



Phytochemical screening and *in vitro* pharmacological activities of methanolic leaves extract of *Caryota mitis*

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Academic Editor: Dr. Md. Abdul Hannan, Dongguk University, South Korea.

Received: 04 January 2020; Accepted: 29 February 2020; Published: 02 May 2020.

ABSTRACT: The present study design to assess the qualitative phytochemical constituents responsible for anti-inflammatory, thrombolytic and cytotoxic activity of methanol extract of *Caryota mitis* leaves. The freshly collected *Caryota mitis* leaves extracted with methanol (MECM) to evaluate the secondary plant metabolites along with anti-inflammatory activity by protein denaturation assay. Thrombolytic and cytotoxic activity investigated using human blood clot lysis and brine shrimp lethality bioassay, respectively. The MECM exhibited several secondary plant metabolites named alkaloid, glycoside, protein, flavonoid, reducing sugar, saponins, and phenolic compound. The MECM extract (62.5-1000 µg/mL) exhibited an extremely significant ($P < 0.0001$) 6.06-45.45 % inhibition of protein denaturation against positive control diclofenac sodium (43.11-85.48%). In thrombolytic, the MECM exhibited 24.29 % significant ($P < 0.0001$) protection against blood clotting, whereas the positive control streptokinase (75.35%). In the cytotoxic study, a significant ($P < 0.05$) percentage of mortality exhibited while 70% mortality of Brine shrimp after 24 h at a concentration of 1000 µg/mL with an LC₅₀ value of 550.57 µg/mL whereas the positive control vincristine sulfate (1.63 µg/mL). The current results suggesting MECM has promising anti-inflammatory, thrombolytic activities with weak cytotoxicity.

KEYWORDS: *Caryota mitis*, anti-inflammatory, thrombolytic, cytotoxicity.

INTRODUCTION

Although the role of inflammation in the pathophysiology of arterial thrombosis has revealed in the last decade, the relationship between them is rarely known. Recently, inflammation has acknowledged as a potential system through which distinctive risk factors trigger thrombus formation in veins. A conventional activation pathway followed for inflammation and coagulation, whereas the compositions of thrombus in vein cause inflammation in the vessel wall. Activation of endothelial cells, platelets, and leucocytes leads to the development of thrombus, with the initiation of inflammation and development of microparticles that trigger the coagulation system through the enlistment of

a tissue factor. In this manner, the critical start of venous thrombus development is most presumably vein wall inflammation [1]. C-reactive protein (CRP) is an acute stage reactant plasma protein that is available in plasma of healthy humans, whereas a significant plasma concentration increases at acute and chronic inflammation [2]. According to several reports, the CRP concentration independently associated with myocardial infarction [3]. Plasma CRP is inadequately associated with atherosclerosis [4]. According to the report, the CRP plays a vital role in the human body by regulating the blood function, platelets, the fibrinolytic system, etc. CRP dependent on inflammation and thrombosis is functioning in two directions due to activation of the blood clotting system (platelets), which regulates CRP structure and genetic function [5, 6].

The brine shrimp lethality bioassay for cytotoxicity is quick (24 h), basic (e.g., no aseptic strategies are required), easy to understand, reasonable, and requires limited quantities of test material [7]. The bioassay has a correlation with cytotoxic effect in solid tumors of humans and with pesticide activity [7, 8]. This test was recommended by (Michael *et al.* 1956) and changed by others. Since its recommendation, this *in vivo* lethality test has progressively utilized for giving a screening for the specific advanced bioassays once the active compounds have been isolated [9].

Traditional medicine has been utilized by most of the population for an extended period. The World Health Organization (WHO) stated that an expected 80 % of the people in a developing country relies upon medicinal plants for their essential therapeutic services [10]. *Caryota mitis* belongs to the *Arecaceae* family, which locally known as fishtail palm. These palm fruits and roots reported to use in folk medicine in Bangladesh and India as constipation, to stop vomiting, stomach aches, piles and arthritis [11, 12]. According to the literature review, the *C. mitis* exhibited antimicrobial and antioxidant activity [13, 14] and there is no report of thrombolytic, anti-inflammatory and cytotoxic activity. So, the present study proves the thrombolytic, anti-inflammatory and cytotoxic activity of methanolic extract of *C. mitis* leaves.

MATERIALS AND METHODS

Chemicals

The chemicals used in the experiment include methanol, sodium citrate, dextrose, citric acid, hydrochloric acid, sodium chloride, albumin, diclofenac sodium from Merck (Mumbai, India) through Taj Scientific Ltd. Bangladesh. UV visible spectrophotometer (Shimadzu, Japan) used to measure the absorbance. Lyophilized streptokinase vial (1500000 IU) obtained from Square Pharmaceuticals Ltd, Bangladesh, and Vincristine sulfate (1 mg/vial) was procured from Beacon Pharmaceuticals Ltd. Bangladesh.

Identification and preparation of plant extract

Leaves of *C. mitis* were gathered from Sitakunda, Chattogram and marking off done by Dr. Shaikh Bokhtear Uddin, Professor, Department of Botany, University of Chittagong, Bangladesh. 5 kg of leaves collected and separated all unwanted plant parts. The collected leaves dried for 15 days and dried into a coarse powder. For extraction, 500 gm of the powder submerged in 2.5 ml methanol and vigorously shaken

for ten days. The plant extract filtrated by using Whatman filter paper (#1) followed by evaporation in a water bath (40 °C). A greenish color of semisolid acquired with a percentage of 7.64 yields of methanolic extract of *C. mitis* leaves.

Phytochemical screening

The methanol extract of *C. mitis* leaves qualitative phytochemical screening was carried out by standard method to evaluate the alkaloid, carbohydrate, glycoside, protein, oxalate, cholesterol, steroid, reducing sugar, tannin, flavonoid, saponins and phenolic compound [15].

Inhibition of protein denaturation

The anti-inflammatory activity of methanolic extract of *C. mitis* (MECM) evaluated by albumin denaturation technique followed the previously described method with minor modifications [16, 17]. The test solution (0.5 mL) included test samples (0.05 mL) and 5% w/v aqueous solution of albumin (0.45 mL). The test control solution (0.5 mL) included of 0.45 mL of albumin solution (5% w/v aqueous solution) and 0.05 mL of distilled water. 5% albumin solution prepared by using, 5 gm albumin in 100 ml distilled water (w/v solution). Different concentrations (62.5, 125, 250, 500 and 1000 µg/mL) MECM and diclofenac sodium were taken. All solutions were adjusted to pH 6.3 using 1N HCl. The samples were incubated at 37 °C for 20 min and afterward kept at 57 °C for 30 min. After cooling, 2.5 mL of phosphate buffer was added and the turbidity measured at 416 nm in a UV-visible spectrophotometer. The control represents all the ingredients except the extract or standard solution. The percentage of inhibition of protein denaturation was calculated as follows:

$$\text{Percentage inhibition of protein} = [(A_c - A_s) / A_c] \times 100$$

Where A_c =absorbance of control and A_s =absorbance of sample.

Thrombolytic activity

Thrombolytic activity test was implemented by Prasad *et al.* [18, 19]. As a stock solution, lyophilized streptokinase vial (1500000 IU) mixed adequately with 5 mL sterile distilled water from which appropriate dilution made. Form healthy volunteers (n=5), 5mL of blood withdrawn by syringe and distributed 0.5 mL per pre-weighted eppendorf tube. At 37 °C for 45 minutes, the eppendorf tubes incubated for the formation of the clot. If any serum formed in the eppendorf tube along with clot removed the serum without disrupting the clot

and again reweighed the eppendorf tube for calculating the clot weight where 100 μ L plants extract of *C. mitis* (10 mg/mL) added. 100 μ L streptokinase (positive control) and 100 μ L distilled water (negative control) were added, respectively and incubated at 37 °C for 90 minutes. After incubation, observed clot lysis and released fluid was removed and reweighed the tube to calculate the difference in weight after clot disruption.

% of clot lysis = (weight of clot after remove of fluid/clot weight) \times 100

Brine shrimp lethality bioassay

The cytotoxicity activity evaluated by brine shrimp lethality bioassay by using *Artemia salina* organisms (shrimp eggs). In the artificial seawater (3.8% NaCl solution), the shrimp eggs hatched for 48 hours for maturing the shrimp called nauplii. The extract serially diluted by using artificial seawater (3.8% NaCl in water) at concentrations of 31.25, 62.5, 125, 250, 500, and 1000 μ g/mL. Vincristine sulfate used as a positive control, whereas a serial concentration dilution 0.25, 0.5, 1, 5, and 10 μ g/mL used. Ten of the nauplii separately distributed in the premeasured concentration and observed for 24 hours. After 24 hours, count the number of living nauplii in each concentration and recorded [20, 21].

% of mortality = $(N_0 - N_1 / N_0) \times 100$

Where, N_0 = the number of nauplii taken; N_1 = the number of nauplii alive.

Statistical analysis

Results represented as the mean \pm SEM (standard error mean). The intergroup comparison was performed using the GraphPad Prism 6 through t-test of one-way ANOVA. P-value (<0.0001) was considered statistically significant.

RESULTS

Phytochemical analysis revealed the presence of secondary metabolites

The qualitative phytochemical analysis revealed several secondary metabolites such as alkaloid, glycoside, protein, flavonoid, reducing sugar, saponins, and phenolic compound. The presence of carbohydrates also exhibited in the Molish test, but the Benedict test not shown carbohydrates (Table 1).

Table 1. Result of phytochemical screening of methanolic extract of *C. mitis* leaves

Test groups	Name of the test	Result
Alkaloid	Wagner test	++
	Mayer test	
Carbohydrate	Molish test	++
	Benedict test	
Glycoside	Glacial acetic acid test	++
	Nitric acid test	+
Oxalate	Alcoholic test	-
Cholesterol	Acetic anhydride Test	-
Steroid	Salkowski reaction	-
Reducing Sugar	Fehling's Test	+
Tannin	Ferric chloride test	-
Flavonoid	Hydrochloric acid test	+
Saponins	Foam test	+
Phenolic Compound	Ferric chloride test	+

(+) = Present; (++) = Rapidly Present; (-) = Absent

Effect of the extract on protein denaturation in vitro

The anti-inflammatory activity evaluated by the protein denaturation method, which summarized in Figure 1. The result exhibited an extremely ($P < 0.0001$) significant inhibition of protein denaturation of *C. mitis* vs. positive control Diclofenac-Na. The anti-inflammatory activities showed a dose-dependent manner activity. The MECM showed 6.06 ± 0.29 , 9.09 ± 0.47 , 19.69 ± 0.39 , 31.81 ± 1.16 and 45.45 ± 1.73 % inhibition of protein denaturation at 62.5, 125, 250 500 and 1000 μ g/mL, respectively, whereas the positive control diclofenac-Na showed maximum 85.48 ± 0.52 % inhibition at a dose of 1000 μ g/mL.

Effect of the extract on clot lysis activity

The results of the clot lysis activity of extracts summarized in Figure 2. The positive control Streptokinase showed an extremely significant ($P < 0.0001$) percentage of clot lysis 75.35 ± 3.38 , whereas the negative control water exhibited negligible clot lysis (3.78 ± 0.53 %). After the treatment of clots with 100 μ L MECM, a significant ($P < 0.0001$) 24.29 ± 1.99 % clot lysis observed.

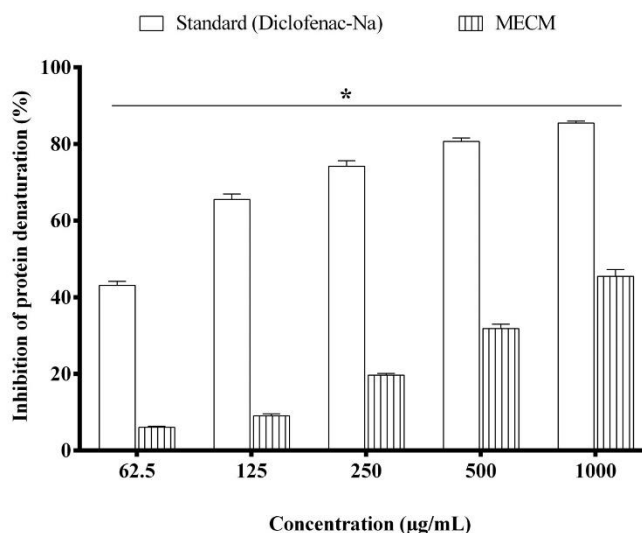


Figure 1. Percentage of inhibition of protein denaturation of anti-inflammatory activity by *C. mitis* leaves at different concentration. Results were represented as the mean \pm SEM. (n = 3). * P < 0.0001 was statistically significant in comparison to Diclofenac Na followed by t-test of one-way ANOVA (GraphPad Prism 6). MECM= Methanolic extract of *C. mitis*.

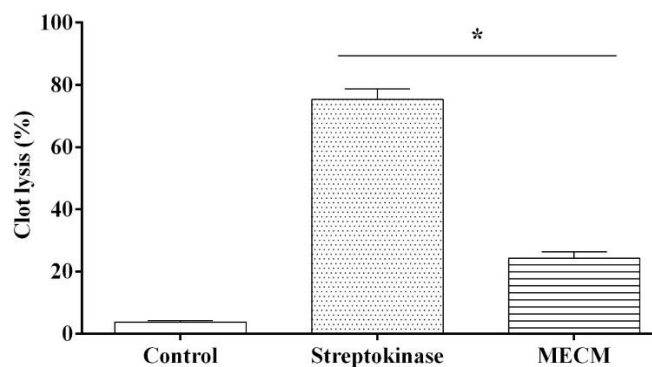


Figure 2. Percentage of clot lysis of human blood by *C. mitis* leaves and positive control streptokinase. Results were represented as the mean \pm SEM. (n = 5). * P < 0.0001 was statistically significant in comparison to negative control water followed by t-test of one-way ANOVA (GraphPad Prism 6). MECM= Methanolic extract of *C. mitis*.

Effect of the extract on the mortality of brine shrimp

Brine shrimp lethality bioassay followed to evaluate the cytotoxicity, which results is summarized in Figure 3. The percent of mortality is significantly (P < 0.05), decreasing with the lowering of concentration in both treatments. The LC₅₀ values of MECM and vincristine sulfate (VCS) were 550.57 µg/mL and 1.63 µg/mL, respectively. MECM is weakly toxic because it exhibited the LC₅₀ between 500-1000 µg/mL.

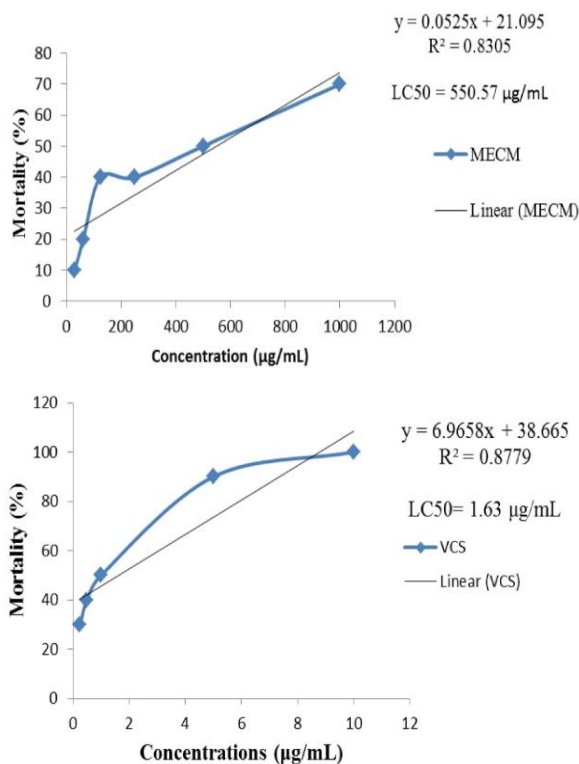


Figure 3. Percentage of mortality of brine shrimp of *C. mitis* leaves and positive control at different concentrations. MECM= Methanolic extract of *C. mitis*, VCS= Vincristine sulfate.

DISCUSSION

Medicinal plants are a potential source of bioactive compounds and have been utilized as a crude material or as pure compounds for treating different diseases [22]. The methanolic extract revealed the presence of alkaloid, glycoside, protein, flavonoid, reducing sugar, saponins, carbohydrates and phenolic compound. They may help to quantitative estimation and in finding the pharmacologically active substance by qualitative phytochemical tests, which are vital and helpful in discovering chemical constituents in the plant material.

Inflammation is a complex biological reaction of vascular tissue to harmful stimuli, pathogens, pain, warmth and swelling [23]. The prolongation of inflammation leads to atherosclerosis, fever and heart problems [24, 25]. Inflammation is well documented due to the denaturation of protein [26]. According to the literature report, denaturation of protein leads to rheumatoid arthritis because of the production of auto-antigens in arthritic diseases [27]. The Mechanism of denaturation is intricate in the modification of electric force, hydrogen, hydrophobic and disulfide bonds. Anti-inflammatory drugs reported having a dose-dependent ability to inhibit the protein denaturation [28]. From the

results, the methanolic extract of *C. mitis* leaves capable of inhibiting protein denaturation, whereas the maximum 45.45 % inhibition exhibited at 1000 µg/mL while the positive control 85.48 %. The extract revealed a dose-dependent manner effect. This effect may be due to the presence of secondary metabolites such as alkaloid and flavonoid [29] and also validated the traditional use of *C. mitis* in folk medicine as arthritis [11, 12].

Thrombogenicity of the atherosclerotic plaque is resolved by the immobility of a fibrous cap and substance of tissue factor in its center, which initiates the coagulation when exposed to blood flow. These components connect with one another and with the blood vessel and under biological circumstances, the blood flow to tissues is unimpaired by coagulating or clotting [30]. Tissue plasminogen activator (tPA), streptokinase (SK) and urokinase-type plasminogen activator (uPA) are thrombolytic drugs available in the market which act by dissolving the blood clot formation by converting plasminogen to plasmin and increased clot lysis [31]. Our result suggests that the methanolic extract of *C. mitis* exhibited an extremely significant ($P < 0.0001$) clot lysis (24.29 %) compared to negative control water (3.78 %), whereas the positive control streptokinase (75.35 %). This effect may be due to the presence of flavonoids which affect embolus and cardiovascular disease by interfering with platelet activation [32] or due to the presence of phenolic compounds [31].

The brine shrimp test characterizes as fast, economical and basic bioassay for testing the plant extract lethality which connects the cytotoxicity and antitumor properties. Yet, a typical biological reaction isn't because of one component instead because of several mixtures of bioactive plant components. Hence, the screening of plant extract is necessary to evaluate the biological activity. The brine shrimp lethality bioassay has demonstrated to be a helpful framework for checking the biological reaction of natural plant products [33]. The lethality of plant extracts directly proportional to the concentration with an LC_{50} of 550.57 µg/mL. This effect may be due to the presence of alkaloids indicates cytotoxic properties [34-48].

CONCLUSIONS

The present study suggests that the methanolic leaves extract of *C. mitis* exhibited secondary metabolites, which also exert weak toxicity. The methanolic extract of *C. mitis* leaves have shown an extremely significant concentration-dependent anti-inflammatory effect on bovine serum albumin. Moreover, the extract

demonstrated a significant thrombolytic activity on human blood clot lysis. The present study of *C. mitis* concludes as the potential source for the potent bioactive substances, especially anti-inflammatory and clot lysis activities as summarized (Figure 4). Further advance study required to identify the mechanism for pharmacological properties.

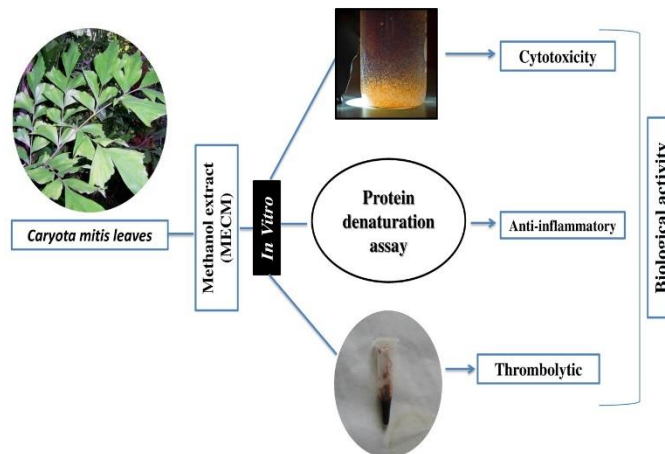


Figure 4. Graphical representation of pharmacological activities of methanolic leaves extract of *C. mitis* through in vitro approach.

ACKNOWLEDGEMENT

Authors are very much thankful to the Department of Pharmacy, International Islamic University Chittagong, Bangladesh for research facilities and other logistic supports.

FUNDING

This work is conducted with the individual funding of all authors.

AUTHOR CONTRIBUTIONS

MRT and AMT together planned and designed the research. MAS arranged the whole facilities for the research and supervised the whole research. IJ, SAS, MHM and MS conducted the entire laboratory works with MRT and AMT. AMT and TBE imparted in study design and interpreted the results putting efforts on statistical analysis and also participated in the manuscript draft and has thoroughly checked and revised the manuscript for necessary changes in format, grammar and English standard. All authors read and agreed on the final version of the manuscript.

CONFLICTS OF INTEREST

Authors declared that they have no conflict of interest.

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