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ANTIOXIDANT POTENTIAL FRACTIONATION FROM METHANOL EXTRACT OF AERIAL PARTS OF *RUELLIA PROSTRATA* POIR (ACANTHACEAE)

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ABSTRACT

Aerial parts of *Ruellia prostrata* were subjected to extraction and further fractionation to obtain antioxidant rich fraction. Different concentrations of methanolic extract and its n-butanolic fraction were subjected to antioxidant assay by DPPH method, Nitric oxide scavenging activity and reducing power assay. The fractions showed dose dependent free radical scavenging property in all the models. IC₅₀ values for methanolic extract and its n-butanolic fraction were found to be 137.89±5.74 and 42.25±3.17 respectively in comparison to L-Ascorbic acid and Rutin as standard with IC₅₀ values of 6.537±0.235 and 5.437±0.206 respectively, in DPPH model. In nitric oxide scavenging activity the IC₅₀ values were found to be 152.22±6.37 and 67.70±2.29, 21.06±0.953 and 29.93±0.324 for methanolic extract and its n-butanolic fraction, L-Ascorbic acid and Rutin respectively. The fractions showed good reducing power with increasing concentration. However, the n-butanolic fraction showed a good reducing power and better free radical scavenging activity as compared to methanolic extract thus its antioxidant potential is comparable to standards. The phytochemical screenings confirm the presence of triterpenoids glycosides and carbohydrates which are responsible for the antioxidant activity. This is the first report on antioxidant activity of *R. prostrata*.

Keywords:

Antioxidant,
R. prostrata,
Free radical

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INTRODUCTION: There is extensive evidence to implicate free radicals in the development of degenerative diseases¹⁻². Almost all organisms possess antioxidant defences that protect against oxidative damage and numerous damage removal and repair enzymes to remove or repair damaged molecules. However, the natural antioxidant mechanisms can be inefficient, hence dietary intake of antioxidant compounds become important³⁻⁵.

Although, synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butyl hydroquinone (TBHQ) have been widely applied in food processing, they have been reassessed for their possible toxic and carcinogenic components formed during degradation⁶. In addition, it has been suggested that there is an inverse relationship between dietary intake of antioxidant rich foods and incidence of number of human diseases⁷⁻⁸. Therefore, search into the isolation of natural antioxidant sources is important.

Ruellia prostrata Poir (Acanthaceae) is a perennial herb, commonly found as a weed throughout India. Reviewing the previous work of the *Ruellia prostrata* P. was found to have very little chemical and biological studies. This plant is widely distributed throughout India from Punjab and Assam to Ceylon, extending to east Africa, central and peninsular India⁹. The plant was well known for its traditional use as an anticancer against the epidermis of nasopharynx region, slightly hypoglycemic, seem to be potent diuretic and possess wound healing properties¹⁰⁻¹². It is also used in gonorrhoea, ear disease, as local drinks and prevents falling of hair^{9, 13-15}. The plant was reported to have for its antifertility activity¹⁶.

Phytochemical review show the presence of Flavonoid glycosides like apigenin 7-glucoside, luteoline 7-glucoside, apigenin 7 β -glucuronide¹⁷ and mixture of sterols like stigmasterol (75.33%), sitosterols (17.61%), 24-methylcholesterol (7.04%),

cholesterol (traces), brassicasterol (traces), amino acids¹³⁻¹⁴ and lupeol¹⁸, mucilage also present¹⁹. So it was found that it is of interest to study this plant and check out scientifically its uses in folk medicine and traced any new biological activities.

MATERIALS AND METHODS:

Source of chemicals: All chemicals used were of analytical grade. 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma Chemical Co. Rutin, L- Ascorbic acid, Tri chloroacetic acid (TCA) and ferric chloride were procured from central drug house (CDH), New Delhi. Sulphanilic acid and naphthylelene diamine dichloride from Rankem, Sodium nitroprusside and potassium ferricyanide were obtained from nice chemicals. Solvents used during the experiment were purchased from Rankem. The other chemicals used in this experiment were of the highest quality available. Absorbance were noted using UV/Visible Spectrophotometer (UV 1700, Pharmaspec, SHIMADZU, Japan)

Plant material: The Aerial parts of *R. prostrata* were collected from near Arawali hills dist. Bhiwani, Haryana in September, 2009. The plant was authenticated by Dr. H. B. Singh from Department of Raw Material Herbarium & Museum, National Institute of sciences Communication and Information Resources, New Delhi under the specimen no. (Ref. NISCAIR/RHMD/CONSULT/2009-10/1275/79 September 23, 2009). The collected aerial parts were cleaned, dried under shade at room temperature, and powdered.

Extraction and fractionation: Coarsely powdered aerial parts (100 g) were defatted with petroleum ether (60°-80°C) and dried marc was further extracted with hydroalcohol (90% methanol) using Soxhlet apparatus. The methanol extract so obtained was freed from solvent in a vacuum evaporator to obtain 10.5 g of extract. This was further extracted with chloroform, ethyl acetate and

n-butanol to obtain their respective fractions. The percentage yields are 0.6% w/w, 0.5% w/w and 10% w/w respectively.

Phytochemical analysis: Methanol, Chloroform, ethyl acetate and n-butanol fractions were subjected to phytochemical analysis for the presence of alkaloids, triterpenoids glycosides, carbohydrates, tannins, flavonoids²⁰.

In-vitro antioxidant studies: Methanolic extract and its n-butanolic fraction were tested for their free radical scavenging property using different *in-vitro* models. All experiments were performed thrice and their results averaged. L-Ascorbic acid and Rutin were used as standard control in each experiment. IC₅₀ values were calculated. The half maximal inhibitory concentration (IC₅₀) is a measure of the effectiveness of a compound in inhibiting biological or biochemical function.

This quantitative measure indicates how much of a particular drug or other substance is needed to inhibit a given biological process by half. In other words, it is the half maximal (50%) inhibitory concentration (IC) of a substance (50% IC, or IC₅₀). It is commonly used as a measure of pharmacological research. Sometimes, it is also converted to the IC₅₀ scale (-log IC₅₀), in which higher values indicate exponentially greater potency. According to the FDA IC₅₀ represents the concentration of a drug that is required for 50% inhibition *in-vitro*.

DPPH radical scavenging activity²¹⁻²²: DPPH radical scavenging activity was performed according to the method of Blois. The 0.1 mM solution of DPPH in methanol (22.2 mg in 1000 ml) was freshly prepared. Different concentrations of extract and standards (5-250 µg/ml) were added at an equal volume to methanolic solution of DPPH. After 30 min at room temperature, the absorbance was recorded at 517nm. Radical scavenging activity was calculated by the following formula (**formula 1**);

$$\% \text{ inhibition} = \frac{A_{\text{control}} - A_{\text{test}}}{A_{\text{control}}} \times 100 \dots \dots \dots 1$$

Where, A_{control} = Absorbance of control reaction and A_{test} = Absorbance in the presence of the samples of extracts.

Nitric oxide radical scavenging activity²³⁻²⁴: Nitric oxide radical scavenging activity was performed according to the method of Garrat. 2 mL of 10 mM sodium nitroprusside in 0.5 mL phosphate buffer saline (pH 7.4) was mixed with 0.5 mL of extract at various concentrations (10-200 µg/ml) and the mixture incubated at 25°C for 150 min. From the incubated mixture 0.5 mL was taken out and added into 1.0 ml sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min., finally, 1.0 mL Naphthylethylenediamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min before measuring the absorbance at 540 nm. The nitric oxide radicals scavenging activity was calculated by the formula 1.

Reducing power assay²⁵⁻²⁶: Reducing power assay was performed according to the method described by Oyaizu. The extract (0.75 mL) at various concentrations (25-400 µg/ml) was mixed with 0.75 mL of phosphate buffer (0.2 M, pH 6.6) and 0.75 mL of potassium ferricyanide (1%, w/v), followed by incubating at 50°C in a water bath for 20 min. The reaction was stopped by adding 0.75 mL of trichloroacetic acid (TCA) solution (10%) and then centrifuged at 3000 r/min for 10 min. 1.5 mL of the supernatant was mixed with 1.5 mL of distilled water and 0.1 mL of ferric chloride (FeCl₃) solution (0.1%, w/v) for 10 min. The absorbance at 700 nm was measured as the reducing power. Higher absorbance of the reaction mixture indicated greater reducing power.

Statistical analysis: All the analysis was done in triplicate and results are expressed as ± mean S.D. IC₅₀ values were determined by interpolations.

RESULTS:

Phytochemical screening: Triterpenoids, glycosides and carbohydrates were found in methanolic and n-butanol fraction.

Antioxidant studies: Several concentrations of methanolic extract and its n-butanol fraction ranging from (5-400 $\mu\text{g/ml}$) were tested for their antioxidant activity in different *in-vitro* models. It has been observed that n-butanol fraction exerted higher antioxidant potential as compared to methanolic extract and its antioxidant activity is comparable to the standards Rutin and L-Ascorbic acid. The antioxidant activity increased with increasing concentration in all the models in both fraction and extract. The percentage inhibition of standards, extract and fraction in various models viz. DPPH and nitric oxide scavenging assay are shown in **figure 1-11**. IC_{50} values of both standards and extracts in the DPPH model and nitric oxide scavenging assay are shown in **table 1** and **2** respectively. Reducing power of fractions was good and increased with increasing concentration. The n-butanol fraction showed a significant reducing ability in comparison to Rutin and L-Ascorbic acid (**table 3**).

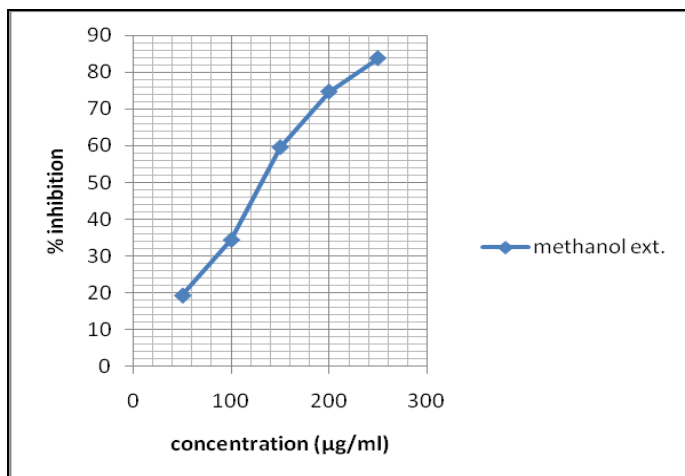


FIG. 1: FREE RADICAL SCAVENGING EFFECT OF METHANOL EXTRACT OF AERIAL PART OF *R. PROSTRATA* BY DPPH

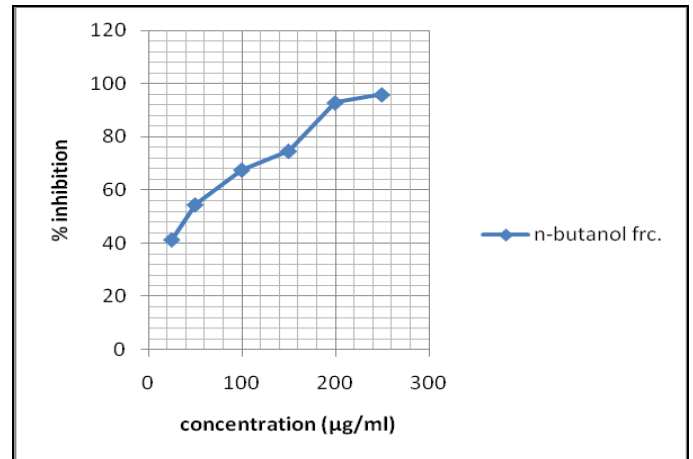


FIG. 2: FREE RADICAL SCAVENGING EFFECT OF N-BUTANOLIC FRACTION OF METHANOL EXTRACT OF AERIAL PART OF *R. PROSTRATA* BY DPPH

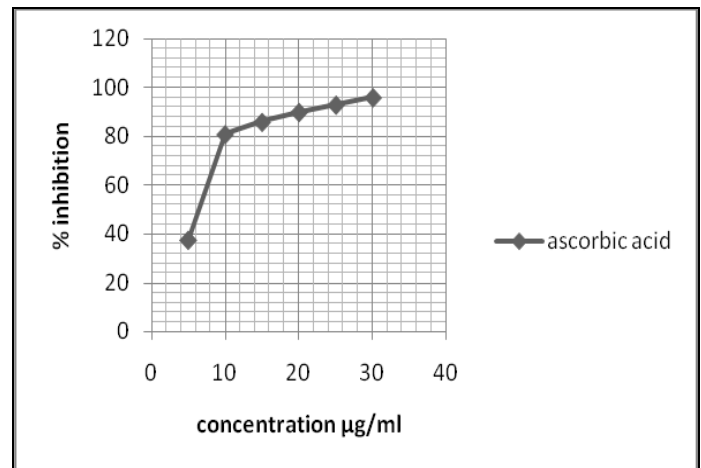


FIG. 3: FREE RADICAL SCAVENGING EFFECT OF THE ASCORBIC ACID BY DPPH

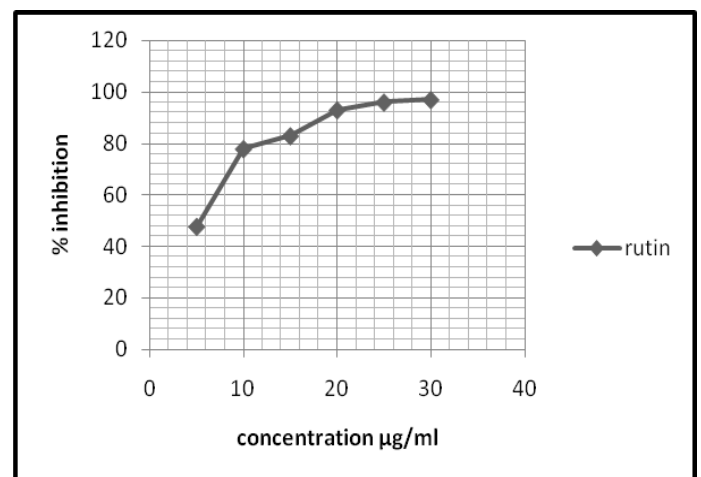


FIG. 4: FREE RADICAL SCAVENGING EFFECT OF THE RUTIN BY DPPH

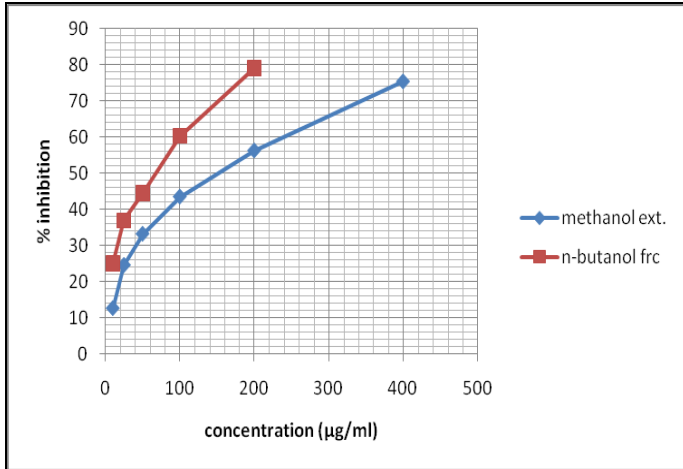


FIG. 5: NITRIC OXIDE SCAVENGING EFFECT OF METHANOL EXTRACT AND ITS N-BUTANOLIC FRACTION OF AERIAL PART OF RUELLIA

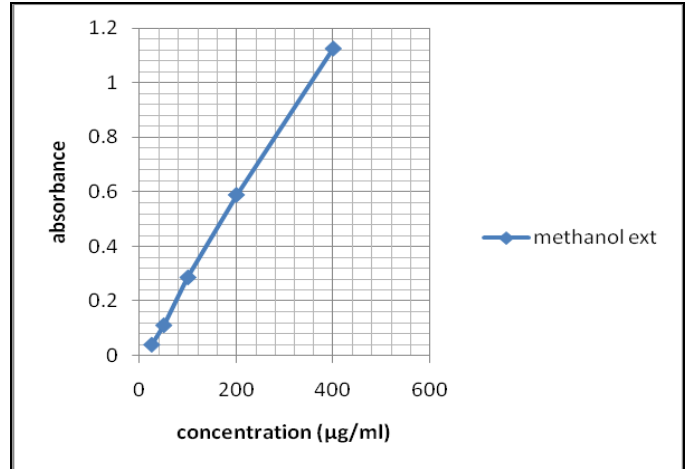


FIG. 8: TOTAL REDUCING POWER OF METHANOL EXTRACT OF AERIAL PART OF *R. PROSTRATA*

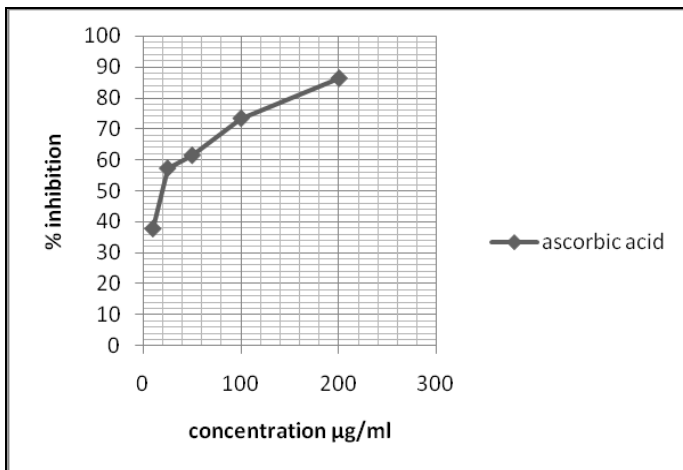


FIGURE 6: NITRIC OXIDE SCAVENGING EFFECT OF THE ASCORBIC ACID

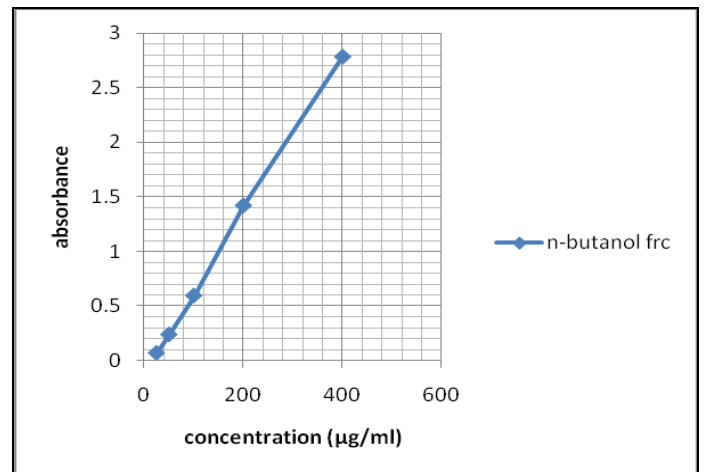


FIG. 9: TOTAL REDUCING POWER OF N-BUTANOLIC FRACTION

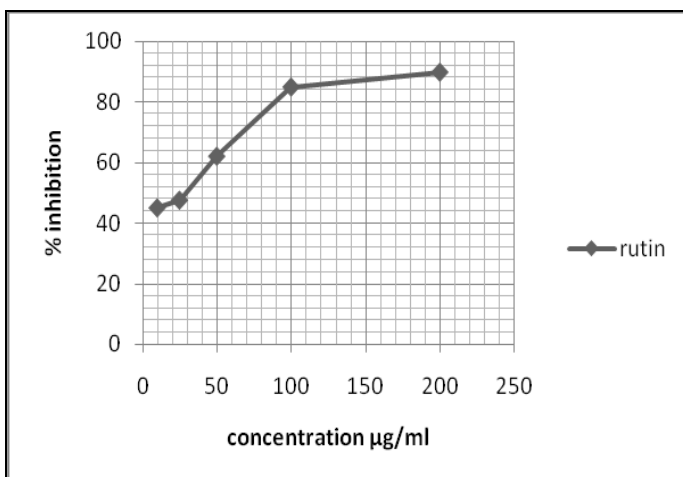


FIG. 7: NITRIC OXIDE SCAVENGING EFFECT OF THE RUTIN

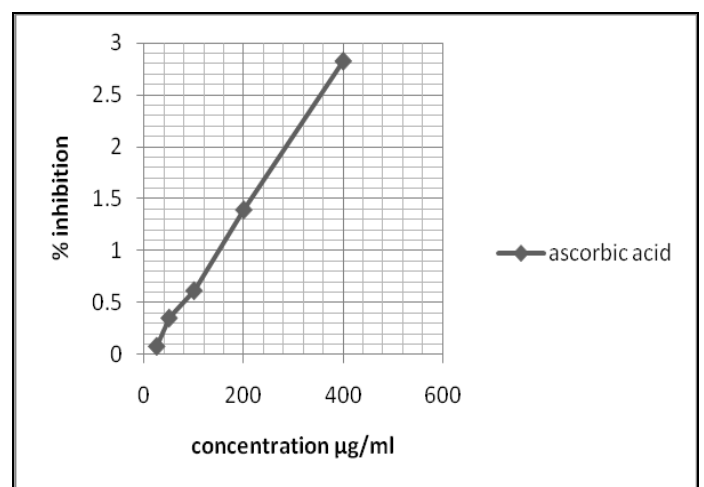


FIG. 10: TOTAL REDUCING POWER OF THE ASCORBIC ACID

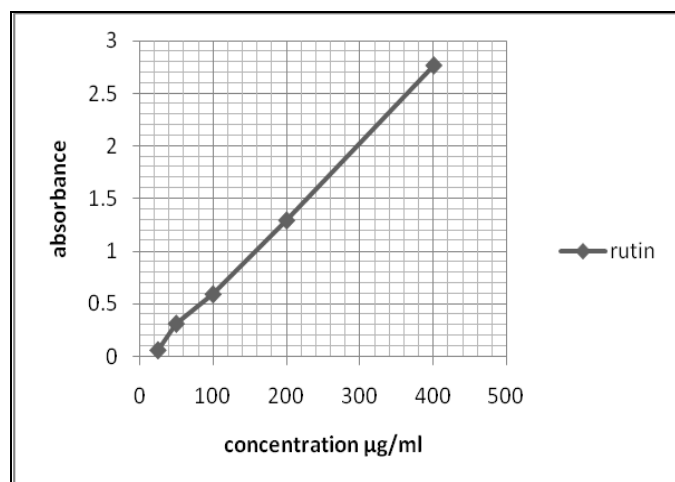


FIG. 11: TOTAL REDUCING POWER OF THE RUTIN

TABLE 1: IC₅₀ VALUES OF FREE RADICAL SCAVENGING EFFECT BY DPPH METHOD OF STANDARDS, EXTRACT AND ITS FRACTIONS (VALUES ARE MEAN ± S.D OF 3 REPLICATES)

Fraction	IC ₅₀ (µg/ml) ± S.D.
Rutin	5.437±0.20
Ascorbic Acid	6.537±0.23
n-butanol fraction	42.25±3.17
Methanolic extract	137.89±5.74

(Values are mean ± S.D of 3 replicates)

TABLE 2: IC₅₀ VALUES OF NITRIC OXIDE SCAVENGING ACTIVITY OF VARIOUS FRACTIONS AND STANDARDS

Fraction	IC ₅₀ (µg/ml) ± S.D.
Rutin	29.93±0.32
Ascorbic Acid	21.06±0.95
n-butanol fraction	67.70±2.29
Methanolic extract	152.22±6.37

(Values are mean ± S.D of 3 replicates)

TABLE 3: REDUCTIVE ABILITY OF *R. PROSTRATA* AND STANDARDS

Conc. (µg/ml)	Ascorbic acid	Rutin	n-butanol fractions	Methanolic extract
25	0.075	0.062	0.067	0.041
50	0.347	0.313	0.236	0.112
100	0.611	0.592	0.592	0.287
200	1.389	1.293	1.418	0.589
400	2.82	2.763	2.782	1.124

(Values are mean ± S.D of 3 replicates)

DISCUSSION: Free radicals are chemical entities that can exist separately with one or more unpaired electrons. The propagation of free radicals can bring about thousands of reactions and thus may cause extensive tissue damage. Lipids, proteins and DNA are all susceptible to attack by free radicals²⁷⁻³⁰. Antioxidants may offer resistance against oxidative stress by scavenging the free radicals and by their reducing ability.

DPPH (1, 1- Diphenyl- 2- picrylhydrazyl) is stable nitrogen centred free radical which can be effectively scavenged by antioxidants and shows strong absorbance at 517nm. DPPH radical accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The change in absorbance of DPPH radical caused by antioxidants is due to the reaction between the antioxidant molecules and the radical, which results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a discoloration from purple to yellow. Extent of DPPH radical scavenged was determined by the decrease in intensity of violet colour in the form of IC₅₀ values³¹.

Hence it has been widely used for rapid evaluation of the antioxidant activity of plant and microbial extracts relative to other methods³². DPPH is also considered as a good kinetic model for peroxy radicals³³. The methanolic extract and the n-butanol fraction showed significant DPPH scavenging activity (Table 1) (IC₅₀=137.89±5.74 µg/ml and IC₅₀=42.25±3.17µg/ml) respectively when compared with the IC₅₀ values of the standards Ascorbic acid and Rutin (IC₅₀=6.537±0.235 and 5.437±0.206 µg/ml respectively).

Nitric oxide (NO) is a potent pleiotropic inhibitor of physiological processes such as smooth muscle relaxation, neuronal signalling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical that plays many roles as an effectors molecule in diverse biological systems including neuronal messenger,

vasodilatation and antimicrobial and antitumor activities³⁴. Although nitric oxide is involved in host defence, over production of these this radical contributes to the pathogenesis of some inflammatory diseases³⁵.

Moreover in the pathological conditions, nitric oxide reacts with superoxide anion and form potentially cytotoxic molecules, peroxy nitrite. Nitric oxide inhibitors have been shown to have beneficial effects on some aspect of inflammation and tissue damage seen in inflammatory diseases²⁷. *Ruellia prostrata* significantly inhibited nitric oxide in a dose dependent manner (Table 2) with the IC 50 being 152.22±6.37 and 67.70±2.29 µg/ml for methanolic and its n-butanolic fraction respectively as compared with the standards Ascorbic acid and Rutin having the IC 50 values of 21.06±0.953 and 29.93±0.324 µg/ml respectively.

Figure 8 show the reductive capability of the methanolic extract is less prominent than n-butanolic fraction with reference to Ascorbic acid and Rutin (standards). The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reducing activities are generally associated with the presence of reductones, which have been shown to exhibit antioxidant action by breaking the chain reactions by donating a hydrogen atom.

Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation³⁶. For the measurement of the reductive ability, we investigated the ferric (Fe³⁺)-ferrous (Fe²⁺) transformation in the presence of extracts using the method of Oyaizu. In this method, antioxidant compounds form a coloured complex with potassium ferricyanide, trichloroacetic acid and ferric chloride that was measured at 700 nm. Increase in absorbance of the reaction mixture indicates the increase in the reducing power of the sample.

CONCLUSION: The antioxidant activity has been attributed to various mechanisms, among which are the prevention of chain initiation, the binding of transition metal ion catalysts, decomposition of peroxides, the prevention of continued hydrogen abstraction, the reductive capacity and free radical scavenging³⁷. Both the n-butanolic and methanolic extract were showing the antioxidant potential but the antioxidant potential of n-butanolic fraction is far higher than the methanolic extract and is comparable to the standards Rutin and L-Ascorbic acid. Hence, the plant *R. prostrata* have antioxidant potential.

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