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Evaluation of antioxidant activity of *Asystasia travancorica* Bedd (Acanthaceae)

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Abstract

In vitro antioxidant activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *Asystasia travancorica* (Acanthaceae) were evaluated by studying 1, 1-diphenyl 2- picrylhydrazyl (DPPH) radical scavenging activity, hydroxyl radical scavenging activity, superoxide radical scavenging activity, ABTs radical cation scavenging activity and reducing power using standard procedures. Total phenolic content was estimated by Folin ciocalteau method. Flavonoid was determined by Aluminium chloride method. The total phenolics and flavonoids in methanol extract were found to be 0.54g100g^{-1} and 0.62g100g^{-1} respectively. Ethanol extract of *A.travancorica* is found to possess higher DPPH radical scavenging activity and reducing ability. Methanol extract of *A.travancorica* exhibited highest superoxide and ABTs radical cation scavenging activity. Petroleum ether extract of *A.travancorica* showed the highest hydroxyl radical scavenging activity. This study indicates significant free radical scavenging potential of *A.travancorica* whole plant which can be exploited for the treatment of various free radical mediated ailments.

Key words : *Asystasia*, total flavonoids, radical scavenging, Methanol.

INTRODUCTION

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. The most common reactive oxygen species (ROS) include superoxide (O^{2-}) anion, hydrogen peroxide (H_2O_2), peroxyl (ROO^-) radicals and reactive hydroxyl (OH^-) radicals. The nitrogen derived free radicals are nitric oxide (NO) and peroxynitrile anion (ONOO) [1]. Under normal state of affairs, the ROS generated are detoxified by the antioxidants nearby in the body and there is symmetry between the ROS generated and the antioxidant present. However, due to ROS over production and / or derisory antioxidant argument, this equilibrium is hindred favouring the ROS gain that culminates in oxidative hassle. The ROS readily attack and induce oxidative damage to various bimolecules including proteins, lipids, lipoproteins and DNA [2]. This oxidative damage is decisive etiological factor concerned in quite a lot of chronic human diseases such as diabetes mellitus, cancer, atherosclerosis, arthritis and neurodegenerative diseases and also into the ageing course [3]. Antioxidants prevent the production of such highly reactive oxygen species, scavenge free radicals, aids in repairing from oxidative damage and effective functioning of naturally prevailing antioxidants in body [4].

At present, most of the antioxidants are manufactured synthetically, several synthetic antioxidants eg. butylated hydroxyanisole (BHA), butylated hydroxytoulene (BHT), tertiary butylated hydroquinone (TBHQ) and gallic acid esters are commercially available. Such synthetic antioxidants are known to have potential side effects and toxicity when taken *in vivo*, hence, their use is being restricted nowadays and there is an increasing interest in finding out safer and bioactive natural antioxidants present in plant species [5-6]. Thus, there is a need of antioxidants of natural origin because they can protect the human body from the diseases caused by free radicals [7-8]. Therefore in current years, substantial attention has been directed towards credentials of plants with antioxidant ability they may be used for human expenditure.

Asystasia includes approximately 70 species of perennial herbs and subshrubs from tropical Africa, India and Asia. *Asystasia* belongs to the family Acanthaceae. Paste of leaves and flowers of *A.travancorica* mixed with honey is taken orally, twice a day, for three weeks for the treatment of rheumatism. [9]. *A.travancorica* whole plant extracts were reported for the biological activities such as anticancer and antiinflammatory activity [10-11]. In view of the above medicinal properties, the present study was designed to investigate the *in vitro* antioxidant activity of different solvent extracts of whole plant of *Asystasia travancorica*.

MATERIALS AND METHODS

The whole plant of *Asystasia travancorica* Bedd were collected from Agasthiarmalai Biosphere Reserve, Western Ghat, Tamil Nadu. 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 whole plant of *Asystasia travancorica* 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 though Whatman No.41 filters 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 by McDonald *et al.* [12] with little modification. To 1mL of each extract (100µg/mL) in methanol, 5mL of Folin-Ciocalteau reagent (diluted 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* [13] 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 [14].

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 [14]. 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.

Percentage of 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 [15]. 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 &800 µg/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* [16]. 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

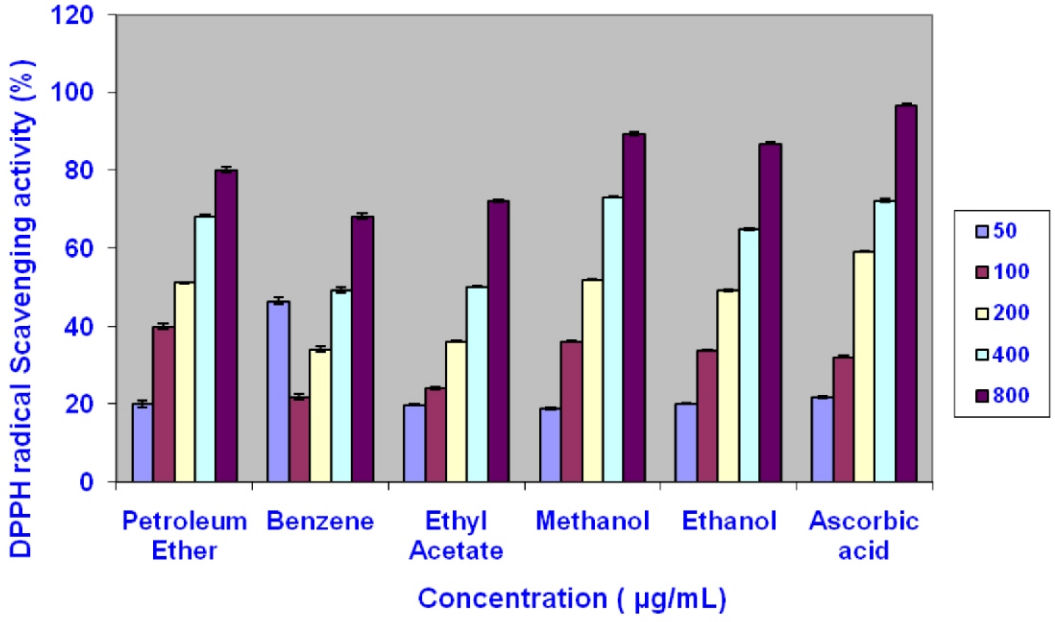

Figure 1: DPPH radical scavenging activity of different extracts of whole plant of *Asystasia travancorica*

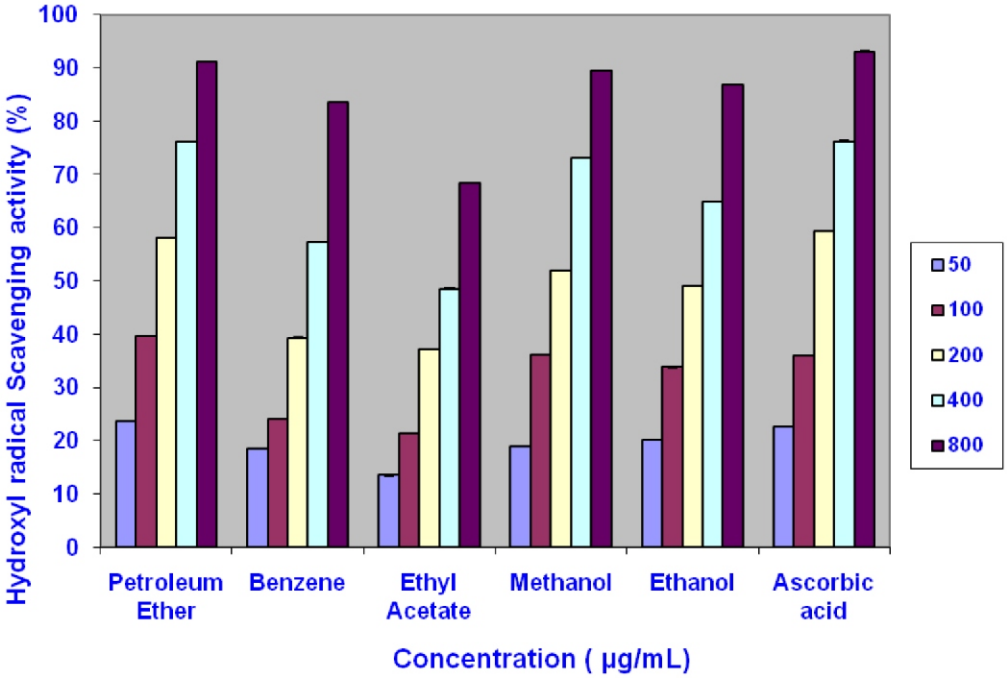

Figure 2: Hydroxyl radical scavenging activity of different extracts of whole plant of *Asystasia travancorica*

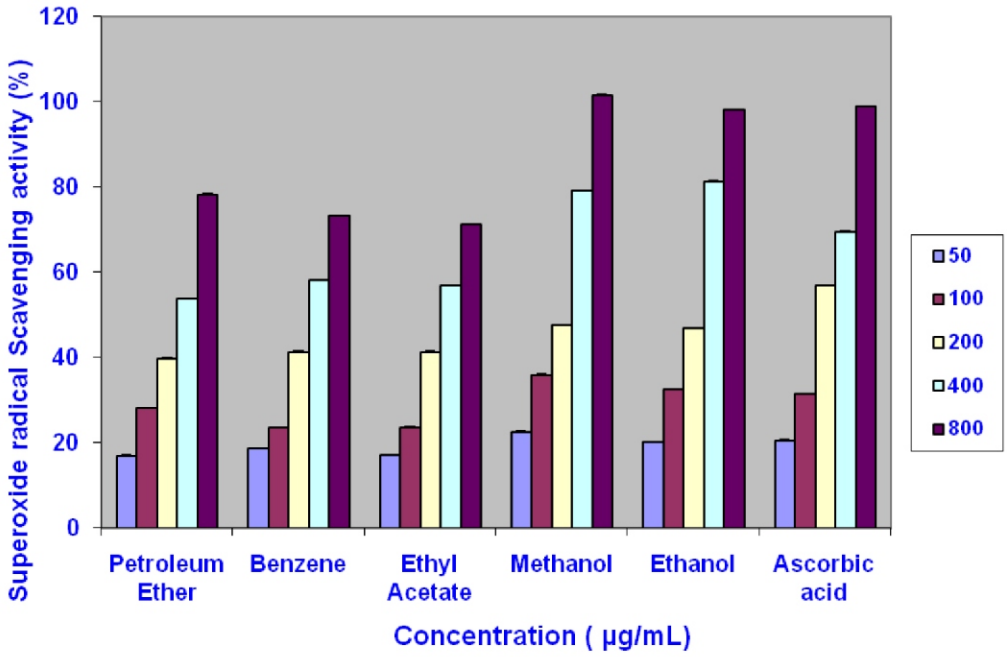

Figure 3: Superoxide radical scavenging activity of different extracts of whole plant of *Asystasia travancorica*

solution (0.12mM) to the mixture, incubated at 25°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* [17]. 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 and Hemalatha [18]. 1.0 mL of solution

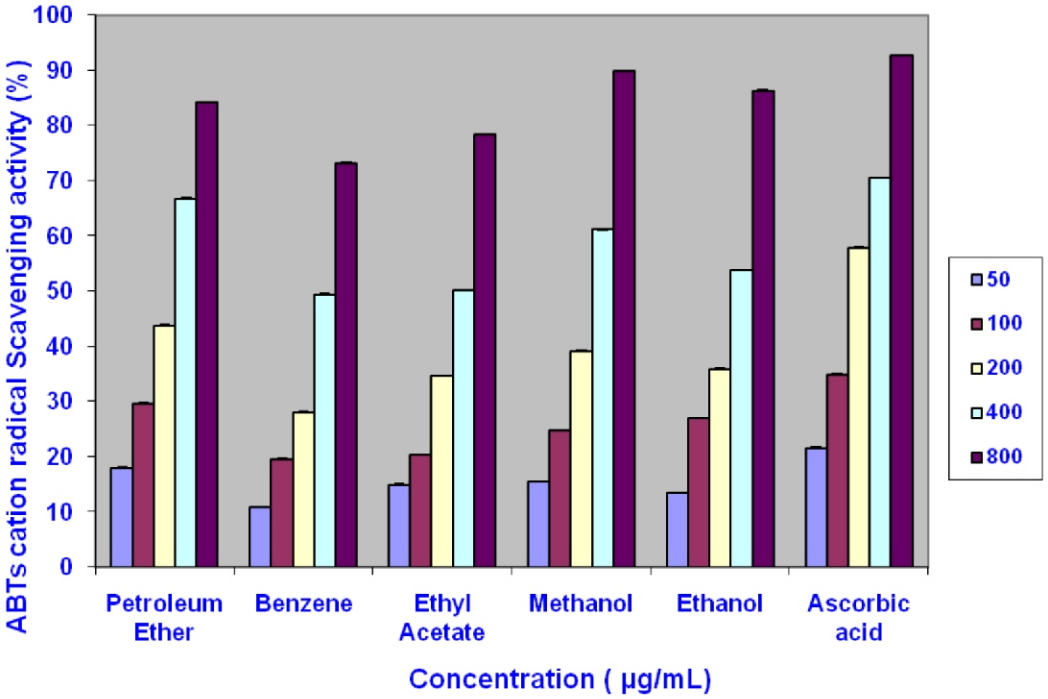

Figure 4: ABTs cation radical scavenging activity of different extracts of whole plant of *Asystasia travancorica*

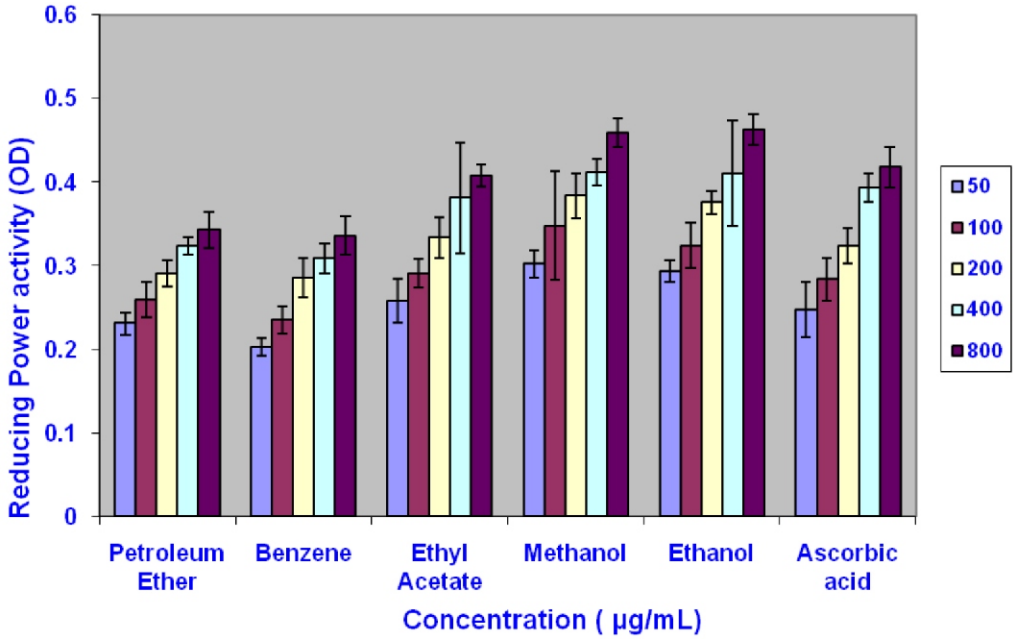

Figure 5: Reducing Power activity of different extracts of whole plant of *Asystasia travancorica*

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.

RESULTS

Total phenolic and flavonoid contents

The total phenolic and total flavonoid contents of the whole

Table 1: IC_{50} values of different solvent extracts of *Asystasia travancorica* whole plant

Solvent	IC_{50} values (µg/ml)			
	DPPH Assay	Hydroxyl Assay	Superoxide assay	ABTS assay
Petrolcum ether	18.41	20.13	20.33	18.09
Benzene	16.84	18.41	19.28	17.42
Ethyl acetate	17.64	14.88	18.94	17.94
Methanol	19.87	19.43	24.97	24.97
Ethanol	20.13	19.16	21.56	21.56
Ascorbic acid	20.94	21.54	22.08	-
Trolox	-	-	-	21.37

plant methanol extract of *A.travancorica* was found to be 0.54g $100g^{-1}$ and 0.62g $100g^{-1}$ respectively.

DPPH radical scavenger activity

DPPH radical scavenging activity of the whole plant petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *A.travancorica* was shown in figure 1. The scavenging effect of all the extracts and also the standard increased with the increase in the concentration. Among the solvents tested, whole plant ethanol extract of *A.travancorica* exhibited highest DPPH radical scavenging activity of 96.36%, the maximum at 800 µg/mL concentration. The concentration of *A.travancorica* whole plant ethanol extract needed for 50% inhibition (IC_{50}) was found to be 20.13µg/mL, whereas 20.94µg/mL needed for ascorbic acid (Table 1).

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of various extracts such as petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of whole plant of *A.travancorica* was shown in figure 2. Among the solvents tested, the whole plant petroleum ether extract of *A.travancorica* exhibited 91.13% hydroxyl radical scavenging activity at 800 µg/mL concentration. The concentration of *A.travancorica* whole plant petroleum ether extract needed for 50% exhibition (IC_{50}) was found to be 18.06µg.mL, whereas 21.54µg/mL (Table 1) needed for ascorbic acid.

Superoxide radical scavenging activity

The different solvent extracts of *A.travancorica* whole plant was subjected to be superoxide radical scavenging activity and the results were shown in figure 3. It indicates that methanol extract of *A.travancorica* whole plant (800µg/mL) exhibited the maximum superoxide radical scavenging activity of 101.50%, which is higher than the standard ascorbic acid whose scavenging activity is 98.86%. The IC_{50} value of the methanol extract of *A.travancorica* whole plant on superoxide radical was found to be 22.81µg/mL and 22.081µg/mL for ascorbic acid, respectively (Table 1).

ABTs radical cation scavenging activity

The different solvent extracts of *A.travancorica* whole plant was subjected to be ABTs radical cation scavenging activity and the results were shown in figure 4. The whole plant methanol extract of *A.travancorica* exhibited highest ABTs radical cation scavenging activity of 89.84% at 800µg/mL concentration. The IC_{50} value of the methanol extract of *A.travancorica* whole plant on ABTs radical cation was found to be 19.18µg/mL and 21.37µg/mL for trolox, respectively (Table 1).

Reducing Power

Figure 5 shows a comparision between the reducing abilities of different solvent extracts of the whole plant of *A.travancorica* and ascorbic acid, the standard. Absorbance of the solution was increased with the concentration. Higher absorbance indicated the higher reducing power. Among the solvents tested, the whole plant ethanol extract of *A.travancorica* exhibited higher reducing activity.

DISCUSSION

Plants are rich sources of natural antioxidants such as phenolics and tocopherols, which are considered to be the best known components [19]. Phenolic compounds are highly reactive towards free radicals. Hence, quantification of these compounds is also an indicative of antioxidant activity [20]. It is known that phenolic compounds from plants are good natural antioxidants. Polyphenolic compounds are antioxidants and may help to prevent diseases associated with oxidative stress, such as atherosclerosis, cancer and neurodegenerative diseases [21-22]. The value obtained for total flavonoid content from this study is high and this is considered an advantage because of the plants containing phenolic compounds, especially the flavonoids have exhibit strong antioxidant properties [23]. It is also reported by Heim *et al*.,[24] that the radical scavenging activity is an indicator of the functionality and antioxidant activity and can be attributed to the contents of total polyphenols and flavonoids in plant foods.

DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH radical was determined by the decrease in its absorbance at 517nm, which is induced by different antioxidants. The decrease in absorbance of DPPH radical caused by antioxidants, because of the reaction between antioxidant molecules and radical progress, results in the scavenging of the radical by hydrogen donation. In the present

study, the extracts had significant scavenging effect on the DPPH radical which was increasing with the increase in the concentration of the sample from 50-800µg/mL. Similar trend of DPPH free radical scavenging activity was reported for the species *Sonerila tinnevelliensis* [25] and *Vernonia cinerea* [26].

Among the reactive oxygen species, the hydroxyl radicals are the most reactive and predominant radicals generated endogenously during aerobic metabolism [27]. A single hydroxyl radical results in the formation of many molecules of lipid hydroperoxides in the cell membrane which may severely, disrupts its function and leads to cell death. In the present study, the extracts had significant scavenging effects on the hydroxyl radical, which was increasing with the increase in concentration from 50 - 800 µg/mL. The petroleum ether whole plant extract of *A.travancorica* possessed higher hydroxyl radical scavenging activity than that of the standard. Daffodil and Mohan [28] and Packia Lincy *et al*. [29] observed similar type of hydroxyl radical scavenging activity for the plants, *Nymphaea rubra* and *Maerua apetala*, respectively.

Superoxide anion plays an important role in the formation of more reactive species such as hydrogen peroxide, hydroxyl radical and singlet oxygen, which induce oxidative damage in lipids, proteins and DNA [30]. Therefore, studying the scavenging activity of plant extracts on superoxide radical is one of the most important ways of clarifying the mechanism of antioxidant activity. In the present study, the superoxide radical scavenging activity of *A.travancorica* whole plant was found to increase in a concentration dependent manner. Methanol extract of *A.travancorica* whole plant showed maximum scavenging activity which was increasing with the increase in the concentration of the sample from 50-800µg/mL. Similar trend of superoxide radical scavenging activity was reported for the species, *Aristolochia bracteata* [31] and *Ruellia tuberosa* [32].

The ABTs method depends on the inhibition of the absorbance of radical cation ABTs, which has a feature wave length at 734nm. Decolorization of ABTs reflects the capacity of the antioxidants species to donate electrons or hydrogen atoms to inactivate these radical actions. In the presence of antioxidant reductant, the colored radical is converted back to colorless ABTs [33]. In the present study, methanol extract is highly potent in neutralizing ABTs cation radicals. The methanol extract, had significant scavenging effects on the ABTs radical which was increasing with the increase in concentration from 50-800 µg/mL. Jegadeeswari *et al*. [34] and Sornalakshmi *et al*. [35] observed similar type of ABTs radical scavenging activity for the plants, *Aristolochia krysagathra* and *Hedyotis leschenaultiana* respectively. ABTs radical scavenging activity is relatively recent, often used for screening complex antioxidant mixtures such as plant extracts and involves a more drastic radical, chemically produced [36].

The reducing power reflects the electron donating capacity of its bioactive components, which serves as a significant indicator of its antioxidant activity. Reduced $Fe3^{+}$ / ferricyanide complex to the ferrous form, which indicated existence of reductants in the sample solution. The presence of these reductants in *A.travancorica* whole plant was considered keys to its reducing power, because they appear as antioxidants by donating a hydrogen atom and breaking the free radical chain [37].

CONCLUSION

In the present study, it is found that whole plant extracts of *A.travancorica* showed concentration dependent free radical scavenging activity and this antioxidant effect may be due to the higher content of phenols and flavonoids. Thus, the *A.travancorica* whole plant extract as promising natural sources of antioxidants can be used in nutritional or pharmaceutical fields for the prevention of free radical mediated diseases.

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