In-Vitro Cytotoxic and Thrombolytic Potential of Methanolic Extract of *Podocarpus neriifolius D. Don* leaves

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Abstract

The present study was aim to evaluate the cytotoxic activity by in-vitro brine shrimp lethality bioassay and thrombolytic potentials of methanolic extract of leaves of *Podocarpus neriifolius*. In brine shrimp bioassay, the crude methanolic extract of leaf showed strong cytotoxic activity with LC_{50} value of 18.24µg/ml compared to that of 0.839 µg/ml exhibited by standard vincristine sulphate. It has significant thrombolytic activity (44.82%) compared to standard streptokinase (77.66%). The results of this study confirm that this plant will be good candidate for future research of anticancer and thrombolytic drugs.

Key words: Brine shrimp, Thrombolytic, Cytotoxic, Clot lysis, *Podocarpus neriifolius.*

1. Introduction

Podocarpus neriifolius is a medium sized to fairly large tree which can reach up to 35(-45) m tall. Its bole is columnar, branchless, for up to 22m and measuring up to 100 cm diameter. It is rarely spurred or even buttressed while the surface of bark is grayish–brown. The foliage buds are ovate, acute or blunt and often with spreading scales. It is the most widespread species of genus, occurring from Nepal, India, Indo-China and Thailand, throughout malesia, towards the Solomon Island and fiji; also planted in garden[1–2]. Eleven compositions isolated from *Podocarpus neriifolius D. Don* were identified as n-C34H69OH, β-sitosteryl stearate, β-sitosterol, sciadopitysin, podocarpusflavone B, robustaflavone-7"-methyl ether, podocarpusflavone A, robustaflavone, p-hydroxyl benzoic acid, 2"-O-rhamnosylscopariu, 2"-O-rhamnosyl vitexin on the basis of physical constants and spectral data[3].

Herbal medicines are assumed to be of great importance in the primary healthcare of individuals and communities in many developing countries[4]**.** Herbal products are often perceived as safe because they are "natural"[5]. In Bangladesh, in recent years, there is increasing research on traditional ayurvedic herbal medicines on the basis of their known effectiveness in the treatment of ailments for which they have been traditionally applied.

Thrombolysis is the breakdown (*lysis*) of blood clots by pharmacological means. It is colloquially referred to as 'clot busting' for this reason. It works by stimulating fibrinolysis by plasmin through infusion of analogs of tissue plasminogen activator (tPA), the protein that normally activates plasmin. In vitro thrombolytic activity of crude extract was enumerated and was compared with streptokinase, which is a well known anticoagulant used in myocardial infarction[6]. Brine shrimp lethality evaluation is a bench top bioassay method for evaluating anticancer, antimicrobial and other pharmacological activity of natural products. Natural products extracts, fractions or pure compounds can be tested for their bioactivity by this method[7].

The aim of our present work was to investigate the cytotoxic and thrombolytic activity of methanolic extracts of *Podocarpus neriifolius D. Don* leaves by using an in vitro procedure.

2.Material and methods

2.1 Plant materials

The leaves of *P. neriifolius* were collected from Chittagong hill tracts area specifically from the area of Chittagong University and it is authenticated by Dr. Shaikh Bokhtear Uddin, Associate Professor, Department of Botany, University of Chittagong, Chittagong-4331, Bangladesh**.**

2.2 Reagents and chemicals

All chemicals i.e. methanol, *DMSO* and other reagents used in these experiments were of the highest analytical grade. Vincristine sulfate (2mg/vial; Techno Drugs Limited Bangladesh) and Streptokinase (1.5 million unit/vial; Sanofi-aventis Bangladesh Limited) were used as positive control for *in-vitro* cytotoxic test and thrombolytic test respectively. In case of brine shrimp lethality bioassay (cytotoxic test), DMSO was used as negative control, while water was used for thrombolytic test.

2.3 Extraction of plant materials

The fresh leaves of *P. neriifolius* were cut, washed and air dried at room temperature (24º±2ºC) for about 10 days. Dried leaves were macerated into coarse powder. Dried powder (500 gm) was then extracted using Methanol. Then methanolic extract was shaken by rotary shaking apparatus for 7 days. The extract was collected using Buckner funnel. The Methanol was evaporated at a temperature below 45ºC and concentrated extract was weighed 25 gm, stored at 4ºC

2.4 *Invitro* Cytotoxic test

Brine shrimp lethality bioassay is widely used in the bioassay for the bioactive compounds[8−9]. Here simple zoological organism (Artemia salina) was used as a convenient monitor for the screening. The dried cyst of the brine shrimp were collected from an aquarium shop (Chittagong, Bangladesh) and hatched in artificial seawater (3.8% NaCl solution) with strong aeration for 48 hours day/dark cycles to mature shrimp called nauplii. The cytotoxicity assay was performed on brine shrimp nauplii using Meyer method[8]. The test sample (extract) were prepared by dissolving them in DMSO (not more than 50 µL in 5 mL solution) plus sea water (3.8% NaCl in water) to attain concentrations of 10, 50, 100, 200, 300 and 500 µg/ml. A vial containing 50 µL DMSO diluted to 5 mL was used as a control. Standard vincristine sulphate was used as positive control. Then matured shrimps were applied to each of all experimental and control vials. After 24 h, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial were counted. From this data, the percent (%) of mortality of the brine shrimp nauplii was calculated for each concentration using the following formula:

% Mortality = N_t* N_0/100

Where, N_t = Number of killed nauplii after 24 hrs of incubation,

N_0 =Number of total nauplii transferred i.e 10.

The LC_{50} (Median lethal concentration) was then determined using Microsoft Excel 2007.

2.5 In vitro Thrombolytic test

The thombolytic activity of this extract was evaluated by the method of Prasad and collaborators (2006)[10] using streptokinase as standard. The dry crude extract (10 mg) was suspended in 10 ml of distilled water and it was kept overnight. Then the soluble supernatant was decanted and filtered. Aliquots (5 ml) of venous blood were drawn from healthy volunteers which were distributed in ten different pre weighed sterile micro centrifuge tube (1 ml/tube) and incubated at 37° C for 45 minutes. After clot formation, the 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 preweighed clot, 100 µl aqueous solutions of the crude extract was added separately. As a positive control, 100 µl of streptokinase (SK) and as a negative non thrombolytic control, 100 µl of distilled water were separately added to the control tubes. All the tubes were then incubated at 37° C for 90 minutes and observed for clot lysis. After incubation, the released of fluid was removed and tubes were again weighed to observe the difference in weight after clot disruption. The differences in weights taken before and after clot lysis were expressed as percentage of clot lysis as shown below:

% of clot lysis = (wt of released clot /clot wt) × 100

2.6 Statistical Analysis

Statistical analysis was performed using Microsoft excel 2007. All the results of thrombolytic test were expressed as mean ± standard error of mean (S.E.M.).

3. Results and Discussion

Brine shrimp lethality bioassay: In brine shrimp lethality bioassay, the methanolic extract of *Podocarpus neriifolius* leaves showed positive result in comparison with the positive control vincristine sulphate. By plotting the concentration versus percent (%) of mortality for all test samples showed an approximate linear correlation. From the graph, the median lethal concentration (LC_{50}) was determined to check the toxic level of the extract. The crude extract of *Podocarpus neriifolius* leave showed significant cytotoxic activity against brine shrimp

nauplii and LC_{50} value was 18.24 µg/ml compared with standard vincristine sulphate (LC_{50} =0.839µg/ml). (Table 1 & Figure 1). DMSO was used as negative control to validate the test method.

Thrombolytic activity assay: 100 µl Stertokinase as a positive control (30,000 I.U.) was added to the clots along with 90 minutes of incubation at 37°C, showed 77.657% clot lysis. Clots when treated with 100 µl sterile distilled water (negative control) showed only negligible clot lysis (3.049%). The *in vitro* thrombolytic activity study revealed that *Podocarpus neriifolius* showed 44.82 % clot lysis. The percentage of weight loss of clot after application of extract solution was taken as the functional indication of thrombolytic activity. % Clot lysis obtained after treating clots with different concentration of sample was shown in (Table 2 & Figure 2).

Herbal preparations are used since ancient times for the treatment of diseases. Phytopharmacological and phytochemical evaluation lead to drug discovery. About 30% of the pharmaceuticals are prepared from plants worldwide [11–12]. A number of studies have been conducted by various researchers to find out the herbs and natural food sources and their supplements having thrombolytic (anticoagulant and antiplatelet) effect and there is evidence that consuming such food leads to prevention of coronary events and stroke [13–16]. Although there are several thrombolytic drugs including those obtained by recombinant DNA technology, but side effects related to some of these drugs that lead to further complications have been reported [17–20]. Brine shrimp lethality bioassay is an easy and straight forward bench top screening method for predicting important pharmacological activities like enzyme inhibition, ion channel interference, antimicrobial and cytotoxic activity [21–23]. The extract showed LC_{50} at a very low concentration with very quick response indicating that the extract is significantly potent. Further investigation is required to find the responsible compound(s) for the cytotoxic activity observed for *Podocarpus neriifolius*. In the thrombolytic bioassay result suggested that the extract showed very potent activity. The plant can be evaluated to further research for thrombolytic activity to a specific disease.

4. Conclusion

From our bioassay, we concluded that *Podocarpus neriifolius* has got the potential as a candidate for future thrombolytic agent. It can also be investigated as a possible source of antitumour drugs. This is only a preliminary study and to make final comment the extract should be thoroughly investigated phytochemically and pharmacologically to exploit their medicinal and pharmaceutical potentials. This study may be helpful for further research works.

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Table 1: Brine shrimp lethality of *Podocarpus neriifolius*

Concentration (µg/ml)	Log C	Total nauplii	No. of nauplii Dead	No. of nauplii live	% of mortality	LC_{50}
10	1	10	05	05	50%	18.24
50	1.69897	10	06	04	60%	
100	2	10	08	02	80%	
200	2.30103	10	09	01	90%	
300	2.47712	10	09	01	90%	
500	2.69897	10	10	0	100%	

Table 2: Thrombolytic activity of *Podocarpus neriifolius*

Volunteer	**MEPN**				**SK**				**Water**			
	% lysis of clot	**Average**	**S.D.**	**S.E.M.**	**% lysis of clot**	**Average**	**S.D.**	**S.E.M.**	**% lysis of clot**	**Average**	**S.D.**	**S.E.M**
0	43.	44.82	10.84325	3.428938	78.	77.66	5.18473	1.639556	5.12	3.049	1.580516	0.499803
0	67.				85.				2.25			
0	41.				76.				1.75			
0	33.				70.				0			
0	35.				73.				2.75			
0	49.				80.				4.45			
0	31.				69.				5.15			
0	42.				77.				3.12			
0	50.				82.				3.15			
1	52.				82.				2.75			

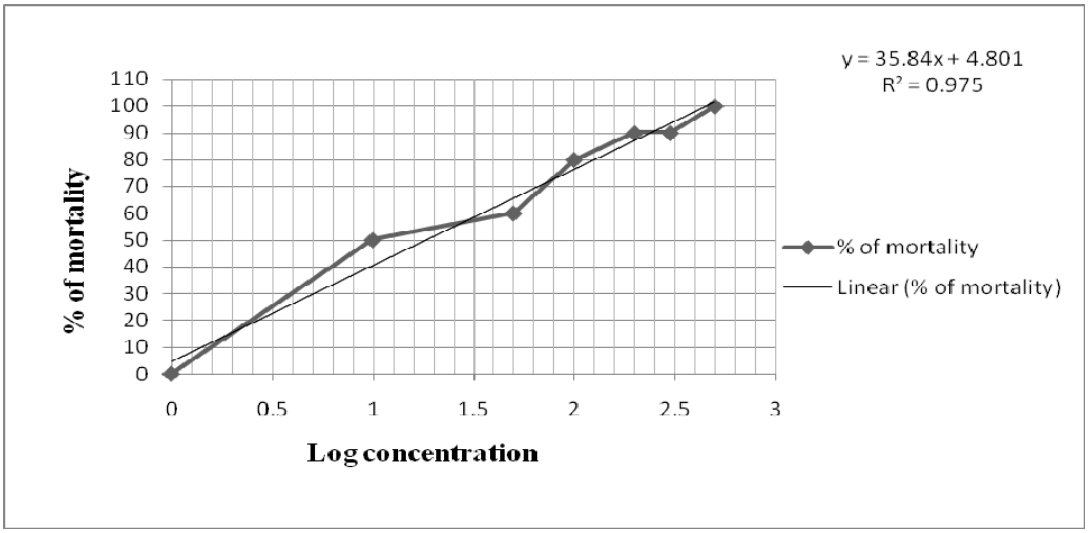

Fig 1: Determination of LC_{50} of methanol extract of *Podocarpus neriifolius*

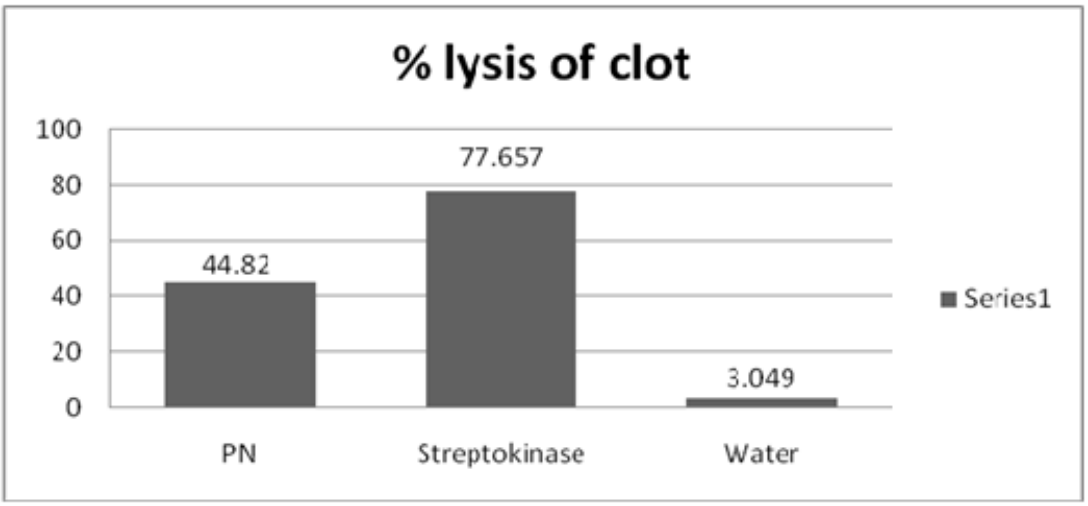

Fig 2: Clot lysis by streptokinase, *Podocarpus neriifolius* and water