ANTIOXIDANT ACTIVITY AND FREE RADICAL SCAVENGING POTENTIAL OF *GMELINA ARBOREA* LINN.

Sangita Shukla*[1], Saluja A K[2], Pandya S S[3]

[1] Department of Pharmacognosy, Indukaka Ipcowala College of Pharmacy, New Vallabh Vidyanagar,P.O. Box 53, Po. Vitthal Udyognagar, Dist. Anand, Gujarat-388121.
Phone: +91 9898047135
shshukla@rediffmail.com
[2] Department of Pharmacognosy, A.R.College of Pharmacy, Vallabh Vidyanagar, Gujarat-388120
[3] Department of Pharm.Chemistry, Babaria Institute of Pharmacy, Varnama, Gujarat-391240

* Corresponding author

Summary

The root of *Gmelina arborea* is used in a number of ayurvedic formulations. It is one of the ingredients of the "Dashmula" of ayurveda. In the present study, antioxidant and free radical scavenging activity of defatted and fractionated methanolic extract of *Gmelina arborea* (MEGA) was evaluated using *in vitro* methods like 1, 1-diphenyl, 2-picryl hydrazine (DPPH) radical scavenging activity, H_2O_2 scavenging activity, Nitric oxide (NO) radical scavenging activity, Nitro Blue Tetrazolium (NBT) reduction assay, β-carotene-lineolate bleaching assay and Total reduction ability by Fe^{3+}-Fe^{2+} transformation (Reducing power assay). Ascorbic acid was used as a reference antioxidant compound. In DPPH radical scavenging activity,H_2O_2 scavenging activity, NO scavenging activity and NBT reduction assay the IC_{50} values obtained for MEGA were found to be 434.56 µg/ml,60.36 µg/ml, 49.54 µg/ml and 67.11 µg/ml respectively and for Ascorbic acid the IC_{50} values were found to be 511.36 µg/ml, 33.06 µg/ml, 42.40 µg/ml and 94.82 µg/ml respectively. In the reduction power assay increase in absorbance was observed in a dose dependant manner. Measurement of total phenolic content using Folin-Ciocalteu phenol reagent showed that 1 mg of the extract contained 85.95 µg/ml total phenolics equivalent to gallic acid. The results obtained in the present study indicate that MEGA can be a potential source of natural antioxidants and the activity may due to the presence of phenolics.

Key Words: *Gmelina Arborea*, *in vitro* antioxidant, total phenolic content

Introduction

Oxidation processes are very important for living organism. The uncontrolled production of Reactive Oxygen Species (ROS) and the unbalanced mechanism of antioxidant protection result in the onset of many diseases and accelerate ageing.

ROS include free radicals such as hydroxyl radicals (OH^-), Superoxide (O_2^-) and non-free radical species such as H_2O_2 and singlet oxygen (O_2) **(1, 2, 3).** These species are highly reactive and potentially damaging transient chemical species. These are continuously produced in the human body, as they are essential for energy supply, detoxification, chemical signaling and immune function. Over production of reactive species, induced by exposure to external oxidant substances or a failure in the defense mechanisms, damage to cell structures, DNA, lipids and proteins **(4)** occur which increases risk of more than 30 different disease processes **(5)**.Interestingly the body possess defense mechanisms against free radical-induced oxidative stress, which involve preventive mechanisms, repair mechanisms, physical defenses and antioxidant defenses. Enzymatic and Non-enzymatic antioxidants [ascorbic acid (Vitamin C), α-tocopherol (vitamin E), glutathione (GSH), carotenoids, flavonoids, etc.] act by one or more of the mechanisms like reducing activity, free radical scavenging, potential complexing of pro-oxidant metals and quenching of singlet oxygen. It is possible to reduce the risks of chronic diseases and prevent disease progression by either enhancing the body's natural antioxidant defenses or by supplementing with proven antioxidants **(6).** Synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) commonly used in processed foods have side effects and are carcinogenic **(7,8).**This is one of the reasons why discovery and synthesis of novel antioxidants is a major active area. The roots of *Gmelina arborea* contains arboreal, isoarboreal, gmelanone **(9),** gmelofuran, cerylalcohol, hentriacontanol-1, β-sitosterol,n-octacosanol, umbelliferone and is reported to be used in hallucination, fever, dyspepsia, hyperdipsia, haemorrhoids, gastralgia, anasarca and in burning sensation. It is acrid, bitter, sweet, tonic, laxative, galactogogue and anthelmintic **(10)**. The tree grows in the hilly areas of the Himalayas, Nilgiri and also on the eastern and western coast of India**(11)**. The present study was taken up to establish the correlation between the pharmacological uses and antioxidant property.

Materials and Methods

Plant Material And Preparation Of Extract.

The roots were collected from Nadiad District of Gujarat, India and authenticated by Pharmacognosist of Pharmacognosy department, Indukaka Ipcowala College of Pharmacy, New Vallabh Vidyanagar. A voucher specimen (IICP/06/01) has been preserved in our laboratory. The collected plant material was cut into small pieces and dried under shade. The material was then powdered (# 60) with mechanical grinder and stored in air tight container. The dry powdered material was defatted using petroleum ether (60-80°) using Soxhlet's extractor. Defatted material was then extracted with chloroform followed by methanol. The solvents were completely removed under reduced pressure The methanolic extract (MEGA) obtained as a semisolid mass and was selected for further studies after preliminary phytochemical analysis of all extracts **(12).**

DPPH Radical Scavenging Activity.

Free radical scavenging potentials of MEGA as tested against a methanolic solution of DPPH **(13, 14).** 0.1 mM solution of DPPH in methanol was prepared and 1.0 ml of this solution was added to 3.0 ml of different concentrations (200-1000 μg/ml) of MEGA prepared in water. It was incubated at room temperature for 30 minutes and the absorbance was measured at 517 nm against the corresponding blank solution. Ascorbic acid was taken as reference. Percentage inhibition of DPPH free radical was calculated based on the control reading, which contain DPPH and distilled water without any extract using the following equation:

% Scavenging Activity = [(Ac- As) / Ac] × 100.

Where, Ac is the absorbance of the control reaction and As is the absorbance in the presence of the sample of the extracts.The antioxidant activity of the extract was expressed as IC_{50}. The IC_{50} value was defined as the concentration (in μg/mL) of extracts that inhibits the formation of DPPH radicals by 50%. The IC_{50} values were calculated from graph by linear regression analysis.

Scavenging Of Hydrogen Peroxide **(15)**

A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). The concentration of hydrogen peroxide was determined by recording the absorbance at 230 nm. Different concentrations of MEGA (20-100 μg/ml) in distilled water added to a hydrogen peroxide solution (0.6 ml, 40 mM). The absorbance of hydrogen peroxide at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenged by the MEGA and ascorbic acid (standard) was calculated using equation.

Nitric Oxide Radical (NO) Scavenging Activity **(16, 17)**

Nitric oxide (NO) radicals were generated from sodium nitroprusside solution at physiological pH. Sodium nitroprusside (1 ml of 10 mM) was mixed with 1 ml of MEGA in different concentrations (20-100 μg/mL) in phosphate buffer (pH 7.4). The mixture was incubated at 25° C for 150 min. To 1 ml of incubated solution, 1 ml of Griess' reagent (1% sulphanilamide, 2% o-phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride) was added. Absorbance was read at 546 nm and percentage inhibition was calculated using formula.

Nitroblue Tetrazolium (NBT) Reduction Assay **(18)**

Antiradical activity is measured by the decrease in absorbance at 590 nm after illumination for 5 mins. Decrease in the absorbance in the standard and extracted was noted.0.2 ml riboflavin (1mg/ml) solution is mixed with 0.4ml EDTA (12mM)+ 5.2 ml extract or ascorbic acid prepared in phosphate buffer(ph 7.4) (20 μg/ml-100 μg /ml)+ 0.2 ml Nitroblue tetrazolium. The reaction mixture was illuminated under light for 5 mins and absorbance measured at 590 nm.

β -Carotene Linoleate Bleaching Assay

The antioxidant activity of the extract was assayed, based on the β -carotene bleaching method **(19).**Ascorbic acid was used as the standard. β-carotene (0.2mg in 1ml chloroform), linoleic acid (0.02ml) and tween-20(0.2ml) were transferred into a round bottom flask. The mixture was then added to 0.2 ml of different concentrations (200-1000 mcg/ml) of extract or standard or methanol (as control). Chloroform was

removed at room temperature under vacuum at reduced pressure using a rotary evaporator. Following evaporation, 50ml of distilled water was added to the mixture, and then shaken vigorously to form an emulsion. 2 ml aliquots of the emulsion were pipetted into test tubes and immediately placed in a water bath at 50° C. The absorbance was read at 30 min intervals for 2h at 470 nm. The antioxidant activity was based upon different parameters, namely antioxidant activity (AA), degradation rates (RD) and oxidation rate ratio (ROR).

Antioxidant activity (AA) was expressed as percent inhibition relative to control, using formula: **(20)**

$AA= [R_{control} - R_{sample\ of\ standard}/ R_{control}] X\ 100$,

Where $R_{control}$ and R_{sample} represent the bleaching rates of β -carotene without and with the addition of antioxidant, respectively.

Degradation rates (RD) were calculated according to first order kinetics: $RD=\ln (A_t/A_x)\ X\ 1/t_x$

Where ln is natural log, At is the absorbance at 470 nm at t=0 and Ax is the absorbance at 470 nm at t= 30, 60, 90, 120 min.

***Reducing Power Assay* (21, 22)**

The different concentration of MEGA (20-100 μg/ml) in 1 ml of deionized water were mixed with phosphate buffer (2.5 ml, 0.2M, pH 6.6) and 1% potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 ml). The mixture was incubated at 50 ^{o}C for 20 minutes. The reaction was stopped by adding trichloroacetic acid (2.5 ml, 10%) to the mixture,2.5ml of this mixture was mixed with distilled water (2.5ml) and ferric chloride (0.5 ml, 0.1%), and the absorbance was measured at 700 nm. Ascorbic acid was taken as reference.

Total phenolic content

The total phenolic content of the extract was determined using the Folin-Ciocalteu reagent **(23).** The reaction mixture contained 1 ml of MEGA, 0.5 ml of the Folin-Ciocalteu reagent, 3 ml of 20% sodium carbonate and 10ml of distilled water. After 2 h of reaction at the ambient temperature the absorbance at 765 nm was measured and used to calculate the phenolic contents using Gallic acid as a standard. The total phenolic content were then expressed as Gallic Acid Equivalent (GAE), in mg/g dry sample.

Results

From the data obtained MEGA showed dose dependant inhibition of DPPH, H_2O_2, NO and Reduction of NBT (Fig 1, 2, 3, 4). The IC $_{50}$ values for MEGA in DPPH, H_2O_2, NO radical scavenging activity and NBT reduction assay were found to be 434.56 μg//ml,60.36 μg/ml, 49.54 μg/ml and 67.11 μg/ml respectively and for Ascorbic acid the IC_{50} values were 511.36 μg/ml, 33.06 μg/ml, 42.40 μg/ml and 94.82 μg/ml respectively. In the reducing power assay there was an increase in the absorbance with increase in concentration of the extract, which is an indicative of increase in reducing power of extract. The results of MEGA showed 50% inhibition at low concentration in DPPH radical scavenging activity and NBT reduction assay as compared to ascorbic acid and *vice versa* in case of H_2O_2 and NO scavenging activity.

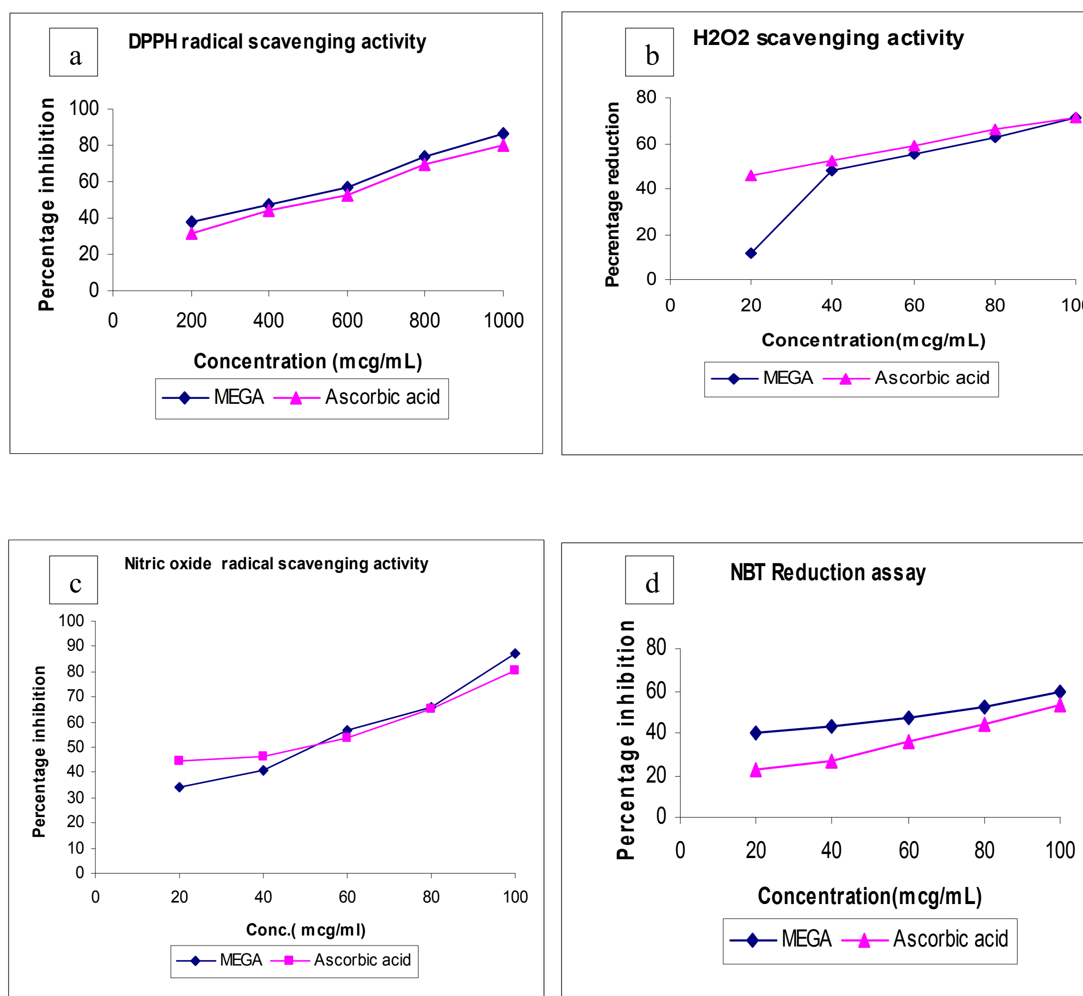

Fig.1. Effect of MEGA and Ascorbic acid on **a)** DDPH radical scavenging activity **b)** H_2O_2 scavenging activity **c)** Nitric oxide scavenging activity **d)** NBT reduction assay.

The reducing power of MEGA was observed to be dose dependant as shown in Table 1.

Concentration in µg/ml	Absorbance in nm MEGA	Absorbance in nm Ascorbic acid
20	0.072	0.232
40	0.114	0.304
60	0.139	0.328
80	0.19	0.433
100	0.232	0.484

Table 1. Absorbance of MEGA and Ascorbic acid at different concentration showing Total reductive ability

In β-carotene bleaching assay there was a co-relation between degradation rate and the bleaching of β-carotene. The extract showed lower β-carotene degradation rate and higher antioxidant activity. (Fig.2a and b)

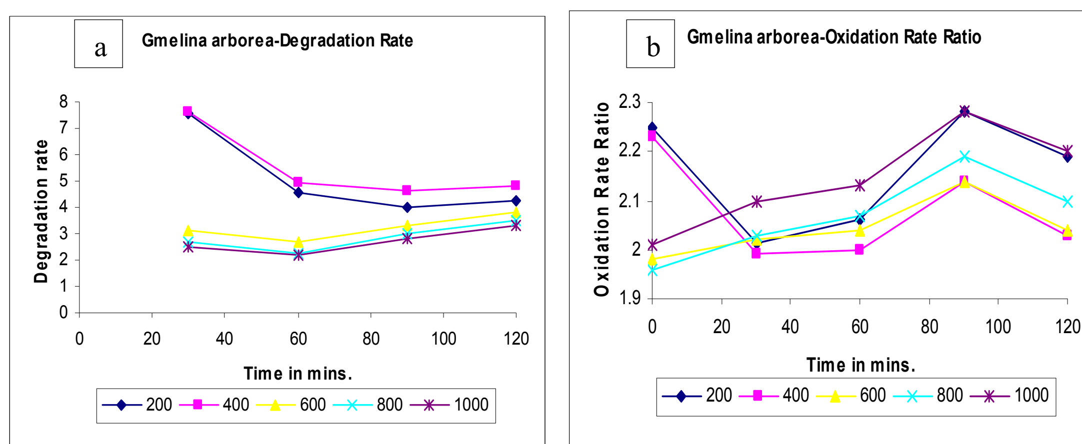

Fig. 2 a and b: Degradation rate and oxidation rate ratio of methanolic extract of *Gmelina arborea* respectively.

Determination of Total Phenolic Content: The absorbance of MEGA (1mg/ml) measured at 765 nm was found to be 0.562.From the standard curve of gallic acid (Fig.3) the amount of total phenolics in MEGA was calculated by linear regression analysis using the equation y=0.0064x-0.0073. 1 mg of MEGA contained 85.95 µg/ml of total phenolics equivalent to Gallic acid.

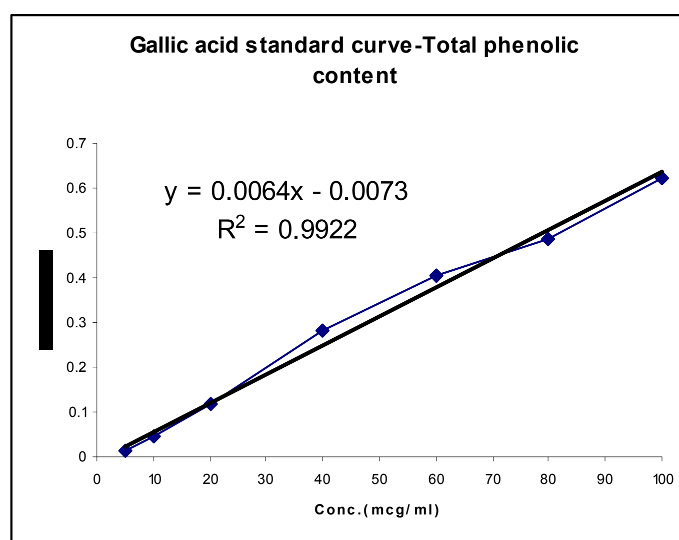

Fig.3 Gallic acid standard curve for determination of total phenolics.

Discussion

DPPH is a free radical compound and has been widely used to test the free radical scavenging ability of extract **(24, 25, 26)**.Anti oxidants on interaction with DPPH· either transfer an electron or hydrogen atom to DPPH· Thus neutralizing its free radical character**(27).** The colour changes from purple to yellow and its absorbance at 517nm decreases. MEGA quenched the DPPH· in a dose dependant manner.

The scavenging of H_2O_2 by MEGA and Ascorbic acid after incubation for 10 min increased with increased concentration. The scavenging of H_2O_2 by phenolic compounds has been attributed to their electron donating ability **(28).**

Nitric oxide (NO) generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions which were measured by the Griess reagent. Nitric oxide is an important chemical mediator generated by endothelial cells, macrophages, neurons, etc. and is involved in the regulation of various physiological processes**(29).**Excess concentration of NO is associated with several diseases **(30,31).** Oxygen reacts with the excess nitric oxide to generate nitrite and peroxynitrite anions, which act as free radicals **(32, 33).** In the present study the extract competes with oxygen to react with NO and thus inhibits the generation of anions.

In NBT reduction assay the antiradical activity is measured by the decrease in absorbance at 590 nm after illumination of the reaction mixture for 5 min. This is caused by the reduction of NBT by riboflavin. The extract required less dose for 50% reduction as compared to ascorbic acid **(18).**

In the β-carotene bleaching assay, linoleic acid produces hydroperoxides as free radicals during incubation at 50 ^{o}C. The presence of antioxidants in the extract will minimize the oxidation of β-carotene by hydroperoxides. Hydroperoxides formed in this system will be neutralized by the antioxidants from the extract. In the present study, we evaluated antioxidant activity of MEGA by β-carotene linoleate bleaching assay because β-carotene shows strong biological activity and is physiologically important compound **(34, 35).**

Phenolic constituents are very important in plants because of their scavenging ability due to their hydroxyl groups **(36).**Phenolic compound are widely distributed in plants**(37)**, which have gained much attention due to their antioxidant activities and free radical scavenging abilities, which potentially have beneficial implications for human health **(38, 39, 40).** One mg of extract contained 85.95µg/ml of GAE of phenols. Phenolic compounds are famous powerful chain breaking antioxidants **(41).**It has been reported that phenolic compounds are associated with antioxidant activity and play a crucial role in stabilizing lipid peroxidation **(42).**

Preliminary phytochemical analysis shows the presence of lignans and flavonoids in the methanolic extract of roots of *Gmelina arborea*.Thus the antioxidant potential of MEGA could be attributed to the presence of these phenolic compounds.

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