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IN VITRO ANTIOXIDANT ACTIVITY OF METHANOLIC EXTRACT OF STEM BARK OF *GMELINA ARBOREA* ROXB. (VERBENACEAE)

Patil S.M., Kadam V.J. and Ghosh R.*

Department of Pharmacology, Bharati Vidyapeeth's College of Pharmacy, Sector 8, CBD Belapur, Navi Mumbai – 400614, Maharashtra, India
E-mail: **rumi1968@hotmail.com**

ABSTRACT: In this study, the antioxidant activity of methanolic extracts of stem bark of *Gmelina arborea* Roxb. (Verbenaceae) (MEGA) was studied using various *in vitro* assays. The antioxidant activity of MEGA was evaluated by using the free radical scavenging activity assay (DPPH method), reducing power assay, nitric oxide scavenging activity, hydroxyl radical scavenging activity and H_2O_2 scavenging activity. Total phenolic content was determined by using gallic acid as a standard. The results of the study show that MEGA possesses significant free radical scavenging properties and a clear correlation exists between the antioxidant activity and phenolic content.

Keywords: *Gmelina arborea*, DPPH method, total phenolic content, nitric oxide, reducing power, hydroxyl radical.

1. INTRODUCTION

Recently, there has been a surge in research on the potential role of antioxidants in the treatment of atherosclerosis, heart failure, liver dysfunction, neurodegenerative disorders, cancer, and diabetes mellitus[1]. The main characteristic of an antioxidant is its ability to trap free radicals. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases[2]. Free radicals which have one or more unpaired electrons are produced during normal and pathological cell metabolism. Reactive oxygen species (ROS) react easily with free radicals to become radicals themselves. ROS are various forms of activated oxygen, which include free radicals such as superoxide anion radicals ($O_2^{\cdot-}$) and hydroxyl radicals ($OH^{\cdot}$), as well as non-free radical species (H_2O_2) and the singlet oxygen ($^1O_2$)[3]. Antioxidants provide protection to living organisms from damage caused by uncontrolled production of ROS and concomitant lipid peroxidation, protein damage and DNA stand breaking[4]. Ethnomedical literature contains a large number of plants that can be used against diseases, in which reactive oxygen species and free radical play important role. There is a plethora of plants that have been found to possess strong antioxidant activity[5].

Gmelina arborea Roxb. (Family: Verbenaceae) is locally known as 'Gambhari'. In English it is known as the 'Candahar tree' or 'White teak'. It is a moderate sized, deciduous tree, found distributed in deciduous forests throughout the greater part of India up to an altitude 1500 meters[6]. The useful parts of the plant are its root, stem bark and fruit. Folklore states that it promotes digestive power, improves memory and is useful alteration of fever, heart disease, nervous disorders and piles. The drug has been known to be used for snake-bites and scorpion-stings. It also has been found to prevent abortions in the early stages of pregnancy[7]. The stem bark and heartwood possess hypoglycaemic action and contain a number of chemical constituents such as lignans, gmelinol, n-hexacosnol, n-octanol along with β-sitosterol[8,9].

The aim of this study was to investigate the antioxidant properties of methanolic extracts of stem bark of *Gmelina arborea* against the free radicals.

***Corres author:**
Address for Correspondence:
Dr. (Mrs.) Rumi Ghosh,
Asst. Professor of Pharmacology,
Bharati Vidyapeeth's College of Pharmacy, Sector-8, Belapur (C.B.D.),
Navi Mumbai (400614)
Email: rumi1968@hotmail.com
Phone No.: (022) 27571122
Mobile No.: 09987584160

2. MATERIALS AND METHODS

2.1 Plant Material

The stem bark was collected from the forests of the Western Ghats in the coastal regions of Maharashtra, India in September 2008. The plant was identified by its vernacular name Gambhari and later authenticated by Dr. A. M. Mujumdar, Head, Plant Science Division, Agharkar Research Institute, Pune, Maharashtra, India (Voucher No. Auth.08-127). A voucher specimen is preserved in our laboratory for further reference at Bharati Vidyapeeth's College of Pharmacy, Navi Mumbai, Maharashtra, India.

2.2 Extract preparation

The stem bark was chopped into small pieces and dried in the shade. The dried stem bark was ground to a coarse powder and extracted (100 g) successively with 600 ml methanol in a Soxhlet extractor at 130°C for 24-30 hrs. The extract was concentrated by using rotary evaporator to yield a light brown solid (12.5% w/w). The extract was preserved in a dessicator till further use.

2.3 Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, gallic acid, Folin-Ciocalteau reagent, potassium ferricyanide, $FeCl_3$, sodium bicarbonate, trichloroacetic acid (TCA), sodium carbonate, hydrogen peroxide, sodium nitroprusside, sulphanilamide, H_3PO_4, napthylethylenediamine dihydrochloride, tribromoacetic acid (TBA). All chemicals used including solvents were of analytical grade.

2.4 Free radical scavenging activity (DPPH method)

The antioxidant activity of the plant extracts and standard were assessed on the basis of the radical scavenging effect of the stable DPPH free radical[10]. About 10-100 µl of each extract or standard was added to 2 ml of DPPH (HiMedia Laboratories Pvt. Ltd., Mumbai) in methanol (0.33%) in a test tube. After incubation at 37°C for 30 minutes the absorbance of each solution was determined at 517 nm using spectrophotometer[11]. The corresponding blank reading were also taken and the remaining DPPH was calculated by using the following formula,

DPPH radical

scavenging activity (%) = [Abs $_{(control)}$ – Abs $_{(standard)}$] × 100.

Where, $Abs_{(control)}$: Absorbance of DPPH radical + methanol

$Abs_{(standard)}$: Absorbance of DPPH radical + extract/standard.

IC_{50} value is the concentration of the sample required to scavenge 50% DPPH free radical.

2.5 Reducing power assay

The reducing power of the MEGA was determined according to the method of Oyaizu (1986)[12]. Different concentrations of the methanolic extract of *Gmelina arborea* (MEGA) (10–100 µg/ml) in 1.0 ml of deionised water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferrocyanide (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion of trichloroacetic acid (2.5 ml, 10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm and compared with standards. Increased absorbance of the reaction mixture indicated increased reducing power.

2.6 Total phenolic content

The total phenolic content of the leaf extract was determined separately using the method of Macdonald et al.[13,14] with modifications. Calibration curve was prepared by mixing methanolic solution of gallic acid (Loba Chemie Pvt. Ltd., Mumbai) (1 ml; 10-100 µg/ml) with 5ml Folin-Ciocalteu reagent (SRL Pvt. Ltd, Mumbai.) (diluted ten fold) and sodium carbonate (4 ml, 0.7 M). We measured absorbance at 765nm and drew the calibration curve. 1 ml of MEGA (100 µg/ml) was also mixed with the reagents above and after 2 h the absorbance was measured to determine total plant phenolic contents. All determinations were carried out in triplicate. The total content of phenolic compounds in the extract in gallic acid equivalents (GAE) was calculated by the following formula:

$$T = \frac{C \cdot V}{M}$$

Where, T=total content of phenolic compounds, milligram per gram plant extract, in GAE; C=the concentration of gallic acid established from the calibration curve, milligram per milliliter; V=the volume of extract, milliliter; M=the weight of ethanolic plant extract, gram.

2.7 Scavenging of Hydrogen peroxide

The ability of the extract to scavenge hydrogen peroxide was determined according to the method of Ruch, Cheng and Klaunig[15]. A solution of hydrogen peroxide (2 mmol/l) (Fine Chem Industries, Mumbai) was prepared in phosphate buffer (pH 7.4). Extracts (10–100 µg /ml) were added to hydrogen peroxide solution (0.6 ml). Absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. For each concentration, a separate blank sample was used for background subtraction. The percentage scavenging activity of hydrogen peroxide by MEGA was calculated using the following formula,

% scavenging activity [H_2O_2]

= [Abs $_{(control)}$ – Abs $_{(standard)}$ / Abs $_{(control)}$] × 100.

Where, Abs $_{(control)}$: Absorbance of the control and

Abs $_{(standard)}$: Absorbance of the extract/standard.

2.8 Nitric oxide radical scavenging activity

Nitric oxide was generated from nitroprusside and measured by the Greiss reaction. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide[16, 17], which interacts with oxygen to produce nitric oxide which, interacts with

oxygen to produce nitric ions that can be estimated by use of Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide[15]. Sodium nitroprusside (5 mM) in phosphate–buffered saline (PBS) was mixed with 3.0 ml of different concentrations (10-100 µg /ml) of the drugs dissolved in the suitable solvent systems and incubated at 25°C for 150 min. The samples from the above were reacted with Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% napthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine was read at 546 nm and referred to the absorbance at standard solutions of potassium nitrite, treated in the same way with Greiss reagent.

NO scavenged (%)

$= [Abs_{(control)} - Abs_{(standard)} / Abs_{(control)}] \times 100.$

Where, $Abs_{(control)}$: Absorbance of the control reaction and

$Abs_{(standard)}$: Absorbance of the extract/standard.

2.9 Hydroxy radical scavenging activity

The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell[18]. The assay was performed by adding 0.1 ml of 1mM EDTA, 0.01 ml of 10 mM FeCl3, 0.1 ml of 10 mM H_2O_2, 0.36 ml of 10 mM deoxyribose, 1.0 ml of different dilutions of the extract (10 – 100 µg/ml) dissolved in distilled water, 0.33 ml of phosphate buffer (50 mM, pH 7.4) and 0.1 ml of ascorbic acid in sequence. The mixture was then incubated at 37 °C for 1 h. A 1.0 ml portion of the incubated mixture was mixed with 1.0 ml of 10% TCA (S. D. Fine Chem. Ltd., Mumbai.) and 1.0 ml of 0.5% TBA (Central Drug House, New Delhi.) (in 0.025M NaOH containing 0.025% BHA) to develop the pink chromogen measured at 532 nm. The hydroxyl radical scavenging activity of the extract is reported as % inhibition of deoxyribose degradation and is calculated as,

OH^- scavenged (%) $= [Abs_{(control)} - Abs_{(standard)} / Abs_{(control)}] \times 100.$

Where, $Abs_{(control)}$: Absorbance of the control reaction and

$Abs_{(standard)}$: Absorbance of the extract/standard.

2.10 Statistical analysis

Results are expressed as mean ± S.E.M. of three determinants. Comparisons among the groups were tested by two-way ANOVA using Graph Pad Prism, Version 5.0 (Graph Pad Software, San Diego, CA, USA). P-values < 0.005 were considered significant.

3. RESULTS AND DISCUSSION

3.1. Free radical scavenging activity

Figure 1 shows the dose-response curve of DPPH radical scavenging activity of the MEGA, compared with ascorbic acid, as standard. At a concentration of 100 µg/ ml, the scavenging activity of methanol extract of stem bark was 85.20%, while at the same concentration, that of the standard ascorbic acid was 89.58%. IC_{50} values of extract and standard were 46.2 ± 1.2 and 28.80 ± 0.50, respectively. The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability[19]. Though the DPPH radical scavenging abilities of the extracts were less than those of ascorbic acid at 100 µg/ ml, the study showed that the extracts have the proton-donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants.

3.2 Reducing power assay

Figure 2 shows the reductive capabilities of the plant extract compared to ascorbic acid. The reducing power of MEGA was very potent and the power of the extract was increased with quality of sample. The plant extract could reduce the most Fe^{3+} ions, which had a lesser reductive activity than the standard of ascorbic acid. Increased absorbance of the reaction indicated increased reducing power.

3.3 Total phenolic content

Phenolic compounds are known to be powerful chain breaking antioxidants and are important constituents of plants. Phenolic compounds may contribute directly to antioxidative action. It is suggested that phenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when ingested up to 1.0 gm daily from a diet rich in fruits and vegetables. The total phenolic content of the MEGA measured by Folin-Ciocalteu reagent in terms of gallic acid equivalent (GAE) was 356 ± 1.4 mg g^{-1}.

3.4 Scavenging of hydrogen peroxide

Hydrogen peroxide itself is not very reactive, but can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells[18]. Scavenging of H_2O_2 by extracts may be attributed to their phenolics, which can donate electrons to H_2O_2, thus neutralizing it to water. The extracts were capable of scavenging hydrogen peroxide in a concentration-dependent manner. From figure 3 shows that MEGA shows less scavenging activity (H_2O_2) than that of Ascorbic acid. The IC_{50} value for scavenging of H_2O_2 for MEGA was 73.6 ± 0.03 µg/ ml while IC_{50} value for ascorbic acid was 62.4 ± 0.12 µg/ ml.

3.5 Nitric oxide scavenging activity

From figure 4, MEGA showed moderately good nitric oxide scavenging activity between 10 and 100 µg/ ml. The percentages of inhibitions were increased with increasing concentration of the extracts. IC_{50} value for scavenging of nitric oxide for MEGA was 93.6 ± 0.98 µg/ ml while IC_{50} value for ascorbic acid was 79.2 ± 0.80 µg/ ml. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological condition.

3.6. Hydroxyl radical scavenging activity

The highly reactive hydroxyl radicals can cause oxidative damage to DNA, lipids and proteins[20]. This assay shows the abilities of the extract and standard

mannitol to inhibit hydroxyl radical-mediated deoxyribose degradation in Fe^{3+}-EDTA-ascorbic acid and H_2O_2 reaction mixture. From figure 5 shows that MEGA shows less scavenging activity (H_2O_2) than that of Ascorbic acid. The IC_{50} values of the extract and standard in this assay were 34 ± 0.82 µg/ ml and 27 ± 0.92 µg/ ml. The IC_{50} value of the extract was less than that of the standard. At 100 µg/ ml, the percentage inhibition values were 63.36% and 66.00% for MEGA and ascorbic acid, respectively.

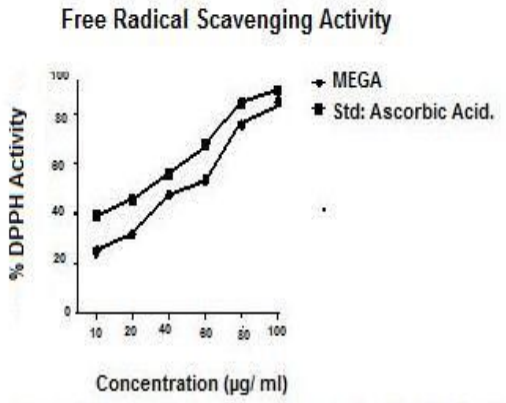

Figure 1: Free radical scavenging activity of MEGA (n=3).

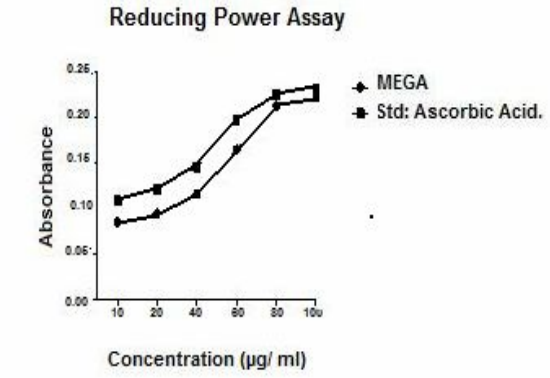

Figure 2: Reducing power assay of MEGA (n=3).

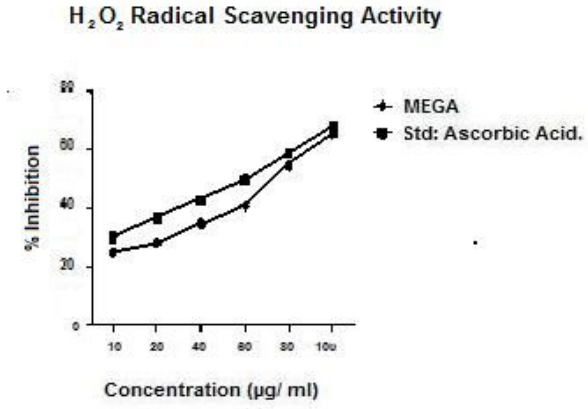

Figure 3: Hydrogen peroxide activity of MEGA (n=3).

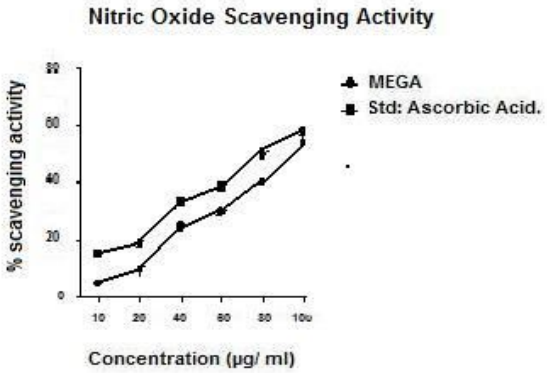

Figure 4: Nitric oxide scavenging activity (n=3).

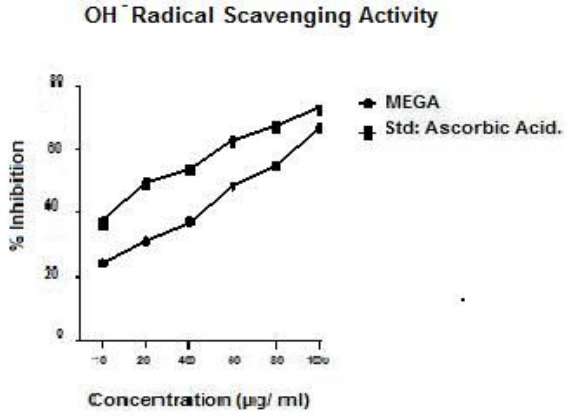

Figure 5: Hydroxy radical sacvenging activity of MEGA (n=3).

4. CONCLUSION

On the basis of the results obtained in the present study, it is concluded that a methanolic extract of Gmelina arborea stem bark, which contains large amounts of phenolic compounds, exhibits high antioxidant and free radical scavenging activities. It also chelates iron and has reducing power. These in vitro assays indicate that this plant extract is a significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses. However, the components responsible for the antioxidative activity are currently unclear. Therefore, further investigations need to be carried out to isolate and identify the antioxidant compounds present in the plant extract. Furthermore, the in vivo antioxidant activity of this extract needs to be assessed prior to clinical use.

5. ACKNOWLEDGMENTS

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