In vitro antioxidant property of the extract of *Excoecaria agallocha* (Euphorbiaceae)

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Received 18 Feb 2008; Revised 11 March 2008; Accepted 27 May 2008

ABSTRACT

Background and objectives: Excoecaria agallocha L. (Family: Euphorbiaceae) is a Bangladeshi medicinal plant found predominantly in the tidal forests and swamps of the Sundarbans and other coastal areas in Bangladesh. As part of our on-going phytochemical and bioactivity studies on medicinal plants from Bangladeshi flora, the *in vitro* antioxidant property on the bark of this plant was evaluated.

Methods: The hydroalcohol extract of the dried and ground bark of *E. agallocha* was assessed for antioxidant activity using a series of well-established assays including the 2,2-diphenyl-1-picrylhydrazyl (DPPH), the lipid peroxidation by thiobarbituric acid (TBA), the reducing power, the nitric oxide (NO.) and the hydrogen peroxide (H_2O_2) scavenging assays.

Results: In the DPPH, the NO and the H_2O_2 scavenging assays, the extract of *E. agallocha* displayed significant antioxidant activities with the IC_{50} values of 179.16, 120.24 and 134.29 µg/ml, respectively. The reducing power of the extract increased dose-dependently, and the extract reduced the most Fe^{3+} ions to the extent less than butylated hydroxy toluene (BHT). In the lipid peroxidation assay, the extract showed significant inhibition of peroxidation effect at all concentrations, with an IC_{50} value of 189.27 µg/ml.

Conclusion: Since reactive oxygen species are important contributors to serious ailments such as atherosclerosis, alcoholic liver cirrhosis and cancer, the antioxidant property of the extract of *E. agallocha* as observed in the present study might be useful for the development of newer and more potent antioxidants.

Keywords: *Excoecaria agallocha*, Euphorbiaceae, antioxidant, lipid peroxidation, DPPH, reactive oxygen species

INTRODUCTION

Excoecaria agallocha L. (Euphorbiaceae) is a small mangrove tree found extensively in the tidal forests and swamps of the Sundarbans and other coastal areas of Bangladesh (1). This plant is also well-distributed in a number of other countries of temperate and tropical Asia, Australasia and Southwestern Pacific (2). This plant has traditionally been used to treat sores and stings from marine creatures, and ulcers, as a purgative and an emetic, and the smoke of its bark has been used to treat leprosy (1). The bark oil has also been found effective against rheumatism, leprosy and paralysis (1). However, the milky sap of this tree can cause temporary blindness if it enters the eyes. The sap can also cause skin blisters and irritation. Clinical trials carried out on this plant have shown its potential anti-HIV, anticancer, antibacterial and antiviral properties (3). Previous phytochemical investigations on this species revealed the presence of diterpenoids (4-10), triterpenoids (11), flavonoid (12) and phorbole esters (13). As part of our on-going phytochemical and bioactivity studies on medicinal plants from Bangladeshi flora (14-28), we now report assessment of *in vitro* antioxidant property of the bark of *E. agallocha*.

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MATERIALS AND METHODS

Plant material

The bark of *Excoecaria agallocha* L. was collected during October 2003 from the Sundarbans of Karomjol, Dacope region. The plant was identified at Bangladesh National Herbarium where a voucher specimen was deposited (Accession No. -30209).

Extraction

The bark of *E. agallocha* (400 g) was dried at room temperature, ground and Soxhlet-extracted with 80% aqueous ethanol. The solvent was completely removed by rotary evaporator to obtain 16.3 g of reddish gummy exudates.

Qualitative DPPH radical scavenging activity

Qualitative assay was performed by the method of Sadhu *et al.,* (29). Test samples were developed on a TLC plate ($CHCl_3$: MeOH , 4:1) and sprayed with 0.004% w/v DPPH solution in MeOH using an atomizer. The positive activity was detected by the discolored (pale yellow) spots on a reddish purple background.

Quantitative DPPH radical scavenging activity

A methanolic solution of DPPH (0.15%) was mixed with serial dilutions (1 µg to 500 µg) of *E. agallocha* extracts and after 30 min, the absorbance was recorded at 515 nm using a spectrophotometer (HACH 4000 DU UV – visible spectrophotometer). Ascorbic acid was used as a positive control. The inhibition curve was plotted and the IC_{50} values were determined.

Nitric oxide radical inhibition assay

Nitric oxide radical scavenging can be estimated by the use of Griess Illosvoy reaction (30). In this study, the Griess-Illosvoy reagent was modified by using naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 1-napthylamine (5%). The reaction mixture (3 ml) containing sodium nitroprusside (10 mM, 2 ml), phosphate buffer saline (0.5 ml) and *E. agallocha* extract (10 µg to 160 µg) or standard solution (BHT, 0.5 ml) was incubated at 25°C for 150 min. After incubation, 0.5 ml of the reaction mixture was mixed with 1 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then, 1 ml of naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 min at 25°C. A pink colored chromophore was formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions. Ascorbic acid and BHT were used as positive controls.

Lipid peroxidation assay

The degree of lipid peroxidation was assessed by estimation the thiobarbituric acid-reactive substances (TBARS) (31-32). Briefly, different concentrations of extracts (50–250 µg/ml) were added to the 10% liver homogenate. Lipid peroxidation was initiated by addition of 100 µl of 15mM $FeSO_4$ solution to 3 ml of liver homogenate (final concentration was 0.5 mM). After 30 min, 100 µl of this reaction mixture was taken in a tube containing 1.5 ml of 0.67% TBA in 50% acetic acid. Samples were incubated at 37°C for 1 hrs, and then lipid peroxidation was measured using the reaction with thiobarbituric acid (TBA). The absorbance of the organic layer was measured at 532 nm. All reactions were carried out in duplicates. Vitamin E was used as the positive control. The percentage of inhibition of lipid peroxidation was calculated, by the formula: Inhibition (%) = (control – test) X 100/ control.

Scavenging of hydrogen peroxide

The ability of the extracts to scavenge hydrogen peroxide (H_2O_2) was determined by the reported method (33). Hydrogen peroxide (43 mM) was prepared in phosphate buffered saline (pH 7.4). Ascorbic acid as positive control and extract solutions were prepared at concentrations of 50 to 250 mM. Aliquots of standard or extract solutions (3.4 mL) were added to 0.6 mL of H_2O_2 solution. The reaction mixture was incubated at room temperature for 10 min, and the absorbance was determined at 230 nm. The percentage of scavenging was calculated (34) as follows: % H_2O_2 Scavenging = 100 x (Absorbance of Control- Absorbance of Sample)/ Absorbance of Control

Reducing power

The reducing power of *E. agallocha* was determined by the method described previously (35). Different concentration of *E. agallocha* extract (100 µg – 1000 µg) 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°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (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. Increased absorbance of the reaction mixture indicated increased reducing power. Butylated hydroxy toluene (BHT) was used as a positive control.

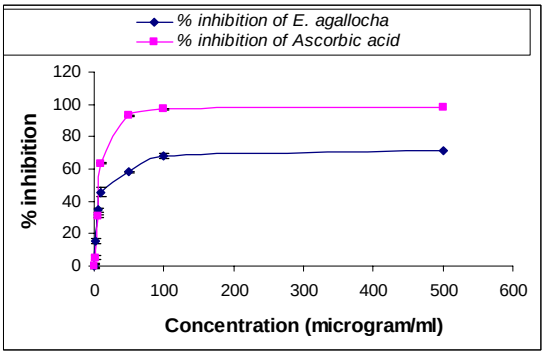

Figure 1. The DPPH scavenging activity of *E. agallocha* (values are the average of triplicate experiments and represented as mean± standard deviation).

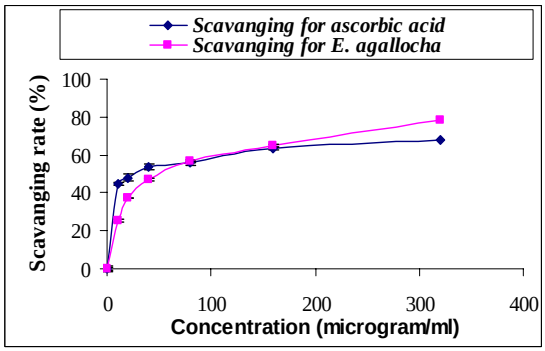

Figure 2. Nitric oxide ($NO^{\cdot}$) scavenging activity of *E. agallocha* (values are the average of triplicate experiments and represented as mean± standard deviation).

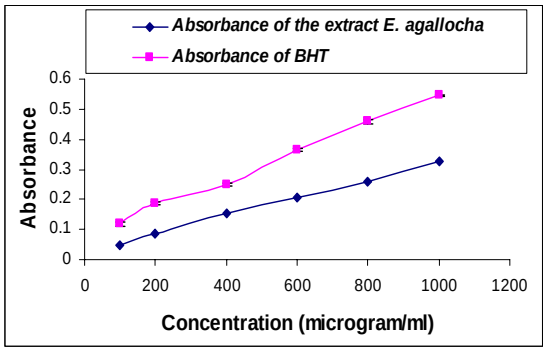

Figure 3. Reducing power of *E. agallocha* (values are the average of triplicate experiments and represented as mean± standard deviation).

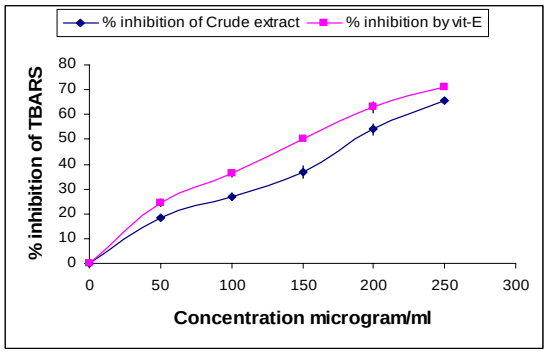

Figure 4. Effect of *E. agallocha* on TBARS on Liver homogenates of Swiss albino mice (values are the average of triplicate experiments and represented as mean± standard deviation).

RESULTS AND DISCUSSION

The DPPH antioxidant assay is based on the ability of 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to be decolorized in the presence of antioxidants (36). The DPPH radical contains an odd electron, which is responsible for the absorbance at 515-517 nm and also for visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized which can be quantitatively measured from the changes in absorbance. The TLC-based qualitative DPPH spray revealed the presence of significant antioxidant activity in the alcoholic extract of *E. agallocha* indicated by the presence of a yellowish spot on the reddish purple back ground of the TLC plate. In the quantitative assay the extract exhibited a notable dose dependent inhibition of the DPPH activity, with a 50% inhibition (IC_{50}) at a concentration of 179.16 µg/ml while the IC_{50} value of the positive control, ascorbic acid, was found 55.91 µg/ml (Figure 1).

Nitric oxide (NO.) and superoxide anion ($O_2^{\cdot-}$) cause ischemic renal injury separately and these radicals work together to bring about further damages. The toxicity and damage caused by $NO\cdot$ and $O_2^{\cdot-}$ is multiplied as they react to produce reactive peroxynitrite ($ONOO^-$), which leads to serious toxic reactions with biomolecules (37).

Suppression of released $NO\cdot$ may be partially attributed to direct $NO\cdot$ scavenging, as the extract of *E. agallocha* decreased the amount of nitrite generated from the decomposition of sodium nitroprusside *in vitro*. The scavenging of nitric oxide (NO.) by the extract was increased in a dose-dependent manner as illustrated in Figure 2. At a concentration of 120.24 µg/ml of the extract, 50% of NO was scavenged. This IC_{50} value of extract was comparable to that of the vitamin C (IC_{50} 107.78 µg/ml) as positive control.

The reducing ability of a compound generally depends on the presence of reductants, which exhibit antioxidant activity by breaking the free radical chain though donation of a hydrogen atom (37). Figure 3 shows the reducing ability of the plant extract compared to that of BHT. The reducing power of the extract increased dose-dependently. However, the extract reduced the most of Fe^{3+} ions to the extent less than BHT.

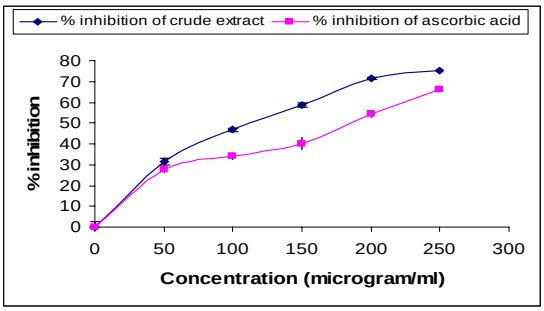

Figure 5. H_2O_2 scavenging activity of *E. agallocha* (values are the average of triplicate experiments and represented as mean± standard deviation)

During lipid peroxidation, low molecular weight end products, generally malonaldehyde, are formed by oxidation of polyunsaturated fatty acids that may react with two molecules of thiobarbituric acid to give a pinkish red chromogen (37). In the Lipid peroxidation assay, the activity of *E. agallocha* extract against non-enzymatic lipid peroxidation in rat liver microsomes was evaluated (Figure 4). Addition of Fe^{2+}/ascorbate to the liver microsomes caused an increase in lipid peroxidation. The extract showed inhibition of peroxidation effect at all concentrations, with an IC_{50} value of 189.27 µg/ml. The IC_{50} value of the vitamin E as positive control was 158.26 µg/ml.

Hydrogen peroxide (H_2O_2) generates a singlet oxygen ($^1O_2$) and a hydroxyl radical (HO·), which then become powerful oxidizing agents. Not only $^1O_2$ and HO·but also H_2O_2 can cross membranes and may oxidize a number of compounds. While H_2O_2 itself is not that reactive, it can generates the highly reactive hydroxyl radicals (HO·) through the Fenton reaction (37). Thus, the scavenging of H_2O_2 is an important antioxidant defense mechanism.

$$Fe_2+ + H_2O_2\text{----------} Fe_3+ + OH + OH^-$$

The decomposition of H_2O_2 to water involves the transfer of electrons.

$$H_2O_2 + 2H+ + 2e\text{- ----------}2H_2O$$

In the H_2O_2 scavenging assay, both ascorbic acid as positive control and the extract dose-dependently scavenged H_2O_2. The extract showed more potent activity with an IC_{50} value of 134.29 g/ml opposed to 179.21 g/ml for the ascorbic acid as positive control (Figure 5).

CONCLUSION

Since reactive oxygen species are important contributors to various serious ailments, the observed antioxidant property of the extract of *E. agallocha* in the present study might be useful for the development of newer and more potent natural antioxidants.

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