ANTIOXIDANT STUDIES OF METHANOLIC EXTRACT OF *RANDIA DUMETORUM* LAM.

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Summary

Antioxidants, or anti-oxidation agents, reduce the effect of dangerous oxidants by binding together with these harmful molecules, decreasing their destructive power. Antioxidants can also help to repair damage already sustained by cells. *Randia dumetorum* Lam. is a plant of medicinal important belongs to the family Rubiaceae. Fruit of this plant has been used as a folk medicine to cures abscess, ulcers, inflammations, wounds, tumors, skin diseases, piles and have antibacterial activity. The qualitative and quantitative estimation of major constituents of crude extract was carried out using standard methods and contained total phenol (0.292 mg gallic acid equivalent) and total triterpenoids (17.66%). Keeping in view of the cited activity, it is contemplated to screen the Methanolic extract of fruit for *in vitro* antioxidant activity using different models *viz.* reducing power assay, DPPH radical scavenging assay, nitric oxide scavenging assay, super oxide scavenging assay, lipid peroxidation assay, total antioxidant capacity and non-enzymatic haemoglobin glycosylation assay . The results were analyzed statistically by regression method. The % scavenging activity at different concentrations was determined and the IC_{50} value of the extracts was compared with that of standard, ascorbic acid. Its antioxidant activity was estimated by IC_{50} value and the values are 50.51 µg/ml (reducing power assay), 41.03 µg/ml, (DPPH scavenging assay), 86.71 µg/ml (nitric oxide scavenging assay), 81.11 µg/ml (super oxide scavenging assay) and 124.39 µg/ml (lipid peroxidation assay). In total antioxidant capacity assay, 1 mg of extract is equivalent to 43 µg of ascorbic acid. It showed 53.74% inhibition of haemoglobin glycosylation. In all the testing, a significant correlation existed between concentrations of the extract and percentage inhibition of free radicals, metal chelation. The antioxidant property may be related to the polyphenols present in the extract. These results clearly indicate that *Randia dumetorum* is effective against free radical mediated diseases.

Keywords: Antioxidant, lipid peroxidation, nitric oxide, *Randia dumetorum*, polyphenols.

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Introduction

The antioxidant prevents the risk of several aging related diseases including cancer, cardio vascular disorder, diabetes, neurodegenerative disorders and others[1]. In recent years, phytochemicals are increasingly purported to exert potent beneficial actions to support health and may play a role in reducing synthetic drug use for the treatment of metabolic complications. To this effect, research has focused on the identification and isolation of compounds from natural products with high antioxidant capacities[2-4]. In Indian system of medicine *Randia dumetorum* (Rubiaceae) is an important medicinal plant and popularly known as emetic nut. It is found in waste places & jungles all over India, extending northwest to the Bias river & ascending to outer Himalaya to 4000 ft. Ceylon, Java, & South China. Also found in Gonda, Garhwal & Baraitch division of Oudit forest[5]. Literature survey reveals that the fruit is bitter, sweet; heating, aphrodisiac, emetic, purgative, carminative, alexiteric, antipyretic; cures abscess, ulcers, inflammations, wounds, tumors, skin diseases, "vata" and "kapha", piles and have antibacterial activity. The pulp of fruit is believed by many practitioners to also have anthelmintic properties, and also used as an abortifacient. Fruit is ground into coarse powder and applied to the tongue and palate, it is highly esteemed as a domestic remedy for the fevers and incidental ailments which children are subjected to, while teething. In colic, the fruit is rubbed to paste with rice water and applied over the naval[5-7]. It contains triterpenoidal saponins (2-3%) in fresh & 10% in dried fruit. They are mostly concentrated in pulp. A mixture of two saponin Randia or neutral saponin (mp-289-90°) & Randia acid (mp-260°) which occurs at all stage of ripening. These all saponins yield oleanolic acid as sapogenin on hydrolysis. In later investigation urosaponin was also isolated. It also contains essential oil, veleric acid, tannins and resin[8].

Materials and Methods

All chemicals and solvents were of analytical grade and were obtained from Ranbaxy Fine Chemicals, Mumbai, India. 1, 1- diphenyl, 2- picryl hydrazyl (DPPH) was obtained from Sigma Chemicals, USA. TBA (Thiobarbituric acid), TCA (Trichloro acetic acid) and BHT (Butylated hydroxytoluene) obtained from Himedia, Mumbai.

The other chemicals used were O-Phenanthroline, ferric chloride, ascorbic acid, sodium nitropruside, dimethyl sulphoxide, NBT (Nitro blue tetrazolium chloride), Folin Ciocalteu's reagent, Gallic acid, sodium carbonate, sodium hydroxide and potassium chloride. UV spectrophotometer (Shimadzu 1650), homogenizer (Remi, India), centrifuge (Remi, India), pH meter (Elico Ltd., India) were the instruments used for the study.

Plant material

Fruits of *Randia dumetorum* were collected during November from Botanical garden of M.S.U. Baroda and were identified by Head of Botany department, M. S. University, Baroda. A voucher specimen has been deposited in the museum of department of Pharmacognosy, M.S.U. Baroda. Voucher specimen (PH-805) was deposited in the herbarium of Pharmacy Department of M.S.U. Baroda.

Plant extract

The fruits were dried in sunlight and reduced to a coarse powder. The powdered materials were subjected to qualitative tests for the identification of various phytoconstituents like alkaloids, glycosides, steroids, terpenoids and flavanoids. Then the powder was subjected to soxhlet extraction with methanol for 72 hours at a temperature of 50-60ºC. The extract was concentrated and the solvent was completely removed. They were freeze dried and stored in the vacuum dessicator until further use.

***Preliminary phytochemical screening*[9]**

Preliminary phytochemical screening revealed the presence of phytosterol, polyphenol, saponins, flavonoids and carbohydrates.

Determination of Total polyphenolic compounds

Total polyphenolic compounds were determined according to a protocol similar to that of *Singleton* and *Rossi*[10]. From the stock solution (1 mg/ml) of the extract, suitable quantity was taken into a 25 ml volumetric flask and mixed with 10 ml of water and 1.5 ml of Folin Ciocalteu's reagent. After 5 minutes 4 ml of 20 % w/v sodium carbonate solution was added and volume was made up to 25 ml with double distilled water.

The absorbance was recorded at 765 nm after 30 minutes. % of total phenolics was calculated from calibration curve of Gallic acid (50-250 µg) plotted by using the same procedure and total phenolics were expressed as % Gallic acid.

***Reducing power ability*[11]**

The reducing power was investigated by the Fe^{3+}-Fe^{2+} transformation in the presence of the extracts. The Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Two ml of the extract (5-1000 µg/ml), 2.5 ml of phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide were incubated at 50°C for 30 min and 2.5 ml of 10% trichloroacetic acid was added to the mixture and centrifuged for 10 min at 3000g. About 2.5 ml of the supernatant was diluted with 2.5 ml of water and is shaken with 0.5 ml of freshly prepared 0.1% ferric chloride. The absorbance was measured at 700 nm. Ascorbic acid (5-500µg/ml) was used as the standard. All tests were performed in triplicate and the graph was plotted with the average of the three determinations.

***DPPH radical scavenging assay*[12-13]**

Different concentrations of standard and test samples (5-1000 µg/ml) 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 methanol as a blank at zero minute. The % scavenging activity at different concentrations was determined and the IC_{50} value of the extracts was compared with that of ascorbic acid which was used as the standard. Experiment was performed in triplicate.

***Nitric oxide scavenging assay*[12]**

Sodium nitroprusside 5mM was prepared in phosphate buffer pH 7.4. To 1 ml of various concentrations of test compound (5-1000 µg/ml), 0.3 ml of sodium nitroprusside was added. The test tubes were incubated at 25 ºC for 5 hr after which, 0.5 ml of Griess reagent was added. The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with napthyl ethylene diamine was read at 546 nm. The experiment was performed in triplicate.

***Superoxide scavenging assay*[14, 16]**

100 µl riboflavin solutions, 200 µl EDTA solution, 100 µl of test sample(5-1000 µg/ml), 200 µl methanol and 100 µl NBT solution was mixed in a test tube and the reaction mixture was diluted up to 3 ml with phosphate buffer. The absorbance of the solution was measured after illumination for 5 minute at 590 nm. For control omit the sample. Higher absorbance indicates lesser antioxidant power. The % scavenging activity at different concentrations was determined and the IC50 value of the extracts was compared with that of ascorbic acid (5-1000 µg/ml), which was used as the standard. Experiment was performed in triplicate.

***Lipid peroxidation assay*[15]**

Egg phosphotidylcholine (20mg) in chloroform (2ml) was dried under vacuum in a rotary evaporator to give a thin homogenous film and further dispersed in normal saline (5ml) with a vortex mixture. The mixture was sonicated to get a homogeneous suspension of liposome. Lipid peroxidation was initiated by adding 0.05 mM ascorbic acid to a mixture containing liposome (0.1ml). 150 mM potassium chloride, 0.2 mM ferric chloride, drug solution (2-64 µg/ml) were added separately in a total volume of 1ml. The reaction mixture was incubated for 40 min at 37ºC. After incubation, the reaction was terminated by adding 1ml of ice cold 0.25M sodium hydroxide containing 20% w/v TCA, 0.4% w/v TBA and 0.05% w/v BHT. After keeping in boiling water bath for 20 min, the samples were cooled. The pink chromogen was extracted with constant volume of n-butanol and absorbance of the upper organic layer was measured at 532nm. The experiment was performed in triplicate.

***Total Antioxidant Capacity*[20]**

The total antioxidant capacity of the extract was determined with phosphomolybdenum using as ascorbic acid the standard. An aliquot of 0.1ml of the extracts (1mg) solution was combined with 1.0 ml of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95^{o}C for 90 min. After the samples had cooled to room temperature, the absorbance was measured at 695 nm against the blank using an UV spectrophotometer.

The blank solution contained 1.0 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions as rest of the sample. The total antioxidant capacity was expressed as µg equivalents of ascorbic acid by using the standard ascorbic acid graph.

***Non-enzymatic haemoglobin glycosylation assay*[17]**

The antioxidant activity of extracts was investigated by estimating degree of non-enzymatic haemoglobin glycosylation, measured colorimetrically. Haemoglobin, 60 mg/100 ml in 0.01 M phosphate buffer (pH 7.4) was incubated in presence of 2 g/100 ml concentration of glucose for 72 h, in order to find out the best condition for haemoglobin glycosylation. The assay was performed by adding 1 ml of glucose solution, 1 ml of haemoglobin solution, and 1 ml of gentamycin (20 mg/ 100 ml), in 0.01 M phosphate buffer (pH 7.4). The mixture was incubated in dark at room temperature for 72 h. The degree of glycosylation of hemoglobin in the presence of different concentration of extracts and their absence were measured colorimetrically at 520 nm. The experiment was performed in triplicate.

Statistical analysis

All results are expressed as mean ± S.E.M. Linear regression analysis (Origin 6.0 version) was used to calculate the IC_{50} values.

Results

Several concentrations ranging from 5-1000 µg/ml of the MeOH extract of fruits of *Randia dumetorum* Lam. were tested for their antioxidant activity in different *in vitro* models. It was observed that free radicals were scavenged by the test compounds in a concentration dependent manner up to the given concentration in all the models. The percentage scavenging and IC_{50} values were calculated for all models. The maximum inhibitory concentration (IC_{50}) in all models viz. reducing power ability, DPPH, nitric oxide scavenging, super oxide scavenging and lipid peroxidation assay were reported in the Table 1.

In total antioxidant capacity assay, it was found that 1 mg of extract is equivalent to 43 µg of ascorbic acid. It showed 53.74% inhibition of haemoglobin glycosylation with a concentration of 1.0 mg/ml in non-enzymatic haemoglobin glycosylation assay. On a comparative basis the extract showed better activity in all in vitro antioxidant models except moderate in lipid peroxidation assay. Total phenolics content was found to be 0.292 mg/ml. The antioxidant activity of the extract is close and identical in magnitude, and comparable to that of standard antioxidant compounds used. IC_{50} value for different assays was calculated (Table-1).

Table 1: IC_{50} value for different assays

Sr. No.	Assay method	MeOH ext. (µg/ml)	Ascorbic acid (µg/ml)
1	Reducing power assay	50.51	6.74
2	DPPH scavenging assay	41.03	3.44
3	Super oxide scavenging assay	81.11	27.23
4	Nitric oxide scavenging assay	86.71	28.44
5	Lipid peroxidation assay	124.39	42.5

Discussion

Phenolic compounds are the principal antioxidant constituents of natural products and are composed of phenolic acids and flavonoids, which are potent radical terminators[18]. They donate hydrogen to radicals and break the reaction of lipid oxidation at the initiation step. Caffeic acid, ferulic acid, and vanillic acid are examples of phenolic acids isolated as natural antioxidants in fruits, vegetables, and other plants[19]. The high potential of polyphenols to scavenge free radicals may be because of their many phenolic hydroxyl groups[20]. Free radicals have been implicated in many disease conditions, the important ones being superoxide radical, hydroxyl radical, peroxyl radical and singlet oxygen. Herbal drugs containing radical scavengers are gaining importance in treating such diseases. Many plants exhibit efficient antioxidant properties owing to their phenolic constituents[21].

The reducing power of MeOH ext. of fruits of R. dumetorum to reduce ferric ions was determined in this study. Extract had significant reducing power and also was in dose dependent manner (fig-1).

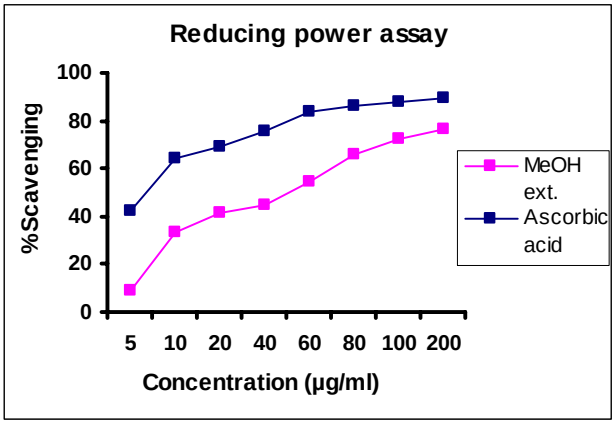

Fig.-1 Antioxidant activity of different concentrations of MeOH extract and ascorbic acid in reducing power method. Each value represents mean ± SEM radical

DPPH is one of the free radicals generally used for testing preliminary radical scavenging activity of a compound or a plant extract. DPPH radical is a stable free radical in an aqueous or methanol solution. Because of the odd electron of DPPH, it gives a strong absorption maximum at 517 nm by visible spectroscopy (purple color). The antioxidant activity measured by the capacity of odd electron of the radical becomes paired off in the presence of extract (hydrogen donor). When it becomes paired off, the absorption strength is decreased, and the resulting decolorization is stoichiometric with respect to the number of electrons captured[22] (fig.-2).

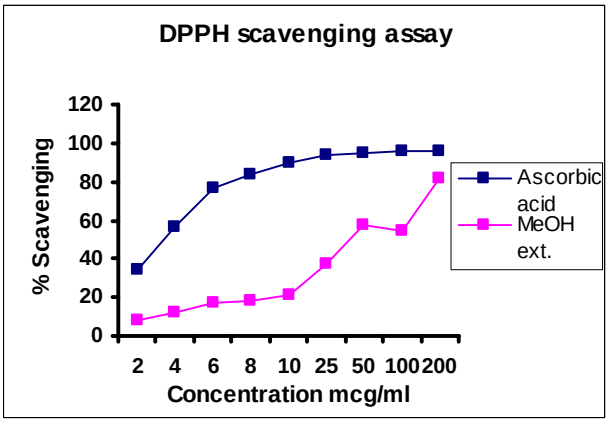

Fig.-2 Antioxidant activity of different concentrations of MeOH extract and ascorbic acid in DPPH scavenging assay. Each value represents mean ± SEM radical.

Nitric oxide (NO), a short lived free radical generated endogenously, is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. Besides mediating normal function, NO has been implicated in pathophysiological states, e.g. DNA fragmentation, cell damage and neuronal cell death. NO generated from sodium nitroprusside in aqueous solution at physiological pH reacts with oxygen to form nitrite ions[23]. MeOH ext. of fruits of R. dumetorum significantly inhibited ($p<0.05$) nitrite formation in dose dependant manner (Fig) This may be due to antioxidant principles in the extract, which compete with oxygen to react with nitric oxide(fig-3).

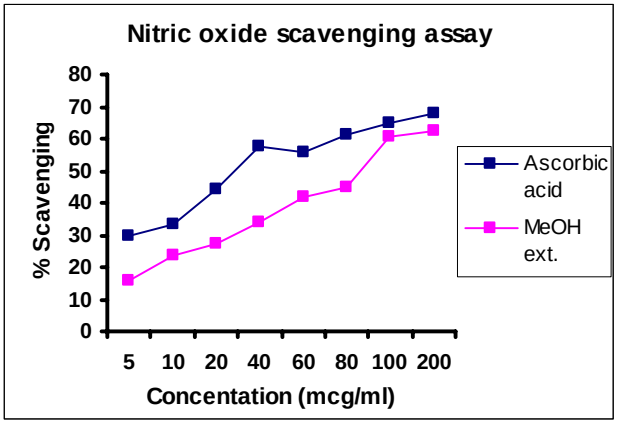

Fig.-3 Antioxidant activity of different concentrations of MeOH extract and ascorbic acid in nitric oxide scavenging method. Each value represents mean ± SEM.

The formation of superoxide radical leads to a cascade formation of other reactive oxygen species in the cell. Endogenously, superoxide could be produced in large amounts by various metabolic and physiological processes[24]. Both aerobic and anaerobic organisms possess super oxide dismutase enzymes, which catalyses the breakdown of superoxide radical[25]. In our study, alkaline DMSO used for superoxide generation. In studying the effect of MeOH extract on superoxide radical, we observed that the extract was significantly (P b .001) more effective in a concentration-dependent manner as a scavenger of the superoxide radical. (Fig.-4).

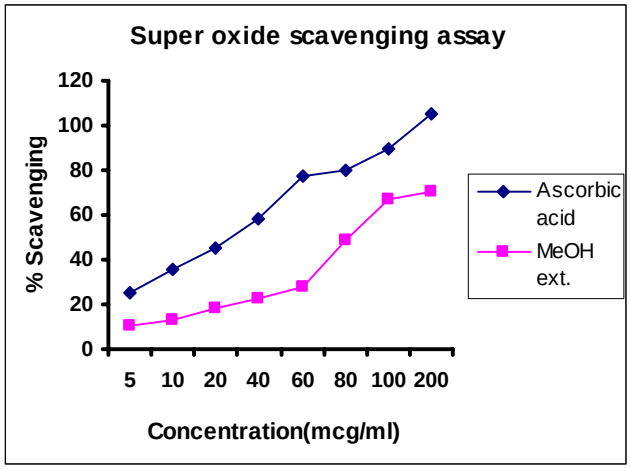

Fig. 4 Antioxidant activity of different concentrations of MeOH extract and ascorbic acid in super oxide scavenging method. Each value represents mean ± SEM.

Initiation of the lipid peroxidation by ferrous sulphate takes place either through ferrylperferryl complex or through OH radical by Fenton's reaction. The lipid radicals thus generated would initiate chain reaction in the presence of oxygen, giving rise to lipid peroxide, which breaks down to aldehydes such as malondialdehyde, which are known to be mutagenic and carcinogenic[26]. Thus the decrease in the MDA level in egg phosphatidylcholine with the increase in the concentration of the extract indicates the role of the extract as an antioxidant (Fig.-5).

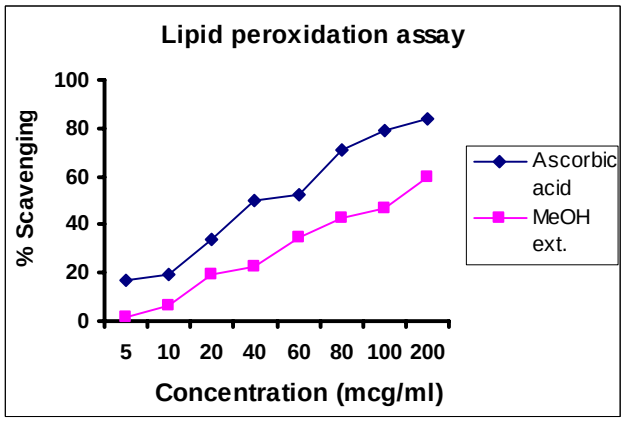

Fig. 5 Antioxidant activity of different concentrations of MeOH extract and ascorbic acid in lipid peroxidation assay. Each value represents mean ± SEM.

The total antioxidant capacity of the extract was calculated based on the formation of phosphomolybdenum complex which was measured spectrophotometrically at 695 nm. Non-enzymatic glycosylation of haemoglobin is an oxidation reaction in which, antioxidant is expected to inhibit the reaction. The degree of haemoglycosylation *in vitro,* in the presence of different concentration of extract, can be measured colorimetrically[17]. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides[27].

Conclusion

In conclusion, the results of the present study show that the extract of fruits of *R. dumetorum* exhibits the better *in vitro* antioxidant effect. From the *in vitro* studies, the antioxidant activity may be due to inhibiting the formation of radicals or scavenge the formed radical and it may be due to the presence of the phenolics compounds. Overall, the plant extract is a source of natural antioxidants that the high polyphenols concentration, as well as the in vitro scavenging activity of free radicals of the fruits extracts, justifies their application in nutrition. *R. dumetorum* could therefore provide a useful source of antioxidants in oxidative stress related disorders, but in vivo studies are needed to confirm this action.

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