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Research Article

IN VITRO ANTIOXIDANT ACTIVITY OF LEAVES OF *ANTHOCEPHALUS INDICUS* A. RICH

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ABSTRACT

Objective: Aim of our study is to evaluate the antioxidant activity of aqueous extract of leaves of *Anthocephaus indicus* A. Rich.

Methods: *Anthocephalus indicus* aqueous leaves extract was screened for its antioxidant activity by total antioxidant assay, ferric reducing antioxidant power and nitric oxide radical scavenging activity. TLC bioautography method was used for rapid screening of antioxidant compounds in the extract. Total phenolic and total flavonoid content was estimated by folin-ciocalteau and aluminium chloride method respectively.

Results: Total antioxidant activity of aqueous leaves extract of *Anthocephalus indicus* was found to be 221.03±2.85 mM of ascorbic acid. As the concentration of extract increases the ferric reducing antioxidant power was found to be increased. The IC_{50} value of extract for scavenging of nitric oxide radicals was found to be 0.36 mg/ml of extract. Total phenolic and flavonoid content was found to be 386.59±0.02 and 320±0.02 mg/g dry weight of leaf powder respectively. TLC bioautography of aqueous leaves extract of *Anthocephalus indicus* revealed four intense greenish blue coloured spots.

Conclusion: The results revealed the significant antioxidant activity of aqueous leaves extract of *Anthocephalus indicus*. Thus the aqueous extract of *Anthocephalus indicus* leaves can be used as easily accessible source of natural antioxidants.

Keywords: *Anthocephalus indicus*, Flavonoids, *In vitro* antioxidant activity, TLC-bioautography

INTRODUCTION

Free radicals are the reactive oxygen and nitrogen species produced in the body due to the oxidative stress. These reactive oxygen and nitrogen species (ROS/RNS) are constantly produced by endogenous processes there is always the potential for ROS/RNS-mediated damage to biomolecules. The imbalance in the body's antioxidant defence system is responsible for this oxidative stress and free radical formation [1]. This oxidative stress is linked with various chronic diseases such as cancer, diabetes, aging, inflammation and neurodegenerative disorders [2] .Thus, in biological system there is a need of molecules or compounds that can readily quench or scavenge these free radicals before they can inflict oxidative damage to the body and causes many chronic diseases [3].

Antioxidants are the compounds that protect the cells against the damaging effects of free radicals by scavenging them [4]. Plants are the valuable source of antioxidants because of the presence of many free radicals scavenging phytoconsituents such as phenols, tannins, flavonoids, carotenoids, coumarins, anthocyanin, and glycosides [1]. Many research studies have indicated that the consumption of food beverages rich in phenolic compounds; flavonoids and polyphenols is correlated with reduced risk of diseases related with oxidative damage by free radicals [5]. They possess ideal structural chemistry for free radical scavenging activity [6, 7]. The cyclic structure and conjugated double bond system are expected to be partially stabilised by electron delocalisation, conferring stability to the radical form [8].

Anthocephalus indicus A. Rich. belongs to the family Rubiaceae commonly known as Kadamba. The tree is a medium to large sized deciduous tree attaining a height of 20-40 m and a girth of about 2-2.5 m with clean cylindrical branches and rounded crown. It is frequently found all over the India on the slopes of evergreen forests up to 500 m. Phytoconstituents in the plant consist of indole alkaloids, terpenoids, phenols, saponins, terpenes, steroids, fats and reducing sugars. It is used as herbal remedy that has been mentioned in ancient Indian medical literatures for the treatment of fever, anaemia, diabetes, uterine and liver complaints, menorrhagia, blood and skin diseases, diarrhoea, colitis, stomatitis, dysentery and in improvement of semen quality [9]. Thus, the present study was planned with an objective to determine the antioxidant activity of leaf extract of *Anthocephalus indicus* in relation with its flavonoid content.

MATERIAL AND METHODS

Collection of plant material

The leaves of *Anthocephalus indicus* A. Rich. were collected from the field grown plants found in Kalyan, Mumbai region. The voucher specimen of the plant was authenticated from Blatter Herbarium, Department of Botany, St. Xavier's College, Mumbai. The leaves were washed properly under running tap water, shade dried, powdered and stored in an airtight bottle.

Preparation of Extract

The dried powder of plant leaves were macerated in the mortar and pestle using distilled water as solvent according to the concentration required for each assay. The mixture was allowed to stand at room temperature for overnight. The aqueous extract was obtained by filtering the mixture through Whatman No. 1 filter paper and used for the analysis [10].

Total antioxidant activity

Total antioxidant activity of aqueous leaf extract was determined according to the method of Prieto *et al*. An aliquot of leaf extract of 1.0 ml (1.0 mg/ml) was combined with 1.0 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated in a boiling water bath at 95 °C for 90 min. Then, the samples were cooled to room temperature and the absorbance was measured at 695 nm against blank prepared in the same conditions by replacing sample with 1.0 ml of distilled water. All the analyses were performed in triplicate and the results were averaged. Antioxidant capacity was expressed as Ascorbic acid equivalents (mmol/g) [11].

Ferric reducing antioxidant power

The reducing power of aqueous leaf extract of *Anthocephalus indicus* and Ascorbic acid was determined according to the method of Oyaizu (1986). 200, 400, 600, 800 and 1000 µg/ml of the extracts and 20, 40, 60 , 80 and 100µg/ml of standard (ascorbic acid) was 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 minutes. 2.5 ml of trichloroacetic acid (10%) was added to the 2.5 ml of the reaction mixture, which was then centrifuged at 3000 g for 10 minutes. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and FeCl3 (0.5 mL, 0.1%) and the absorbance was measured at 700 nm against blank prepared in the same conditions by replacing sample with 1.0 ml of distilled water. All the analyses were performed in triplicate and the results were averaged. Increased absorbance of the reaction mixture indicated increasing reducing power [12].

Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity of leaf extract was estimated by Griess Illosvoy reaction. In this study, the Griess-Illosvoy reagent was modified by using naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 1- napthylamine (5%). The reaction mixture (3 ml) containing sodium nitroprusside (10 mM, 2 ml), phosphate buffer saline (0.5 ml) and 1.0 ml of different concentrations of extract (0.025, 0.05, 0.1, 0.2, 0.4 and 0.8 mg/ml) and standard Ascorbic acid (0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 mg/ml) was incubated at 25°C for 150 min. After incubation, 0.5 ml of the reaction mixture was mixed with 1 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for complete diazotization. Then, 1 ml of naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 min at 25°C. A pink colored chromophore was formed in diffused light. The absorbance of these solutions was measured at 540 nm against blank prepared in the same conditions by replacing sample with 1.0 ml of distilled water [13].

Rapid screening of antioxidant compounds by TLC-Bioautography method

Screening of antioxidant compounds in the leaves extract was evaluated using TLC bioautography method. Silica gel G 60 F254 (Merck) was used as a stationery phase. 20 µl of aqueous leaves extract of *Anthocephalus indicus* was applied in band form with a capillary. Plates were developed vertically, in a twin trough chamber previously saturated with mobile phase vapour for 15 min at room temperature. Ethyl acetate: Glacial acetic acid: Formic acid: Water (100:11:11:26) was used as mobile phase [14]. After development the plates were dried at room temperature, a mixture of 1 volume of 1 in 100, 10% ethanol solution of ferric chloride and 1 volume of 1 in 100, 50% ethanol solution of potassium ferricyanide was used as a spraying agent for the detection. Presence of antioxidant compounds were detected by greenish-blue spots against a yellow background [15].

Total flavonoid estimation

Total flavonoid content was measured by aluminium chloride colorimetric assay. 1ml of aqueous leaf extract and different dilution of standard solution of Quercetin (200, 400, 600, 800 and 1000 µg/ml) were added to 10ml volumetric flask containing 4ml of water. To the above mixture, 0.3ml of 5% NaNO2 was added. After 5 minutes, 0.3ml of 10% AlCl3 was added. After 6 min, 2ml of 1 M NaOH was added and the total volume was made up to 10ml with distilled water. Then the solution was mixed well and the absorbance was measured against a freshly prepared reagent blank at 510 nm. Total flavonoid content of the extracts was expressed as mg of Quercetin equivalent per g dry weight of sample [16].

Total phenol estimation

Total phenol was estimated by Standard Folin-Ciocalteau method. 1 ml of Folin-Ciocalteau's reagent, previously diluted (1:20), was added to 1 ml of samples (100µg/ml) and mixed thoroughly. To the mixture, 4 ml of sodium carbonate (75 g/L) and 10 ml of distilled water were added and mixed well. The mixture was allowed to stand for 2 h at room temperature. Contents were then centrifuged at 2000 g for 5 min and the absorbance of the supernatant was taken at 760 nm. A standard curve was obtained using various concentrations of Gallic acid. Results were expressed as Gallic acid equivalents (GAE) per gram of leaf powder [17].

RESULT AND DISCUSSION

Total antioxidant activity

Total antioxidant activity is a quantitative assay, since the antioxidant activity is expressed as the number of equivalents of Ascorbic acid. The assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of a green phosphate/Mo (V) complex at acidic pH with the maximal absorption at 695nm. The linear equation of ascorbic acid for total antioxidant activity was found to be y=2.676x with r^2= 0.9979 (Fig. 1). The antioxidant activity of leaf extract was found to be 221.03±2.85 mM Ascorbic acid/ g dry weight of leaf powder.

Ferric reducing antioxidant power

The reducing ability of a compound generally depends on the presence of reductants which have been exhibited antioxidative potential by breaking the free radical chain, donating a hydrogen atom [18]. The presence of reductants (i.e. antioxidants) in plant extract causes the reduction of the Fe3+/ferricyanide complex to the ferrous form. Therefore, the Fe2+ can be monitored by measuring the formation of Perl's Prussian blue colour at 700 nm. Increase in absorbance at 700 nm reflects an increase in reductive ability [19]. As the concentration of leaf extract of *Anthocephalus indicus* and ascorbic acid increases the ferric reducing antioxidant power increases (Fig. 3). The ferric reducing capacity of leaf extract was compared with standard antioxidant Ascorbic acid. The reducing power of extract of was very potent and it increases as the quantity of sample increases.

Nitric oxide radical scavenging activity

Nitric oxide (NO) is a potent pleiotropic inhibitor of physiological processes such as smooth muscle relaxation, neuronal signalling, and inhibition of platelet aggregation and regulation of cell mediated toxicity [20]. Excess production of these radicals leads to production of peroxynitrite radicals by reacting with oxygen molecules. These peroxynitrite radicals lead to serious toxic reactions with biomolecules and cause renal injury [21]. In the assay sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be determined by the use of the Griess Illosvoy reaction. Nitric oxide radical scavenging activity of the leaf extract is related with the suppression in production of NO radicals due to decomposition of sodium nitroprusside by the extract [20]. The scavenging of NO radicals by the extracts was increased in dose dependent manner. The nitric oxide radical scavenging activity of leaf extract was compared with standard antioxidant ascorbic acid. The IC_{50} value of Ascorbic acid and leaf extract to inhibit the nitric oxide radicals were found to be 0.07 and 0.36 mg/ml respectively. As the concentration of leaf extract of *Anthocephalus indicus* and Ascorbic acid increases the nitric oxide radical scavenging activity increases (Fig. 3)

Rapid screening of antioxidant compounds by TLC-Bioautography method

TLC-bioautography method is used to obtain the preliminary information on the presence of antioxidant compounds in the plant extracts[1]. Screening of antioxidant compounds in the leaves extract was evaluated using TLC-FRAP bioautography method. Spraying the plates with 10% ethanol solution of ferric chloride and 50% ethanol solution of potassium ferricyanide revealed four intense greenish blue coloured spots indicating the presence of number of antioxidants compounds in leaf extract [22] (Fig. 4).

Total phenol and flavonoid estimation

Phenolic and flavonoids comprise the largest group of plants secondary metabolite that have been reported to have multiple biological effects, including antioxidant property [23]. The linear equation of Gallic acid and Quercetin for total flavonoid and phenol content was found to be y=0.0005x with r^2= 0.9983 and y=0.0194x with r^2=0.9989 respectively (Fig. 5 and 6). Total phenol and flavonoid content of the aqueous leaf extract of *Anthocephalus indicus* was found to be 386.59±0.02 and 320±0.02 mg/g dry weight of leaf powder respectively. Due to the presence of high flavonoid content, the leaf extract act as good source of antioxidant agent.

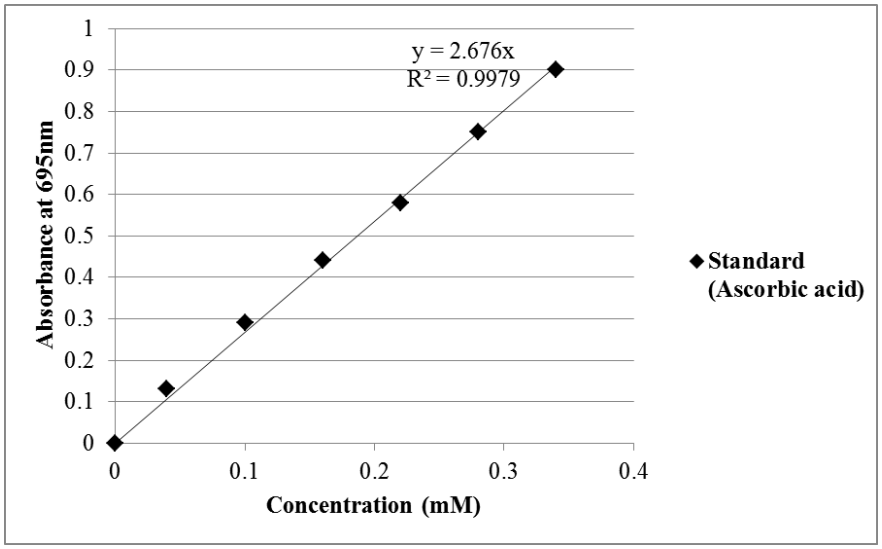

Fig. 1: Standard calibration curve of Ascorbic acid for total antioxidant activity analysis

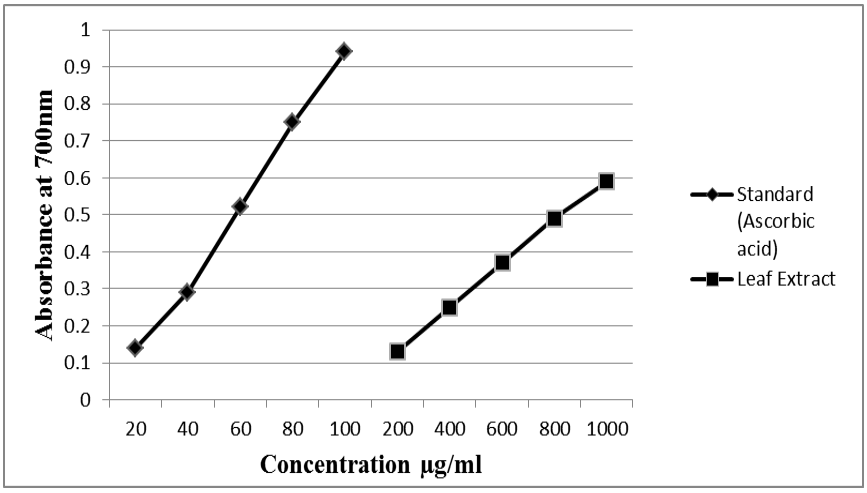

Fig. 2: Ferric reducing antioxidant power of aqueous leaf extract of *Anthocephalus indicus* A. Rich. and Ascorbic acid

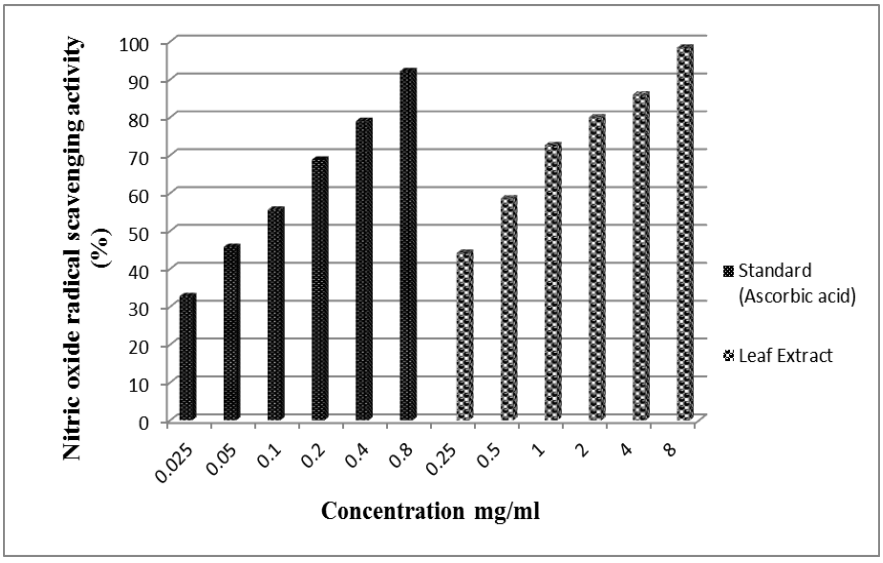

Fig. 3: Nitric oxide radical scavenging activity of aqueous leaf extract of *Anthocephalus indicus* A. Rich. and Ascorbic acid

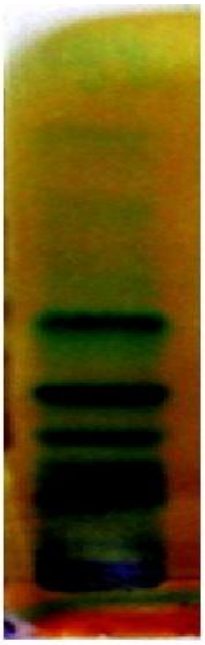

Fig. 4: TLC chromatogram of aqueous extract of *Anthocephalus indicus* A. Rich. leaves for detection of antioxidant compounds

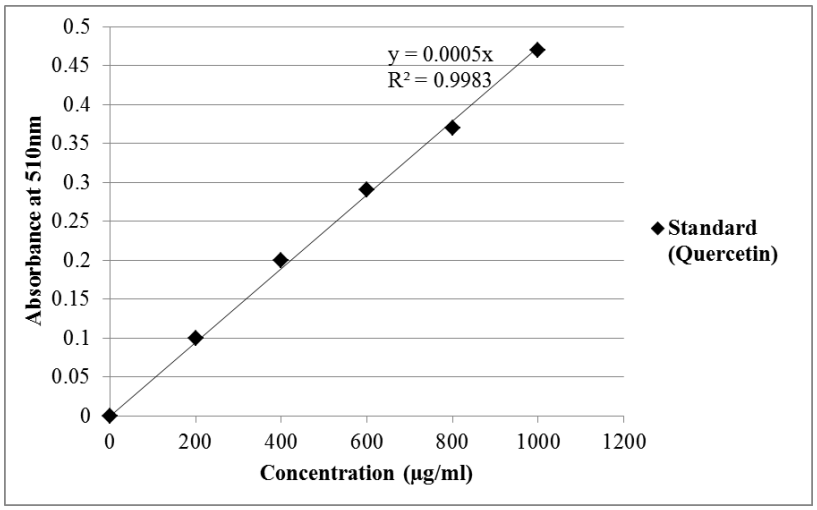

Fig. 5: Standard calibration curve of Quercetin for total flavonoid analysis

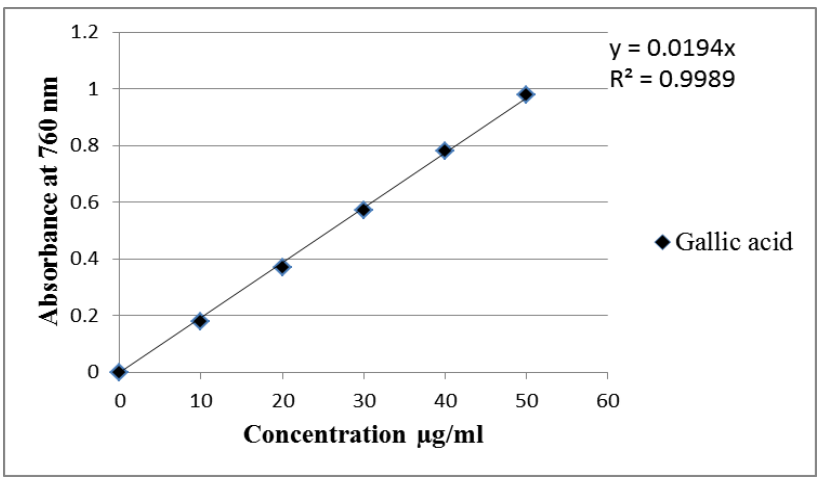

Fig. 6: Standard calibration curve of Gallic acid for total phenol analysis

CONCLUSION

The aqueous extract of *Anthocephalus indicus* leaves showed potent antioxidant activity by total antioxidant assay, by inhibiting nitric oxide radicals and reducing ferric ions. In addition, the extract found to contain a noticeable amount of total phenols and flavonoids, which play a major role in controlling antioxidants. The results of this study show that the aqueous extract of *Anthocephalus indicus* leaves can be used as easily accessible source of natural antioxidants. TLC-bioautography method revealed the presence of four major antioxidant compounds in the extract. Therefore, further works should be performed on the isolation and identification of the antioxidant components in aqueous extract of *Anthocephalus indicus* leaves.

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