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Original Article

Antioxidant and Free Radical Scavenging Activities of *Ervatamia coronaria* Stapf. leaves

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Abstract

The present study was carried out to evaluate the antioxidant and free radical scavenging activity of methanolic extract of *Ervatamia coronaria* leaves (Apocynaceae) in various 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, 250 and 500 µg of methanol extract of *Ervatamia coronaria* (MEEC) showed 61.33, 66.21, 72.04 and 76.83% inhibition respectively on peroxidation of linoleic acid emulsion. Like antioxidant activity, the effect of MEEC on reducing power increases in a dose dependent manner. In DPPH radical scavenging assay the IC_{50} value of the extract was found to be 167.09 µg/ml. MEEC was found to inhibit the nitric oxide radicals generated from sodium nitroprusside. The IC_{50} value was found to be 83.375 µg/ml, whereas the IC_{50} value of curcumin was 20.4 µg/ml. Moreover, the MEEC was found to scavenge the superoxide generated by PMS/NADH-NBT system. MEEC was also found to inhibit the hydroxyl radical generated by Fenton's reaction, where the IC_{50} value of MEEC was found to be more than 1000 µg/ml and for catechin the IC_{50} value was found to be 5 µg/ml, which indicates the prooxidant activity of MEEC. The amounts of total phenolic compounds were also determined in this study. The results obtained in the present study indicate that the MEEC can be a potential source of natural antioxidant.

Keywords: Ervatamia coronary; Antioxidant activity; Lipid peroxidation; Free radical scavenging; DPPH assay.

Introduction

The effects of free radicals on human beings are closely related to toxicity, disease and aging (1). Most living species have an efficient defense system to protect themselves against the oxidative stress induced by Reactive Oxygen Species (ROS) (2). Recent investigations have shown that the antioxidant properties of plants could be correlated with oxidative stress defense and different human diseases including cancer, atherosclerosis and the aging process (3-5). The antioxidants can interfere with the oxidation process by reacting with free radicals, chelating free catalytic metals and also by acting as oxygen scavengers.

Ervatamia coronaria Stapf (Synonym : *Tabernaemontana divaricata*) (6) belongs to the family Apocynaceae, is a glabrous, evergreen tree indigenous to India and is cultivated in gardens for its ornamental and fragrant flowers. This species has been extensively investigated and a number of chemical constituents such as alkaloids (7-10), triterpenoids (11-13), steroids (12, 14), flavonoids (15), phenyl propanoids (14, 15) and phenolic acids (8) were isolated from leaves, roots and stems of the plant. In Indian traditional system of medicine the plant

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material is widely used as a purgative, tonic to the brain, the spleen and the liver; in the treatment of cancer, wounds and inflammations (16, 17). The plant extract was also found to possess analgesic, antipyretic, vasodilator and CNS depressant effects (18), antispasmodic, hypotensive activity (19), antiinflammatory (8), uterine stimulant effect (20) and cytotoxic activity (21).

Furthermore, literature survey of *E. coronaria* revealed that no researcher has yet reported antioxidant activities of this plant. Therefore, it is worth conducting an investigation on the *in vitro* antioxidant activities of methanolic extract of *E. coronaria* leaves (MEEC).

Experimental

Plant material

The plant *Ervatamia coronaria* was collected in March 2003 from the Kolli Hills, Tamil Nadu, India. The plant material was taxonomically identified by the Botanical Survey of India, Shibpur, Kolkata, India, and the Voucher specimen (No. GMG1) was retained in our laboratory for future reference. The leaves were dried under shade with occasional shifting and then powdered with a mechanical grinder and stored in an airtight container. The dried and powdered leaves (500 g) were defatted with petroleum ether (60-80°C) in a Soxhlet apparatus. The defatted powder material thus obtained was further extracted with methanol in the Soxhlet for 72 h. The solvent was removed by distillation under suction and the resulting semisolid mass was dried using the rotary flash evaporator to yield (14.51%) a solid residue (methanolic extract). The dried MEEC was suspended in distilled water and used for further studies.

Materials

Ammonium thiocyanate was purchased from E. Merck, Germany. Ferrous chloride, ferric chloride, 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), nicotinamide adenine dinucleotide (NADH), EDTA, butylated hydroxy toluene (BHT), butylated hydroxy anisole (BHA), α-tocopherol, ascorbic acid, quercetin, catechin, pyrocatechol, curcumin, nitroblue tetrazolium, thiobarbituric acid, 2-deoxy-2-ribose, trichloroacetic acid, phenazine methosulphate and potassium ferricyanide were all purchased from Sigma Chemical Co. Ltd, USA. All the other unlabeled chemicals and reagents were of analytical grade.

Antioxidant activity

The antioxidant activity of MEEC was determined according to the thiocyanate method (22). About 10 mg of MEEC was suspended in 10ml water. Various concentrations (50, 100, 250 and 500 μg/ml) of MEEC 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 5 ml with potassium phosphate buffer (0.04 M, pH 7.0). Then the mixed samples were incubated at 37 °C in a glass flask for 60 h to accelerate the oxidation process (23, 24). Each 12 h, 1 ml of the incubated sample was removed and 0.1 ml of FeCl2 (0.02 M) and 0.1ml 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. In order 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 the average of triplicate analysis. The percentage inhibition of lipid peroxide generation was measured by comparing the absorbance values of control and those of test samples.

Reducing power

The reducing power of MEEC was determined according to the method of Oyaizu (25). 10 mg of MEEC extract in 1ml of distilled water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K3Fe(CN)6] (2.5 ml, 1%). The mixture was incubated at 500C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 g for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl3 (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. BHT was used as the reference material. All the tests were performed in triplicate and the graph was plotted with the average of three observations.

Inhibition of DPPH radical

The free radical scavenging activity of MEEC was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH) using the method of Blois (26). 0.1 mM solution of DPPH in methanol was prepared and 1ml of this solution was added to 3 ml of various concentration of MEEC and the reference compound (50, 100, 150, 200 and 250 µg). After 30 min, absorbance was measured at 517 nm. BHA was used as the reference material. All the tests were performed in triplicate and the graph was plotted with the mean values. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples.

Inhibition of Nitric oxide radical

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 (27, 28). The reaction mixture (3 ml) containing sodium nitroprusside (10 mM) in phosphate buffered saline (PBS) and MEEC and the reference compound in different concentrations (10, 25, 50, 75 and 100 µg) were incubated at 25°C for 150 min. Each 30 min, 0.5 ml of the incubated sample was removed and 0.5 ml of the Griess reagent (1% sulphanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H3PO4) was added. The absorbance of the chromophore formed was measured at 546 nm. All the tests were performed in triplicate and the results averaged. The percentage inhibition of nitric oxide generated was measured by comparing the absorbance values of control and test sampels Curcumin was used as a positive control compound.

Inhibition of Superoxide anion radical

Measurement of superoxide anion scavenging activity of MEEC was performed based on the method described by Nishimiki (29) and slightly modified. About 1 ml of nitroblue tetrazolium (NBT) solution containing 156 µM NBT which is dissolved in 1.0 ml of phosphate buffer (100 mM, pH 7.4), 1 ml of NADH solution containing 468 µM of NADH which is dissolved in 1 ml of phosphate buffer (100 mM, pH 7.4) and 0.1 ml of various concentration of MEEC and the reference compounds (10, 25, 50, 75 and 100 µg) were mixed and the reaction started by adding 100 µl of phenazine methosulphate (PMS) solution containing 60 µM of PMS 100 µl of phosphate buffer (100 mM, pH 7.4). The reaction mixture was incubated at 250C for 5 min and the absorbance at 560 nm was measured against the control samples. BHT and quercetin were used as the reference compounds. All the tests were performed in triplicate and the results averaged. The percentage inhibition was calculated by comparing the results of control and test samples.

Inhibition of Hydroxyl radical

Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the test compounds (MEEC) for hydroxyl radical generated by Fe3+-Ascorbate-EDTA-H2O2 system (Fenton reaction) according to the method of Kunchandy and Rao (30). The reaction mixture contained in a final volume of 1.0 ml, 100 µl of 2-deoxy-2-ribose (28 mM in KH2PO4-KOH buffer, 20 mM, pH 7.4), 500mcl of the various concentrations of MEEC and the reference compound (1, 100 and 1000 µg) in KH2PO4-KOH buffer (20 mM, pH 7.4), 200 µl of 1.04 mM EDTA and 200 µM FeCl3 (1:1 *v/v*), 100 µl of 1.0 mM H2O2 and 100 µl of 1.0 mM ascorbic acid was incubated at 370C for 1h. 1.0 ml of thiobarbituric acid (1%) and 1.0 ml of trichloroacetic acid (2.8%) were added to the test tubes and incubated at 100°C for 20 min. After cooling, absorbance was measured at 532 nm against a control sample containing deoxyribose and buffer. Catechin was used as a positive control. Reactions were carried out in triplicate. The percentage inhibition was determined by comparing the results of the test and control compounds.

Amount of total phenolic compounds

Total soluble phenolics present within in the MEEC were determined using the Folin-Ciocalteu reagent, according to the method of a Slinkard and Singleton (31). 0.1 ml of suspension of 1mg of MEEC in water was totally transferred into 100 ml Erlenmeyer flask. Then the final volume was adjusted to 46 ml by the addition of distilled water. Afterwards, 1 ml of Folin - Ciocalteu reagent (FCR) was added to this mixture and after 3 min, 3 ml of Na2CO3 (2%) was added. Subsequently, the mixture was shaken on a shaker for 2 h at room temperature and then its absorbance measured at 760 nm. All the tests were performed in triplicate and the results averaged. The concentration of total phenolic compounds in MEEC was determined as microgram of pyrocatechol equivalent by using an equation that was obtained from the standard pyrocatechol graph. The equation is given below;

$$\text{Absorbance} = 0.001 \times \text{Pyrocatechol } (\mu g) + 0.0033$$

Statistical analysis

Experimental results were mean±SD of three parallel measurements. Statistical analysis was performed according to the student t-test. and ANOVA procedure. The values for $P < 0.05$ were regarded as significant and the values for $P < 0.01$ as highly significant.

Results And Discussion

Lipid peroxidation has been defined as the biological damage caused by free radicals, wich are formed under oxidative stress (32). The antioxidative activity of natural sources is due to the active compounds present in the plants. Most natural antioxidants are found in wood, bark, stem, leaf, fruit, root, flower and seed (33). Most of these compounds are normally phenolic or polyphenolic in nature eg, tocopherols, flavonoids and derivatives of cinnamic acid, phosphatidic and other organic acids.

Antioxidant activity

In this study the antioxidative activity of the leaf extract of *Ervatamia coronaria* was measured using the ammonium thiocyanate method. This method was used to measure the peroxide level during the initial stages of lipid oxidation. The antioxidant activity of MEEC might be due to the reduction of hydroperoxide, inactivation of free radicals or complexation with metal ions, or combinations thereof. This good antioxidant activity of MEEC might be attributed to the presence of phytochemicals, such as flavonoids and biflavones (34). Figure 1 illustrates the antioxidative activities of various concentrations of MEEC (50, 100, 250 and 500 µg/ml). The different concentrations of MEEC (50, 100, 250 and 500 µg/ml) showed antioxidant activities in a dose dependent manner and had 61.33, 66.21, 72.04 and 76.83% inhibition respectively on the lipid peroxidation of linoleic acid system. MEEC, at a concentration of 500 µg/ml showed 76.83% inhibition, which is more or less equal to the antioxidant activity of 500 µg/ml of α-tocopherol (77.13%). The IC_{50} value of MEEC on lipid peroxidation was found to be 40.76 µg/ml. The results indicate that methanolic extract of *Ervatamia coronaria* significantly ($P<0.05$) inhibits the linoleic acid peroxidation. The antioxidative activity of the leaves of *Ervatamia coronaria* may be due to the reduction of hydroperoxides, inactivation of free radicals, chelation of metal ions or combinations thereof.

Reductive ability

The antioxidant activity has been reported to be concomitant with the development of reducing power (35). However this pattern was not observed in this research. Okuda et al.

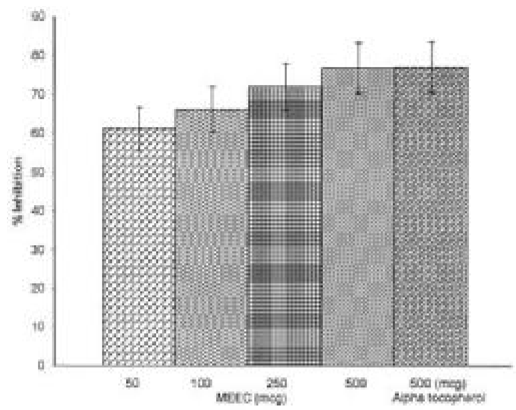

Figure 1. Inhibition (%) of lipid peroxidation of alpha tocopherol and different doses of MEEC in the linolic acid emulsion.

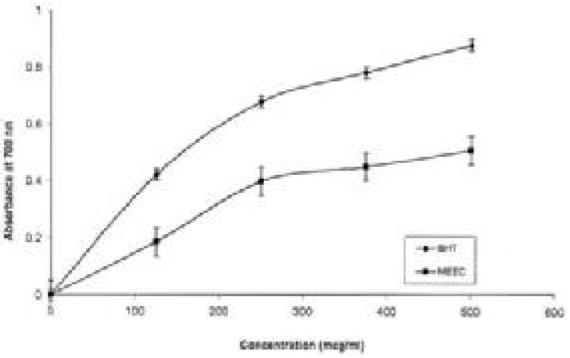

Figure 2. Reducing power of MEEC and BHT.
Results are mean SD of three parallel measurements.

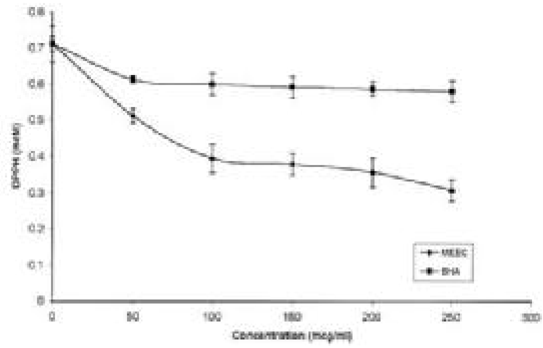

Figure 3. Free radical Scavenging activity of MEEC and BHA by 1,1-diphenyl-2-picryl hydrazyl radicals.
Results are mean SD of three parallel measurements.

reported that the reducing power of tannins prevents liver injury by inhibiting the formation of lipid peroxides. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (36). Figure 2 shows the reductive capabilities of MEEC compared with BHT. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (27). Like antioxidant activity, the reducing power of MEEC increased with increasing amount of sample. All MEEC showed concentrations higher activities than the control and these differences were statistically highly significant ($P < 0.01$).

Inhibition of DPPH radical

The DPPH radical is considered to be a model for a lipophilic radical. A chain reaction in lipophilic radicals was initiated by the lipid autoxidation. The radical scavenging activity of the crude plant extract was determined from the reduction in the optical absorbance at 517 nm due to scavenging of stable DPPH free radical. Positive DPPH test suggests that the samples are free radicals scavengers. The scavenging effects of MEEC and BHA on DPPH radical are compared and shown in Figure 3. MEEC had significant scavenging effects on the DPPH radical and the effects increasing with increasing concentration in the range of 50-250 μg/ml. Compared with that of BHA, the scavenging effect of MEEC was lower. The IC$_{50}$ value of MEEC on DPPH radical scavenging assay was found to be 167.09 μg/ml. The results were found to be statistically significant ($P<0.05$).

Inhibition of Nitric oxide radical

It is well known that nitric oxide has an important role in various types of inflammatory processes in the animal body. In the present study, the crude extract of the leaves was checked for its inhibitory effect on nitric oxide production. Figure 4 illustrates the percentage inhibition of nitric oxide generation by MEEC. Curcumin was used as a reference compound. The concentration of MEEC needed for 50% inhibition was found to be 83.38 μg/ml, whereas 20.4 μg/ml was needed for curcumin. The results were found to be statistically significant ($P<0.05$).

Inhibition of Superoxide anion radical

Superoxide anions indirectly initiated lipid oxidation as a result of superoxide and hydrogen peroxide serving as precursors of singlet oxygen and hydroxyl radicals (37). Robak and

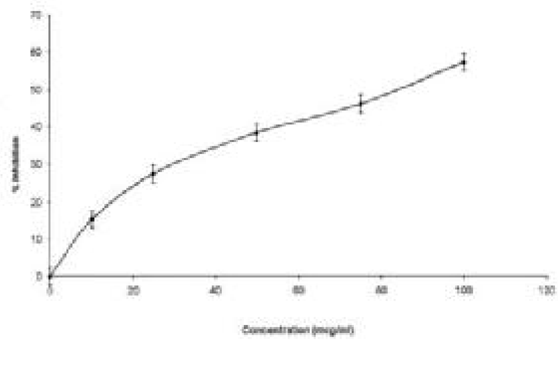

Figure 4. Inhibition of nitric oxide radical by MEEC
Results are mean SD of three parallel measurements.

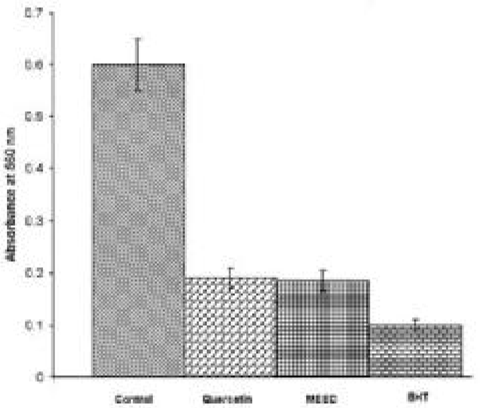

Figure 5. Superoxide anion scavenging activity of MEEC and some doses of quercetin and BHT by PMS/NADH-NBT method.
Results are mean SD of three parallel measurements.

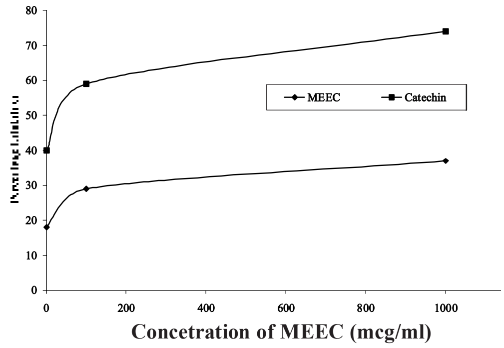

Figure 6. Hydroxyl radical scavenging activity of MEEC and Catechin on deoxyribose damage.

Glyglewski reported that the antioxidant properties of flavonoids are effective mainly via the scavenging of superoxide anion. MEEC was found to possess good scavenging activity on superoxide anions at all the tested concentrations (38). MEEC at concentrations from 10-100 µg/ml, inhibited the production of superoxide anion radicals by 11.09-42.67%. MEEC also showed a strong superoxide radical scavenging activity. The results are presented in Figure 5. The IC_{50} value of MEEC on superoxide radical scavenging activity was found to be 118.77 µg/ml, whereas the IC_{50} value of BHT and quercetin was found to be 22.77 and 31.58 µg/ml respectively. The results were found to be statistically significant (P<0.05).

Inhibition of hydroxyl radical

Hydroxyl radicals are the major active oxygen species causing lipid oxidation and enormous biological damage (39). Ferric-EDTA was incubated with H2O2 and ascorbic acid at pH 7.4. Hydroxyl radicals were formed in free solution and detected by their ability to degrade 2-deoxy-2-ribose into fragments that on heating with TBA at low pH form a pink chromogen (40, 41). When MEEC and the reference compound, catechin, added to the reaction mixture they removed hydroxyl radicals from the sugar and prevented degradation. The results are shown in Figure 6. MEEC was also capable of reducing DNA damage at all concentrations used. Catechin used as a standard was highly effective in inhibiting the oxidative DNA damage. The IC_{50} value of MEEC on hydroxyl radical scavenging assay was found to be 1833.73 µg/ml. The results were found to be statistically significant (P<0.05).

Amount of total phenolic compounds

Phenols are very important plant constituents because of their scavenging ability, which is due to their hydroxyl groups (42). In MEEC (1 mg) 64.7 µg pyrocatechol equivalent of phenols was detected. The phenolic compounds may contribute directly to the antioxidative action (43). The result indicates strong association between antioxidative activities and phenolic compounds (r^2 = 0.9983), suggesting that phenolic compounds are probably responsible for the antioxidative activities of *Ervatamia coronaria*. Phenolic compounds are effective hydrogen donors, making them good antioxidants (44). Similarly Shahidi and Naczk reported that naturally occurring phenolics exhibit antioxidative activity (45). Thus, therapeutic properties of E. Coronaria may be possibly attributed to the phenolic compounds present.

Conclusion

It is well known that free radicals are one of the causes of several diseases, such as Parkinson disease (46), Alzheimer type dementia (47) etc. The production of free radicals and the activity of the scavenger enzymes against those radicals, such as superoxide dismutase (SOD) is correlated with the life expectancies (48). Polyphenols, tannins and flavonoids are very valuable plant constituents in the scavenging action due to their several phenolic hydroxyl groups (49). The exact constituents of MEEC, which shows free radical scavenging action, are unclear. However, the phytoconstituents like polyphenol and flavonoids present in the plant extract may be responsible for antioxidant and free radical scavenging activities.

Thus, the radical scavenging activity, reductive capability and anti-lipoperoxidant activity strongly suggests that MEEC has antioxidant and anti-lipoperoxidant activities. Further studies are needed to evaluate the *in vivo* antioxidant potential of this extract in various animal models and to isolate the active component.

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