



## Anxiolytic and thrombolytic investigation of methanol extract of *Piper nigrum* L. fruits and *Sesamum indicum* L. seeds

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**ABSTRACT:** The study was intended to compare and evaluate the *in vitro* thrombolytic activity and *in vivo* anxiolytic activities of methanol extracts of *Piper nigrum* fruits (MEPNF) and *Sesamum indicum* seeds (MESIS). An *in vitro* thrombolytic activity was employed by investigating the clot lysis effect of MEPNF, MESIS, and their combinations. *In vivo* anxiolytic activity of MEPNF and MESIS was examined by using the Elevated Plus Maze (EPM) test considering diazepam (1 mg/kg) as the standard drug. In the thrombolytic test, MEPNF and MESIS showed a moderate ( $P < 0.01$ ) clot lysis activity. Besides, MEPNF and MESIS showed significant ( $P < 0.001$ ) dose-dependent anxiolytic activity. Since MEPNF or MESIS 200 and 400 (mg/kg, b.w; p.o) has been administered, the time spent in the open arms, and the number of entry into the open arms were increased. The finding suggests that MEPNF and MESIS may be the source for biological activity and therefore further research is required for confirmation.

**KEYWORDS:** *Piper nigrum*, *Sesamum indicum*, Streptokinase, Thrombolytic, Anxiolytic activity.

### INTRODUCTION

Cardiovascular disorders are one of the principal reasons beyond death instead of any other reason which is rising dramatically. The principal reason for the cardiovascular disease is because of thrombus (blood clot), which inhibits the blood circulation simply by constriction of the blood vessel and deprives the normal blood supply and oxygen to the tissue [1]. Thrombolytic/fibrinolysis representatives are utilized to combat thrombosis and remove the clots [2]. As tissue Urokinase (UK), streptokinase (SK), plasminogen activator (tPA), [3] are frequently using a thrombolytic agent which used to treat fibrin or clot disorders. Therefore, specialists worldwide are using thrombolytic agents for the treatment of heart, and artery complications [4]. Some thrombolytic substances have been obtained from several sources. Some of these thrombolytic agents are

also assessed to indicate their direct and compelling development [5]. Therefore, clot lysis is useful for clot-related disorders such as, deep vein thrombosis, thrombo-embolic strokes, myocardial infarction, and pulmonary embolism to eliminate a clogged artery that avoids permanent tissue damage [6].

Besides, according to the World Health Report [7], approximately 450 million people suffer from a mysterious or behavioral sickness, yet only a small minority of them receive even the most basic treatment. This amounts to 12.3% of the global burden of disease and will rise to 15% by 2020 [8]. Anxiety is a persistent emotion, but when it occurs frequently then it turns into a horrific psychological condition. The presence of anxiety due to depression results in symptomatic complications, low accurate prognoses, worse medication response, and an increased risk of suicide [9]. Worldwide medicinal plant work has progressed in the quest for alternative therapeutic agents for psychiatric

disorders, the pharmacological effects of certain plants were demonstrated in many animal models. Medicinal plant products extracted from natural products are used extensively around the world to treat these chronic diseases and for the treatment of mild and life-threatening illness [10]. Bangladeshi people living in rural areas consume medicinal plants as their principal source of health care and play a key role in treating many diseases [11]. From that, point of view *Piper nigrum* (Family: Piperaceae) which is popularly called “Golmarich, Kalimeris” in West Bengal, India, and in Bangladesh. It is known as a hefty climbing bush. Leaves of this plant are extensively praised, whole, adjusted at the base, in a matter of seconds taper at zenith. *P. nigrum* contains alkaloids, aflatoxin, greasy oil oleoresin, and gum [12]. The fruit of the plant has been used as carminative, anti-periodic, rubefacient, stimulant, and aphrodisiac; used mostly for cough and cold recovery [13].

Another one is *Sesamum indicum* (Family: Edaliaceae) is commonly called “Sesame Benne”. Plants can be branched or un-branched. Seeds are remarkably rich in a fixed oil (up to 55%) comprising fundamentally of glycerides of oleic and linoleic acids and palmitic, stearic and myristic acids. Additionally, it also contains strong fats, stearin, palmitin, and myrisin, a crystalline substance, sesamin; sesamol, which separates to a phenolic substance, sesamol, sesamin, protein, vitamin A and E, folic acid and minerals. Leaves contain a sticky substance and mucilage [14]. A flavonoid glycoside - pedalin has been segregated from leaves [13]. The plant has been traditionally used to treat clot lysis, high calories, ingestion, swelling, ulcers, asthma, and cough. The leaves and seeds are astringent. The combination of leaves with water was used to treat child cholera, diarrhea, dysentery, catarrh, and bladder disorders. [15]. The existing research is to be investigated the thrombolytic and anxiolytic activities of methanol extracts of *Piper nigrum* fruits, *Sesamum indicum* seeds, and their combinations. Also, the study covers the anxiolytic activities of methanol extracts of *Piper nigrum* fruits and *Sesamum indicum* seeds.

## MATERIALS AND METHODS

### Collection and identification of the plants

In April 2019, *Piper nigrum* L. fruits and *Sesamum indicum* L. seeds were collected from the nearby forest of Chittagong, Bangladesh for the experiment. After that, the taxonomy of the plants has been confirmed by Dr. Sheikh Bokhtear Uddin, (Professor, Department of Botany, University of Chittagong, Chittagong-4331, Bangladesh) and stored the plant specimen for the further analysis (accession no: CTGUH-1335 and

CTGUH-2712). After washing, the obtained plant fruits and seeds were dried throughout shade at low temperatures for twenty days and ground using a suitable blender machine. The coursed powder materials were then put in an impermeable container and kept in a cool, safe, and warm place until further study. In the case of ethical considerations, all the study protocols have been approved and the protocol was approved by the P&D committee (Pharm-P&D17/08<sup>7</sup>-19) of the department of pharmacy, International Islamic University Chittagong.

### Animals

For the experiment, Swiss-albino mice (25-30 g) were obtained from the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). Before the study, for one week, all animals were accustomed to the new environment. The animals were kept at 25 ° C temperature, comparative humidity (55-65%), and 12 h day/light cycle in a well-ventilated animal house during the experimental phase. Standard laboratory food and drinking water (*Ad libitum*) were given to the experimental animals.

### Drug and chemicals

The drug that was exploited for the analyses of anxiolytic activity diazepam were purchased from Square Pharmaceutical Ltd, Bangladesh. For thrombolytic activity, economically available lyophilized Streptokinase (SK) vials (Polamin Werk GmbH, Herdecke, Germany) of 1500000 IU was borrowed from Sanofi Bangladesh Ltd. Besides, all other chemicals used in the experiment were pharmaceutical grade.

### Extraction process

The granulated powder substance approximately (800 g) was soaked in 2.4 liters of methanol and kept in a glass bottle. The bottle was then shaken regularly and continued about 15 days for complete mixing. Then, it was filtered by the Whatman filter paper (Bibby RE200, Sterilin Ltd, UK) and concentrated by using a water bath at 60-65 °C temperature and finally, 11.27 g crude extract was obtained from *Piper nigrum* L. fruits and 8.2 g crude extract have been obtained *Sesamum indicum* L. seeds. The concentrate was then kept at 4 °C for the reservation as well as for further investigation.

## ***In vitro* thrombolytic test**

### *Preparation of extract*

10 mL of distilled water is used to suspend 10 mg of the extract and vigorously shaken by the vortex mixer. By that point, the suspension was vacuumed to remove the insoluble elements supernatant and was removed by filter paper (Whatman No. 1). The extracts were then prepared to evaluate the *in vitro* thrombolytic activity [4].

### *Streptokinase (SK) solution preparation*

5 mL of clean distilled water was added to the commercially available SK-15, 00,000 I.U. (Polamin Werk GmbH) bottle that was then properly mixed. From the suspension, 100  $\mu$ L (30,000 I.U) was used as a stock solution for the *in vitro* thrombolysis investigation. [4].

### *Specimen for thrombolytic test*

The experiment was performed using the previously established protocol mentioned by Emon et.al; [16] and the study was approved by the Ethics Committee, Department of Pharmacy, International Islamic University Chittagong. For this research, blood (5 mL) were taken from the physically strong human volunteers (n = 10) having no history of NSAID and anticoagulants for 10 days. 500  $\mu$ L of blood has been transferred to the early measured Eppendorf and sustained in the incubator at 37 °C for 45 minutes. After the formation of coagulation, the serum was completely separated from the Eppendorf. Each tube containing only coagulation has been weighed further in order to determine the exact weight of the coagulation. Then 100  $\mu$ L of plant extract was added to the tubes. The test tube containing plant extract was suspended in the incubator and fixed the temperature at 37 °C for 90 minutes. The blood serum has been withdrawn after the clot lysis and the tube has been measured once again to track the weight difference after the formation of clot lysis.

$$\% \text{ clot lysis} = \frac{\text{Weight of the lysis clot}}{\text{Weight of clot before lysis}} \times 10$$

## **Acute oral toxicity test**

A single oral dose limit of 2000 (mg/kg, b.w; p.o) for employing the acute oral toxicity test was carried out in compliance with the OECD 425 guidelines (OECD, 236) [17]. For this study, five swiss albino mice have randomly selected. One animal was first dosed at the target test dose and was then monitored for 24 hours.

The remaining four animals were also dosed to 2000 mg/kg sequentially, for recording the overall testing of 5 animals. At least one in the first 30 minutes of dosage, regularly during the first 24 hours and daily for the next 13 days, for a total of 14 days, each animal was monitored individually for toxicity signs.

## ***In vivo* anxiolytic activity**

### *Elevated plus maze test (EPM)*

The study for estimating anxiolytic activity has been employed by using the method which was listed in [18, 19]. In an elevated plus-maze test, the instrument was formed with two open and two shut arms of wood over one another individually shaping a figure like plus-sign. The EPM (EPM; 30 cm  $\times$  6 cm, each arm) was arranged 50 cm over the ground. The mice were decorated into six groups where each group consisting of three mice. Group I received control solutions (Tween-80, 10 mg/mL, b.w; p.o) and standard drug diazepam (1 mg/kg, b.w; i.p), has been administered to group II. Besides, group III-VI received MEPNF and MESIS (400 and 200 mg/kg; p.o) respectively. After the application of the medication, every animal was put at the focal point of the instrument to face one of the closed arms. The number of the entry in the open and close arm and time spent in the open arm has been recorded for 5 min after administration of the test samples [20].

Percentage of the period spent in the arm =

$$\frac{\text{Time in open arm}}{\text{Open arm time} + \text{close arm time}}$$

## **Statistical analysis**

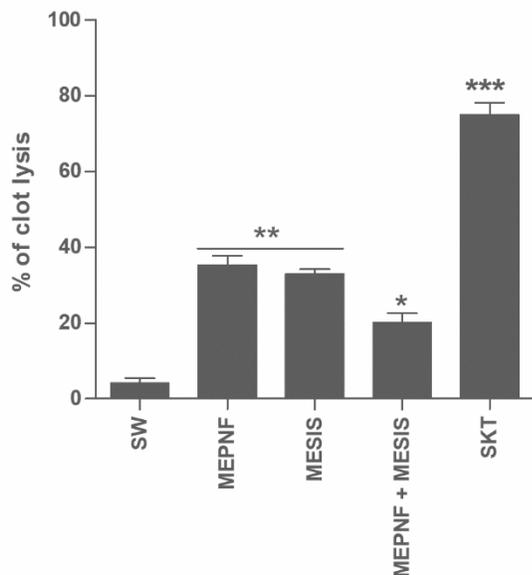
Graph pad prism version 5.0 was used to analyze experimental results. Data was submitted as SEM  $\pm$  Mean. One-way ANOVA followed by Dunnett's Multiple Comparison Test has performed for statistical analysis. The  $P < 0.05$ ,  $P < 0.01$ , and  $P < 0.001$  were considered statistically significant.

## **RESULTS**

### **Effect of extracts on thrombolytic activity and acute toxicity**

The expansion of 100  $\mu$ L SK, a positive control (30,000 I.U.) to the coagulations alongside an hour and a half of brooding at 37 °C, showed 75.01  $\pm$  3.20 % ( $P < 0.001$ ) clot lysis. The *in vitro* thrombolytic action study uncovered that MEPNF and MESIS showed 35.4  $\pm$  2.4% ( $P < 0.01$ ) and 32.94  $\pm$  1.23 % ( $P < 0.01$ ) activity and the summary of the clot lysis of MEPNF and MESIS

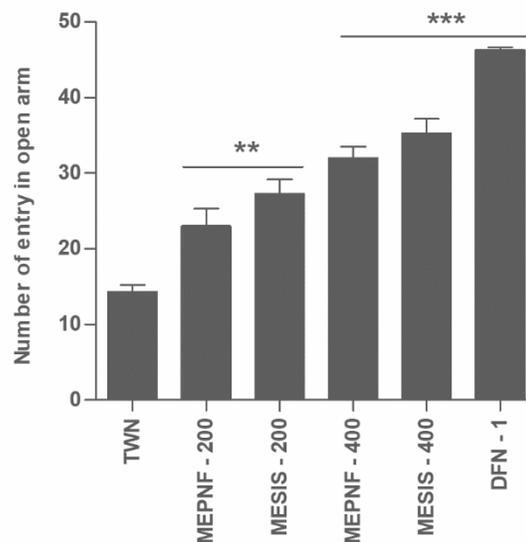
and their combination has been shown in Figure 1. Further, no morbidity, skin deterioration, or any physical degradation has appeared as explained by acute toxicity.



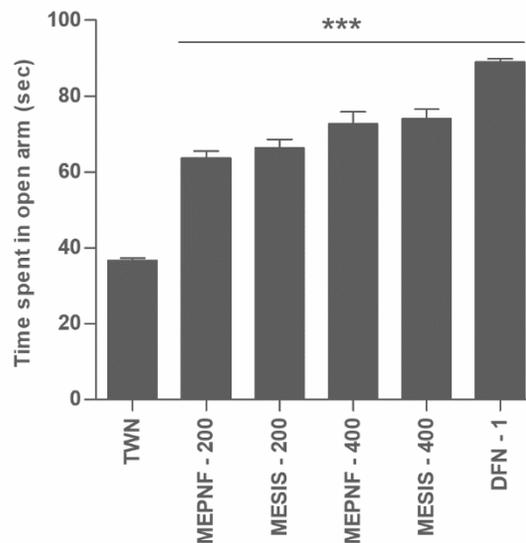
**Figure 1.** *In vitro* thrombolytic activity of test samples. Values are presented as mean  $\pm$  SEM; One-way analysis of variance (ANOVA) followed by Dunnett's test. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  is considered as significant compared with the control. Note: MEPNF = Methanol extract of *Piper nigrum* fruits and MESIS = Methanol extract *Sesamum indicum* seeds, SW = 1% Tween 80, and SKT = Streptokinase.

### Effect of extracts on elevated plus maze (EPM)

In the EPM, after the administration of test drugs, the number of open arm entries and spent time in the open arm has been increased significantly ( $P < 0.1$ , and  $P < 0.01$ ). MEPNF and MESIS showed dose dependent reduction of the number of entrances in the close arm and the spent of time in the close arm. After the administration of MESIS 400 mg/kg, the mice showed maximum entrance ( $35.33 \pm 1.85$ ,  $P < 0.001$ ) and spent comparatively high time ( $74.06 \pm 2.51$  sec,  $P < 0.001$ ) in the open arm. The overall result has been shown in Figures 2 and 3.



**Figure 2.** *In vitro* thrombolytic activity (number of the entry in open arms) of test samples. Values are presented as mean  $\pm$  SEM; One-way analysis of variance (ANOVA) followed by Dunnett's test. \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  is considered as significant compared with the control. Note: MEPNF = Methanol extract of *Piper nigrum* fruits and MESIS = Methanol extract *Sesamum indicum* seeds, TWN = 1% Tween 80, and DFN = Diazepam.



**Figure 3.** *In vivo* anxiolytic activity (time spent in open arms) of test samples. Values are presented as mean  $\pm$  SEM; One-way analysis of variance (ANOVA) followed by Dunnett's test. \*\*\* $P < 0.001$  is considered as significant compared with the control. Note: MEPNF = Methanol extract of *Piper nigrum* fruits and MESIS = Methanol extract *Sesamum indicum* seeds, TWN = 1% Tween 80, and DFN = Diazepam.

## DISCUSSION

Phytopharmaceutical work has now developed a new field for the discovery of drugs from plant derivative that cure certain disorders and renew the focus in herbal medicines. Around 30 % of medicines are made from plant derivatives [21]. This study examined the thrombolytic and anxiolytic effect of methanol extract of *Piper nigrum* fruits (MEPNF), methanol extract of *Sesamum indicum* seeds (MESIS), and their combination. Several researchers have conducted a series of studies to identify plants and natural food resources which contains thrombolytic (anti-platelet, and anti-coagulant) activity and the finding suggests that the intake of such a food contributes to strokes and cardiac disorder to prevention [22]. There are several plant products with a thrombolytic capacity. These are Garlic (*Allium sativum*) [23], *Flammulina velutipes* [24], *Pleurotus ostreatus* [25], *Crocus sativus* Linn (indraceae) [4], Ginger (*Zingiber officinale*) [26], *Lumbricus rubellus* [27], and *Ganoderma lucidum* [28], chungkook-jang [29], *Spirodela polyrhiza* [28], and natto [30] correspondingly. Our analysis of MEPNF, MESIS, and MEPNF + MESIS indicates a moderate probability of clot lysis as opposed to saline water. Compared to the controls, the rise in clotting lysis by MEPNF, MESIS, and MEPNF+ MESIS indicates its potential application in coagulation-related disorders. The plasminogen enzyme stimulates the majority of thrombolytic agents and removes the related fibrin in order to restore blood supply into blood congested vessels [31]. The clot lysis activity was observed both for the MEPNF and MESIS, which mean methanol soluble compounds of *Piper nigrum* fruits and *Sesamum indicum* seeds contain thrombolytic responsible constituents [32]. The plasminogen cell-binding surface is readily stimulated through plasmin that can result in fibrinolysis whereas other plants are using their enzymes as a fibrinolysis effect [16]. Furthermore, the plasminogen activator bacterial, Staphylokinase streptokinase, serves as cofactor molecules that help to form exosites and enhance the enzyme's substrate presentation. Staphylokinase stimulates plasminogen to dissolve the blood clotting and also destroys cellular extra-cellular matrix and fibrin fibers [33].

The EPM is a widely recognized assessment and is particularly susceptible to the actions of both anxiolytic and anxiogenic medications on type A (GABAA) gamma amino-butyric corrosive benzodiazepine complex [34]. In EPM, normally standard mice would recommend passing much of their time in the closed arms. That preference seems to reflect an aversion to open arms generated by open space fears. A drug such as diazepam that improves open arm exploration is considered anxiolytic and the reverse is true for

anxiogenic substances [35]. The methanol extract of *Piper nigrum* fruits and *Sesamum indicum* seeds (200 and 400 mg/kg b.w) has had an anxiolytic effect in mice because it increases open arm entry and spends more time in open arms of the EPM. These results may be linked to the effects of extract components that have on the behavior of  $\gamma$ -aminobutyric acid transaminase (GABA-t). They are supposed to increase the levels of GABA in the brain to suppress anxiety [36]. Acute therapy of methanol extract or dried leaves has been shown to improve calmness, modulate moods, and alleviate experimental tension in healthy young participants [37]. In our study, the MEPNF, MESIS, and MEPNF+MESIS showed moderate thrombolytic activity which supposed to their phytochemicals. Besides, MEPNF and MESIS showed significant anxiolytic activity in animal model which may be possible because of the presence of phytochemicals that increase the level of GABA in the brain.

## CONCLUSIONS

This study tends to be presumed that, the methanol extracts of *Piper nigrum* fruits and *Sesamum indicum* seeds have significant possibilities as a candidate for anxiolytic activity having moderate thrombolytic properties and fascinated to discover the dynamic component(s) in charge of the anxiolytic and thrombolytic effect of these plants. This is just an initial report so that, the author suggests to investigate in further to identify the medicinal and medical possibilities.

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## AUTHORS CONTRIBUTIONS

The research protocol was conceptualized, prepared, and designed by NUE. SA and MNI supervised the investigations. Along with NUE the laboratory experiment performed by MJU, MFIK, MAJ, SMT, ANMR. Together, MI and MK collected and analyzed the information. Data processing and software analysis were performed by NUE and MI. In preparation of the draft manuscript, NUE and MK participated. SA and MNI reviewed and corrected the error of the draft and with NUE they finalized the draft. A native English

speaker then reviewed the text, and all the authors agreed eventually to publish the manuscript draft.

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## CONFLICTS OF INTEREST

Authors declared that they have no conflict of interest.

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