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Preliminary Free Radical Scavenging, Brine Shrimp Lethality, Antimicrobial and Thrombolytic Activities of *Aganosma dichotoma* (Roth) K. Schum.

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Abstract

Bangladesh is a good repository of natural products. Numerous plants are available to facilitate the traditional treatments. Proper scientific evaluations are essential to explore the plant derived drugs. With this view, the crude methanol extract of leaves of *Aganosma dichotoma* (Roth) K. Schum. and its Kupchan fractions were screened for free radical scavenging, brine shrimp lethality, antimicrobial and thrombolytic activities. Among all extractives, the chloroform soluble fraction demonstrated the highest free radical scavenging activity with IC_{50} value 18.21 µg/ml. Moreover, the chloroform soluble fraction showed significant brine shrimp lethality having LC_{50} value of 3.98 µg/ml. On the other hand, the petroleum ether, carbon tetrachloride and chloroform soluble materials revealed mild to moderate antimicrobial activity with the zone of inhibition ranging from 7 to 14 mm. In the thrombolytic assay, the carbon tetrachloride soluble partitionate showed the highest clot lysis (30.48%). This is the first report of the comprehensive investigations of different extractives of *A. dichotoma* for the above-mentioned bioassays.

Key words: *Aganosma dichotoma*, Apocynaceae, free radical scavenging, antioxidant, cytotoxic, antimicrobial, thrombolytic

Introduction

Natural products have earned an admirable place in drug discovery. Screening of plants focusing on bioactivities is essential for exploring the desired drug molecules (Hung *et al.*, 2012; Newman and Cragg, 2012). Free radicals are considered as notorious oxidizing elements to promote ageing, lipid peroxidation, inflammation and other pathologies. Plants are good sources of antioxidants (Flora, 2007; Pham-Huy *et al.*, 2008). Brine shrimp lethality bioassay is well accepted by the natural product scientists as a bench-top tool to find out bioactive elements (Meyer *et al.*, 1982; Rahman *et al.*, 2008a). Besides, secondary metabolites of plants are worthy to screen out for searching anti-infective agents. There are the life saving elements available in market but drug-resistance issue promotes the need for new antimicrobials (Drago *et al.*, 2014; Gonzalez and Cortes, 2014). Thrombus formation may lead to death by worsening the cardiovascular situation. Better drug is required to overcome limitations of the currently available drugs (Kline, 1990; Prasad *et al.*, 2006).

The plant *A. dichotoma* (Roth) K. Schum. (Family- Apocynaceae, Bengali Name- Gandhomalati) is a large climber. It has a very stout stem and milky latex. Its leaves are 10-12.5 cm long, coriaceous, ovate or elliptic, acute or obtuse. The plant is grown all over Bangladesh (Patel *et al.*, 1972; Banglapedia, 2012). It is used as antiseptic, anthelmintic, psychoactive and emetic. Previous phytochemical investigations on *A. dichotoma* led to the isolation of quercetin, rutin, kaempferol and phenolic acids (Khare, 2007; Sandhya *et al.*, 2010; Subramanian *et al.*, 2014).

The present study has been undertaken as part of our regular research (Ara *et al.*, 2006; Begum *et al.*, 2010; Rahman *et al.*, 2011) and we, herein, report the comprehensive studies of free radical scavenging, brine shrimp lethality, antimicrobial and thrombolytic activities of the leaves of *A. dichotoma* for the first time.

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Materials and Methods

Plant materials: The leaves of *A. dichotoma* were collected on June, 2013 from Sylhet, Bangladesh and a voucher specimen (DACB accession no. 39645) has been deposited at Bangladesh National Herbarium, Mirpur, Dhaka for future reference.

Extraction and fractionation: The collected plant parts were sun dried for several days and then oven dried for 24 hours at 40° C to facilitate grinding. The powdered leaves (650 g) of *A. dichotoma* was extracted with 1.8 L methanol for 7 days and then filtered through a cotton plug followed by whatman filter paper number 1. The extract was then concentrated by using a rotary evaporator at reduced temperature (40-45°C) and pressure. The concentrated methanol extract (5 g) was fractionated by modified Kupchan method (VanWagenen *et al.*, 1993) and the resultant partitionates i.e., petroleum ether, carbon tetrachloride, chloroform and aqueous soluble materials were used for different biological screenings.

Antioxidant activity: The free radical scavenging activity of the plant extractives was determined on the stable radical produced by 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Parvin *et al.*, 2009; Chowdhury *et al.*, 2010).

Brine shrimp lethality: This technique (Meyer *et al.*, 1982; Rahman *et al.*, 2008a) was applied for the determination of general toxic property of the plant extractives indicating bioactive materials using *Artemia salina* in a 1-day *in vivo* assay. Vincristine sulfate was used as positive control.

Antimicrobial activity: The antimicrobial activity of the extractives was determined by the disc diffusion method (Bauer *et al.*, 1966; Rahman *et al.*, 2008b) against 5 gram positive bacteria and 8 gram negative bacteria (Table 4). The organisms were collected as pure cultures from the Institute of Nutrition and Food Sciences (INFS), University of Dhaka. Standard disc of Ciprofloxacin (5 µg/disc) was used as standard.

Thrombolytic activity: The *in vitro* thrombolytic assay was performed by the method developed by Prasad *et al*., 2006. In the present assay, human venous blood was used.

Statistical Analysis: Three replicates of each sample were used and the values are reported as mean ± standard deviation (SD).

Results and Discussion

The different partitionates of methanol extract of *A. dichotoma* were tested for free radical scavenging activity. The IC_{50} values of the extractives were found in the range of 18.21 µg/ml to 81.12 µg/ml. The chloroform soluble fraction showed highest (IC_{50} = 18.21) free radical scavenging activity (Table 1).

Table 1. IC_{50} values of standard and different partitionates of *A. dichotoma* in DPPH assay.

Samples	IC_{50} (µg/ml)
Tert- butyl-1-hydroxytoluene (standard)	27.23 ± 0.44
Crude methanol extract	49.34 ± 0.35
Petroleum ether soluble fraction	81.12 ± 0.38
Carbon tetrachloride soluble fraction	19.99 ± 0.51
Chloroform soluble fraction	18.21 ± 0.34
Aqueous fraction	40.50 ± 0.47

In the brine shrimp lethality screening, median lethal concentration (LC_{50}) of the test samples after 24 hours was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration and the best-fit line was obtained from the graph by means of regression analysis. The chloroform soluble materials exhibited highest lethality with LC_{50} value of 3.98 µg/ml (Table 2).

Table 2. LC_{50} values of standard and different partitionates of *A. dichotoma* in brine shrimp lethality bioassay.

Samples	Regression line	R^2	LC_{50} (µg/ml)
Vincristine sulfate (standard)	y = 30.8x + 60.64	0.96	0.47 ± 0.11
Crude methanol extract	y = 17.11x- 6.347	0.88	1964.36 ± 0.64
Petroleum ether soluble fraction	y = 21.74x - 11.12	0.87	647.75 ± 0.52
Carbon tetrachloride soluble fraction	y = 29.79x - 15.16	0.93	153.93 ± 0.47
Chloroform soluble fraction	y = 27.78x + 33.34	0.91	3.98 ± 0.32

Table 3. Antimicrobial activity of *A. dichotoma* extractives at 400 µg/disc.

Test microorganisms	Diameter of zone of inhibition (mm)				
	ME	PE	CT	CL	CF
Gram positive bacteria					
Bacillus sereus	-	-	-	-	44 ± 0.23
B. megaterium	-	-	14 ± 0.21	12 ± 0.41	44 ± 0.34
B. subtilis	-	10 ± 0.34	10 ± 0.22	-	44 ± 0.21
Staphylococcus aureus	-	10 ± 0.20	12 ± 0.30	12 ± 0.46	46 ± 0.33
Sarcina lutea	-	-	7 ± 0.24	12 ± 0.12	46 ± 0.20
Gram negative Bacteria					
Escherichia coli	-	10 ± 0.51	8 ± 0.35	10 ± 0.34	45 ± 0.12
Pseudomonas aeruginosa	-	-	8 ± 0.54	9 ± 0.23	46 ± 0.10
Salmonella Paratyphi	-	9 ± 0.19	-	-	45 ± 0.21
S. Typhi	-	-	12 ± 0.21	12 ± 0.54	45 ± 0.40
Shigella dysenteriae	-	-	8 ± 0.40	12 ± 0.41	48 ± 0.04
S. boydii	-	-	-	-	46 ± 0.42
Vibrio mimicus	-	12 ± 0.23	12 ± 0.041	13 ± 0.22	45 ± 0.21
V. parahemolyticus	-	10 ± 0.25	10 ± 0.32	12 ± 0.42	45 ± 0.21

'-' indicates no response, ME- crude methanol extract, PE- petroleum ether soluble fraction, CT- carbon tetrachloride soluble fraction, CL- chloroform soluble fraction, CF- ciprofloxacin (5 µg/disc)

The crude extract and its different partitionates were subjected to antimicrobial screening at 400 µg/disc. The petroleum ether, carbon tetrachloride and chloroform soluble fractions displayed weak to moderate antimicrobial activity with the zone of inhibition ranging from 7 to 14 mm (Table 3).

In the thrombolytic assay, the carbon tetrachloride soluble fraction revealed the highest thrombolytic activity (30.48%) followed by chloroform (27.09%) and aqueous (26.17%) soluble fractions (Table 4).

Table 4. Thrombolytic activity (in terms of % clot lysis) of *A. dichotoma.*

Sample	% of clot lysis
Water (Blank)	1.81 ± 0.38
Streptokinase (Standard)	65.27 ± 0.51
Crude methanol extract	12.00 ± 0.74
Petroleum ether soluble fraction	11.26 ± 0.42
Carbon tetrachloride soluble fraction	30.48 ± 0.32
Chloroform soluble fraction	27.09 ± 0.37
Aqueous fraction	26.17 ± 0.43

Bangladesh is located in tropical zone facilitating the growth of numerous medicinal plants. The current experiment was designed to screen the free radical scavenging, brine shrimp lethality, antimicrobial and thrombolytic activities of *A. dichotoma* growing in Bangladesh. The experimental data of the assays have been summarized in Tables 1 to 4.

Excess free radicals and oxidants produce oxidative stress causing injury to cellular proteins, lipids, lipoproteins and deoxyribonucleic acid. If not controlled, oxidative stress can induce a variety of chronic and degenerative diseases, aging process, trauma, inflammatory damages etc. (Arrigoni-Martelli, 1985; Hewitt *et al.*, 1987; Hamvas *et al.*, 1992). In the current study, *A. dichotoma* significantly scavenged the free radicals generated by DPPH to demonstrate its antioxidant activity (Table 1).

The bioactivities of plants are always generated from secondary metabolites. The lethality of brine shrimp due to toxicity is considered as an indicator of the presence of bioactive compounds (Meyer *et al.*, 1982; McLaughlin *et al.*, 1998). This current assay showed the presence bioactive natural compounds in the test samples of *A. dichotoma* (Table 2).

Thrombus formation in the circulatory system causes vascular blockage leading to death. Currently used thrombolytic agents that include tissue plasminogen activator, urokinase, streptokinase etc. are used but still associated with risk of hemorrhage, anaphylactic reaction and lack specificity. So, attempts are still ongoing around the world to develop improved thrombolytic agents

(Wilson and Chaikof, 2008; Rodriguez *et al.*, 2012; Sikder *et al.*, 2012). With this view, this investigation was done on *A. dichotoma*. The carbon tetrachloride, chloroform and aqueous fractions displayed moderate thrombolytic activity (Table 4).

Conclusion

A. dichotoma has many bioactive secondary metabolites as evident from brine shrimp lethality bioassay. Besides, it can provide antioxidants and thrombolytic agents. In addition, moderate antimicrobial and thrombolytic constituents can be expected from the extractives of this plant. Further phytochemical investigation is required to isolate the bioactive molecules from this plant.

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