ISSN: 2394-1391

Original Article

Indian Journal of Biology
Volume 10 Number 2/July - December 2023
DOI: http://dx.doi.org/10.21088/ijb.2394.1391.10223.2

Antioxidant Activity of Bark and Leaf Extracts of *Anthocephalus cadamba* (Roxb.) Mique using FRAP Assay

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How to cite this article:
Nand Lal/Antioxidant Activity of Bark and Leaf Extracts of *Anthocephalus cadamba* (Roxb.) Mique using FRAP Assay/Indian J Biol 2023; 10(2):69–72.

Abstract

Phytochemicals derived from various herbs hold a promise in treating the oxidative stress in various diseases. The aim of this study was to measure the antioxidant activity of various extracts prepared from the bark and leaves of *Anthocephalus cadamba* (Roxb.) Mique (Rubiaceae) plant. The Ferric reducing ability of plasma (FRAP, also Ferric ion reducing antioxidant power) assay was used to assess the oxidative potential of various extracts. The results show that methanol extracts of the bark and the leaves give the higher TAC FRAP values. The results obtained have the potential of bark and leaf extracts of *A. cadamba* to be exploited pharmacologically in future.

Keywords: Invitro; Antioxidant Activity; Anthocephalus Cadamba; Bark and Leaves.

Introduction

The process of oxidative metabolism is crucial for the survival of most cells. However, the indispensability of oxygen as the element that gives life has a flip side to it. The fame of oxygen (O_2) stems from its capacity to generate free radicals, which, in the majority of instances, are associated with a multitude of negative outcomes. The examples of such reactive oxygen species are highly reactive radicals having surplus of electrons such as hydroxyl electron ($OH^{\cdot}$), the superoxide radical (O_2), the nitric oxide radical ($NO^{\cdot}$) and lipid peroxyl radical ($LOO^{\cdot}$)[1] (Bagchi and Puri, 1998).

Cells are proving to be adversely affected by the reactive oxygen species which may be generated as unwanted products of oxidative metabolism in cells. Cells have evolved a variety of mechanisms to protect themselves from the oxidative stress they may be subjected to either during the course of disease or as a part of their routine metabolism. Some of the most important defences of a cell against the oxidative damage resolve around the antioxidant activities of vitamins such as ascorbic acid, tocopherol, coenzyme Q or glutathione etc. Other vital players in the antioxidant defence of our body are the enzymes superoxide dismutase (SOD), catalase and peroxidase etc.

Plants, being a rich source of antioxidant substances, have been frequently used to repair oxidative damage occurring during the course of various diseases[2] (Singh *et al*., 2000). Plants are a rich source of antioxidants because they possess

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Received date: 18.09.2023

Accepted date: 14.10.2023

numerous phytochemicals which may have the ability to remove or neutralize harmful radicals. Plant metabolites are considered to be as superior to modern medicines because their consumption pre-empts a variety of side effects which are associated with synthetic medicines. This is the reason why natural products are in demand these days, and this demand is growing day by day (Gupta, 1994).

Anthocephalus cadamba is one such herb that has been extensively used in traditional medicine to treat various conditions.

Description of the Plant

The diverse culture of our country is a rich source of traditional medicines, many of which are of plant origin. *Anthocephalus cadamba* (Roxb.) Mique, also known as Kadam or Kadamba, is a tropical tree species that is native to South Asia and Southeast Asia, including Indonesia. In India, it is found on the slopes of evergreen forests up to 500 meters. It is commonly distributed in the sub-himalayan tract from Nepal eastwards on the lower hills of Darjeeling terai in West Bengal; in Chhota Nagpur (Bihar), Orissa, and Andhra Pradesh; in damp places along large streams in the Andamans; in Karnataka and Kerala on the west coast, and at low level in wet places of the western ghats. The bark is gray, smooth in young trees, but rough and longitudinally fissures in old trees. Leaves simple, opposite, elliptic–oblong; flowers in solitary globose head, orange and yellow; fruits are pseudocarps[4,5] (Anonymous, 1985; Naithani and Sahni, 1997).

In folk medicine *Anthocephalus cadamba* is used in the treatment of fever, uterine complaints, blood diseases [6] (Majumdar, 2002), skin diseases[7] (Bhandary *et al*., 1998), eye inflammation and diarrhoea[8] (Pal and Jai, 2000). anaemia, leprosy, dysentery and stomatitis, menorrhagia and in improvement of semen quality[9] (Salkar *et al*.,1992).

Chemical constituents

The major constituents of bark have reported triterpenes, triterpenoids, glycosides, saponins, indole alkaloids, cadamabine, 3 alpha (symbol) dihydrocadambine, isocadambine and isodihydrocadambine[10,11] (Sahua *et al*., 2000; Rastogi *et al*., 1993). Quercetin-3-rhamnoglucoside, kaempferol and chromogenic acid are isolated from the leaves[12] (Kapil *et al*., 1995).

Materials and Methods

Collection of Plant Sample

Fresh bark and leaves of *Anthocephalus cadamba* were collected from the medicinal plants garden, C.S.J.M. University, Kanpur. Collected samples were immediately broken into small fragments in the field to hasten drying and reduce bulks. Packed samples were further chopped to reduce size for drying and then processed for drying under shade. Before further size reduction of the samples by grinding, the crude chopped samples (100-200 gm) were preserved in plastic bags with proper label and kept as voucher samples. They were completely dried and then mounted on a sheet with proper labelling. To convert dried samples into coarse powder, grinding of samples was done with the help of a milling machine. After grinding, the samples were kept in a cool and dry place for further use.

Chemicals

The chemicals used in the experiment were TPTZ (2,4,6–tripyridyl–s–triazine), Ferrous Sulphate and Ferric Chloride for antioxidant estimation were purchased from Sigma Solvents. Hexane, Methanol, Chloroform, Ethyl Acetate used for extraction was purchased from Merck Chemicals Ltd. All chemicals used were of analytical grades.

Method of Extraction

Extraction was done by Soxhlet apparatus using four different solvents in increasing order of their polarity: Hexane, Chloroform, Ethyl Acetate and Methanol. Finally, the aqueous extract was prepared and immediately 5.0 ml methanol was added to avoid fungal growth in aqueous extracts. These extracts were properly weighed, labelled and kept in refrigerator at low temperature for further use in estimating in vitro antioxidant activity.

Method for in vitro Antioxidant Assay

For in vitro antioxidant assays the most widely used assay – Ferric reducing ability of plasma (FRAP) as µmol ferrous ion equivalents was performed for measuring the total antioxidant activity[13] (Benzie and Strain, 1966).

Reagents for FRAP Assay

a. Acetate buffer 300mM, pH 3.6: Weigh 3.1 g Sodium acetate trihydrate and added 16 ml of glacial acetic acid and made the volume to 1.0 L with distilled water.

b. TPTZ (2,4,6–tripyridyl–s–triazine): (MW 312.34), 10 mM in 40 mM HCl (MW 36.46). 0.031 g of TPTZ was added to 10 ml of 40 mM HCl and dissolved at 50 °C.

c. $FeCl_3.6H_2O$: (MW 270.30), 20 mM. 0.054 ***g of*** $FeCl_3$ was dissolved in 10 ml of distilled water.

The usable FRAP reagent was prepared by mixing a, b and c in the ratio of 10:1:1 just before the test. The standard was $FeSO_4.7H_2O$:0.1 – 1.0 mM in methanol.

FRAP Assay Procedure

FRAP solution (3.6 mL) was added to distilled water (0.4 mL), incubated at 37 °C for 5.0 minutes and this solution was mixed with a certain concentration of the plant extract (80 mL) and incubated at 37 °C for 10 minutes. The absorbance of the reaction mixture was measured at 593 nm. For the construction of the calibration curve, five concentrations of $FeSO_4.7H_2O$ (0.1, 0.2, 0.4, 0.6, 0.8 and 10 mM) were used, and the absorbance values were measured for the sample solutions. Table 1 shows the amount of plant extract, distilled water and FRAP reagents required for the assay.

Table 1: The amount of plant extract, distilled water and FRAP reagents taken for the assay

Reagents	Samples	Standards	Blank
Plant extract	20 µL	20 µL ($FeSO_4.7H_2O$)	x
Deionized water	30 µL	30 µL	30 µL
FRAP reagents	3000 µL	3000 µL	3000 µL

Results and Discussion

The results for total antioxidant capacity (TAC) of *Anthocephalus cadamba* bark and leaves by the FRAP method are presented in Table 2. The antioxidant activities were expressed as the concentrations of antioxidant having a ferric reducing ability equivalent to that of 1 mM of $FeSO_4$.

The sensitivity of the method is determined by the strong absorbance of FRAP. After observing the results of FRAP assay between different extracts, the highest TACFRAP value is obtained for methanol extract of leaves of *Anthocephalus cadamba* followed by Methanol extract of bark of *Anthocephalus cadamba* > Chloroform extract of leaves of *Anthocephalus cadamba* > Water extract of leaves of *Anthocephalus cadamba* > Hexane extract of leaves of *Anthocephalus cadamba* > Water extract of bark of *Anthocephalus cadamba* > Ethyl acetate extract of leaves of *Anthocephalus cadamba* > Hexane extract of bark of *Anthocephalus cadamba* > Ethyl acetate extract of bark of *Anthocephalus cadamba* > Chloroform extract of bark of *Anthocephalus cadamba* recorded lowest TACFRAP value.

Despite the fact that *Anthocephalus cadamba* and several other herbs have extensively been used in medicinal preparations of Ayurveda, the biochemical basis of the beneficial effects of such herbs remains unexplored in most of the cases. These necessities the need to identify phytochemicals present in various parts of a medicinal plant and the effects they may have on cells. Such knowledge may be of critical use in developing herbal drugs for ailments.

Table 2: FRAP activity of different extracts of bark and leaves of *Anthocephalus cadamba.*

Sample	0 sec	1 min	2 min	3 min	4 min	5 min	6 min	7 min	8 min	Blank
KBM	0.509	0.538	0.557	0.567	0.576	0.584	0.589	0.595	0.601	0.180
KBH	0.383	0.387	0.391	0.393	0.394	0.397	0.398	0.399	0.401	0.176
KBC	0.384	0.383	0.383	0.384	0.383	0.384	0.387	0.387	0.388	0.176
KBEA	0.395	0.398	0.399	0.402	0.402	0.403	0.406	0.406	0.406	0.178
KBW	0.332	0.334	0.336	0.340	0.344	0.346	0.349	0.352	0.353	0.180
KLM	0.606	0.662	0.691	0.716	0.733	0.748	0.762	0.773	0.784	0.173
KLH	0.353	0.360	0.363	0.367	0.371	0.372	0.373	0.375	0.377	0.174
KLC	0.388	0.406	0.418	0.420	0.423	0.425	0.426	0.428	0.430	0.174
KLEA	0.370	0.376	0.380	0.383	0.384	0.386	0.387	0.389	0.390	0.181
KLW	0.367	0.372	0.375	0.379	0.381	0.384	0.387	0.391	0.393	0.179

KBM - KadambaBark Methanolic extract; KBH - Kadamba Bark Hexane extract; KBC - Kadamba Bark Chloroform extract; KBEA - Kadamba Bark Ethyl Acetate extract; KBW - Kadamba Bark Water extract; KLM - Kadamba Leaf Methanolic extract; KLH - Kadamba Leaf Hexane extract; KLEA - Kadamba Leaf Ethyl Acetate extract; KLW - KadambaLeaf Water extract.

The past few decades have been a burst of knowledge regarding the processes of ROS generations and disposal. Some plant metabolites (e.g. Phenolic compounds and flavonoids) have been shown to be excellent scavengers of ROS produced in vitro such as superoxide radical (O_2), H_2O_2, hydroxyl radical ($OH^{\cdot}$) and singlet oxygen[14] (Rice – Evans *et al.*, 1996).

The FRAP assay, which tests the ability of herbal extracts to reduce ferric ions (Fe^{3+}), has been used to measure the antioxidant potential of various plant extracts. The test relies on changer in absorbance at 593 nm owing to the formation of blue coloured Fe^{2+}/ 2,4,6–tripyridyl–S–triazine from the colourless ferric ions (Fe^{3+}). The FRAP values are early obtained by measuring absorbance and are reproducible. Besides, the reducing power of extracts linearly increases with the concentration of phytochemicals that are implicated in antioxidant activity.

There are many possible ways in which the plant extracts can prevent oxidative damage. These include chelation of metal ions involved in catalysis of oxidation reactions, decomposition of peroxides, scavenging of free radicals and interruption of chain initiation reactions triggered by free radicals.

The in vitro assays performed in this study establish the efficacy of various *A. cadamba* bark and leaf extracts in managing oxidative stress of cells and could serve as free radical inhibitors or scavengers, which may act as primary antioxidants. The fact that methanol extracts of bark and leaves have high antioxidant activity is of great pharmacological interest. Further studies in this line can help in tapping the therapeutic potential of this herb.

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