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Biological and Preliminary Phytochemical Investigations of *Tiliacora acuminata* Miers

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ABSTRACT

The crude methanol extract of leaf of *Tiliacora acuminata* Miers as well as organic solvent (*n*-hexane, carbon tetrachloride and chloroform) fractions and methanolic fraction (aqueous) were subjected to screening for total phenolic content, antioxidant, cytotoxic, thrombolytic and antimicrobial activity. In total phenolic content analysis, the *n*-hexane soluble fraction of leaf was found to contain highest amount of phenolic content (TPC, 132.18 mg/gm of dry weight of extract, expressed as gallic acid equivalents). In the DPPH assay, the *n*-hexane soluble fraction of leaf displayed the highest free radical scavenging activity with IC_{50} value 64.54 µg/ml as compared to 27.5 µg/ml produced by butylated hydroxyl toluene. A positive correlation was evident between total phenolic content and free radical scavenging activity of *T. acuminata* having correlation coefficient (R^2) of 0.91. In cytotoxicity screening, the crude methanol extract of leaf demonstrated strong cytotoxic activity with LC_{50} value of 2.40 µg/ml as compared to 0.451 µg/ml produced by vincristine sulphate. During assay for thrombolytic activity, the crude methanol extract revealed 32.5% lysis of clot while standard streptokinase and water, used as positive and negative controls, demonstrated 70.4% and 3.62% lysis of clot, respectively. In antimicrobial assay by disc diffusion method, all the samples exhibited moderate to significant antimicrobial activity (zone of inhibition = 10.0-20.0 mm) against all the test organisms. Among all the samples, the carbon tetrachloride soluble fraction displayed strong antimicrobial activity against *Escherichia coli* (20.0 mm).

Keywords: *Tiliacora acuminata*, Total Phenolic Content, Cytotoxicity, Antioxidant, Thrombolytic, Antimicrobial.

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INTRODUCTION

Bangladesh is a rich source of medicinal plants which are frequently used by our local people for the treatment of various diseases such as jaundice, cough, cold, skin disease, diarrhea, fever, etc. These medicinal plants have immensely contributed to the development of human health & welfare. The active principles of any drugs found in plants are secondary metabolites. Isolation of these active compounds from the natural source and structural characterization of the purified lead compounds would be considered as a success of the research[1].

Tiliacora acuminata Miers (Bengali name- Teliakora; Family- Menispermaceae) is a large woody climber which is widely distributed through Bangladesh (especially in Dhaka, Bagerhat, Kushtia, Khulna, Faridpur, Rajshahi), India, Nepal, Laos, Vietnam, Indonesia. This plant has been used as an ingredient in many of the ayurvedic preparations and regard as an antidote for snake bite[2,3]. The previous phytochemical investigations of this plant led to isolation of novel ester *i.e.* octyl(benzoylamino)acetate and heptadeca-4-ene-acetate[4], lactone[2], alkaloids *i.e.* tiliaresine and (+) *N*-methyltiliamosine[5,6].

As part of our ongoing research with medicinal plant of Bangladesh[7,8] the present study has been undertaken to evaluate the antioxidant, brine shrimp lethality, thrombolytic and antimicrobial activities of *T. acuminata* as well as to find out the logical evidence for its folk uses.

MATERIALS AND METHODS

Plant material

The leaves of *T. acuminata* were collected from the area of Matlab Thana under Chandpur district in January 2012. A voucher specimen for this plant has been maintained in Bangladesh National Herbarium, Dhaka, Bangladesh for future reference.

Reagents and chemicals

All chemicals *i.e.* methanol, *n*-hexane, carbon tetrachloride, chloroform and other reagents such as 1,1-diphenyl-2-picrylhydrazyl, butylated hydroxy toluene, vincristine sulfate, streptokinase, dimethyl sulfoxide etc used in these experiments were of the highest analytical grade.

Extraction and fractionation

The samples were sun dried for several days and then oven dried for 24 hours below 40 °C to facilitate grinding. The powdered materials (550 gm) were macerated in 1.8 L of methanol for 7 days and then filtered through a cotton plug followed by Whatman filter paper number 1. The extract was concentrated with a rotary evaporator at low temperature (40-45 ºC) and reduced pressure. The concentrated methanol extract was partitioned by the modified Kupchan method[9]

and the resultant partitionates i.e., *n*-hexane (1.3 gm), carbon tetrachloride (1.2 gm), chloroform (700 mg), and aqueous soluble (1.1 gm) fractions were used for the experiment.

Preliminary phytochemical investigation

For preliminary phytochemical investigation the crude extract was subjected to various tests (Table 1) to determine the chemical nature of the extractive[10].

Table 1. Chemical groups present in the extract of *T. acuminata.*

Test for	Test performed	Crude methanol extract
Alkaloids	Meyer's test	+
	Dragendorff's test	+
	Wagner's test	-
	Hager's test	-
	Tannic acid test	+
Steroids	Salkowski test	+
	Libermann-Burchared test	+
Tannins	Ferric chlorides test	+
	Potassium dichromate test	+
Flavonoids	Conc. HCl and alcoholic test	-
Saponins	Shake test (aq. solution)	-
Reducing sugars	Fehling's test	+
	Benedict's test	+
Gums	Molisch's test	+

Determination of Total Phenolic Content

The total phenolic content of *T. acuminata* was measured by employing the method[11] involving Folin- Ciocalteu reagent as an oxidizing agent and gallic acid as standard. To 0.5 ml of extract solution (2.0 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. After 20 minutes of incubation at room temperature, the absorbance was measured at 760 nm using a UV-visible spectrophotometer. Total phenolics were quantified with the help of calibration curve obtained from gallic acid (0-100 µg/ml). The phenolics content of the sample was expressed as mg of GAE (gallic acid equivalent)/gm of the dried extract.

Determination of free radical scavenging activity

The free radical scavenging activity (antioxidant capacity) of the plant extractives on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was estimated by the method of Brand-Williams[12]. Here, 2.0 ml of a methanol solution of the sample (extractive/ Standard) at different concentration (500 to 0.977 µg/ml) were mixed with 3.0 ml of a DPPH methanol solution (20 µg/ml). After 30 min of reaction at room temperature in dark place the absorbance was measured at 517 nm against methanol as blank by UV spectrophotometer. Inhibition of free

radical DPPH in percent (I %) was calculated as follows:

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

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test material). Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted with inhibition percentage against extractive/standard concentration.

Cytotoxicity screening

Dimethyl sulfoxide **(**DMSO) solutions of the extractives were applied against *Artemia salina* in a one-day *in vitro* assay[13]. For the experiment, 4 mg of each of the Kupchan fractions was dissolved in DMSO and solutions of varying concentrations such as 400, 200, 100, 50, 25, 12.50, 6.25, 3.125, 1.563, 0.78125 µg/ml were obtained by serial dilution technique. Vincristine sulphate and DMSO were used as the positive and negative control, respectively.

Thrombolytic activity

The thombolytic activity of all extractives was evaluated by the method[14] using streptokinase as standard. The dry crude extract (100 mg) was suspended in 10 ml of distilled water and it was kept overnight. Then the soluble supernatant was decanted and filtered. Aliquots (5 ml) of venous blood were drawn from healthy volunteers which were distributed in five different pre weighed sterile micro centrifuge tube (1 ml/tube) and incubated at 37 °C for 45 minutes. After clot formation, the serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight (Clot weight = weight of clot containing tube – weight of tube alone).

To each micro centrifuge tube containing pre-weighed clot, 100 µl aqueous solutions of different partitionates along with the crude extract was added separately. As a positive control, 100 µl of streptokinase (SK) and as a negative non thrombolytic control, 100 µl of distilled water were separately added to the control tubes. All the tubes were then incubated at 37 °C for 90 minutes and observed for clot lysis. After incubation, the released of fluid was removed and tubes were again weighed to observe the difference in weight after clot disruption. The differences in weights taken before and after clot lysis were expressed as percentage of clot lysis as shown below:

$$\% \text{ of clot lysis} = (\text{wt of clot after lysis /clot wt}) \times 100$$

Antimicrobial activity

The preliminary antimicrobial activity of the extractives was determined at 400 µg/disc by the disc diffusion method[15] against a number of Gram positive and Gram negative bacteria and fungi

(Table-3). The bacterial and fungal strains used in this experiment were collected as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka, Bangladesh. Here, standard Ciprofloxacin and fluconazole (30 µg) disc were used as reference.

RESULTS AND DISCUSSION

Preliminary phytochemical screening

The crude extractive when tested with various chemical reagents demonstrated the presence of alkaloids, steroids, tannins, reducing sugars and gums as shown in Table 1.

Biological studies

In total phenolic content analysis, the amount of total phenolic content varied for different partitionates ranging from 56.23 to 132.18 mg of GAE/gm of dried extract (Table 2). The highest total phenolics were found in *n*-hexane soluble fraction (132.18 gm of GAE/100 gm of dried extract) and the lowest in crude methanol extract (56.23 gm of GAE/100 gm of dried extract).

In the DPPH free radical scavenging assay the *n*-hexane soluble fraction showed the highest free radical scavenging activity with IC_{50} value 64.54 µg/ml while the chloroform soluble fraction displayed moderate antioxidant activity with IC_{50} value 97.44 µg/ml when compared to 27.5 µg/ml exhibited by butylated hydroxyl toluene (Table 2)

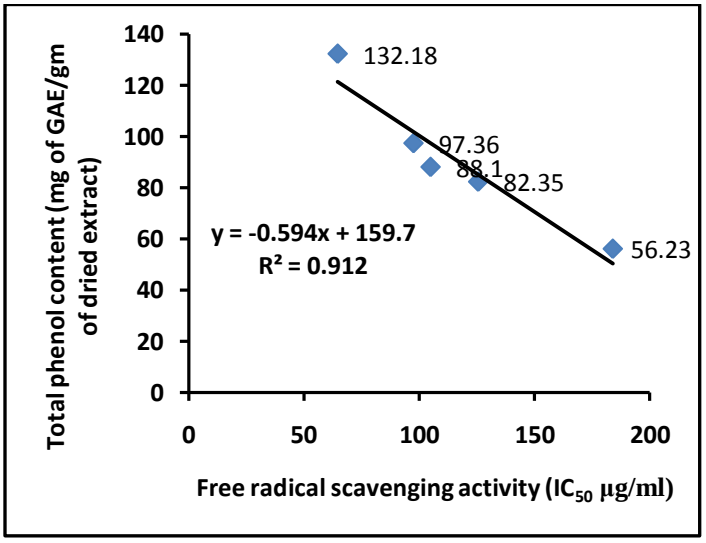

Figure 1: Correlation between the total phenolic content and free radical scavenging activity

The correlation analysis revealed that positive correlation exists between total phenolic content and free radical scavenging activity. The correlation coefficient (R2) for the total phenolics and free radical scavenging (Figure 1) was 0.91 indicating a positive relationship between the total phenolics and free radical scavenging activity. This result suggests that 91% of the free radical

scavenging activity might be the contribution of the phenolic compounds[16]. Different secondary metabolites, such as volatile oils, carotenoids and vitamins may also contribute to the antioxidant capacity[17].

Bioactive compounds are almost always toxic at higher dose. Thus, *in vivo* lethality in a simple zoological organism can be used as a convenient monitor for screening and fractionation of crude extracts in the discovery of new bioactive natural products. In the present bioactivity study all the crude extracts and their *n*-hexane, carbon tetrachloride, chloroform, and aqueous soluble fractions of leaf showed positive results indicating that the test samples are biologically active. Each of the test samples showed different mortality rates at different concentrations. Plotting of log of concentration versus percent mortality for all test samples showed an approximate linear correlation. From the graphs, the median lethal concentration (LC_{50}, the concentration at which 50% mortality of brine shrimp nauplii occurred) was determined for the samples.

The crude methanol extract of leaf and its *n*-hexane and chloroform soluble fraction demonstrated strong cytotoxic activity with LC_{50} value of 2.40, 4.79, 6.42 µg/ml, respectively while the carbon tetrachloride soluble fraction was also significantly cytotoxic with LC_{50} values of 34.91 µg/ml as compared to 0.451 µg/ml produced by vincristine sulphate (Table 2).

Table 2: Antioxidant (IC_{50} µg/ml), cytotoxic (LC_{50} µg/ml) and thrombolytic activity (% Clot lysis) of different Kupchan fractions of *T. acuminata.*

Sample	Total phenolic content (mg of GAE/gm of dried extract)	Antioxidant activity (IC_{50} µg/ml)	Cytotoxic activity (LC_{50} µg/ml)	Thrombolytic activity (% Clot lysis)
BHT	ND	27.5±0.54	ND	ND
VS	ND	ND	0.451± 0.24	ND
SK	ND	ND	ND	70.4 %±1.20
CME	56.23 ± 0.76	183.92±1.26	2.40±0.96	32.5 %±1.15
HSF	132.18 ± 1.12	64.54±1.19	4.79±1.34	20.0 %±0.82
CTSF	82.35 ± 0.24	125.5±0.82	34.91±0.67	18.0±1.32
CSF	97.36 ± 1.24	97.44±1.35	6.42±0.57	27.5 %±0.84
AQSF	88.10 ± 1.56	104.85±0.57	78.79±1.13	8.2±0.46

Here, BHT = Butylated Hydroxy Toluene; VS= Vincristine sulphate, SK = Streptokinase, CME = Crude methanolic extract; HSF = Hexane soluble fraction; CTSF = carbon tetrachloride soluble fraction; CSF= chloroform soluble fraction; AQSF= aqueous soluble fraction of the methanolic extract of *T. acuminata*; ND = Not determined

In order to identify the drugs with the ability to promote lysis of blood clot from natural sources, the extractives of *T. acuminata* were assessed for thrombolytic activity. Addition of 100 µl streptokinase (SK), a positive control (30,000 I.U.) to the clots of human blood and subsequent incubation for 90 minutes at 37 °C, showed 70.4% lysis of clot. On the other hand, distilled

water when treated as negative control, showed negligible lysis of clot (3.62%). The mean difference in percentage of clot lysis between positive and negative control was found to be statistically significant. In this study, the crude methanol extract and its chloroform soluble fractions exhibited 32.5 and 27.5% clot lysis, respectively (Table-2).

During screening for antimicrobial activity, all the samples exhibited moderate antimicrobial activity (zone of inhibition = 10.0-20.0 mm) against the test organisms(Table 3). The crude methanol extracts exhibited significant antimicrobial activity against *Bacillus subtilis* (17.0 mm), *Aspergillus niger* (17.0 mm), *E.coli* (16.0 mm), *V. parahemolyticus* (16.0 mm) while the *n*-hexane soluble fraction showed maximum activity against *Bacillus subtilis* (16.0 mm). Among all the samples, the carbon tetrachloride soluble fraction displayed strong antimicrobial activity against *E. coli* (20.0 mm). The chloroform and aqueous soluble fraction of the crude methanol extract revealed little activity against the test organisms.

Table 3: Antimicrobial activity of *T. acuminate* extractives at 400 µg/disc.

Test microorganisms	**Diameter of zone of inhibition (mm)**					
	CME	**HSF**	**CTSF**	**CSF**	**AQSF**	**Ciprofloxacin**
Gram positive bacteria						
Bacillus megaterium	13	11	12	10	12	40
B. cereus	14	14	15	12	14	40
B. subtilis	17	16	15	11	10	42
Sarcina lutea	15	13	13	12	11	41
Staphylococcus aureus	13	13	15	12	13	41
Gram negative bacteria						
Escherichia coli	16	12	20	12	14	40
Pseudomonas aeruginosa	15	10	12	11	10	42
Salmonella Paratyphi	15	11	10	10	12	40
Salmonella Typhi	14	12	11	10	12	38
Shigella boydii	13	13	16	10	13	41
Sh. dysenteriae	14	13	16	13	10	42
Vibrio mimicus	13	15	15	12	12	40
V. parahemolyticus	16	14	16	12	10	41
Fungi Fluconazole						
Aspergillus niger	17	13	12	10	12	40
Candida albicans	13	10	12	12	11	42
Sacharomyces cerevisiae	12	10	14	13	12	40

CONCLUSION

In conclusion, we can say that *Tiliacora acuminata* Miers contains chemical constituents having antioxidant, cytotoxic, thrombolytic and antimicrobial activity. This could provide a rationale for traditional uses of this plant and suggests for further investigation and isolation of biologically active constituents responsible for the activity.

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