

***In vitro* thrombolytic and cytotoxic evaluation of *Mentha arvensis* L., *Mentha spicata* L. and *Mentha viridis* L.**

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Abstract: Atherothrombotic diseases or coronary artery thrombosis are common disorders which are treated by streptokinase (SK), urokinase (UK) or tissue plasminogen activators (t-PA). Because of the high risk of bleeding, severe anaphylactic shock, intracranial hemorrhage and lacks of specificity of these drugs, they are restricted to those patients who have undergone surgery or those with a history of gastrointestinal bleeding or hypertension. Therefore, plant based drugs are used because they are cheap, safe, low side effects and effective against many diseases. The study was carried out to check the clot lysis effect and cytotoxic effects of *Mentha arvensis* L., *Mentha spicata* L. and *Mentha viridis* L. using streptokinase as a positive control and water as a negative control. In this experiment, the *M. arvensis*, *M. spicata* and *M. viridis* showed 32.56%, 30.89%, 30.29% clot lysis activity in case of methanol extract, 32.04%, 30.37%, 30.02% clot lysis activity in case of ethanol extract, 31.87%, 29.77%, 29.05% clot lysis activity in case of chloroform extract and 30.29%, 28.45%, 27.55% clot lysis activity in case of acetone extract respectively. In brine shrimp cytotoxic assay the methanol extracts *M. arvensis*, *M. spicata* and *M. viridis* showed LC_{50} values of 2.088, 1.964 and 1.812 $\mu\text{g/ml}$, respectively, which was referred to Vincristine sulfate (LC_{50} 1.160). From this study, it can be said that the *M. arvensis*, *M. spicata* and *M. viridis* has clot lysis activity and low cytotoxic activity. So, these plants could be incorporated as a thrombolytic agent with *In vivo* effects to improve the atherothrombotic patients.

Keywords: Thrombolysis, Cytotoxicity, *Mentha arvensis* L., *Mentha spicata* L., *Mentha viridis* L., Streptokinase.

I. Introduction

Atherothrombotic diseases or coronary artery thrombosis are common disorders in the world. Now a days atherothrombotic diseases are treated by streptokinase (SK), urokinase (UK) or tissue plasminogen activators (t-PA) which are also used as a clinical thrombolytic agent for the treatment of severe or myocardial infarction, massive deep venous thrombosis, pulmonary embolism and occluded intravenous or dialysis cannulas [1]. Streptokinase (SK) and urokinase (UK) are widely used in Bangladesh and other developing countries because of their availability and lower cost [2] as compared to other thrombolytic drugs. Although UK and SK are wonder drug [3] but it has high risk of bleeding, severe anaphylactic shock, intracranial hemorrhage and lacks specificity [4]. For these reasons patients who have undergone surgery or those with a history of gastrointestinal bleeding or hypertension have not used these drugs [4]. However, as a result of immunogenicity multiple treatments with SK in a given patient are restricted [5]. Genetically Modified t-PA is effective in acute myocardial infarction, but the action of reperfusion rate is limited and slow. Moreover, the platelet-rich thrombi are highly resistant to lysis by t-PA [6].

During this process, the analysis of the efficacy of plant-based drugs used in the traditional medicine have been paid great attention because they are cheap, have slight side effects and according to WHO still about 80% of the world population trust mainly on plant-based drugs [7]. Herbal products are often perceived as safe because they are "natural" [8]. Bangladesh harbors a large number of medicinal plants, but the therapeutic potential of many of these important medicinal plants are yet to be revealed. Medicinal plants and their formulations are used enormously for treating a range of illness in ethnic medical practices [9]. Considerable efforts have been directed towards the discovery and development of natural products from various plants sources which have antiplatelet [10,11], anticoagulant [12,13], antithrombotic [14], and thrombolytic activity. Epidemiologic studies have provided evidence that foods with experimentally proved thrombolysis effect could reduce the threat of thrombosis. Herbs showing thrombolytic activity have been studied and some important observations have been reported [15].

The plant genus *Mentha* belongs to the family Lamiaceae (Labiatae), and consists of about 25-30 species and mostly used as a medicinal plant in all over the world [16, 17]. The plant is found across Asia, Australia, Africa, Europe, and North America. It has different uses in all over the world. It has used as a medicinal herb which used to treat stomach and chest pain [18]. Most of spearmint and peppermint are used in the toothpastes and other oral products [19]. The essential oils from *Mentha* showed a broad spectrum antibacterial activity (both Gram-positive and gram-negative bacteria) like *Staphylococcus aureus*, *S.*

epidermis, *Bacillus cereus*, *Streptococcus faecalis* and *B. subtilis* etc [20-22]. *Mentha* has anti-fungal property which can kill fungus [23, 24]. It also is reported that insecticidal activity is persisted in *Mentha* oil [25-27]. It belongs to most flavoring agents in the world. Among the species, *M. spicata* has antifungal, antioxidant activity [28]. *M. arvensis* are used in food, drinks, cough medicines, creams and cigarettes, treat flatulence, digestional problems, gall bladder problems etc. It also contains menthol, limonene, menthone, methyl acetate, beta-pinene, beta-caryophyllene, isomenthone, neomenthol, piperitone, alpha-pinene, tannins and flavonoids [29]. *M. viridis* (Spearmint) has carminative, antispasmodic and stimulant characteristics. It has used in gonorrhoea, strangury, gravel and hemorrhoids.

The aim of the study was to investigate herbal preparation (organic extract) of *M. arvensis*, *M. spicata* and *M. viridis* for their clot lysis property (thrombolytic activity) and cytotoxic properties by using *In vitro* models.

II. Materials and Methods

Plant collection and identification

The fresh and healthy whole plants of *Mentha arvensis* L., *Mentha spicata* L. and *Mentha viridis* L. were collected from Khulna region, Bangladesh. The plants were taxonomically classified by the standard taxonomical method.

Extract preparation

The fresh leaves were washed with clean water immediately after collection, then collected leaves were chopped into small pieces, sun dried for about 3 days and grinded into powder with the help of mechanical grinder and stored in a suitable airtight poly bag for the extraction process. The multiple solvent (methanol, ethanol, chloroform and acetone) were used to prepare the extract by the supplier [30]. 150 gm powder was macerated in 1.0 L 95% pure methanol, 99.50% ethanol, 95% chloroform and acetone (Sigma Chemicals Co., USA) for 15 days at room temperature ($25 \pm 2^\circ$ C) in a dark place with occasional stirring. After 15 days, each extract was shaking and filtered gently with Whatman No.1 filter paper. The extract was evaporated through the rotary vacuum evaporator and the temperature kept below 40° C [31]. The concentrated extract was collected in a Petri dish and allowed to air dry for complete evaporation of solvent. The whole process was repeated four times and finally, approximately 16 gm blackish-green colored, concentrated plant extract was obtained for each of the plant extracts, which was preserved in the refrigerator at 4° C.

A 100 mg each of the plant extracts was suspended in 10 ml double distilled water and the suspension was shaken on a vortex mixer and the suspension was kept overnight. The suspension was filtered through a 0.22- μ m syringe filter, which remove the soluble supernatant. To check thrombolytic activity, 100 μ l of this aqueous preparation was added to the microcentrifuge tubes containing the clots. The same concentration (10 mg/ml) of extracts was prepared for screening the cytotoxic properties.

Chemicals and reagents

To the commercially available lyophilized Streptokinase (SK) vial (Durakinase, Dongkook Phama. Co. Ltd, South Korea) of 15,00,000 I.U., 5 ml sterilized deionized water was added and mixed properly. This suspension was used as a stock from which 100 μ l (30,000 I.U) was used for *In vitro* thrombolysis. Absolute ethanol (99.50%), methanol (95%), chloroform (95%), acetone, DMSO and vincristine sulfate (VS) were purchased from Sigma-Aldrich, Munich, Germany.

Blood Specimen

Whole blood (4 ml) was drawn from healthy human volunteers ($n = 20$) without a history of oral contraceptive or anticoagulant therapy (using a protocol approved by the Institutional Ethics Committee of Chittagong University, faculty of medicine) [32]. 500 μ l of blood was transferred to each of the ten previously weighed microcentrifuge tubes to form clots.

Clot lysis

Experiments for clot lysis were carried out as reported earlier [33]. Briefly, 4 ml venous blood drawn from healthy volunteers was distributed in ten different pre weighed sterilized microcentrifuge tube (500 μ l /tube) and incubated at 37° C for 45 minutes. After clot development, 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 holding pre-weighed clot, 100 μ l of methanol, ethanol, chloroform and acetone extract of *M. arvensis*, *M. spicata* and *M. viridis* were added separately. As a negative non thrombolytic control, 100 μ l of distilled water and as a positive control, 100 μ l of Streptokinase were separately added to the control tubes numbered. All the tubes were then incubated at 37° C for 90 minutes and detected for clot lysis. After incubation, fluid was removed and tubes were again weighed to detect the difference in weight after clot disruption. Difference obtained in weight taken before and after clot

lysis was expressed as percentage of clot lysis. The experiment was repeated 20 times with the blood samples of 20 volunteers.

Cytotoxicity assay

Brine shrimp bioassay was carried out with the method as described by Meyer et al. (1982) [34] to investigate the cytotoxicity of the extracts. In a small beaker, 50 mg of the sample was accurately weighed and dissolved in 5ml DMSO (Dimethylsulfoxide) to obtain a solution of 10mg/ml. From this stock, 1ml of the sample was taken and 19 ml water was added to give a final working concentration of 500µg/ml. A 5.0 ml of artificial sea water was added into all the test tubes. Simple zoological organism (*Artemiasalina*) was used as a convenient monitor for cytotoxic screening. The brine shrimp was collected from an aquarium shop (Katabon, Dhaka, Bangladesh) and hatched in artificial seawater (prepared by using sea salt 38 g/L and adjusted to pH 8.5 using 1N NaOH or 1N HCl) under constant aeration for 24 h under the light. The hatched shrimps were allowed to grow by 48h to get shrimp larvae called nauplii. After 48 h, nauplii were then separated from the eggs by aliquoting them in another glass petri dish containing artificial sea water and used for the assay. Suspension containing 10 nauplii was added into each test tube and then plant extract was added at different concentration and was incubated at room temperature (25±1°C) for 12 h under the light. The tubes were then examined after 24h and the number of surviving larvae in each tube was counted with the aid of a 3X magnifying glass. Experiments were conducted along with vincristine sulfate (VS) in a set of three tubes per dose. The concentration that would kill 50% of the nauplii (LC₅₀) was determined from a linear regression equation using “Microsoft Excel-2010”.

Statistical analysis

The significance between % clot lysis by Streptokinase and plant extracts, LC₅₀ values by VS and extracts was tested by the paired t-test analysis using the software Microsoft Excel-2010. Data are expressed as mean ± standard deviation. The main difference between positive and negative control was considered significant at p < 0.05.

III. Results

Thrombolysis test:

The addition of 100µl SK (positive control) to the slits along with 90 min of incubation at 37°C, showed 75 ± 3.04% clot lysis. However, distilled water (negative control) treated-clots showed only negligible clot lysis (3.29 ± 0.58%). The mean difference in clot lysis percentage between positive and negative control was very significant (p value < 0.0009). Treatment of clots with *M.arvensis*, *M.spicata* and *M. viridis* showed 32.56%, 30.89%, 30.29% clot lysis activity in case of methanol, 32.04%, 30.37%, 30.02% clot lysis activity in case of ethanol, 31.87%, 29.77%, 29.05% clot lysis activity in case of chloroform and 30.29%, 28.45%, 27.55% respectively it was statistically very significant (p value < 0.0001) compared to those of both positive control SK and negative control water is shown in Table 1. Percentages of clot lysis obtained after treating the clots with different organic extracts and appropriate controls is shown in Figure-1.

Table 1: Effect of different herbal extracts (10 mg/ml) on invitro clot lysis.

Treatments	% of clot lysis (Mean ± S.D.)	P value when compared to negative control
Streptokinase (Positive control)	75 ± 3.04	< 0.0009
Distilled Water (Negative control)	3.29 ± 0.58	
<i>Mentha arvensis</i> L.	Methanol	32.56 ± 3.87
	Ethanol	32.04 ± 3.98
	Chloroform	31.87 ± 4.03
	Acetone	30.29 ± 4.09
<i>Mentha spicata</i> L.	Methanol	30.89 ± 3.77
	Ethanol	30.37 ± 3.87
	Chloroform	29.77 ± 4.94
	Acetone	28.45 ± 3.01
<i>Mentha viridis</i> L.	Methanol	30.29 ± 4.78
	Ethanol	30.02 ± 4.89
	Chloroform	29.05 ± 3.92
	Acetone	27.55 ± 5.01

Statistical representation of the effective clot lysis percentage by herbal preparations, positive thrombolytic control (Streptokinase) and negative control (sterile distilled water) done by paired t-test analysis; clot lysis % is represented as mean ± S.D.

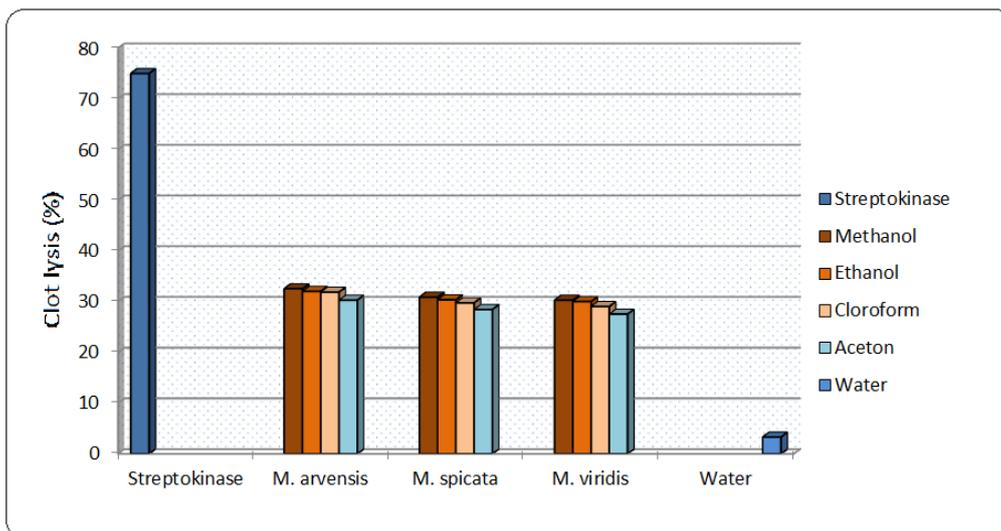


Figure 1: Clot lysis by Streptokinase, water and various herbal preparations.

Effects of drugs on dissolution of clots prepared from blood of normal individuals. Maximum clot lysis ($75 \pm 3.04\%$) was observed in clot treated with streptokinase (SK). Among herbal drugs *M. arvensis*, *M. spicata* and *M. viridis* showed 32.56%, 30.89%, 30.29% clot lysis activity in case of methanol, 32.04%, 30.37%, 30.02% clot lysis activity in case of ethanol, 31.87%, 29.77%, 29.05% clot lysis activity in case of chloroform and 30.29%, 28.45%, 27.55% clot lysis respectively. Water (as a negative control) showed 3.29% clot lysis.

Cytotoxic test:

The regression analysis for brine shrimp bioassay was presented in Table-2. Comparative mortality of brine shrimps and LC_{50} values for different extracts in reference to control (VS) was shown in Figures-2. No extract was found to be significantly toxic compared to positive control. The *M. arvensis*, *M. spicata* and *M. viridis* of methanol extract had a smallest LC_{50} value of 2.088, 1.964 and 1.812 $\mu\text{g/ml}$ which was significantly ($p < 0.01$) different from that (1.160 $\mu\text{g/ml}$) of positive control indicating that the extract is not toxic (Figure 2).

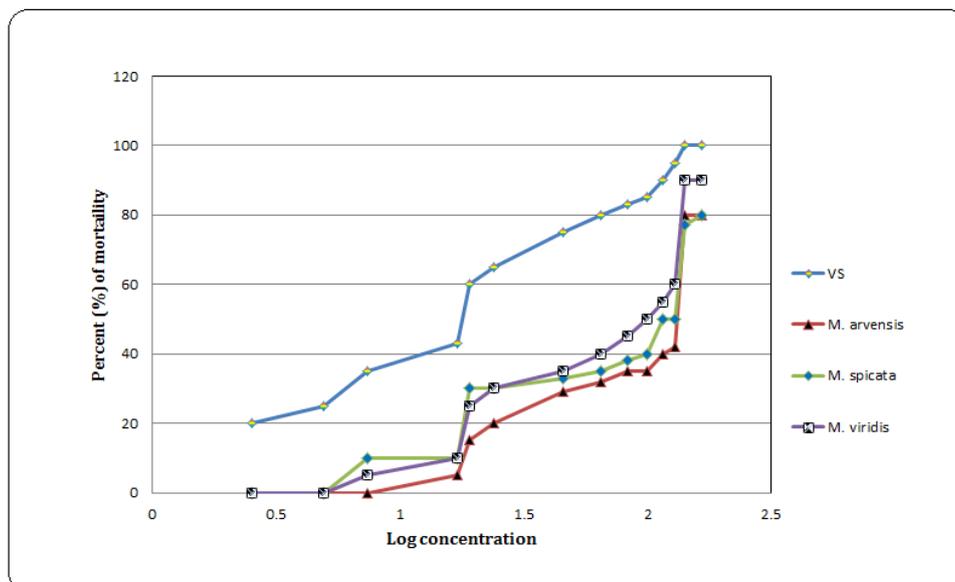


Figure 2: Brine shrimp mortality by VS and different organic extracts.

Percent mortality of brine shrimps of three plant extracts and standard cytotoxic agent vincristine sulfate (VS). Data are shown as mean \pm SD of ten shrimps for each concentration. Mortality achieved by the extracts of *M. arvensis*, *M. spicata* and *M. viridis* are lower than that by VS.

Table 2: Calculation of LC₅₀ values, confidence limits, regression equations and chi square values for different extracts with reference to vincristine sulfate

Sample	LC ₅₀ (µg/ml)	Regression equation	Chi square
VS	1.160	y = 46.212x - 3.6068	0.9765
<i>M. arvensis</i>	2.088	y = 38.482x - 30.367	0.7499
<i>M. spicata</i>	1.964	y = 37.998x - 24.614	0.8152
<i>M. viridis</i>	1.812	y = 45.882x - 33.165	0.838

IV. Discussion

Plant derived materials have been used as a medicine for many years for prevention and treatment of human disease. The herbal preparation is popular now a day because it has no adverse effect. A key role for plant-derived complexes based on the testified immunomodulatory properties has developed in recent times and has directed to the laborious scientific examination to determine efficacy and safety. Many herbal plants have clot lysis activity. Interestingly, some of them are also used as food complements. Different plant source, particularly several fruits, vegetable have been considered for their supplements having fibrinolytic, antiplatelet and anticoagulant activity, and there is indication that consuming such food leads to prevention of coronary events and stroke. Some of these plant derivatives are altered further by recombinant technology to make them more active and site specific.

The aim of the study was to conclude that whether these extracts have clot lytic activity or not. We used streptokinase as a positive control and water as a negative control. In our thrombolytic assay, the assessment of positive control with negative control clearly demonstrated that when water was added to the clot there was no clot dissolution occurs.

When we compare the clot lysis percentage obtained through Streptokinase and water, a tremendously significant (p value < 0.0001) thrombolytic activity was detected after treating the clots with *M. arvensis*, *M. spicata* and *M. viridis* extracts. Staphylokinase triggers plasminogen to dissolve clots, also terminates the extracellular matrix and fibrin fibers that hold cells together. From this study it can be said that, the *M. arvensis*, *M. spicata* and *M. viridis* plant extract presented moderate to good clot lysis activity. However, the tremendously significant effect of Mentha demonstrates that to be the best thrombolytic factor for further processing.

Now a days toxicity of plant materials is a chief concern to scientists and medical consultants [35- 37] and hence our study was to determine the toxicity profile of the plant extracts through the Brine Shrimp Lethality (LC₅₀, 24 h) assessment. Lagarto [38] established a good relationship (r = 0.85; P < 0.05) between the LC₅₀ of the brine shrimp lethality test and the serious oral toxicity test in mice. Based on that relationship, brine shrimp lethality LC₅₀ < 10µg/ml (LC₅₀ between 100 and 1000 mg/kg) is considered as the cut off significance of cytotoxicity. According to the LC₅₀ values of the extracts, there was no report or no one was found severely lethal or toxic to be administered as pharmaceutical products in thrombolytic uses.

V. Conclusion:

In conclusion, on the basis of beneficial effects of *M. arvensis*, *M. spicata* and *M. viridis*. Bangladeshi traditional medicine, which was validated in this study, these plant extracts possessed significant blood clots lytic activity *In vitro*. However, *In vivo* clot dissolving property and active component(s) of this organic extracts for clot lysis are yet to be investigated. Further work will establish whether or not, phytochemicals derived from this plant could be incorporated as a thrombolytic agent for the improvement of the patients suffering from atherothrombotic diseases.

VI. Competing interests

The authors declare that they have no competing interests.

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