IN VITRO ANTIOXIDANT ACTIVITY, TOTAL PHENOLIC AND TOTAL FLAVONOID CONTENTS OF AERIAL PART EXTRACTS OF *DAPHNIPHYLLUM NEILGHERRENSE* (WT.) ROSENTH

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ABSTRACT

The aim of this study was to screen various solvent extract of aerial part of *Daphniphyllum neilgherrense* to display potent antioxidant activity *in vitro*, total phenolic and total flavonoid contents in order to find possible sources of future novel antioxidants in food and pharmaceutical formulations. The total phenolic content of the extracts was determined, spectrophotometrically according to the folin–ciocalteu procedure. The total flavonoid content of extracts was determined by aluminium chloride spectrophotometric assay. Antioxidant activity was studied using 1,1-Diphenyl-2-picrylhydrazyl (DPPH), hydroxyl, superoxide and 2, 2´azinobis (3-ethtylbenzthiazoline-6-sulphonic acid) (ABTS) cation scavenging activity and reducing power assay. Results showed that methanol and ethanol extract of *Daphniphyllum neilgherrense* aerial part exhibited significant, DPPH, hydroxyl, superoxide and ABTS radical cation scavenging activity. From the results, it is concluded that phenolic and flavonoid present in the *Daphniphyllum neilgherrense* aerial part extract may be responsible for the activity.

Keywords: *Daphniphyllum neilgherrense*, Antioxidant, DPPH, Flavonoid, ABTS

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INTRODUCTION

Antioxidants are compounds that inhibit or set back the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. Exogenous chemical and endogenous metabolic processes in the human body or in food system might produce highly reactive free radicals, especially oxygen derived radicals which are capable of oxidizing biomolecules, resulting in cell death or tissue damage. Oxidative damage plays a significant pathological role in human diseases. 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, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, ageing, inflammatory response syndrome, respiratory diseases, liver diseases, cancer and AIDS (Halliwell & Gutteridge 1984; Halliwell & Gutteridge 1989; Pourmorad *et al*., 2006).

Many herbal plants contain antioxidant compounds and these compounds protect cells against the damaging effects of reactive oxygen species (ROS), such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite (Dasgupta 2004; David *et al*., 2004). Drugs from plant origin are relied upon by 80% of the world's population. In India, the use of herbal drugs is an important component of the traditional system of medicine. Knowing the fact that several diseases have been treated by the administration of plant extracts from medicinal plants (Borek 1997).

Daphniphyllum neilgherrense is a shrub or small tree found in Indo-Malaysian region. It is a type genus of the family Daphniphyllaceae. The plants related to the genus *Daphniphyllum* are reported to be used in folklore medicines in South-East Asia and Southern China for the treatment of various ailments. Many of the plants of this genus are used in the treatment of asthma, cough, rheumatism, inflammation, fever, fractures and snake bites (Kothiyal *et al*., 2011). Recently, few members of the genus become famous for their anti-tumour, antioxidant, anti-platelet aggregation, vasorelaxant and insecticidal properties (Zhen *et al.,* 2009). It also has some aesthetic and religious uses in this region (Avasthe *et al*., 2004). Over 200 *Daphniphyllum* alkaloids have been isolated from the different species of the genus which are biosynthesized from six molecules of mevalonic acid (Niwa *et al*., 1973).

The present investigation was aimed at evaluating the *in vitro* antioxidant potential of different solvent extracts of *Daphinphyllum neilgherrense*. The literature survey indicates that no reports are available regarding *in vitro* antioxidant activity of *Daphniphyllum neilgherrense*.

MATERIALS AND METHODS

The aerial parts of *Daphniphyllum neilgherrense* (Wt.) Rosenth were collected from Kothagiri, Nilgiri Biosphere Reserve, Western Ghats, Tamil Nadu, India. The collected samples were cut into small fragments and shade dried until the fracture is uniform and smooth. The dried plant material was granulated or powdered by using a blender and sieved 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 plant extract

The coarse powder (100g) of aerial part of *Daphniphyllum neilgherrense* was extracted successively with petroleum ether, benzene, ethyl acetate, methanol and ethanol, each 250 mL in a Soxhlet apparatus for 24 hrs. All the extracts were filtered through Whatman No.41 filter paper. All the extracts were concentrated in a rotary evaporator. The concentrated extracts were used for *in vitro* antioxidant activity. The methanol extract was used for the estimation of total phenolics and flavonoids.

Estimation of total phenolic content

Total phenolic contents were estimated using Folin-Ciocalteau reagent based assay as previously described (McDonald *et al*., 2001) with little modification. To 1mL of 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 flavonoids content was determined according to Eom *et al*., (2007). An aliquot of 0.5ml 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. This mixture was vortexed 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 (Shen *et al*., 2010).

The free radical scavenging activity of all the extracts was evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) according to the previously reported method (Shen *et al*., 2010). 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 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 *et al* (1987). Stock solutions of EDTA (1mM), $FeCl_3$ (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*., (2007). 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*., (2011). 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 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 & Hemalatha (2011). 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^{o}C for 20 minutes. Then 5mL of 10% trichloroacetic acid was added and centrifuged at 980 g (10 minutes at 5^{o}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

Total phenol and flavonoid content

The total phenolic content and total flavonoid content of *D. neilgherrense* aerial part methanol and ethanol extract were found to be 2.12 and 2.72 g $100g^{-1}$ and 1.90 and 2.42g 100 g^{-1} respectively.

DPPH radical scavenging activity

Figure 1 shows the scavenging effects of aerial part of *D. neilgherrense* on DPPH radical and were in the following order: methanol> ethanol> ethyl acetate> petroleum ether> benzene. The IC_{50} values of scavenging DPPH radicals for the methanol and ethanol extracts was 40.16 and 34.90 µg/mL respectively (Table-1). Through the antioxidant potential of methanol and ethanol extract was found to be high than those of ascorbic acid, the study revealed that methanol and ethanol extract have prominent antioxidant activity.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of different solvent extracts of *D. neilgherrense* aerial part can be ranked as ethanol>methanol>petroleum ether> benzene > ethyl acetate (Figure 2). All the extracts showed antioxidant activity in dose dependent manner at concentration 50-800µg/mL. The IC_{50} value of hydroxyl radical scavengeing activity for the ethanol and methanol was 38.41 and 36.88µg/mL respectively (Table 1). The markedly strong antioxidant response of ethanol and methanol in comparison with ascorbic acid might be helpful in characterizing the significant sources of natural antioxidant reaction.

Superoxide radical scavenging activity

The superoxide radical scavenging effect of different solvent extracts was compared with the same doses of ascorbic acid ranging from 50-800µg/mL. The scavenging activity for superoxide radical of various solvent extracts from *D. neilgherrense* aerial part was in the order of methanol>ethanol>petroleum ether> ethyl acetate>benzene respectively. The IC_{50} value of superoxide radical scavenging activity for the methanol and ethanol was 49.36 and 43.16 µg/mL respectively (Table 1).

ABTS radical cation scavenging activity

All the extracts of *D. neilgherrense* aerial part scavenged ABTS radical in a concentration dependent way (50-800µg/mL) (figure 4). Present results showed that the ABTS radical scavenging ability of samples can be ranked as methanol> ethanol >ethyl acetate >benzene > petroleum ether. The IC_{50} values of ABTS radical cation scavenging activity for the methanol and ethanol extract was 42.86 and 40.84µg/ml respectively (Table 1).

Reducing power activity

Figure 5 shows the dose response curves for the reducing power of all extracts (50-800µg/mL) from *D. neilgherrense*. It was found that the reducing power increased with concentration of each sample. The ranking order for reducing power was methanol> ethanol>ethyl acetate>benzene> petroleum ether.

DISCUSSION

Plant materials rich in phenolics are increasingly being used in the food industry because they retard oxidative degradation of lipids and improve the quality and nutritional value of food (Kahkonen *et al.*, 1999). Phenolic compounds are considered as secondary

Solvent	IC_{50} (µg/mL)			
	DPPH	Hydroxyl	Superoxide	ABTS
Petroleum ether	27.36	31.46	35.18	30.16
Benzene	25.94	28.24	33.16	32.92
Ethyl acetate	31.56	26.88	34.16	38.16
Methanol	40.16	36.88	49.36	42.86
Ethanol	34.90	38.41	43.16	40.84
Ascorbic acid	33.15	32.88	41.16	-
Trolox	-	-	-	37.24

Table 1: IC_{50} values of different solvent extracts of aerial part of *D. neilgherrense* *

All the values are mean by triplicate determines*

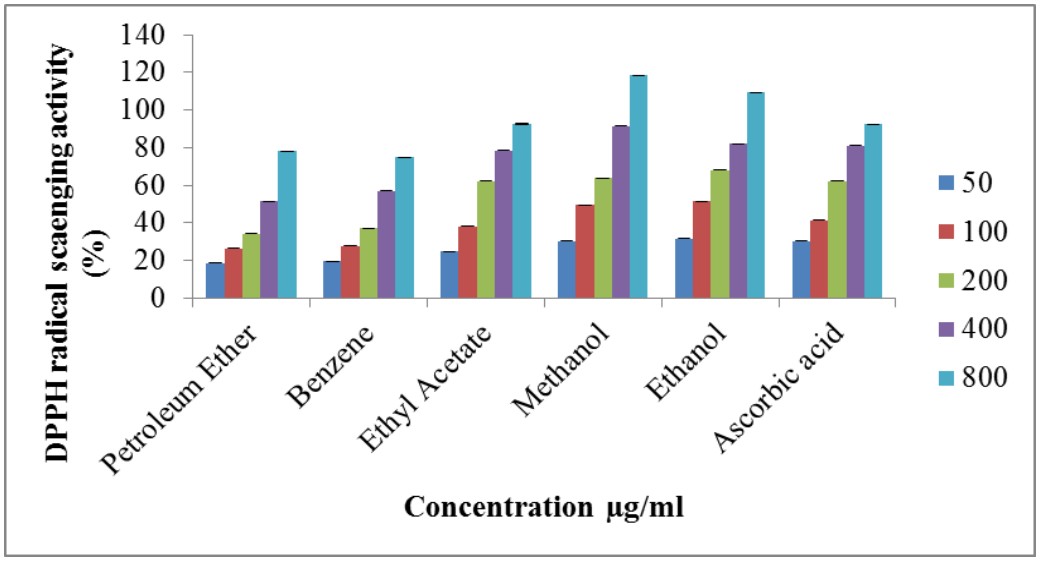

Fig 1: DPPH radical scavenging activity of different extracts of *D. neilgherrense* aerial part

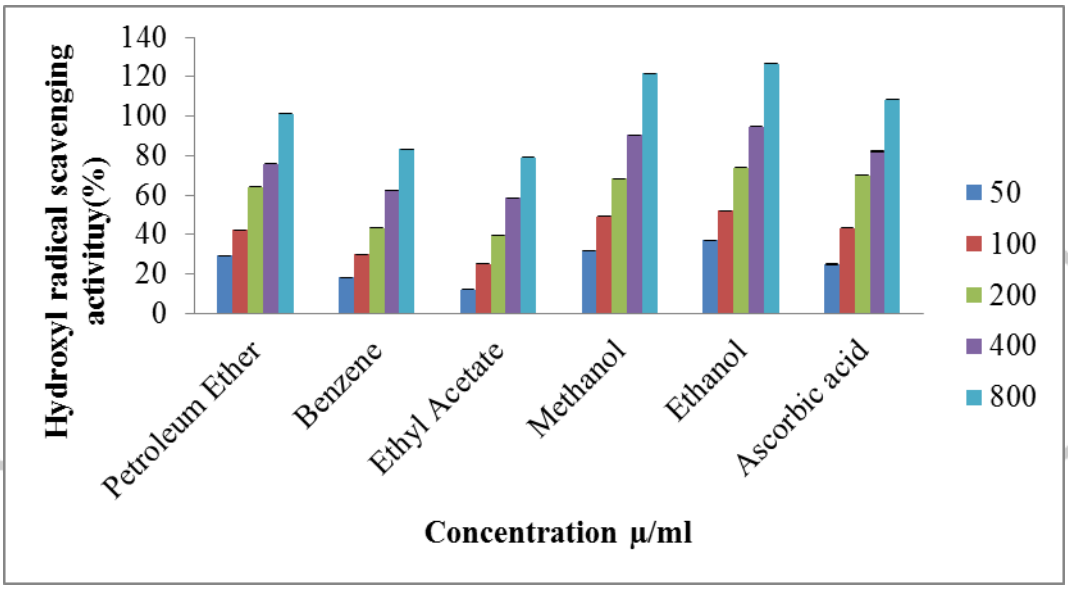

Fig 2: Hydroxyl radical activity of different extracts of *D. neilgherrense* aerial part

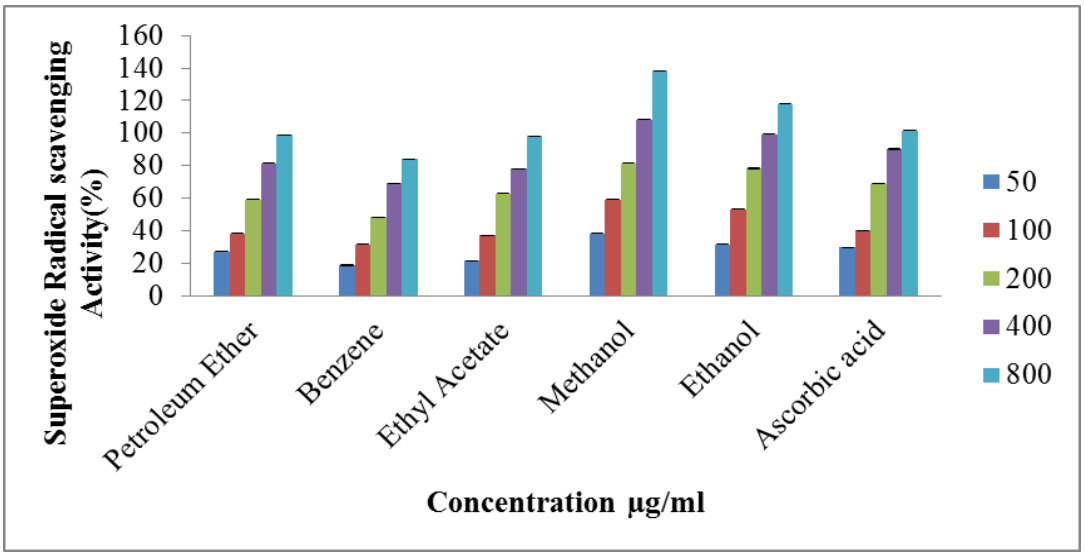

Fig 3: Superoxide radical scavenging activity of different extracts of *D. neilgherrense* aerial part

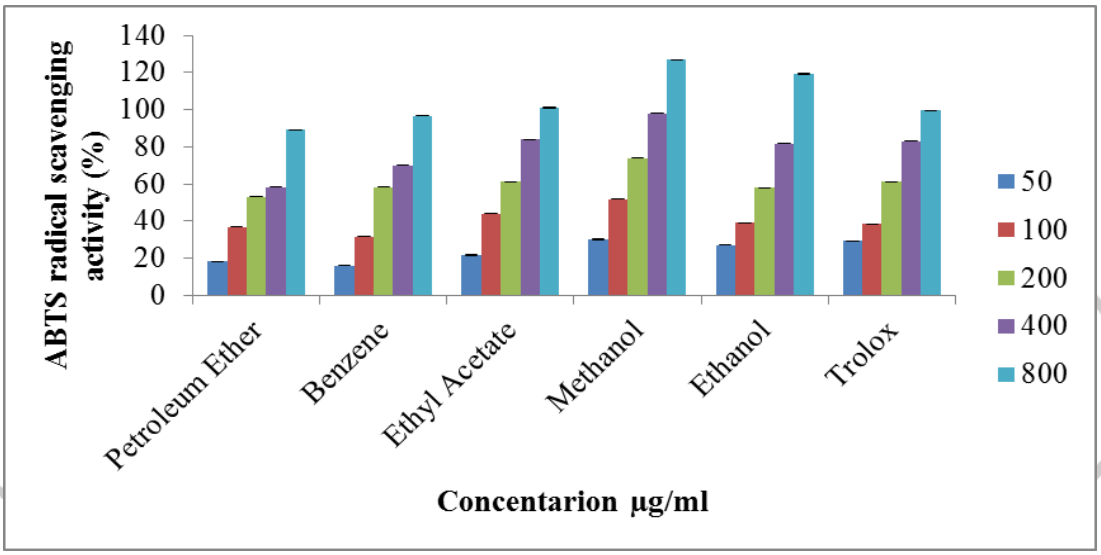

Fig 4: ABTS radical cation scavenging activity of different extracts of *D. neilgherrense* aerial part

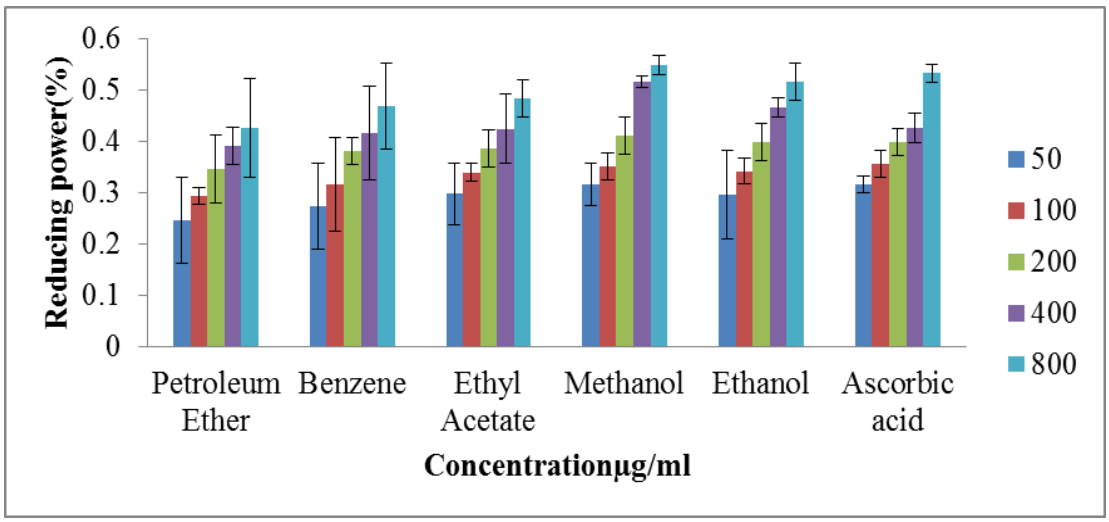

Fig 5: Reducing power ability of different extracts of *D. neilgherrense* aerial part.

metabolites and these phytochemical compounds derived from phenylalanine and tyrosine occur ubiquitously in plants and are diversified (Naczk & Shahidi, 2004). In the present study the methanol extract exhibited the highest total phenolic content. Phenolic compounds of plants are also very important because their hydroxyl groups confer scavenging ability.

Phenolic compounds of plants fall into several categories; chief among these are the flavonoids which have potent antioxidant activities. (Nunes *et al*., 2012) Flavonoids are naturally occurring in plants and are thought to have positive effects on human health. Studies on flavonoidic derivatives have shown a wide range of antibacterial, antiviral, anti-inflammatory, anticancer and antiallergic activities (Dicarlo *et al*., 1999; Montoro *et al.*, 2005). Flavonoids have been shown to be highly effective scavengers of most oxidising molecules, including singlet oxygen and various free radicals implicated in several diseases (Bravo, 1998). So comparable with the findings in the literature for other extracts of plant products (Sahreen *et al*., 2011) our results suggested that phenolics and flavonoids may be the major contributors for the antioxidant activity.

Several techniques have been used to determine the antioxidant activity *in vitro* in order to allow rapid screening of substances, since substances that have low antioxidant activity *in vitro*, will probably show little activity *in vivo* (Nunes *et al*., 2012). 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 mechanism (Umamaheswari & Chatterjee, 2008)

The electron donation ability of natural products can be measured by 2, 2'-diphenyl -1-picrylhydrazyl radical (DPPH) purple –coloured solution bleaching (Nunes *et al*., 2012). This method is based on scavenging of DPPH through the addition of a radical species or antioxidant that decolourizes the DPPH solution. The degree of colour change is proportional to the concentration and potency of the antioxidants. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound under test (Krishnaiah *et al*., 2011). In the present study among all the solvents tested, methanol and ethanol showed significantly higher inhibition percentage. Results of this study suggest that the plant extract contain phytochemical constituents that are capable of donating hydrogen to a free radical to scavenge the potential damage.

Hydroxyl radical is one of the potent reactive oxygen species in the biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell (Halliwell & Gutteridge, 1981, Khan *et al*., 2012). The hydroxyl radical is regarded as a detrimental species in pathophysiological processes and capable of damaging almost every molecule of biological system and

contributes to carcinogenesis, mutagenesis and cytotoxicity (Babu *et al*., 2001). Hydroxyl radical scavenging capacity of an extract is directly proportional to its antioxidant activity which is depicted by the low intensity of red colour (Gulcin *et al*., 2005). In the present study ethanol and methanol extracts of *D. neilgherrense* aerial part when added to the reaction mixture actively scavenged the hydroxyl radicals and prevented the degradation of 2-deoxyribose.

Superoxide radical is considered a major biological source of reactive oxygen species (Alves *et al*., 2010). 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 (Meyer and Isaksen, 1995). In the present study, methanol and ethanol extracts had effective capacity of scavenging for superoxide radical.

ABTS radical cation 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 spectrophotometrically at 745 nm. In the present study, among all the solvents tested, methanol and ethanol exhibited significantly higher ABTS radical cation scavenging activity.

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. (Packia lincy *et al*., 2015). Increasing absorbance at 700 nm indicates an increase in reducing ability. The antioxidants present in the extracts of *D. neilgherrense* caused their reduction of Fe3+/ ferricyanide complex to the ferrous form, and thus proved the reducing power.

The present study reported the antioxidant activity, total phenolic and flavanoid contents of *Daphniphyllum neilgherrense* aerial part. In order to realize and health benefits from potential plants sources, it is important to measure the antioxidant activity using various radicals and oxidation systems. This study reported that tested plant materials have significant antioxidant activity and free radical scavenger activity. The result of the present study suggested that *Daphniphyllum neilgherrense* aerial part can be used as a source of antioxidants for pharmacological preparations which is very well evidenced by the present work.

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