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Research Article

EVALUATION OF *IN-VITRO* (NON & SITE-SPECIFIC) ANTIOXIDANT POTENTIAL OF *MIMOSA PUDICA* ROOTS

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ABSTRACT

The *in-vitro* antioxidant potential of petroleum ether extract, methanol extract and its ethyl acetate fraction of *Mimosa pudica* (Mimosaceae) roots were evaluated by various established *in-vitro* systems, including antioxidant capacity by 2,2-diphenyl-1-picryl hydrazyl (DPPH) free radical, superoxide (SO) radical, nitric oxide (NO) radical scavenging (non-site specific) and inhibition of lipid peroxidation in rat liver homogenate (site specific). These results were compared to standard antioxidants ascorbic acid, rutin and butylated hydroxy anisole (BHA). The ethyl acetate fraction exhibited high reduction capacity and powerful free radical scavenging against DPPH, SO radical scavenging, NO radical inhibition and inhibition of lipid peroxidation assay with IC_{50} values 58.88 ± 0.87, 85.85 ± 0.84, 67.52 ± 0.76 and 129.25 ± 1.09 µg/ml, respectively. Whereas, the petroleum ether extract was found to be inactive in all the tested methods and methanol extract showed moderate antioxidant activity with IC_{50} values 162.54 ± 1.26, 221.98 ± 1.66, 206.23 ± 1.33 and 176.5 ± 1.24 µg/ml, respectively. These results clearly reveals that ethyl acetate fraction act as most active non-site/site specific antioxidant for the biological systems susceptible to free radical-mediated reactions *viz.* hepatotoxicity, aging-related diseases etc. The maximum antioxidant potential of the ethyl acetate fraction may be attributed to the presence of high concentration of phenolic compounds (78.93 ± 2.73 mg gallic acid/g equivalent) such as flavonoids (39.29 ± 1.76 mg rutin/g equivalent) as evident by colorimetric determination. In addition, superimposed spectra with standard quercetin in HPTLC study confirm the presence of quercetin in ethyl acetate fraction. Based on these data, free radical-scavenging property due to presence of phenolic compounds such as flavonoids may be one of the mechanisms which clearly justify the medicinal claims attributed to this plant.

Keywords: Antioxidant, Free radical scavenging, HPTLC, Lipid peroxidation, *Mimosa pudica*

INTRODUCTION

Antioxidants provide protection to living organisms from damage caused by uncontrolled production of free radicals/reactive oxygen species (ROS), which initiates lipid peroxidation, protein damage and DNA strand breakings[1]. ROS are well known inducers of cellular and tissue pathogenesis leading to several human diseases such as hepatotoxicity, neurodegenerative disorders, cancer, cardiovascular diseases, atherosclerosis, cataracts, inflammatory disorders, as well as in aging processes[2, 3].

The antioxidants neutralise their effect by directly reacting with ROS, quenching them and/or chelating the catalytic metal ions[4]. It has been shown that a dynamic relationship exists between the extent of ROS production and antioxidant capacity of a cell system[5, 6]. Imbalance between the antioxidant defense systems and the formation of ROS results in oxidative stress, this stress may damage essential biomolecules such as proteins, DNA and lipids[7, 8]. Several synthetic antioxidants e.g. Butylated hydroxyl anisol (BHA) and butylated hydroxytoluene are commercially available but are quite unsafe and toxic[9]. Natural antioxidants, especially phenolics and flavonoids have been reported to possess antioxidant and antiradical properties, and are quite safe to be used[10, 11]. Therefore, in recent years, considerable attention has been directed towards identification of plants with antioxidant ability that may be used for human consumptions.

Mimosa pudica Linn. (Fabaceae) is creeping perennial herb, commonly known as touch-me-not, is traditionally used as an agent for birth control among rural people[12]. The roots extracts of *M. pudica* in combination with other medicinal plants are traditionally used in the treatment of diseases like diarrhea, amebic dysentery, headache, migraine, insomnia, cobra bite, fever, piles, fistula gynecological disorders, skin diseases and blood pressure disorders[13, 14]. The phytochemical characterization shows the presence of carbohydrates, tannins, steroids, alkaloids, triterpenes and flavonoids, glycosides, C-glycosylflavones[15, 16]. Other reported phytoconstituents are phenolics, norepinephrine and a non-protein amino acid leucenine (mimosine). A saponin and a bufadienolide are also reported in *M. pudica* seeds[17]. In present study, we evaluated the antioxidant and free radical scavenging potential of *Mimosa pudica* employing various *in-vitro* assay systems, *viz.* antioxidant capacity 2,2-diphenyl-1-picryl hydrazyl (DPPH) free radical, superoxide and nitric oxide radical scavenging. Further, inhibition of lipid peroxidation was evaluated in order to explore the mechanism of its antioxidant activity.

MATERIALS AND METHODS

Plant material

The root of *Mimosa pudica* was collected from Corbet National Park, Ramnagar, Uttrakhand, India and authenticated by Dr. H.B. Singh, Scientist F & Head, Raw Material Herbarium & Museum, National Institute of Science Communication and Information Resources (NISCAIR) New Delhi, India (Voucher specimen - NISCAIR/RHMD/Consult/-2010-11/1646/244).

Extraction and fractionation

The roots were chopped to small pieces and dried in shade. The dried powdered roots were passed through sieve no. 20 and extracted to exhaustion in a Soxhlet apparatus (200 g) with petroleum ether and methanol. The extracts were concentrated to dryness under reduced pressure and controlled temperature (40-50^{0}C). The methanol extract so obtained was further fractionated with ethyl acetate by sonication. The extracts and fraction were preserved in a refrigerator till further use.

Chemicals and reagents

2,2-Diphenyl-1-picryl hydrazyl (DPPH) was obtained from Sigma–Aldrich Co., St. Louis, USA. Rutin was obtained from Acros Organics, NJ, USA. Naphthyl ethylene diamine dihydrochloride (NEDD) was from Roch-Light Ltd., Suffolk, UK. Ascorbic acid, nitro blue tetrazolium (NBT) and butylated hydroxy anisole (BHA) were from SD Fine Chemicals Ltd., Mumbai, India and 2-deoxy-d-ribose was from Hi-Media Laboratories Pvt. Ltd., Mumbai, India. Sodium nitroprusside was from Ranbaxy Laboratories Ltd., Mohali, India. Sulphanilic acid used was from E-Merck (India) Ltd., Mumbai, India. All chemicals used were of analytical grade.

Instruments and softwares

UV-1700 Pharmaspec UV/VIS spectrophotometer model Shimadzu, HPTLC instrument set-up (Camag, Switzerland) with Linomat 5 sample applicator and scanner III with WinCats 4.02 integration software was employed. TLC Si-gel 60_{F-254} (20 × 20 cm × 0.25 mm)

aluminum plates were of E. Merk, Germany. Statistical analysis was performed using Graph Pad Instant Software, (Version 3.01).

Phytochemical screening

Screening of phytoconstituents present in the methanol extract and its ethyl acetate fraction were carried out by using respective testing reagents[18].

Preparation of test and standard solutions

Both the extracts and ethyl acetate fraction of *Mimosa pudica* root and the standard antioxidants (ascorbic acid, rutin and BHA) were dissolved in dimethyl sulphoxide (DMSO) separately and used for the *in-vitro* antioxidant assays. The stock solutions were serially diluted with the respective solvents to obtain lower dilutions.

Estimation of total flavonoids and total phenolics contents (colorimetric method)

Total flavonoids quantification was done on the basis of a standard curve of rutin. The absorbance of the reaction mixture of standard and test was measured at 415 nm (n=3). The results were expressed as mg rutin/g equivalents (RE) and percentage w/w[19]. The quantification of total phenolics was assessed on the basis of gallic acid standard curve. The absorbance of the resulting blue colour in standard and test was measured at 765 nm after 30 min (n=3). The results were expressed as mg gallic acid/g equivalents (GAE) and percentage w/w[20].

High performance thin layer chromatographic study (HPTLC)

HPTLC analysis of ethyl acetate fraction (10mg/ml MeOH) was performed by applying 8mm bands (application rate 80nLs^{-1}) on precoated TLC plate. Linear ascending development was carried with optimized mobile phase $CHCl_3$/MeOH/HCO_2H (9:1:0.1 v/v/v) at room temperature (25±2^0 C) and relative humidity (55± 5%). Standard quercetin (1mg/ml MeOH) was used and densitometric scanning was carried out at 365 nm in absorbance mode (slit dimension 6 × 0.30 mm at 20 mms^{-1}).

Free Radical Scavenging Assays

DPPH radical scavenging activity (non-site-specific)

In an ethanol solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical (final concentration: 1.0×10^{-4} M), test or the standard samples at different concentrations were added. The reaction mixtures were shaken vigorously and then kept in the dark for 30 min. The absorbance of the resulting solutions was measured at 517 nm, against blank without DPPH. Decreasing of DPPH solution absorbance indicates an increase of DPPH radical scavenging. The percentage inhibitions were plotted against log concentration and the IC_{50} was calculated[21].

Superoxide radical scavenging activity (non-site-specific)

1ml of alkaline DMSO (1ml DMSO containing, 5mM NaOH in 0.1ml water) was added to the reaction mixture containing 0.1ml of NBT (1 mg/ml solution in DMSO) and different concentrations of tests & standard samples in DMSO, to give a final volume of 1.4ml. The absorbance was measured at 560 nm. The decrease in the extent of NBT reduction measured by the absorbance of the reaction mixture correlates with the superoxide radical scavenging activity of extracts and fraction. The percentage inhibition was calculated and IC_{50} values were calculated by plotting % inhibition against log concentration[22].

Nitric oxide radical inhibition assay (non-site-specific)

The reaction mixture (6 ml) containing sodium nitroprusside (10 mM, 4 ml), phosphate buffer saline (1ml) and the tests and standard solutions (1ml) were incubated at 25^0C for 150 min. After incubation, 0.5 ml of the reaction mixture was removed and 1 ml of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) was mixed and allowed to stand for 5 min. For the completion of diazotization reaction, 1-naphthylethyelenediamine dihydrochloride (NEDD) was added and allowed to stand for another 30 min. The absorbance of the chromophore that formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with NEDD was immediately read at 540 nm against the corresponding blank. The NO radical percentage inhibition was calculated and IC_{50} values were calculated by plotting % inhibition against log concentration[23, 24].

Lipid peroxidation (Site-specific)

Lipid peroxidation was induced and assayed in rat liver homogenate. The reaction mixture, in a total volume of 1.0 ml, contained 0.58 ml phosphate buffer (0.1 M, pH 7.4), 0.2 ml of hepatic homogenate (10%, w/v), 0.02 ml ferric chloride (100 mM) and 0.1 ml of the different concentration of the tests and standard samples were incubated at 37^0C in a shaking water bath for 1 h. The reaction was stopped by the addition of 1.0 ml trichloroacetic acid (TCA; 10%, w/v). Following, to each tube 1.0 ml thiobarbituric acid (TBA; 0.67% w/v) was added and were placed in a boiling water bath for 20 min. At the end, the tubes were shifted to an ice-bath and centrifuged for 10 min. The amount of malonaldehyde formed in each of the samples was assessed by measuring the optical density of the supernatant at 535 nm against a reagent blank and from these values corresponding percentage inhibition were calculated, percentage inhibitions were plotted against log concentration to calculate IC_{50} values[25].

RESULTS

Phytochemical investigations revealed the presence of carbohydrates, proteins, alkaloids, phenolic compounds and flavonoids in the methanol extract. Ethyl acetate fraction extracted from methanol extract showed the presence of phenolic compounds and flavonoids. The petroleum ether extract showed the presence of steroids and triterpenes. Total phenolic and flavonoid contents by colorimetric quantification were estimated to be 78.93 ± 2.73 mg of gallic acid/g and 39.29 ± 1.76 mg of rutin/g equivalent of the dried ethyl acetate fraction, respectively (Table 1). In addition, HPTLC study showed the superimposed spectra of quercetin in the test with the standard quercetin at Rf 0.48 confirms the presence of quercetin in ethyl acetate fraction (Fig 2).

Among the two extracts and ethyl acetate fraction tested for *in-vitro* antioxidant activity, ethyl acetate fraction exhibited potent antioxidant activity in DPPH, superoxide radical scavenging, nitric oxide radical inhibition and inhibition of lipid peroxidation assays and the IC_{50} values were found to be 55.88 ± 0.87, 85.85 ± 0.84, 67.52 ± 0.76 and 129.25 ± 1.09 µg/ml, respectively. The values were found to be comparable to those obtained for the standards used. The petroleum ether extract was found to be inactive in all the methods tested. The methanol extract showed moderate antioxidant activity with IC_{50} value 162.54 ± 1.26, 221.98 ± 1.66, 206.23 ± 1.33 and 176.5 ± 1.24 µg/ml in DPPH, superoxide, nitric oxide radical scavenging and inhibition of lipid peroxidation assay, respectively (Table 2).

Table 1: Estimation of total flavonoids and total phenolics contents in methanol extract & ethyl acetate fraction

Test Sample	Total Phenolics (mg gallic acid/g equivalents)	Total Flavonoids (mg rutin/g equivalents)
Methanol extract	26.74 ± 1.41	15.43 ± 2.13
Ethyl acetate fraction	78.93 ± 2.73	39.29 ± 1.76

(Data are mean ± SEM values; n=3)

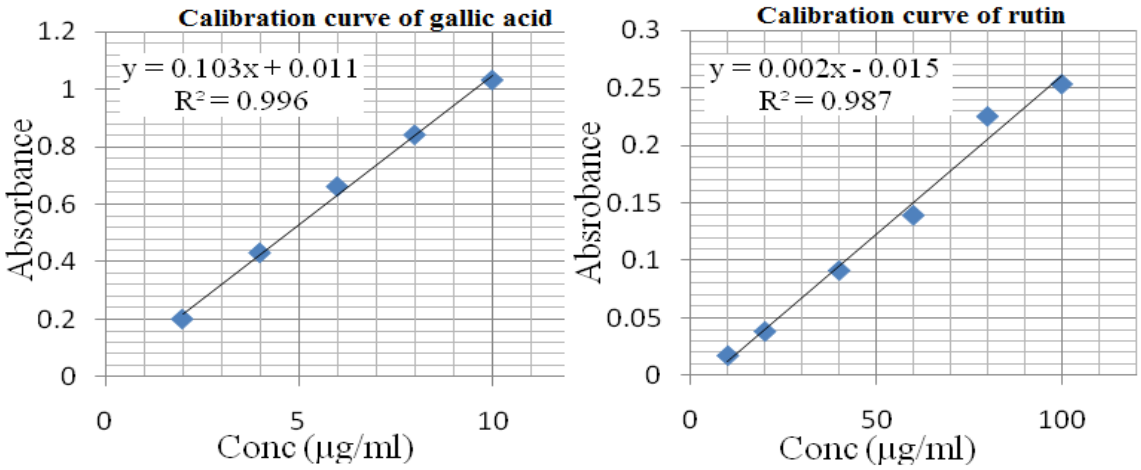

Fig. 1: Shows the calibration curve of standard rutin and gallic acid

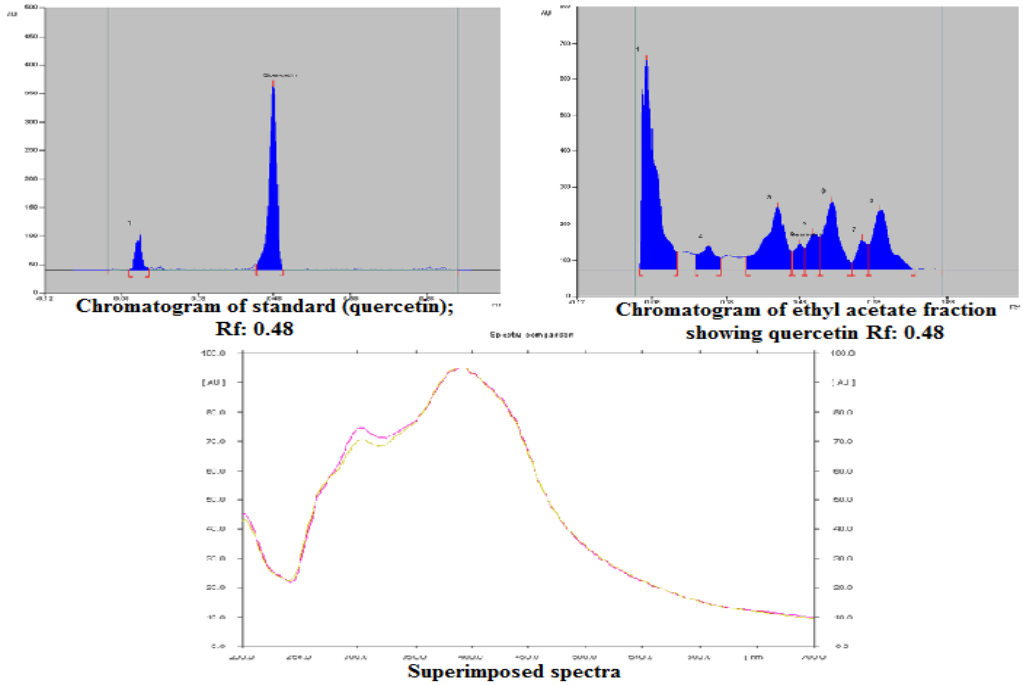

Fig. 2: HPTLC chromatogram showing the peak of quercetin (Rf 0.48) in ethyl acetate fraction and superimposed spectra with standard quercetin

Table 2: *In-vitro* antioxidant activity of *M. pudica* roots extracts (petroleum ether & methanol) and ethyl acetate fraction of methanol extract

Concentration (µg/ml)	IC_{50}values (µg/ml) DPPH	Super oxide	Nitric oxide	Lipid peroxidation
Pet. ether (25-400)	>1000.00	>1000.00	>1000.00	>1000.00
Methanol (25-400)	162.54 ± 1.26	221.98 ± 1.66	206.23 ± 1.33	176.5 ± 1.24
Eth. acetate fraction (25-400)	55.88 ± 0.87	85.85 ± 0.84	67.52 ± 0.76	129.25 ± 1.09
Ascorbic acid (5-25)	8.92 ± 0.29	12.36 ± 0.46	9.35 ± 0.27	-
Rutin (2-10)	3.67 ± 0.11	-	4.08 ± 0.06	-
BHA (10-160)	17.75 ± 0.21	24.07 ± 0.39	-	101.96 ± 1.17

(Data are mean ± SEM values; n=3)

DISCUSSION AND CONCLUSION

The methanol extract and its ethyl acetate fraction of *M. pudica* roots exhibited different levels of antioxidant activity in all the models studied. The results from various free radical-scavenging systems revealed that the ethyl acetate fraction had significant antioxidant activity and free radical-scavenging activity, with effective scavenging activity against free radicals such as DPPH, superoxide and NO. DPPH is usually used as a substrate to evaluate antioxidative activity of antioxidants[26]. The method is based on the reduction of methanolic DPPH solution in the presence of a hydrogen donating antioxidant, due to the formation of the non-radical form DPPH-H by the reaction. The methanol extract and its ethyl acetate fraction were able to reduce the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine. It appears that the ethyl acetate fraction possesses more hydrogen donating capabilities and acts as an effective antioxidant.

Superoxide radical is known to be very harmful to cellular components as a precursor of more reactive oxygen species[5]. Photochemical reduction of flavins generates O_2^- , which reduces NBT, resulting in the formation of blue formazan[27]. In addition to superoxide radical reactive species, a nitric oxide (NO) radical is also implicated in inflammation, hepatotoxicity, cancer and other pathological conditions[28]. The methanol extract and ethyl acetate fraction have the property to counteract the effect of SO and NO radicals formation and in turn may be of considerable interest in preventing the ill effects of excessive SO and NO radicals generation. Further, methanol extract and ethyl acetate fraction inhibited peroxides in rat liver homogenate & our results were comparable to previous studies reported[29]. Lipid peroxidation is an important biological consequence of oxidative cellular damage and aging. The polyunsaturated fatty acyl side chains, because of their susceptibility to oxidative damage in membrane phospholipids, pose a constant threat to cellular integrity and function[30].

These results clearly reveal that ethyl acetate fraction exhibited high reduction capacity and powerful free radical scavenging and act as a non-site/site specific potential antioxidant for biological systems susceptible to free radical-mediated reactions. Therefore, it might also act as hepatoprotective, reduce the risk of aging-related diseases and/or promote general human health. Based on these data, free radical-scavenging property attributable to the presence of high concentration of phenolic compounds such as flavonoids may be one of the mechanisms by which this drug is useful as a traditional medicine. The antioxidative potential of phenolic compounds is mainly due to their redox properties, which play an important role in neutralizing free radicals, quenching singlet and triplet oxygen species, or decomposing peroxides[31].

However, further studies are under process to characterise the additional bioactive compounds responsible for the observed behaviors in *M. pudica*.

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List of Abbreviations

DPPH: 2,2-diphenyl-1-picryl hydroxyl, SO: Superoxide, NO: Nitric oxide, BHA : Butylated hydroxyl anisol, ROS: Reactive oxygen species, NEDD: Naphthyl ethylene diamine dihydrochloride, NBT: Nitro blue tetrazolium, DMSO: Dimethyl sulphoxide, TCA: Trichloroacetic acid, TBA: Thiobarbituric Acid.

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