European Journal of Medicinal Plants
10(2): 1-9, 2015, Article no.EJMP.17121
ISSN: 2231-0894

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Study of Cytotoxic, Thrombolytic and Anthelmintic Activity of Extract of *Neolamarckia cadamba* (Roxb.) Leave

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Authors' contributions

This work was carried out in collaboration between all authors. Authors ATMMK and KAAC designed and supervised the study, authors MMR, AI, EAK and Anaytulla performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MAH and MMHC managed the analyses of the data with interpretation and the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/EJMP/2015/17121
Editor(s):
(1) Marcello Iriti, Professor of Plant Biology and Pathology, Department of Agricultural and Environmental Sciences, Milan State University, Italy.
Reviewers:
(1) Anonymous, National Research Centre, Egypt.
(2) Anonymous, Universidad de Talca Talca, Chile.
(3) Anonymous, Mexico.
Complete Peer review History: http://sciencedomain.org/review-history/10606

Original Research Article

Received 26th February 2015
Accepted 1st April 2015
Published 19th August 2015

ABSTRACT

Aim of the Study: The study was aimed to evaluate the cytotoxic, thrombolytic and anthelmintic activity of methanolic extract of the stem of *Neolamarckia cadamba* (Roxb.).
Place and Duration of Study: The research was carried out in Department of Pharmacy, International Islamic University Chittagong, Bangladesh between January 2013 and November 2014.
Methodology: The study adopted cytotoxic activity using brine shrimp lethality, thrombolytic activity using red blood cell of human and anthelmintic activity by using aquarium

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worm *Tubifex tubifex.*
Results: The brine shrimp lethality bioassay was used to determine the cytotoxic activity of crude extract and LC_{50} values of the extract was found 130.617±0.82 µg/ml. Here vincristine sulphate was used as standard and the result of this positive control was found 8.50±0.16 µg/ml. The extract showed clot lytic activity (39.97±4.67%) as compared to standard streptokinase's (48.82±2.35%) clot lytic activity in case of thrombolysis assay. The evaluation of anthelmintic activity was determined by using *Tubifex tubifex* by using three concentrations *viz.,*5, 8 and 10 mg/ml of the extract was studied which was mainly concerned with the determination of time require for paralysis and that of death of the worms. The result showed that as the dose increases, the activity also increases gradually. At highest concentration of 10 mg/ml the anthelmintic activity of the extract's activity was found significant when compared with standard reference levamisole (1 mg/ml) and distilled water was used as control.
Conclusion: The results of this study substantiates that the methanol extract of the stem of *Neolamarckia cadamba* (Roxb.) have moderate cytotoxic activity and significant thrombolytic activity and have anthelmintic activity in dose dependent manner, which could be further exploited for their potential biological activity and might overcome the ever expensive synthetic.

Keywords: Neolamarckia cadamba (Roxb); cytotoxic activity; thrombolytic activity; anthelmintic activity methanol extract.

1. INTRODUCTION

The foods that are obtained from plant sources contains abundant amount of bioactive compounds, which is beneficial for health along with basic nutrition. Epidemiological evidence suggests that if people take diet filled of vegetables and fruits that may provide positive implications on their health [1]. The World Health Organization reported that 80% of the world populations rely chiefly on indigenous medicine and that the majority of traditional therapies involve the use of plant extracts or of their active constituents [2,3] and over 25% of modern medicines of medical science that are used worldwide for mankind contains compounds extracted from medicinal plants [4]. In Bangladesh there is abundant of medicinal plants and ninety percent of the medicinal plants are wild sourced [5,6].

During recent decades, the demand for finding newer and safer chemotherapeutic agents are increasing rapidly [7]. Cancer is the third leading cause of death worldwide, only preceded by cardiovascular disease, infectious and parasitic disease [8]. Extracts derived from medicinal plants contain an abundant of polyphenolic, flavonoids, alkaloids, terpenoids and saponin compounds having therapeutic properties and can resist cancer formation [9,10]. Over 60% of current cytotoxic agents are obtained from natural sources, which includes plants, marine organisms and microorganisms, either directly or by chemical synthesis based on natural lead compounds [7,11,12]. Therefore, natural products have a wide application in cancer chemotherapy [12].

Cardiovascular disease caused by blood clot (thrombus) formation is one among the most severe diseases which are increasing at an alarming rate in the recent years. Homeostasis maintains the integrity of circulatory system after damaging of the vascular channel. Thrombus development is a critical event in the arterial diseases associated with myocardial infarction, anoxia, hypertension, stroke, and also reduce the blood supply to the liver [13] and venous thromboembolic disorders that account for considerable number of deaths worldwide [14,15]. Remarkable efforts have been made towards the discovery and development of natural constituents from various plant and animal sources which have antiplatelet, anticoagulant, antithrombotic and thrombolytic activity. Thrombolytic agents are used to dissolve clot and in the management of thrombosis in patients [16]. Thrombolytic agents such as tissue plasminogen activator (t-PA), Urokinase (UK), streptokinase (SK) [17] etc, are used worldwide for the ailment but their use is associated with hyper risk of haemorrhage, anaphylactic reaction and lacks specificity [17,18]. Because of the shortcomings in the existing thrombolytic agents, a number of researches are underway to improve the variants of these drugs for their better effective nature [17].

Helminthic infestations are now being recognized as a cause of chronic ill health and sluggishness amongst the children [19]. World Health

Organization estimated 2 billion people infected with helminthes and it was also estimated that 100% of all age group of school children are at risk of morbidity [20]. The major phyla of helminthes are nematodes (round worms) which are soil transmitted helminths that mostly cause the intestinal infection, filarial worms cause the onchocerciasis and lymphatic filariasis, while platyhelminths (flatworms) also known as trematodes like schistosomes and cestodes causes cyticerosis [21,22]. Worm infects different organ of the body and more than half of the people of the world is infected with intestinal helminths, such as *Ascaris*, hookworms, *Trichuris*, *Enterobius*, *Strongyloides*, and tapeworms, and the people of remote rural areas of the developing countries are becoming infected most [21,23]. In case of other animals also gastrointestinal parasites causes infections that diminish the animal survival, growth rates and reproductive performance. Morbidity from nematodes is common with diabetes and lung cancer [24]. The helminths parasites mainly subsist in human body in intestinal tract, but they are also found in tissue, as their larvae migrate towards them [25,26]. Chemical control of helminthes coupled with improved management has been the important worm control strategy throughout the world [25]. The anthelmintic drug used for the prevention or cure the helminth infection have some common side effect including intestinal gastro-intestinal disturbances, nausea and giddiness and various studies and reviews have showed that the resistance of anthelmintic drug is increasing day to day [27,28]. Henceforth it is important to look for alternative strategies against gastrointestinal nematodes, which have led to the proposal of screening medicinal plants for their anthelmintic activity [25].

Neolamarckia cadamba (Roxb.) belonging to the family Rubiaceae, known as Kodom in the Bengali language, is grown commonly in different parts of Bangladesh, Nepal, India, Myanmar, Sri Lanka, the Philippines, Indonesia, and Papua New Guinea [29,30]. The Kadamba tree grows up to 45 m high. The large tree, having a broad crown and straight cylindrical bole, is quick growing and grows rapidly in the first 6-8 year. The trunk of the tree has a diameter of 100-160 cm and leaves are 13-32 cm long and flowering usually begins at 4–5 years old. The literature review informs that the plant contain saponins, indole and quinoline alkaloids, secoiridoids and triterpenes [29]. In folk medicine it is used in the treatment of fever, uterine complaints, blood diseases, skin diseases, eye inflammation, diarrhoea, anaemia, leprosy, dysentery and stomatitis [31]. The pharmacological studies have revealed that the extract of the plant have antimicrobial, antioxidant, and wound healing as well as anti-diarrheal properties [32]. The present study was undertaken to investigate the cytotoxic, thrombolytic and anthelmintic activity of leave extract of this plant.

2. MATERIALS AND METHODS

2.1 Chemicals

Lyophilised streptokinase vial (1 500 000 IU) was purchased from Square Pharmaceuticals Ltd, Bangladesh. Methanol was purchased from Merck, Germany. Normal saline solution was purchased from Beximco Infusion Ltd. Levamisole was purchased from ACI Limited, Bangladesh. All chemicals used were of analytical reagent grade.

2.2 Plant Materials

Fresh stem of *C. reflexa* for this study were collected from the local area of Chittagong, Bangladesh and were authenticated by Dr. Sheikh Bokhtear Uddin, Associate Professor, Department of Botany, University of Chittagong, Chittagong-4331, Bangladesh.

2.3 Preparation of Crude Extract

The collected stem were dried for a period of 2 weeks under shade and ground. The ground stem (750 gm) were soaked in sufficient amount of methanol for one week at room temperature with occasional shaking and stirring. The sediments were filtered and the filtrates were dried at 40°C in a water bath. The solvent was completely removed by filtering with What man number-1 filter paper. The solvent was evaporated under reduced pressure at room temperature to yield semisolid. The extract was then preserved in a refrigerator till further use [33].

2.4 Brine Shrimp Lethality Assay

The assay was carried out according to the principle and protocol previously described by Meyer et al. [34], with slight modifications. Here simple zoological organism (*Artemia salina*) was used as a convenient monitor for the screening. Dried cysts of *Artemia salina* were collected from an aquarium shop (Chittagong, Bangladesh) and

hatched in artificial seawater (3.8% NaCl solution) for 48 h to mature shrimp called nauplii. After hatching, active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the assay.

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, 25, 50, 100, 200, 300, 500 and 800 µg/ml. A vial containing 50 µL DMSO diluted to 5 mL was used as a control. Vincristine sulphate was used as positive control. After 24 hours the number of survival of nauplii was counted and percentage of mortality was determined using the equation:

% mortality = (no. of dead nauplii/ initial no. of live nauplli) x 100.

Statistical method of probit analysis (Finney's table) [35] was used to calculate LC_{50}. Criterion of toxicity for fractions was established according to Déciga-Campos et al. [36]. LC_{50} values > 1000 µg/mL (non-toxic), ≥ 500 ≤ 1000 µg/mL (weak toxicity) and < 500 µg/mL (toxic).

2.5 Thrombolytic Test

This test was performed according to the method described by Prasad et al. [37]. In the commercially available lyophilised streptokinase vial (1 500 000 IU), 5 mL sterile distilled water was added and mixed properly. This suspension was used as a stock solution from which appropriate dilution was made. Five milliliter of venous blood was drawn from the healthy volunteers (n=10) without the history of oral contraceptive or anticoagulant therapy and was distributed (0.5 mL/tube) to each ten previously weighed sterile micro centrifuge tube and incubated at 37°C for 45 min to form the clot. After the clot formation, serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight. A volume of 100 µL of methanol extract (10 mg/ mL) was added to each micro centrifuge tube containing pre weighed clot. As a positive control, 100 µL of streptokinase and as a negative control 100 µL of distilled water were separately added to the control tube numbered. All the tubes were then incubated at 37°C for 90 min and observed for clot lysis. After incubation, fluid released was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis.

2.6 Anthelmintic Assay

The anthelmintic activity of methanolic extract of stem of *C. reflexa* was carried out as per the procedure of Ajaiyeoba et al. [38] with some minor modifications. The aquarium worm *Tubifex tubifex* were used in the present study because it has anatomical similarity and belongs to the same group of intestinal worm i.e. annelid [39-41]. The worm were collected from the local market of Chittagong, average size of worms 2-2.5 cm. were taking study. The standard drug levamisole and three different concentrations of methanol extracts (2.5, 5 and 10 mg/ml) in double distilled water [42,43] were prepared freshly and used for the study of anthelmintic activity. One group was composed of water and it was considered as controlled group. The anthelmintic activity was determine at two different stage 'time of paralysis' and 'time of death' of the worms. Time for paralysis was noted when no movement of any sort could be observed except when the worms were shaken vigorously. Death was concluded when the worms lost their motility followed with fading away of their body colors. Death was also confirmed by dipping the worms in slightly warm water. The mortality of parasite was assumed to have occurred when all signs of movement had ceased [44].

3. RESULTS

The lethality was determined on *Artemia salina* after 24 h of exposure of the crude extract of leave of *N. cadamba*. The LC_{50} value (Fig. 1) of the extract was found 130.617±0.82 µg/ml and that for standard vincristine sulphate was 8.50± 0.16 µg /ml and the control group shows no mortality, using DMSO and sea water. The clot lysis activity of the plant extract was showed moderate (39.97±4.67%) as compared to standard streptokinase's clot lysis (48.82±2.35%) activity (Fig. 2). Results of study were recorded by considering the time required to get consecutive attacks of paralysis and finally the time required for complete death of parasite. From the observations made, higher concentration of extract produced paralytic effect much earlier and the time to death was shorter for all worms. From the above study it was seen that the methanolic extract showed dose dependent anthelmintic/antihelminthic activity as compared to a standard drug levamisole (Fig. 3). The extract showed paralyzing time of *Tubifex tubifex* with the dose of 2.5, 5 and 10 mg/ml were found to be 55.19±3.204, 4.76±1.134 and

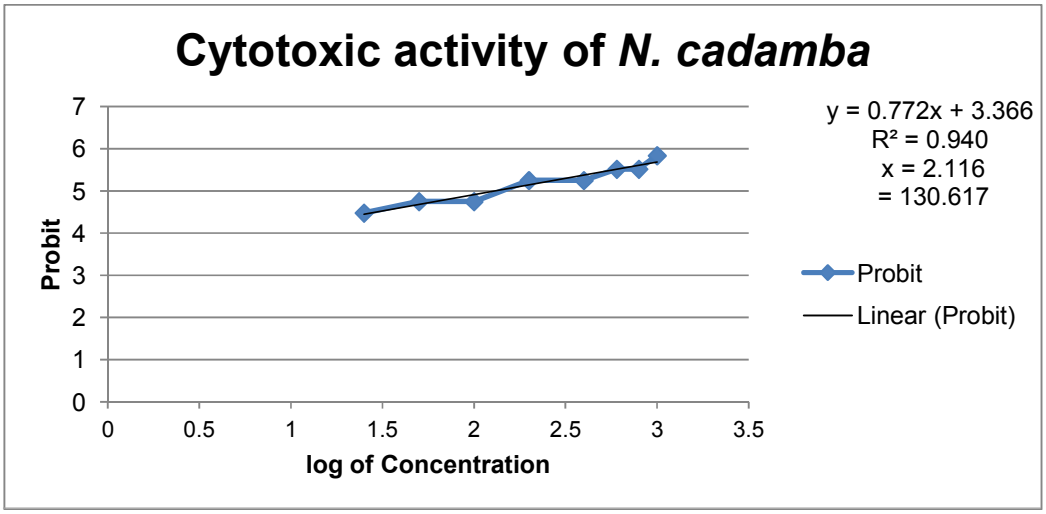

Fig. 1. Toxicity assay of *N. cadamba* on brine shrimp. The results are expressed as mean±SEM of three measurements

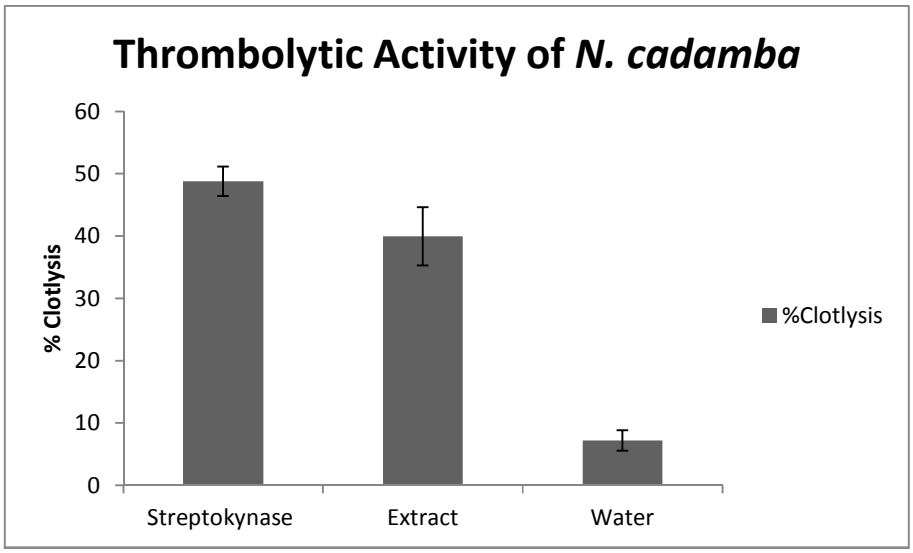

Fig. 2. The clot lysis activity of *N. cadamba* extract and streptokinase. All results are mean±SEM of three consecutive experiments

1.25±0.352 minutes respectively. In the meantime levamisole at a dose of 0.5, 0.8 and 1 mg/ml causes paralysis in the above helminth in 14.41±2.643, 6.26±1.261 and 3.30±0.645 minutes respectively. The mean death time of *Tubifex tubifex* with the extract at dose of 5, 8 and 10 mg/ml were found to be 90.18±5.326, 10.38±2.642 and 2.54±0.219 minutes respectively and the standard levamisole at a dose of 0.5, 0.8 and 1 mg/ml causes death in the above helminth in 51.32±3.825, 12.21±3.512 and 6.50±1.314 minutes respectively. No paralysis or death was observed in case of control (water).

3.1 Statistical Analysis

All the results obtained by in vitro experiment were expressed as mean±SEM of three measurements followed by Dunnet's test where $P<0.01$ was considered as statistically significant.

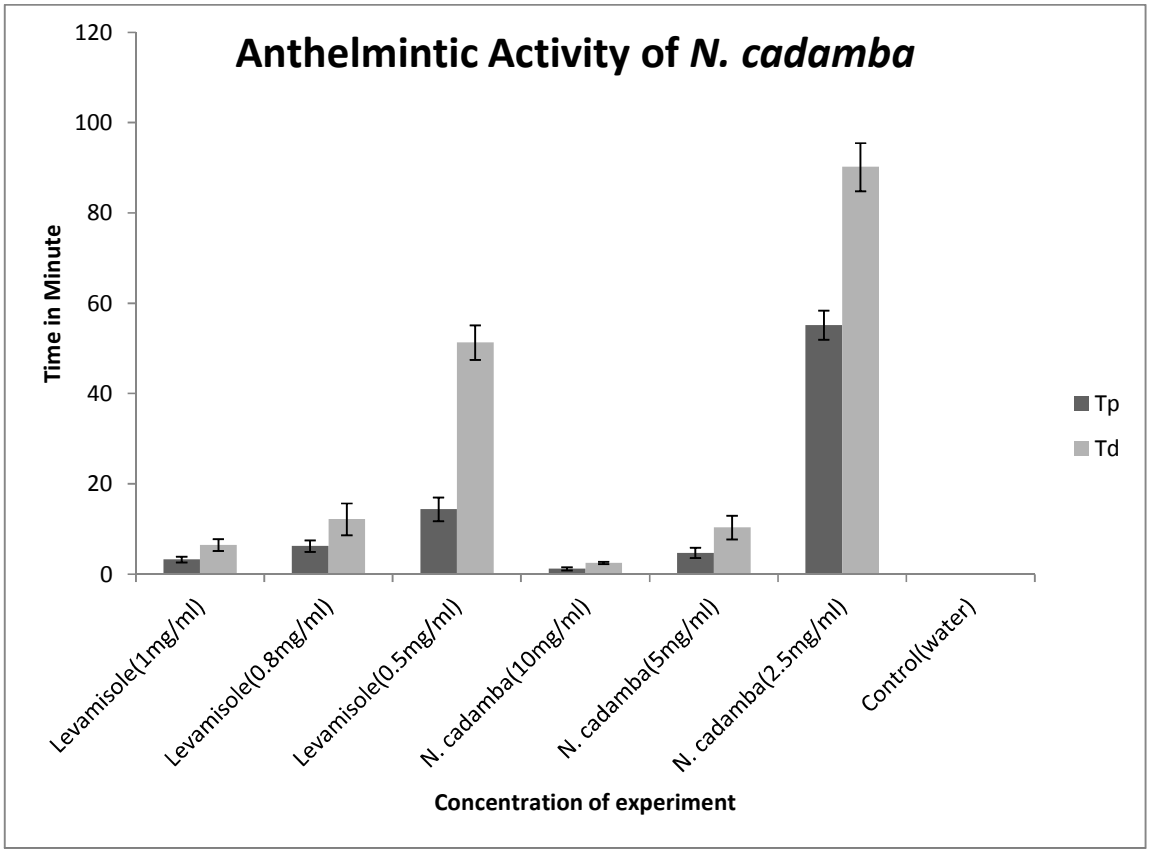

Fig. 3. The anthelmintic activity of *N. cadamba* extract and levamisole. All results are mean±SEM of three consecutive experiments

4. DISCUSSION

Ideally, any agent that is used in the treatment of cancer should not be toxic for normal cell. However, in reality, the anticancer agents are often shows toxicity to normal cells, particularly towards the rapidly growing cell [45,46]. That is why the extract of the plant should be tested against various cancer cell line as well as against normal cell line so as to justify the potential of the plant for anticancer activity. It is necessary to test this extract to evaluate its potency and also against various cancer cell lines as well as normal cell line so justify the potential to further investigate this plant for anticancer activity.

Most thrombolytic agents works by activating the enzyme plasminogen that clears the cross-linked fibrin mesh responsible for clot formation by making the clot soluble and subject to further proteolysis by other enzymes, and restores blood flow over occluded blood vessels. Thus thrombolytic agents are useful for the treatment of different disease like myocardial infarction, thromboembolic strokes, deep vein thrombosis and peripheral embolism, to clear a blocked artery and avoid permanent damage to the perfused tissue (e.g. myocardium, brain, and leg) [14]. Anthelmintics are the drugs that shows their activity by expelling out parasitic worms (helminthes) from the body by either causing paralysis or by directly killing them [47,48] by damaging its cuticle and leads to digestion partially or rejection by immune mechanisms [49]. Levamisole works as a nicotinic acetylcholine receptor agonist that causes continued stimulation of the parasitic worm muscles, leading to paralysis. The literature have been reported that some compounds like flavonoids, tannins and polyphenolic [50], are responsible for anthelmintic activity by binding to free protein in the gastrointestinal tract of host animal or glycoprotein on the cuticle of the parasite [19] and thereby leading to death of the parasite [51,52]. Some synthetic phenol anthelmintics e.g. niclosamide, oxyclozanide and bithionol provide their activity by interfering with energy generation in anthelmintic parasites by

uncoupling oxidative phosphorylation and phosphorylation [53].

5. CONCLUSION

The study concludes that the leave of *Neolamarckia cadamba* has found to possess moderate cytotoxic, weak thrombolytic and significant anthelmintic activity in dose dependent manner. It might have potential to prevent disease and might be a candidate for the development of useful, economic and safe therapeutics in future. But it demands more thorough study to find out the exact chemicals responsible for the activity and the probable mechanism of action and to check how friendly the component to the internal organ of the body are.

ACKNOWLEDGEMENT

The author would like to thank the authority of Department of Pharmacy of International Islamic University Chittagong for their support throughout.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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