Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 8, 2014

Original Article

ANTIOXIDANT ACTIVITES AND PHYTOCHEMICAL ANALYSIS OF METHANOL EXTRACT OF LEAVES OF *ARTOCARPUS HETEROPHYLLUS* LAM

N. RAAMAN*, C. SIVARAJ, TENZING

Natural Products & Tissue Culture Laboratory, Centre for Advanced Studies in Botany, University of Madras, Guindy campus, Chennai- 600 025, India.
Email: raaman55@gmail.com

Received: 23 May 2014 Revised and Accepted: 28 June 2014

ABSTRACT

Objective: The aim of the present investigation was to evaluate the phytochemical constituents, free radical scavenging activities and antioxidant properties of the methanol extract of leaves of *Artocarpus heterophyllus*.

Methods: The phytochemicals in the methanol extract of leaves of *A. heterophyllus* were determined qualitatively and quantitatively using standard methods. The antioxidant activities were carried out by DPPH free radical scavenging assay, $OH^{\bullet}$ radical scavenging assay, $NO^{\bullet}$ radical scavenging assay, Fe^{3+}reducing power assay and phosphomolybdenum reduction assay methods.

Results: The methanol extract of leaves of *A. heterophyllus* showed good radical scavenging activities and reducing power activities which were found to increase with the increasing concentration of the extract. The study indicated that the presence of the major phytochemicals *viz.* flavonoids and phenols in the methanol extract of leaves of *A. heterophyllus* were 86.75 mg/g and 524.86 mg/g, respectively.

Conclusion: The present study revealed that the methanol extract of leaves of *A. heterophyllus* showed significant antioxidant activities as well as phenolic content.

Keywords: *Artocarpus heterophyllus*, Phytochemicals, Phenol, Flavonoids, Antioxidant, DPPH, $OH^{\bullet}$, $NO^{\bullet}$, reducing power.

INTRODUCTION

Artocarpus heterophyllus Lam. belongs to the family Moraceae. *Artocarpus heterophyllus* is a monoecious evergreen tree grown in several tropical and subtropical regions. Different parts of this tree have been used for ailments; leaves for stimulating lactation in women and animals, seeds for relieving sick, constipation and diarrhoea and the roots for alleviating asthma and fever [1]. Wood chips yield a dye, which gives orange-red colour to the robes of Buddhist priests. The leaves are also useful to treat fever, ulcers, boil wounds, skin diseases and are antidiarrhoeal, analgesic and immune modulator. The ripe fruits are sweet, cooling, laxative, aphrodisiac and tonic. The plant is known to produce prenyl flavonoids, stilbenes, triterpenes, and sterols. Some of these compounds have exhibited interesting biological activities such as cytotoxicity, antioxidant activity, anti-inflammatory activity and antimalarial activity, inhibition of tyrosinase and melanin biosynthesis and inhibition of 5α-reductase [2]. Hence, in the present investigation, we evaluated the phytochemical constituents, free radical scavenging activities and antioxidant properties of the methanol extract of leaves of *Artocarpus heterophyllus*.

MATERIALS AND METHODS

Chemicals and reagents

All the chemicals used in the study were of analytical grade and procured from Merck India Pvt. Ltd.

Plant material

Leaves of *Artocarpus heterophyllus* were collected from Maduravoyal at Chennai, India. The plant (Fig.1) was authenticated by Prof. Dr. N. Raaman, Director, Centre for Advanced Studies in Botany, University of Madras, Guindy, Chennai, India. Jackfruit is an important tree in home gardens in India, the Philippines, Thailand, Sri Lanka and other regions where Jackfruit is grown commercially and is perhaps the most widespread and economically important *Artocarpus* species. This family encompasses about 1,000 species in 67 genera, mostly tropical shrubs and trees. The tree is monoecious, producing male and female flowers. Stem of this plant is straight and rough whereas bark is green or black, 1.25 cm thick and exudes milky latex; leaves broad obovate, elliptic, decurrent, glabrous, entire; inflorescence solitary axillary, cauliferous and ramflours on short leafy shoots. The evergreen leaves are oblong, oval or elliptic in shape, 10-15 cm in length, alternate, glossy and dark green in colour. The juvenile leaves are lobed. The cut bark of Jackfruit trees produces a milky juice [3]. The tree grows well in equatorial to subtropical maritime climates at elevations of 1–1600 m and average rainfall of 1000–2400 mm.

Fig. 1: Habitat of *Artocarpus heterophyllus*

Preparation of the extract

The collected leaves were subjected to shade drying for 20 days. The dried plant material was powdered mechanically and stored in air tight container for further analysis. The powdered leaves of *A. heterophyllus* were extracted with methanol and concentrated at room temperature. The extract obtained was stored in a refrigerator for further analysis.

Qualitative phytochemical screening

Chemical tests for screening and identification of bioactive chemical constituents present in the methanol extract of leaves of *Artocarpus heterophyllus* were carried out using the standard procedures [4-6].

Quantitative phytochemical estimation

Estimation of total phenol by Folin-Ciocalteu reagent method

Folin-ciocalteu method was used to determine the total phenolic compounds [7] with slight modifications. Methanol extract (0.05 mL) of leaves of *A. heterophyllus* was mixed with 0.5 mL of Folin Ciocalteu reagent (1:10 diluted with distilled water). After 5 min, 1 mL of aqueous Na_2CO_3 (20%) was added. The mixture was then allowed to stand for 30 min incubation in dark. The quantification of phenolic compounds was performed spectrophotometrically by measuring the absorbance in UV-VIS spectrophotometer at 765 nm. The total phenolic content was expressed in terms of Gallic acid equivalent (mg/g of dry mass), which is a common reference compound.

Estimation of total flavonoids by $AlCl_3$ method

Aluminium chloride colorimetric method was used to determine the total flavonoids [8] with slight modifications. Methanol extract (0.1 mL) of leaves of *A. heterophyllus* was mixed with 0.5 mL of 10 % aluminium chloride, 0.5 mL of 1 M potassium acetate and 0.5 mL of distilled water. It was incubated at room temperature for 30 min. The absorbance of the reaction mixture was measured by spectrophotometer at 415 nm. The total flavonoid content was expressed in terms of quercetin equivalent, which is a common reference standard.

***In vitro* antioxidant assay**

DPPH radical scavenging assay

The antioxidant activity of methanol extract of leaves of *A. heterophyllus* was measured on the basis of the scavenging activity of the stable 1, 1- diphenyl 2-picrylhydrazyl (DPPH) free radical with slight modifications [9, 10]. One mL of 0.1 mM DPPH solution in methanol was mixed with 1 mL of various concentrations (10-70 µg/mL) of methanol extract of leaves of *A. heterophyllus*. The mixture was then allowed to stand for 30 min incubation in dark. Ascorbic acid was used as the reference standard. Mixer of 1 mL methanol and 1 mL DPPH solution was used as the control. The decrease in absorbance was measured using UV-Vis spectrophotometer at 517 nm. The percentage of inhibition was calculated using the following formula

$$\% \text{ of DPPH radical inhibition} = \frac{\text{Control - Sample}}{\text{Control}} \times 100$$

Hydroxyl radical ($OH^{\bullet}$) scavenging activity

Various concentrations (2-14 µg/mL) of methanol extract (1 mL) of leaves of *A. heterophyllus* were added with 1.0 mL of iron-EDTA solution (0.13% ferrous ammonium sulphate and 0.26% EDTA), 0.5 mL of EDTA solution (0.018%), and 1.0 mL of dimethyl sulphoxide (DMSO) (0.85% v/v in 0.1 M phosphate buffer, pH 7.4). The reaction was initiated by adding 0.5 mL of ascorbic acid (0.22%) and incubated at 80-90°C for 15 min in a water bath. After incubation, the reaction was terminated by the addition of 1.0 mL of-ice cold TCA (17.5% w/v). An amount of 0.5 mL of Nash reagent (75.0 g of ammonium acetate, 3.0 mL of glacial acetic acid, and 2 mL of acetyl acetone were mixed and made up to 1 L with distilled water) was added and left at room temperature for 15 min. The reaction mixture without sample was used as control. The intensity of the colour formed was measured spectroscopically at 412 nm. Ascorbic acid was used as the reference standard. The percentage of inhibition was calculated using the following formula

$$\% \text{ of } OH^{\bullet} \text{ radical inhibition} = \frac{\text{Control - Sample}}{\text{Control}} \times 100$$

Nitric oxide radical scavenging assay

Different concentrations (2-14 µg/mL) of methanol extract (1 mL) of leaves of *A. heterophyllus* were mixed with 360 µL of sodium nitroprusside (5 mM) in phosphate buffer (pH 7.4, 0.1 M). Then, 216 µL of Greiss reagent [1% sulphanilamide, 2% *o*-phosphoric acid and 0.1% of N-(1- naphthyl) ethylenediamine dihydrochloride] was added and the tubes were incubated at 25°C for 1 h. After incubation, 1 mL of water was added. Control tube was maintained with all chemicals excluding *A. heterophyllus* extract. The absorbance of the chromophore formed was read at 546 nm. The percentage of NO radical scavenging activity was calculated using the following formula

$$\% \text{ of } NO^{\bullet} \text{ radical inhibition} = \frac{\text{Control - Sample}}{\text{Control}} \times 100$$

Ferric (Fe^{3+}) reducing power assay

The reducing power of methanol extract of leaves of *A. heterophyllus* was determined by slightly modified method of Oyaizu [11]. One mL of each plant extract of different concentrations (10 - 70 µg/mL) was mixed with phosphate buffer (1 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (1 mL, 1 %). The mixtures were then incubated at 50°C for 20 min. One mL of trichloroacetic acid (10 %) was added to each mixture, which were then centrifuged for 10 min at 1036 x g. The upper layer of the solutions (1 mL) were mixed separately with distilled water (1 mL) and $FeCl_3$ (0.5 mL, 0.1 %), and the absorbances were measured at 700 nm using a spectrophotometer. Ascorbic acid was used as the standard reference.

Phosphomolybdenum reduction assay

The antioxidant capacity of the methanol extract of leaves of *A. heterophyllus* was assessed as described by Prieto *et al*. [12]. The methanol extract of leaves of *A. heterophyllus* in dilution from 10 to 70 µg/mL was combined with reagent solution containing ammonium molybdate (4 mM), sodium phosphate (28 mM) and sulphuric acid (600 mM). The reaction mixture was incubated in a water bath at 90°C for 90 min. The absorbance of the coloured complex was measured at 695 nm. Ascorbic acid was used as standard reference. Increased absorbance of the reaction mixture indicates the increase in phosphomolybdenum reduction.

RESULTS AND DISCUSSION

The methanol extract of leaves of *A. heterophyllus* was taken for various qualitative phytochemical tests and it showed the presence of various phytoconstituents such as terpenoids, flavonoids, steroids, phenolic compounds, glycosides, carbohydrates and saponins as depicted in Table 1.

Table 1: Qualitative phytochemical screening of methanol extract of leaves of *Artocarpus heterophyllus*

S. No.	Phytoconstituents	Chemical reagents	Results
1.	Terpenoids	$CHCl_3$ + conc. H_2SO_4	++
2.	Flavonoids	NaOH solution	+
3.	Phenols	$FeCl_3$ solution	+
4.	Steroids	Acetic anhydride + conc. H_2SO_4	+
5.	Glycosides	5% NaOH + Fehling's solution	+
6.	Carbohydrates	α-Naphthol + conc. H_2SO_4	+
7.	Saponins	Foam test	+

+ Present ++ Strongly present

The total phenolic content (estimated by Folin-Ciocalteu method) in the methanol extract of leaves of *A. heterophyllus* was 524.86 mg/g and the total flavonoid content (estimated by $AlCl_3$ method) was 86.75 mg/g (Table 2). Free radicals are produced under certain

environmental conditions and during normal cellular function in the body; these molecules are missing in an electron, giving them an electric charge. To neutralize this charge, free radicals try to withdraw an electron from, or donate an electron to a neighbouring molecule. The newly create free radical, in turn, looks out for another molecule and withdraws or donates an electron, setting off a chain reaction that can damage hundred of molecules. Antioxidants such as phenolic compounds and flavonoids halt this chain reaction. These antioxidants are themselves free radical, donating electrons to stabilize and neutralize the dangerous free radicals. The phenolic compounds and flavonoids present in the methanol extract of leaves of *A. heterophyllus* work against the molecules that form free radicals, destroying them before they can begin the domino effect that leads to oxidative damage [13].

Table 2: Quantitative phytochemical estimation of phenols and flavonoids of methanol extract of leaves of *Artocarpus heterophyllus*

S. No.	Component	Quantity (mg/g of extract)
1.	Phenols	524.86
2.	Flavonoids	86.75

DPPH is a stable free radical, usually used as a substrate to evaluate antioxidant activity. DPPH assay is based on the measurement of the scavenging ability of antioxidants present in the methanol extract of leaves of *A. heterophyllus* towards the DPPH free radical. The method is based on the reduction of purple colored methanol solution of DPPH radical in the presence of methanol extract of leaves of *A. heterophyllus*, which is having hydrogen donating antioxidants, by the formation of yellow colored non radical form of DPPH. Lower absorbance indicates higher DPPH free radical scavenging activity [14]. The methanol extract of leaves of *A. heterophyllus* was able to reduce purple colored 1,1-diphenyl-2-picryl hydrazyl (DPPH) to yellow colored 1,1-diphenyl-2-picryl hydrazine. The maximum DPPH radical scavenging activity was 63.97% of inhibition at 70 µg/mL [Table 3]. The IC_{50} of methanol extract of leaves of *A. heterophyllus* was 42.59 µg/mL while that of the standard ascorbic acid was 8.25 µg/mL [Table 4].

Table 3: DPPH radical scavenging activity of methanol extract of leaves of *A. heterophyllus*

S. No.	Concentration (µg/mL)	% of inhibition
1	10	21.05 ± 1.47
2	20	29.15 ± 2.04
3	30	38.05 ± 2.66
4	40	46.96 ±3.28
5	50	57.49 ± 4.02
6	60	61.54 ± 4.30
7	70	63.97 ± 4.47

Table 4: DPPH, $OH^{\cdot}$ and $NO^{\cdot}$ radical scavenging activity of standard (Ascorbic acid)

S. No.	Concentration (µg/mL)	% of inhibition		
		DPPH	**$OH^{\cdot}$**	**$NO^{\cdot}$**
1	2	11.98 ± 0.83	22.00 ± 1.54	27.14 ± 1.89
2	4	17.70 ± 1.23	36.04 ± 2.52	35.26 ± 2.46
3	6	25.48 ± 1.78	49.14 ± 3.43	48.00 ± 3.36
4	8	37.25 ± 2.60	56.89 ± 3.98	54.28 ± 3.79
5	10	60.62 ± 4.24	62.24 ± 4.35	60.00 ± 4.20
6	12	70.95 ± 4.96	74.23 ± 5.19	65.85 ± 4.60
7	14	79.28 ± 5.54	87.67 ± 6.13	69.14 ± 4.83

The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells [15]. This radical has the capacity to join nucleotides in DNA and cause strand breakage, which contributes to carcinogenesis, mutagenesis, and cytotoxicity. In addition, this species is considered to be one of the quick initiators of the lipid peroxidation process, abstracting hydrogen atoms from unsaturated fatty acids.

Hydroxyl radical scavenging activity was estimated by generating the hydroxyl radicals using ascorbic acid-iron EDTA. The hydroxyl radicals formed by the oxidation react with dimethyl sulfoxide (DMSO) to yield formaldehyde, which provides a convenient method to detect hydroxyl radicals by treatment with Nash reagent. The maximum $OH^{\cdot}$ radical scavenging activity was 51.60 % of inhibition at 14 µg/mL concentration [Table 5]. The IC_{50} of methanol extract of leaves of *A. heterophyllus* was 13.57 µg/mL, whereas it was 6.11 µg/mL for the standard ascorbic acid [Table 4].

Table 5: Hydroxyl ($OH^{\cdot}$) and nitric oxide ($NO^{\cdot}$) radical scavenging activities of methanol extract of leaves of *A. heterophyllus*

S. No.	Concentration (µg/mL)	% of inhibition	
		$OH^{\cdot}$	**$NO^{\cdot}$**
1	2	5.91 ± 0.41	10.87 ± 0.76
2	4	11.26 ± 0.78	33.69 ± 2.35
3	6	23.49 ± 1.64	43.48 ± 3.04
4	8	25.34 ± 1.77	55.43 ± 3.88
5	10	30.61 ± 2.14	60.87 ± 4.26
6	12	46.14 ± 3.22	65.22 ± 4.56
7	14	51.60 ± 3.61	68.48 ± 4.79

Nitric oxide is implicated in diseases such as cancer and inflammation [16]. It also mediates smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated cytotoxicity [17]. Sodium nitroprusside spontaneously generates nitric oxide at physiological pH, in aqueous solutions. The nitric oxide generated is converted into nitric and nitrous acids on contact with dissolved oxygen and water. The liberated nitrous acid was estimated using a modified Griess-Illosvoy method. Nitrous acid reacts with Griess reagent to form a purple azo dye. In the presence of antioxidants, the amount of nitrous acid will decrease and the degree of decrease in the formation of purple azo dye will reflect the extent of scavenging. The maximum NO• radical scavenging activity showed was 68.48% of inhibition at 14 µg/mL concentration [Table 5]. The methanol extract of leaves of *A. heterophyllus* showed significant nitric oxide radical scavenging effect with the IC_{50} of 7.21 µg/mL, whereas that of standard ascorbic acid was 6.25 µg/mL [Table 4]. The reducing properties are generally associated with the presence of reductones such as flavonoids and phenolic compounds, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom [18]. Reductones are also reported to react directly with peroxides and also with certain precursors of peroxides, thus preventing peroxide formation [19]. In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of methanol extract of leaves of *A. heterophyllus*. Presence of reducers causes the conversion of the Fe^{3+}/ferricyanide complex to ferrous form.

Fe^{+3} reduction is often used as an indicator of electron- donating activity, which is an important mechanism of phenolic antioxidant action, and can be strongly correlated with other antioxidant properties [20]. By measuring the formation of Pearl's Prussian blue at 700 nm, it is possible to determine the concentration of ferrous ions. Increase in absorbance of the reaction mixture indicated increase in reducing power (0.221) of the extract which is shown in Table 6. The methanol extract of leaves of *A. heterophyllus* showed good ferric reducing power (0.221) at 70 µg/mL concentration, when compared with the standard (0.308) ascorbic acid [Table 7].

Table 6: Ferric (Fe^{3+}) reducing power and phosphomolybdenum reduction activities of methanol extract of leaves of *A. heterophyllus*

S. No.	Concentration (µg/mL)	Absorbance	
		Fe^{3+} reducing power (700 nm)	Phosphomolybdenum reduction (695 nm)
1	10	0.057 ± 0.003	0.002 ± 0.000
2	20	0.122 ± 0.008	0.005 ± 0.000
3	30	0.168 ± 0.011	0.008 ± 0.001
4	40	0.180 ± 0.012	0.010 ± 0.001
5	50	0.185 ± 0.012	0.012 ± 0.001
6	60	0.195 ± 0.013	0.015 ± 0.001
7	70	0.221 ± 0.015	0.027 ±0.002

Table 7: Ferric (Fe^{3+}) reducing power and phosphomolybdenum reduction of standard (Ascorbic acid)

S. No.	Concentration (µg/mL)	Absorbance	
		Fe^{3+} reducing power (700 nm)	Phosphomolybdenum reduction (695 nm)
1	10	0.154 ± 0.011	0.130 ± 0.009
2	20	0.189 ± 0.013	0.159 ± 0.011
3	30	0.209 ± 0.015	0.172 ± 0.012
4	40	0.214 ± 0.015	0.205 ± 0.014
5	50	0.247 ± 0.017	0.317 ± 0.022
6	60	0.289 ± 0.020	0.359 ± 0.025
7	70	0.308 ± 0.022	0.398 ± 0.028

Phosphomolybdenum assay revealed the reduction of MO (VI) to MO (V) by the the methanol extract of leaves of *A. heterophyllus* and formation of a MO (V) complex at acidic pH. Increase in absorbance of the reaction mixture indicates increase in reducing power [21].

The significant increase in absorbance of extract was found to be 0.027 [Table 6], which was compared with the standard (0.398) ascorbic acid [Table 7]. The result obtained was confirmed by the high potency of the methanol extract of leaves of *A. heterophyllus* towards the reduction of transition metal ions. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants.

CONCLUSION

The results of the present work indicated that the methanol extract of leaves of *A. heterophyllus* is a potential source of natural antioxidants and could dose-dependently and significantly inhibit free radicals. The difference in the antioxidant activity may be ascribed to their different group of phenolic and flavonoids compounds. The methanol extract of leaves of *A. heterophyllus which* showed higher phenolic content contributes to the higher antioxidant activity. Based on the results obtained, it can be concluded that the plant contains essential phytochemical constituents and possesses active antioxidant property. Further investigations on the isolation of the active component of the extract will throw more information on the mechanism of action.

CONFLICT OF INTERESTS

Declared None

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