In vitro anti-oxidant activity of *Ruellia tuberosa* root extracts

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ABSTRACT

Background: The present study was aimed to evaluate *in vitro* anti-oxidant activity of the *Ruellia tuberosa* (Acanthaceae) roots. **Materials and Methods:** Anti-oxidant activity was evaluated by using DPPH free radical scavenging activity and reducing power by $FeCl_3$. The methanolic extract (ME), water extract (WE), ethyl acetate extract (EAE) and ethyl acetate fraction of methanol extract (EAFME) of root were tested. The Ascorbic acid was used as positive control. Total phenolic and total flavonoid content were also determined by Folin-Ciocalteu reagent and complementary colorimetric methods (aluminum chloride method and 2, 4-dinitrophenylhydrazine method respectively. **Results:** The EAFME of root showed the highest concentration of phenolic, flavonoid content, free radical scavenging activity and reducing power. The various extract showed a significant anti-oxidant activity when ($P < 0.05$) compared with standard**. Conclusion:** It is concluded that the *R. tuberosa* root possess anti-oxidant activity. Further studies are suggested to isolate the active principle responsible for the activity.

Keywords: Anti-oxidant activity, DPPH free radical scavenging activity**,** *Ruellia tuberosa*, total phenolic.

INTRODUCTION

Reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide anion (O_2–), hydroxyl (.OH) radical and hydrogen peroxide (H_2O_2) are often generated as by -products of biological reactions or from exogenous factors. These reactive species exert oxidative damaging effects by reacting with nearly every molecule found in living cells. Such species are considered to be important causative factors in the development of diseases such as diabetes, stroke, arteriosclerosis, cancer, cardiovascular diseases and the aging process.[1–6] Human body has multiple mechanisms especially enzymatic and non-enzymatic anti-oxidant systems to protect the cellular molecules against reactive oxygen species (ROS) induced damage.[7] However, the innate defense may not be enough for severe or continued oxidative stress. Hence, certain amounts of exogenous anti-oxidants are constantly required to maintain an adequate level of anti-oxidants in order to balance the ROS in human body. Many synthetic anti-oxidants such as butylated hydroxyanisole (BHA) and butylated hydroxyl toluene (BHT) are very effective and are used for industrial processing but they possess potential health risk like carcinogenicity and toxic properties to human health and should be replaced with natural anti-oxidants.[8,9] Among the various natural anti-oxidants, phenolic compounds in herbs act as anti-oxidants due to their redox properties, allowing them to act as reducing agents, hydrogen donors, free radical quenchers and metal chelators.[10–12] Several natural anti-oxidants have already been isolated from plant materials, such as oil seeds, cereal crops, vegetables, fruits, leaves, roots, spices, and herbs.[13–17]

Ruellia tuberosa Linn. belongs to family Acanthaceae, a native of Central America, introduced into Indian garden as an ornament. It is used medicinally in West Indies, Central America, Guiana and Peru. *R. tuberosa* is commonly known as 'Cracker plant' 30–45 cm height,

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erect, sub-erect or diffuse perennial herbs with tuberous roots. Leaves are opposite, inflorescence in axillary 1–3 flowered cymes, flower bluish, purple or deep blue; Fruit bluish, purple or deep blue seeds.[18–21] Root infusion is used for kidney diseases; in the form of a syrup for whooping cough; infusion or decoction for a diabetes remedy; tubers in a tea used for cleansing the blood. Root and leaf used in form of tea alleviates retention of urine and it is suggested as a remedy to weakness.[22–24] The Roots used for oestrus induction and as an anthelmintic.[25] Dried and ground root in dose of two ounces cause abortion and also used in sore eyes. The herb also exhibits emetic activity and employed substitute of ipecac, also used in bladder stones and decoction of leaves used in treatment of Bronchitis.[26] The tubers of the plant were reported to contain n-alkanes, triterpenoids and phytosterols, lupeol. In which n-alkanes (C_{23}–C_{33}) with maximum occurrence of n-nonacosane (C_{29}: 44.83%), n-hentriacontane (26.52%), Sterol-stigmasterol, B-sitosterol, campesterol.[27] Methanolic extract and various fraction of methanolic extract of stem of *Ruellia tuberosa* have reported anti-oxidant activity.[28] *R. tuberosa* roots remain unexplored. Therefore, the present study was aimed on evaluating their phenolic constituents, flavonoid content, anti-oxidant potential and free radical scavenging capacity of roots of *R. tuberosa*.

MATERIALS AND METHODS

Plant material

Fresh plant of *Ruellia tuberosa* was collected from the campus of The M. S. University of Baroda in the month of August-2008. Plant was authenticated at Botany department of The M. S. University. Voucher specimen (PHR/HDT/DC-RT-08) was stored in herbarium of our laboratory. Roots were separated and sun dried. Dried plant material was powdered.

Chemicals

Gallic acid, Folin-Ciocalteu reagent, sodium carbonate, quercetin, aluminum chloride, potassium acetate, naringin, 2, 4-dinitrophenylhydrazine reagent, potassium hydroxide, α, α diphenyl – β picryl hydrazyl (DPPH), ascorbic acid were obtained from E. Merck (Darmstadt, Germany), Hi-Media lab. Ltd (Mumbai) and Sigma (Chemical Co, St. Louis, MO, USA). All other reagents were analytical grade. All UV–Vis measurements were recorded on a Shimadzu UV–1800.

Preparation of extracts

About 50 gm of powdered drug roots were extracted in soxhlet apparatus for 3 days with methanol and ethyl acetate separately. The extract was filtered, concentrated by evaporation under vacuum and completely dried *in vacuo*.

Preparation of Water extract: Root powder was macerated in water for 24 h. Extract was filtered and evaporated to dryness.

Ethyl acetate fraction: Dried methanol extract were dissolved in ethyl acetate. Insoluble part of extract was removed by filtration. It was concentrated by evaporation under vacuum and completely dried *in vacuo*.

Phytochemical screening[29]

Prepared Extracts were screened for the presence of various class of phytoconstituent by employing chemical tests. It was further confirmed by thin later chromatographic studies.

HPTLC fingerprinting of phenolics compounds[30,31]

The HPTLC Fingerprint of various extract and fraction were developed using $CHCl_3$: ethyl acetate: Formic acid (1:9:0.4) as mobile phase, stationary phase; (pre-coated plate) Silica gel G 60 F_{254}, sample applicator; CAMAG LINOMAT 5, developing chamber; twin trough chamber. The plate was scanned at 366 nm under fluorescence mode and in visible mode after derivatization with alcoholic $FeCl_3$ using CAMAG TLC SCANNER-3.

Determination of total phenolics content[32]

Total Phenolic content was determined as method described by Singleton and Rossi (1965). The method was performed for methanol extract (ME), Ethyl acetate extract (EAE), water extract (WE), and Ethyl acetate fraction of MeoH extract (EAFME) of roots. Total phenolic was determined with Folin–Ciocalteu reagent according to the method using Gallic acid as a standard phenolic compound. The calibration curve of Gallic acid was taken using methanol.

About 1.0 ml of extract solution containing 1 mg extract in a volumetric flask was diluted with 10 ml of distilled water. To this, 1.5 ml of Folin–Ciocalteu reagent was added. The above mixture was kept for 5 min. and then 4 ml of 20% sodium carbonate solution was added and made the volume up to 25 ml with the distilled water. This mixture

was kept for 30 min and the absorbance of the blue color developed was measured at 765 nm, using Shimadzu 1800 spectrophotometer. The percentage of total phenolics was calculated from calibration curve of Gallic acid and total phenolics were expressed as % Gallic acid.

Determination of total Flavonoid content[33,34]

The total flavonoids determined by two complementary colorimetric methods, aluminum chloride method and 2, 4-dinitrophenylhydrazine method and the values obtained were summed up to give the final value. The method was performed for Methanol extract (ME), Ethyl acetate fraction of methanol extract (EAFME), Water extract (WE) of root of *Ruellia tuberosa.*

I) Aluminum chloride colorimetric method

Quercetin was used to make the calibration curve. 1 mg of Quercetin was dissolved in 100 ml methanol to produce (10 µg/ml). From this solution 0.1, 0.2, 0.3, 0.5, 0.8, 1, 1.5 ml taken and diluted up to 10 ml methanol to produce 1, 2, 3, 5, 8, 10, 15 µg/ml concentrations respectively. The standard solution was separately mixed with 1.5 ml of 95% Methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the absorbance of reaction mixture was measured at 415 nm with Shimadzu 1800 spectrophotometer. The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank.

About 1.0 ml of extract solution containing 4 mg extracts was reacted with Aluminum chloride for determination of flavonoids content as described in above procedure; the percentage of total flavonoids was calculated from calibration curve.

II. 2, 4-Dinitrophenylhydrazine colorimetric method

Naringin was used to make the calibration curve. Naringin was dissolve in methanol to10 mg/mL standard solution. From the standard stock solution 0.25, 0.5, 1, 1.5, 2 ml, were taken and diluted up to 10 ml methanol to give 250, 500, 1000, 1500, 2000 µg/mL concentration respectively. One ml of the each standard solution was separately mixed with 2 ml of 1% 2, 4 di nitro phenyl hydrazine reagent and 2 ml of methanol, and then kept at 50°C for 50 min. After cooling to room temperature, the reaction mixtures were mixed with 5 ml of 1% potassium hydroxide in 70% methanol and incubated at room Temperature for 2 min. Then, 1 ml of the mixture was taken, mixed with 5 ml of methanol and Centrifuged at 1000 rpm for 10 min. to remove the precipitate. The supernatant was collected and adjusted to 25 ml. The absorbance of the supernatant was measured at 495 nm by using Shimadzu 1800 spectrophotometer.

Similarly, 4 mg/ml sample solution were reacted with 2, 4-dintophenylhydrazine for determination of flavonoid content as described in the above procedure. The percentage of total flavonoids was calculated from calibration curve.

Anti-oxidant activity *in vitro*

DPPH free radical scavenging activity[35,36]

Required quantity of Ascorbic acid (standard) was dissolved in methanol to give the concentration of 10, 20, 40, 60, 80 and 100 µg/ml. The test samples methanol extract and ethyl acetate fraction of methanol were dissolved in methanol to give stock solution of 1000 µg/ml. 50, 75, 100, 150, 200, 300 µg/ml methanol extract and ethyl acetate fraction of methanolic and water extract 50, 100, 250, 500, 750, 1000 µg/ml concentration of the test samples were prepared by proper dilution of the stock solution with methanol. Accurately weighed 1.3 mg of DPPH was dissolved in 1 ml of methanol, protected from the light by covering the test tube with aluminum foil.

Protocol for estimation of DPPH free radical scavenging activity

A 75 µl DPPH solution was added to 3 ml methanol and the absorbance was taken immediately at 516 nm for control reading. Different concentrations of standard and test samples were diluted with methanol up to 3 ml and 75 µl of DPPH was added. The absorbance was taken immediately after addition of DPPH solution at 516 nm using Shimadzu 1800 spectrophotometer. Methanol was used as a blank. Decrease in absorbance in presence of test samples at different concentration was noted after 10, 20 and 30 min. The % reduction and IC_{50} were calculated as follows

$$\% \text{ Anti-oxidant activity} = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \times 100$$

Reducing power by $FeCl_3$[37,38]

Required quantity of Ascorbic acid (standard) and test samples were dissolved in methanol. The reducing power of methanolic, water extract and ethyl acetate fraction of methanol extracts of the roots of *R. tuberosa* was determined according to the method of Oyaizu (1986). Sample solutions at different amounts were mixed with 2.5 mL of

0.2 M phosphate buffer (pH 6.6) and 2.5 mL of potassium ferricyanide (1%). After the mixture was incubated at 50°C for 20 min, 2.5 mL of TCA (10%) were added and the mixture was centrifuged at 3000 rpm for 10 min. Supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and 0.5 mL of ferric chloride (0.1%), kept for 10 min. Control was prepared in similar manner excluding samples and the absorbance was measured at 700 nm and compared with standard. Higher absorbance of the reaction mixture indicates greater reducing power.

RESULTS AND DISCUSSION

Phytochemical screening

It was found that methanolic extract of roots contain carbohydrates, Saponins, flavonoid, phenolics compound and sterols. Water extracts contain phenolic compound, saponins and carbohydrates.

HPTLC fingerprinting for phenolics compounds: Figure 1

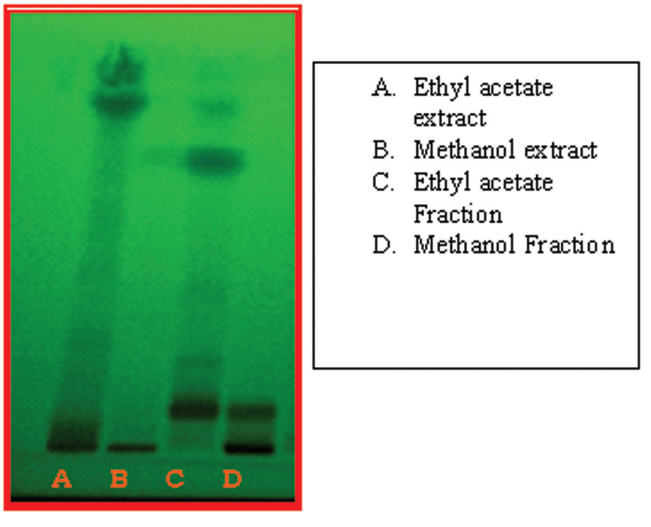

Figure 1. HPTLC Fingerprinting of phenolics compounds of *R. tuberosa* root extracts.

Total phenolics content

Folin–Ciocalteu reagent is mixture of phosphomolybdate and phosphotungstate. Phenolic compound cause the reduction of phosphotungstate-phosphomolybdate complex to blue reaction products in alkaline conditions and the estimation of phenolics involves the measurement of the absorbance of this colored complex at 765 nm. It measures the amount of the substance need to inhibit the oxidation of the reagent. The total phenolic content was expressed in terms of % Gallic acid. Results were reported in Table 1.

Methanol extract (ME), Ethyl acetate fraction of methanol extract (EAFME), Water extract (WE), Ethyl acetate extract (EAE).

Total flavonoid content

Flavones, flavonols and isoflavones forms complex mainly with Aluminum chloride ($AlCl_3$) while flavonones strongly reacted only with 2, 4-dinitrophenylhydrazine (2, 4 DNPH), the results obtained by two methods were added [Table 1], to evaluate the total flavonoids content.

Method I. Aluminum chloride colorimetric method

The principle of aluminum chloride colorimetric method is that aluminum chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl

Table 1 Total phenolic, Flavonoid content and IC_{50} of *R. tuberosa* root extracts

Extracts	%W/W (phenolic content)	Flavonoid compounds %W/W		IC_{50} (µg/ml) Value
		Method-I	Method-II	DPPH Assay
ME	3.84 ± 0.060	0.042 ± 0.012	1.1 ± 0.226	26.17 ± 1.14
EAE	7.65 ± 0.140	—	—	571.00 ± 0.53
WE	3.84 ± 0.035	0.027 ± 0.010	0.75±0.056	60.28 ± 0.90
EAFME	14.04 ± 0.078	0.74 ± 0.047	1.5 ± 0.272	30.60 ± 0.88

Values represent mean ± standard deviation (n = 3)

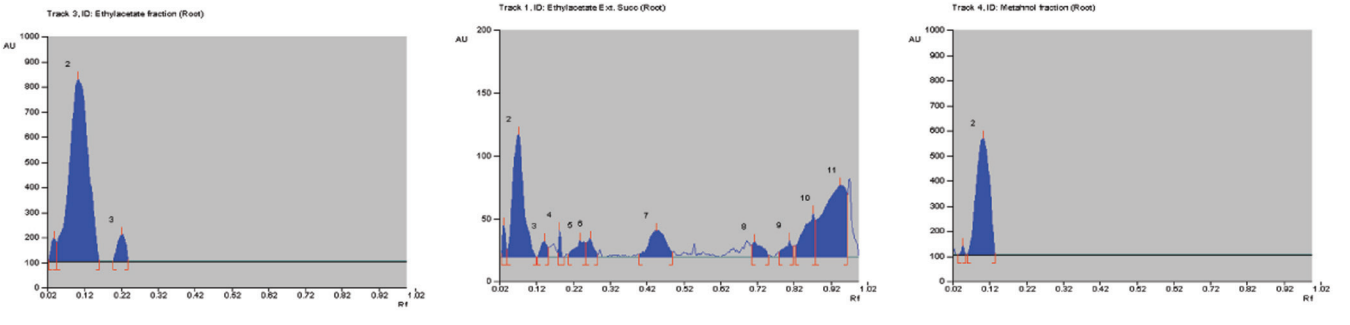

Figure 2. HPTLC Chromatogram for phenolic compounds of Ethyl acetate fraction, ethyl acetate extract and MeOH Fraction at 366 nm.

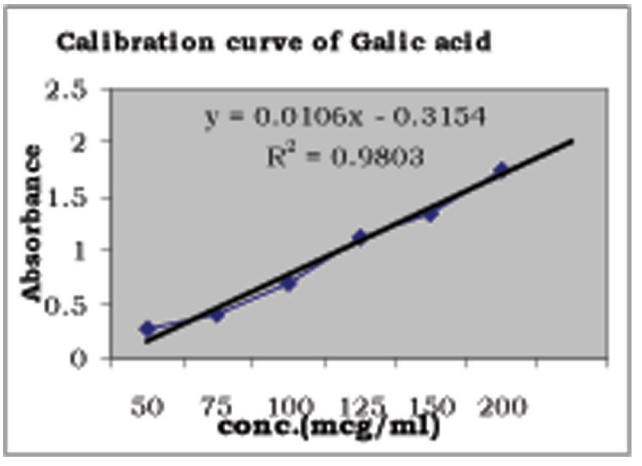

Figure 3. Calibration curve of Gallic acid.

group of flavones and flavonols. In addition, aluminum chloride forms acid labile complexes with them ortho-dihydroxylgroups in the A- or B-ring of Flavonoid.[39] Total flavonoid content was calculated as % quercetin.

Method II. 2, 4-Dinitrophenylhydrazine colorimetric method

The principle of 2, 4-Dinitrophenylhydrazine method is that 2, 4-dinitrophenyl hydrazine reacts with ketones and aldehydes to form 2, 4-dinitrophenylhydrazones, and show maximum absorbance at 495 nm. Naringin was used as a standard.

Results show that the ethyl acetate fraction of methanol extract (EAFME) of root contains higher amount of total phenolics, total flavonoid content as compared to methanol extract and water extract of root.

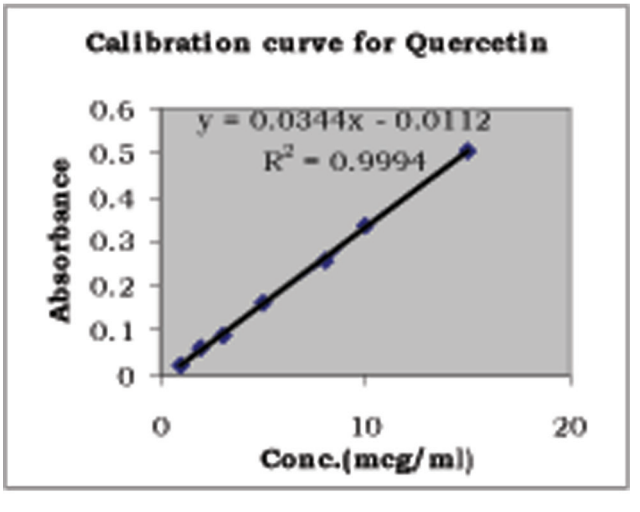

Figure 4. Calibration curve of quercetin.

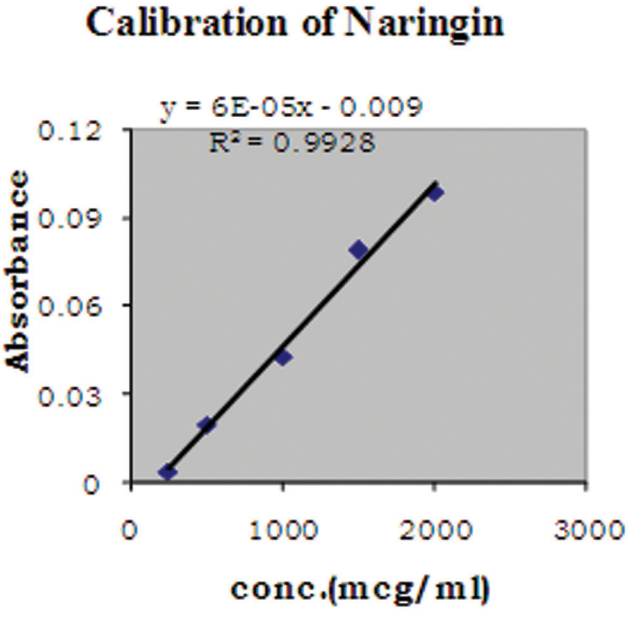

Figure 5. Calibration curve of Naringin.

Anti-oxidant activity *in vitro*

In vitro anti-oxidant activity for water extract, methanol extract and ethyl acetate fraction of methanol extract of root part of *Ruellia tuberosa* were carried out as per reported method.

DPPH free radical scavenging activity

DPPH (1, 1-diphenyl-2-picrylhydrazyl) is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule yellow-colored diphenylpicrylhydrazyl.[40,41] Ascorbic acid was used as standards. The reduction capability of DPPH radical is determined by the decrease in absorbance at 516 nm induced by anti-oxidants. It has been found that cysteine, glutathione, ascorbic acid, tocopherol, flavonoids, tannins, and aromatic amines (p-phenylenediamine, p-aminophenol, etc.), reduce and decolorize DPPH by their hydrogen donating ability.[42,43] This activity was expressed as decrease in absorbance of the samples at a different concentration levels. Comparison data of various extract of *R. tuberosa* roots of DPPH free radical scavenging activity were tabulated in Table 1. The IC_{50}: radical-scavenging activity (concentration in µg required for 50% inhibition of DPPH radical) was calculated from the graph [Figure 6]. A higher DPPH radical scavenging activity is associated with a lower IC_{50} value. The scavenging effect of extracts and standards with the DPPH radical is in the following order: AA> EAFME>Me> WE. EAFME shows higher radical-scavenging activity as

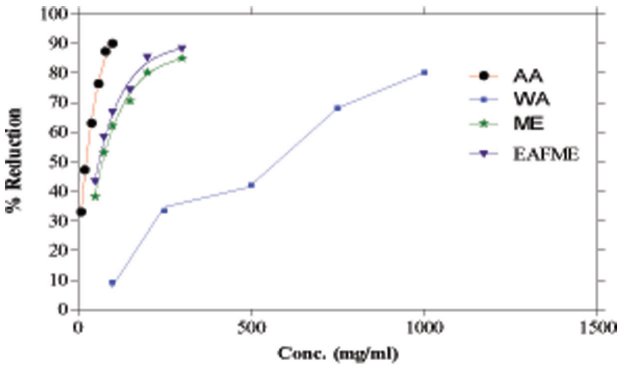

Figure 6. DPPH free radical scavenging activity *R. tuberosa* roots extract and standard.

compared to methanol and water extract en compared with Ascorbic acid [Table 1].

Ethyl acetate fraction of methanol extract of root show significant higher anti-oxidant activity as compared to methanol extract and water extract when ($P < 0.05$) compared with standard.

Reducing power by $FeCl_3$

In the present study, assay of reducing activity was based on the reduction of Fe3+/ferricyanide complex to the ferrous form in presence of reductants (anti-oxidants) in the tested samples. The Fe2+ was then monitored by measuring the formation of Perl's Prussian blue at 700 nm.[44] Figure 7 shows the reducing power of the extracts of *R. tuberosa* and standards, using the potassium ferricyanide reduction method. The reducing power of the extracts increased with increasing concentration.

Ethyl acetate fraction of methanol extract of root show higher anti-oxidant activity as compared to methanol extract and water extract when ($P < 0.05$) compared with standard.

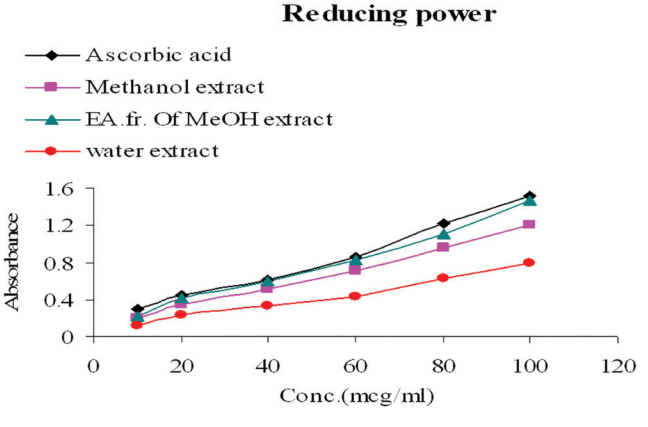

Figure 7. Reductive potential of *R. tuberosa* roots extracts and standard.

Statistical analysis

Results of estimation various phytochemical parameters have been reported as mean ±SD. The variation in a set of data has been estimated by performing one way analysis of variance (ANOVA) using Graph Pad Prism version 3.00 for Windows 97 and MS excel 2007. Value of $P < 0.05$ was considered as significant difference.

CONCLUSION

The root of *Ruellia tuberosa* has exhibited significantly anti-oxidant activity when tested with different *in vitro* assay. The ethyl acetate fraction of methanol extract (EAFME) showed better activity than Water extract and methanol extract of root. Also, amounts of both total phenolic and total flavonoid contents of EAFME were higher than methanol extract and water extract of root. The methanol extract and ethyl acetate fraction of methanol extract were showed significantly anti-oxidant activity.

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