ORIGINAL ARTICLE

Antioxidant potential of hydro-methanolic extract of seed of *Caesalpinia bonduc*: An *in vitro* study

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ABSTRACT

It is well known that the over production of reactive oxygen species is harmful for living organisms and it damages major cellular constituents such as DNA, protein, and lipid. At present, searching of new plant sources having free radical scavenging activity is an important field of research in phytomedicine as natural products are safe and relatively low cost. In this respect, attention has been focused to evaluate the antioxidant potential of hydro-methanolic extract of seed of *Caesalpinia bonduc* (Caesalpenacae) using different *in vitro* models. To evaluate the antioxidant activity, extract was examined on 2, 2-diphenyl-1-picrylhydrazyl radical scavenging effect, scavenging of hydrogen peroxide, hydroxyl radical scavenging potential, and anti-lipid peroxidation activity by biochemical methods. Total phenol and flavonoids contents in the said extract were measured biochemically as per standard methods. Results were compared with butylated hydroxyl toluene and α-tocopherol. Results indicated that hydro-methanolic extract has strong scavenging activity on 2, 2-diphenyl-1-picrylhydrazyl radical with IC_{50} value 157.4 μg/ml, hydroxyl radical with IC_{50} value 61.9 μg/ml and hydrogen peroxide with IC_{50} value 64.32 μg/ml. Hydro-methanolic extract also showed notable inhibition in lipid peroxidation having IC_{50} value 58.87 μg/ml. Phytochemical study focused that the extract is rich in phenolic compounds (24.66 mg gallic acid equivalent/g dried extract) and flavonoids (136.65 μg quercetin equivalent/g dried extract). Findings of the experiment indicated that the hydro-methanolic extract of seed of *Caesalpinia bonduc* is a source of natural antioxidants.

Key words: Antioxidant, *Caesalpinia bonduc*, free radicals, lipid peroxidation

INTRODUCTION

Majority of the diseases or disorders including diabetes mellitus, arthritis, cancer, ageing processes are often connected with reactive oxygen species (ROS) and lipid peroxidation.[1,2] The main contributor of oxidative stress is uncontrolled generation of free radical together with reduced levels of antioxidative vitamins and enzymes.[3] These free radicals interfere with biochemical processes and represent an essential part of aerobic life and metabolism.[4] Therefore, antioxidant with free radical scavenging activities may have great relevance for the management and therapeutics of free radical inducing diseases. Polyphenolic compounds like flavonoids and phenolic acid commonly found in plant have multiples biological effects including antioxidant activity, free radical scavenging abilities, anti inflammatory, and anti carcinogenic activities.[5] Currently, available synthetic antioxidants like butylated hydroxytoluene (BHT), tertiary butylated hydroquinone, and gallic acid esters have suspected to cause or prompt negative health effects.[6] Hence, strong restrictions have been placed on their application and there is a trend to substitute them with phyto-antioxidants. Neutraceuticals having antioxidant properties are non toxic or may have minimum side effects than synthetic compounds. In this concern, our attempt is to search out the neutraceuticals

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for substitute of synthetic antioxidant drug. Therefore, the aim of the present study is to investigate the antioxidant activity of hydro-methanolic extract of seed of *Caesalpinia bonduc* (*C. bonduc*) on different *in vitro* models such as 2, 2-diphenyl-1-picrylhydrazyl (DPPH), hydrogen peroxide, hydroxyl radical, and lipid peroxidation inhibition activity. The antioxidant activities of the said plant part have been expressed in term of percentage of inhibition on free radical generation on the different *in-vitro* experimental models.

C. bonduc is a medicinal plant belonging to the family of Caesalpenacae. In Indian traditional plant medicine, it has been considered as an important remedy for the treatment of filarial infection, tumor, asthma, and diabetes.[7] This plant part also has a remedial effect on hyperglycemic and hyperlipidemic state in diabetic rats which was noted in our previous report.[8]

MATERIALS AND METHODS

Chemicals

Chemicals like 2, 2-diphenyl-1-picrylhydrazyl (DPPH), trichloroacetic Acid (TCA), thiobarbituric Acid (TBA), gallic acid, ascorbic acid, α–tocopherol, butylated hydroxytoluene (BHT), and folin-ciocalteu (FC) reagent were used in this experiment 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. Aluminium chloride ($AlCl_3$) was obtained from Sd Fine Chemicals Ltd, Mumbai, India.

Collection of Plant Material

The dried seeds of *C. bonduc* were collected from village area of Paschim Medinipur district, West Bengal, India, in the months of July-September. The plant was identified by the taxonomist, Prof. R. K. Bhakat, Department of Botany and Forestry, Vidyasagar University, Midnapore, West Bengal, and the voucher specimen was deposited having the Reference No. Bio-Med/V.U/C.B/24/10.

Preparation of Hydro-Methanolic Extract of Seed of *C. bonduc*

Hydro-methanolic extract of seeds of *C. bonduc* was prepared as per the standard method.[9] In brief, fresh seeds of *C. bonduc* were dried in an incubator for 2 days at 40°C, then crushed in an electric grinder and pulverized. From this powder, 50 g was suspended in the mixture solvent consisting of 80 ml of water and 120 ml methanol in a container for 48 hrs at room temperature and then the supernatant was filtered through No. 3 Whatman filter paper. The filtrate was concentrated and the collected residue was preserved in a refrigerator at 2-8°C for use in the experiments.

Phytochemical Screening

Qualitative tests for phytochemicals of the seeds of *C. bonduc* were performed as per the standard methods.[10]

Determination of Total Flavonoid Content

The total flavonoid content was determined with the $AlCl_3$ method[11] using quercetin as a standard. The seed extract (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 min at room temperature, the reaction mixture was treated with 0.5 ml of 1mM NaOH. Finally, the reaction mixture was diluted with 275 µl of distilled water. Further incubation for 20 min 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 quercertin equivalents (QE) per gram (g) of dried extract.

Determination of Total Phenolic Content

Total phenolic content was determined using the Folin-Ciocalteu (FC) reagent method[12] with slight modification. Briefly, the seed extract (0.5 ml) was mixed with 0.5 ml of FC reagent (previously diluted with 1:1 with distilled water) and incubated for 5 min at room temperature, and then 1 ml of 2% Na_2CO_3 solution was added. After incubation at room temperature for 10 min, the absorbance was noted. Gallic acid monohydrate was used as the standard. The phenolic content was expressed as mg of gallic acid equivalents (GAE) per g of dried extract.

Assessment of *in-vitro* Antioxidant Activity of Hydro-Methanolic Extract

DPPH radical scavenging assay

The radical scavenging activity of *C. bonduc* against DPPH was determined spectrophotometrically.[13] 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 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) and 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 extract. 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: % 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 extract.

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 extract or standard in hydro-methanol (1 ml) were added to 2 ml of hydrogen peroxide solution in PBS. After 10 min 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 using the formula given before.

Hydroxyl radical scavenging assay

Hydroxyl radical inhibitory activity was performed as per 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), ascorbic acid (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 extract or standard in DMSO were added to give a total volume of 1.1 ml. The solutions were then incubated for 30 min 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 min, 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.

Lipid Peroxidation Inhibitory Activity

The lipid peroxidation inhibitory activity of hydro-methanolic extract was studied *in-vitro* following the modified method.[16,17] Rats were killed by cervical dislocation (NIH, 1985), the liver tissue was excised, rinsed in ice-cold saline solution, and blotted dry. Then, 0.5 gm 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 ascorbic acid (AA), 4 mM $FeCl_2$, and 0.05 ml of various concentration of extract (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% SDS, 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 extract. The result was compared with BHT as a standard. The percentage inhibition of lipid peroxidation was calculated by comparing the results of test with those of controls not treated with the extract as per the formula:

% 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 extract.

Statistical Analysis

Statistical analysis was performed by software (Origin-8.1). Data was expressed as means ± SD of three measurements. Data was analyzed using analysis of variance (ANOVA) followed by multiple comparison two tail '*t*-test'. The results obtained were considered statistically significant if the *P*-value was <0.05. The amount of extract 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 has been implicated in the pathology of many diseases and conditions including diabetes, cardiovascular disease, inflammatory condition, cancer, ageing etc.[18,19] Antioxidants may offer resistance against the oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation, and by many other mechanisms and thus prevent diseases.[20] Consequently, we studied the antioxidant activities of hydro-methanolic extract by a series of *in-vitro* protocols using some biologically relevant models.

The findings of the phytochemical screening indicated that the seeds of *C. bonduc* are rich in flavonoids, phenols, and saponins which may be responsible for the antioxidative efficacy as these phytochemicals act as antioxidants.[21-23]

Phenolic compounds may contribute directly to the antioxidative action. The total phenolic content was 24.66 mg GAE/g dried extract. The total flavonoids content of the hydro-methanolic extract was 136.65 µg QE/g dried extract [Table 1]. Due to redox properties, phenolic compounds play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides.[24,25] It has been also recognized that flavonoids show antioxidant activity through scavenging or chelating process and their effects on human nutrition and health are considerable.[26]

The scavenging ability of hydro-methanolic extract on DPPH radical is represented by line diagram [Figure 1]

Table 1: Total phenolic and flavonoids contents of hydro-methanolic extract of seed of *C. bonduc*

Samples	Total phenolic content (mg GAE/g dried extract)	Total flavonoids (µg QE/g dried extract)
Hydro-methanolic extract of seed of *C. bonduc*	24.66	136.65

GAE: Gallic acid equivalents; QE: Quercertin equivalents

and compared with BHT. The scavenging activity of the investigated extract varied widely from 23.32% to 75.92% (IC_{50} value 157.4 µg/ml) and in standard 26.78% to 79.88% (IC_{50} value 145.89 µg/ml). From the result, we say 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.[27] It was observed that the radical scavenging activity is increasing with the increase of phenolic compound content.[28] The two separate studies were also reported a high concentration between DPPH radical scavenging potential and total phenolic content.[29,30]

Hydrogen peroxide neutralization ability of the extract [Figure 2] varied from 11.22% to 80.53% (IC_{50} value 64.32 µg/ml) and in standard 18.26% to 85.16% (IC_{50} value 57.06 µg/ml). The ability of the said extract to neutralize hydrogen peroxide was dose dependent. Hydrogen peroxide is important because of its ability to penetrate biological membranes. Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells.[31] Scavenging of hydrogen peroxide by *C. bonduc* 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 hydro-methanolic seed extract was shown in [Figure 3] and was compared with α-tocopherol. The extract inhibited the degradation of deoxyribose in dose dependent manner. Thereby, hydroxyl radical neutralization values ranges from 14.21% to 87.82% (IC_{50} value 61.9 µg/ml) and in standard from 17.98% to 92.66% (IC_{50} value 54.97 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 in 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.[33,34]

Inhibition percentage in lipid peroxidation varies widely in different doses ranges from 27.23% to 91.26% (IC_{50} value 58.87 µg/ml) in hydro-methanolic extract and 32.18% to 94.54% (IC_{50} value 51.34 µg/ml) in case of standard [Figure 4]. Lipid peroxidation has been broadly defined as the oxidative deterioration of polyunsaturated fatty acids and involves formation of lipid radicals leading to membrane damage. Free radicals induced lipid peroxidation mainly occurs in brain and liver due to presence of polyunsaturated lipid.[35] Increased lipid peroxidation is a salient characteristic of chronic diabetes which impairs membrane function by reducing the activity of enzymes as well as receptors.[36] Results focused that the hydro-methanolic extract of the seeds of *C. bonduc* inhibit lipid peroxidation under *in vitro* conditions indicating the anti-lipid peroxidant effect of the seed of *C. bonduc*.

CONCLUSION

The results of the study clearly indicate that hydro-methanolic extract of seed of *C. bonduc* possess *in vitro*

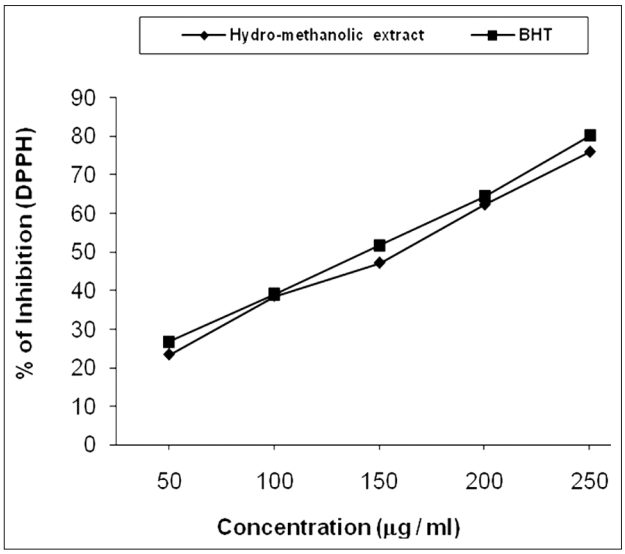

Figure 1: Inhibition in DPPH radical by hydro-methanolic extract of seed of *C. bonduc* and standard BHT. The IC_{50} value of the extract was 157.4 µg/ml

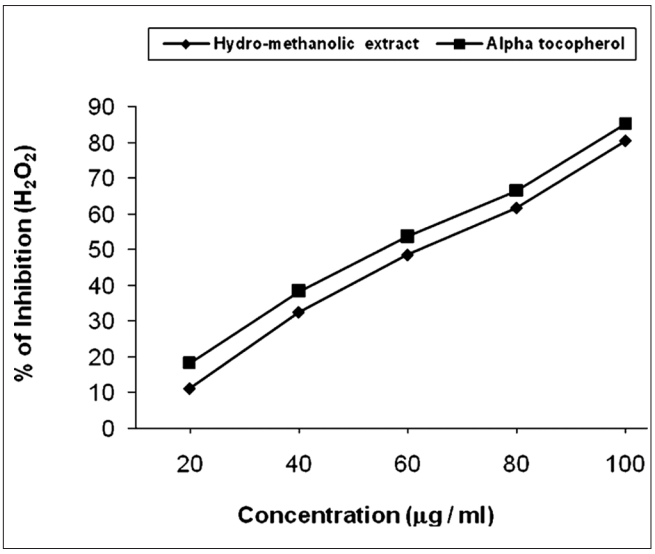

Figure 2: Scavenging of hydrogen peroxide by hydro-methanolic extract of seed of *C. bonduc* and standard α–tocopherol. The IC_{50} value of the extract was 64.32 µg/ml

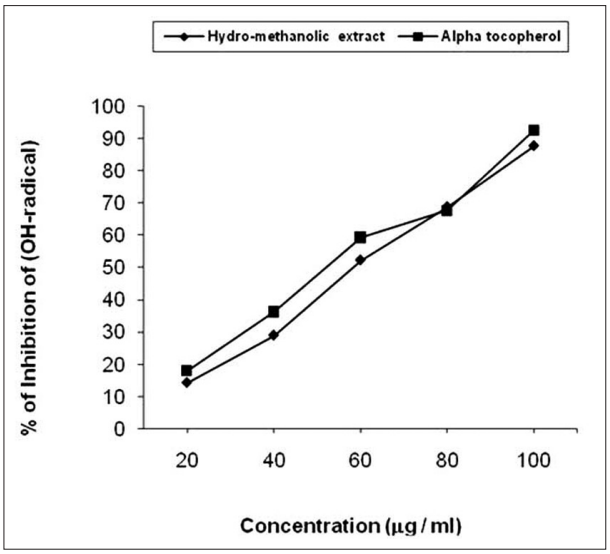

Figure 3: Hydroxyl radical scavenging ability of hydro-methanolic extract of seed of *C. bonduc* and α-tocopherol as a standard. The IC_{50} value of hydro-methanolic extract was 61.9 µg/ml

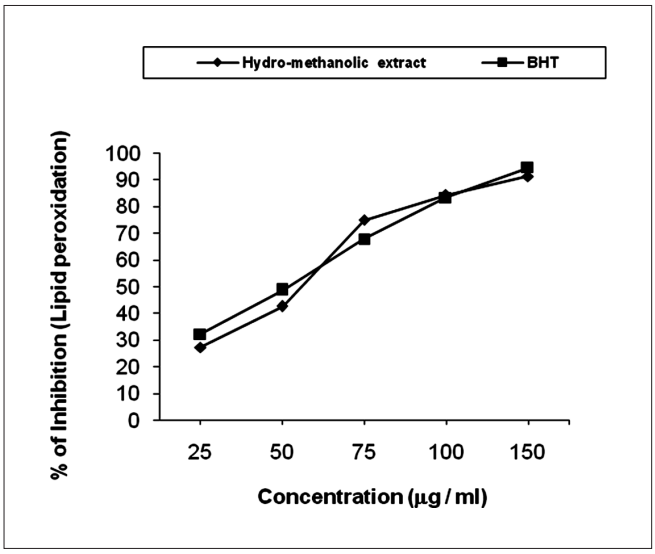

Figure 4: Inhibition in lipid peroxidation by hydro-methanolic extract of seed of *C. bonduc* and standard BHT. The IC_{50} value of the extract was 58.87 µg/ml

antioxidant activity. The encouraging results of this extract in various *in vitro* tests proved that the plant seeds 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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