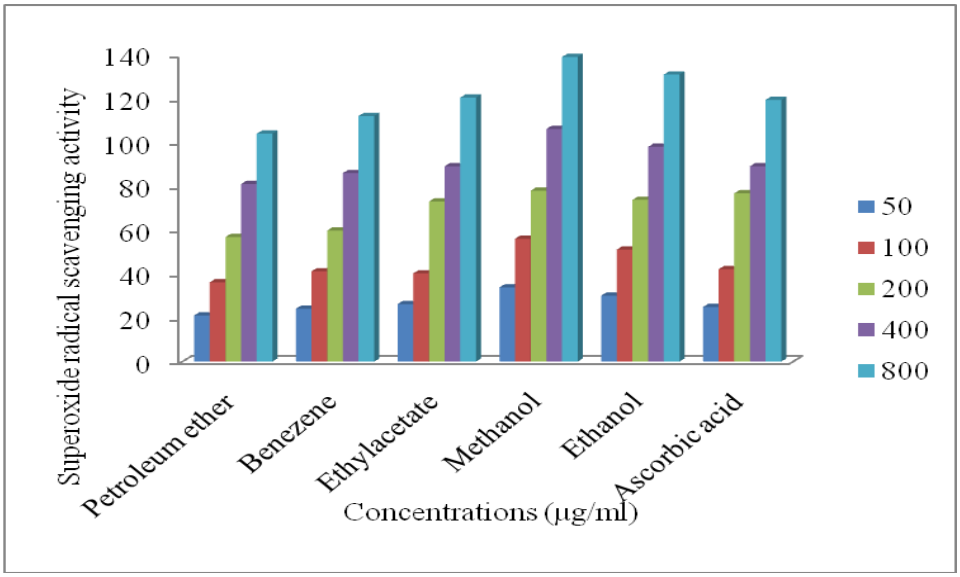


Figure 4: Effect of different solvent extract of *N. apetala* stem on ABTS assay

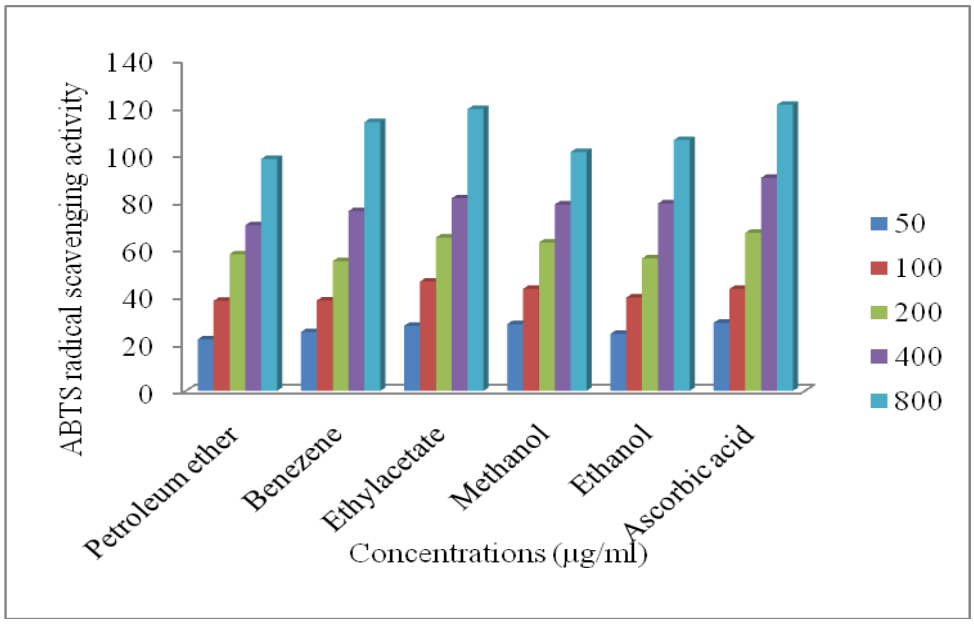
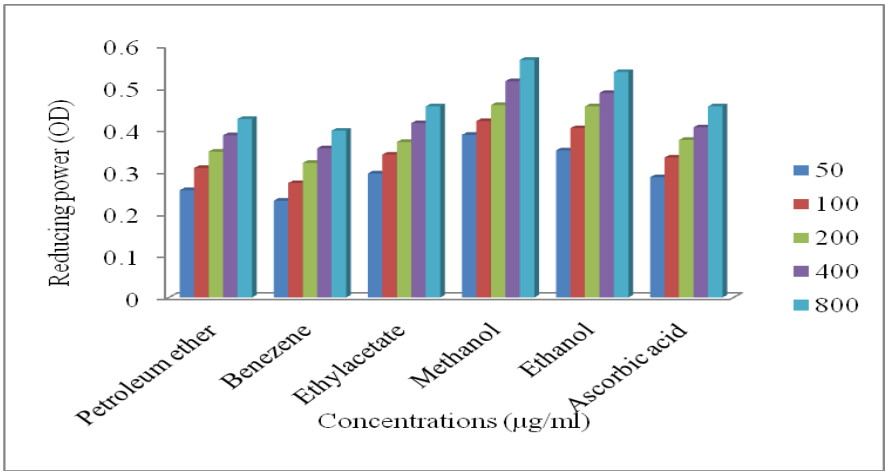


Figure 5: Effect of different solvent extract of *N. apetala* stem on reducing power assay



The results of the present study revealed that *N. apetala* stem extracts possessed potent free radical scavenging ability in methanol and ethanol extracts. The activity observed may be attributed to the presence of phenolic and flavonoid contents in the methanol and ethanol extracts and further we conclude that this plant is a potential candidate for natural antioxidant.

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