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Evaluation of anti-arthritic, thrombolytic and cytotoxic activities of methanolic and ethanolic extract of *Macaranga denticulata* leaves

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Abstract
For the evaluation of potential anti-arthritic, thrombolytic and cytotoxic activities of methanolic and ethanolic leaf extract of *Macaranga denticulata,* bovine serum albumin induced anti-arthritic method, clot lysis method and brine shrimp lethality bioassay was performed. In the case of protein denaturation, methanolic and ethanolic leaf extract of *M. denticulata* showed 68.40±0.92%* and 63.35±1.22%* whereas standard diclofenac sodium exhibited 90.58±0.82% at 1000 µg/ml (highest conc.) respectively. At lowest concentration (31.25 µg/ml), methanolic and ethanolic leaf extract demonstrated 26.31±0.31%* and 22.44±0.42%* whereas diclofenac sodium showed 51.75±0.92 respectively. Both methanolic and ethanolic leaf extract exhibited promising thrombolytic activity which were 38.23±0.88*% and 33.52±1.64**% respectively whereas standard streptokinase showed 63.54±2.61%**. Methanolic and ethanolic leaf extract of this plant showed significant cytotoxic activity. LC_{50} value of methanolic leaf extract was 74.30 µg/ml and ethanolic leaf extract was 97.77 µg/ml whereas 12.59 µg/ml cytotoxic effect was observed for the standard vincristine sulphate.

Keywords: *Macaranga denticulata*, Protein denaturation, bovine serum albumin, streptokinase, vincristine sulphate.

Introduction
Over twenty five percent of modern medicines that are commonly used worldwide contains compounds extracted from medicinal plants [1]. A report from World Health Organization states that 80% of the world populations rely chiefly on indigenous medicine and majority of traditional therapies involves the use of plant extracts or of their active constituents [2]. A significant amount of bioactive compounds, which provide desirable health benefits are seen to be contained by Plant-based medicines. The active principles differ from plants to plants due to their biodiversity and produce a definite physiological action on the human body that develops interest on their medicinal properties [3]. In recent years, there has been a revival in the use of traditional medicinal plants and therefore, pharmaceutical companies are investing a lot of money in developing natural products extracted from plants [4]. In Bangladesh thousands of plant species are known to have medicinal value [5] and a lot of medicines are discovered from medicinal plants. *Macaranga denticulata* Muell. Arg. (Euphorbiaceae) is a low evergreen tree and also a common pioneer species in moist open areas and secondary forests [6]. *M. denticulata* is used as a fallow enriching species by Karen hill tribe farmers In the mountains of Northern Thailand [7]. In Bangladesh, it is widely distributed in the forests of Chittagong and Chittagong hill tracts. Fresh or dried leaves of some *Macaranga* species are found to be used to treat swellings, cuts, sores, boils and bruises by traditional healers in folk medicine [8]. The juice of the leaves and flowers is administered in cases of constipation, mucous stool and colic in Jointiapur of Sylhet described by Yusuf *et al*. A phytochemical review of literatures indicates the genus *Macaranga* contains high amount of isoprenylated, farnesylated and geranylated flavonoids and stilbenes. Furthermore, more classes of secondary metabolites like terpenes, tannins, coumarins and other types of compounds are known to be isolated from different species of the genus *Macaranga*. Flavonoids and stilbenes are regarded as the major constituents and are most likely responsible for most of the activities found in the plants of this genus. Notable phytochemical constituents such as 3-acetylaleuritolic acid, oleanolic acid, macarangin, scopoletin, β-sitosterol, stigmasterol were isolated from *M. denticulata* [9]. The main purpose of this comparative study is to identify the anti-arthritic, thrombolytic and cytotoxic activities of methanolic and ethanolic extract of *Macaranga denticulata*.

Materials and Methods

Collection and proper identification of plant

Macaranga denticulata was collected from Chittagong Hill Tracts area, Chittagong. The sample was identified by Dr. Shaikh Bokhtear Uddin, Associate Professor, Department of Botany, University of Chittagong.

Preparation of extract

Plant extract was dried and ground (Moulinex Blender AK-241, Moulinex, France) into powder (40 to 80 mesh, 500 g) and soaked for 7 days with 2 to 3 days interval both in 2.0 L of methanol and ethanol at room temperature (23 ± 0.5 °C). Filtrate obtained through cheese cloth and Whatman filter paper No. 1 was concentrated under reduced pressure at a temperature below 50 °C using a rotary evaporator (RE 200, Sterling, UK). The extracts (yield 4.4 to 5.6% W/W) were all placed in glass petri dishes (90 × 15 mm, Pyrex, Germany). A 100 mg each of the extracts was suspended in 10 ml distilled water and the suspension was shaken vigorously by using a vortex mixer. In this way, the concentration (10 mg/ml) of both methanolic and ethanolic extracts were prepared for screening the anti-arthritc, thrombolytic and cytotoxic properties. The crude methanol extract was suspended with distilled water (150 ml) and partitioned with ethanol and chloroform. The resultant partitionates i.e. ethanol was used for the biological screenings.

Chemicals and reagents

The chemicals used were bovine serum albumin (BSA), diclofenac sodium, absolute methanol and ethanol (99.50%) and vincristine sulfate (VS) were purchased from Sigma-Aldrich, Munich, Germay. Lyophilized Streptokinase vial (Durakinase, Dongkook Pharma. Co. Ltd, South Korea) of 15 00000 I.U., 5 ml sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100 µl (30,000 I.U) was used for in vitro thrombolysis. All chemicals in this investigation were of analytical reagent grade.

Inhibition of protein denaturation

For the evaluation in vitro anti-arthritic activity of *M. denticulata*, the method used was "inhibition of protein denaturation" with diclofenac sodium as a standard [10-13]. The test solution (0.5 ml) consists of 0.45 ml. of bovine serum albumin (5% w/v aqueous solution) and 0.05 ml of methanol and ethanol extract of *M. denticulata*. The control solution (0.5 ml) consists of 0.45 ml of bovine serum albumin (5% w/v aqueous solution) and 0.05 ml of distilled water. Product control (0.5 ml) consists of 0.45 ml of distilled water and 0.05 ml of methanolic and ethanolic extract of *M. denticulata*. Standard solution (0.5 ml) consists of 0.45 ml of bovine serum albumin (5% w/v aqueous solution) and 0.05 ml of diclofenac sodium. Various concentrations (31.25, 62.5, 125, 250, 500, and 1000 µg/ml) of both methanol and ethanol extracts of *Macaranga denticulata* (MD) and diclofenac sodium (standard) were used respectively. All the solutions were adjusted to pH 6.3 using 1 N HCL. Samples were kept in incubator at 37 °C for 20 min and the temperature was increased to keep the samples at 57 for 3 min. 2.5 ml of phosphate buffer was added to the previous solutions after cooling. The absorbance was determined by UV-Visible spectrophotometer at 416 nm. The control represents 100% protein denaturation. The results were compared with diclofenac sodium used as a standard. The percentage inhibition of protein denaturation of different concentrations is tabulated in Table 1.

The percentage inhibition of protein denaturation can be calculated as:

% inhibition = [100 - (OD of test solution - OD of product control)] ×100

Where OD = optical density.

The control represents 100% protein denaturation. The results were compared with diclofenac sodium [14, 15].

Thrombolytic activity

Blood specimen

Total blood (2 ml) was drawn from healthy human volunteers (n = 5) without a history of oral contraceptive or anticoagulant therapy. A 500 µl of blood was transferred to each of the three previously weighed microcentrifuge tubes to form clots.

Clot lysis

Experiments for clot lysis were carried as reported earlier [16]. Briefly, 2 ml venous blood drawn from the healthy volunteers was distributed in three different pre weighed sterile microcentrifuge tube (0.5 ml/tube) and incubated at 37 °C for 45 min. After clot formation, 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 microcentrifuge tube containing pre-weighed clot, 100 µl of methanol and ethanol extracts of *M. denticulata* were added separately. 100 µl of streptokinase as positive control and 100 µl of distilled water as negative control were used and separately added to the marked control tubes. All the tubes were then incubated at 37 °C for 90 min and observed for clot lysis. After incubation, fluid released was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference in weight before and after clot lysis was expressed as percentage of clot lysis as shown below:

Percent (%) of clot lysis = (Weight of released clot /clot weight) ×100

The experiment was repeated with the blood samples of the 5 volunteers [17, 18].

Cytotoxicity screening

Assesment of cytotoxic effect of methanolic and ethanolic extracts were performed by brine shrimp lethality bioassay, which is mostly used for bioactive compound screening [19, 20]. In this experiment *Artemia salina* used as zoological organism. At first, eggs of brine shrimps were collected from an aquarium shop (Dhaka, Bangladesh). Then brine shrimps were hatched in artificial seawater (3.8% NaCl solution) for 48 h and developed from brine shrimp eggs to larval shrimp (nauplii). Meyer's method was used for this cytotoxic assessment of brine shrimp nauplii. Tested samples (methanolic and ethanolic extracts) were prepared by dissolving them in dimethyl sulfoxide which was not more than than 50 µL in 5 mL solution. Seawater (3.8% NaCl solution) was added so that concentrations of 10, 50, 100, 150, 200, 300 and 500 µg/mL were attained. After that, a vial with 5 mL dimethyl sulfoxide was taken and used as control. Vincristine sulphate (standard drug) was used as positive control. Mature shrimps were placed in each of the experimental vials. Then vials were inspected after 24 h by using magnifying glass and the number of surviving nauplii in each vial was counted. Those data represented the percentage of lethality of brine shrimp nauplii, from which each concentration can be evaluated by using the formula below:

$$\%\ of\ mortality = \frac{Nt}{No} \times 100$$

Where, Nt is the number of dead nauplii after 24-hour incubation; No is the total number of nauplii transferred, i.e., 10.

The LC_{50} was determined from the log concentrations versus percentage of mortality curve [17].

Statistical Analysis

The results were expressed as mean of the three repetitions and standard deviations as well as standard errors were calculated. Statistical comparisons were made using the Independent t-test and *P<0.05 was considered as significant for the anti-arthritic activity. Statistical significance between % of clot lysis by streptokinase and plant extracts was performed by paired t-test analysis. All of the tests were performed by using the software SPSS version 20.0 (SPSS for Windows, IBM Corporation, New York, USA). Expression of data was expressed as mean ± SD. The mean difference between positive and negative controls was considered significant at *P<0.05, **P<0.001.

Results and Discussion

Anti- arthritic activity

Different concentrations of methanolic and ethanolic leaf extracts of *M. denticulata* and diclofenac sodium were tested for anti-arthritic activity and found significant percentage inhibition in protein denaturation (Table 1). In this study, methanolic and ethanolic extract of *M. denticulata* showed 26.31±0.31* and 22.44±0.42*, where the standard drug diclofenac sodium showed 51.75±0.92 of inhibition at 31.25 µg/ml. Methanolic and ethanolic leaf extract of *M. denticulata* exhibited 68.40±0.92* and 63.35±1.22* inhibition, while the diclofenac sodium exhibited 90.58±0.82 inhibition of protein denaturation at 1000 ug/ml.

Table 1: Percent of inhibition of protein denaturation of two fraction of *M. denticulate*

Percent of inhibition of protein denaturation			
Conc.	**MD (MeOH)**	**MD (EtOH)**	**Diclofenac Na**
31.25	26.31±0.31*	22.44±0.42*	51.75±0.92
62.5	30.98±0.65*	27.80±0.64*	60.55±1.01
125	42.02±0.79*	36.30±0.85*	69.44±0.29
250	49.11±0.95*	42.52±1.19*	76.25±0.96
500	57.60±1.33*	52.43±0.56*	83.76±1.33
1000	68.40±0.92*	63.35±1.22*	90.58±0.82

Values are expressed as mean±SEM of three replicate (n=3). *P<0.05

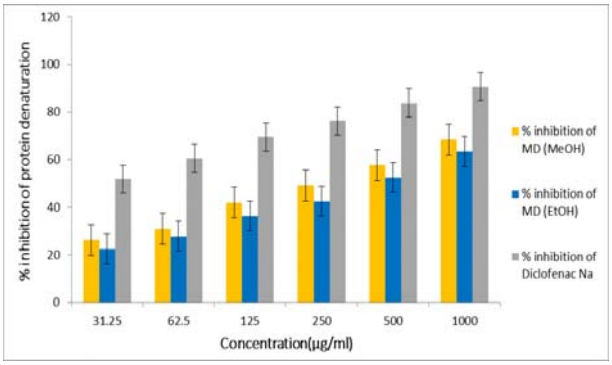

Fig 1: Inhibition of protein denaturation (%) of fractions of *M. denticulata* leaf and Diclofenac sodium

Thrombolytic activity

Addition of 100 µl streptokinase (positive control) to the clots along with 90 minutes of incubation at 37 °C, showed 63.54 ± 2.61% clot lysis. However, distilled water (negative control) treated-clots showed only negligible clot lysis (4.21 ± 0.73%). The mean difference in clot lysis percentage between positive and negative control was very significant (**P<0.001). Treatment of clots with *M. denticulata* methanolic and ethanolic leaf extracts provided the clot lysis 38.23 ± 0.88*% and 33.52 ± 1.64**%, respectively. (*P<0.05, **P<0.001).

Table 2: Effect of both fractions (10 mg/ml) on in-vitro clot lysis

Treatment	% of clot lysis (Mean ± S. D.)
Streptokinase (Positive Control)	63.54 ± 2.61**
Distilled water (Negative Control)	4.21 ± 0.73**
MD (MeOH)	38.23 ± 0.88*
MD (EtOH)	33.52 ± 1.64**

*MD= *Macaranga denticulata*, MeOH= Methanol, EtOH= Ethanol, Positive control= Streptokinase, Negative control= Distilled Water. Statistical representation was performed by using paired t-test. Percentage of clot lysis is represented as mean ± S.D. *P<0.05, **P<0.001.

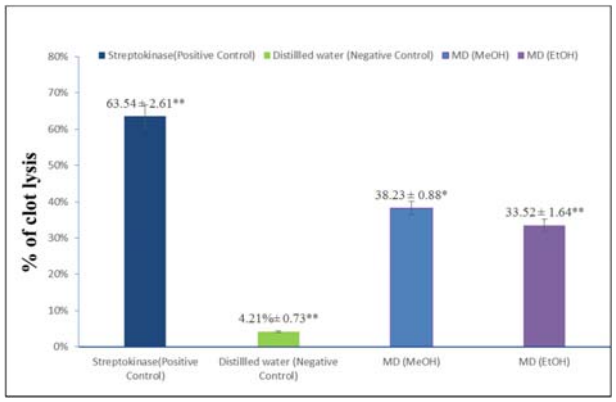

Fig 2: Clot lysis by streptokinase, water and two fraction of *Macaranga denticulata*. Effects of drugs on dissolution of clots prepared from blood of normal individuals

Cytotoxic activity

Brine shrimp lethality bioassay was used to evaluate the cytotoxic activity of methanolic and ethanolic fractions of *M. denticulata* leaves. Following the procedure of Meyer, the lethality of methanolic and ethanolic extracts of *M. denticulata* leaves were determined on Artemia salina after sample exposure for 24 h. Determination of the cytotoxic effect of the extracts was conducted by making comparison between negative control (dimethyl sulfoxide only) and positive control (vincristine sulphate). Table 3 represents the percentage of mortality of brine shrimp caused by the plant extracts at six different concentrations (10 to 500 µg/mL) of the extracts. It was precise that the percentage of lethality was directly proportional to the concentrations of extracts. LC_{50} values of methanolic and ethanolic extracts of *M. denticulata* obtained in the present experiment were 74.30 and 97.77 µg/mL, respectively. In case of vincristine sulphate (positive control), the LC_{50} value was 12.59 µg/mL. However, no mortality was obtained from the negative control.

Table 3: Percent of mortality of the extract at six concentrations

Percent of mortality of *M. denticulata*				
Conc. (µg/ml)	**Log C**	**(MeOH)**	**(EtOH)**	**Vincristine Sulphate**
10	1	20	20	40
50	1.699	40	30	80
100	2	70	50	100
200	2.301	80	100	100
300	2.477	100	100	100
500	2.699	100	100	100
LC_{50}		74.30	97.77	12.59

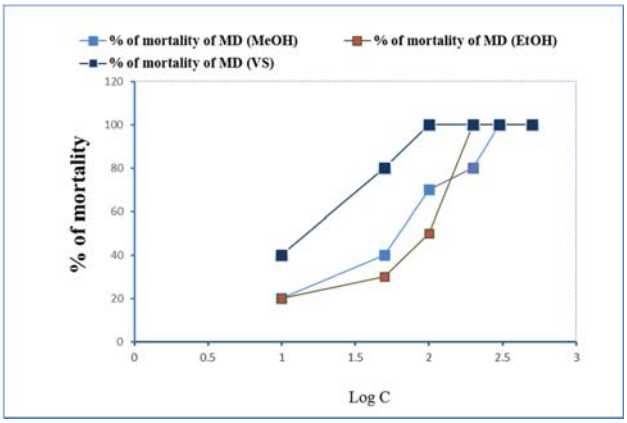

Fig 3: Brine shrimp lethality bioassay. Determination of LC_{50} values for methanol and ethanol extract of *M. denticulata* between log concentrations versus percent of mortality

In conclusion, methnolic and ethanolic extract of *Macaranga denticulata* leaf possess significant anti-arthritic, thrombolytic and cytotoxic activities in vitro. It would be fascinating to investigate the mechanism underlying percentage of inhibition of protein denaturation, clot lytic effects and cytotoxic activity demonstrated by the two fractions of *M. denticulata* extract. However, these activities might be due to the presence of bioactive or inhibitory compounds or synergism by the existence of some compounds. A lot of constituents might be present in this extract, such as tannin, polyphenols flavonoids, alkaloids, saponin and so on [21]. In addition, comprehensive research are required to figure out the specific activity of anti-arthritic, thrombolytic, and cytotoxic compounds present in these methanolic and ethanolic leaf extracts.

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Conflict of interest statement
Authors have none to declare.

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