

Research Article

Antioxidant Activity of Tuber of *Ruellia tuberosa* L. (Acanthaceae)

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ABSTRACT

In vitro antioxidant activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *Ruellia tuberosa* (Acanthaceae) was 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 procedure. Among the solvents tested, methanol and ethanol extracts of tuber of *R. tuberosa* showed potent *in vitro* antioxidant activities. The results clearly indicated extracts of tuber of *R. tuberosa* is effective in scavenging free radicals and has the potential to be a powerful antioxidant.

Keywords: *Ruellia tuberosa*, DPPH, ABTS, hydroxyl radical scavenging activity, reducing power.

INTRODUCTION

Oxidative stress results from an imbalance between the generation of reactive oxygen species and endogenous antioxidant systems. ROS are major sources of primary catalysts that initiate oxidation *in vivo* and *in vitro*. There ROS creates oxidative stress which results in numerous disease and disorders such as cancer, cardiovascular disease, neural disorders, Alzheimer's disease, mild cognitive impairment, Parkinson's disease; alcohol induced liver disease, ulcerative colitis, ageing and atherosclerosis. The compounds from natural sources are capable of providing protection against free radicals. This has attracted a great deal of research interest in natural antioxidants. It is necessary to screen out the medicinal plants for their antioxidant potential^{1,2}.

Plants have great importance due to their nutritive value and they are the major source of medicines which play an important role in the human history³. Plants synthesize primary metabolites (proteins, fats, nucleicacids and carbohydrates) by simple substances such as water, carbon dioxide, nitrogen and a number of inorganic salts in small amounts. These primary metabolites are transformed into secondary metabolites (alkaloids, steroids, terpenoids, saponins, flavonoids etc.) that are used as drugs⁴. *Ruellia tuberosa* L. is a tropical plant and widely distributed in Southeast Asia. In folk medicine, it has been used as anti-diabetic, antipyretic, analgesic, antihypertensive, thirst- quenching, and antidotal agent⁵. Perusal of literature reveals that information on the *in vitro* antioxidant activity of tuber of *R. tuberosa* is totally lacking. Hence, the objective of the present study is to evaluate the *in vitro* antioxidant activity using different models viz. DPPH, Hydroxyl, Superoxide and ABTS.

MATERIALS AND METHODS

The tubers of *Ruellia tuberosa* L. were collected from Government Girl's Higher Secondary School campus, Barugur, Krishnagiri District, Tamil Nadu. They were shade dried at room temperature for 10-15 days. The dried plant material was 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 Extracts: The coarse powder (100g) 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 papers. All the extracts were concentrated in a rotary evaporator. The concentrated extracts were used for *in vitro* antioxidant activity.

Estimation of Total Phenolics: Total phenolic content was estimated using Folin-Ciocalteu reagent based assay as previously described⁶ with little modification. To 1mL of each extract (100µg/mL) in methanol, 5mL of Folin-Ciocalteu reagent (diluted ten-fold) and 4mL (75g/L) of Na₂CO₃ were added. The mixture was allowed to stand at 20°C 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*⁷. An aliquot of 0.5 mL of sample (1mg/mL) was mixed with 0.1mL of

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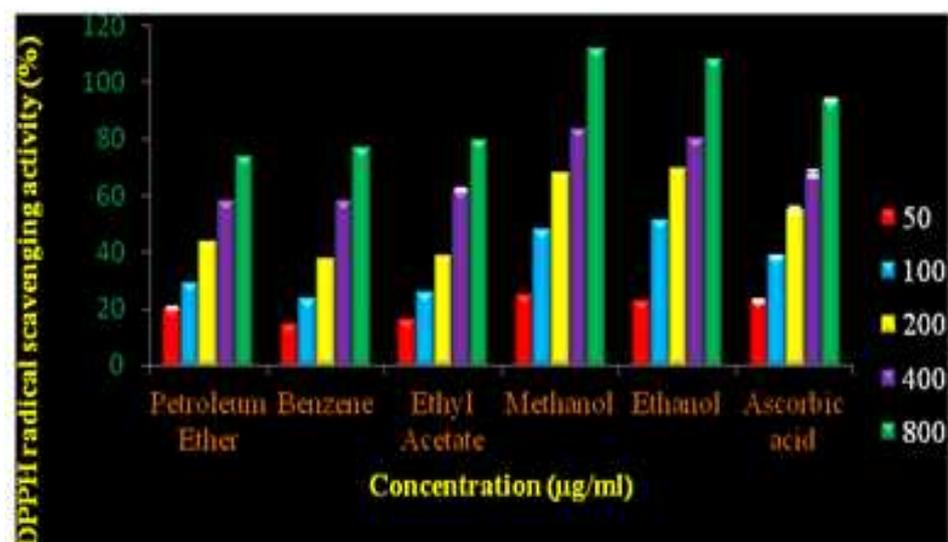


Fig 1: DPPH radical scavenging activity of different extracts of *R. tuberosa*

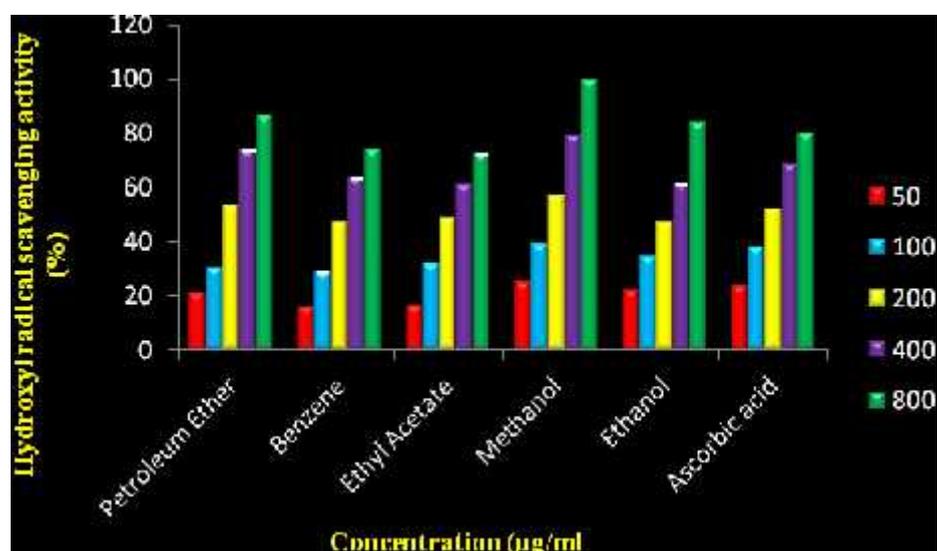


Fig 2: Hydroxyl radical scavenging activity of different extracts of *R. tuberosa*

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 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-H⁸.

The free radical scavenging activity of all the extracts was evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) according to the previously reported method⁸. 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 of scavenging the DPPH radical was calculated by using the following formula.

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

Where, A₀ is the absorbance of the control reaction, and A₁ 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*⁹. Stock solutions of EDTA (1mM), FeCl₃ (10mM), Ascorbic Acid (1mM),

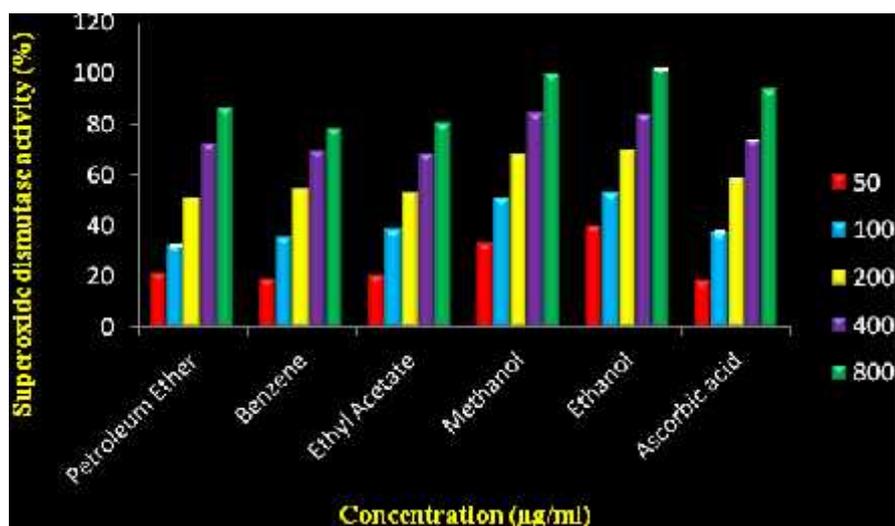


Fig. 3: Superoxide radical scavenging activity of different extracts of *R. tuberosa*

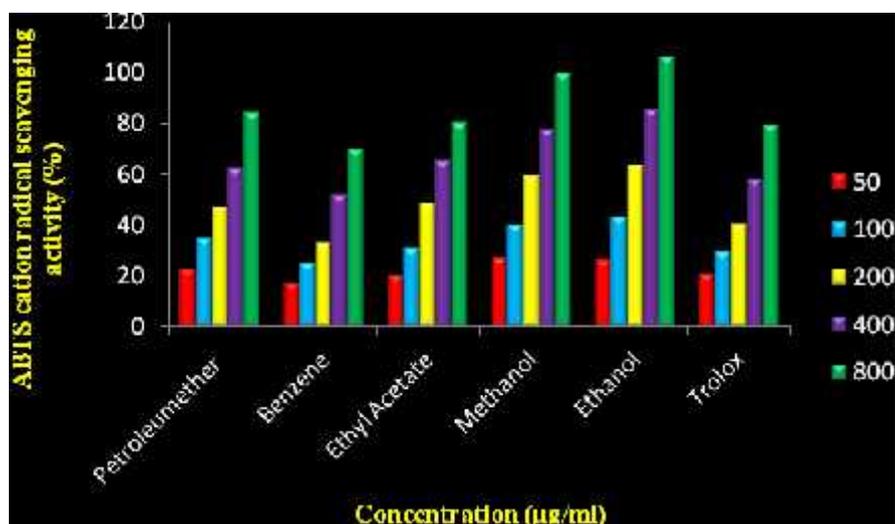


Fig 4: ABTS radical cation scavenging activity of different extracts of *R. tuberosa*

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°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*¹⁰. The superoxide anion radicals were

generated in 3.0 ml of Tris – HCL buffer (16 mM, pH 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°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*¹¹. 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

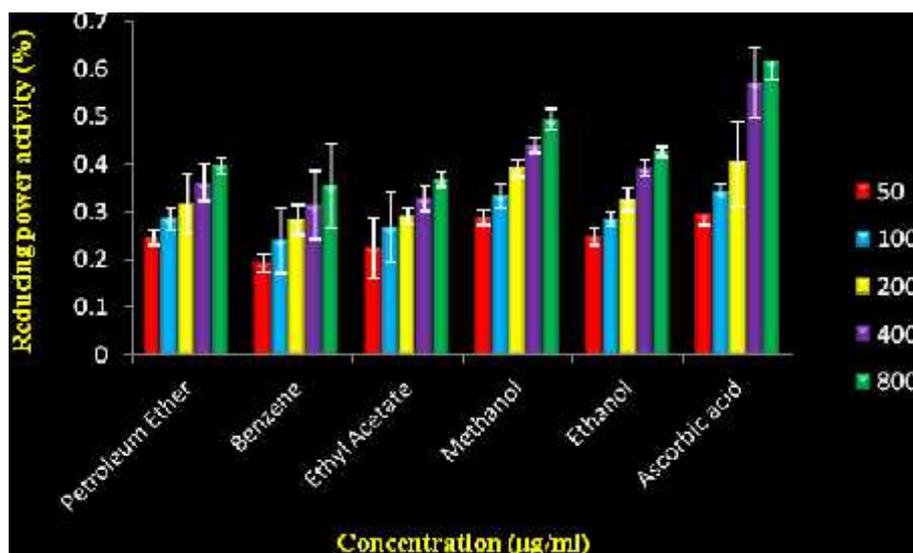


Fig 5: Reducing power ability of different extracts of *R. tuberosa*

Table1. IC₅₀ values of different extracts of *R. tuberosa*

Antioxidant activity	Different solvent extract						Trolox
	Ethanol	Methanol	Petroleum Ether	Benzene	Ethyl Acetate	Standard (Ascorbic acid)	
DPPH assay	22.90	23.66	18.04	18.31	18.86	20.11	-
Hydroxyl assay	20.24	22.66	20.93	19.22	18.24	19.46	-
Superoxide	24.98	24.19	22.18	21.03	21.54	22.18	-
ABTS assay	23.41	22.16	20.16	18.36	20.91	-	20.67

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 Hemalatha¹². 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.

RESULTS

Total phenolics and total flavonoid content: The total phenolic content and total flavonoid content of the ethanol extract of *R. tuberosa* tuber were found to be 0.94g100g⁻¹ and 1.26g100g⁻¹ respectively.

DPPH radical scavenging activity: DPPH radical scavenging activity of petroleum ether, benzene, ethyl acetone, methanol and ethanol extracts of *R. tuberosa* tuber was shown in Fig.1. The scavenging effect increased with the concentration of standard and samples. Among the solvent tested, petroleum ether extract exhibited highest DPPH radical scavenging activity. At 800 µg/ml concentration methanol extract of *R. tuberosa* tuber possessed 112.16% scavenging activity on DPPH.

Hydroxyl radical scavenging activity: Hydroxyl radical scavenging activity of petroleum ether, Benzene, ethyl acetate, methanol and ethanol extracts of *R. tuberosa* tuber was shown in Fig. 2. Petroleum ether extracts showed very potent activity. At 800 µg/ml concentration, petroleum ether extract of the tuber possessed 99.33% scavenging activity on hydroxyl radical.

Superoxide radical scavenging activity: The tuber extracts were subjected to the superoxide scavenging activity and the results were shown in Fig. 3. It indicated that ethanol extract of tuber (800 µg/ml) exhibited the maximum superoxide scavenging activity of 101.88% which is higher than the standard ascorbic acid whose scavenging effect is 93.51%.

ABTS radical cation scavenging activity: The tuber extracts were subjected to the ABTS radical cation scavenging activity and then results were presented in Fig. 4. The ethanol extract exhibited potent ABTS radical cation scavenging activity in concentration depended

manner. At 800 µg/ml concentration, *R. tuberosa* tuber possessed 106.27% scavenging activity in ABTS which is higher than the standard trolox whose scavenging activity is 79.16%.

Reducing Power: Figure 5 showed the reducing ability of different extracts of *R. tuberosa* tuber compared to ascorbic acid. Absorbance of the solution was increased when the concentration increased. A highest absorbance indicated a higher reducing power. Among the solvents tested, methanol extract exhibited higher reducing activity (0.493 OD) which was higher than the standard ascorbic acid whose reducing ability is 0.616 OD.

IC₅₀ values: IC₅₀ values of petroleum ether extract of the tuber and standard ascorbic acid for DPPH, hydroxyl, superoxide radical scavenging and trolox for ABTS radical cation scavenging were found to be 18.04 µg/ml and 20.11 µg/ml; 20.93 µg/ml and 19.46 µg/ml; 22.18 µg/ml, 22.18 µg/ml and 20.16 µg/ml and 20.67 µg/ml respectively. IC₅₀ values of benzene extract of *R. tuberosa* tuber and standard ascorbic acid for DPPH, hydroxyl, superoxide radical scavenging and trolox for ABTS radical cation scavenging were found to be 18.31 µg/ml and 20.11 µg/ml; 19.22 µg/ml and 19.46 µg/ml; 21.03 µg/ml and 22.18 µg/ml and 18.36 µg/ml and 20.67 µg/ml respectively. IC₅₀ values of ethyl acetate of *R. tuberosa* tuber and standard ascorbic acid for DPPH, Hydroxyl, superoxide radical scavenging and trolox for ABTS radical cation scavenging were found to be 18.86 µg/ml and 20.11 µg/ml; 18.24 µg/ml; 19.46 µg/ml and 21.54 µg/ml and 22.18 µg/ml and 20.91 µg/ml and 20.67 µg/ml respectively. IC₅₀ values of methanol extract of *R. tuberosa* tuber and standard ascorbic acid for DPPH, Hydroxyl, superoxide radical scavenging and trolox for ABTS radical cation scavenging were found to be 23.66 µg/ml and 20.11 µg/ml; 22.66 µg/ml and 19.46 µg/ml; 24.19 and 22.18 µg/ml and 22.16 µg/ml and 20.67 µg/ml respectively. IC₅₀ values of ethanol extracts of *R. tuberosa* tuber and standard ascorbic acid for DPPH, hydroxyl, superoxide radical scavenging and trolox for ABTS radical cation scavenging were found to be 22.90 µg/ml and 20.11 µg/ml; 20.24 µg/ml and 19.46 µg/ml; 24.98 µg/ml and 22.18 µg/ml and 23.41 µg/ml and 20.67 µg/ml respectively (Table 1).

DISCUSSION

Phenolics have been considered classic defence compounds for protecting plants from herbivores, ever since plant secondary metabolites were suggested to have evolved for that reason. In contrast to these compounds, it has been suggested that the main role of many plants phenolics may be to protect leaves from photodamage, not herbivores; they can achieve this by acting as antioxidants; and their levels may vary with environmental conditions in order to counteract this potential photodamage¹³. Phenolics are an important class of secondary plant metabolites possessing an impressive array of pharmacological activity with excellent radical scavenging ability. The antioxidant activities of phenolics are due to their redox properties. The phenol moiety (hydroxyl group on aromatic ring) helps them to work as

reducing agents, hydrogen donors and singlet oxygen quenchers^{14, 15}. Phenolics compounds such as flavonoids, phenolic acids and tannins are considered to be the major contributors to the antioxidant activity of vegetables, fruits or medicinal plants. These compounds have been effective in many health related properties such as anticancer, antiviral, anti-inflammatory activities, effects on capillary fragility and ability to inhibit human platelet aggregation¹⁶. Flavonoids are important secondary metabolites of plant modulating lipid peroxidation involved in atherogenesis, thrombosis and carcinogenesis. It has been confirmed that pharmacological effects of flavonoids is correlating with their antioxidant activities¹⁷.

Free radical is a molecule with an unpaired electron and is involved in bacterial and parasitic infections, lung damage, inflammation, reperfusion injury, cardiovascular disorders, atherosclerosis, aging and neoplastic diseases. They are also involved in autoimmune disorders like rheumatoid arthritis etc.¹⁸.

In vitro antioxidant activity of the petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *R. tuberosa* tuber were investigated in the present study by DPPH, hydroxyl, superoxide and ABTS radical cation scavenging activities. These methods have proven the effectiveness of the extracts in comparison to that of the reference standard antioxidants, ascorbic acid and trolox. DPPH radical is one of the few stable organic nitrogen free radicals, which has been widely used to determine the free radical scavenging ability of the various samples. The method is based on the reduction of an alcoholic DPPH solution in the presence of a hydrogen donating antioxidant due to the formation of the non-radical form. DPPH-H by the reaction^{19,20}. In the present study, the percentage of scavenging effect on the DPPH radical was concomitantly increased with the increase in the concentration of tuber of *R. tuberosa* extracts from 50-800µg/mL. Among the tested extracts, methanol extract tuber of *R. tuberosa* exhibited maximum DPPH radical scavenging activity.

The hydroxyl radical is one representative reactive oxygen species generated in the body. These radicals are produced through various biological reactions is the Iron (II)- based Fenton reaction. Among the tested extracts, methanol extracts tuber of *R. tuberosa* exhibited the strongest hydroxyl radical scavenging activity (99.33% at 800µg/mL) standard ascorbic acid showed the least radical scavenging activity (79.84% at 800µg/mL). Hydroxyl radical scavenging activity of different extracts tuber of *R. tuberosa* and standard is presented in the following order: methanol> petroleum ether> ethanol> ascorbic acid> benzene> ethyl acetate. The radical scavenging capacity may be attributed to phenolic compounds in tuber extracts with the ability to accept electrons, which can combine with free radical²¹.

Endogenously, superoxide could be produced in large amounts by various biological processes. It is known to be very harmful to cellular components as a precursor of the most reactive oxygen species (ROS), contributing to tissue damage and various diseases. In the present study,

among the tested extracts, ethanol extract tuber of *R. tuberosa* showed 101.88% superoxide inhibition at the concentration of 800µg/mL. These extracts exhibited higher ability in scavenging superoxide radical, when compared to the standard ascorbic acid (93.51%). The results reveal that the *R. tuberosa* extracts have superoxide radical scavenging activity which can be of significant interest in health point of view in reducing the level of superoxide radical which is elevated during oxidative stress in the body.

The decolorization of ABTS radical reflects the capacity of an antioxidant species to donate electrons or hydrogen atoms to inactivate this radical species. The ABTS radical cation is generated from the reaction of ABTS with potassium persulfate overnight in water²². In the present study, among the tested extracts, ethanol extract of tuber of *R. tuberosa* showed higher ABTS radical scavenging activity. This study indicated that the extracts have the hydrogen donating ability and could serve as free radical scavenging by acting as primary antioxidants.

The reducing ability of a compound generally depends on the presence of reductants which have been exhibited antioxidative potential by breaking the free radical chain, donating a hydrogen atom²³. In the present study, the results revealed the dose dependent reducing ability for all the extracts. Among the tested extracts, methanol extracts of tuber of *R. tuberosa* exhibited greater reducing power ability.

The results obtained from this study clearly indicate that the extracts from the tuber of *R. tuberosa* were found to possess strong antioxidant activity and scavenging effects on free radicals. These *in vitro* assays indicate that this plant extract is a significant some of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses. Therefore, further investigations need to be carried out to isolate and identify the antioxidant compounds present in the plant extract. Furthermore, the *in vivo* antioxidant activity of this extract needs to be assessed prior to clinical use.

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