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RESEARCH ARTICLE

Phytochemical Analysis and *In vitro* Antioxidant Activity of *Ochna obtusata*

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ABSTRACT

The present study was carried out to evaluate the phytochemical constituents and *in vitro* antioxidant potential of *Ochna obtusata*. Qualitative phytochemical analysis was performed using extracts of five solvents methanol, ethanol, petroleum ether, chloroform and water to check for the presence of phytochemical constituents. Among the five extracts obtained, methanolic extract possessed to have the maximum phytochemical constituents comparatively and therefore used for further study. The antioxidant activity of methanolic extracts of *Ochna obtusata* was evaluated by various antioxidant assays which include DPPH assay, nitric oxide assay, reducing power assay and H_2O_2 assay. In all the tested methods *Ochna obtusata* has been found to possess the antioxidant activity in a dose-dependent manner. The present study revealed that *Ochna obtusata* is very rich in Phytochemicals and a good source of natural antioxidants.

KEYWORDS

Ochna Obtusata, Anti-Oxidant, Free Radical Scavenging, DPPH Assay

INTRODUCTION

Oxygen though is vital for aerobic life process only 5% or more of the inhaled oxygen is converted to reactive oxygen species (ROS). Under normal circumstances, the free radicals which are defined as chemical species possessing an unpaired electron positively charged, negatively charged or electrically neutral; are detoxified by the antioxidants present in the body and there is equilibrium between the ROS generated and detoxified by the antioxidants present. When generation of ROS overtakes the antioxidant defence of the cells, the free radicals start attacking the cell proteins, lipids and carbohydrates and this leads to a number of physiological disorders[1,2]. Free radicals have been implicated in the pathogenesis of diabetes, liver damage, nephrotoxicity, inflammation, cancer, cardiovascular disorders and neurological disorders and in the process of aging[3]. Antioxidants are chemical substances that donate an electron to the free radical and convert it to a harmless molecule. They may reduce the energy of the free radical or suppress radical formation or break chain propogation or repair damage and reconstitute membranes. These anti-oxidants are found to be present abundantly in nature which certainly may have free-radical scavengers, reducing agents, potential complexes of pro-oxidant metals, quenches of singlet oxygen etc[4].

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Ochna is a genus comprising 86 species of evergreen trees, shrubs and shrublets. This family is characterized by the presence of Flavonoids and biflavonoids and terpenoids as main secondary metabolites[5] and several studies on other Ochna species were conducted and revealed that the phytochemicals contained within this genus constitutes mainly glycosides, saponins, steroids, flavones and fatty acids[6]. *Ochna obtusata,* (Family - Ochnaceae). Habit: Small trees up to 8 m tall. Trunk & Bark: Bark greyish, smooth; blaze pinkish. Branches and branchlets: Branchlets terete, lenticellate, glabrous. Leaves : Leaves simple, alternate, distichous; stipules caducous and leaving scar; petioles ca. 0.4 cm long, planoconvex, glabrous; lamina 16 x 5 cm, elliptic or elliptic-oblong to obovate, apex acute to rounded, base acute to rounded, margin serrate, shining above, chartaceous, glabrous beneath; midrib raised above; secondary nerves ca. 12 pairs, ascending towards apex; tertiary nerves slender, reticulo-percurrent. Inflorescence / Flower: Inflorescence axillary or lateral racemes; flowers yellow; pedicels up to 2.5 cm long. Fruit and Seed: Drupe, 3-5 distinct drupes seated on the enlarged disk; seeds 1 drupe. Distribution: South Asia; in the Western Ghats- South, Central and Maharashtra Sahyadris. The leaves and roots of Ochna obtusata is used for ulcer, asthma and bronchitis[7]. With respect to the literature review and relevant traditional approaches on the plant drug the study was made to investigate the phytochemicals in leaf extracts of *Ochna obtusata* using various solvents and then study *In vitro* anti-oxidant activity of extract possessing large number of phytochemicals.

MATERIALS AND METHOD

Collection of Plant

The fresh leaves of *Ochna obtusata* were collected from the forest of Tirumala Hills. The leaves were authenticated by Dr. Madhava Shetty, Taxonomist, Department of Botany, Sri Venkateswara University; Andhra Pradesh. The leaves were examined carefully for any unwanted objects. These fresh leaves were washed under fresh water and shade dried for four weeks until the leaves lost its moisture and the leaves could be crushed to a coarse powder.

Extraction Procedure

The powdered plant material was subjected to successively Soxhlet extraction with solvents such as methanol, ethanol, petroleum ether, chloroform, and water. After concentration of the extract they were dried and tests for identification of phytoconstituents. Phytochemical screening for major phytoconstituents of the plant extract was undertaken using standard qualitative methods. The plant extract were screened for the presence or absence of biologically active compounds like Alkaloids, Steroids, Tannins, Flavonoids, Glycosides, Anthraquinones, Saponins, Sugars etc.

Phytochemical Screening

Test for Alkaloids (*Dragendroff's Test)*

A 0.1 ml diluted hydrochloric acid, and 0.1 mL Dragendroff's reagent was added in 2 ml of extracts in test tubes. Formation of orange brown coloured precipitate indicated the presence of alkaloids.

Mayer's Test

A 2 ml of each extract were taken in test tube then 0.2 ml of diluted hydrochloric acid and 0.1 ml of Mayer's reagent were added. Formation of yellowish buff coloured precipitate indicated the presence of alkaloids.

Test for Steroids (*Salkowski Test)*

A 1 ml of concentrated sulphuric acid was added to 10 mg of extracts, dissolved in 1 ml of chloroform. A reddish brown colour exhibited by chloroform layer and green fluorescence by the acid layer suggested the presence of steroids.

Liebermann-Burchard Test

A 10 mg extracts were dissolved in 1 ml of chloroform. A 1 ml of acetic anhydride was added following the addition of 2 ml of concentrated sulphuric acid from the side of the test tube. Formation of reddish violet colour at

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the junction indicated the presence of steroids, triterpenoids and cardiac glycoside.

Test for Tannins (*Ferric chloride Test*)

A 5 ml of each extract solution was allowed to react with 1 ml of 5% ferric chloride solution. Greenish black colouration indicated the presence of tannins.

Potassium Dichromate Test

A 5 ml of each extract was treated with 1 ml of 10% of aqueous potassium dichromate solution. Formation of yellowish brown precipitate suggested the presence of tannins.

Test for Flavonoids (*Lead acetate Test*)

A 5 ml of each extract was treated with 1 ml of 10 % aqueous lead acetate solution. Development of yellow colour precipitate indicated the presence of tannins.

Test for Cardiac glycosides (*Keller kiliani's Test*)

A 5 mg of each extract was treated with 1 ml of glacial acetic acid and few drops of ferric chloride solution in a test tube. 2 ml of concentrated sulphuric acid was added carefully along the sides of the test tubes. The formation of a reddish brown colour at the junction of two layers and formation of bluish green upper layer indicated the presence of de-oxy sugar and hence cardiac glycosides are present.

Test for Anthraquinones (*Modified borntrager's Test*)

A 5 ml of the extract was dried and shaken with 3 ml petroleum ether. The filtrate was added to 2ml of a 25 % ammonia solution. The mixture was shaken and a red coloration observed was taken as an indication of the presence of anthraquinone.

Test for Coumarins

A 1 g of test residue moistened with water was taken in a test tube. The mouth of test tube was covered with paper moistened with dilute sodium hydroxide solution. The covered test tube was placed in boiling water bath for 15 min. The paper was removed and exposed to ultra-violet light. Yellowish green fluorescence confirmed the presence of coumarins.

Test for Phenolics

Two ml of ethanol was added to the test solution and few drops of ferric chloride solution. Blue coloration indicates the presence of phenolics.

Test for Saponins (*Foam formation test*)

A 1 ml solution of the extracts were diluted with distilled water up to 20ml and shaken in a graduated cylinder for 15 min. The development of stable foam indicated the presence of saponins.

Haemolysis Test

Test sample was dissolved in normal saline in such a way that 5 ml of the solution represented 1g of the crude drug. In a series of five test tubes, doses of 0.2, 0.4, 0.6, 0.8 and 1.0 ml were added and the volume was made up to 1 ml in each case with normal saline. 1 ml of diluted blood (0.5 ml of rabbit's blood diluted to 25 ml with normal saline) was added to each tube and the changes were observed. The occurrence of haemolysis of blood indicated the presence of saponins.

Test for Sugars (*Molisch's test*)

A 2 ml of each extract solution was treated with few drops of 15 % ethanolic alpha-naphthol solution in a test tube and 2 ml of concentrated sulphuric acid was added carefully along the sides of the test tubes. The formation of a reddish violet ring at the junction of two layers indicated the presence of carbohydrates.

Fehling's Test

A 5 ml of each extract solution was mixed with 5 ml of Fehling's solution (equal mixture of Fehling's solution A and B) and boiled. Formation of brick red precipitate indicated the presence of reducing sugars.

Study of *In Vitro* Antioxidants Analysis

DPPH Radical Scavenging Assay

The 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) assays was performed by using the method followed by Brand-Williams et al.,[8]. Plant

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extracts of five different concentrations viz. 250, 500, 750, 1000, 1500 was prepared and each added to 1.0 ml of the 0.004% methanol solution of DPPH. An equal amount of methanol and DPPH served as control. The mixture was vortexed thoroughly and left at room temperature for 30 min in the dark. The absorbance was measured by using a spectrophotometer at 517 nm and found to be 1526 for control. Radical scavenging activity was calculated as percent inhibition using formula: % inhibition = [Abs control – Abs sample] / [Abs control] x 100.

Nitric Oxide Scavenging Activity

The method of Fan et al.,[9] was adopted to evaluate the scavenging activity of *Ochna obtusata* against the nitric oxide radicals. A volume of 2 ml Sodium Nitroprusside (10 mM) was prepared in 0.5 mM phosphate buffer saline (pH 7.4) and was mixed with 0.5 ml of leaf extract at various concentrations as mentioned above and incubated at 25°C for 2.5 hrs. After incubation, 0.5 ml of the reaction mixture was removed; 1 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) was mixed and allowed to stand at room temperature for 5 min for complete diazotization reaction. 1 ml of naphthyl ethylene diamine dichloride (0.1%w/v) was added and the mixture was allowed to stand for 30 min at room temperature. Absorbance was measured at 540 nm. Nitric oxide percent inhibition = [Abs control – Abs sample] / [Abs control] x100.

Ferric Reducing Power Assay

The reducing power of methanolic extract of *Ochna obtusata* was evaluated according to the method of Wu et al.,[10]. Different concentrations of the extracts and standard drugs were mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium hexacyanoferrate II. The mixture was incubated at 50°C for 20 min, 2.5 ml of 10% trichloroacetic acid was added to the mixture and centrifuged at 3000 rpm for 10 min. The supernatant was mixed with 2.5 ml distilled water and 0.5 ml of 0.1% $FeCl_3$. The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated stronger reducing power.

Hydrogen Peroxide Scavenging Activity

The method of Hemwimol et al.,[11] was used to assess the ability of the extracts to scavenge hydrogen peroxide. A volume of 0.6 ml of 4 mM H_2O_2 solution prepared in 0.1M phosphate buffer (pH 7.4) was mixed with different concentrations of the extracts. The absorbance of the solution was measured at 230 nm after 15 min against a blank solution containing phosphate buffer without H_2O_2. The scavenging activity of the plant extract on H_2O_2 was expressed as: % scavenged [H_2O_2] = [Abs control-Abs sample]/ [Abs control] x100.

RESULTS AND DISCUSSION

The result of phytochemical screening of *Ochna Obtusata* is tabulated as in Table 1. The tests confirm the presence of various phytochemicals such as Alkaloids, Anthraquinones, Coumarins, Flavonoids, Glycosides, Steroids, Saponins, Sugars and Tannins in methanolic extract of leaves of *Ochna obtusata.* Therefore the methanolic extract was chosen to study the anti-oxidant activity. Table 2 shows the *in vitro* anti-oxidant assay of methanolic extract of leaves of *Ochna obtusata* whereas the scavenging ability of same extract on hydrogen peroxide is shown Table 3. DPPH radical scavenging activity of the methanolic extracts which was found to increase with increase in the concentration is indicated in Table 2. Highest inhibition was recorded at a dose of 1500 µg/ml and the lowest at 250µg/ml. DPPH is a relatively stable free radical and it is reduced by antioxidants to hydrazine by converting the unpaired electrons in it to paired ones thereby making it stable. The dose dependent inhibition of DPPH radicals indicates that methanolic extract of *Ochna obtusata* causes reduction of DPPH radical in a stoichometric manner which confirms the presence of anti-oxidants in the extract.

Similarly nitric oxide scavenging capacity of *Ochna obtusata* was found to be increasing remarkably following increase in extract concentration.

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Table 1: Phytochemical characteristics of leaves extracts of *Ochna obtusata*

Phytoconstituent	Methanol	Ethanol	Pet Ether	Chloroform	Water
Alkaloids	+	+	-	-	-
Anthocyanin	+	-	-	-	-
Coumarin	+	+	-	+	-
Flavonoids	+	+	+	-	+
Glycosides	+	+	-	+	-
Phenols	-	-	+	-	+
Proteins	-	-	-	-	-
Saponins	+	+	+	+	-
Steroids	+	+	+	-	-
Sugars	+	+	-	-	-
Tannins	+	-	+	-	-

a) + = indicates positive test (presence of compound)

b) – = indicates negative test (absence of compound)

Table 2: *In vitro* antioxidant assay of leaves extracts of *Ochna obtusata*

Plant Concentration (µg/ml)	DPPH assay (%)	Nitric Oxide assay (%)	Reducing power assay (%)
250	20.74	22.75	52.18
500	41.10	35.94	58.84
750	58.39	46.88	68.52
1000	73.11	54.69	77.60
1500	89.76	65.63	90.44
IC_{50}	680	942	178

Table 3: *In vitro* antioxidant assay of *Ochna obtusata* leaves extract (H_2O_2 assay)

Plant Concentration	H_2O_2 assay (%)
200	21
400	37
600	52
800	68
1000	90
IC_{50}	560

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The highest inhibition was recorded at a dose of 1500 µg/ml and the lowest at 250µg/ml. Nitric oxide being an important chemical mediator is involved in regulation of various physiological processes and excess of nitric oxide leads to several diseases. During excess of nitric oxide oygen reacts with it to form free radicals (nitrites and peroxynitrite anions). In the present study, the extract competes with oxygen to react with nitric oxide and thus inhibits the generation of free radicals.

The ability of *Ochna obtusata* extracts to reduce ferric cyanide to its ferrous form is also presented in the Table 2. Like the previously performed assays it showed significantly higher reducing power of the extract in a dose dependant manner. The highest inhibition was recorded at a dose of 1500µg/ml and the lowest at 250µg/ml.

Table 3 shows the scavenging ability of methanolic extract of *Ochna obtusata* leaves extract on hydrogen peroxide. Ferrous salts can react with hydrogen peroxide to form hydroxyl radicals via Fenton's reaction. The hydroxyl radicals thus produced may attack the sugar of DNA base causing sugar fragmentation thereby strand breakage. Therefore it is needed to minimise or stop the formation of hydroxyl radicals. The table shows a significant decrease of hydroxyl radicals in a dose dependant manner which may be due to proton donating capabilities of the phenolic contents of the methanolic extract. The above findings therefore confirm that the Phytochemicals present in the extract possess anti-oxidant activity.

CONCLUSION

The study confirms that the methanolic extract of Leaves of *Ochna obtusata* possess significant anti-oxidant activity in a dose dependant manner. The reason behind this may be the presence of several phytochemical constituents in the methanolic extract. Fractionation of these extracts to isolate and identify the most effective bioactive phytoconstituents responsible for the observed activities as well as their interactive mechanisms is highly advocated.

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