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Research Article

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In vitro and *in vivo* antioxidant potential of *Gmelina arborea* stem bark

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ABSTRACT

Reactive oxygen species (ROS) are involved in a wide spectrum of diseases including chronic inflammation and cancer. In the present study, the 70 % methanolic extract of Gmelina arborea (GA) stem bark was tested for its antioxidant activities in both in vitro and in vivo models. The GA extract was shown to possess antioxidant activities as it scavenged superoxide radical and inhibited lipid peroxidation. The extract also showed reducing property as evidenced by ferric reducing power assay. The sodium fluoride (600 ppm/day for 14 days) induced oxidative stress model was used for evaluating the in vivo antioxidant potential of the plant extract. The sodium fluoride intoxication resulted in the impairment of antioxidant defense system of blood and liver as evidenced by the reduced levels of superoxide dismutase, glutathione peroxidase and glutathione. The lipid peroxidation in the liver tissue was elevated indicating the free radical mediated membrane damage. The treatment with plant extract could reduce lipid peroxidation and enhance antioxidant defense system in a significant manner.

Keywords: *Gmelina arborea*, free radical, antioxidant, lipid peroxidation

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INTRODUCTION

A free radical is defined as any chemical species that contains unpaired electrons. These unpaired electrons produces a highly reactive free radical which react with inhaled oxygen in our biological system and produces reactive oxygen species (ROS) and reactive nitrosative species (RNS) which is commonly termed as oxidative stress and nitrosative stress [1].It is well known that reactive oxygen species (ROS), such as superoxide anion (O_2•-), hydroxyl radicals (OH•), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2), play a major role in the development of oxidative stress that can lead to many illnesses including cardiovascular diseases, diabetes, inflammation, degenerative diseases, cancer, anemia and ischemia [2].

In humans, there is a complex endogenous defense system designed to protect tissues from ROS/RNOS induced cell injury. Special enzymes such as superoxide dismutase, catalase and glutathione peroxidase (including their cofactors selenium, zinc, manganese, and iron), sulfhydryl group donors (i.e., glutathione) and vitamins (i.e., vitamins E, C, and β carotene) form a network of functionally overlapping defense mechanisms [3]. Since endogenous anti-oxidant defenses are inadequate to prevent damage completely, diet derived anti-oxidants are important in maintaining health [4]. Thus, pharmacologists and biologists are searching for scavengers, especially naturally occurring anti-oxidants which can inhibit oxidative damage by means of ROS.

Medicinal plants are considered to be an important source of antioxidant compounds and the therapeutic benefit of many medicinal plants is often attributed to their antioxidant properties [5]. *Gmelina arborea*, family Verbenaceae is

having tremendous therapeutic potential and is known to have been used in traditional Indian medicine. A study on the composition of secondary metabolites and antioxidant activity of *G. arborea* showed its richness in phenolic compounds and natural antioxidant substances that play an important role in pharmacology [6]. Therefore, the present study aims to evaluate the *in vitro* antioxidant activity and ameliorative effect on*in vivo*sodium fluoride (NaF) induced oxidative stress of the 70% methanolic extract of *G. arborea* stem bark.

EXPERIMENTAL SECTION

1.1. Chemicals and reagents

2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) was obtained from Sigma-Aldrich.Nitrobluetetrazolium salt and thiobarbituric acid were purchased from Himedia Laboratories. All the other reagents and chemicals used were of analytical grade.

1.2. Animals

Female Sprague Dawley rats (200 - 220 g) were purchased from the Small Animal Breeding Station, Kerala Veterinary and Animal Sciences University, Mannuthy, Kerala, India. The animals were maintained under standardized environmental conditions (temperature: 22 - 30°C, relative humidity: 60 - 70% and 12 hrs of dark/light cycle) with free access to standard rat feed (Lipton, India) and water *ad libitum*. All animal experiments were conducted during the present study got prior permission from Institutional Animal Ethical Committee and followed the internationally accepted laboratory animal use and care guide lines and rules of IAEC.

1.3. Plant collection and extraction

The stem bark of the plant *Gmelina arborea* was collected from the Ayurvedic garden, Amala Cancer Research Centre, Thrissur, Kerala. Stem bark was dried at 45 - 50 ºC for 7 days, powdered and extracted with 70% methanol in a soxhlet apparatus. The extract was evaporated to dryness to remove the traces of the solvent

1.4. *In vitro* antioxidant studies

1.4.1. Superoxide radical scavenging assay

The superoxide radical scavenging ability of the extract was determined according to the McCord and Fridovich [7] method based on the ability of the drug to inhibit reduction of nitrobluetetrazolium (NBT) by superoxide radicals which are generated during photo oxidation of riboflavin. Different concentrations of the extract was added to the reaction mixture containing 200 µl of 0.0015% KCN in 0.1 M EDTA, 100 µl of 1.5 mM NBT and the total volume was adjusted to 2950 µl with 67 mM K-Na phosphate buffer (pH 7.8). The reaction mixture without the addition of the extract was served as control. The initial absorbance of the reaction mixture was taken at 560 nm immediately after the addition of 50 µl of 0.12 mM riboflavin. The tubes were then uniformly illuminated for 15 min with an incandescent lamp. The absorbance of the final reaction was taken at 560 nm. Percentage inhibition was calculated after comparing the absorbance of the extract with that of the control using the formula described and the concentration of the extract to scavenge 50% of the generated superoxide anion (IC_{50}) was also calculated.

$$\% \text{ Inhibition} = \frac{(\text{Absorbance of control - Absorbance of test})}{\text{Absorbance of control}} \text{ X } 100$$

1.4.2. Inhibition of lipid peroxidation

The evaluation of the lipid peroxidation inhibitory potential of the extract was determined by adding different concentrations of the extract to the reaction system contained 100 µl of 25% (w/v) rat liver homogenate in 0.1 M Tris-HCl buffer (pH 7.0), 100 µl of 0.06 mM ascorbic acid, 100 µl of 0.16 mM$FeSO_4(NH_4)_2SO_4.6H_2O$, 100 µl of 30 mMKCl and the volume was adjusted to 500 µl with 0.2 M Tris-HCl buffer (pH 7.0). The reaction was incubated at 37°C for 1 hr and the TBARS reacting substance was measured according to Ohkawa [8]. In short, to 100 µl of the reaction 200 µl of 8.1% SDS, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% TBA was added and the volume was made up to 4 ml by the addition of 400 µl distilled water. The reaction mixture was incubated at 95°C for 1hr in boiling water bath. After the incubation period, the reaction mixture was cooled under tap water and 1 ml of distilled water was added to make up the volume to 5 ml. To this, 5 ml of pyridine:butanol (15:1) was added, mixed by vortexing and centrifuged at 3000 rpm for 10 min. The absorbance of the clear supernatant was measured at 532 nm. The percentage inhibition of lipid peroxidation was calculated based on the above formula and the IC_{50} was also determined.

1.4.3. Ferric reducing antioxidant power assay (FRAP)

The evaluation of the GA extract on the effectiveness of ferric ion (Fe^{3+}) reduction to ferrous (Fe^{2+}) form was based on the method of Pulido [9]. The FRAP reagent contained 2.5 ml 10 mM 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) in 40 mMHCl, 2.5 ml of 20 mM $FeCl_3$. $6H_2O$ and 25 ml of 300 mM acetate buffer (pH 3.6) resulting in the formation of Fe^{3+} - TPTZ complex. Different concentrations of the extract was incubated with 900 µl of freshly prepared FRAP reagent at 37°C for 20 min and the absorbance was determined at 595 nm. Based on the reducing ability of the extract, the Fe^{3+} - TPTZ complex will get reduced to its ferrous form (Fe^{2+}) resulting in the formation of blue color and therefore the absorbance at 595 nm is directly proportional to the reducing power of the extract. The percentage increase in the reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) was calculated using the formula and the half maximal effective concentration (EC_{50}) was also determined.

$$\% \text{ Increase} = \frac{(\text{Absorbance of test - Absorbance of control})}{\text{Absorbance of test}} \text{ X } 100$$

1.5. *In vivo* antioxidant activity against NaF induced oxidative stress

Thirty female Sprague Dawley rats were divided into five groups consisting of six animals in each.Group I: Normal – Untreated; Group II: Control – NaF alone (600 ppm); Group III: Standard –Vitamin C (10 mg/kg b.wt.,i.o.) + NaF (600 ppm); Group IV: GALC – GA extract low concentration (250 mg/kgb.wt.,i.o.) + NaF (600 ppm); Group V: GAHC - GA extract high concentration (500 mg/kgb.wt., i.o.) + NaF (600 ppm). The animals in group III, IV and V were pretreated for seven days with respective drug doses. All the animals except in group I received NaF (600 mg/l) through drinking water for seven days. The drug treatment was continued for another seven days along with the administration of NaF. The next day, all the animals were sacrificed by ether anesthesia.

The blood was collected by heart puncture into heparinized tubes, hemolysate was prepared and used for the estimation of superoxide dismutase (SOD) [7] and reduced glutathione (GSH) [10]. The Liver was collected, washed and 25% homogenate was prepared in 0.1M Tris buffer (pH 7) which was used for the estimation of lipid peroxidation (LPO) [8]. The supernatant of the homogenate was collected after centrifugation at 10,000 rpm for 60 min and used for the estimation of superoxide dismutase (SOD) [7], glutathione peroxidase (GPx) [11] and GSH [10].

1.6. Statistical analysis

The values were expressed as mean ± SD of 3 independent experiments (for *in vitro* studies) or 6 animals per group(for *in vivo* studies). All groups were analyzed for one way ANOVA by Dunnett's test using Graph Pad Instat 3 software. The values with $p \leq 0.05$were considered significant.

RESULTS AND DISCUSSION

Oxidative stress induced by imbalance between production of reactive oxygen species (ROS) and antioxidants have been associated with pathogenic processes including carcinogenesis and inflammation [12, 13]. Radical scavenging activity is very important owing to the deleterious role of free radicals in biological systems and generally proceeds through hydrogen atom transfer or donation of electrons [14]. The GA extract was found to have good *in vitro* antioxidant activity as evidenced by the superoxide radical scavenging, inhibition of lipid peroxidation and ferric reducing activity.

Superoxide anion is an oxygen-centered radical with selective reactivity. This species is produced by a number of enzyme systems in auto-oxidation reactions and by non-enzymatic electron transfers that univalently reduce molecular oxygen [15]. The extract showed significant activity in scavenging the superoxide radicals generated by the photo reduction of riboflavin (Figure 1A). The concentration of the extract required to scavenge 50% of the superoxide anion (IC_{50}) was found to be 184.16 µg/ml. The superoxide radical if not scavenged will give rise to more toxic hydroxyl radicals, singlet oxygen and hydrogen peroxide which can damage macromolecules of the body [16] mainly lipids and consequently resulting in lipid peroxidation. The addition of Fe^{3+} ions with a reductant(ascorbate) could significantly induce lipid peroxidation in rat liver homogenate as revealed by the increased level of TBARS that is MDA in the control tubes. The addition of the extract was effective in reducing the level of MDA formation thus revealing its inhibitory potential on free radical mediated lipid peroxidation. The GA extract exhibited an $IC_{50}$of 266.93 µg/ml (Figure 1B). The reducing power assay is often used to evaluate the ability of the antioxidant to donate an electron [17].During the assay, the GA extract was able to reduce Fe^{3+} to its ferrous

form (Fe^{2+}) in a concentration dependent manner and the EC_{50} for the extract was found to be 34.17 µg/ml (Figure 1C).

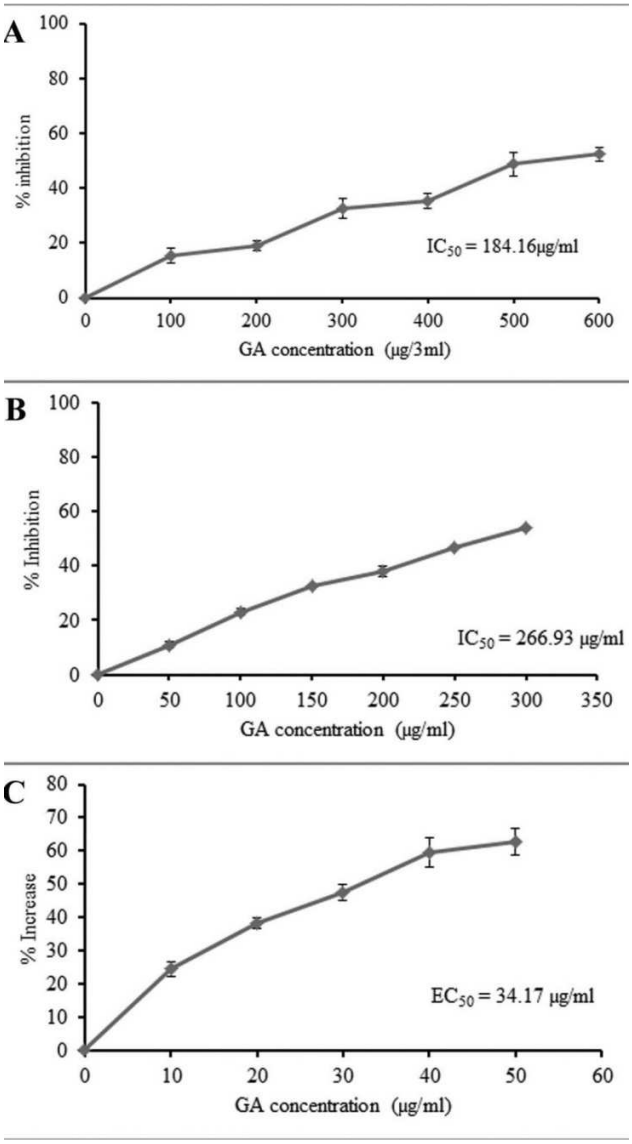

Figure 1:*In vitro* antioxidant potential of *G. arborea*stem bark. A) Superoxide radical scavenging activity; B) Inhibition of lipid peroxidation; C) Ferric reducing activity. Values are expressed as mean ± SD for 3 experiments

In vivo free radical scavenging ability of the extract was also evaluated using NaF induced oxidative stress model. Over production of free radicals by fluoride can induce lipid peroxidation, mediated by the reduced antioxidant enzyme activity [18]. Similar to this NaF administration during the current study has resulted in the reduction of antioxidant defense system of blood and liver. The blood antioxidant enzyme SOD has a significant reduction (24.43±3.05 U/g Hb) after fluoride intoxication when compared to the normal animals (33.12±3.21 U/g Hb). The GALC and GAHC treated groups could enhance the enzyme activity to a level of 26.56±3.16 and 31.23±3.74 U/g Hb, respectively (Figure 2A). Similar result was observed for the blood GSH level. The augmented level of GSH content during NaF administration (8.93±0.79 nmol/g Hb) was enhanced by the treatment with vitamin C (13.42±0.97 nmol/g Hb), GALC (11.32±0.8 nmol/g Hb) and GAHC (12.86±0.86 nmol/g Hb) in a significant manner ($p \leq 0.01$), when compared to the normal GSH level (14.46±1.21 nmol/g Hb) (Figure 2B).

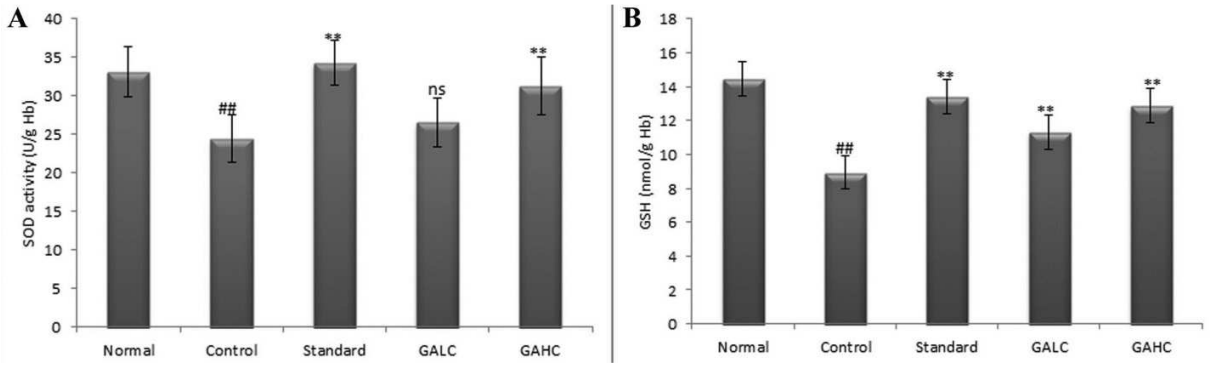

Figure 2:Effect of GA extract on blood antioxidant status of rats treated with NaF. A) Superoxide dismutase activity; B) Glutathione.Values are expressed as mean ± SD for 6 animals; ##P≤0.01, when compared to Normal; **P ≤0.01, when compared to Control;nsnon significant

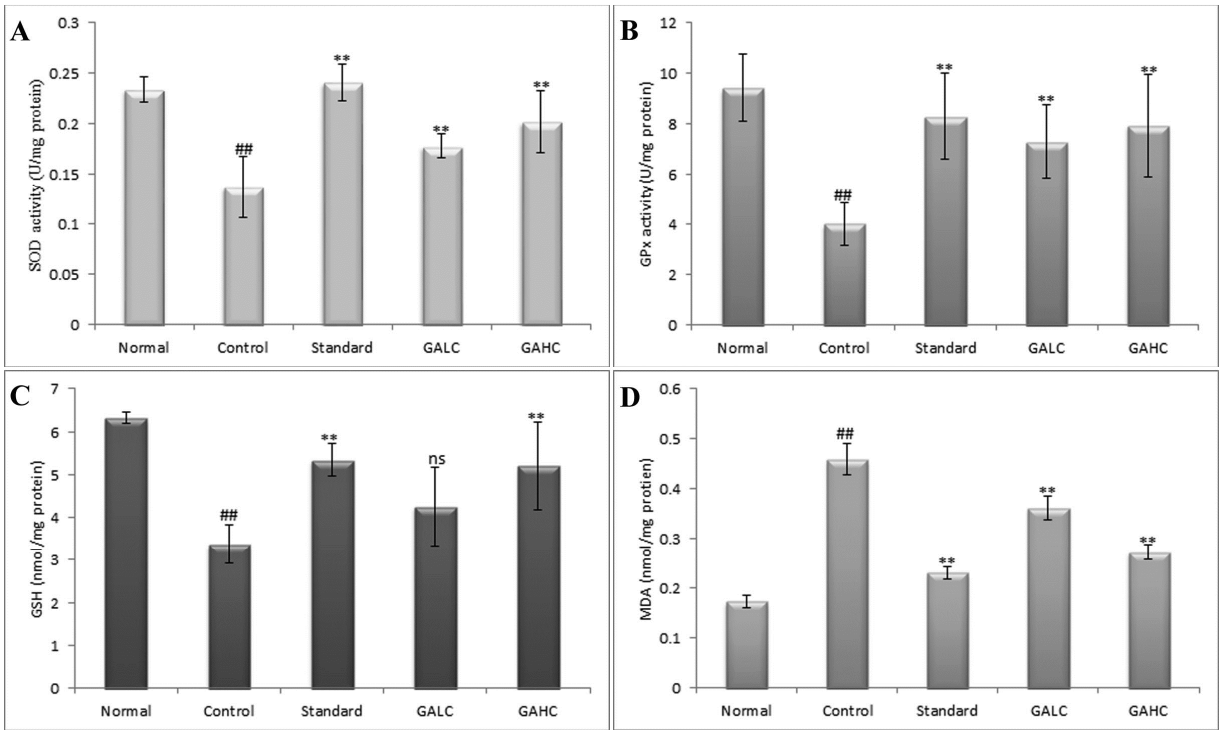

Figure 3:Effect of GA extract on liver antioxidant status and lipid peroxidation of rats treated with NaF. A) Superoxide dismutase; B) Glutathione peroxidase; C) Glutathione; D) Lipid peroxidation. Values are expressed as mean ± SD for 6 animals; ##P≤0.01, when compared to Normal; **P ≤0.01, when compared to Control;nsnon significant

Since liver is the detoxification system of the body, it is the organ which is affected mainly by NaF induced free radical damage. The antioxidant defense system of liver tissue is altered by the fluoride toxicity. The superoxide enzyme activity in liver was reduced from 0.234±0.01 U/mg protein to 0.137±0.03 U/mg protein during NaF administration in the control group. There was a significant increase in the enzyme activity of the standard ($p \leq 0.01$), GALC ($p \leq 0.05$) and GAHC ($p \leq 0.01$) treated groups (Figure 3A). Another antioxidant enzyme glutathione peroxidase activity (9.43±1.31 U/mg protein) was also reduced in the liver tissue after NaF administration (4.02±0.84 U/mg protein). A significant ($p \leq 0.01$) enhancement of the enzyme activity was observed during the treatment with GA extract (Figure 3B). The GSH content in the liver tissue (6.32±0.12 nmol/mg protein) was also reduced in the control group (3.37±0.45 nmol/mg protein). The vitamin C treated standard group (5.34±0.38 nmol/mg protein) and GAHC group (5.21±1.02 nmol/mg protein) could significantly ($p \leq 0.01$) restore this GSH level (Figure 3C).

During the study, the fluoride intoxication induced a significant ($p \leq 0.01$) increase in hepatic lipid peroxidation (0.459±0.03 nmol MDA/mg protein), when compared with the untreated normal group (0.173±0.01 nmol MDA/mg protein). The increased liver lipid peroxidation during fluoride intoxication is observed in number of studies [19-21]. The treatment with GA extract showed a significant protection of lipid peroxidation ($p \leq 0.01$) with GALC and GAHC groups showing only 0.362±0.02 and 0.272±0.01 nmol MDA/mg protein, respectively (Figure 3D).

CONCLUSION

These results indicate the antioxidant potential of the plant *G. arborea* stem bark. Therefore, the observed amelioration of sodium fluoride induced oxidative damage by the extract can be attributed to its free radical scavenging ability. Further studies are required in order to isolate the active phytochemical compound which imparts the antioxidant potential to the plant.

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