Research Article

Antioxidant Activity of Methanolic Extract from the Stem Bark of the *Holoptelea integrifolia* Planch

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ABSTRACT

The present study was carried out to determine in-vitro antioxidant potential of methanolic extract of *Holoptelea integrifolia* Planch stem bark (HME), which compared with antioxidant compound ascorbic acid. The antioxidant activity of HME was evaluated by measuring inhibition activity of DPPH, nitric oxide, superoxide radical and its reducing power activity. The HME at different concentration (10-100 µg/ml) exhibited DPPH radical scavenging activity ranged from 17.07 to 69.78% inhibition, while ascorbic acid from 22.36 to 93.68% inhibition respectively. HME also exhibited nitric oxide and superoxide radical scavenging activity in the range of 25.23 to 77.01 % inhibition and 15.51 to 64.35 % inhibition respectively. The IC 50 values of HME for DPPH, nitric oxide and superoxide radical were found to be 53.67, 45.46, and 64.05µg/ml. However for ascorbic acid were 33.67, 24.94, 48.59 µg/ml respectively. Reducing power activity of extract was increased with increase in concentration. At higher concentration reducing power activity of HME was 1.199 respectively. Total phenolic content of the methanolic extract *H. integrifolia* was measured by using Folin–Ciocalteau reagent. The total phenolic content of aqueous extract of *H. integrifolia* was found to be 91.18±0.14 mg GAE/g.The results obtained from the study indicate that HME are a potential source of natural antioxidants.

Keywords: Antioxidant, DPPH, Free radical, *Holoptelea integrifolia*, Nitric oxide.

INTRODUCTION

The oxidation is an essential process to the living organism, plays an important role in the production of energy to the biological system. However as a result of this process, uncontrolled production of oxygen derived free radicals and reactive oxygen species (ROS). When production of ROS excess, can cause oxidative damage to DNA, proteins, lipids and causing the oxidative stress related various diseases such as cancer arthritis, cirrhosis, arteriosclerosis, diabetes, neurons related Parkinson's and Alzheimer disease.[1] Free radicals are also responsible for food deterioration during storage process.[2] Antioxidants are substance that can prevent or delay oxidative damage of lipids, proteins and DNA, caused by the reactive oxygen species such as superoxide, hydroxyle, peroxyle, alkoxyl, and nonradical hydrogenperoxide, hypochlorous in chain reaction etc.[3-9] Besides this antioxidants are also play key roles in the improvement of nutritional quality of the food by preventing the nutritional loss and formation of harmful substances during the storage of the food.

Recently there has been increase much interest in the potential of Plant bioactive components as antioxidants useful for preventing various oxidation stress related loss. Therefore, screening of plants on the basis of their antioxidants activity is becomes a challenge for the scientists.

Many medicinal plant screened for their antioxidant activities and results showed that individual chemical constituent more effective than synthetic antioxidants e.g. butylated hydroxyl anisol (BHA), butylated hydroxyl toluene (BHT) or vitamin E.[10-12] These synthetic antioxidants may have carcinogenic and other harmful effects on the lungs and livers.[13-15] Hence, natural antioxidant compounds especially phenolics and flavonoids capable of protecting against ROS mediated damage may have potential application in prevention diseases and also can replaced the harmful synthetic antioxidants.[16]

The Indian elm *Holoptelea integrifolia* (Planch) is an ornamental road side tree and grows up to 30-35 m in height and 3 m girth.[17] It belongs to family Ulmaceae which comprises 15 genera and 200 species and commonly known as Chilbil, Kanju in Hindi, Chirivilva, Poothigam in Sanskrit and Indian Elm tree in English.[18] *H. integrifolia* is distributed over tropical and temperate region of northern hemisphere.[19] It is native to Asian – Tropical region including India, Nepal, Srilanka, Cambodia, Laos, Myanmar, Vietnam, and China.[20] The stem bark contains the triterpenoidal fatty acid esters, holoptelin-A (epi-friedelinol palmitate) and holoptelin-B (epi-friedelinol stearate), friedelin and epi-friedelinol.[21] Two new medicinal pentacyclic triterpenods, betulinic acid (3β- Hydroxy- lup-20(29)-en-28-oic acid) and betulin (Lup-20(29)-ene-3β, 28-diol) were isolated from bark of plant.[22] Stem bark is whitish or yellowish grey, exfoliating in irregular flakes and with an offensive smell when freshly cut. Leaves are simple, alternate, elliptic-ovate, entire glabrous with cordate base, acuminate, Its flower is greenish yellow. Fruit a one seeded samara; light brown, obliquely elliptic or orbicular. The flower bears on the tree in month of January to February, whereas, fruiting is seen in April to May.[23]

Bark and leaves are bitter, astringent, acrid, anti-inflammatory, digestive, carminative, laxative depurative and urinary astringent.[24] Dried bark is taken as an oxytoxic for pregnant ladies.[25] The paste of Stem bark and seed is used for treating ringworms, eczema and cutaneous affection when applied externally. Stem bark is externally used for treatment in inflammation of lymph gland, for fever [26], and scabies. The paste of Leaf bud with lime juice is used externally for treatment of hair loss due to infection and treatment of herpes infection.[27] Ethno-medicinally, the leaves and stem bark of *H. integrifolia* are used for cancer treatment.[28] Stem bark is taken as an anti-inflammatory agent for eyes.[29] Leaves are used for treating oedema, leprosy, and other skin diseases, intestinal disorders, piles, and sprue.[30-32]

MATERIALS AND METHODS

Chemicals

Nitroblue tetrazolium (NBT), ferrozine and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma–Aldrich (Steinheim, Germany). Ascorbic acid, o-phosphoric acid, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, was purchased from Merck, Mumbai, India. Gallic acid, potassium ferricyanide, sodium carbonate, sodium nitroprusside, trichloroacetic acid (TCA), and ferric chloride were purchased from SD fine chemicals, India. All other reagents were of analytical grade.

Collection of plant material

The bark of *H. integrifolia* were collected in May 2011 District, Varanasi, India and identified by Prof. Dr. K.N. Dwivedi. A voucher specimen was deposited in the herbarium of the Faculty of Dravyagun, faculty of medicinal science B.H.U. Varanasi; herbarium code number: KND/JS09.

Preparation of the plant extract

The air-dried stem bark of *H. integrifolia* Planch (100 g) were powdered and then extracted with 500 ml of methanol by soxhlet process. The crude extract was concentrated to dryness under reduced pressure to give a viscous dark mass with a percentage yield of 11.24% (w/w). The extract was stored at 4°C for further use. This crude extract was dissolved in solvent and used for the assessment of antioxidant activity.

Determination of total phenolic content

Determination of total phenolic content was performed according to the method of using Folin-Ciocalteu reagent.[33] Briefly, 0.5 ml of each extract was mixed with 5 ml of Folin-Ciocalteu reagent (1:10 with distilled water), and then 4 ml of 1 M Na_2CO_3 was added to the mixture and the mixture was allowed to stand for 15 min at room temperature. The absorbance of the mixtures was measured at 765 nm. A standard curve was prepared by using gallic acid in various concentrations (50, 100, 150, 200, and 250 mg/ml). All measurements were carried out in triplicate and the results were expressed as gallic acid equivalents per gram of dry weight (mg GAE/g dry weight). Results represented as mean ± standard deviation.

DPPH radical scavenging activity assay

DPPH free radicals scavenging activity was measured according to the procedure described by Blios.[34] The HME at different concentration (10-100µg/ml) were added with 3ml of .1mM methanolic solution of DPPH. The mixture was shaken and allowed to react in dark at 37°C for 30 minute. After 30 min. incubation, reduction of DPPH was determined at 517 nm using spectrophotometer. The experiment was carried out in triplicate. Methanol is served as blank and ascorbic acid as standard. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound. The DPPH radical scavenging activity was calculated by using the following formula

$$\% \text{ inhibition} = (Ac - As / Ac) \times 100$$

Where Ac was the absorbance of the control (blank, without extract) and As was the absorbance in the presence of the extract. All the tests were performed in triplicate and the graph was plotted with the mean values.

Nitric oxide scavenging activity assay

The procedure is based on the method, where sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated using greiss reagent.[35] Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10mM) in phosphate buffered saline was mixed with different concentrations of aqueous extract of *H. integrifolia* dissolved in methanol and incubated at room temperature for 150 min. After the incubation period, 0.5ml of griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride was added. The absorbance of the blue chromophore formed was read at 546 nm.

$$\% \text{ inhibition} = (Ac - As / Ac) \times 100$$

Where Ac was the absorbance of the control (blank, without extract) and as was the absorbance in the presence of the extract. All the tests were performed in triplicate and the graph was plotted with the mean values.

Superoxide anion ($O_2^{\cdot-}$) radical scavenging activity

Measurement of superoxide anion scavenging activity of ethanol and water extracts was based on the method described by Liu.[36] Superoxide radicals are generated in PMS-NADH systems by oxidation of NADH and assayed by the radiation of NBT. In this experiment, the superoxide radicals were generated in 3ml of Tris-HCl buffer (100mM, pH 7.4) containing 0.75ml of NBT (300µM)

solution, 0.75ml NADH (936µM) solution and 0.3ml of different concentration (10-100µg/ml) of sample extract. The reaction was started by adding 0.75ml of PMS solution (120µM) to the mixture. The reaction mixture was incubated at 25˚C for 5min and the absorbance was measured at 560nm against blank sample. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. The inhibition percentage of superoxide anion generation was calculated by using the following formula.

$$\% \text{ inhibition} = (Ac - As / Ac) \times 100$$

Where Ac was the absorbance of the control (blank, without extract) and As was the absorbance in the presence of the extract. All the tests were performed in triplicate and the graph was plotted with the mean values.

Reducing power assay

The reducing power of the extracts was determined according to the method of Oyaizu.[37] 1 ml of the different concentration (100- 1000µg/ml) extract was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide (1%). The mixture was incubated at 50°C for 30 min. After cooling the mixture, 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of the upper layer was pipette out and mixed with 2.5 ml of distilled water and 0.5 ml of ferric chloride (0.1%) was added. The absorbance was measured at 700 nm. These reducing powers of extract compare with standard ascorbic acid. The intensity of reducing power is directly proportional to the absorbance of the reaction mixture.

RESULTS AND DISCUSSION

Total phenolic content

There is growing interest in phenolic compounds because of exhibiting antioxidant properties by scavenging free radicals and provide protection against oxidation. Phenolic compounds have ability to scavenge free radicals due to presence of one and more aromatic rings bearing hydroxyl groups that potentially able to act as reducing agents, antioxidants and singlet oxygen quenchers.[38] The mean of total phenol content per mg of the *H. integrifolia* was found to be 91.18±0.14 mg gallic acid equivalents of phenols. The results suggest that antioxidant activity of plant due to presence of higher amount of phenolic compounds. This result was indicated that phenolic compounds are major contributors to antioxidant activity.

DPPH scavenging activity

DPPH is a stable free radical at room temperature and widely accepted for evaluating free radical scavenging activity of natural antioxidants. The DPPH scavenging capability is based on the reduction of methanolic DPPH solution in the presence of a hydrogen donating antioxidant, due to formation of the non radical form DPPH-H by the reaction with a color change from deep violet to light yellow color. This can be quantified spectrophotometrically at 518 nm to indicate the extent of DPPH scavenging activity by the plant extracts.[34] Positive test of DPPH test suggested that the sample were free radical scavenger. At different concentration (10- 100 µg/ml) methanolic extract of bark of *H. integrifolia* (HME) showed significant antioxidant activities in a dose dependent manner (17.57, 34.50, 47.52, 58.76, 65.55, 69.74% inhibition) respectively (Figure 1). Methanolic extract of bark of *H. integrifolia* (HME) showed significant DPPH radical scavenging effect with increasing concentration in the range of 10-100 µg/ml. Extract showed lower DPPH radical scavenging activity when compared with ascorbic acid. At higher concentration (100µg/ml) of methanolic extract of bark and ascorbic acid inhibition was found to be 69.74 and 93.68 % inhibition respectively. The IC50 values of HME and standard in DPPH radical scavenging were found to be 53.67µg/ml and 33.67µg/ml respectively (Table 1). The results were found to be statistically significant ($P < 0.05$) by using PRISM software.

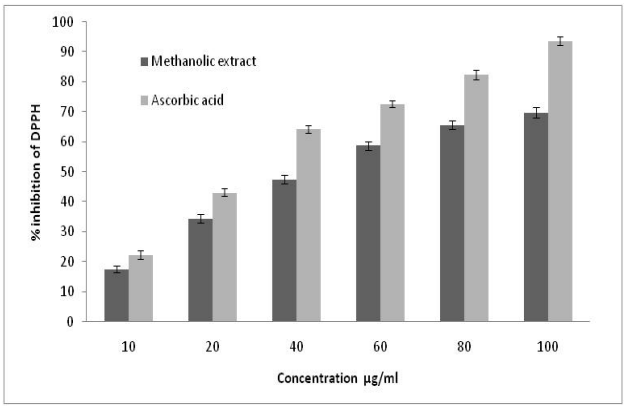

Figure 1: DPPH radical scavenging activities of the methanolic stem bark extract of *H. integrifolia* (HME). Ascorbic acid was used as the positive control.

Nitric oxide scavenging activity

Nitric acid plays an important role as chemical mediator, generated by endothelial cells, macrophages, neurons and involved in various inflammatory processes in the animal body. Nitric oxide is used in various types of disorders like AIDS, cancer, Alzheimer, and arthritis by cytotoxic effects.[39] DNA fragmentation, neural cell death, and cell damage occurs as the toxicity of over production of NO.[40] In the present study the methanolic extract of *Holoptelea integrifolia* stem bark have the properties to counteract the effect of NO production and preventing the ill effects of excessive NO generation in the human body. The HME at different concentration showed significant nitric oxide activities (25.23, 36.2, 46.40, 64.93, 72.62, 77.01% inhibition) respectively (Figure 2). At higher concentration (100µg/ml) of HME and ascorbic acid exhibited 77.01 and 91.25 % inhibition respectively. The IC 50 value of HME and standard in nitric oxide radical scavenging assay exhibited 45.46µg/ml and 24.94µg/ml respectively (Table 1). The results were found to be statistically significant ($P < 0.05$) by using PRISM software.

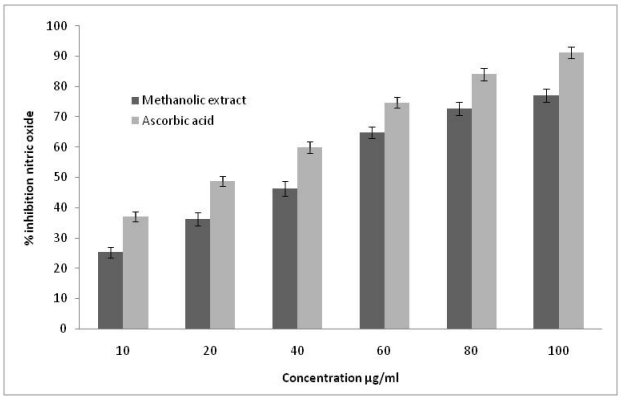

Figure 2: Nitric oxide radical scavenging activities of the methanolic stem bark extract of *H. integrifolia* (HME). Ascorbic acid was used as the positive control.

Superoxide scavenging activity

Superoxide anion radical is one of the strongest reactive oxygen species among the free radicals and very harmful to cellular components, act as a precursor of more reactive oxygen species, contributing in tissue damage and various diseases.[41] In the PMS-NADH-NBT system, Superoxide radical generated from dissolved oxygen by PMS-NADH coupling reaction and reduces NBT.[42] Different concentration of extract of *H. integrifolia* showed 15.51, 27.82, 35.05, 51.37, 62.98, 64.35 % inhibition respectively (Figure 3). The result indicated that % inhibition was increased with increasing concentration of the extract. At higher concentration 100µg/ml of HME and ascorbic acid exhibited 74.06 and 80.81% inhibition respectively. IC 50 value of HME were 64.05µg/ml whereas ascorbic acid 48.59µg/ml respectively (Table 1). The results were found to be statistically significant ($P < 0.05$) by using PRISM software.

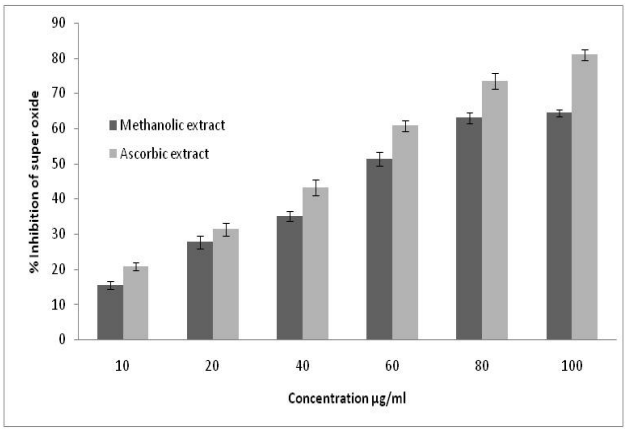

Figure 3: Superoxide anion scavenging activities of the methanolic stem bark extract of *H. integrifolia* (HME). Ascorbic acid was used as the positive control.

Table 1: Effects of methaonolic extract of stem bark of *Holoptelea integrifolia* (HAE) on different radical scavenging activity.

	DPPH radical scavenging activity	AA	Nitric oxide radical scavenging activity	AA	Superoxide radical scavenging activity	AA
IC50 values Of HAE	53.67 %	33.67%	45.46.%	24.94 %	64.05 %	48.59%

Reducing power activity

Reducing properties of the phytochemical responsible for scavenging of free radicals and associated with antioxidant activity, may serve as a significant indicator of potential of antioxidant activity for antioxidants.[43] In the presence of antioxidants the yellow color of sample changes in various shades of blue or green color, depending on reducing ability. The presence of reducers causes reduction of the Fe^{3}+/ferricyanide complex to the ferrous form with it formation of Perl's Prussian blue: this reaction was monitored spectrophotometrically by recording the absorbance of the reaction mixture at 700 nm.[44] Reductones have been shown to exert antioxidant activity by breaking the free radical chain by donating a hydrogen atom. Reductones also react with certain precursors of peroxide, thus preventing the formation of peroxide.[45] The reducing activity of HME and the reference compounds ascorbic acid increased steadily with the increasing concentration. Reducing activity of the HME at lower (100µg/ml) concentration was 0.121 and at higher concentration (1000µg/ml) was 1.199 (Figure 4).

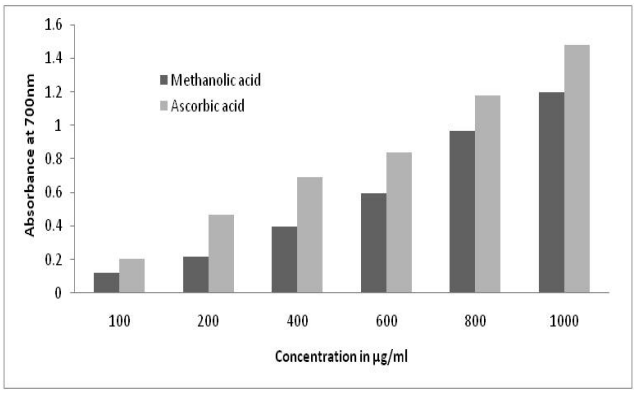

Figure 4: Reducing power activities of the methanolic stem bark extract of *H. integrifolia* (HME). Ascorbic acid was used as the positive control.

CONCLUSION

The present study demonstrated that methanolic stem bark extract of *Holoptelea integrifolia* (HME) contained higher levels of total phenolic content and neutralizing free radicals, quenching singlet, triplet oxygen to terminate the radical chain reaction and acting as antioxidants. In addition HME showed significant dose dependent antioxidant activity by inhibiting DPPH, nitric

oxide, superoxide radical and its reducing power activity in all the models studied. The activity of extract compared with standard ascorbic acid. Thus it can be concluded that methanolic stem bark extract of *Holoptelea integrifolia* used as a potential source of natural antioxidants, which might be helpful in preventing or slowing the progress of various oxidative stress related diseases. Further investigation on the isolation and identification of antioxidant compounds, their in vivo antioxidant activities and different antioxidant mechanisms is needs to be done.

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