JOURNAL OF PURE & APPLIED MICROBIOLOGY, Nov. 2014. Vol. **8**(Spl. Edn. 2), p. 775-780

Phytochemical Screening and *In vitro* Antioxidant Activity of Ethanolic Extract of *Ormocarpum sennoides*

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(Received: 14 August 2014; accepted: 06 October 2014)

The ethanolic extract of the leaves of *Ormocarpum sennoides(Os)* were analyzed for the presence of phytochemicals by standard qualitative analysis and evaluated the invitro antioxidant activity by total antioxidant capacity, 1.1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, hydrogen peroxide radical scavenging activity , superoxide scavenging activity and Ferric reducing anti oxidant potential. The results showed the ethanolic extract of *Os* was able to efficiently scavenge the free radicals in a dose dependant manner. The results were compared with the standard antioxidant ascorbic acid. The results have shown that ethanolic extract of the leaves of *Os* can be used as a chemo protective agent and an antistressor.

Key words: *Ormocarpum sennoides,* phytochemicals, antioxidant activity.

Oxidative Stress, a physiological stress is caused due to a homeostatic imbalance between the production of oxidants called reactive oxygen species (ROS) and the antioxidants ROS is a collective term given to a group of free radicals like superoxide anions, hydrogen peroxide, and hydroxyl, nitric oxide and peroxy nitrite radicals that plays a vital role in the pathogenesis of neurodegenerative disorders, atherosclerosis, diabetes, inflammation[1], aging[2] cancer, coronary heart disease and Alzheimer's disease[3]. Antioxidants are compounds which act as radical scavengers when added to the food products and prevent the radical chain reaction of oxidation, delay or inhibit the oxidation process and increase the shelf life by retarding the processes of lipid per oxidation[4]. So, search for a novel antioxidant that can combat against these free radicals is being a current trend of research. Antioxidant-based drug formulations in allopathy, a modern trend in medicine have been very well developed as a modality to combat against any form of stress. But herbal medicines used from our traditional India as a folk medicine is preferable due to low toxicity and cost effectivity. Ancient Indian medicine, Siddha and Ayurveda commonly uses herbal extracts for innumerable disorders. These herbs possess a good amount of bioactive compounds that make them effective as a potent medicinal plant. In this regard, we have chosen *Ormocarpum sennoides* (fabaceae family) which is found growing in the scrub jungles of Coromandel, in India. This plant being known to the people as bone-knit and used traditionally for fracture healing[5] and there is not much information regarding its medicinal uses in the literature. In this study the leaf extracts were analyzed to reveal its phytochemicals and antioxidant property.

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MATERIALS AND METHODS

Chemical reagents

The solvents used were of HPLC grade. The standards (BHT, ±-tocopherol, L-Ascorbic acid and gallic acid) and chemicals used were obtained from Hi-Media lab. Ltd, Mumbai, India. 1,1 diphenyl-2-picryl hydrazyl (DPPH) radicals were purchased from Sigma Chemical Co, St. Louis, MO, USA.

Preparation of plant extract

Healthy leaves of *Os* were collected and washed with double distilled water, dried under shade and powdered. The dried powder was extracted using 95% ethanol using Soxhlet apparatus. Using rotary evaporator, the final trace of the solvent was removed. The dried crude ethanolic extract was stored at 4 º C until use.

Phytochemical screening

The plant extracts were tested for the presence of bioactive compounds such as alkaloids, anthocyanin and betacyanin, cardiac glycosides, coumarins, flavonoids**,** phenols quinines, saponins, steroids, tannins, terpenoids by standard methods[6].

Test for alkaloids

About 0.5 g of extract was dissolved in 5% HCl, filtered and tested with Dragendorff's reagent and Mayer's reagent separately. Any precipitate or turbidity with the reagents suggests the presence of alkaloids.

Test for anthocyanin and betacyanin

About 2mL of leaf extract was added with 1 mL of 2N NaOH and heated for 5 min at 100°C. Formation of bluish green color indicates the presence of anthocyanin and formation of yellow color indicates the presence of betacyanin.

Test for cardiac glycosides (Keller-Killiani test)

To 0.5 mL of the leaf extract, 2 mL of glacial acetic acid and few drops of 5% ferric chloride were added and 1 mL of concentrated sulphuric acid were added along the side of the test tube. Formation of brown ring at the interface indicates the presence of cardiac glycosides.

Test for coumarins

Moistened plant extract (0.5 g) was shaken in a small test tube and covered with filter paper moistened with 1N NaOH. The test tube was kept in boiling water for few min. Then the filter paper was removed and examined in UV light for yellow flourescence to indicate the presence of coumarins.

Test for flavonoids

Prepared plant extract (0.5 g) was shaken with petroleum ether to remove the fatty materials. The defatted residue was dissolved in 20 mL of 80% ethanol and filtered. The filtrate was used for the following test:

i) About 3 mL of filtrate was mixed with 4 mL of 1% AlCl in MeOH in a test tube. Formation of yellow colour was observed to indicate the presence of flavonoids, flavones or chalcones.

ii) About 3 mL of filtrate was mixed with 4 mL of 1% KOH. A dark yellow colour was observed to indicate the presence of Flavonoids

Test for phenol (Ferric chloride test)

To 1 mL of the leaf extract, 2 mL of distilled water was added followed by few drops of 10% ferric chloride. Formation of blue or black colour indicates the presence of phenols.

Test for quinones

To 1 mL of the leaf extract, 1 mL of concentrated sulphuric acid was added. Formation of red colour indicates the presence of quinones.

Test for saponins (Foam test)

Plant extract (0.5 g) was dissolved in 2 mL of boiling water in a test tube, allowed to cool and shaken to mix thoroughly. Foam appears for 10 min indicating the presence of saponins.

Test for steroids

To 0.5 mL of leaf extract, 2 mL of chloroform and 1 mL of sulphuric acid were added. Formation of reddish brown ring at interface indicates the presence of steroids.

Test for tannins

About 0.5 g of plant leaf extract was boiled in 20 mL of distilled water in a test tube and then filtered, 1 mL of 0.1% $FeCl_3$ was added to the filtrate. Appearance of brownish green or blue black colour indicates the presence of tannins[7].

Test for terpenoids (Salkowski test)

To 0.5 mL of the leaf extract, 2 mL of chloroform and 0.5 ml of concentrated sulphuric acid was added carefully. Formation of reddish brown coloration at the interface indicates the presence of terpenoids.

***In vitro* anti oxidant activity**

DPPH radical scavenging activity

The scavenging activity for DPPH free radicals was measured according to the procedure described in[8]. An aliquot of 3 ml of 0.004% DPPH

solution in ethanol and 0.1 ml of plant extract/ ascorbic acid at various concentrations were mixed. The mixture was shaken vigorously and allowed to reach a steady state at room temperature for 30 min. Decolorization of DPPH was determined by measuring the absorbance at 517 nm. A control was prepared using 0.1 ml of respective vehicle in the place of plant extract/ascorbic acid. The percentage inhibition of DPPH radicals by the extract/compound was determined by comparing the absorbance values of the control and the experimental tubes.

$$\text{DPPH scavenged (\%)} = \frac{(A\,cont - A\,test)}{A\,cont} \times 100$$

Where, A cont is the absorbance of the control reaction and Atest is the absorbance for the presence of the sample in the extracts. The antioxidant activity of the extract was expressed as IC50. The IC50 value was defined as the concentration (in µg/ml) of extract that inhibits the formation of DPPH radicals by 50%. The inhibition curve was plotted for duplicate experiments and represented as % of mean inhibition ± standard deviation. IC_{50} values were obtained by probit analysis[9].

Determination of H_2O_2 radical scavenging activity

Scavenging activity of hydrogen peroxide (H_2O_2) by the plant extract was determined by the method explained in[10]. Plant extract (4 ml) prepared in distilled water at various concentrations were mixed with 0.6 ml of 4 mM H_2O_2 solution prepared in phosphate buffer (0.1 M pH 7.4) and incubated for 10 min. The absorbance of the solution was taken at 230 nm. Ascorbic acid was used as a positive control compound. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples.

$$H_2O_2\ \text{Scavenged (\%)} = \frac{(A\,cont - A\,test)}{A\,cont} \times 100$$

Where A cont is the absorbance of the control reaction and A test is the absorbance for the presence of the sample in the extracts. The inhibition curve was plotted for duplicate experiments and represented as % of mean inhibition ± standard deviation. IC_{50} values were obtained by probit analysis[9].

Determination of total antioxidant capacity

The antioxidant activity of the extract was evaluated by the phosphomolybdenum method according to the procedure described in[11]. The assay is based on the reduction of molybdenum by the extract and subsequent formation of a green phosphate, phosphomolybdenum complex at acid pH. A 0.3 ml extract was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer against blank after cooling to room temperature. Ethanol (0.3 ml) in the place of extract is used as the blank. The antioxidant activity is expressed as the number of gram equivalents of ascorbic acid.

Ferric reducing anti oxidant power (FRAPASSAY)

The reducing powers of the extracts were determined by the method of Oyaizu *et al*[12]. Various concentrations of Os extracts were prepared in 1ml of distilled water, mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 RPM for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Ascorbic acid was used as the standard. Phosphate buffer (pH 6.6) was used as blank solution. All the tests were performed in triplicate and the graph was plotted with the average of three observations.

Superoxide scavenging

Alkaline DMSO was used as a super oxide generating system. To 0.5 ml of different concentrations of the Os extract, 1 ml of alkaline DMSO and 0.2 ml of NBT 20 mM in phosphate buffer pH 7.4 was added. The experiment was performed in triplicate[13].

$$\text{Scavenged (\%)} = \frac{(A\,cont - A\,test)}{A\,cont} \times 100$$

Where A cont is the absorbance of the control reaction and A test is the absorbance for the presence of the sample in the extracts. The

amount of sample required to decrease the absorption of DPPH, FRAP, H2O2, total antioxidant capacity and superoxide by 50% were calculated graphically (% of inhibition was plotted against the concentration in µg/ml). DPPH, FRAP, H_2O_2 superoxide radical scavenging activity, and total antioxidant capacity was compared with a reference standard ascorbic acid.

RESULTS

In the present study Os was analyzed for its phytochemicals and antioxidant property. The experiments were carried out in triplicate and the results were expressed as mean±standard deviation, Table [2 to 6] and Figure [1A to 1E] shows the DPPH, H_2O_2, total antioxidant capacity, FRAP, and superoxide radical scavenging activity with a reference standard ascorbic acid used as positive control. The antioxidant property was expressed as inhibition concentration, IC_{50}. The concentration of extract was used to calculate the inhibition concentration IC_{50} in µg/ml.

Table. 1. Phytochemical analysis of *Ormocarpum sennoides*

S.no	Phytochemicals	Analysis
1	Alkaloids	Present
2	Betacyanin	Present
3	Cardiac glycosides	Absent
4	Coumarins	Present
5	Flavonoids	Present
6	Phenol	Present
7	Steroid	Absent
8	quinones	Absent
9	Saponins	Present
10	Tannins	Present
11	Terpenoids	Present

Table 3. Hydrogen peroxide scavenging activity

Conc (µg/ml)	Ascorbic acid % inhibition	Os% inhibition
50	40.33+0.90	27.58+1.90
100	43.09+0.51	32.63+1.30
200	43.44+0.30	35.51+0.68
300	44.49+0.49	36.93+0.60
400	45.58+0.32	38.61+0.50
500	45.65+0.34	39.53+0.63
IC 50 (µg/ml)	102.25	172

DISCUSSION

Table [1] shows the phytochemical analysis of Os extract. Plant-derived antioxidants such as tannins, phenolic acids, flavonoids, anthocyanins and proanthocyanins, lignans, stilbenes, coumarins, quinones, xanthones, catechins, etc., could delay or prevent the onset of degenerative diseases because of their redox properties, which allow them to act as hydrogen donors, reducing agents, hydroxyl radicals (-OH.) or superoxide radical (O_2) scavengers. Ethanolic extract of *Os* which was qualitatively assessed for phytochemical analysis revealed the presence of Alkaloids, Betacyanin, Coumarins, Flavanoids, Phenol, Saponins, tannins and terpenoids. These bioactive compounds may be the probable reason for the free radical scavenging activity of the extract.

Os was able to effectively scavenge the free radicals in different concentrations in a dose dependant manner in all the assays. DPPH free radical scavenging activity is one of the commonly accepted model against lipid oxidation. The effect of antioxidants on DPPH radical scavenging was

Table. 2. DPPH Radical scavenging activity

Conc (µg/ml)	Ascorbic acid % inhibition	Os% inhibition
50	81.17+2.40	64.66+1.23
100	89.83+2.23	67.91+1.19
200	92.85+0.37	73.26+1.37
300	95.97+1.67	74.79+1.37
400	101.93+2.72	78.09+1.57
500	103.11+2.46	82.78+1.69
IC_{50} (µg/ml)	142	264

Table 4. Total antioxidant capacity

Conc (µg/ml)	Ascorbic acid % inhibition	Os% inhibition
50	18.47+1.08	23.51+1.19
100	30.64+1.62	34.87+1.77
200	45.12+1.94	45.53+2.33
300	46.73+1.37	52.82+1.77
400	53.75+1.82	58.13+0.81
500	60.72+1.09	64.25+0.03
IC 50 (µg/ml)	284	321

Table 5. Ferric reducing anti oxidant potential (FRAP)

Conc (µg/ml)	Ascorbic acid % inhibition	Os% inhibition
50	86.08+1.64	62.89+0.69
100	92+1.95	65.99+1.61
200	94.42+1.06	72.71+1.68
300	95.58+0.73	75.63+0.54
400	96.24+0.31	87.72+1.57
500	96.72+0.43	89.29+0.86
IC 50 (µg/ml)	226	265

Table 6. Superoxide scavenging activity

Conc (µg/ml)	Ascorbic acid % inhibition	Os% inhibition
50	86.37+0.91	68.59+0.84
100	93.13+0.64	72.90+0.66
200	94.14+0.18	75.41+0.67
300	95.35+0.33	78.17+1.01
400	96.39+0.34	83.54+0.50
500	96.93+0.04	85.42+0.42
IC 50 (µg/ml)	251	266

thought to be due to their hydrogen donating ability. Thus Os caused a very strong anti oxidant activity against DPPH radicals showing hydrogen donating ability.

Superoxide ($O2^{-}$•) is accepted to be a very harmful radical that can be converted into more reactive species, such as hydroxyl radical or peroxynitrite, contributing to tissue damages and various diseases. Superoxides are produced from molecular oxygen due to oxidative enzymes of the body by autooxidation of catecholamines[14]. The scavenging activity of *Os* may be due to inhibitory effect of generation of superoxides in an invitro reaction mixture.

Hydrogen peroxide (H_2O_2) is a weak oxidizing agent and crosses cell membranes rapidly to enter the cell. There, H_2O_2 can react with Fe^{+2} ions to generate the hydroxyl radical and this may be the origin of many of its toxic effects[15]. H_2O_2 is relatively stable in the absence of reducing compounds. Scavenging of H_2O_2 by *Os* may be attributed to their electron donating abilities[16]

FRAP assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} and is generally associated with the presence of reductones, that exert a very strong antioxidant action by breaking

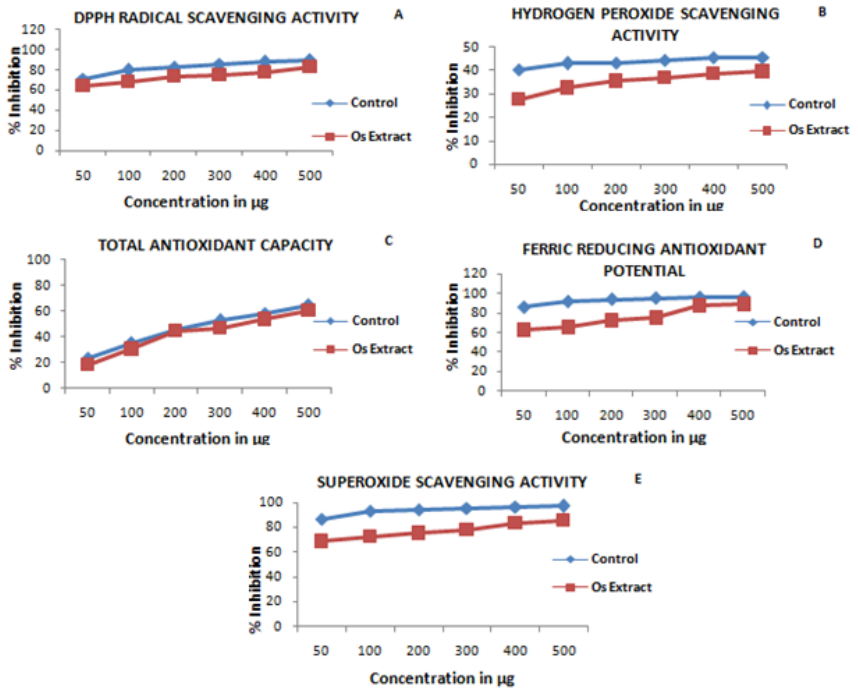

Fig. 1. Antioxidant potential of *Ormocarpum sennoides*

the free radical chain through donating a hydrogen atom[17]. The total anti oxidant capacity of OS extract reveal that it exhibited a very strong anti oxidant potential .The FRAP activity and the total anti oxidant capacity is correlated to high phenolic and flavonoid compounds namely quercetin, and kempferol. Phytochemical analysis on the crude extract of *Os* showed the presence of such phenolic and flavonoid compounds in the extract.

CONCLUSION

In conclusion, it can be stated that the ethanolic extract of *Ormocarpum sennoides* possesses a antioxidant activity which may be potentially responsible for its free radical scavenging capacity, and thus may be recommended its use as an antistressor.

ACKNOWLEDGMENTS

The author's S.Srividya and G.Sridevi gratefully acknowledge Sathyabama University Dental College and Hospital for providing necessary facility for this research work.

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