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In Vitro Antioxidant and Thrombolytic Activities of *Bridelia* Species Growing in Bangladesh

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Abstract

The organic soluble extractives of three *Bridelia* species, *B. verrucosa*, *B. stipularis* and *B. tomentosa* growing in Bangladesh were subjected to screening for free radical scavenging activity, total antioxidant capacity and total phenolic content. All of the methanol extracts of the these plants and their kupchan fractions showed moderate to strong free radical scavenging activity, the total antioxidant capacity and total phenolic content, of which the methanol extract of the leaf of *B. verrucosa* revealed highest activity having IC_{50} value of 6.35 µg/ml. All the extractives of three plants were also studied for their thrombolytic potential. Among the three plants the carbon tetrachloride soluble fraction and methanol extract of leaf and aqueous soluble fraction of bark of *B. tomentosa*, methanol extract of bark of *B. stipularis* and carbon tetrachloride soluble fraction of leaf of *B. verrucosa* exhibited highest thrombolytic activity with clot lysis value of 41.46%, 34.85%, 37.04%, 36.45% and 33.72%, respectively. Standard streptokinase was used as positive control which exhibited 61.50% lysis of clot while the negative control water revealed 2.56% lysis of clot.

Keywords: Antioxidant; Free radical scavenging; Total antioxidant capacity; Phenolic content; Thrombolytic activity; *Bridelia*.

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1. Introduction

Free radicals are highly reactive particles with an unpaired electron and are produced by radiation or as by-products of metabolic processes. They initiate chain reactions, which lead to disintegration of cell membranes and cellular compounds, including lipids, proteins and nucleic acids [1]. A serious imbalance between the production of free radicals and the antioxidant defense system is responsible for oxidative stress [2] which is related to the ageing process and some chronic diseases such as cancer, cardiovascular disorders and diabetes [3].

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The active nitrogen and oxygen species may induce some damage to the human body. Over production of various forms of activated oxygen species, such as oxygen radicals and non-free radical species are considered to be the main contributors to oxidative stress, which have been linked to several diseases like atherosclerosis, cancer, and tissue damage in rheumatoid arthritis [4, 5]. Antioxidants are vital substances which possess the ability to protect the body from such damage caused by free radical induced oxidative stress [6].

Sources of natural antioxidants are mainly plant phenolics found in all parts of plants such as fruits, vegetables, nuts, seeds, leaves, roots and barks [7]. However, some synthetic antioxidants such as butylhydroxyanisole and butylhydroxytoluene need to be replaced with natural antioxidants due to their potential health risks and toxicity [8].

In Africa and Asia around 60 species of *Bridelia*, (Phyllanthaceae) are found and several species are used as folkloric medicines due to their antiamebic, antianemic, antibacterial, anticonvulsant, anti-diabetic, antidiarrhoeal, antihelmintic, anti-inflammatory, antimalarial, antinociceptive, antiviral and hypoglycemic properties [9]. The three *Bridelia* species of the Phyllanthaceae family available in Bangladesh, *Bridelia verrucosa (*Haines), *Bridelia stipularis* (L) and *Bridelia tomentosa (*Blume) are shrubs or small evergreen trees [10]. *B. verrucosa* (Haines) (Synonym: *B. montana* Willd, *B. sikkimensis* Gehrmann) is a large shrub or straggling tree without thorns which is widely distributed in the Chittagong Hill Tracts [10, 11]. The root and bark are much used as astringent in Bombay and Goa. The plant has been credited with anthelmintic properties [10, 12, 13]. Previous phytochemical studies with the leaves of this plant showed the presence of sitosterol, its glucoside, hexacosanol and Naringin [13, 14]. *B. stipularis* (L) (Synonym: *Clutia stipularis* L., *B. scandens*, Local name: Pat khowi) is a large more or less climbing shrub, which grows in shady and moist forest floors. It is distributed in the forest areas of the central and eastern parts of Bangladesh. It is also found in India and Myanmar. The plant is used in the treatment of amoebic dysentery, chest pain, constipation, diarrhea, leucoderma and strangury [15]. Decoction of bark is used for cough, fever and asthma. It also showed hypotensive and hypoglycaemic actions on animals. Leaves are used for jaundice [16]. *B. tomentosa* (Blume) (Synonym: *B. lanceaefolia*, *B. monoica*; Local name: Khy, Serai) is a large shrub or small evergreen tree and in Bangladesh it is distributed in the forest areas of Srimangal, Sylhet, Dinajpur and Chittagong district. It is also found in India, Khasia Mountains, Andaman Islands and distributed in Malay Islands, China, Philippines and Northern Australia [17]. The bark of *B. tomentosa* is astringent and used in colic [16] while the leaves are used as herbal medicine for traumatic injury. The roots are used in epidemic influenza and neurasthenia. The bark is known to contain 8% of tannins [18].

As part of our continuing studies on medicinal plants of Bangladesh specially the *Bridelia* species growing in Bangladesh [19] we investigated the antioxidant potential in forms of free radical scavenging activity, total antioxidant capacity and total phenolic content and also the thrombolytic property of *B. verrucosa*, *B. stipularis* and *B. tomentosa* extractives for the first time, and we, herein, report the results of our preliminary studies.

2. Materials and Methods

2.1. *Collection of plant materials*

Leaf and stem bark of *B. verrucosa*, *B. stipularis* and *B. tomentosa* were collected from the village of Panchouri, Khagrachhori District in February 2007 and identified in Bangladesh National Herbarium where voucher specimens have been maintained representing these collections (Accession No. DACB - 31376, 31378 and 31377, respectively). The sun dried leaf and stem bark were cut into small pieces, cleaned, oven dried and pulverized.

2.2. *Extraction and fractionation*

The powdered stem bark of *B. verrucosa* (550 g), *B. stipularis* (550 g) and *B. tomentosa* (575 g) was separately soaked in 1.5 L methanol and 325 g of powdered leaf of each plant was also separately soaked in 750 mL methanol for seven days, filtered through fresh cotton bed and finally with Whatman No. 1 filter paper and concentrated by using a rotary evaporator at low temperature (36 – 40 ºC) and reduced pressure. A portion (10 g) of the concentrated methanol extract (Me) of both stem bark and leaf of all the three plants was separately fractionated by the modified Kupchan partitioning method [20] into *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous (AQSF) soluble fractions and the yields are summarized in Table 1.

Table 1. Kupchan partitioning of crude extracts (10 gm each) of the stem bark and leaf of *Bridelia* species growing in Bangladesh.

Soluble fractions	*B. verrucosa*		*B. stipularis*		*B.tomentosa*	
	Stem bark (g)	Leaf (g)	Stem bark (g)	Leaf (g)	Stem bark (g)	Leaf (g)
n-hexane	1.75	3.1	2.75	3.3	2.25	2.7
Carbon tetrachloride	0.62	0.62	0.65	0.81	0.62	0.83
Chloroform	0.80	0.62	0.92	0.71	0.85	0.69
Aqueous	5.7	4.0	4.3	4.2	4.3	4.5

3. Biological Assays

3.1. *Free radical scavenging activity*

The free radical scavenging (antioxidant) activity of the plant extractives on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was estimated by the method established by Brand-Williams *et al.* [21]. Two ml of a methanol solution of the sample

(extractive/standard) at different concentrations (500 µg/ml to 0.977 µg/ml) were mixed with 3.0 ml of a DPPH methanol solution (20 µg/ml). After 30 mins of reaction at room temperature in dark place the absorbance was measured at 517 nm against methanol as blank by using a spetrophotometer. Then inhibition of free radical DPPH in percent (I%) was calculated as follows:

$$(I\ \%) = (1 - A_{sample}/A_{blank}) \times 100$$

where, A_{blank} is the absorbance of the control (containing all reagents except the test material and A_{sample} is the absorbance of the sample extractive.

Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted inhibition percentage inhibition against extractive/standard concentration.

3.2. *Total phenolic content (TPC)*

Total phenolic content of methanol extractives of the leaf and stem bark of *B. verrucosa*, *B. stipularis* and *B. tomentosa* along with their *n*-hexane, carbon tetra chloride, chloroform and aqueous soluble fractions were measured by employing the method described by Skerget *et al*. [22] involving Folin-Ciocalteu reagent as an oxidizing agent and gallic acid as a standard. To 0.5 ml of extract solution (2 mg/ml) in water, 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with water) and 2.0 ml of sodium carbonate (7.5 % w/v) solution were added. The mixture was then incubated for 20 minutes at room temperature. After 20 minutes, the absorbance was measured at 760 nm using a visible spectrophotometer. Total phenolics were quantified by calibration curve obtained from measuring the absorbance values from known concentrations of gallic acid (0-100 µg/ml). The phenolic contents of the sample were expressed as gm of GAE (gallic acid equivalent) / 100 gm of the dried extract.

3.3. *Phosphomolybdenum assay*

The total antioxidant activity of the plant extracts was evaluated by the phosphomolybdenum method [23] based on the reduction of Mo (VI) to Mo (V) by the extracts and subsequent formation of a green phosphate-Mo (V) complex in acidic condition. A 0.3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4mM ammonium molybdate), and the reaction mixture was incubated at 95ºC for 90 minutes. Then the absorbance of the solution was measured at 695 nm using a UV-visible spectrophotometer against blank after cooling to room temperature. The antioxidant capacity was expressed as the number of milligrams equivalent of ascorbic acid per gram of dried extract.

3.4. *Thrombolytic activity*

The thrombolytic activity of all the plant extracts of was evaluated by the method developed by Daginawala [24] and slightly modified by Kawsar [25] using streptokinase

(SK) as the standard. Commercially available lyophilized Altepase (Streptokinase) vial (Beacon pharmaceutical Ltd.) of 15,00,000 I.U. was collected and 5 ml sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100µl (30,000 I.U) was used for *in vitro* thrombolysis.

Blood (*n*=5) was drawn from healthy human volunteers without a history of oral contraceptive or anticoagulant therapy and 1ml of blood was transferred to the previously weighed microcentrifuge tubes and was allowed to form clots.

Three replicates of each sample were used for statistical analysis and the values were reported as mean±SD. Correlation analysis of free radical scavenging activity versus total phenolic content was carried out using the correlation and regression program.

4. Results and Discussions

The present study was undertaken to evaluate the antioxidant and thrombolytic activities of different partitionates of the methanol extracts of the stem bark and leaf of three Bangladeshi *Bridelia* species. The results are given in Tables 2 to 5. The amount of total phenolic content varied for different partitionates ranging from 2.95 mg to 98.76 mg of GAE/gm of dried extract. The highest total phenolic content was found from aqueous soluble fraction of the leaf of *B. stipularis* (98.76 mg of GAE/gm of dried extract) and the lowest in the crude methanol extract of stem bark of *B. verrucosa* (2.95 mg of GAE/gm of dried extract). Smaller IC_{50} value corresponds to a higher activity of the plant extracts [26]. Free radical scavenging activity of the methanol extract of the leaf of *B. verrucosa* was highest having IC_{50} value of 6.35 µg/ml and lowest activity was shown by the aqueous soluble fraction of *B. tomentosa* being 26.4 µg/ml. The other extractives of the three plants demonstrated moderate to high free radical scavenging activity with the IC_{50} value ranging from 21.3 µg/ml to 7.34 µg/ml, as compared to the standards, i.e. *tert*-butyl-1-hydroxytoluene (BHT), (IC_{50} = 24.35 µg/ml) and ascorbic acid, ASA (IC_{50} = 5.80 µg/ml).

Table 2. Free radical scavenging activity (IC_{50} in µg/ml) of extractives of stem bark and leaf of three *Bridelia* species growing in Bangladesh.

Test material	*B. verrucosa*		*B. stipularis*		*B. tomentosa*	
	Stem bark	Leaf	Stem bark	Leaf	Stem bark	Leaf
Me	11.6±0.39	6.35± 0.80	9.10±0.40	13.9±0.80	7.55±0.27	9.70±0.25
HSF	10.51±0.43	25.8 ±0.21	10.20±0.55	11.8±0.55	10.35±0.48	11.20±0.50
CSF	11.65±0.80	9.9±0.85	15.60±0.50	9.75±0.74	10.50 ±0.44	8.8±0.10
CHSF	15.8±0.15	8.25±0.10	13.2±0.10	7.34±0.46	7.35±0.40	8.9±0.80
AQSF	15.5±0.36	15.8±0.40	21.3±0.40	21.3±0.60	17.60±0.33	26.4±0.45

The total antioxidant capacity of the extractives expressed as the mg of ascorbic acid/gm of plant extract was determined by phosphomolybdenum assay where the highest value was found in methanol extract of leaf of *B. tomentosa* followed by methanol extracts of bark and leaf of *B. verrucosa* as evident from 612.54 mg, 542.88 mg and 484.43 mg equivalents of ascorbic acid, respectively.

Table 3. Total phenolic content (mg of GAE/gm of dried extract) of extractives of stem bark and leaf of three *Bridelia* species growing in Bangladesh.

Test material	*B. verrucosa*		*B. stipularis*		*B. tomentosa*	
	Stem bark	Leaf	Stem bark	Leaf	Stem bark	Leaf
Me	2.95±0.22	7.54±0.77	18.67±1.84	33.38±1.33	30.59±0.68	25.65±0.99
HSF	59.15±0.46	63.18±0.37	63.90±1.99	45.85±0.77	48.86±0.81	45.09 ±0.71
CSF	63.03±0.65	45.79±0.16	16.08±1.22	50.71±1.13	76.39±4.20	38.01±0.23
CHSF	68.14±6.02	3.26±0.65	20.22±1.11	87.31±0.59	74.78±1.33	24.97±0.74
AQSF	89.14±0.62	80.63±0.74	86.59±0.58	98.76±1.27	95.81±1.54	87.05±1.24

Table 4. Total antioxidant capacity (mg of ascorbic acid/gm of dried extract) of extractives of stem bark and leaf of three *Bridelia* species growing in Bangladesh.

Test material	*B. verrucosa*		*B. stipularis*		*B. tomentosa*	
	Stem bark	Leaf	Stem bark	Leaf	Stem bark	Leaf
Me	542.88±1.61	484.43±1.11	248.99±0.75	392.54±2.32	442.77±3.93	612.54±1.79
HSF	217.32±0.91	161.88±1.02	142.66±0.63	243.54±0.73	254.32±0.83	210.99±1.38
CSF	257.1±1.36	208.43±0.77	226.32±0.4	192.77±1.32	211.66±0.98	185.77±0.61
CHSF	151.88±0.76	148.43±0.85	181.21±1.08	226.32±0.95	153.77±0.65	152.21±1.24
AQSF	462.88±2.65	279.99±1.83	285.66±0.30	293.32±2.40	254.32±0.81	287.10±0.54

As a part of discovery of cardio-protective drugs from natural sources the extractives of plants were assessed for thrombolytic activity and the results are presented in Table 5. Addition of 100 µl streptokinase (SK), a positive control (30,000 I.U.), to the clots and subsequent incubation for 90 minutes at 37 °C, showed 61.5% lysis of clot. At the same time, distilled water was treated as negative control which exhibited negligible lysis of clot (2.56%). In this study, the carbon tetrachloride soluble fraction (CSF) of the leaf of

B. tomentosa exhibited highest thrombolytic activity (41.46%). However, significant thrombolytic activity was demonstrated by the aqueous soluble fraction (AQSF) of the stem bark of *B. tomentosa* (37.04%), methanol extract (Me) of leaf of *B. tomentosa* (34.85%), carbon tetrachloride soluble fraction (CSF) of leaf of *B. verrucosa* (33.72%), methanol extract (Me) of stem bark of *B. stipularis* (36.35%).

Table 5. Thrombolytic Activity (In terms of % lysis) of *B. verrucosa*, *B. stipularis*, and *B. tomentosa*.

	B. verrucosa stem bark			
Fractions	W_1	W_2	W_3	% of lysis
Me	4.8956 ± 0.15	5.3520 ± 0.06	5.2165 ± 0.02	29.69 ± 0.97
HSF	4.8682 ± 0.00	5.4435 ± 0.10	5.3428 ± 0.09	17.50 ± 0.54
CSF	4.8954 ± 0.17	5.2189 ± 0.04	5.1467 ± 0.02	22.32 ± 0.95
CHSF	4.8154 ± 0.05	5.2185 ± 0.08	5.1255 ± 0.01	23.07 ± 0.86
AQSF	4.7817 ± 0.18	5.3107 ± 0.08	5.2130 ± 0.13	18.47 ± 2.24
	B. verrucosa leaf			
Fractions	W_1	W_2	W_3	% of lysis
Me	4.8756 ± 0.08	5.3386 ± 0.03	5.2271 ± 0.01	24.08 ± 0.68
HSF	4.8682 ± 0.06	5.4435 ± 0.00	5.3428 ± 0.04	17.50 ± 0.41
CSF	4.9762 ± 0.09	5.2781 ± 0.10	5.1763 ± 0.02	33.72 ± 1.05
CHSF	4.8813 ± 0.10	5.3892 ± 0.03	5.3438 ± 0.09	8.94 ± 0.22
AQSF	4.6689 ± 0.11	5.2056 ± 0.07	5.1560 ± 0.13	9.24 ± 1.86
	B. stipularis stem bark			
Fractions	W_1	W_2	W_3	% of lysis
Me	4.7870 ± 0.10	5.1582 ± 0.04	5.0229 ± 0.08	36.45 ± 0.66
HSF	4.7747 ± 0.09	5.2446 ± 0.10	5.1151 ± 0.04	27.56 ± 0.73
CSF	4.8069 ± 0.01	5.2529 ± 0.06	5.1196 ± 0.01	30.06 ± 0.55
CHSF	4.6709 ± 0.10	5.0970 ± 0.09	5.0039 ± 0.02	21.85 ± 0.47
AQSF	4.7420 ± 0.08	5.3244 ± 0.10	5.1721 ± 0.01	28.73 ± 0.86
	B. stipularis leaf			
Fractions	W_1	W_2	W_3	% of lysis
Me	4.9863 ± 0.12	5.4876 ± 0.05	5.3486 ± 0.04	27.73 ± 0.92
HSF	4.8854 ± 0.11	5.3218 ± 0.06	5.1987 ± 0.01	28.21 ± 0.82
CSF	4.8639 ± 0.11	5.3465 ± 0.07	5.2188 ± 0.03	26.46 ± 0.73
CHSF	4.9769 ± 0.07	5.4712 ± 0.05	5.3982 ± 0.02	14.77 ± 0.51
AQSF	4.8792 ± 0.11	5.3478 ± 0.03	5.2168 ± 0.03	27.96 ± 0.79

Table 5 (*contd*.)

B. tomentosa stem bark				
Fractions	W_1	W_2	W_3	% of lysis
Me	4.8708 ± 0.11	5.4598 ± 0.05	5.3391 ± 0.01	20.49 ± 0.72
HSF	4.7375 ± 0.10	5.2563 ± 0.09	5.2020 ± 0.02	10.43 ± 0.48
CSF	4.7678 ± 0.10	5.1586 ± 0.06	5.0724 ± 0.01	22.06 ± 0.32
CHSF	4.7813 ± 0.09	5.2239 ± 0.03	5.1609 ± 0.03	16.13 ± 0.43
AQSF	4.8321 ± 0.03	5.2587 ± 0.05	5.1007 ± 0.01	37.04 ± 0.78
B. tomentosa leaf				
Fractions	W_1	W_2	W_3	% of lysis
Me	4.7583 ± 0.09	5.2786 ± 0.03	5.0973 ± 0.01	34.85 ± 1.00
HSF	4.8953 ± 0.17	5.3865 ± 0.11	5.2804 ± 0.08	21.60 ± 1.37
CSF	4.8537 ± 0.00	5.2895 ± 0.09	5.1087 ± 0.06	41.46 ± 0.98
CHSF	4.9765 ± 0.07	5.4487 ± 0.15	5.3786 ± 0.02	14.85 ± 1.11
AQSF	4.8569 ± 0.04	5.2786 ± 0.19	5.1874 ± 0.01	21.63 ± 2.33
Blank	4.8880±.001	5.4730±.02	5.1130±.01	2.56±0.79
SK	4.8850±.009	5.4670±.02	5.4530±.02	61.50±0.17

5. Conclusion

The results obtained indicate that extractives of three *Bridelia* species are the potential sources of natural antioxidants and cardio-protective drugs. Further studies are underway to isolate and characterize the compounds responsible for these activities.

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