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Anti-Arthritic, Anti-Inflammatory, Thrombolytic, Membrane Stabilizing, Antifungal and Cytotoxic Activity of *Polyscias scutellaria* Leaf Extract: An *In-vitro* Analysis

Jakiya Akter [a++], Hasib K. Shomudro [a++] and Sadia A. Chowdhury [a#*]

[a] *Department of Pharmacy, Stamford University Bangladesh, Dhaka, Bangladesh.*

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The objective of this investigations was to evaluate *in Vitro* anti-arthritic, anti-inflammatory, thrombolytic, membrane stabilizing, antifungal and cytotoxic activities from the methanolic leaf extract of *Polyscias scutellaria*. Primary evaluation of methanolic extract of *Polyscias scutellaria* leaf (MEPSL) was performed via phytochemical screening. Presence of Alkaloids, flavonoids, saponins,

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++ *Student Bachelor of Pharmacy (Honors);*
\# *Associate Professor;*
**Corresponding author: E-mail: sadia.afreen.chowdhury@stamforduniversity.edu.bd;*

glycosides, carbohydrates and reducing sugars were observed by phytochemical screening, among other secondary compounds. Using in vitro methods of protein denaturation, the anti-arthritic and anti-inflammatory properties of MEPSL was investigated. The results showed that the extracts significantly slowed down arthritis and inflammation with the percent inhibition of 94.59% and 86.33% at the conc. of 1000 µg/mL compared to the standard diclofenac sodium (98.19%) and acetyle salicylic acid (98.56%) at same concentration respectively. The thrombolytic activity of the extracts was additionally examined by clotlysis method, and the results showed that the ability to break up blood clots increased with the amount of extract used with the value of 97.32% which is very significant compared to the standard streptokinase which showed clotlysis of 91.304%. MEPSL was also showed action that stabilized membranes by using heat induced hemolysis method, which could be helpful in treating conditions like bleeding and swelling with the percent of protection value of 58.87% when compared to standard diclofenac sodium with the percent of protection value 73.63%. Antifungal action was also seen, which shows that it could be used to treat diseases caused by fungi with the zone of inhibition 7-36 mm varying on the type of fungi. Lastly, in vitro method was used to investigate the extracts' damaging effect on *Artemia salina* by using shrimp lethality assay. The results showed significant cytotoxicity with the LC_{50} value of 1.057 µg/mL compared to the standard vincristine sulphate (LC_{50} value of 0.608 µg/mL). To sum up, it is clear that the phytochemical found in this plant can be used for wide range of drug discovery field due to its potent pharmacological actions.

Keywords: Anti-arthritic; thrombolytic; membrane stabilizing; antifungal; cytotoxic activity.

1. INTRODUCTION

A large number of today's modern medicines are derived from the plant resources. In the past, people uses different plant parts for therapeutic purposes [1]. Herbal drugs are used because there is widespread faith and reliance on this type of drugs for being cheap and convenient; also, do not contain any adverse effect. Besides, many of the few potent medicines were plant derived. Examples include morphine (originated from the opium poppy), digoxin (originated from foxglove), aspirin (originated from willow bark), and quinine (originated from cinchona bark) [2]. Since pharmacology enlarged itself such a guiding principle of curative therapy, the use of herbal treatment went into prompt decline. But this scenario has begun to change over the span of years. Nowadays herbal medicines are extensively used by widespread US population and it is expanded by 380% between 1990 and 1997 (from a 1-year frequency of 2.5–12.1%), as an example [3]. A number of chronic or persistent diseases like arthritis, diabetes, AIDS or cancer are often not possible to cure with allopathic system of medicine and for this reason, people are now depending on herbal medicine [4]. On the contrary, herbal medicines can also have some interactions and for this reasons doctor, pharmacists and many other health care professionals should be well informed to consult laibly to the patients [2].

Polyscias scutellaria (Burm.f.) Fosberg also known as shield aralia, plum aralia which is a small shrub or bush reaching 2-6 meters in height. The genus Polyscias of family Araliaceae comprises about 116 species that are widely used for ornamental purposes, some with potential medicinal value. It is commonly growing in pacific country. It has significant anti-inflammatory properties [5]. Traditionally, this plant was used to treat breast inflammation, wounds, urinary tract problem and body odor.

The motive behind this analysis is to find out in vitro anti-arthritic, anti-inflammatory, thrombolytic, membrane stabilizing, antifungal and cytotoxic activities of *Polyscias scutelaria* from its methanolic leaf extract along with its phytochemical screening.

2. MATERIALS AND METHODS

2.1 Plant Materials

The sample plant *Polyscias scutellaria* (Burm.f.) Fosberg was collected in November, 2022 from Ramna Park, Moulana Bhashani Road, Dhaka, Bangladesh. Then the plant (accession number: DACB 88046) was precisely recognized by the professionals at the Bangladesh National Herbarium in Mirpur, Dhaka. In the meantime, plant's leaves had been stored and dried in shade and powder was made from these dried leaves.

2.2 Reagents

Methanol, concentrated H_2SO_4, Diluted HCl acid, acetic acid and NaOH was supplied by Sigma Chemical Co., USA. From Polysciences, Inc. India, Bovine Serum Albumin was bought. Streptokinase was purchased from Incepta Pharmaceuticals Ltd, Bangladesh. Square Pharmaceuticals Ltd manufactures diclofenac sodium injections. The sterile saline solution was obtained through Orion Infusion Ltd. Vincristine Sulphate was taken from Celon Laboratories Pvt. Ltd. India.

2.3 Preparation of Plant Extract

The extraction of plant was obtained by using cold maceration method [6]. About 80g powder of *Polyscias scutellaria* leaf was soaked in 600 mL of methanol for 10 days in a round bottom flask sealed with a stopper and wrap [7]. Then the mixture was filtered and air dried for further 7 days. After drying, overall weight of 16.15g of leaf extract was obtained.

2.4 Phytochemical Screening Test

Many therapeutic characteristics of the plants are obtained from its chemical constituents [8]. Freshly prepared MEPSL was screened qualitatively for the presence of phytochemicals such as alkaloids, carbohydrates, saponins, glycosides, reducing sugar, flavonoids, tannins and steroids.

2.5 *In vitro* Anti-Arthritic Test

Rheumatoid arthritis (RA) is one of the prevalent autoimmune disorder which is accompanying with systemic difficulty, progressive impairment, premature death and socioeconomic cost [9]. About 0.3-1% people across the world are affected by rheumatoid arthritis and among them males are three times less prone to RA then females [10].

Anti-arthritic activity is tested by using protein denaturation assay by bovine serum albumin method [11]. Bovine serum albumin (5% aqueous solution) of 0.45 mL and MEPSL of 0.05 mL are together formed 0.5 mL of test solution and as a standard drug, 0.05 mL of Diclofenac sodium were used. MEPSL and Diclofenac sodium are sampled in different concentration (62.5, 125, 250, 500, 1000 µg/mL). Small amount of 1N HCl is added to modify the pH of the solution to 6.3. After that, for 20 minutes at 37°C the samples were incubated and heated for 3 minute at 57°C. Then 2.5 mL phosphate buffer was added after cooling the solution. Finally, at a wavelength of 416 nm the absorbance of the solutions was taken by using UV-Visible spectrometer. Here, 0.05 mL of distilled water is used as a test control instead of utilizing BSA (Bovine Serum Albumin) for control. For comparison, Diclofenac sodium is used in this study. Equation used for calculating percentage of inhibition of protein denaturation as follows:

$$\% \text{ Inhibition} = \frac{(\text{OD of Control} - \text{OD of sample})}{\text{OD of control}} \times 100$$

Here, OD means optical density.

2.6 *In vitro* Anti-Inflammatory Test

For this test, various concentrations of 62.5, 125, 250, 500 and 1000 µg/mL mixture was prepared which consist of total 5 mL of reaction mixture containing 2.8 mL of phosphate buffered saline (PBS, pH 6.4), 0.2 mL of egg albumin (from a hen's egg), and 2 mL of MEPSL. Double-distilled water was used at equal amount for control group. At 70°C, the mixtures were heated for 5 minutes after incubating the mixture at (37±2)°C, using Biological Oxygen Demand (BOD) incubator for a time period of 15 minutes. After cooling, absorbance of the mixtures was taken at 660 nm. For comparison, Acetyl Salicylic acid also used in equal concentration as a standard [12]. Fractional equation for calculating percentage of inhibition of protein denaturation as follows:

% Inhibition = Absorbance of control - Absorbance of sample / Absorbance of control * 100

2.7 *In vitro* Thrombolytic Test

2.7.1 Blood sample

4 mL of venous blood was drawn from healthy human volunteers (n=15), whom had never consumed blood thinners, nicotine and oral contraceptives, and this process was aided by a medical professional. Then, total of 15 micro centrifuge tubes was filled with 500 µL of fresh blood.

2.7.2 Clotlysis methods

The method used for determining the percentage of clotlysis was obtained previously published research paper [13]. In short, 2.5 mL of fresh

blood was filled in 15 discrete pre-weighed sterile micro centrifuge tubes (0.5 mL/tube) and at 37°C, it was incubated for 45 minutes. After incubation, serum was deliberately drained out from the tubes without disturbing the clot. For calculating the clot weight, tubes were weighted again (Clot weight = weight of clot containing tube – weight of tube without clot). Then 100 µL of MEPSL was added to each micro centrifuge tube which contain pre-weighted clot. By adding 2.5 mL of PBS, lyophilized streptokinase vial was reconstituted and was mixed properly. In the volume of 100 µL of this suspension was filled to the tube as a positive control. For negative control, distilled water of 100 µL was used. Clotlysis was checked in each tube after incubating at 37°C for 90 minutes. After incubation, the tubes were weighted again to observe the weight changed for clot disruption. Finally, by measuring the variation in weight before and after the clotlysis, the percentage of clotlysis was calculated and the equation used for this determination as follows:

$$\text{\% of Clotlysis} = \frac{A}{B} \times 100$$

Here, A and B represent the weight of released clot before and after treatment.

2.8 Membrane Stabilizing Activity Test

2.8.1 Preparation of Human Red Blood Cells (HRBC) suspension

2% dextrose, 0.8% sodium citrate, 0.05% citric acid, and 0.42% sodium chloride in water used for making a sterile Alsever solution which was mixed with equal quantity of freshly sampled human blood. Then centrifugation of that blood was performed at 3000 rpm for 10minutes and combining with isosaline (0.85%, pH 7.2), packed cells were washed three times. Reconstitution as 10% suspension was performed with isosaline and the volume of blood was measured [14].

2.8.2 Heat induced hemolysis

The fundamental principle of this method is the stability of human red blood cell membrane through hypotonicity induced hemolysis. As reaction mixture, 0.15M phosphate buffer (1 mL, pH 7.4), 0.36% hyposaline (2 mL), 10% v/v HRBC suspension (0.5 mL) with plant extracts (0.5 mL) and diclofenac sodium used as a standard drug and distilled water instead of hypo saline to produce 100 % hemolysis used as a control group and incubation performed at 37°C for 30 min and centrifugation respectively. Spectrophotometer at 560 nm was used to determine the hemoglobin content in the suspension. The formula used for estimating the percentage of hemolysis of HRBC membrane as follows:

% Hemolysis = (Optical density of Test sample / Optical density of Control) X 100

The equation utilized for determining the percentage of HRBC membrane stabilization:

% Protection = 100 – [(Optical density of Test sample / Optical density of Control) X 100]

2.9 Antifungal Susceptibility Test

2.9.1 Fungal strains

From Microbiology Department of Stamford University Bangladesh, pure culture of fungi (*Penicillium chrysogenum*, *Aspergillus niger*, *Mucor hiemalis* and *Saccharomyces cerevisiae*) was obtained.

2.9.2 Disc diffusion methods

Antifungal activity of MEPSL was carried out by using disc diffusion assay [15]. In this method, a solid agar medium was formed in a Petri Dish. Then 1 mL culture of each fungus was spread uniformly throughout the medium. Sterile filter paper disc of 6 mm in diameter was used and this disc was saturated with diluted MEPSL of 10 µL, setting on the top of each agar plate. In this test, MEPSL was taken in several concentration (300, 500, 700 µg/mL). Then the plates were put on the incubator for next 24 hours. Griseofulvin containing disc was used as an antifungal agent for positive control, while methanol containing disc was used for negative control. After 24 hours, based on the size of inhibition zone surrounding the disc, measured in mm, antifungal activity was determined [16].

2.10 *In vitro* Cytotoxic Activity Test

Cytotoxic activity of MEPSL was investigated using the brine shrimp lethality test, a standard bioassay for screening bioactive compound [17]. *Artemia salina* (zoological organism) used as a model for this research. At first, from a pet store (Dhaka, Bangladesh) shrimp eggs were bought. Hatching of shrimp eggs were performed in artificial seawater (3.8% NaCl solution) after incubating 48 hours in it and larval shrimp (nauplii) was grown. By applying Meyer's

approach brine shrimp nauplii can be evaluated for cytotoxic activity. Test sample of MEPSL was prepared by dissolving it in a dimethyl sulfoxide solution that cannot be more than 50 µL per 5 mL. Then artificial seawater was mixed up to 5 mL for making desirable concentration (1.95, 3.91, 7.81, 15.625, 31.25, 62.5, 125, 250, and 500 µg/mL). For positive control, Vincristine sulphate was employed. Then 10 mature shrimp nauplii were added in test tube. Test tubes were observed by using magnifying glass after 24 hours to see how many nauplii had survived. By utilizing a logarithmic plot of concentration against mortality rate, LC_{50} was calculated.

2.11 Statistical Analysis

All experimental data were handled in triplicate, and mean, standard deviation was used to express tubular data. Excel also used for statistical analyses.

3. RESULTS

3.1 Phytochemical Screening Test

From this screening test, it was identified that alkaloids, carbohydrates, saponins, glycosides, reducing sugars, and flavonoids were present, wheareas, tannins and steroids were absent in MEPSL (Table 1.)

Table 1. Qualitative phytochemical analysis of MEPSL.

Phytochemical constituent	MEPSL
Alkaloid	++
Carbohydrate	++
Saponin	++
Glycoside	+
Reducing Sugar	+
Flavonoid	+
Tannin	-
Steroid	-

Here, (++) indicates a higher amount, (+) indicates a moderate amount, and (–) indicates absence

3.2 *In vitro* Anti-Arthritic Test

Denaturation of BSA property compared to the standard drug has been shown in Table 2 and Fig. 1.

3.3 *In vitro* Anti-Inflammatory Test

The percentage of proteinase inhibition carried out by MEPSL shows a dose dependent rise which is in moderate level compared to the standard and shown in Table 3 and Fig 2.

Table 2. *In vitro* anti-arthritic test results

Samples	Concentrations (µg/mL)	% of inhibition
Diclofenac Sodium	62.5	89.19
	125	91.89
	250	93.69
	500	94.59
	1000	98.19
MEPSL	62.5	83.78
	125	84.68
	250	87.38
	500	93.69
	1000	94.59

Table 3. Protein denaturation (egg albumin) assay results

Samples	Concentrations (µg/mL)	% of inhibition
Acetyl Salicylic acid	62.5	93.52
	125	94.96
	250	95.68
	500	97.84
	1000	98.56
MEPSL	62.5	79.86
	125	80.58
	250	82.01
	500	82.73
	1000	86.33

Table 4. Percentage of clot lysis, n=15 (mean value)

Sample	% of clot lysis
Negative control	7.296
Streptokinase	91.304
MEPSL	97.32

3.4 *In vitro* Thrombolytic Test

Thrombolytic activity of MEPSL is very significant than compared to standard drug (Table 4). So, it can be assumed that MEPSL can be used as a drug like plasmin which can reduce blood clots.

3.5 Membrane Stabilizing Activity Test

Percentage of hemolysis and protection of MEPSL compared to the standard is measured in this test which is deliberated in Table 5.

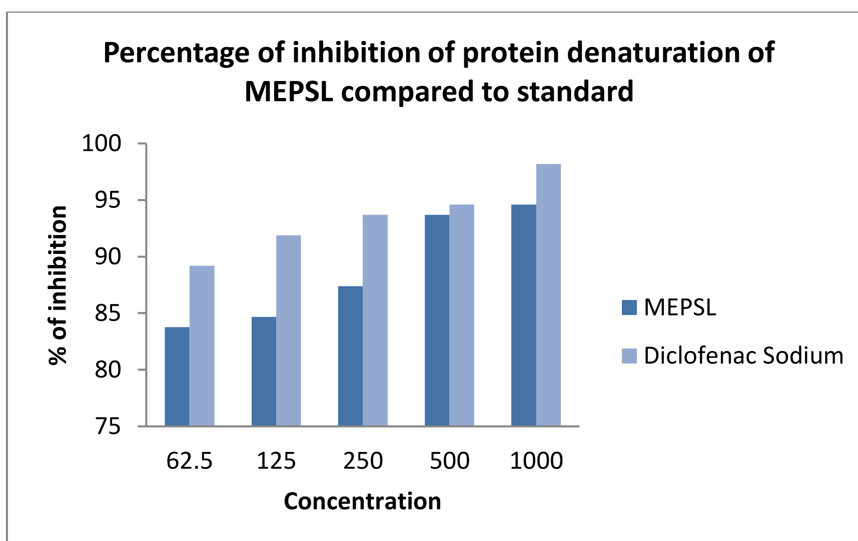

Fig. 1. Percentage of inhibition of MEPSL compared to standard

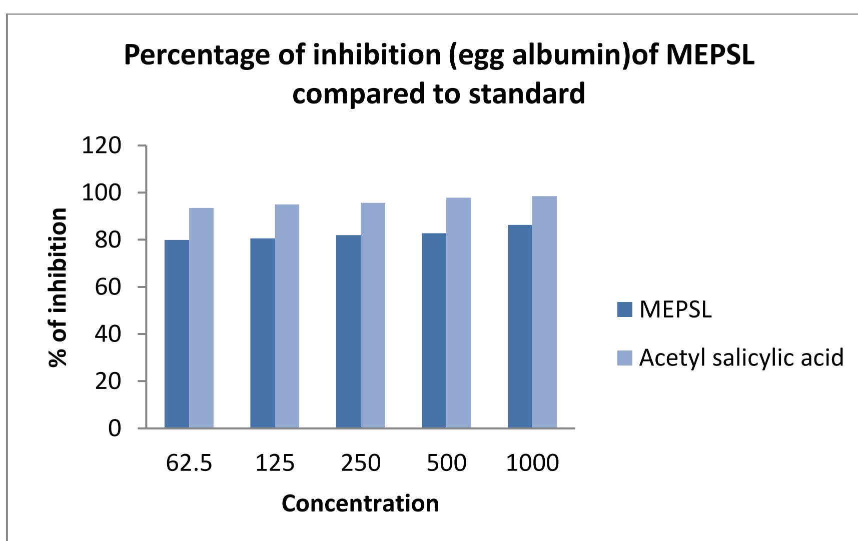

Fig. 2. Percentage of inhibition of MEPSL compared to standard using egg albumin

3.7 *In vitro* Cytotoxic Activity Test

In Table 7. The cytoxic activity of MEPSL to brine shrimp nauplii is summarized and standard calibration curve of standard and MEPSL, which shows the effect of both on brine shrimp nauplii, illustrated in Fig 3 and Fig 4 respectively.

Table 5. Percentage of hemolysis of RBC by MEPSL extract

Sample	% of hemolysis	% of protection
Diclofenac Sodium	26.36	73.63
MEPSL	41.18	58.87

3.6 Antifungal Susceptibility Test

A moderate antifungal activity has obtained compared to the standard drug which is demonstrated in Table 6.

4. DISCUSSION

This investigation has performed for determining the effect of MEPSL on variety of pharmacological tests such as anti-arthritic, anti-inflammatory, antifungal, membrane stabilizing, thrombolytic and cytotoxic activity along with its phytochemical screening. In Table 1, it has been shown that alkaloids, carbohydrates, saponin are present at a higher amount on MEPSL. Alkaloids

posses' anti-inflammatory and analgesic properties which helps in reducing pain and enhances immune response. MEPSL has significant quantity of alkaloids which can be used to skin diseases, asthma and snake bite. The appearance of saponins in higher amount on MEPSL is an excellent indication that this plant can be used as a medicinal importance because saponin shows anti-cancer, antioxidant, antimicrobial, anticonvulsant, anthelmintic, anti-inflammatory, analgesic, and cytotoxic effect [17]. Glycosides, reducing sugar, and flavonoids are also present in a moderate amount (Table 1). Flavonoids are generally found in plants, fruits and vegetables which possess antibacterial and antioxidant properties. Flavonoids structures are responsible for anti-bacterial characteristics [18]. The existence of this phytochemical in MEPSL exerts the medicinal importance of this species whereas; tannins and steroids are not identified in this screening process.

In economically evolved countries, about 1% of populations are affected by Rheumatoid arthritis (RA) which is one type of inflammatory disease. Lack of mobility, hyperalgesia, and pause in body weight gain are the signs of acute RA [19]. In this investigation, it has been shown that the MEPSL has significant anti-arthritic value of 94.59% in the concentration of 1000 µg/mL, which is very close when compared to Diclofenac sodium's value of 98.19% in 1000 µg/mL concentration (Table 2, Fig 1). Because of its significant anti-arthritic value, it can be used to treat Rheumatoid arthritis in future.

Inflammation is physiologic response to tissue injury and infection; it occurs due to the production of prostaglandins through cyclooxygenase pathway. In this in vitro anti-inflammatory test, we found that the MEPSL has the properties of inhibiting inflammation 86.33% in the doses of 1000 µg/mL, by a percentage close to the inhibition emerged by the extensively recognized NSAIDS such as aspirin (acetyl salicylic acid) 98.52% in the doses of 1000 µg/mL (Table 3, Fig 2).Aspirin is the oldest class of NSAID which targets and inhibit cyclooxygenase (COX) pathway, the rate limiting enzyme in the production of prostaglandins. As the MEPSL has inhibition value close to the Acetyl salicylic acid, this study clearly manifested that the MEPSL has cyclooxygenase inhibitory properties by inhibiting in vitro conversion of arachidonic acid to PGE2 [20].

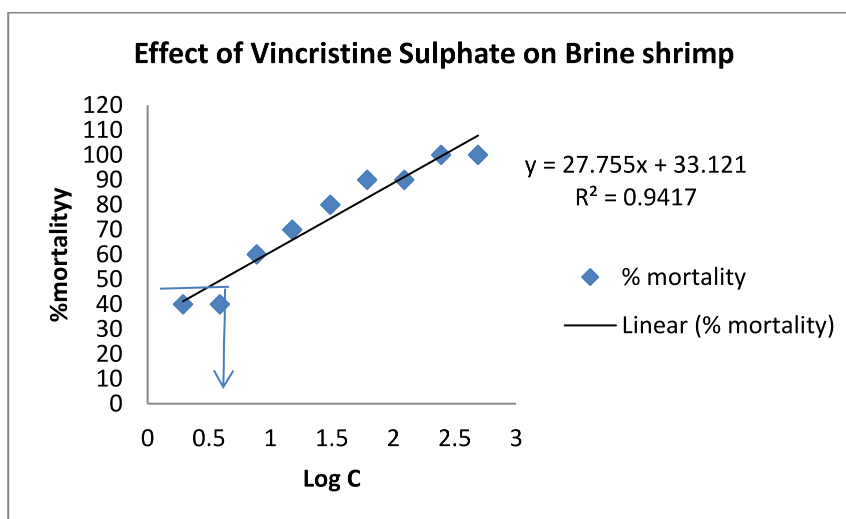

Fig. 3. Cytotoxic activity of vincristine sulphate on brine shrimp nauplii

Table 6. Results of antifungal activity of MEPSL (mm)

Diameter of Zone of Inhibition (mm)				
Test organisms	**MEPSL (300 µg/disc)**	**MEPSL (500 µg/disc)**	**MEPSL (700 µg/disc)**	**Griseofulvin (50µg/disc)**
Penicillium chrysogenum	**07**	**08**	**10**	**19**
Aspergillus niger	**07**	**11**	**13**	**20**
Mucor hiemalis	**08**	**10**	**15**	**21**
Saccharomyces cerevisiae	**10**	**20**	**36**	**21**

Table 7. Brine shrimp assay (Mortality %, LC_{50} value)

Sample	Concentration (C) (μg/mL)	Mortality %	LC_{50} value
Vincristine Sulphate	1.95	40	
	3.91	40	
	7.81	60	
	15.325	70	0.608
	31.25	80	
	62.5	90	
	125	90	
	250	100	
	500	100	
MEPSL	1.95	20	
	3.91	30	
	7.81	40	
	15.625	60	
	31.25	70	1.057
	62.5	80	
	125	90	
	250	100	
	500	100	

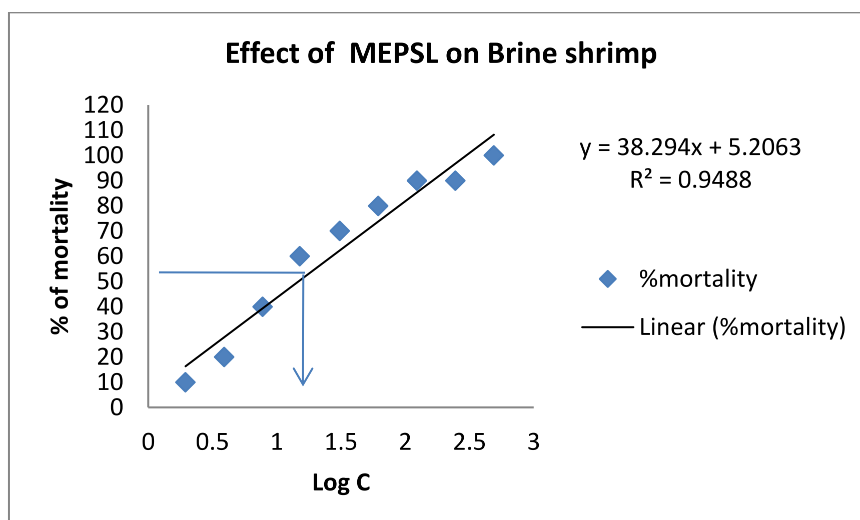

Fig. 4. Cytotoxic activity of MEPSL on Brine shrimp nauplii

Different kinds of research have been carried out to determine which supplements, herbs and natural food sources have thrombolytic activity to treat coronary events and strokes. This investigation determined the thrombolytic potential of MEPSL. Thrombolytic potential of MEPSL was rapid and the value is 97.32% compared to standard 91.304% (Table 4). This value obtained because MEPSL diminish coagulation of human blood in vitro, so it can be claimed as cardio protective. As the MEPSL has significant value, it may have important implication in cardiovascular health and this may lead to the formation of novel thrombolytic agents from *Polyscias scutellaria* leaf [21].

The percentage of membrane stabilization for MEPSL and Diclofenac sodium were done by the inhibition of HRBC membrane lysis i.e., stabilization HRBC membrane induced by hypotonicity. MEPSL are efficacious in suppressing the heat induced hemolysis of HRBC as shown in Table 5. This indicated the range of protection 58.87% of MEPSL compared to Diclofenac sodium 73.63%, which declare the considerable membrane stabilizing property of *Polyscias scutellaria* leaf. It can be said that flavonoids are responsible for this type activity. Hence, *Polyscias scutellaria* can be used as an anti-inflammatory agent.

Antifungal activity of MEPSL was shown in Table 6, using 4 fungi. According to Table 6, MEPSL exerts several degrees of antifungal activity for each fungus. It was found that MEPSL have stronger fungicidal activity than Griseofulvin against *Saccharomyces cerevisiae* like fungi. In case of *Penicillium chrysogenum*, *Aspergillus niger*, *Mucor hiemalis*, it was found that the zone of inhibition is close to the standard. So it can be said that MEPSL can be used as an antifungal agents [22].

Brine shrimp assay is low cost and simple method for determining cytotoxic properties of plant extract. The cytotoxic activity of MEPSL was tested by this method and the results are summarized in Table 7. The LC_{50} values for MEPSL, and standard drug Vincristine Sulphate was 1.057 µg/mL and 0.608 µg/mL respectively (Fig 3 and Fig 4). Moreover, several dosage levels of test solution were shown to have several degrees of mortality to *Artemia salina*. The values of LC_{50} ranged from 1.95 µg/mL (significant) to 500 µg/mL (very significant), declaring a genuine connection between concentration and LC_{50}. Percentage mortality was highest at a concentration of 500 µg/mL and conversely lowest at a concentration of 1.95 µg/mL. So it can be said, when the concentration of the test samples rises, the percentage of mortality also increases and vice versa. Compared to the standard vincristine sulphate (0.608 µg/mL), the MEPSL exhibit substantial cytotoxic activity against brine shrimp nauplii with LC_{50} value of 1.057 µg/mL. The MEPSL shows significant cytotoxicity compared to the standard vincristine sulphate which can be taken into consideration for further research to be used as an antitumor and pesticides compound [23,24].

5. CONCLUSION

As in many other countries, *Polyscias scutellaria* can be found growing wild in Bangladesh. The above description makes it very evident that *Polyscias scutellaria* is rich in phytochemicals and serves several pharmacological purposes. Previously, it was hypothesized that the crude methanolic extract of *Polyscias scutellaria* would have anti-inflammatory, anti-arthritic, and cytotoxic activities; the current research confirms that these hypotheses are correct. Compared to the standard Griseofulvin, the extract showed significant fungicidal activity against some yeast like fungi. Thrombolytic properties of the extract are also remarkable than the standard streptokinase. Membrane stabilizing properties are also significant. This data suggests that *Polyscias scutellaria* may have potential in the pharmaceutical industry. This makes the plant an excellent candidate for more systematic, chemical, and biological testing to isolate the active ingredient. It is possible that GC-MS analysis and in-vivo studies may be required in the future for confirmation by researchers.

CONSENT

Every single donor was supplied with a written consent form that narrated the purpose of this research, title of this project, and the volume of blood that will be drawn. The illustration of this research includes whether or not volunteers will consume any therapy, any kind of irritation to the piercing area and the time period for blood collection.

ETHICAL APPROVAL

This research followed all rules set forth by the US Food and Drug Administration, the Declaration of Helsinki, and the International Conference on Harmonization. Stamford University Bangladesh's Faculty of Science examined and accepted the research procedure and written consent form (reference number: SUB/ERC/202302).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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