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In vitro antioxidant and free radical scavenging activities of *Galega purpurea* root

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ABSTRACT

This study was designed to determine the antioxidant and free radical scavenging properties of methanol extract of *Galega purpurea* (MEGP) (Papilionaceae) root in various in vitro systems. DPPH radical, superoxide anion radical, nitric oxide radical and hydroxyl radical scavenging assays were carried out to evaluate the antioxidant potential of the extract. The antioxidant activity of methanolic extract increased in a dose dependent manner. About 50, 100, 200, 300, 400 and 500 µg of methanol extract of *Galega purpurea* (MEGP) showed 38.11, 45.71, 51.32, 58.04, 63.44 and 70.28 % inhibition respectively on peroxidation of linoleic acid emulsion. Like antioxidant activity, the effect of MEGP on reducing power increases in a dose dependent manner. In DPPH radical and nitric oxide radical scavenging assays, MEGP exhibited maximum activity of 71.32 and 76.14 % inhibition respectively at the concentration of 125 µg/mL. Moreover, the MEGP was found to scavenge the superoxide generated by PMS/NADH-NBT system. MEGP was also found to inhibit the hydroxyl radical generated by Fenton's reaction, where the IC_{50} value of MEGP was found to be 61.36 µg/mL and for catechin the IC_{50} value was found to be 5.27 µg/mL, which indicates the prooxidant activity of MEGP. The amounts of total phenolic compounds were also determined in this study. The results obtained in the present study indicate that the MEGP can be a potential source of natural antioxidant.

KEY WORDS: *Galega purpurea*, Antioxidant activity, DPPH assay

INTRODUCTION

Free radicals have aroused significant interest among scientists in the past decade. Their broad ranges of effects in biological systems have drawn on the attention of many experimental works. Highly reactive free radicals, especially oxygen-derived radicals, which are formed by exogenous chemicals or endogenous metabolic processes in the human body, are capable of oxidizing biomolecules, resulting in cell death and tissue damage. Oxidative damage plays a significantly pathological role in human diseases. Cancer, emphysema, cirrhosis, atherosclerosis and arthritis have all been correlated with oxidative damage (1). Almost all organisms are well protected against free radical damage by enzymes such as superoxide dismutase and catalase or compounds such as ascorbic acid, tocopherol and glutathione (2). When the mechanism of antioxidant protection becomes unbalanced by factors such as ageing, deterioration of physiological functions may occur resulting in diseases and accelerating ageing. However, antioxidant supplements may be used to help the human body to reduce oxidative damage.

Synthetic antioxidants are widely used because they are effective and cheaper than natural types. However, the safety and toxicity of synthetic antioxidants have been important concerns (3). Much attention has been focused on the use of antioxidants especially natural antioxidants to inhibit lipid peroxidation or to protect the human body from the oxidative damage by free radicals.

Galega purpurea (Papilionaceae) a plant thrives in Southern parts of India and grows on hard stony ground too difficult to be rooted. Various parts of the plant are widely used in the folk medicine for the treatment of cough, asthma, bilious febrile attacks, arthritis and rheumatism. Decoction of the root useful in the management of enlargement and obstruction of the liver, spleen and kidney. The root is useful in the treatment of dyspepsia, chronic diarrhea and ulcers

(4). Therefore our aim in this study is to evaluate the antioxidant and antimicrobial properties of methanol extract of *Galega purpurea* root.

MATERIALS AND METHODS

Plant material

The roots of the plant *Galega purpurea* were collected from Erode district of Tamilnadu, India. The plant material was taxonomically identified by Botanical Survey of India, Kolkata. A voucher specimen (No. GMG 03/05) has been preserved in our laboratory for future reference. The collected plant material was dried under shade and then powdered with a mechanical grinder and stored in an air-tight container. The dried powder material of the root was defatted with petroleum ether and the marc thus obtained was then extracted with methanol in a Soxhlet apparatus. The solvent was completely removed under reduced pressure and a semisolid mass was obtained. The dried MEGP was dissolved in water and used for the present study.

Chemicals

Ammonium thiocyanate was purchased from E. Merck. Ferrous chloride, ferric chloride, polyoxyethylene sorbitan monolaurate (Tween-20), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), nicotinamide adenine dinucloetide (NADH), EDTA, butylated hydroxy toluene (BHT), butylated hydroxy anisole (BHA), α-tocopherol, ascorbic acid, quercetin, catechin, pyrocatechol, nitrobluetetrazolium, thiobarbituric acid, 2-deoxy-2-ribose, trichloroacetic acid, phenazine methosulphate and potassium ferricyanide were purchased from Sigma Chemical Co. Ltd, USA. All other unlabeled chemicals and reagents were ofanalytical grade and were used without further purification.

Antioxidant activity

The antioxidant activity of MEGP was determined according to the thiocyanate method (5). About 10 mg of MEGP was dissolved in 10 mL distilled water. Various concentrations (50, 100, 200, 300, 400 and 500 μg/mL) of MEGP were added to linoleic acid emulsion (2.5 mL, 0.04 M, pH 7.0) and phosphate buffer (2 mL, 0.04 M, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.2804 g of linoleic acid, 0.2804 g of Tween 20 as emulsifier and 50 mL phosphate buffer and then the mixture was homogenized. The final volume was adjusted to 5mL with potassium phosphate buffer (0.04 M, pH 7.0). Further on the mixed samples were incubated at 37^0C in a glass flask for 60 hours to accelerate the oxidation process (6,7). Each 12 hours, 1 mL of the incubated sample was removed and 0.1 mL of $FeCl_2$ (0.02 M) and 0.1 mL of ammonium thiocyanate (30%) were added. The amount of peroxide was determined by measuring the absorbance at 500 nm. Alpha tocopherol was used as the reference compound. To eliminate the solvent effect, the control sample, which contains the same amount of solvent added into the linoleic acid emulsion in the test sample and reference compound was used. All the data are expressed as mean of triplicate determinations. The percentage of inhibition of lipid peroxide generation was measured by comparing the absorbance values of control and those of test samples.

Reductive ability

The reducing power of MEGP was determined according to the method of Oyaizu (1986) (8). 10 mg of MEGP extract in 1 mL of distilled water was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 mL, 1%). The mixture was incubated at 50^0C for 20 minutes. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 g for 10 minutes. 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. BHT was used as reference compound. All the analyses were performed in triplicate and the results were averaged. Increased absorbance of the reaction mixture indicated increasing reducing power.

DPPH radical scavenging effect

The free radical scavenging activity of MEGP was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH) using the method of Blois (1958) (9). Used as a reagent, DPPH evidently offers a convenient and accurate method for titrating the oxidizable groups of natural or synthetic antioxidants (10). 0.1 mM solution of DPPH in methanol was prepared and 1 mL of this solution was added to 3 mL of MEGP suspension in water at different concentrations (10, 25, 50, 75, 100 and 125 μg). After 30 minutes, absorbance was measured at 517 nm. Alpha tocopherol was used as the reference material. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. All the tests were performed in triplicate and the results averaged. The percentage inhibition was calculated by comparing the absorbance values of control and samples.

Nitric oxide radical scavenging effect

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions which were measured by the Griess reaction (11,12). The reaction mixture (3 mL) containing sodium nitroprusside (10 mM) in

phosphate buffered saline (PBS) and the MEGP in different concentrations (10, 25, 50, 75, 100 and 125 μg) were incubated at 25 oC for 150 minutes. Every 30 minutes, 0.5 mL of the incubated sample was removed and 0.5 mL of Griess reagent (1% sulphanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H_3PO_4) was added. The absorbance of the chromophore formed was measured at 546 nm. All the analyses were performed in triplicate and the results were averaged. The percentage inhibition of nitric oxide generated was measured by comparing the absorbance values of control and test. Curcumin was used as a reference compound.

Superoxide anion radical scavenging effect

Measurement of superoxide anion scavenging activity of MEGP was done based on the method described by Nishimiki et al (1972) (13) and slightly modified. About 1 mL of nitroblue tetrazolium (NBT) solution (156 μM NBT in 100 mM phosphate buffer, pH 7.4), 1 mL of NADH solution (468 μM in 100 mM phosphate buffer, pH 7.4) and 0.1 mL of sample solution of MEGP (10, 25, 50, 75, 100 and 125 μg) in distilled water were mixed and the reaction started by adding 100 μl of phenazine methosulphate (PMS) solution (60 μM PMS in 100 mM phosphate buffer, pH 7.4. The reaction mixture was incubated at 25^0C for 5 minutes, and the absorbance at 560nm was measured against blank samples. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. Curcumin was used as reference compound. All the experiments were performed in triplicate and the results were averaged. The percentage of inhibition was determined by comparing the results of control and test samples.

Hydroxyl radical scavenging effect

Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and test compounds for hydroxyl radical generated by Fe^{3+}-Ascorbate–EDTA–H_2O_2 system (Fenton reaction) according to the method of Kunchandy and Rao (1990) (14). The hydroxyl radicals attack deoxyribose that eventually results in TBARS formation.

The reaction mixture contained in a final volume of 1.0 mL, 100 μl of 2-deoxy-2-ribose (28 mM in KH_2PO_4-K_2HPO_4 buffer, pH 7.4), 500μl solutions of various concentrations of MEGP (10, 25, 50, 75, 100 and 125 μg) in KH_2PO_4-KOH buffer (20 mM, pH 7.4), 200 μl of 1.04 mM EDTA and 200 μM $FeCl_3$ (1:1 v/v), 100 μl of 1.0 mM H_2O_2 and 100 μl of 1.0 mM ascorbic acid was incubated at 37^0C for 1 hour. The free radical damage imposed on the substrate, deoxyribose was measured as TBARS by the method of Ohkawa et al (1979) (15). 1.0 mL of thiobarbituric acid (1%) and 1.0 mL of trichloroacetic acid (2.8%) were added to the test tubes and were incubated at 100^0C for 20 minutes. After cooling absorbance was measured at 532 nm against control containing deoxyribose and buffer. Catechin was used as a positive control. All the experiments were performed in triplicate and the results were averaged. The percentage inhibition was determined by comparing the results of the test compounds and control.

Amount of total phenolic compounds

Total soluble phenolics in the MEGP were determined with Folin–Ciocalteu reagent according to the method of Slinkard and Singleton (1977) (16). In brief, 0.1 mL of extract in distilled water (contains 1 mg of MEGP) was transferred into 100 mL Erlenmeyer flask then final volume was adjusted to 46 mL by addition of distilled water. Afterwards, 1 mL of Folin – Ciocalteu reagent (FCR) was added to this mixture and after 3 minutes. 3 mL of Na_2CO_3 (2%) were added. Subsequently, mixture was shaken on a shaken for 2 hours at room temperature and then absorbance was measured at 760 nm. All experiments were performed in triplicate and the results were averaged. The concentration of total phenolic compounds in MEGP was determined as microgram of pyrocatechol equivalent by using an equation that was obtained from standard pyrocatechol graph. The equation is given below;

Absorbance = 0.001 x Pyrocatechol (μg) + 0.0033

Statistical analysis

Statistical analysis of difference between groups was evaluated by one-way ANOVA followed by student t-test. The values $P<0.05$ were regarded as significant and the values $P<0.01$ were considered as highly significant.

RESULTS AND DISCUSSION

Antioxidant activity

The most commonly used method for determining antioxidant activity is to measure the inhibitory degree of autoxidation of linoleic acid (5). The results were shown in figure 1. The different concentration of MEGP 50, 100, 200, 300, 400 and 500 μg/mL showed antioxidant activities in a dose dependent manner and had 38.11, 45.71, 51.32, 58.04, 63.44 and 70.28 % inhibition respectively on lipid peroxidation of linoleic acid system. At the same time α-tocopherol at the concentration 500 μg/mL showed 81.21 % inhibition.

Fig 1. Percentage Inhibition of lipid peroxidation by Alpha tocopherol and different doses of MEGP in the linoleic acid emulsion.

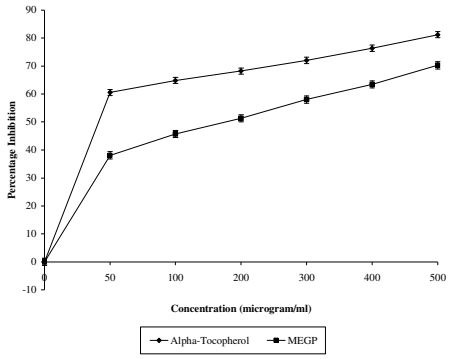


Fig 2. Reductive ability of BHT and different doses of MEGP

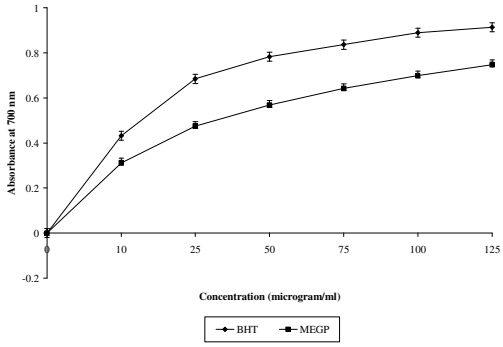


Fig 3. Percentage Inhibition of DPPH radicals by Alpha tocopherol and different doses of MEGP

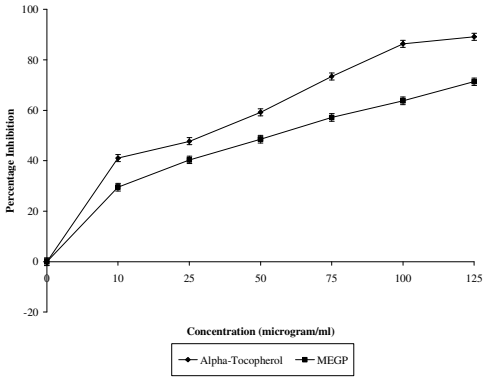


Fig 4. Percentage Inhibition of nitric oxide radicals by curcumin and different doses of MEGP

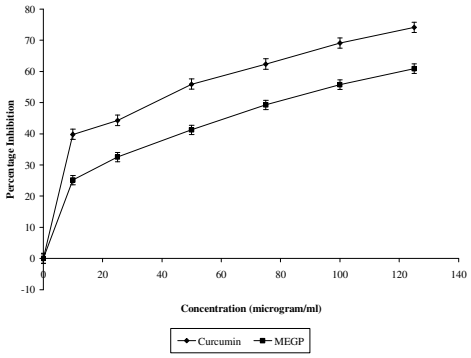


Fig 5. Percentage Inhibition of superoxide radicals by curcumin and different doses of MEGP on PMS/ NADH-NBT system

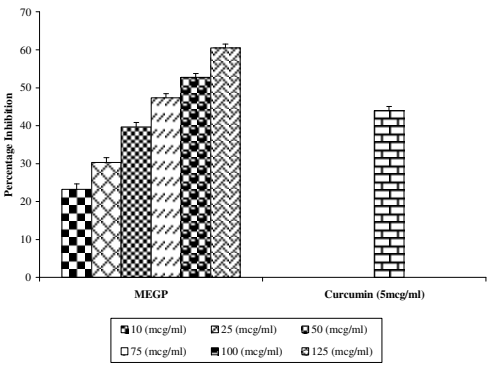
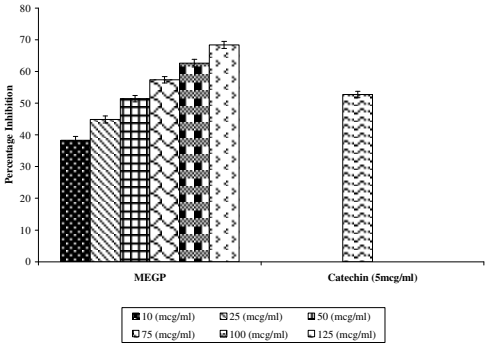


Fig 6. Percentage Inhibition of hydroxyl radicals by catechin and different doses of MEGP



Reductive ability - The antioxidant activity has been reported to be concomitant with the development of reducing power (17). The reducing power of MEGP might be due to its hydrogen donating ability, as described by Shimada et al (1992) (18). The reducing power of MEGP and BHT is shown in figure 2. The reducing power increased as the extract concentration increased, indicating some compounds in *Galega purpurea* is both electron donors and could react with free radicals to convert them in to more stable products and to terminate radical chain reactions. For the measurements of the reductive ability, we investigated the Fe^{3+}-Fe^{2+}, transformation in the presence of the MEGP using the method Oyaizu (1986) (8). All the amounts of MEGP showed higher activities than control and these differences were statistically highly significant ($P< 0.01$).

DPPH radical scavenging effect

DPPH scavenging activity has been used by various researchers as a quick and reliable parameter to assess the in vitro antioxidant activity of crude plant extracts. (19, 20). In DPPH test the ability of a

compound to act as donor for hydrogen atoms or electrons was measured spectrophotometrically. The scavenging activities of DPPH exerted by MEGP as well as α-tocopherol were summarized in figure 3. MEGP at the concentration of 125 μg/mL exhibited 71.32 % inhibition, where standard drug α-tocopherol at the same concentration exhibited 89.17 % inhibition. In the present investigation MEGP at different doses demonstrated significant DPPH scavenging activity indicating their abilities to act as radical scavengers.

Nitric oxide radical scavenging effect

Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons, etc. and is involved in the regulation of various physiological processes (21). Excess concentration of NO is associated with several diseases (22,23). Oxygen reacts with the excess nitric oxide to generate nitrite and peroxynitrite anions, which act as free radicals (24,25). In the present study, the extract competes with oxygen to react with nitric oxide and thus inhibits generation of the anions. Figure 4 illustrates the percentage inhibition of nitric oxide generation by MEGP. Curcumin was used as a reference compound. The IC_{50} value of MEGP was found to be 76.14 μg/mL whereas the IC_{50} value of curcumin was found to be 20.4 μg/mL.

Superoxide anion radical scavenging effect

Superoxides are produced from molecular oxygen due to oxidative enzymes (25) of body as well as via non-enzymaticreaction such as autoxidation by catecholamines (26). In the present study, superoxide radical reduces NBT to a blue colored formazan that is measured at 560 nm (27). Figure 5 shows the superoxide scavenging effect of MEGP and curcumin on the PMS/NADH-NBT system. The decrease of absorbance at 560nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. MEGP at concentration from 10-125 μg/mL inhibited the production of superoxide anion radicals by 23.25-60.52 %. MEGP had strong superoxide radical scavenging activity. The IC_{50} value of MEGP on superoxide radical scavenging activity was found to be 94.77 μg/mL and for curcumin 5.95 μg/mL.

Hydroxyl radical scavenging effect

Hydroxyl radicals are the major active species causing lipid oxidation and enormous biological damage (28,29). The deoxyribose method is a simple assay to determine the rate constants for reactions of hydroxyl radicals (30). Ferric_EDTA was incubated with H_2O_2 and ascorbic acid at pH 7.4. Hydroxyl radicals were formed in free solution and were detected by their ability to degrade 2-deoxy-2-ribose in to fragments that on heating with TBA at low pH form a pink chromogen (30,31). Any hydroxyl radical scavenger added to the reaction would compete with deoxyribose for the availability of hydroxyl radicals, thus reducing the amount of MDA formation. We herein tested the scavenging activity of MEGP along with positive control catechin. The concentration of MEGP needed for 50% inhibition was found to be 61.36 μg/mL. Catechin, used as a standard was highly effective in inhibiting the oxidative DNA damage, showing an IC_{50} = 5.27μg/mL (Figure 6).

Amount of total phenolic compounds

Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups (32). In the MEGP (1 mg) 161.02 μg pyrocatechol equivalent of phenols was detected. The phenolic compounds may contribute directly to antioxidative action (33). It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when ingested up to 1g daily from a diet rich in fruits and vegetables (17).

CONCLUSION

Medicinal herbs are known to contain a variety of antioxidants. Numerous substances have been suggested to appear as antioxidants. The most detailed investigations so far were concerned with reactions involving phenolic compounds ranging from polymer chemistry to biochemistry and food chemistry (34). It has been revealed that various phenolic antioxidants such as flavonoids, tannins, coumarins, xanthones and more recently procyanidins scavenge radicals dose-dependently, thus they are viewed as promising therapeutic drugs for free radical pathologies (35).

The results of the present study, which demonstrate the radical scavenging of MEGP, indicate that the use of *Galega purpurea* for the treatment of various inflammatory diseases seems quite useful and reasonable. The antioxidant and free radical scavenging activities of *Galega purpurea* root might be due to the presence phenolic compounds in methanol extract which is confirmed by FCR reagent test. Further studies are in progress in our laboratory to evaluate the in vivo antioxidant potential of this extract in various animal models and phytochemical studies are required to establish the types of compounds responsible for the bioactivity of this medicinal plant and to determine the value of the ethnobotanical approach for the screening of plants as potential source of bioactive substances.

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