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Evaluation of Phenolics Content and Invitro Antioxidant Activities of Methanolic extract of *Amoora rohituka* Bark

M. K. Umesh, C.B. Sanjeevkumar and Ramesh L Londonkar*

Department of P.G. Studies and Research in BioTechnology, Gulbarga University, Kalaburagi-585106, Karnataka, India
**Corresponding author*

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A B S T R A C T

Numerous oxidative stress related diseases are gaining importance as a result of accumulation of free radicals in the body. A lot of researches are going on worldwide aimed towards finding natural antioxidants of plants origins. The aims of this study were to evaluate phenolic content and in vitro antioxidant activities of the methanolic extract of *Amoora rohituka* bark. The antioxidant and free radical scavenging activity of methanolic extract of bark were assessed against 2,2-diphenyl-1 picrylhydrazyl (DPPH), 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), Hydrogen peroxide (H_2O_2), ferric reducing, Phosphomolybdenum assay and metal chelation spectroscopically and results were compared with that of Butylated hydroxyl toluene (BHT) and Ascorbic acid and EDTA as standards. The IC_{50} value of the DPPH, ABTS, H_2O_2 and metal chelation are 73µg/ml, 73µg/ml, 103 µg/ml and 101µg/ml respectively. Our findings offer confirmation that the crude methanol extract of *A.rohituka* is a potential source of natural antioxidants, and this justified its uses in folkloric medicines.

Introduction

The oxidation process is highly reactive consisting harmful chain reactions that generate free oxygen radicals, which in turn causes damage to living organism. The oxygen centered free radicals and other reactive oxygen species (ROS), which are continuously produced, may result in cell death or tissue damage. The human body has its own composite system of natural enzymatic and non-enzymatic antioxidant defense mechanism which neutralizes the harmful effect of free radicals and other oxidants (1). ROS including superoxide free radical, hydroxyl free radical, hydrogen peroxide and singlet oxygen play a key role in the oxidative damage of various diseases. Since these free radicals are responsible for causing a large number of diseases including aging (2), alcohol induced liver disease (3), Alzheimer's disease (4), atherosclerosis (5),

cancer (6), cardiovascular disease (7), mild cognitive impairment (8), neural disorders, Parkinson's disease (9) and ulcerative colitis (10).

The screening of various plants for medicinal properties has attracted an increasing curiosity since last couple of decades because of their potent pharmacological activities, convenience to use, economic viability and low toxicity (11). Since from ancient times, the medicinal properties of plants have been investigated for scientific development throughout the world due to their strong radical scavenging activities. Defense against the free radicals can be enhanced by sufficient intake of dietary antioxidants. Antioxidant is a molecule which terminates the chain reaction by removing free radical intermediates. They will be of great benefit in improving the quality of life by preventing or postponing the onset of degenerative diseases. In addition, they have a potential for substantial savings in the cost of health care delivery (1). As antioxidants have been reported to prevent oxidative stress and damage caused by free radical and also interfere with the oxidation process by reacting with free radicals, chelating agents, catalytic metals and also by acting as oxygen scavengers (12).

Aphanamixis polystachya (Wall) Parker (Meliaceae) is also known as *Amoora rohituka* is a traditional plant used as medicine extensively across the Asian countries (13, 14). *Amoora rohituka* has shown a high potential biological activity and evaluated for the presence of number of secondary compounds with their therapeutic properties (15). *Amoora rohituka* is widely distributed in higher altitudes in Western Ghats and Eastern Ghats of South India, rarely found in Sub Himalayan tracks from upper area going eastwards to Bengal, Sikkim and Assam 14, 16, 17). The plant extract possess Anti-cancer (18, 19), Antifeedant (20), Anti-oxidant, Thrombolytic activity (21), Insecticidal, Laxative (17) and Anti-microbial agents (22, 23). This study is aimed to evaluate the in-vitro antioxidant effect of methanolic extract of *Amoora rohituka* (MEAR) in comparison with commercial standards.

Collection and Extraction of Plant Materials

Stem bark of *Amoora rohituka* was collected from in and around *Thiruvananthapuram, Kerala, INDIA* in the month of march 2013 and the plant was duly identified and authenticated by Dr.Kotresha, Associate professor, Taxonomy and Floristic Laboratory, Department of Botany, Karnataka Science College, Dharwad, Karnataka, INDIA.

The stem bark was allowed to dry in shade for two to four weeks. Precaution was taken to avoid direct sun light otherwise it will destroy the active compounds of bark. After drying, the bark were grinded finely and stored in airtight container. The air dried bark powder (150 g) was successively extracted by soxhlet extraction with solvents of increasing polarity i.e., petroleum ether (60-80 C), benzene, chloroform and methanol. The extracts were dried and stored in a sterile container for further use.

Determination of Total Phenolic Content

The amount of total soluble phenolic content in methanolic extract of *A ruhiatuka* bark was determined according to Folin-Ciocalteu method (24) with minor modifications. Briefly, 100mg/ml stock solution was prepared from which the 20 µL was mixed with 200 µL of Folin-Ciocalteu reagent. After 10 min of incubation, 600 µL

of 20% Na_2CO_3 solution was added and the volume was adjusted to 2 mL using distilled water. The mixture was incubated in dark for 2 h and the absorbance was measured at 765 nm using a UV-Vis spectrophotometer against blank sample. The total phenolic content was measured as gallic acid equivalents (mg GAE)/gram of dry weight (dw) and the values were presented as means of triplicate analysis.

DPPH Radical Scavenging Activity

The antioxidant activity of the plant extracts was assessed on the basis of the radical scavenging effect using stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) (25). DPPH solution (0.004% w/v) was prepared in 95% methanol and serial dilutions were carried out with the stock solutions (20 mg/mL) of the extracts. Various concentrations of extracts were mixed with DPPH solution (900 µL), incubated in dark for 30 min and absorbance was measured at 517 nm. Methanol (95%) was used as blank; DPPH solution was used as a reference whereas ascorbic acid was used as standard.

ABTS Radical Scavenging Assay

The ability of the extract to scavenge for the ABTS radical was determined by the method (26). Accordingly the working solution was prepared by mixing two stock solutions i.e, 7mM ABTS and 2.4mM potassium persulphate and keeps it in dark at room temperature for 16 hours. It was further diluted with 80% ethanol to obtain an absorbance value of 700±0.005. The extract at different concentration (300 µL) was incubated with 2.7mL of ABTS mixture at 30˚C for 30 min and the absorbance was recorded at 734nm. BHT at the same concentration is used as a reference. The radical scavenging activity was calculated as follows:

Scavenging rate = ((As − Ai)/As)×100,

where As is the absorbance of pure ABTS mixture and Ai is the absorbance of ABTS mixture in the presence of extract.

Hydrogen Peroxide-scavenging Activity

The ability of the *Amoora rohituka* extract to scavenge hydrogen peroxide was determined according to the method of Ruch et al. (27). Briefly, 2 mM hydrogen peroxide solution was prepared in 50 mM phosphate buffer (pH 7.4). Hydrogen peroxide concentration was determined spectrophotometrically at 230 nm, using the molar extinction coefficient for H_2O_2 of 81 mol^{-1} cm^{-1}. Different concentrations of extract (0.050–0.250 mg/ml) and ascorbic acid (0.025–0.800 mg/ml) were transferred into the test tubes, and their volumes were made up to 800µl with 50 mM phosphate buffer (pH 7.4). Add 1.2 ml hydrogen peroxide solution to the tubes and vortex the tubes. Absorbance of the hydrogen peroxide was determined at 230 nm after 10 min incubation, against a blank. 50 mM phosphate buffer without hydrogen peroxide was used as blank. Hydrogen per oxide scavenging ability was calculated by the following equation:

Hydrogenperoxide scavenging activity = (1 – absorbance of sample/absorbance of control)X100

IC_{50} was calculated through graph prism pad software.

Ferric Reducing Power

The reducing power of the MEAR was determined by the method of Jungmin et al (28) with minor modifications. An aliquot (250 µl) of sample, 250 µl of sodium phosphate buffer (200 mM, pH 6.6), and 250

µl of potassium ferricyanide (1%) were mixed and incubated in a water bath at 50 °C for 20 min. The reaction was terminated by adding 250 µl of trichloroethanoic acid solution (10%, w/v). Take 750µl of the mixture and then add equal volume of distilled water and 75 µl of ferric chloride solution (0.1%, w/v) then incubate it for 10 min at room temperature. Methanol was used as blank. The intensity of the Prussian blue color was measured at 700 nm using a spectrophotometer. Results are expressed as the mean absorbance value.

Phosphomolybdenum Assay

The antioxidant activity of samples was evaluated by the green phosphomolybdenum complex according to the method of Prieto et al (29). An aliquot of 100 µL of sample solution was combined with 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in a 4 mL vial. The vials were capped and incubated in a water bath at 95 ◦C for 90 min. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 695 nm against a blank. The total antioxidant activity was expressed as the absorbance of the sample at 695 nm. The higher absorbance value indicated higher antioxidant activity (30).

Metal Chelating Activity

The chelating activity of the extracts for ferrous ions (Fe^{2+}) was measured according to the method of Dinis et al. (31). The extracts (100 µL) were added to a solution of 2 mmoL/L $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5 mmoL/L ferrozine (0.2 mL) and the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The chelating activity was calculated as follows:

Chelating rate = (As − Ai)/As X 100

where As is the absorbance of the control and Ai is the absorbance in the presence of the extract. Ethylene diaminetetraacetic acid (EDTA) was used for comparison

Results and Discussion

The phenolic compounds may contribute directly to the antioxidative action (32). It is recommended that polyphenolic compounds might have inhibitory effects on mutagenesis and carcinogenesis in humans, when up to 1.0 g daily are ingested from a diet rich in fruits and vegetables (33). Besides, it was reported that the phenolic compounds were associated with antioxidant activity and play an important role in stabilizing lipid peroxidation (34). Total phenol content was estimated as 168.4±2.32 mg gallic acid equivalents/g (dry weight) extract

DPPH Radical Scavenging Activity

The DPPH radical inhibition assay is a widely used and comparatively simple method for the evaluation of antioxidant activity. DPPH accepts hydrogen radicals or electrons to produce stable diamagnetic molecules. Based on their hydrogen-donating ability, the antioxidative property of proton-donating substances was demonstrated using DPPH based assay. The DPPH radical scavenging ability of methanolic extract of *Amoora rohituka* and ascorbic acid is shown in the figure 1. The MEAR showed significant DPPH radical activity comparable to Ascorbic acid a standard antioxidant drugs used in this study. IC_{50} of extract was found to be 73µg/mL. Results are presented as mean ± SD (n=3)

ABTS Radical Scavenging Assay

The ABTS radicals are determined by the decolonization of the ABTS, which was measured spectrophotometrically at 734 nm. The % inhibition of absorbance was calculated and plotted as a function of concentration of the extract and standard as shown in Figure 2. The percentage inhibition of ABTS radical scavenging activity was concentration-dependent with increased in the reaction mixture for the extract and the standard. The IC_{50} was determined as 73µg/mL.

Hydrogen Peroxide-scavenging Activity

Dose-dependent H_2O_2 scavenging activity of the MEAR is shown in Figure 3. At 200µg/mL concentration, the H_2O_2 scavenging activity of MEAR increased rapidly with an increase in concentration. However, at concentration >200µg/ml, the rate of increase in the scavenging activity with increase in concentration was relatively slower. It is perhaps that, at low concentrations MEAR has scavenged H_2O_2 efficiently. At increasing concentrations, the absorbance decreased slowly indicating that the increase in the scavenging activity. At the highest evaluated concentration (800µg/mL), the MEAR was found to scavenge 88.5±1.55% of the hydrogen peroxide and the determined IC_{50} value of MEAR is 103µg/mL.

Reducing Activity

The dose-response curve for the reducing activity of the MEAR was determined as described by Jungmin et al (28) with minor modifications. Fig 4 shows the reductive capabilities of the plant extract at each concentration, in the range of 25 to 800 µg/ml compared to BHT. The absorbance of the MEAR increased abruptly when the concentration increases from 25µg/ml to 100µg/ml while further increase in the concentration faintly increased in the absorbance. The higher absorbance indicates a relative stronger reducing activity, hence the study indicates that the extract possess a moderate reducing activity.

Phosphomolybdenum Method

The phosphomolybdenum method is based on the reduction of molybdenum (VI) to molybdenum (V) by the antioxidant compounds and the formation of a green molybdenum (V) complex, which has a maximal absorption at 695 nm. The reducing activity of MEAR and BHT are presented in Figure 5 which clearly depicting the dose dependent curve.

Fe^{2+} chelation

Ferrozine produces a violet complex with Fe2+. In the presence of a chelating agent, complex formation is interrupted and as a result the violet color of the complex is decreased. The results demonstrate that the formation of ferrozine-Fe2+ complex is inhibited in the presence of the test and reference compounds. Dose-dependent Fe2+ chelation activity of the MEAR is shown in Figure 6. At 800µg of extract significantly chelates 81±1.87% of the divalent iron and the determined IC_{50} value of MEAR is 101µg/mL.

The methanolic extract of the Amoora rohituka was investigated which has showed a pronounced antioxidant activity. A number of bioactive compounds such as alkaloids, flavonoids, phenol, tannins, terpenes and flavonoids reported from this plant could be responsible for the observed activities.

DPPH is considered to be the model of the lipophilic radical. In this mode, the

scavenging activity was attributed to H^+ donating ability of the antioxidants(35). DPPH radical is commonly used as a substrate to evaluate antioxidant activity; it is a stable free radical that can accept an electron to become a stable molecule (36). Figure 1 shows that the dose-response curve of DPPH radical scavenging activity of the methanol extract of *Amoora rohituka*. At a concentration of 800 µg/ml, the scavenging activity of methanol extract has shown 92.8%, while at the scavenging ability of the extract was less than that of ascorbic acid (99.4%) at the same 800 µg/ml.

Figure.1 DPPH Scavenging Activity of Methanolic Extract of *Amoora rohituka* Bark

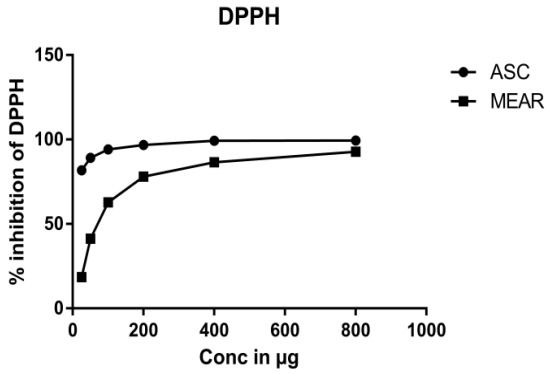


Figure.2 ABTS Scavenging Activity of Methanolic Extract of *Amoora Rohituka* Bark

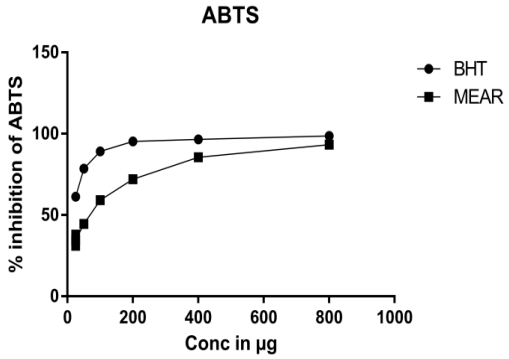


Figure.3 H2O2 Scavenging Activity of Methanolic Extract of *Amoora Rohituka* Bark

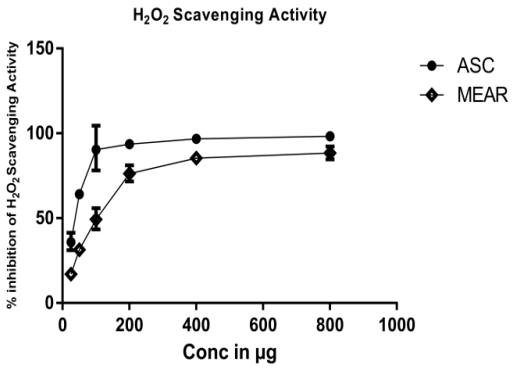


Figure.4 Ferric Reducing Power of Methanolic Extract of *Amoora Rohituka* Bark

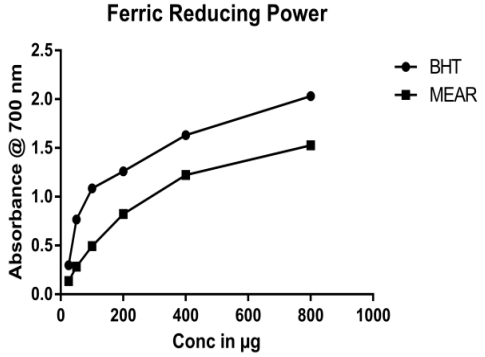


Figure.5 Phosphomolybdenum Assay of Methanolic Extract of *Amoora Rohituka* Bark

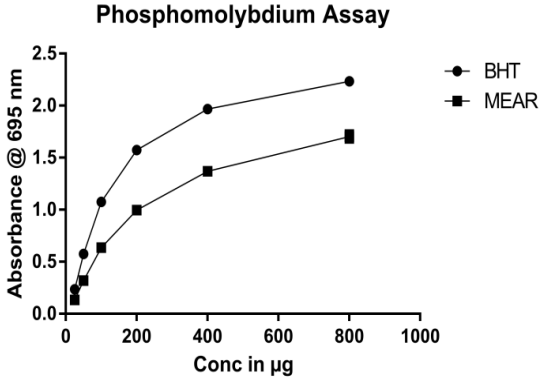
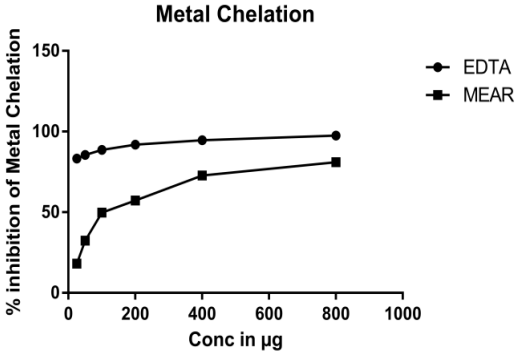


Figure.6 Metal Chelation of Methanolic Extract of *Amoora rohituka* Bark



The decolorization of ABTS•+ cation radical is an unambiguous way to measure the antioxidant activity of phenolic compounds. The percentage inhibition of ABTS radical scavenging activity was concentration-dependent with increased in the reaction mixture for MEAR and standard. The positive correlations between phenolic content and antioxidant activity tested using the Oxygen Radical Absorbance Capacity (ORAC), ABTS and the DPPH assays was studied by

Awika et al (37). Results of the present study revealed The scavenging capacity of the MEAR was 44.6% at 50µg and 59.2% at 100 µg. The IC_{50} value indicated that the ABTS free radical scavenging activity with a 50% inhibition (IC_{50}) at a concentration of 72 µg/ml of MEAR was nearly higher than BHT (>25 µg/mL).

Hydrogen peroxide occurs naturally at low concentration levels in the environment, human body, plants, microorganisms and in food (38). Hydrogen peroxide is a known antimicrobial agent with cleansing property when it was initially introduced into clinical practice (39). Hydrogen peroxide is an oxidant that is being continuously generated in living tissues as a result of several metabolic processes. H_2O_2 is rapidly decomposed into oxygen and water and further may produce hydroxyl radicals (•OH) that can initiate lipid peroxidation and cause DNA damage (40). As shown in Figure 3 methanolic extract of *Amoora rohituka* have an effective radical scavenging activity for H_2O_2in a concentration dependent manner which is compared with the ascorbic acid. The MEAR was found to scavenge 88.5±1.55% at 800µg/mL of extract and the IC_{50} value of MEAR was found to be 103µg/mL.

Their antioxidant activity is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers, which can play an important role in adsorbing and neutralizing free radicals (41,42). The Figure 4 indicates the reductive capabilities of the MEAR at different concentration which ranges from 25 to 800 µg/ml compared to BHT. The reducing power of MEAR was increased with quantity of sample. The plant extract could reduce the most Fe^{3+} ions, which had a lesser reductive activity than the standard of BHT. The methanolic extract of *A.rohituka* exhibited a significant dose dependent inhibition of reducing power activity.

The phosphomolybdenum method is based on the reduction of molybdenum (VI) to molybdenum (V) by the antioxidant compounds and the formation of a green molybdenum (V) complex, which has a maximal absorption at 695 nm. It is clear from the Figure 5 that, the methanol extract has showed an increase in antioxidant capacity with increase in concentration of sample.

The chelating of ferrous ions by the MEAR was estimated by the method of Jungmin et al (28). Ferrozine can quantitatively form complexes with Fe2+. In the presence of chelating agents, the complex formation is disrupted, resulting in a decrease in the red colour of the complex. The Metal chelating capacity is significant since it reduces the concentration of the catalyzing transition metal in lipid peroxidation (32). As shown in Figure 6, the percentage of Fe^{2+} – ferrozine complex was linearly increased dose dependently (from 25 to 800 µg/ml). The The IC_{50} value of the metal chelation was found to be 101 µg/mL.

The differential response of the extract in various antioxidant tests may be explained by the fact that the transfer of electrons/hydrogen from antioxidants occur at different redox potential in various assay systems and the transfer also depends on the structure of the antioxidants (Loo et al., 2008).

In conclusion, the present study reveals the in vitro antioxidant activities methanolic extract of *Amoora rohituka* in terms of total phenolics content, DPPH, ABTS radical scavenging activity, reducing power, hydrogen peroxide, phosphomolybdium and metal chelating activity. Results from the

present study confirm that the methanolic extract of A rohituka acts effectively as scavenger of reactive oxygen species. However more research is needed to characterize the chemical compositions and structures that contribute to the total antioxidant activities. The results of this study is likely to provide sufficient baseline information for further exploration of this Amoora rohituka bark for medicinal purposes as well as for developing new drugs. However, further research on the chemical composition and in vivo studies should be carried in the future.

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