

## PHYTOCHEMICAL ANALYSIS AND ANTIOXIDANT ACTIVITY OF *BARRINGTONIA ACUTANGULA* (L.) GAERTN. LEAVES

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### ABSTRACT

The proposed study on the potential valued bioactive benefits of *Barringtonia acutangula* leaf extracts, on the basis of phytochemical characteristics has explored their untapped properties. *Barringtonia acutangula* has exhibited its synergistic antioxidant activity, which is evidenced by some useful estimation like phenolic, flavonoid, tannin, flavonol, protein, carbohydrate and Vitamin C contents. For all these estimations methanol extract has exhibited significant effects compared to chloroform and petroleum ether extracts. *In vitro* assays like DPPH - radical scavenging and reducing power activity revealed the promising potential in methanol extract. IC<sub>50</sub> and EC<sub>50</sub> values in methanol extract for the above assays were 0.15 and 0.11 mg. Significant contents for phenolic, flavonoid, flavonol and tannin were 79.71, 109.52, 91.18 and 105.52 µg respectively. Hence, this piece of work acclaims the therapeutic potential of *Barringtonia acutangula* leaf extract indicating its pharmacological applications.

**Keywords:** *Barringtonia acutangula*, Phytochemical, IC<sub>50</sub> and EC<sub>50</sub>, DPPH, *In Vitro*.

### INTRODUCTION

Plants have been associated with the human health from very ancient and they are the important sources of medicines. Medicinal plants possess secondary metabolites like alkaloids, glycosides, steroids and flavonoids which are the important sources of drugs. Approximately one third of all pharmaceuticals are of plant origin<sup>1</sup>. In aerobic environment all animals and plants require oxygen and hence the reactive oxygen species are ubiquitously present. It is well that excess generation of ROS is involved in the structural alterations of cellular molecules leading to cytotoxicity and cell death<sup>2</sup>. Antioxidants are the substances used by the body to protect itself from the damage caused by oxidation. Oxidation reactions can produce free radicals, which can start chain reactions that damage cells of big molecules i.e. protein, lipids etc., Excess of free radicals has been linked to many diseases, such as heart diseases<sup>3</sup>, liver diseases, cancers etc<sup>4</sup>. Hence the research has been focussed on the use of a natural antioxidant, which may inhibit reactive oxygen species and may display protective effects. Plant phenolics, in particular phenolic acids, flavonoids are known to be potent antioxidants that occur in vegetables, flowers, fruits, nuts, seeds, roots and barks<sup>5</sup>. In the past few years the suspected toxicity of some synthetic compounds used in food has increased interest in natural products<sup>6</sup>. Some industries such as those related to food additive production, cosmetics and pharmaceuticals have raised their efforts in preparing bioactive compounds from natural products by extraction and purification.

*Barringtonia acutangula* an evergreen tree of moderate size is called as Hijja or Hijjala in Sanskrit. The fruit is spoken of as samudra-phala and various part of this plant used as a folklore medicine for curing various ailments like hemiplegia, pain in joints, eye diseases, stomach disorders, anthelmintic, diarrhoea, cough, dyspnoea, leprosy, intermittent fever, and spleenic disorders<sup>7</sup>. An aqueous extract of the bark is found hypoglycemic and is reported to be used in pneumonia, diarrhea, asthma and leaf juice is given for diarrhea<sup>8</sup>. Fruit is bitter, acrid, anthelmintic, emetic, expectorant and vulnerary. It is prescribed in gingivitis, as an astringent and tonic. Whole plant was reported to possess flavonols, phenolic acids, triterpenoids, tannins and steroidal compounds such as barringtogenic acid, tanguic acid and acutangulic acids. The fruit possessed saponins based on barringtogenol B, C and D<sup>9</sup>. The therapeutic potential of this plant were reported to be antitumor<sup>10</sup>, antibiotic<sup>11</sup>, inhibit growth of *Helicobacter pylori*<sup>12</sup> and antifungal activities<sup>13,14</sup>. Objective of the present study is set to investigate the antioxidant propensities of *Barringtonia acutangula* leaves in various extracts. Further, their carbohydrate, protein and vitamin C contents were assessed which would additionally ascribe their positive utility.

### MATERIALS AND METHODS

#### Chemicals

Folin-Ciocalteu's reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), Gallic acid, (±)-Catechin, Rutin, α-Tocopherol, Ascorbic acid, Bovine serum albumin, Glucose, Tannic acid, solvents and other reagents used were of analytical grade purchased from Sigma Chemical Co. (St. Louis, MO, USA).

#### Plant collection and extraction

Healthy *Barringtonia acutangula* leaves were collected from Kunnathur village, Paramathi velur, Namakkal district, Tamil Nadu State, India. The plant materials were identified and confirmed by Botanical Survey of India (BSI), Coimbatore, Tamil Nadu, India. The voucher specimen number (BSI/SRC/5/23/10-11/Tech.1788).

The plant materials were washed with tap water, prior to distilled water, shade dried and powdered. The powdered plant materials were subjected to successive extraction with petroleum ether, chloroform and methanol using 'Soxhlet Extractor'. The extracts were dried in vacuum pump at 40°C. The dried crude extracts were stored in freezer at 0°C for future use.

#### Phytochemical screening

The preliminary phytochemical screening tests were carried out to identify the useful constituents by standard methods<sup>15</sup>.

#### Determination of total phenolic contents

The total phenolics in the extracts were estimated by spectrophotometric assay<sup>16</sup>. One mL of sample (concentration 1 mg/mL) was mixed with 1 mL of Folin and Ciocalteu's phenol reagent. After 3 min, 1 mL of saturated sodium carbonate solution was added to the mixture and it was adjusted to 10 mL with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm. Gallic acid was used for constructing the standard curve (20-100 µg/mL, Y=0.094x+0.0117, R<sup>2</sup>= 0.9673) and the results were expressed as µg of gallic acid equivalents/mg of extract (GAEs).

#### Determination of total flavonoid contents

Flavonoid contents in the extracts were determined by spectrophotometric method<sup>16</sup>. The (250 µL) extract (concentration 1 mg/mL) was mixed with 1.25 mL of distilled water and 75 µL of a 5% NaNO<sub>2</sub> solution. After 5 min, 150 µL of 10% AlCl<sub>3</sub> solution was added. After 6 min, 500 µL of 1 M NaOH and 275 µL of distilled water were added to prepare the mixture. The solution was mixed well and the absorbance was read at 510 nm. (±)-Catechin was used to

calculate the standard curve (20-120 µg/mL,  $Y=0.0085x+0.0375$ ,  $R^2=0.999$ ) and the results were expressed as µg of (±)-catechin equivalents (CEs) per mg of extract.

#### Estimation of total flavonol contents

One mL of leaf extract (concentration 1 mg/mL) was mixed with 1 mL aluminium trichloride (5 mg/mL) and 3 mL sodium acetate (25 mg/mL). The absorbance read at 440 nm was read after 2.5 h. The absorption of standard rutin solution (0.5 mg/mL) in methanol was measured under the same conditions<sup>17</sup>. All determinations were carried out in duplicates. The amount of flavonols in plant extracts in rutin equivalents (RE) were calculated by the following formula.  $X = (A_0 m_0) / (A_m m)$ , where X is the flavonol content, mg/mg plant extract in RE, A is the absorption of plant extract solution,  $A_0$  is the absorption of standard rutin solution, m is the weight of plant extract (mg),  $m_0$  is the weight of rutin in the solution (mg).

#### Estimation of tannins content

Tannin content of the extracts was measured by Folin-Denis method<sup>18</sup>. The various extracts (50 µL) were made upto 7.5 mL by adding double distilled water. Then 0.5 mL Folin-Denis reagent and 1 mL of  $Na_2CO_3$  were mixed with it. Again volume was made upto 10 mL by double distilled water. Absorption was recorded at 700 nm. Tannic acid was used to calculate the standard curve (20-120 µg / mL,  $Y=0.0069x+0.0091$ ,  $R^2=0.9985$ ) and the results were expressed as µg of tannic acid equivalents per mg of extract.

#### DPPH Radical Scavenging Activity

Various concentrations of *Barringtonia acutangula* leaf extracts (0.3 mL) were mixed with 2.7 mL of methanol solution containing DPPH radicals ( $6 \times 10^{-5}$  mol/L). The mixture was shaken vigorously and allowed to stand for 60 min in the dark. The reduction of the DPPH radical was determined by reading the absorbance at 517 nm<sup>19</sup>. The radical-scavenging activity (RSA) was calculated as a percentage of DPPH discoloration, using the equation:  $\%RSA = [(A_{DPPH} - A_s) / A_{DPPH}] \times 100$ , where  $A_s$  is the absorbance of the solution when the sample extract is added at a particular level and  $A_{DPPH}$  is the absorbance of the DPPH solution. The extract concentration providing 50% of radical-scavenging activity ( $IC_{50}$ ) was calculated from the graph of RSA percentage against extract concentration. Ascorbic acid and  $\alpha$ -tocopherol were used as standards.

#### Reducing power

The reducing power of *Barringtonia acutangula* leaf extracts was determined<sup>18</sup>. Various concentration of different solvent extract (1 mL), phosphate buffer (1 mL, 0.2M,  $p^H=6.6$ ) and potassium

ferricyanide (1 mL, 10 mg/mL) were mixed together and incubated at 50°C for 20 min. Trichloroacetic acid (1 mL, 100 mg/mL) was added to mixture and centrifuged at 8,000 rpm for 5 min. The supernatant (1 mL) was mixed with distilled water (1 mL) and ferric chloride (0.1 mL, 1 mg/mL) and then the absorbance was measured at 700 nm.

#### Estimation of carbohydrate content

Total carbohydrate contents were estimated by Anthrone method<sup>20</sup>. Glucose was used to calculate the standard curve (20-120 µg/mL,  $Y=0.0263x+0.0532$ ,  $R^2=0.9992$ ) and the results were expressed as µg of glucose equivalents per mg of extract.

#### Estimation of protein content

Total proteins were estimated by Lowry's method<sup>21</sup>. Bovine serum albumin was used to calculate the standard curve (20-160 µg / mL,  $Y=0.0159x+0.0319$ ,  $R^2=0.9569$ ) and the results were expressed as µg of bovine serum albumin equivalents per mg of extract.

#### Estimation of ascorbic acid content

One mg of various extract was treated with 4.0 mL of 10% Trichloroacetic acid and centrifuged for 20 min at 3500 rpm and 0.5 mL of supernatant was then, mixed with 0.1 mL DTC reagent (2, 4-Dinitrophenylhydrazine-thiourea-copper sulphate reagent). The tubes were incubated at 37°C for 3 h. Ice cold 65%  $H_2SO_4$  (0.75 mL) was added and the tubes were allowed to stand at room temperature for an additional 30 min. The colour developed was read at 520 nm<sup>22</sup>. Ascorbic acid was used to calculate the standard curve (20-120 µg/mL,  $Y=0.0308x+0.1156$ ,  $R^2=0.9998$ ) and the results were expressed as µg of ascorbic acid equivalents per mg of extract.

#### Statistical Analysis

The results are expressed as mean values and standard error (SE) or standard deviation (SD), n=3. Data were analysed using one way analysis of variance (ANOVA) followed by Turkey's multiple comparison post hoc test using SPSS software 16.0 versions. Values of  $p < 0.05$  were considered statistically significant.

## RESULTS

#### Qualitative Screening

The phytochemical screening of *Barringtonia acutangula* leaf in various extract revealed the presence of numerous secondary metabolites to evidence their antioxidant properties is laid in Table.1.

**Table 1: Phytochemical screening of *Barringtonia acutangula* leaves using various solvents**

Phytochemical constituents	Tests	Various solvent extracts		
		Petroleum ether	Chloroform	Methanol
Alkaloids	Mayers test	-	+	+
	Wagners test	-	+	+
	Hagers test	-	+	+
Phenolics / Tannins	FeCl <sub>3</sub> test	+	+++	+++
	Lead acetate test	+	+++	+++
	K <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub> test	-	+++	+++
Flavonoids	Shinoda test	+	+++	+++
Proteins / Amino acids	Ninhydrin test	+	+	++
	Biuret test	+	+	++
Carbohydrates	Molisch's test	+	++	++
	Fehling's test	+	++	++
	Barfoed's test	-	++	++
Fats / Oils	Sudan IV test	+++	+	-
Steroids	Salkowski test	-	+	+
	Libermann's test	-	+	+
Saponins	Foam test	++	+	+
Terpenoids	Knollar's test	-	++	++
Cardiac glycoside	Keller-Killiani test	-	-	-
Anthraquinones	Borntreger's test	-	++	++

+++ = Copiously present, ++ = Moderately present, + = Slightly present, - = Absent.

### Total phenolic content

All the extracts experimented for their phenolic content showed an appreciable record of about 41.76, 79.71 and 18.77  $\mu\text{g} / \text{mg}$  of extract for chloroform, methanol and petroleum ether extracts respectively. It can be noted that the highest content was produced by methanolic extract comparatively.

### Total flavonoid content

An effective content of about 109.52  $\mu\text{g}$  was observed for methanolic extract. Whereas the chloroform and petroleum ether extracts recorded 79.35 and 13.21  $\mu\text{g} / \text{mg}$  of extract respectively.

### Estimation of total Flavonol content

Flavonol content was rich in methanol extract (91.18  $\mu\text{g}$ ) compared to chloroform (56.87  $\mu\text{g}$ ) and petroleum ether (22.63  $\mu\text{g}$ ) extracts.

### Total Tannin content

A significant content was produced by the methanol leaf extract compared to other two extracts. About 105.41  $\mu\text{g}$  of tannin content of the methanol extract had offered the effective antioxidant activity of *Barringtonia acutangula*, 68.25 and 15.54  $\mu\text{g}$  was produced by the chloroform and petroleum ether extracts.

### DPPH radical scavenging activity

The radical scavenging activity values of the extracts had shown a uniform increase with the increase in concentration as shown

in Fig.1. The  $\text{IC}_{50}$  values calculated had revealed good values of about 0.15 mg for methanol, 0.88 mg for chloroform extract and 1.70 mg for petroleum ether against the positive control ascorbic acid (0.18 mg).

### Reducing power

Increase in absorption with increase in concentration for all extracts was observed as depicted in Fig.2. It was obvious that the high activity was exhibited by the methanol extract, with 0.5 absorption ( $\text{EC}_{50}$ ) of 0.11 mg chloroform and petroleum ether extract showed 0.31 and 1.02 mg as  $\text{EC}_{50}$  value giving their low reducing ability in contrast.

### Total Carbohydrate content

The methanolic extract showed higher carbohydrate content (98.53  $\mu\text{g}$ ), when compared to chloroform extract (29.51  $\mu\text{g}$ ). And the least content was recorded by the petroleum ether extract (16.78  $\mu\text{g}$ ).

### Estimation of Total protein content

Once again methanol extract had proved their potency of bearing about 131.30  $\mu\text{g}$  standing ahead to chloroform (68.12  $\mu\text{g}$ ) and petroleum ether extract (23.47  $\mu\text{g}$ ).

### Estimation of Total ascorbic acid content

Methanol extract has higher ascorbic acid content when compared to chloroform and petroleum ether extracts as shown in Table 2.

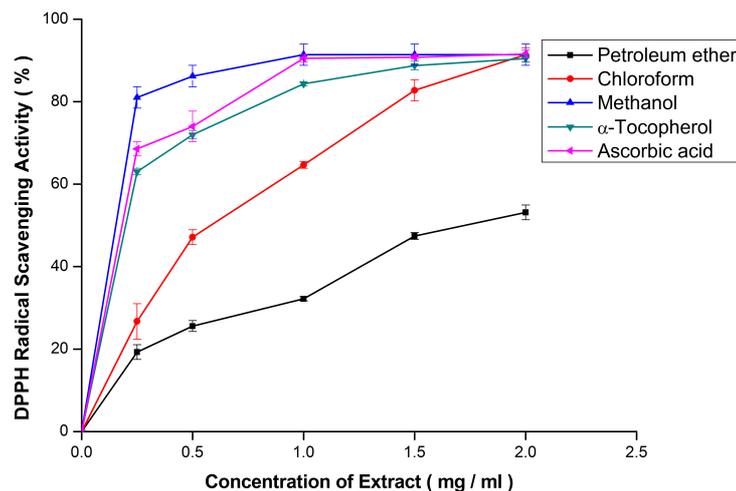


Fig. 1: DPPH radical scavenging activities of *Barringtonia acutangula* leaves in different concentrations.

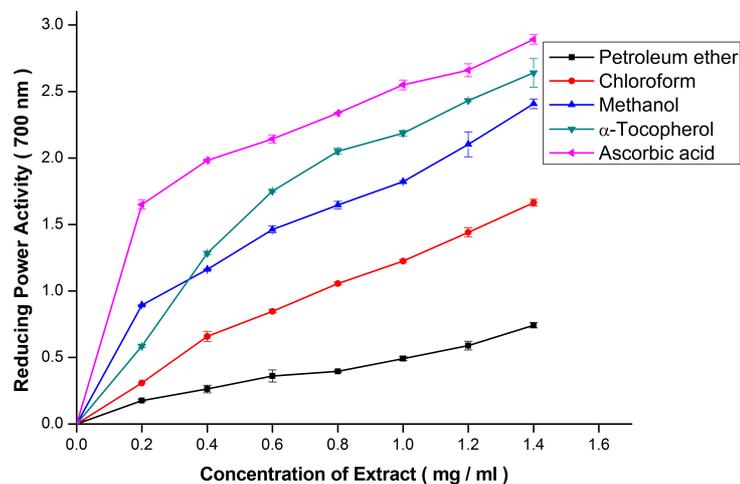


Fig. 2: Reducing power of *Barringtonia acutangula* leaves in different concentrations.

Table 2: Estimations of various content of *Barringtonia acutangula* leaves by various solvents.

Phytochemical constituents	Various solvent extracts		
	Petroleum ether	Chloroform	Methanol
Total phenolic content ( $\mu\text{g/g}$ extract)*	18.77 $\pm$ 1.09	41.76 $\pm$ 1.97	79.71 $\pm$ 1.97
Total flavonoid content ( $\mu\text{g/mg}$ extract)*	13.21 $\pm$ 0.48	79.35 $\pm$ 4.86	109.52 $\pm$ 4.59
Total flavonol content ( $\mu\text{g/mg}$ extract)*	22.63 $\pm$ 2.78	56.87 $\pm$ 2.52	91.18 $\pm$ 0.55
Total Tannin content ( $\mu\text{g} / \text{mg}$ extract)*	15.39 $\pm$ 1.30	68.21 $\pm$ 1.93	105.52 $\pm$ 1.27
Total ascorbic acid content ( $\mu\text{g/mg}$ extract)*	16.35 $\pm$ 2.20	53.60 $\pm$ 2.64	79.69 $\pm$ 2.23
Total protein content ( $\mu\text{g} / \text{mg}$ extract)*	23.47 $\pm$ 4.35	68.12 $\pm$ 2.34	131.30 $\pm$ 3.69
Total carbohydrate content ( $\mu\text{g} / \text{mg}$ extract)*	16.78 $\pm$ 1.17	29.51 $\pm$ 1.12	98.53 $\pm$ 1.27

\*The values are mean of three replicates with standard errors (Mean  $\pm$  S.D),  $p < 0.05$ .

## DISCUSSION

The qualitative and quantitative phytochemical analysis of various solvent extracts of *Barringtonia acutangula* leaves has thrown light over its antioxidant propensities. Many useful chemical constituents that has vast pharmacological properties has been screened out, which thereby paved way to estimate their free radical scavenging activities. Phenolic compounds, the biologically active components, are the main agents that can donate hydrogen to free radicals and thus break the chain reaction of lipid oxidation at the first initiation step. This high potential of phenolic compounds to scavenge radicals may be explained by their phenolic hydroxyl groups<sup>23</sup>. From the results, it can be noted that the flavonoid content of the methanolic extract had an immense potency which reasoned the significant antioxidant activity. In addition, the phenolic content of the methanol extract had been a greater support to this activity. Comparatively, both chloroform and petroleum ether extracts have shown lesser contents which may be due to the lesser amount of phenolics and flavonoids that have got extracted or present in the leaves matching to the polarity of these solvents. On the otherhand it reveals that highly polar phenolics would have got extracted in methanol, offering higher contents. Furthermore, the highest tannin content was also observed for the methanol extract in contrast to the other extracts. Though the other contents like protein, carbohydrate, vitamin C, tannin have been determined, the phenolics, tannins and flavonoids stood much important for the concept of free radical scavenging activities. Recent studies showed that a number of plant products contain polyphenolic substances such as flavonoids and tannins. These natural antioxidative substances usually have a phenolic moiety in their molecular structure. They have been found among flavonoids, tocopherols and catechines. Organic acids, carotenoids, protein hydrolysates and tannins can act as antioxidants or can have synergistic effects when used together with phenolic antioxidants. Phenolic antioxidants are potent free radical terminators<sup>24</sup>.

DPPH free radical scavenging activity has been considered as the most important determination, which showed an increase of absorption with increase in concentration, as depicted in Fig.1. IC<sub>50</sub> values of the crude extracts have been considered to be the main factor to comment the antioxidant activity range. Thus the 50% inhibition range among the extracts tested, methanol gave the least value, which has shown its capacity of having scavenged the free radicals efficiently.

Reducing power of any extract will be given by the amount of reductones present in them. The ability of the hydroxyl groups present in the flavonoids / phenolics to reduce the free radicals by donating their electrons will determine their activity. Plant phenolics contribute a major group of compounds that act as primary antioxidants<sup>25</sup>, which can react with hydroxyl radical [ $\cdot\text{OH}$ ], superoxide anion radicals [ $\text{O}_2^-$ ] and lipid peroxy radicals. Hence, such a strong reducing activity is given by the methanol extract of *Barringtonia acutangula* leaves. An effective concentration (EC<sub>50</sub>) value of about 0.3274 mg obtained gives an idea about its greater reducing power at such a lower concentration.

Typical compounds that contribute to both the antioxidant property and nutritional value have been characterized as vitamin C, protein and carbohydrate compounds. It was reasonable to investigate their total level in leaf extracts. Carbohydrates plays an important role in immunomodulatory, free radical scavenging activity<sup>26</sup> and

antibacterial activity<sup>27</sup>. The bioactivities and medicinal purposes of carbohydrates have reported them to be as diluents, disintegrant as an energy store<sup>28, 29</sup>. The content of vitamin C present in the extracts varied from the range 16 to 79  $\mu\text{g}$ . The highest amount was found in the methanol extract followed by chloroform extract. A correlation can be observed between the vitamin C content can be ascribed to the nutritional value of the extracts, in particular. The highest content obtained for the methanolic extract comparatively was found likely to contribute to the potential source as a food / drug. However, the other extracts recorded a lesser content. Good contents of proteins were also obtained for leaf extracts, especially in methanol extracts. A content of 13% in methanol extract is highly sufficient for the nutritional value of *Barringtonia acutangula*. Crude protein contents of plants are influenced by a lot of environmental factors and the protein contents in different organs of plants in general are different. When a large part of the protein contents of plants (70 to 90%) consists of amino acids, the remaining portion and the part called as non-proteins are formed from ammonium and nitrate salts. As can be seen, protein content is an important parameter in the determination of food values of plant nutrients<sup>30</sup>.

The results in this study demonstrated that the leaf extracts contain an appreciable amount of carbohydrates, ascorbic acid, proteins, tannins revealing a high level of antioxidant activity too. This reveals that the antioxidant activity is not limited to phenolic content alone. The significant content of methanol extract contributed by the phenolics, flavonoids, tannins proves that this leaf extracts consists potential antioxidant activity, which is proved by the DPPH scavenging activity and reducing power activity. Uniformly, for all the determinations the methanolic leaf extract revealed its role against the free radicals. This antioxidant activity may also come from the other secondary metabolites such as vitamins, proteins, carbohydrates etc.

## CONCLUSION

The results obtained in this study are noteworthy, not only with respect to the antioxidant activities, but also with respect to its various valuable contents. The significant activity of the methanol extract shall be attributed particularly to the phenolic and polyphenolic compounds, which forms the base of the scavenging and reducing properties. Based on the promising pharmacological property of the *Barringtonia acutangula* leaf extract, it can be used as a safe nutritional supplement without any adverse side effects.

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