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Original Article

Evaluation of Antioxidant Properties of *Elaeocarpus ganitrus* Roxb. Leaves

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Abstract

Ethanolic extract of leaves of *Elaeocarpus ganitrus* was analyzed for their total antioxidant capacity, reducing power, metal chelating, $ABTS^+$ (2, 2-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical scavenging and hydroxyl radical scavenging activities. The extract at 500 µg/ml showed maximum Iron chelating activity (76.70%) followed by the scavenging of the $ABTS^+$ radical (55.77%) at the same concentration. However, the extract showed only moderate hydroxyl radical scavenging activity (13.43%). Total antioxidant capacity was found to be 24.18 mg ascorbic acid equivalents at 500 µg/ml extract concentration. There was a positive correlation between the total phenolic content and antioxidant capacity, $R^2 = 0.8547$, whereas the correlation between the total flavonoids and antioxidant capacity was determined to be $R^2 = 0.8413$. The results suggest that phenolics and flavonoids in the leaves provide substantial antioxidant activity.

Keywords: Antioxidants; Total phenolic content; Flavonoids; *Elaeocarpus ganitrus.*

Introduction

Oxygen is essential for the survival of all living creatures on this earth. During the process of oxygen utilization in normal physiological and metabolic processes approximately 5% of oxygen gets univalently reduced to oxygen derived free radicals (1, 2) like superoxide, hydrogen peroxide, hydroxyl and nitric oxide radicals. All these radicals known as reactive oxygen species (ROS) exert oxidative stress towards the cells of human body rendering each cell to face about 10000 oxidative hits per second (3). When generation of ROS overtakes the antioxidant defense of the cells, the free radicals start attacking the cell proteins, lipids and carbohydrates (4-6) and this leads to a number of physiological disorders. Many plants often contain substantial amounts of antioxidants including vitamin C and E, carotenoids, flavonoids and tannins etc., and thus can be utilized to scavenge the excess free radicals from human body (7).

Elaeocarpus ganitrus (syn: *Elaeocarpus sphaericus*; Elaeocarpaceae) is a large evergreen broad-leaved tree which grows in the area from the Gangetic Plain to the foothills of the Himalayas. *Elaeocarpus ganitrus* is commonly known as Rudraksha tree in India. Rudraksha is used in Ayurveda for mental diseases, epilepsy, asthma, hypertension, arthritis and liver diseases (8). There is no information about the antioxidant activity of plant mentioned above. The purpose of the present study is to evaluate the antioxidant properties of ethanolic extract of leaves of *Elaeocarpus ganitrus*.

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Experimental

Chemicals

All chemicals used were of analytical grade. 2,2-azinobis-(3-ethylbenzothiozoline-6-sulphonate) (ABTS) was obtained from Sigma Chemicals, USA. Rutin, 2-thiobarbituric acid, mannitol, 2-deoxyribose, ferrozine, ferrous chloride, vitamin C, Folin-Ciocalteu reagent, 2,4- dinitrophenyl hydrazine, ferric chloride, potassium ferricyanide, hydrogen peroxide, aluminum chloride, sodium carbonate, sodium nitrite, ammonium persulphate, sodium hydroxide and ethanol were obtained from HiMedia Chemicals, Mumbai, India.

Plant material and its extraction

The leaves of *Elaeocarpus ganitrus* Roxb. were collected from Nilgiris, India. The species was identified and confirmed at Botanical Survey of India (BSI), Southern Circle, Coimbatore, India and the Voucher specimen (No. MPG 04) was retained in our laboratory for future reference. About 5 g of air dried leaves were dissolved in 50 ml of ethanol and kept in an orbital shaker for overnight. The obtained extracts were filtered with Whatman no.1 filter paper and the filtrate was collected. The ethanol was then removed under reduced pressure at 50°C to obtain the concentrated extract.

Antioxidant activities assays

Determination of total antioxidant capacity

The method described by Prieto et al. (9) was used to determine the total antioxidant capacity of the plant extract. The tubes containing 0.2 ml of *E. ganitrus* (100-500 µg/ml), 1.8 ml of distilled water and 2 ml of phosphomolybdenum reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) were incubated at 95°C for 90 minutes. After the mixture had cooled to room temperature, the absorbance of each solution was measured at 695 nm using an UV/Vis spectrophotometer (Beckman DU-530). The antioxidant capacity was expressed as ascorbic acid equivalent (AAE) by using the standard ascorbic acid graph.

Reducing power assay

A method developed by Oyaizu (10) was adopted for the determination of reducing power. 2.5 ml of different concentrations of *E. ganitrus* (100-400 µg/ml) were mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 minutes, then rapidly cooled, mixed with 2.5 ml of 10% trichloroacetic acid and centrifuged at 3000 rpm for 10 minutes. 2.5 ml of supernatant was taken. 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride were added to it. Mixed well and allowed to stand for 10 minutes. The absorbance was measured at 700 nm.

Ferrous ion chelating ability

The method proposed by Decker and Welch (11) was espoused to determine the metal chelation ability of the plant extract. 2 ml of *E. ganitrus* (100-500 µg/ml) was mixed with 0.1 ml of 2 mM $FeCl_2$ and 0.2 ml of 5 mM ferrozine solutions and allowed to react for 10 minutes at room temperature. The absorbance at 562 nm of the resulting solutions were measured and recorded. The $FeCl_2$ and ferrozine acted as control solution. The percentage inhibition of the ferrous ion was calculated by comparing the results of the test with those of the control using the formula (12)

Percentage inhibition=

[1−(absorbance of test/absorbance of control)]×100..... (1)

$ABTS^+$ radical scavenging assay

$ABTS^+$ radical scavenging activity was determined according to Re et al (13). $ABTS^+$ radical was freshly prepared by adding 5 ml of 4.9 mM ammonium persulfate solution to 5 ml of 14 mM ABTS solution and kept for 16 h in dark. This solution was diluted with ethanol (99.5%) to yield an absorbance of 0.70±0.02 at 734 nm and the same was used for the assay. To 950 µl of ABTS radical solution, added 50 µl of *E. ganitrus* (100-500 µg/ml) and the reaction mixture was vortexed for 10 sec. After 6 minutes the absorbance was recorded at 734 nm and compared with the control ABTS solution. Percentage inhibition was calculated from the formula (1).

Hydroxyl radical scavenging activity

A modified deoxyribose method determining thiobarbituric acid reactive substances (TBARS) proposed by Res_at Apak et al. (14), 2006, used to determine the hydroxyl radical scavenging activity. To a test tube added 3 ml of phosphate buffer (pH 7.0), 1 ml of 10 mM 2-deoxy-D-ribose, 0.5 ml of 20 mM Na_2-EDTA, 0.5 ml of 20 mM $FeCl_2$ solution, 3.8 ml distilled water, 0.2 ml of *E. ganitrus* (100-500 µg/ml) and 1 ml of 10 mM H_2O_2 in the order given, and the mixture (total volume of 10 ml) was incubated for 4 h at 37°C in a water bath. At the end of the period, the reaction was arrested by adding 5 ml of 2.8% TCA. To this added 5 ml of 1% TBA and the reaction mixture was kept in a boiling water bath for 10 minutes. The mixture was cooled under running tap water, and the absorbance at 520 nm was recorded. Percentage inhibition was calculated from the formula (1).

Quantitative analysis of antioxidative components

Total phenolics (TPC)

Total phenolics were quantified and expressed as gallic acid equivalents according to a method proposed by Singleton et al. (15), 1999. To 0.1 ml of *E. ganitrus*, added 3.9 ml of distilled water and 0.5 ml of Folin-Ciocalteau reagent. The tube was incubated at room temperature for 3 minutes. To this added 2 ml of 20% sodium carbonate and kept at boiling water bath for 1 minute. The blue color formed was read at 650 nm.

Total flavonoids (TFC)

TFC was estimated colorimetrically based on the method modified by Zhishen et al. (16), 1999. To 0.1 ml of *E. ganitrus* in a 10 ml volumetric flask, distilled water was added to make the volume to 5 ml and 0.3 ml 5% $NaNO_2$ was added to this. 3 ml of 10% $AlCl_3$ was added 5 minutes later. After 6 minutes, 2 ml of 1 M NaOH was added and the absorbance was measured at 510 nm. Rutin was used as a standard for constructing a calibration curve.

Statistical analysis

The results were expressed as means±SD.

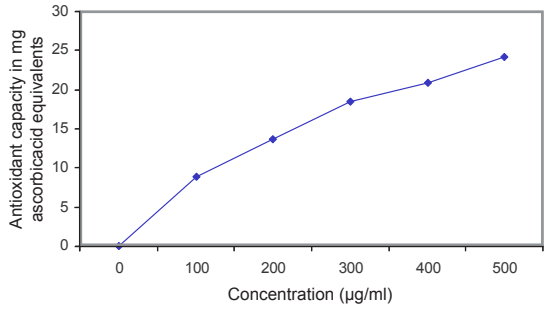

Figure 1. Antioxidant capacity of ethanolic extract of *E. ganitrus*.

Linear regression analysis was used to calculate IC_{50} values whenever needed. Correlation analysis of total antioxidant capacity versus TPC and TFC were carried out using the correlation programme in the EXCEL program.

Results and Discussion

Phosphomolybdenum assay used to determine the total antioxidant capacity is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH (9). Figure 1 illustrates the antioxidative capacities of various concentrations of *E. ganitrus* (100-500 µg/ml). Total antioxidant capacity of *E. ganitrus* was found to be 24.18 mg ascorbic acid equivalents at 500 µg/ml extract concentration. This good antioxidant activity might be attributed to the presence of phytochemicals, such as flavonoids and biflavones (17). This assay has been successful in the quantification of vitamin E antioxidant activity (9) and it was efficient to extend its application to plants polyphenols (18).

Fe^{3+}/Fe^{2+} transformation was investigated in the presence of samples for the measurements of the reductive ability. The reducing power of *E. ganitrus* ranged from 1.112 to 1.973 Abs for 100 µg/ml to 400 µg/ml of extract (Figure 2). Okuda et al 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 (19).

The chelation of Fe^{2+} ions was estimated by the method of Decker and Welch in which

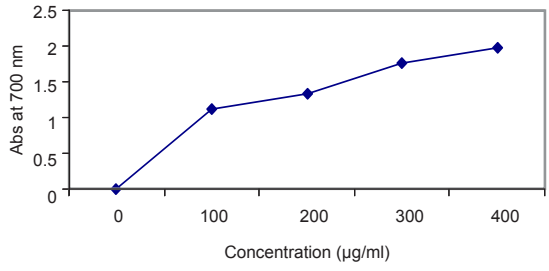

Figure 2. Reducing power of *E. ganitrus*.

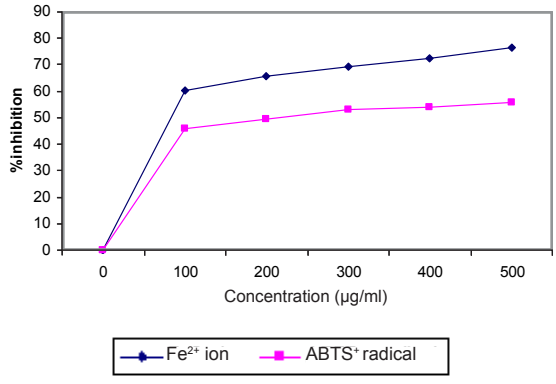

Figure 3. Fe^{2+} ion chelating activity and $ABTS^{+}$ radical scavenging activity of *E. ganitrus*.

ferrozine quantitatively forms complexes with Fe^{2+}. In the presence of chelating agents, the formation of this complex is disrupted, thereby impeding the formation of red color imparted by the complex as well. Measurement of this color change therefore allows for the estimation of the chelating activity of the coexisting chelator. As shown in Figure 3, the formation of Fe^{2+}-ferrozine complex is not complete in the presence of extract, indicating that *E. ganitrus* chelate the iron. 76.70 % inhibition was noted with 500 µg/ml of *E. ganitrus*. IC_{50} value was found to be 211.73 µg/ml *E. ganitrus*. Metal chelating agents reduce the concentration of catalyzing transition metal in lipid peroxidation by forming sigma bonds with metals, reducing the redox potential, thereby stabilizing the oxidized form of the metal ion (20).

The decolorization of $ABTS^{+}$ radical is an unambiguous way to measure the antioxidant activity of phenolic compounds. Recently Awika et al. (21), 2003 found positive correlations between the determination of phenolic antioxidant using the oxygen radical absorbance capacity (ORAC), $ABTS^{+}$, and the 1,1-diphenyl-2,2picrylhydrazyl (DPPH) assays. Thus monitoring the antioxidant activity by $ABTS^{+}$ radical scavenging assay gives good prediction of their ORAC and DPPH radical scavenging capacity. *E. ganitrus* showed potential activity in $ABTS^{+}$ decolorization. 55.77 % inhibition was noted with 500 µg/ml of *E. ganitrus*. The dose-dependent results observed were shown in Figure 3. IC_{50} value was found to be 297.12 µg/ml *E. ganitrus*. Decolorization of $ABTS^{+}$ in the present study reflects the capacity of an antioxidant species to donate electrons or hydrogen atoms to inactivate this radical cation.

Hydroxyl radical is the most reactive free radical and can be formed from superoxide anion and hydrogen peroxide, in the presence of metal ions, such as copper or iron. Hydroxyl radicals have the highest 1-electron reduction potential (2310 mV) and can react with everything in living organisms at the second-order rate constants of 10^{9}-10^{10} mol/s. Hydroxyl radicals react with lipid, polypeptides, proteins, and DNA, especially thiamine and guanosine. When a hydroxyl radical reacts with aromatic compounds, it can add across a double bond, resulting in hydroxycyclohexadienyl radical. The resulting radical can undergo further reactions, such as reaction with oxygen, to give peroxyl radical, or decompose to phenoxyl-type radicals by water elimination (22). The ability of the extracts to scavenge these radicals was evaluated by the Fenton-mediated 2-deoxyribose assay. The result of present study does not show any promising hydroxyl radical scavenging property. Only 13.43% inhibition was noted with 500 µg/ml of *E. ganitrus* (Figure 4). Although the measurement of aromatic hydroxylation with HPLC/electrochemical detection is more specific than the low-yield TBARS test, it requires sophisticated instrumentation.

Recent studies have shown that many flavonoids and related polyphenols contribute significantly to the total antioxidant activity of many plants (23). Total phenolic content in *E. ganitrus* was found to be 56.79±1.6 mg gallic acid equivalents/g of dry material. Total flavonoids in *E. ganitrus* were found to be 18.58± 0.3 mg rutin equivalents/g of dry material. There was a positive correlation between the total phenolic content and antioxidant capacity,

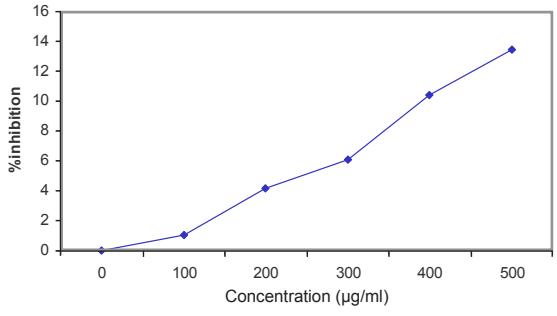

Figure 4. Effect of *E. ganitrus* on hydroxyl radical model.

R=0.8501, whereas the correlation between the total flavonoids and antioxidant capacity was determined to be R^2=0.8413. These results suggest 85% of the antioxidant capacity of *E. ganitrus* is due to the contribution of phenolics and flavonoid components. In addition, the antioxidant activity may be due to enzymatic and other non-enzymatic antioxidants, which needs further analysis.

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