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Antioxidant potential of polar and non-polar extracts of *Aphanamixis polystachya In vitro*

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Abstract

Aphanamixis polystachya (Wall.) Parker, vernacularly called "*Amoora rohituka*" is a medicinal plant belonging to the family of Meliaceae and appearing among Indian natural drugs used as remedy as an astringent and applied on swelling after a fall. In recent times anti carcinogenic ingredients are found from this plant. The search for safe therapeutic molecules suitable for long term use are most needed today to prevent the progression of free radical mediated diseases. Hence, the antioxidant activity of polar and non-polar solvent extracts of the leaf of *Aphanamixis polystachya* was evaluated in a series of *in vitro* assay involving free radicals and reactive oxygen species. The extract exhibited its scavenging effect in concentration dependent manner depending on the solvent extract on superoxide anion radicals, DPPH radical scavenging and property of metal chelating and reducing power. The extract has shown considerably good value for total reducing power, total phenolics, flavanoids and flavanol content

Keywords: Antioxidant activity, *Aphanamixis polystachya*, *Amoora rohituka*, Free radicals.

Introduction

Aphanamixis polystachya (syn. *Amoora rohituka*), is a species of tree in the family Meliaceae. *A.polystachya* is a medicinal tree employed in our indigenous system of medicine as it is known to exhibit laxative, antihelmintic and antiulcer properties. Powdered bark of the plant is effective in hepatomegaly and also used in the treatment of spleen diseases, tumors and abdominal complaints [1]. The plant triterpenic acid, amooranin, extracted from the bark of *A.polystachya* trees, has shown to possess anticancer potential [2]. In earlier reports, amooranin has been shown to induce apoptosis in breast carcinoma through caspase activity [3] and is known to be effective against breast cancer, cervical cancer, colon cancer and leukemia cell panels [4]. Chan et al., [5] have further contributed that *Amoora* extracts have specificity towards targeting only the cancerous cells and do not harm the healthy tissues. Their work has shown that *A.polystachya* possess anticancer potential against two breast cancer (MCF-7 and HTB-126) and three pancreatic cancer (Panc-1, Mia-Paca 2 and Capan-1) cell lines. Chemical investigations on this species growing in different parts of the world has led to the isolation of bioactive compounds which includes alkaloids [6], flavonoids [7], sesquiterpenoids [8], lignan, polystachyol, two lignan glycosides, sterol, stigmasterol, oleic and linoleic acids [9].

So far, *in vitro* and *in vivo* antioxidant studies of the stem bark of *A.polystachya* have been undertaken and shown to exhibit excellent scavenging ability [10, 11]. The present investigation was undertaken to assess the *in vitro* antioxidant activity of the different solvent extracts of the leaf of *A.polystachya* which has not been studied so far. Further research needs to be focused on the isolation and characterization of bioactive molecules responsible for antioxidant activity.

Oxidative stress, defined as an imbalance between free radical production and antioxidant defense system favoring oxidation, plays a significant role in the development of many diseases [12]. Overproduction of the free radicals leads to tissue injury, cancer, aging, heart diseases etc. Antioxidants are substances capable to mop up free radicals and prevent them from causing cell damage. Antioxidants render protective effect by neutralizing free radicals, which are toxic byproducts of natural cell metabolism [13]. The human body naturally produces antioxidants but the process is not 100 per cent effective in case of overwhelming production of free radicals and that effectiveness also declines with age [14]. Hence, it becomes essential to supplement our diet with foods that enhance antioxidant activity. Extensive research shows that phytoconstituents are effective radical scavengers and with cytotoxic studies such compounds can be considered as safe antioxidant supplements for human use. Our study focuses on the isolation of one or more of such bioactive compound with antioxidant ability from the leaf of *A.polystachya*.

Materials and methods

Plant material and Extraction

The leaves of *Aphanamixis polystachya* was collected from Mudigere, Chikamagalur District, Karnataka. Collected samples were identified by consulting taxonomists and the herbaria of the plant was deposited in Herbarium Collection Centre, Department of Studies in Microbiology, University of Mysore and the accession number given to the herbarium specimen being *A.polystachya* (MGMB/001/2011-12). The leaf material was air dried and ground into a fine powder. 100 g of the powder was then extracted with 500 ml methanol, filtered, squeezed off and evaporated under reduced pressure in a rotary evaporator to obtain crude extract.

Free radical scavenging activity

DPPH radical scavenging assay

DPPH is a stable free radical that has been extensively used to determine free-radical scavenging ability of various compounds. It has maximum absorbance at 515 nm. Absorbance decreases when antioxidants donate protons to DPPH [15]. The antioxidant activity using DPPH (1,1-diphenyl-2-picrylhydrazyl) assay was assessed as per the protocol of Sultanova et al, [16]. The reaction mixture contained 300 μM DPPH in methanol in different concentrations of extract. Absorbance at 517 nm was determined after 20 minutes incubation at room temperature and the radical scavenging activity was calculated as a percentage of the radical reduction. The experiment was performed in triplicate and Ascorbic acid was used as a reference compound. The percentage radical scavenging activity was calculated using the formula:

% scavenging activity = [(A of control – A of sample) / A of control] X 100

Reducing power assay

The reducing power of the plant extract was determined by the method of Yen and Chen, [17]. Varying concentration of the plant extract (2 – 10 μg/ml) was mixed with phosphate buffer (0.2 M, pH 6.6) and 1 % of potassium ferricyanide. The mixtures were incubated at 50^0c for 20 minutes. After incubation 10% trichloroacetic acid (TCA) were added to the mixture and centrifuged at 3000g for 10 minutes. The upper layer of the solution was taken and mixed with 0.1% ferric chloride and 1.5 ml distilled water. The absorbance was measured at 700 nm in a spectrophotometer. Butylatedhydroxyanisole (BHA) was used as a positive control.

Metal Chelation assay

The chelating activity of the extracts for ferrous ions Fe^{2+} was measured according to the method of Dinis et al, [18] with slight modifications. To 100 μl of different concentration of the extract, 135 μl distilled water was added along with 2 mM $Fecl_2$ 5 mM ferrozine. The reaction mixture was incubated for 10 minutes at room temperature, the absorbance of the Fe^{2+} - Ferrozine complex was measured at 562 nm. EDTA (Ethylenediaminetetraaceticacid) was used as positive control. The chelating activity of the extract for Fe^{2+} was calculated as

Chelating rate (%) = $(A_0 – A_1) / A_0$ X 100

Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the extract.

Superoxide radical scavenging assay

The scavenging activity of the extract towards superoxide anion radicals was measured by the method of Liu et al., [19]. Superoxide anions were generated in a non-enzymatic phenazine methosulphate – nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). In this experiment, the superoxide anion was generated in 3 ml of Tris-Hcl buffer containing 0.75 ml of NBT, 0.75 ml of NADH solution and 0.3 ml different concentrations of the extract. The reaction was initiated by adding 0.75 ml of PMS to the mixture. After 5 minutes of incubation at room temperature, the absorbance at 560 nm was measured in spectrophotometer. BHA was used as a positive control. The superoxide anion scavenging activity was calculated according to the following equation

% inhibition = $[(A_0 – A_1) / A_0$ X 100]

Where A_0 was the absorbance of the control and A_1 was the absorbance in presence of the extract.

Phytochemicals

Total flavonoid content

Total flavonoid content was determined as described by Sudha et al, [20]. 0.25 ml of extracts was diluted with 1.25 ml of distilled water. 75 μl of 5% $NaNO_2$ solution were added and after 6 minutes 150 μl of 10% $Alcl_3.H_2O$ were added and mixed. After 5 minutes, 0.5 ml of 1 M NaOH was added. The absorbance was measured immediately against the prepared blank at 510 nm. Quercitin was used as a standard and the results were expressed as mg of Quercitin equivalent per gram of dry extract.

Total flavonol content

The total content of flavonol was assessed as described by Miliauskas et al, [21]. To 2 ml of the plant extract, 2 ml of $AlCl_3$ and 6 ml of sodium acetate was added. The reaction mixture was allowed to stand for 2.5 h at 20^0c and absorbance was measured at 510 nm using UV-Vis spectrophotometer. The total flavonol content was determined as mg of Quercitin equivalent per gram of the extract.

Total phenolic content

Total phenol content was determined by the method adapted from Singleton and Rossi, [22] with some modifications using the Folin – Ciocalteu reagent. 200 μl of the extract was mixed with 1.5 ml of Folin-Ciocalteu reagent. After 5 minutes, 1 ml of saturated Na_2CO_3 was added to the mixture. The mixture was incubated for 90

minutes at room temperature in the dark. The absorbance was measured at 725 nm against the blank. The total phenolic content is expressed as mg of gallic acid equivalents (GAE) per gram of dry weight.

Statistical analysis

Results were expressed as mean ± SD. The statistical comparison among the groups were performed with one way ANOVA followed by Tukey's HSD as Post Hoc Test (Whenever F value was significant) using statistical presentation system software version 16.

Results

DPPH radical scavenging ability of the different solvent extracts of *Aphanamixis polystachya* was compared against Ascorbic acid. The assay shows that methanol extract possesses the highest scavenging activity of 76.06% at a concentration of 50 µg/ml (Fig 1).

The reductive ability of the extracts was assessed taking BHA as reference standard. The reducing power of all the extracts increased with increasing extract concentration, indicating that reducing agents were present in these extracts. The methanol extract exhibited the highest reducing capability followed by chloroform, water and hexane extracts (Fig 2).

Our results are indicative that all the extracts have substantial capacity for iron-binding with the methanol extract showing the highest chelation with an IC_{50} value of 92.3 µg/ml followed by chloroform, water and hexane extracts as assessed by the metal chelation assay (Fig 3).

Aphanamixis polystachya showed a significant dose-dependent superoxide anion scavenging activity with the water and methanol extract exhibiting excellent anion scavenging with an IC_{50} value of 752.2 and 760.2 µg/ml respectively (Fig 4).

IC_{50} value is a parameter to measure antioxidant activity. A lower IC_{50} value is suggestive of a higher antioxidant activity. Table 1 shows the IC_{50} value of the different solvent extracts of *Aphanamixis polystachya*. The lowest IC_{50} value was observed in case of methanol extract of *Aphanamixis polystachya*, indicating it to be the most potential solvent for extraction of bioactive molecules with antioxidant activity.

The results hence indicate that the methanol extract of the leaf of *Aphanamixis polystachya* possesses the highest antioxidant activity in comparison with the other solvent extracts. It could be said that methanol was the most effective solvent for the extraction of bioactive antioxidant agents from *Aphanamixis polystachya.* This further suggests that compounds with high polarity were responsible for the potential antioxidant activity of *Aphanamixis polystachya*.

Hence, the methanol extract was evaluated for the concentration of total phenolics, total flavonoids and flavonols. Total phenolic content was found to be 39.5 mg GAE/g extract. The content of total flavonoids and flavonols was found to be 37.5mg and 23 mg GAE/g extract respectively.

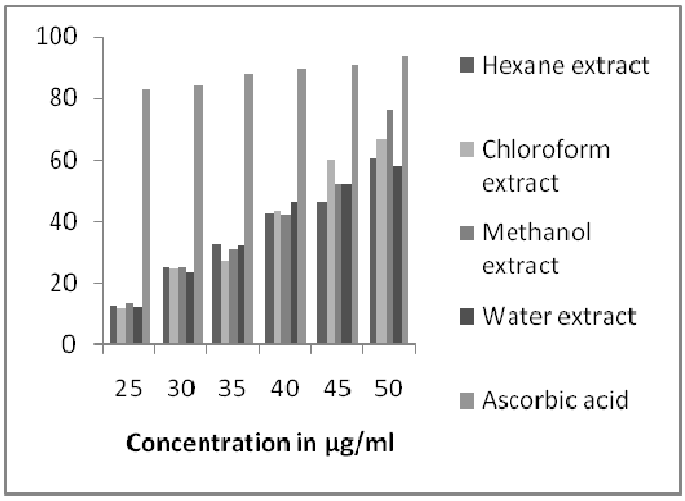

Fig 1. DPPH radical scavenging activity of A.polystachya to that of Ascorbic acid. Each value is expressed as mean ± S.D. (n=3)

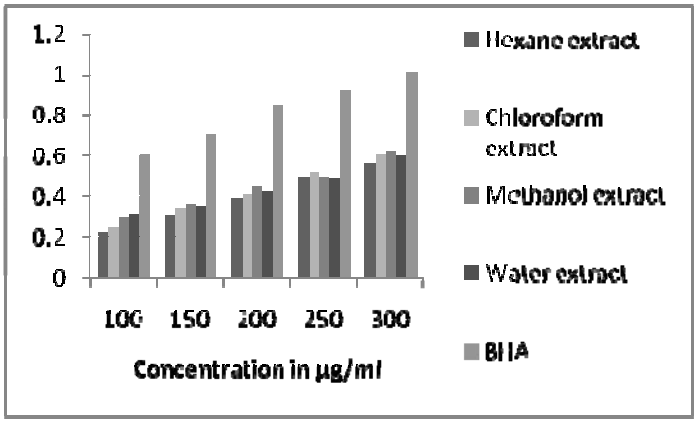

Fig 2. Reducing power of *A.polystachya* to that of BHA.

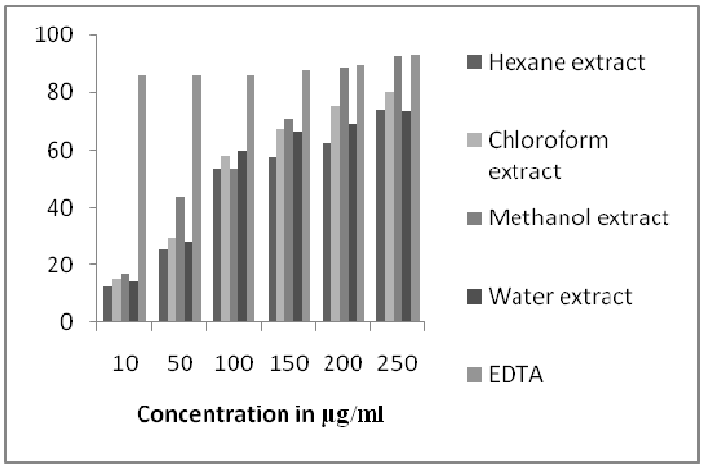

Fig 3. Metal chelation ability of *A.polystachya* to that of EDTA. Each value is expressed as mean ± S.D. (n=3)

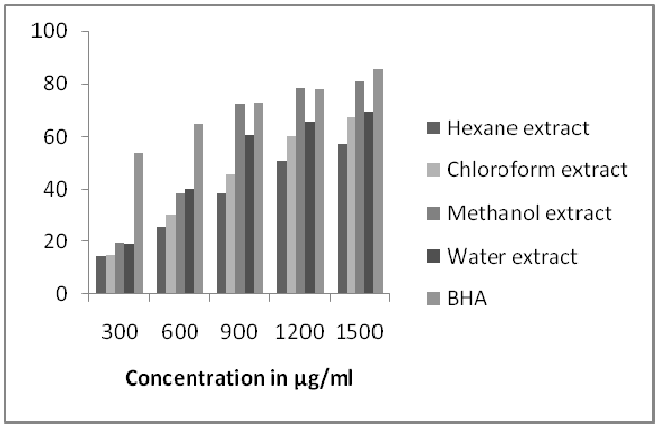

Fig 4. Superoxide anion scavenging assay of *A.polystachya* to that of BHA. Each value is expressed as mean ± S.D. (n=3)

Discussion

The present study investigated the *in vitro* antioxidant efficacy of the different polar and non-polar solvent extracts of the leaves of *A.polystachya*. The plant has exhibited significant antioxidant potency when compared against the standard antioxidant reference compounds such as Vitamin C, BHA etc.

DPPH radical is considered to be a model of lipophilic radical. In this mode, scavenging activity is attributed to hydrogen donating ability of the antioxidants [23]. In the present study, methanol and hexane extract showed significant radical scavenging activity.

The reducing power is indicative of the reducing agent having the availability of atoms which can donate electron and hence react with free radicals and convert it into a much stable form [24]. Going by this, it could be concluded that *A.polystachya* extract in general and methanol extract in particular contains considerable amount of reductants which may react with the free radicals to stabilize and terminate free radical chain reaction.

Chelating agents that form – bonds with metal ions are effective as secondary antioxidants since they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion [25]. The methanol and chloroform extract have shown excellent chelating activity in comparison with the other extracts.

Superoxide anion radical is one of the strongest reactive oxygen species among the free radicals that are generated after oxygen is taken into living cells. Superoxide anion changes to other harmful Reactive Oxygen Species (ROS) and free radicals such as hydrogen peroxide and hydroxyl radical, which induce oxidative damage [26]. The water and methanol extracts have proven to be the best scavengers of superoxide anion radicals.

In the present investigation, we evaluated comparative antioxidant activity of various *A. polystachya* extracts in comparison to Vitamin C, BHA and EDTA. Methanol extract exhibited the best efficacy in all the *in vitro* antioxidant assays carried out. In the longer term, further characterization of the bioactive compounds from *A.polystachya* which has shown appreciable antioxidant activity as identified by the present investigation may prove to be of value for treatment of disorders such as Alzheimer's disease, in which free radical mediated induced tissue damage has been implicated. Increasing knowledge in antioxidant phytoconstituents and including them in daily diet can provide sufficient defense to human body to fight against free radical mediated diseases. The exploration of antioxidant principles from natural resources; identification and isolation of those phytoconstituents are simultaneously presenting enormous scope for their better therapeutic application for treatment of human disease.

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