

## *In vitro* antioxidant activities of stem bark, leaves and callus extracts from *Amoora rohituka*

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### Abstract

The present work examined the *in vitro* antioxidant activity of sequentially extracted, solvent extracts (Petroleum ether, chloroform and Methanol) of stem bark, leaves and callus from *Amoora rohituka*. The antioxidative capacity of the extracts were examined by using DPPH (1,1-diphenyl,2-picryl hydrazyl), ABTS (2,2'-azinobis-3-ethyl-benzothiozoline-6-sulphonic acid) and NOS (Nitric oxide scavenging) assays by using Gallic acid as standard control. Among the tested extracts, methanol extract of stem bark showed better antioxidative capacity. The total antioxidant activity was expressed as  $\mu\text{g}$  equivalent of Gallic acid per gram on dry weight basis.  $\text{IC}_{50}$  values for methanol extract of stem bark were 7.53  $\mu\text{g}/\text{ml}$ ; 34.92  $\mu\text{g}/\text{ml}$  and 56.31  $\mu\text{g}/\text{ml}$  in ABTS, DPPH and NOS assays respectively. Whereas, leaves and callus extracts showed moderate antioxidative properties.

**Keywords:** *Amoora rohituka*, Antioxidant activity, Petroleum ether, Chloroform and Methanol.

### Introduction

Active oxygen species and free radical mediated reactions have been implicated in degenerative or pathological processes such as aging [1], cancer, coronary heart disease and Alzheimer's disease [2]. To protect their possible damages to biological molecules, all oxygen-consuming organisms are endowed with a well-integrated antioxidant system, including enzymatic and non-enzymatic components. Plants constitute an important source of active natural products which differ widely in terms of structure and biological properties. They have played a remarkable role in the traditional medicine of various countries. The protective effects of plant products are due to the presence of several components which have distinct mechanisms of action; some are enzymes and proteins and others are low molecular weight compounds such as vitamins [3], carotenoids [4], flavonoids [5], anthocyanins and other phenolic compounds [6]. The importance of the antioxidant

constituents in the maintenance of health and protection from coronary heart disease and cancer is also raising interest among scientists, food manufacturers and consumers as the trend of the future is moving toward functional food with specific health effects [7].

*Amoora rohituka* is distributed throughout India in evergreen forests and is a member of the family Meliaceae. The stem bark and seeds of this plant have been reported to be useful in splenomegaly, liver disorders, tumors and also reported to possess various pharmacological properties for human disorders [8]. The stem bark has been found to be useful in certain tumors including liver and spleen [9][10]. The alcoholic extracts of the stem has been reported to show anticancer activity against Friends Leukemia and Ehrlich as cites carcinoma in mice [11]. Because of the presence of amoorastatin and 12x-hydroxyamoorastatin, the stem bark extract exhibits cytotoxic and growth inhibitory effects in murine P388 lymphocytic leukaemia cell lines [12]. The seeds and stem bark contain isomeric rohitukin alkaloids and a range of complex

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limonoids like, polystachin, prierianin and hispidin C [13] and three flavonone glycosides, [14] and anthraquinone and naringeninaglycone [9]. In earlier studies, naringin protected the mice bone marrow cells against the radiation-induced chromosomal damage [15].

Therefore, it is of great interest to evaluate the antioxidant activity of *Amoora rohituka*. For this purpose, *Amoora rohituka* stem bark, leaves and callus were selected in the present study for the analysis of their antioxidant activity.

## Materials and methods

### Plant materials collection

*Amoora rohituka* tree growing along the banks of Kukkarahalli lake (Mysore, Karnataka, India) were surveyed. Mature tree (5-6 years) was selected; stem bark and leaves were collected for *in vitro* studies.

### Induction of callus

Young leaves of *Amoora rohituka* were collected and surface sterilized by running tap water treatment for 40 min, bavistin treatment for 30 min, antibiotic treatment for 20 min followed by 0.1% mercuric chloride treatment for 10 min. Finally, the explants were washed with sterile distilled water successively three times and were inoculated on Murashige and Skoog [16] medium supplemented with 2.0 mg/l NAA (1-naphthaleneacetic acid), 0.5 mg/l BAP (6-benzyladenine purine), 30 g/l sucrose, 9 g/l agar at pH 5.8 in glass jars for the production of callus. The cultures were incubated at 25±2°C, 3000 lux intensity for 16 h photoperiod. Callus was subcultured onto fresh medium of the same composition for a period of six weeks before being analyzed.

### Extraction of Stem barks leaves and callus

Collected plant materials (stem bark and leaves) and *in vitro* grown callus were cleaned and air dried under shade, and coarsely powdered in a ballmill. The powdered materials were sequentially extracted with petroleum ether, chloroform and finally with methanol in a soxhlet apparatus at 4°C for 30 cycles. After each solvent extraction the marc was made free from traces of solvent by drying it at 40°C overnight. The extracts were concentrated and dried under reduced pressure and controlled temperature (40°C – 50°C) in a rotary evaporator. The extract was preserved in a refrigerator at 4°C until further use.

### Antioxidant Assays

#### Sample preparation

In these assays Gallic acid was used as control. Gallic acid and extracts kept under 4°C were dissolved in DMSO (10mg/10ml). These samples were further serially diluted to prepare 640, 320, 160, 80, 40, 20 and 10 µg/ml. These dilutions were used for the assays.

#### DPPH Assay

DPPH assay was carried out according to method of Vani *et al.* [17]. In brief, 2 ml of methanol or test solution and 0.5 ml of DPPH solution were incubated at 25°C for 20 min, after which the absorbance was measured at 510 nm.

#### ABTS Assay

The assay was carried out according to Auddy [18]. In brief, 0.2 ml of various concentrations of test solution and 2.30 ml of ABTS radical solution were incubated at room temperature in dark for 16 h. The absorbance is measured at 734 nm.

#### NOS Assay

Nitric oxide scavenging assay was carried out as the method of Sreejayan and Rao [19]. In brief, 50 µl of 10 mM sodium nitroprusside and 50 µl of test solution/positive control of various concentrations are kept at room temperature for 15 min. After incubation, 125 µl of Griess reagent was added and incubated for 10 min at room temperature. The color developed was measured at 546 nm.

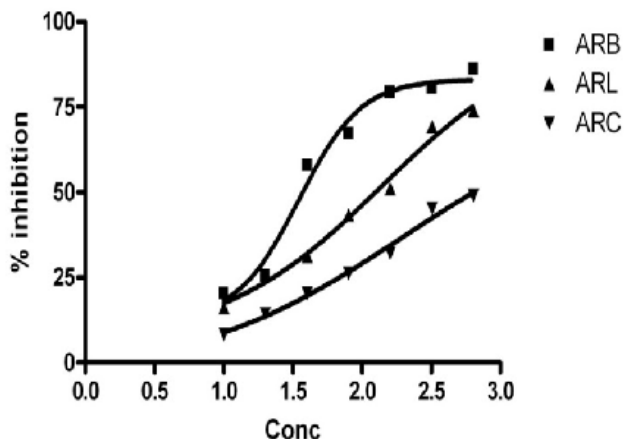
### Calculations and Statistical analysis

The percentage inhibition of all the three assays calculated by using the formula  $C-T/Cx100$ , where, C is absorbance of the control, T is absorbance of the test sample. IC<sub>50</sub> was calculated by using graph pad prism using non-linear regression.

## Results

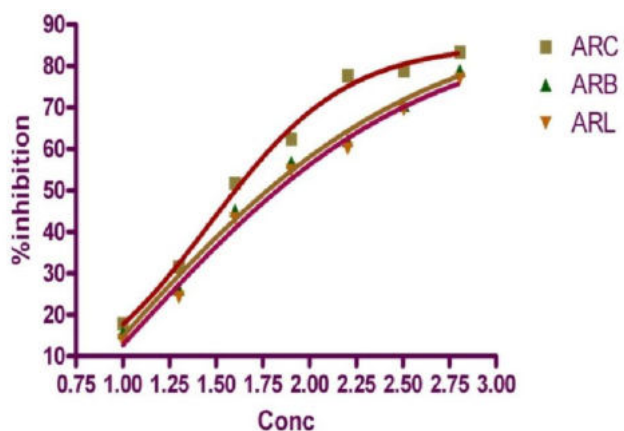
Several concentrations ranging from 10-640 µg/ml of the different solvent (petroleum ether, chloroform and methanol) extracts of stem bark, leaves and callus from *Amoora rohituka* were tested for their antioxidant activity in different *in vitro* models. It was observed that free radicals were scavenged by the test extracts in a concentration dependent manner up to the given concentrations in all the models.

In DPPH assay the maximum IC<sub>50</sub> (Inhibitory Concentration) was shown by methanol extract of stem bark (34.92 µg/ml); next was methanol extract of leaves (138.5 µg/ml) and then methanol extract of callus (198.2 µg/ml) (Figure-1).



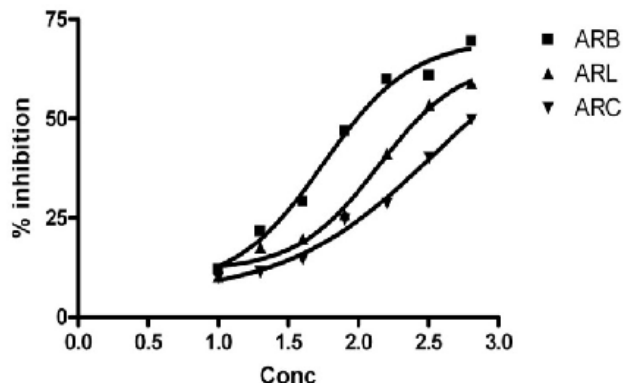
**Figure 1.** IC<sub>50</sub> values of methanol extracts of *Amoora rohituka* for DPPH assay. ARB= *Amoora rohituka* stem Bark (IC<sub>50</sub>= 34.92 µg/ml), ARL= *Amoora rohituka* Leaves (IC<sub>50</sub>= 138.5 µg/ml) and ARC = *Amoora rohituka* callus (IC<sub>50</sub>= 198.2 µg/ml).

In ABTS assay the maximum IC<sub>50</sub> was shown by methanol extract of stem bark (7.53 µg/ml), next was methanol extract of leaves (10.36 µg/ml) and then methanol extract of callus (29.34 µg/ml) (Figure-2).



**Figure.2.** IC<sub>50</sub> values of methanol extracts of *Amoora rohituka* for ABTS assay. ARB= *Amoora rohituka* stem Bark (IC<sub>50</sub>= 7.53 µg/ml), ARL= *Amoora rohituka* Leaves (IC<sub>50</sub>= 10.36 µg/ml) and ARC = *Amoora rohituka* callus (IC<sub>50</sub>= 29.34 µg/ml).

In NOS assay maximum IC<sub>50</sub> was shown by methanol extract of stem bark (56.31 µg/ml), next was methanol extract of leaves (139.7 µg/ml) and then methanol extract of callus (361.3 µg/ml) (Figure-3).



**Figure 3.** IC<sub>50</sub> values of methanol extracts of *Amoora rohituka* for NOS assay. ARB= *Amoora rohituka* stem Bark (IC<sub>50</sub>= 56.31 µg/ml), ARL= *Amoora rohituka* Leaves (IC<sub>50</sub>= 139.7 µg/ml) and ARC = *Amoora rohituka* callus (IC<sub>50</sub>= 361.3 µg/ml).

### Discussion

The present study assess the antioxidant activity of the plant *Amoora rohituka*, and the findings describe that the extracts posses considerable antioxidant property. Since free radicals are involved in a variety of pathological events, free radical scavenging activity has great role in normal biological function. Due to this high reactivity, the Reactive Oxygen Species (ROS) readily combine and oxidize biomolecules.

DPPH is relatively stable free radical. The assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH [20]. According to the studies carried out by Yoshie *et al.* [21], IC<sub>50</sub> value of dulse, *P. palmata*, extract was 12.5 mg/mL and Tepe *et al.* [22] have recorded IC<sub>50</sub> 140, 125, 110 mg/mL for their experiment conducted with various extracts of *Salvia tomentosa* Miller (Lamiaceae). Siriwardhana *et al.* [23] have also reported higher DPPH scavenging activities for a water and methanol extract of *Hizikiatusiformis* (a brown alga), while ethanol, chloroform and ethyl acetate extracts also indicated strong inhibition activities over 50%.

In previous studies, ethyl acetate extract of stem bark of *Amoora rohituka* inhibited the induction of DPPH radicals up to 38% by 30 µg/ml in a dose dependent manner [24]. In the present study, methanol extract of stem bark showed significant activity in DPPH scavenging when compared with other crude extracts.

The ABTS assay is based on the inhibition of the absorbance of the radical cation ABTS<sup>+</sup>, which has a characteristic long wavelength absorption spectrum [25]. The ABTS chemistry involves direct generation of ABTS radical mono cation with no involvement of any intermediary radical. The decolorization of ABTS cation radical is an unambiguous way to measure the antioxidant activity of phenolic compounds. Recently, Awika *et al.* [26] found positive correlations between the determination of phenolic antioxidant using the oxygen radical absorbance capacity (ORAC), ABTS and the 1,1-diphenyl 2-picryl hydrazyl (DPPH) assays. Thus monitoring

the antioxidant activity of phenolic compounds by their ability to scavenge ABTS radical was demonstrated to give good prediction of their ORAC. Jagetia and Venkatesha, [25] showed 59% ABTS<sup>+</sup> radicals were inhibited by 30 µg/ml of ethyl acetate extract of *Amoora rohituka* stem bark. The present study showed significant ABTS scavenging activity in methanol extracts of stem bark, leaves and callus extracts of *Amoora rohituka* when compared with other crude extracts.

Nitric oxide (NO) is a free radical produced in mammalian cells, involved in the regulation of various physiological processes. However, excess production of NO is associated with several diseases [27]. Nitric oxide is a very unstable species under aerobic condition. It reacts with O<sub>2</sub> to produce stable product nitrate and nitrite through intermediates NO<sub>2</sub>, N<sub>2</sub>O<sub>2</sub> and N<sub>3</sub>O<sub>4</sub>. The toxicity and

damage caused by NO and O<sub>2</sub> is multiplied as they react to produce reactive peroxynitrite (ONOO), which leads to serious toxic reactions with biomolecules, like protein, lipids and nucleic acids [28][29][30]. Suppression of NO released may be partially attributed to direct NO scavenging, as methanol extract of *Amoora rohituka* stem bark decreased the amount of nitrite generated from the decomposition of sodium nitroprusside *in vitro*.

These results showed that free radicals scavenging antioxidants are abundantly present in *Amoora rohituka* stem bark, leaves and callus. Further studies have to be conducted to evaluate the exact mode of action of this material and its usefulness as an antioxidant agent.

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