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Free Radical Scavenging Activity of *Mussaenda glabra*

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Abstract: The flowers of *Mussaenda glabra* occurring in white and pink have been evaluated for their anti oxidant property. The white (MGW) and the pink (MGP) varieties showed considerable NO scavenging activity. The IC_{50} values were found to be 92.79 and 122.45 μg mL^{-1}, respectively. MGW inhibited lipid peroxidation to a greater extent when compared to MGP. MGW showed 50% inhibition at the concentration of 112.4 μg mL^{-1} while MGP at 53.42 μg mL^{-1}. The reducing power was found in a dose dependent manner. The pink variety showed greater reducing power when compared to white variety. Both the extracts showed hydroxyl radical and ABTS radical scavenging property. The total phenolic content was 17.5 and 8.3 mg g^{-1} in MGP and MGW, respectively. The HPTLC fingerprinting showed peaks due to flavonoids. The results show that both are having considerable anti oxidant property. The abundance of this flower throughout the year can be used for evaluating its medicinal value.

Key words: *Mussaenda glabra*, ABTS, LPO, HPTLC

INTRODUCTION

Many ornamental plants have lines or cultivars with different flower colours. The varieties of flower colours is mainly due to the chemical structures of different anthocyanins accumulated in the flower (Kohei Kazuma *et al*., 2003). *Mussaenda glabra* (Rubiaceae), an ornamental plant widespread in India and Malaysia, is tolerant to cold (Huxley *et al*., 1999). The plant is in full bloom throughout the year. The flowers of the plant are rich in flavonoids. Flavonoids have been considered to function as antioxidants and UV filters in higher plants (McClure, 1986). It has been reported that a series of human illness such as cancer (Grunberger *et al*., 1988), diabetes (Lean *et al*., 1999), immune system impairment neurodegenerative diseases such as Parkinson's and Alzheimer's diseases and arthritis, as well as premature body aging, can be linked to the damaging action of extremely reactive free radicals (Paladini *et al*., 1999). Flavonoids have been found to possess free radical scavenging activity (Van Acker *et al*., 1998). Flavonoids are widely distributed in many flowers. Some rare flavonoids have been reported from Rubiaceae. There are no previous report on the medicinal properties of *Mussaenda glabra*. Hence the hydroalclcoholic extracts of pink and white flowers of *Mussaenda glabra* was tested for its antioxidant properties in the various *in vitro* methods. Fingerprinting of the two extracts which help in authentification have been done using HPTLC. The total phenolic content was also estimated in the two extracts.

MATERIALS AND METHODS

All solvents and Chemicals used were of analytical grade, obtained from Merck, India. Deoxyribose, 2, 2azinobis-)3-(ethylenzothiazoline-6-sulfonate) and Rutin (0.98% HPLC) were purchased from Sigma, USA. This study was conducted in the laboratory of Centre for Advanced Research In Indian System of Medicine, SASTRA Deemed University.

Plant material: The fresh flowers of MGP and MGW were collected during the month of September from Thanjavur. The plant material was authenticated by botanists at Rabinat Herbarium, St. Joseph College, Tiruchirapalli. A voucher specimen has been deposited in the herbarium of CARISM, SASTRA Deemed University.

Preparation of extract: The fresh flowers (MGW and MGP) of *Mussaenda glabra* (100 g) was extracted by macerating in 500 mL of 70% methanol at room temperature for 72 h. It was concentrated *in vaccuo*. The yield obtained in each case was found to be 0.69% (w/w) and 0.48% (w/w).

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HPTLC fingerprinting: HPTLC fingerprinting helps in the authentification of the plant material (Aggarwal, 2001; Spreeman and Gaedeke, 2000) Chromatography was performed on a 10*10 cm preactivated HPTLC Silica gel $60F_{254}$ plate. Samples were applied to the plate as 6 mm band using CAMAG Linomat 5 applicator. The slit dimension was kept at 6 *0.45 mm and 20 mm/s scanning speed was employed. The hydroalcoholic extracts of the flowers have been prepared at a concentration of 1 mg/1 mL in alcohol. The chromatogram was developed by using Ethyl acetate: Ethyl Methyl Ketone: Water: Formic acid (5:3:1:1) as the mobile phase.

Determination of total phenolic content: Total soluble phenolics in the extracts were determined with Folin-ciolcateau reagent (Slinkard and Singleton, 1977) using vanillin as a standard phenolic compound. 1.0 mL of Folin-Ciocalteau reagent was added to 1 mL of sample solution. After 3 min 3.0 mL of 2% sodium carbonate was added and incubated for 20 min. The absorbance of the blue color developed was read at 760 nm.

Determination of NO radical scavenging activity: Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction (Green *et al*., 1982). Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide sodium nitroprusside (5 mM) in phosphate buffered saline was mixed with 3 mL of different concentrations (30-1000 μg mL^{-1}) of the extracts dissolved in methanol and incubated at 25°C for 150 min. The sample from the above were reacted with Greiss reagent (1% Sulphanilamide, 2% O-Phosphoric acid and 0.1% Naphthylethylenediaminehydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine was read at 546 nm and referred to the absorbance of standard solutions of potassium nitrite, treated in the same way with Greiss reagent.

NO scavenged = [(A control-A test)/A control]* 100

where A control is the absorbance of the control reaction and A test is the absorbance in the presence of sample of the extracts.

Lipid peroxidation: Liver homogenate was prepared from male albino Wistar rats. The liver was quickly excised after decapitation and exanguinations and washed several times with ice cold saline solution (0.15 M KCl, pH 7.4). The liver homogenate was prepared at a protein concentration of 500 μg mL^{-1}. Lipid peroxidation (Kornblat *et al*., 1980) was initiated by the addition of 25 μM $FeSO_4$, 100 mM ascorbate and 10 mM KH_2PO_4. The homogenates were incubated at 37°C for 30 min with different concentrations of MGP and MGW (30-1000 μg mL^{-1}). Lipid peroxidation was measured by the estimation of thiobarbituric acid reactive substances (TBARS) according to the method of Ohkawa *et al*. (1979).

Determination of reducing power: The reducing power of the extracts was determined according to the method of Oyaizu (1986). Various concentrations (30-1000 μg mL^{-1}) of the extracts in 1.0 mL of deionized water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and 1% potassium ferricyanide (2.5 mL). The mixture was incubated at 50°C for 20 min. Aliquots of 10%TCA (2.5 mL) were added to the mixture, which was then centrifuged at 1036×g for 10 min. The upper layer of reaction mixture was mixed with distilled water (2.5 mL) and freshly prepared $FeCl_3$ solution (0.5 mL, 0.1%). The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Determination of hydroxyl radical scavenging activity: The hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and $Fe3^+$/ascorbate/EDTA/H_2O_2 system (Halliwell *et al*., 1981). The hydroxyl radicals attack deoxyribose, which eventually results in TBARS formation. The reaction mixture contained deoxyribose (2.8 mM), $FeCl_3$(0.1 mM), EDTA (0.1 mM),H_2O_2 (1 mM), ascorbate (0.1 mM), KH_2PO_4-KOH (20 mM, pH -7.4). The reaction mixture was incubated at 37°C for 1 h. Deoxyribose degradation was measured as TBARS by the method of Ohkawa *et al*. (1979).

ABTS radical scavenging activity: Method developed by van den Berg *et al*. (1999) was used with slight modification in this experiment (Re *et al*., 1999). 1.0 mM ammonium persulphate was mixed with 2.5 mM ABTS as diammonium salt in phosphate-buffered saline solution (100 mM phosphate buffer, pH-7.4, containing 150 mM NaCl). The mixture was heated in a 68°C water bath for 30 min. The concentration of the resulting blue-green ABTS radical solution was adjusted to an absorbance of 0.650±0.020 at 734 nm. Various concentrations of the sample solution of 20 μL. were added to 980 μL of the resulting blue green solution. The mixture was incubated in darkness in a 37°C water bath for 10 min and the decrease of absorbance at 734 nm was measured. A control solution consisted of 20 μL 50% methanol and 980 μL of ABTS radical solution.

Statistical analysis: All determinations were carried out in triplicate and the mean of the values are represented. IC50 values have bee calculated using Prism Graphed Software.

RESULTS

The preliminary phytochemical analysis showed the presence of polyphenolics flavonoids, carbohydrates, terpenoids and amino acids. HPTLC fingerprinting showed several quenching zones due to flavonoids (Wagner *et al*., 1995) (Fig. 4-7). The total phenolic content of the MGP was 17.50 mg g^{-1} of the dried extract and that of MGW was 8.3 mg g^{-1} of the dried extract (Fig. 8). The plant MGP showed greater LPO inhibition with IC_{50} value of 53.42 μg mL^{-1}. The extract showed negligible activity at the higher concentration tested (1000 μg mL^{-1}). The extracts were able to scavenge hydroxyl radical also. The extracts showed more than 80% hydroxyl radical scavenging ability at the dose of 500 μg mL^{-1} (Fig. 1). The IC_{50} values of the MGP and MGW were found to be 48.67 and 124.56 μg mL^{-1}, respectively. (Fig. 2). The extracts also showed significant NO scavenging activity. The IC_{50} values of the MGP and MGW were found to be 92.79 and 122.45 μg mL^{-1}, respectively. As the trend observed reducing power of MGP was also nearer to Rutin (Fig. 3). The ABTS scanvenging property was higher at the lower

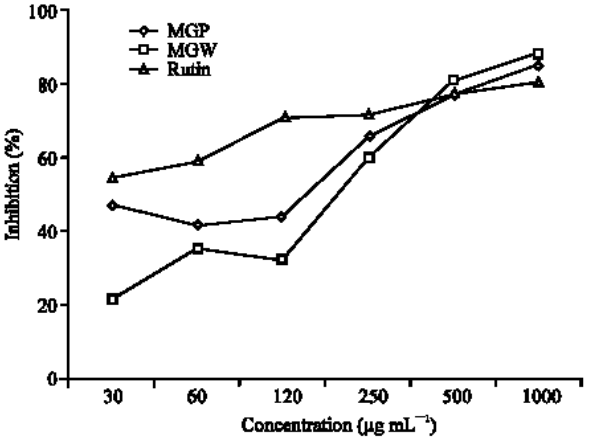

Fig. 1: Hydroxyl radical scavenging property of MGP, MGW and Rutin

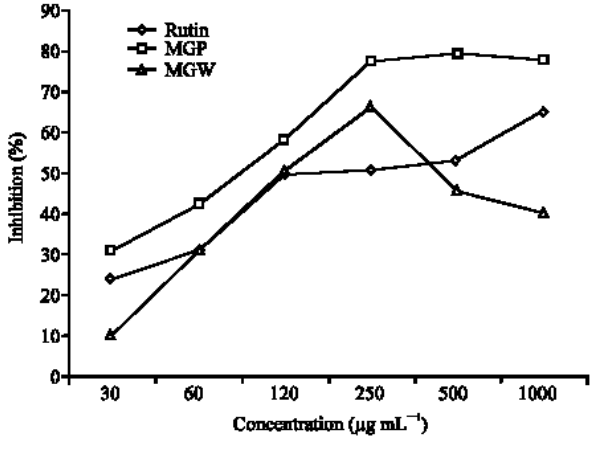

Fig. 2: Nitric oxide radical scavenging property of MGP, MGW and Rutin

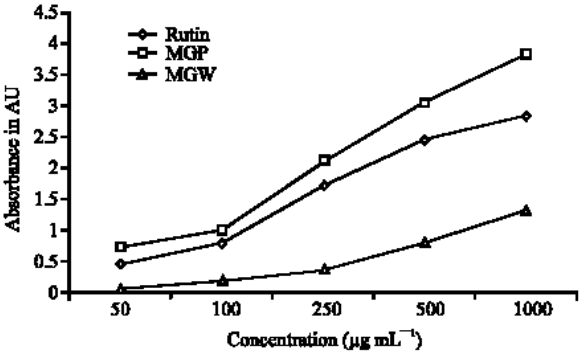

Fig. 3: Reducing power of MGP, MGW and Rutin

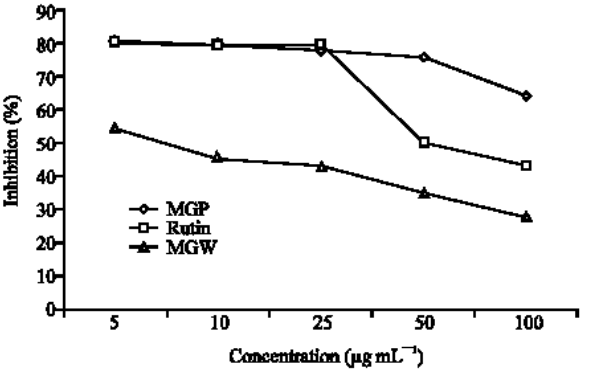

Fig. 4: ABTS radical scavenging property of Rutin, MGP and MGW

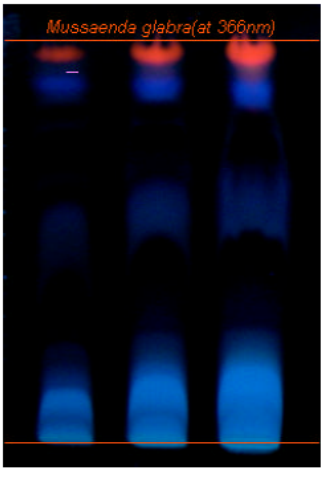

Fig. 5: MGP At 366 nm

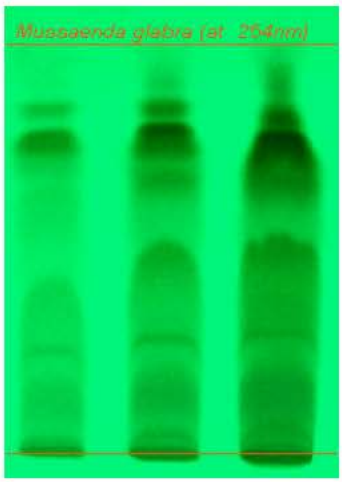

Fig. 6: MGP At 254 nm

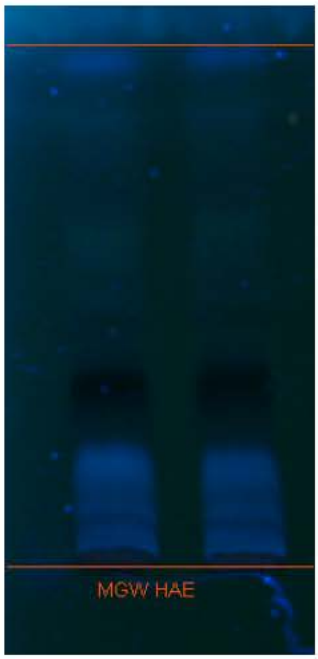

Fig. 7: MGW At 366 nm

Table 1: IC_{50} values of MGP and MGW

Sample	LPO	NO	OH	ABTS
MGP ($\mu g\ mL^{-1}$)	112.4	48.67	92.79	125.54
MGW ($\mu g\ mL^{-1}$)	53.42	124.56	122.45	255.32

dose 80% at 5 $\mu g\ mL^{-1}$ (Fig. 4). The activity decreased with increase in concentration with IC_{50} values observed at 125.54 $\mu g\ mL^{-1}$ for MGP and 255.32 $\mu g\ mL^{-1}$ for MGW (Table 1).

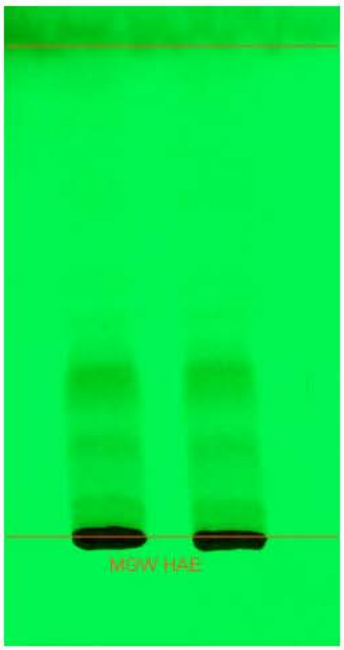

Fig. 8: MGW At 254 nm

DISCUSSION

Lipid of cell membranes are potential target site for free radical attack (Halliwell, 1991; Halliwell and Chirico, 1993). Lipid peroxidation has been implicated in several pathologic conditions including aging, hepatotoxicity, hemolysis, cancer, tumour promotion, inflammation and iron toxicity (Bus and Gibson, 1979; Plaa and Witschi, 1976). Peroxides are usually decomposed to compounds of lower molecular weight which are measured by TBA method (Kikuzaki and Nakatani, 1993). Both the extracts showed moderate LPO inhibition. The moderate activity of the extract may probably be due to the rapid and extensive degradation of the antioxidant principles in an *ex vivo* state thereby corroborating the finding that was observed in a study carried out in Australia with a group of human volunteers (Pryor, 1986). The LPO inhibition by MGP is significantly greater than that of Rutin whose IC_{50} value is 93.5 $\mu g\ mL^{-1}$ and that of Silibin is 98.5 $\mu g\ mL^{-1}$ (Bing Yang *et al*., 2001). The ability of the flavonoids to interact with the lipid bilayers including their incorporation rate into cells and their orientation was suggested to be an important factor of their antioxidant activity in biological systems. The flavonoids react rapidly with OH radical because of the generally high reactivity of this radical with the aromatic compounds (Saija *et al*., 1995).

Nitric oxide is a reactive free radical produced by phagocytes and vascular endothelial cells, to yield an even more reactive species, peroxynitrite which can decompose to form OH radical in a reaction independent of transition metal ions (Michel and Bors, 1991; Beckman *et al*., 1990). Several flavonoids, including quercetin, result in a reduction in ischemia-reperfusion injury by interfering with inducible nitric-oxide synthase activity (Shoskes *et al*., 1998). When flavonoids are used as antioxidants, free radicals are scavenged and therefore can no longer react with nitric oxide, resulting in less damage (Shutenko *et al*., 1999). Interestingly, nitric oxide can be viewed as a radical itself and it is was reported that nitric oxide molecules are directly scavenged by flavonoids (van Acker *et al*., 1995). Therefore, it has been speculated that nitric oxide scavenging plays a role in the therapeutic effects of flavonoids

The ABTS assay is based on the inhibition of the absorbance of the radical cation $ABTS^{+}$, which has a characteristic long wavelength absorption spectrum. The results obtained imply the activity of the extracts either by inhibiting or scavenging the $ABTS^{+}$ radicals (Sanchez-Moreno, 2002). The ABTS scavenging property of MGP was comparable to that of Rutin. The extracts of MGP and MGW were the most active: they nearly fully scavenged $ABTS^{+}$. It also should be noted that the reaction with $ABTS^{+}$ was quite fast and was completed in 0.25-0.5 min.

The extracts showed considerable free radical scavenging activity. As this flower is blossoming throughout the year, this can be used as a source of flavonoids and its anti oxidant potential also indicates that it can be explored for the treatment of other diseases.

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