



STUDY OF CYTOTOXIC, THROMBOLYTIC AND ANTHELMINTIC ACTIVITY OF STEM OF *CUSCUTA REFLEXA* ROXB.

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ABSTRACT

The study was aimed to evaluate the cytotoxic, thrombolytic and anthelmintic activity of methanol extract of the stem of *Cuscuta reflexa* Roxb. The cytotoxic activity of crude extract was determined using brine shrimp lethality bioassay and LC_{50} values of the sample was $181.134 \pm 0.43 \mu\text{g/ml}$ whereas for standard vincristine sulphate was $8.50 \pm 0.16 \mu\text{g/ml}$ as a positive control. The extract showed $(12.89 \pm 1.12\%)$ clot lytic as compared to standard streptokinase's $(30.26 \pm$

$0.67\%)$ clot lytic activity in case of thrombolysis assay. The anthelmintic activity was done 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 of paralysis and time of death of the worms. The gradual increased in a dose exhibited, a gradual increase in the activity. The extract exhibited significant anthelmintic activity at highest concentration of 10 mg/ml as compared with levamisole (1 mg/ml) was evaluated as standard reference and distilled water as control.

KEYWORDS: *Cuscuta reflexa*, Cytotoxic activity, Thrombolytic activity, anthelmintic activity Methanol extract.

INTRODUCTION

Plant-based foods contain significant amounts of bioactive compounds, which provide desirable health benefits beyond basic nutrition. Epidemiological evidence suggests that consumption of a diet rich in vegetables and fruits has positive implications for human health.

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^[1] and over 25% of modern medicines that are commonly used worldwide contains compounds extracted from medicinal plants.^[2] In Bangladesh there is abundant of medicinal plants and ninety percent of the medicinal plants are wild sourced.^[3, 4]

During recent decades, there has been an increasing demand for finding newer and safer chemotherapeutic agents. Cancer is the third leading cause of death worldwide, only preceded by cardiovascular disease, infectious and parasitic disease ^[5]. Extracts of medicinal plants are believed to contain a wide spectrum of polyphenolic, flavonoids, alkaloids, terpenoids and saponin compounds, which might have therapeutic properties and hinder cancer formation.^[6] Over 60% of current cytotoxic agents have been derived from natural sources including plants, marine organisms and microorganisms, either directly or by chemical synthesis based on natural lead compounds.^[7,8] Therefore, natural products have a wide application in cancer chemotherapy.^[8]

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, reduction of the blood supply to the liver^[9] and venous thromboembolic disorders that account for considerable number of deaths worldwide.^[10] 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.^[11] Thrombolytic agents such as tissue plasminogen activator (t-PA), Urokinase (UK), streptokinase (SK) etc, are used all over the world for the treatment but their use is associated with hyper risk of haemorrhage, anaphylactic reaction and lacks specificity.^[12] 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.

Helminthic infestations are now being recognized as a cause of chronic ill health and sluggishness amongst the children. 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.^[13] 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 platihelminths (flatworms) also known as trematodes like schistosomes and cestodes causes cyticercosis.^[14,15] Current estimates suggest that over half of the world population is infected with intestinal helminths, such as *Ascaris*, hookworms, *Trichuris*, *Enterobius*, *Strongyloides*, and tapeworms, and that most of these infected people live in remote rural areas in the developing countries.^[14] 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.^[16] The helminths parasites mainly subsist in human body in intestinal tract, but they are also found in tissue, as their larvae migrate towards them.^[17] Chemical control of helminthes coupled with improved management has been the important worm control strategy throughout the world. Side effects of anthelmintic commonly include intestinal gastro-intestinal disturbances nausea and giddiness, while various studies and reviews have showed the resistance to anthelmintic is increasing day to day.^[18] 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.

Cuscuta reflexa Roxb, is a leafless, delicate yellow coloured total stem parasite, belonging to the plant family Convolvulaceae. . It is widespread in temperate and tropical regions and commonly found throughout Bangladesh. It grows on different host plants.^[19] The tiny white flowers appear in bunches and the fruit are pea shaped and seeds are black in colour. Phytochemical investigation of *C. reflexa* indicates the presence of kaempferol-3-O-glucoside, astragallin, myrecetin, benzopyrones, glucopyranosides, propenamide, flavonols, quercetin and quercetin-3-O-glucoside, sitosterol, and bergenin.^[20] Traditionally, it used in treatment of protracted fever, diaphoretic, and as demulcent and as purgative.^[21] The present study was undertaken to investigate the cytotoxic, thrombolytic and anthelmintic activity of stem extract of this plant.

MATERIALS AND METHODS

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.

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.

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 Whatman 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.^[22]

Brine shrimp lethality assay

The assay was carried out according to the principle and protocol previously described by Meyer *et al.*^[23], 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:

$$\% \text{ mortality} = (\text{no. of dead nauplii} / \text{initial no. of live nauplii}) \times 100.$$

Statistical method of probit analysis (Finney's table)^[24] was used to calculate LC₅₀. Criterion of toxicity for fractions was established according to Déciga-Campos *et al.*^[25] LC₅₀ values > 1000 µg/mL (non-toxic), $\geq 500 \leq 1000$ µg/mL (weak toxicity) and < 500 µg/mL (toxic).

Thrombolytic test

This test was performed according to the method described by Prasad *et al.*^[26] 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.

Anthelmintic assay

The anthelmintic activity of methanolic extract of stem of *C. reflexa* was carried out as per the procedure of Ajaiyeoba *et al.*^[27] 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.^[28,29,30] 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^[31,32] 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 determined at two different stages '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 by fading away of their body colors. Death was also confirmed by dipping the worms in slightly warm water. The mortality of the parasite was assumed to have occurred when all signs of movement had ceased.^[33]

RESULTS

The lethality of the crude extract of *C. reflexa* flower to brine shrimp was determined on *Artemia salina* after 24 h of exposure to the samples, the negative control DMSO and sea water and vincristine sulphate used as standard. This technique was applied for the determination of general toxic property of the plant extract. The LC_{50} value (Figure 1) of the extract was $181.134 \pm 0.43 \mu\text{g/mL}$ and that for standard vincristine sulphate was $8.50 \pm 0.16 \mu\text{g/mL}$. No mortality was found in the control group, using DMSO and sea water. The plant extract showed moderate clot lysis activity ($12.89 \pm 1.12\%$) as compared to standard streptokinase's clot lysis ($30.26 \pm 0.67\%$) activity (Figure 2). Results of the study were recorded as shown in table-1 as in the form of time required to get consecutive attacks of paralysis and at the end time required for complete death of the parasite. From the observations made, higher concentration of extract produced a 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 anti-helminthic activity as compared to a standard drug levamisole. The extract showed paralyzing time of *Tubifex tubifex* with the dose of 5, 8 and 10 mg/ml were found to be 25.42 ± 2.204 , 14.32 ± 2.134 and 7.21 ± 1.352 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 ± 1.643 , 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 57.59 ± 5.326 , 44.16 ± 3.642 and 28.32 ± 2.219 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 ± 4.825 , 12.21 ± 2.512 and 6.50 ± 1.314 minutes respectively. No paralysis or death was observed in case of control (water).

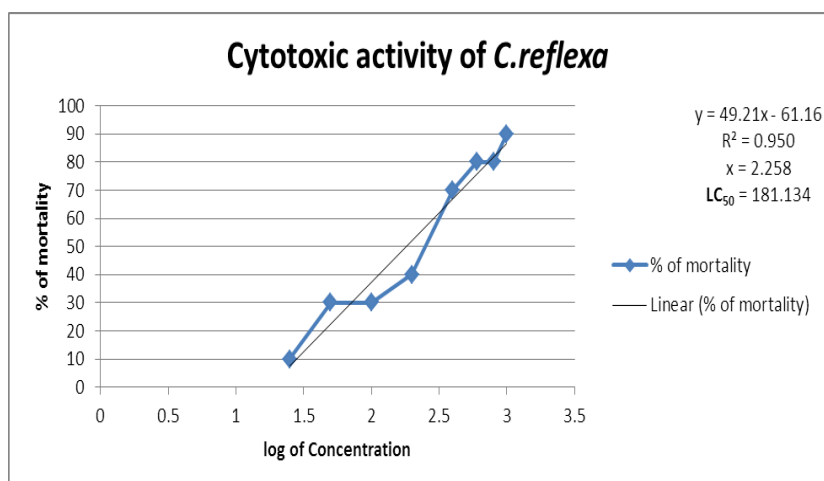


Figure 1. Toxicity assay of *C.reflexa* on brine shrimp. The results are expressed as mean±SEM of three measurements.

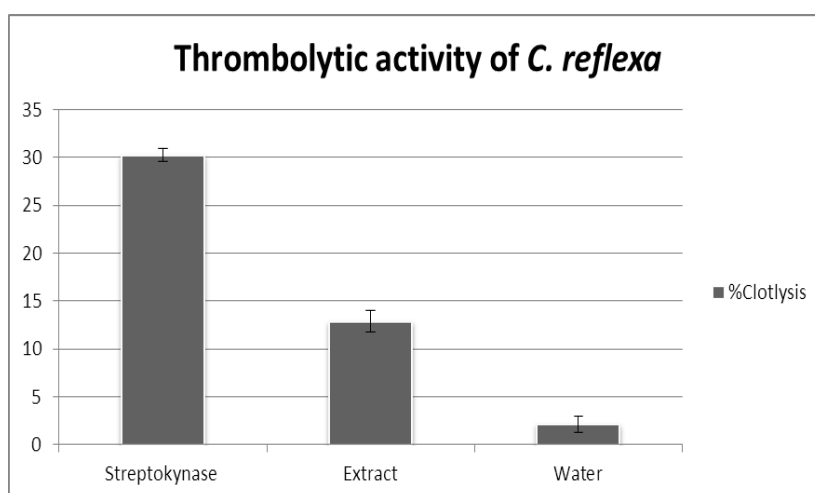


Figure 2. The clot lysis activity of *C. reflexa* extract and streptokinase. All results are mean±SEM of three consecutive experiments.

Table-1: Anthelmintic activity of *C. reflexa*. All results are mean±SEM of three consecutive experiments.

Test Sample	Concentration Mg/ml	Time taken for paralysis (min)	Time taken for Death (min)
<i>C. reflexa</i>	10	7.21 ± 1.352	28.32 ± 2.219
	8	14.32 ± 2.134	44.16 ± 3.642
	5	25.42 ± 2.204	57.59 ± 5.326
Standard (Levamisole)	1	3.30 ± 0.645	6.50 ± 1.314
	0.8	6.26 ± 1.261	12.21 ± 2.512
	0.5	14.41 ± 1.643	51.32 ± 4.825
Control (water)		--	--

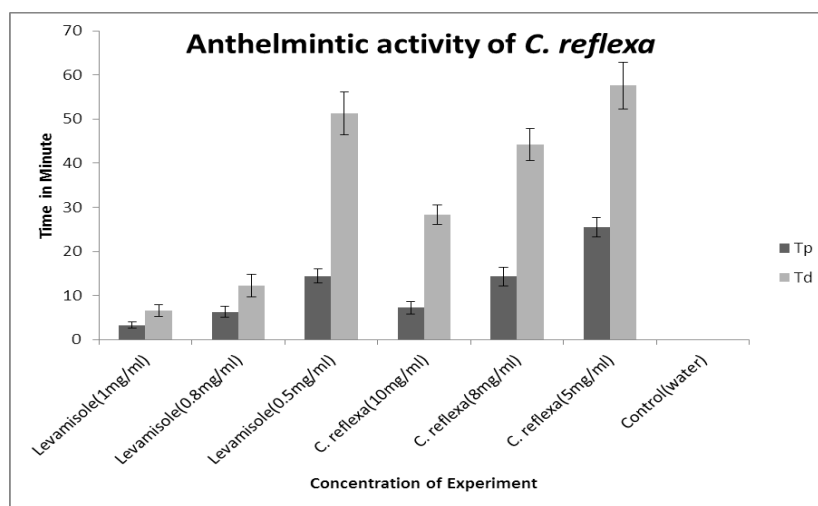


Figure 3. The anthelmintic activity of *C.reflexa* extract and levamisole. All results are mean $\pm$ SEM of three consecutive experiments.

Statistical analysis

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

DISCUSSION

Ideally, any agent useful in the treatment of cancer should not be toxic to normal cell. However, in reality, anticancer agents are often toxic to normal cells, particularly towards rapidly growing cells.^[34] It is necessary to test this extract to evaluate its potency and also against various cancer celllines as well as normal cell line so justify the potential to further investigate this plant for anticancer activity.

Most thrombolytic agents work by activating the enzyme plasminogen, which clears the cross-linked fibrin mesh responsible for clot formation. This makes 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).

Anthelmintics are the drugs that expel out parasitic worms (helminthes) from the body by either causing paralysis or by directly killing them^[35] by damaging its cuticle, leading to

partial digestion or rejection by immune mechanisms.^[36] 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 the presence of flavonoids, tannins and polyphenolic compounds show anthelmintic activity^[37], as they can bind to free protein in the gastrointestinal tract of host animal or glycoprotein on the cuticle of the parasite and thereby causes death.^[38] Some synthetic phenol anthelmintics e.g. niclosamide, oxclozanide and bithionol are shown effects to interfere with energy generation in antihelminth parasites by uncoupling oxidative phosphorylation and phosphorylation.^[39] Finally study concludes that the plant under study has found to possess moderate cytotoxic, weak thrombolytic and significant anthelmintic activity in dose dependent manner . The plant might have potential to be developed as useful economic and safe anthelmintic alternative, but it demands more thorough study to find out the exact chemical responsible for anthelmintic activity of plant so as to isolate and extract it separately so as to improve the potency.

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