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Research Article

IN VITRO ANTIOXIDANT POTENTIALITY OF ETHYL ACETATE FRACTION OF SEED OF *EUGENIA JAMBOLANA*

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ABSTRACT

Objective: Over production of reactive oxygen species is dangerous for all living organisms which can damage the key cellular constituents such as protein, lipid, and DNA. Recently, searching of different new plant sources having free radical scavenging activity is an interesting field of research in the pharmaceutical area, as natural products are harmless and low cost. In this connection, we focused to evaluate the antioxidant potential of ethyl acetate fraction of seed of *Eugenia jambolana* (*E. jambolana*) using different *in vitro* tests.

Methods: To assess the antioxidant activity, said fraction was examined on scavenging of hydrogen peroxide, hydroxyl radical scavenging potential, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging effect, and anti-lipid peroxidation activity by biochemical methods. We also measured total phenol and flavonoids contents in the said fraction.

Results: Results indicated that ethyl acetate fraction has strong scavenging activity on hydroxyl radical with inhibitory concentration (IC_{50}) value 54.34 µg/ml, hydrogen peroxide with IC_{50} value 63.25 µg/ml, DPPH radical with IC_{50} value 154.95 µg/ml and the said fraction also showed remarkable inhibition in lipid peroxidation having IC_{50} value 59.42 µg/ml. In another phytochemical study focused that the fraction is rich in phenolic compounds (18.54 mg gallic acid equivalent/g dried extract) and flavonoids (122.32 µg quercetin equivalent/g dried extract).

Conclusion: The findings indicated that the ethyl acetate fraction of seed of *E. jambolana* is a rich source of natural antioxidants.

Keywords: Antioxidant, *Eugenia jambolana,* Free radicals, Lipid peroxidation.

INTRODUCTION

Free radicals or reactive oxygen species (ROS) exert oxidative stress toward the cells of the body by various mechanisms and cause damage to cellular proteins, nucleic acids, membrane lipids, and eventually cell death. All cells in the body have an enzymatic antioxidant defense system such as superoxide dismutase, catalase, glutathione-s-transferase, etc. and non-enzymatic antioxidant defense such as vitamin E and vitamin C to combat oxidative stress [1]. Most of the diseases or disorders such as diabetes mellitus, arthritis, cancer and ageing processes are closely related with ROS and lipid peroxidation [2,3]. Uncontrolled generation of free radical together with reduced levels of anti-oxidative vitamins and enzymes are the main contributor of oxidative stress [4]. These free radicals are mainly hampering biochemical processes and which is an essential part of aerobic life and its metabolism [5]. Hence, antioxidants with free radicals scavenging activities are important for the management of free-radical inducing diseases. Different polyphenolic compounds like flavonoids and phenolic acid usually present in plant parts, which have various biological activities including free radical scavenging abilities, antioxidant activity, anti-carcinogenic activities, and anti-inflammatory [6]. At present, different synthetic antioxidants such as butylated hydroxytoluene (BHT), tertiary butylated hydroquinone and gallic acid esters which have assumed to causes of harmful health effects [7]. Hence, our attempt is to search out the natural nutraceuticals for alternate of synthetic antioxidant drug. Therefore, the aim of the present study was to investigate the antioxidant activity of ethyl acetate fraction of seeds of *Eugenia jambolana* on different *in vitro* models such as hydrogen peroxide, hydroxyl radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and lipid peroxidation inhibition activity. The antioxidants activities of said fraction expressed as a percentage of inhibition on free radical production on the different *in-vitro* experimental model.

E. jambolana has been widely used in Indian traditional medicine for a remedy of various ailments. Different parts of *E. jambolana* such as seeds, kernel, leaves, and septum have a significant anti-hyperglycemic effect. For example, an ethyl acetate fraction of *E. jambolana's* seed has anti-hyperglycemic effect on diabetic rats and human [8,9]. The anti-hyperglycemic activity of ethyl acetate fraction of seed of *E. jambolana* has been reported previously by us [10-12] as well as by others [13]. But *in vitro* antioxidant activity of ethyl acetate fraction of seeds of *E. jambolana* has not been evaluated presently. Hence, our study has been focused on *in vitro* antioxidant activity of the said fraction of seeds of *E. jambolana* through different biochemical methods.

METHODS

Chemicals

Chemicals were used in this experiment like DPPH, trichloroacetic acid (TCA), thiobarbituric acid (TBA), gallic acid, ascorbic acid (AA), α-tocopherol, BHT, Folin–Ciocalteu (FC) reagent, and these were purchased from Himedia Laboratories Pvt. Ltd., Mumbai, India. Ethylene diamine tetra acetic acid (EDTA), hydrogen peroxide, sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3), ferric chloride ($FeCl_2$), and sodium nitrite ($NaNO_2$) were purchased from Sisco Research Laboratories (SRL) Pvt. Ltd. Mumbai, India. Aluminum chloride ($AlCl_3$) was obtained from SD Fine Chemicals Limited, Mumbai, India.

Bioactivity-guided ethyl acetate fractionation of hydro methanolic (2:3) extract of seed of *E. jambolana*

For the preparation of ethyl acetate fraction, the seeds of *E. jambolana* were collected in rainy season (July-August) from the local market at Midnapore town, West Bengal, India. The specimen was authenticated by the taxonomist in the Department of Botany and Forestry, Vidyasagar University, Midnapore, where a voucher specimen was preserved having Ref. No.- BMLSM-10/06. The preparation of crude extract using hydro-methanol (2:3) as solvent mixture followed by its fractionation using ethyl acetate was performed according to our previously reported method [12].

Phytochemical screening

Hydrogen peroxide scavenging assay

A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffer saline (PBS) (pH-7.4). Various concentrations (20, 40, 60, 80, and 100 µg) of fraction or standard in ethyl acetate (1 ml) were added to 2 ml of hydrogen peroxide solution in PBS. After 10 minutes of incubation, the absorbance was measured at 230 nm, against a blank solution containing phosphate buffer without hydrogen peroxide [14]. The result was compared with alpha-tocopherol, as a standard. The percentage of inhibition was calculated according to the following equation:

$$\% \text{ Inhibition} = \{(A_0 - A_1)/A_0 \times 100\}$$

where A_0 is the absorbance of the control (without extract) and A_1 is the absorbance in the presence of the fraction.

Hydroxyl radical scavenging assay

Hydroxyl radical inhibitory activity was performed as per the deoxyribose method [15]. To the reaction mixture containing deoxyribose (3 mM, 0.2 ml), $FeCl_2$ (0.1 mM, 0.2 ml), EDTA, disodium salt (0.1 mM, 0.2 ml), AA (0.1 mM, 0.2 ml) and hydrogen peroxide (2 mM, 0.2 ml) in PBS (pH, 7.4, 20 mM), various concentrations (20, 40, 60, 80, and 100 µg) of 0.2 ml of the fraction or standard in DMSO were added to give a total volume of 1.1 ml. The solutions were then incubated for 30 minutes at 37°C. After incubation, ice-cold TCA (0.2 ml, 15% w/v) and TBA (0.2 ml, 1% w/v) in 0.25 N hydrochloric acid (HCl) were added. The reaction mixture was kept in a boiling water bath for 30 minutes, cooled in room temperature and the absorbance was measured at 532 nm with reagent blank containing water in the place of extract. Alpha-tocopherol was used as the standard for the comparison. The percentage of inhibition was calculated using the formula given before.

Assessment of in-vitro DPPH radical scavenging assay

The radical scavenging activity of *E. jambolana* against DPPH was determined spectrophotometrically [16]. DPPH reacts with an antioxidant compound that can donate hydrogen and thereby DPPH is reduced. Change in color of the solution, (from deep violet to light yellow) was measured. The intensity of the yellow color depends on the amount and nature of radical scavenger present in the sample and standard compounds. The reaction mixture containing 1 ml of 0.1 mM DPPH, various concentrations of extract (50, 100, 150, 200, and 250 µg) were made up to 3 ml with water. Then the tubes were incubated for 10 minutes. Once the blue color chromophore was formed, the absorbance of this solution was measured at 517 nm, against reagent blank containing water in place of the fraction. BHT was used as the standard for the comparison. The ability to scavenge the DPPH radical in terms of percentage of inhibition was calculated according to the following equation:

$$\% \text{ Inhibition} = \{(A_0 - A_1)/A_0 \times 100\}$$

where A_0 is the absorbance of the control (without fraction) and A_1 is the absorbance in the presence of the fraction.

Lipid peroxidation inhibitory activity

The lipid peroxidation inhibitory activity of ethyl acetate fraction was studied *in-vitro* following the modified method [17,18]. Rats were killed by cervical dislocation, the liver tissue was excised, rinsed in ice-cold saline solution and blotted dry. Then 0.5 g of the liver was sliced and homogenized with 10 ml of 150 mM KCL-Tris-HCl buffer (pH-7.2). The reaction mixture was composed of 0.25 ml of liver homogenate, Tris-HCl buffer (pH-7.2), 0.1 mM AA, 4 mM $FeCl_2$ and 0.05 ml of various concentration of fraction (25, 50, 75, 100, and 150 µg). The mixture was incubated at 37°C for 1 hr in capped tubes. Then, 0.5 ml of 0.1 N HCl, 0.2 ml of 9.8% sodium dodecyl sulphate, 0.9 ml of distilled water and 2 ml of 0.6% TBA were added to each tube and the tubes were vigorously shaken. All the tubes were then placed in boiling water bath at 100°C for 30 minutes. The tubes were allowed to keep at room temperature and centrifuged at 3000 rpm for 20 minutes. The absorbance of the supernatant was measured at 532 nm, against reagent blank containing water in place of the fraction. The result was compared with BHT, as a standard. The percentage inhibition of lipid peroxidation was calculated by comparing the results of the test with those of controls not treated with the fraction as per the formula:

$$\% \text{ Inhibition} = \{(A_0 - A_1)/A_0 \times 100\}$$

where A_0 is the absorbance of the control (without fraction) and A_1 is the absorbance in the presence of the fraction.

Qualitative tests for phytochemicals of the seeds of *E. jambolana* were performed as per the standard methods [19]

Determination of total flavonoid content

The total flavonoid content was determined with the $AlCl_3$ method [20] using quercetin as a standard. The fraction (0.25 ml) was added to 1.25 ml of distilled water followed by addition of 75 µl of 5% $NaNO_2$. The preparation was allowed to incubate at room temperature for 5 minutes and then, $AlCl_3$ (0.15 ml, 10%) was added. After a further incubation for 6 minutes at room temperature, the reaction mixture was treated with 0.5 ml of 1 mM NaOH. Finally, the reaction mixture was diluted with 275 µl of distilled water. Further incubation for 20 minutes at room temperature was performed, and the absorbance was measured at 510 nm. All tests were performed in triplet. The flavonoid content was expressed as µg of quercetin equivalents per gram (g) of the dried fraction.

Determination of total phenolic content

Total phenolic content was determined using the FC reagent method [21] with slight modification. Briefly, the seed fraction (0.5 ml) was mixed with 0.5 ml of FC reagent (previously diluted with 1:1 with distilled water) and incubated for 5 minutes at room temperature, and then 1 ml of 2% Na_2CO_3 solution was added. After incubation, at room temperature for 10 minutes, the absorbance was noted. Gallic acid monohydrate was used as a standard. The phenolic content was expressed as mg of gallic acid equivalents per gram of the dried fraction.

Statistical analysis

Statistical analysis was performed by software (Origin-6.1). Data were expressed as means±SD of three measurements. Data were analyzed using ANOVA followed by multiple comparison two-tail "t-test." The results obtained were considered statistically significant if the $p<0.05$. The amount of fraction needed to inhibit free radicals concentrations by 50%, IC_{50} was performed by software (STATISTICA) based on the percentage of inhibition in different doses or concentration.

RESULTS AND DISCUSSION

Oxidative stress plays a major role in pathology of various diseases and condition including cancer, diabetes, cardiovascular disease, ageing, etc. [22,23]. Different antioxidants make protection against the oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation or such other mechanisms and thus prevent diseases [24]. Therefore, we studied the antioxidant activities of ethyl acetate fraction by a series of *in-vitro* protocol using some biochemical experimental models.

The results showed that the seeds of *E. jambolana* are rich in flavonoids, phenols, and saponins, which may be liable for the antioxidative efficacy as these phytochemicals act as antioxidants [25-27].

Phenolic compounds directly act as antioxidants. Due to redox properties, phenolic compounds play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides [28,29]. On the other hand, it has been also acknowledged that flavonoids show antioxidant activity through scavenging or chelating process and their effects on human nutrition and health are considerable (Table 1) [30].

Table 1: Total phenolic and flavonoids contents of ethyl acetate fraction of seed of *E. jambolana*

Samples	Total phenolic content (mg GAE/g dried extract)	Total flavonoids (µg QE/g dried extract)
Ethyl acetate fraction of seed of *E. jambolana*	18.54	122.32

GAE: Gallic acid equivalents, QE: Quercertin equivalents,
E. jambolana: Eugenia jambolana

The neutralization ability hydrogen peroxide of the fraction (Fig. 1) varied from 13.5% to 82.22% (IC_{50} value 63.25 µg/ml) and in standard 18.22-84.4% (IC_{50} value 54.85 µg/ml). Hydrogen peroxide can penetrate biological membranes, and it is not very reactive, but it can sometimes be toxic to a cell because it may give rise to hydroxyl radical in the cells [31]. Scavenging of hydrogen peroxide by *E. jambolana* may be attributed to their phenolic compound, which could donate electron to hydrogen peroxide. Thus, it is neutralizing to water.

Hydroxyl radical scavenging ability of ethyl acetate fraction was shown in Fig. 2 and was compared with α-tocopherol. The extract inhibited the degradation of deoxyribose in dose-dependent manner. Thereby, hydroxyl radical neutralization values ranges from 13.88% to 85.34% (IC_{50} value 54.34 µg/ml) and in standard from 16.6% to 88.2% (IC_{50} value 49.72 µg/ml). Hydroxyl radical is an extremely reactive free radical formed in biological systems and has the capacity to cause DNA strand breakage, which contributes to carcinogenesis, mutagenesis, and cytotoxicity [32]. Like many free radicals, hydroxyl radical can be neutralized if it is provided with hydrogen atoms. Oxygen radical may attack DNA either in sugar or base, giving rise to a large number of products. Phytochemical study of seed extract revealed the presence of phenolic compounds which may responsible for the hydroxyl radical scavenging activity (Fig. 3) [33,34].

The scavenging ability of the investigated fraction varied widely from 19.47% to 71.64% (IC_{50} value 154.95 µg/ml) and in standard 24.31-77.75% (IC_{50} value 149.7 µg/ml). From the result, we predict that DPPH antioxidant assay is based on the ability of DPPH, a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for a 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 [35]. It was noted that if radical scavenging activity is increasing then phenolic compound content also increased [36]. Both studies were also reported a high concentration between DPPH radical scavenging potential and total phenolic content (Fig. 2) [37,38].

Lipid peroxidation inhibition percentage varies widely in different doses of ethyl acetate fraction ranges from 22.74% to 82.8% (IC_{50} value 59.42 µg/ml) and in case of standard ranges from 27.94% to 89.1% (IC_{50} value 55.33 µg/ml) (Fig. 4). Lipid peroxidations mainly defined as oxidative deterioration of polyunsaturated fatty acids and involve structure of lipid radicals leading to damage the cell membrane. Due to the presence of polyunsaturated lipid in liver and brain, the free radicals-induced lipid peroxidation has been occurred [39]. Increased lipid peroxidation is a salient characteristic of chronic diabetes leads to, which impair membrane function by reducing the activity of enzymes as well as receptors [40]. Results focused that the ethyl acetate fraction of the seeds of *E. jambolana* inhibits lipid peroxidation under *in vitro* conditions, indicating the anti-lipid peroxidant effect of the seed of *E. jambolana*.

CONCLUSION

The results of the study clearly indicate that ethyl acetate fraction of seed of *E. jambolana* possesses *in vitro* antioxidant activity. The

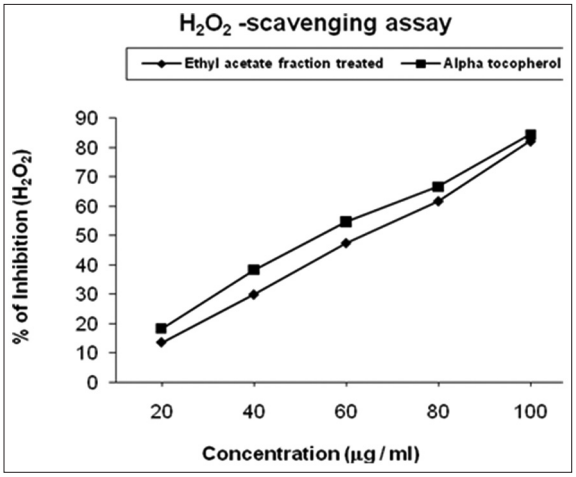

Fig. 1: Scavenging of hydrogen peroxide by ethyl acetate fraction of seed of *Eugenia jambolana* and standard α-tocopherol. The inhibitory concentration 50 value of the fraction was 63.25 µg/ml

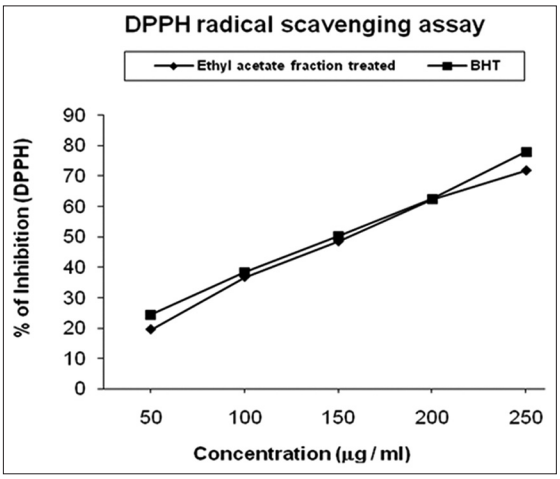

Fig. 2: Inhibition in 2,2-diphenyl-1-picrylhydrazyl radical by ethyl acetate fraction of seed of *Eugenia jambolana* and standard butylated hydroxytoluene. The inhibitory concentration 50 value of the fraction was 154.95 µg/ml

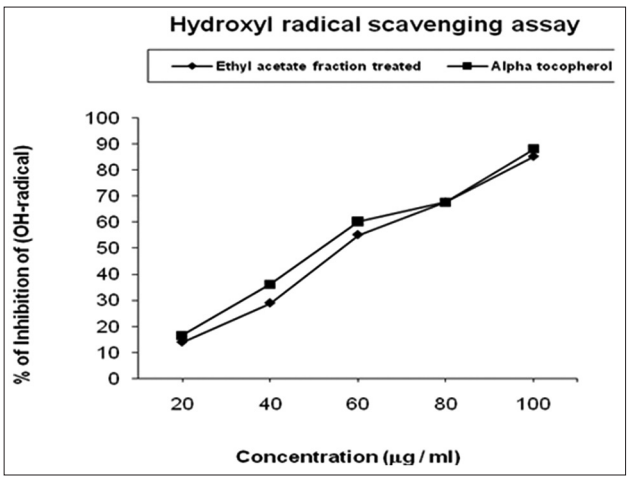

Fig. 3: Hydroxyl radical scavenging ability of ethyl acetate fraction of seed of *Eugenia jambolana* and α-tocopherol as a standard. The inhibitory concentration 50 value of ethyl acetate fraction was 54.34 µg/ml

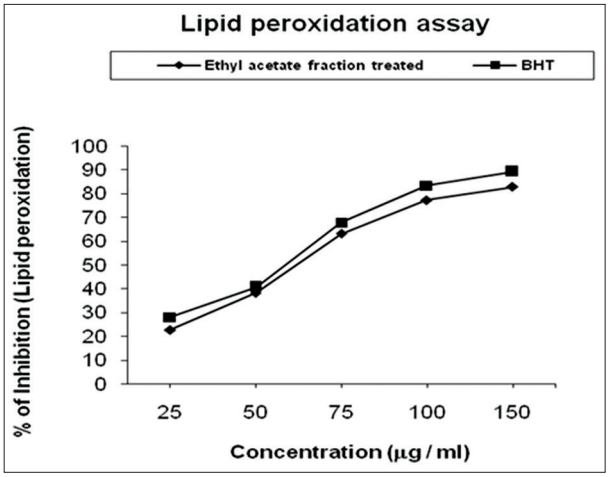

Fig. 4: Inhibition in lipid peroxidation by ethyl acetate fraction of seed of *Eugenia jambolana* and standard butylated hydroxytoluene. The inhibitory concentration 50 value of the fraction was 59.42 µg/ml

encouraging results of this extract in various *in vitro* tests proved that the plant seeds fraction act as a reducing agent, its hydrogen donating ability, and effectiveness as scavengers of hydrogen peroxide and hydroxyl radical. Hence, it is worthwhile to isolate and elucidate the bioactive principle(s), responsible for the antioxidant activity of the extract which is underway in our laboratory.

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