

Evaluation of *In vitro* Anthelmintic Activity, Total Phenolic Content and Cytotoxic Activity of *Crinum latifolium* L. (Family: Amaryllidaceae)

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ABSTRACT

Purpose: *Crinum latifolium* is a widely used plant in Asian folk and traditional medicine. In the present study, we have tried to find out the anthelmintic activity, total phenolic contents and cytotoxicity of the methanolic extract of the target plant.

Methods: Anthelmintic activity was assessed applying five different concentrations of the plant extract and recording the time of paralysis and death. Total phenolic contents were determined using Folin-Ciocaltu method, using Gallic acid as standard; while brine shrimp lethality test (BSLT) method was used to evaluate the cytotoxicity of the plant extract, where vincristine sulphate and DMSO was used as positive and negative control respectively.

Result: The lowest time for paralysis and death of worms, for test sample at highest concentration (50mg/ml), were found 24 ± 0.45 and 46.4 ± 0.60 min respectively, which gradually increased with the decrease of concentration. On the other hand, albendazole, which was used as standard, caused paralysis and death of worms at 56.2 ± 0.20 min and 77.4 ± 0.24 min respectively; whereas no mortality of the worms was observed, when distilled water was used as control. The crude methanolic extract exhibited lower amount of total phenolic content (17.50 ± 2.64 mg/ml). In case of cytotoxicity measurement, the crude methanolic extract showed positive result (with LC_{50} 15.652 μ g/ml) compared to standard Vincristine sulphate (0.839 μ g/ml); which indicated that the leaves of *Crinum latifolium* possess mild cytotoxic principles.

Conclusion: Therefore, further studies are suggested to evaluate the possible mechanism of action and the active compounds responsible for the biological activities of the plant extract.

Introduction

From the ancient time, herbal medicines have been used for the welfare of mankind as medicines to cure the series of diseases. New drugs of plant derivation are crucial because they are cheap, have little side effects and in accordance with WHO, about 80% of the world population are still depends mainly on plant based drugs.^{1,2} As of now, it is well established that medicinal plants serve as lead molecules in modern medicines and nutraceuticals because of their derived phyto-constituents.³ That involves with the development of essential drugs against a variety of pharmacological targets.

Amaryllidaceae is a widely spread family all over the world and contains about 90 genera and 1310 species.⁴ The genus *Crinum* represents an important part in family Amaryllidaceae with broad geographical distribution throughout the tropics, subtropics and warm moderate regions.⁵ *Crinum latifolium* L., a member of Amaryllidaceae family and popularly known as 'Sukhdarshan', has been used in Asian folk and traditional medicine as rubefacient,

tonic and for management of allergic disorders and tumor diseases⁶ and also used in the treatment of serious health situation like prostatitis adenoma, benign prostate enlargement.^{7,8} Several studies reported that, extracts of *Crinum* species used to possess antitumor, immune stimulating, painkiller, anti-viral, anti-bacterial, anti-fungal effects, uterine fibroids, hypoxia, irritation, detoxification, tissue regeneration, hormone balancing, to improve cell-mediated immunity and effective T-lymphocyte activator properties.⁹⁻¹¹

It is evident that the Indian species *Crinum latifolium* L. was used traditionally to take care of rheumatism, fistula, tumors, earaches, rubefacient, tubercle and whitlow.^{12,13} It was also found to have good thrombolytic activity.¹⁴ But no study is found regarding the investigation of anthelmintic, antioxidant and cytotoxic activities of *Crinum latifolium* L. leaves in Bangladesh. Therefore, in this present study, we tried to assess the anthelmintic

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activity, total phenolic content and cytotoxic effects of powdered *Crinum latifolium* L. leaves extract.

Materials and Methods

Plant materials

For this study, leaves of *Crinum latifolium* L. were collected from Noakhali region of Bangladesh, in August 2012. After collection the taxonomic identification was carried out with the help of taxonomist of Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh (Accession No. DACB: 37652).

Chemicals and Reference drug

All the chemical reagents used in the analysis of phytochemicals were purchased from Sigma Chemical Co. Ltd, (St. Louis, MO, USA) and E. Merck (Germany). For the anthelmintic test, the methanolic extract of leaves of *Crinum latifolium* was tested using different concentrations. Distilled water was used as control and Albendazole (Batch no: ALF0171, Square Pharmaceuticals Ltd., Bangladesh) was used as the standard drug for evaluation of anthelmintic activity. For the assessment of cytotoxic activity, Vincristine Sulphate (VS) was used as standard drug and DMSO was used as control.

Extraction of plant materials

For methanolic extraction 450 g of air dried powder of leaves was immersed in 3000 ml of distilled water in a beaker and kept for maceration for 10 days with occasional shaking. At the end of 10th day it was filtered using filter cloth and Whatman[®] filter paper (Sargent-Welch, USA) and allowed to evaporate.¹⁵ Thus a greenish black colored semisolid mass of the extract was obtained (yield 24 gms).

Collection of worms

The earthworms belonging to species *Pheretima posthuma* (Annelida), about 3-5 cm in length and 0.1-0.2 cm in width, & weighing about 0.8-3.04 g, were collected from the moist soil of Noakhali Science and Technology University, Sonapur, Noakhali, Bangladesh.

Evaluation of anthelmintic activity

The assay for anthelmintic activity was carried out as per the method of Ajaiyeoba et al.¹⁶ with insignificant modifications. The test was conducted by using the adult earthworm, *Pheretima posthuma*, because of its anatomical and physiological similarity with the intestinal round-worm parasite of human beings.¹⁷⁻¹⁹ Earthworms have been used widely for the preliminary evaluation of anthelmintic activity in vitro because of easy availability and ease of use.²⁰⁻²² All the worms were washed with normal saline water to remove all fecal matters. Extract was weighed and

dissolved in 10 mL of distilled water to obtain the concentrations of 10, 20, 30, 40 and 50 mg/ml. Earthworms were divided into seven groups (each containing five worms) in petri-dish. The methanolic extract of *Crinum latifolium* leaves was applied to the petri-dishes and the time of paralysis and death was determined. Time for paralysis was noted when no movement of any sort could be observed except when the worms were shaken vigorously. Time for death of worms was recorded after ascertaining that worms neither moved when shaken vigorously nor when dipped in warm water (50 °C) followed with fading away of their body colors.

Total phenolic content determination

The amount of total phenol contents in extract was calculated using the Folin-Ciocalteu reagent method.²³ In this method Gallic acid was used as standard and the total phenolic contents were expressed as mg/g Gallic acid equivalents (GAE). Different concentrations, 6.25, 12.5, 25, 50, 100µg/ml, of standard Gallic acid and concentration of 1µg/ml of crude methanolic plant extract were prepared and 0.5ml of each sample were taken into test tubes and thoroughly mixed with 2.5 ml of a 10 fold diluted Folin-Ciocalteu reagent and 2ml of 7.5% w/v sodium carbonate. Before the absorbance was read at 760 nm spectrometrically (using UV spectrophotometer, Shimadzu) the tubes were enclosed with para film and allowed to stand at normal room temperature for 30 minutes. All values were determined in triplicate.²⁴

Evaluation of cytotoxicity

The cytotoxicity was conducted using brine shrimp lethality test following the method of Firdaus et al.²⁵ The brine shrimp eggs were placed in 1 L of sea water, aerated and hatched for 48 hrs at 37 °C, to become nauplii. After 48 hr, ten brine shrimp nauplii were placed in a small container filled with seawater. Methanolic extract of *Crinum latifolium* L. leaves, serially diluted with DMSO (Dimethyl Sulfoxide), was then added to the container. The mortality of brine shrimp was observed after 24 hrs of treatment was given. An approximate linear correlation was observed when logarithm of concentration versus percentage of mortality was plotted and the values of LC₅₀ were calculated using Microsoft Excel[®] 2007. Vincristine sulphate was used as positive control.

Statistical analysis

Data were processed and analyzed using both Microsoft Office Excel[®] version 7 and SPSS (version 16.0). The LC₅₀ value of plant extracts, from the 24 h counts and the dose-response data were transformed into a straight line by means of a trend line fit linear regression analysis (Microsoft Office Excel[®] version 7) and finally the LC₅₀ was derived from the best-fit line obtained. The data of anthelmintic studies were

reported as mean ± standard deviation, while the data of total phenolic contents determination was expressed as mean ± standard deviation. For determining the statistical significance, standard error mean and analysis of variance (ANOVA) at 5 % level significance was employed. P<0.05 was considered significant (shown as *).²⁶

Results

Anthelmintic activity

The results of anthelmintic activity of the methanolic extract of leaves of *Crinum latifolium* revealed that, it possesses varying degree of anthelmintic activity; i.e. the extract exhibited not only paralysis but also death of worms at all the tested concentrations. From the above study, it was observed that the methanolic extract of *Crinum latifolium* leaves was a potent anthelmintic, when compared to the standard drug in a dose dependent manner (Table 1 and Figure 1). The highest activity (shortest time for paralysis and death of worms) of the plant extract was found at the concentration of 50 mg/ml, as compared to the standard drug albendazole at 10 mg/ml, which gradually decreased with the decrease of concentration of the plant extract. Thus it was also clear from the study (Table 1) that, the concentration of the extract has an inversely proportional relationship with the time of paralysis and death of worms.

Table 1. Evaluation of anthelmintic activity of methanolic extract of *C. latifolium* L. leaves

| Test Substance | Concentration (mg/ml) | Time taken for paralysis (min) | Time taken for death (min) |
|---------------------------|-----------------------|--------------------------------|----------------------------|
| Control (Distilled water) | - | - | - |
| Standard (Albendazole) | 10 | 56.2±0.20 | 77.4±0.24 |
| | 10 | 84±0.84*** | 125.8±1.16*** |
| Methanolic extract | 20 | 62.6±0.40** | 104.6±0.40*** |
| | 30 | 46.8±0.86** | 88.2±1.61** |
| | 40 | 37.2±0.97*** | 71.8±1.68* |
| | 50 | 24±0.45*** | 46.4±0.60*** |

Values are expressed as mean ± standard deviation (SD). Values were found out by using ONE way ANOVA followed by Student's *t*-test. Significance level *P = ≤0.05, **P = ≤0.01, ***P = ≤0.001

Total phenolic content determination

Table 2 shows the total phenol contents of methanolic extracts of *C. latifolium* leaves. Total phenol compounds were reported as gallic acid equivalents by reference to a standard curve ($y=0.002x+0.107$; $R^2 = 0.889$).

The results showed that, the total phenol contents of methanolic extract was found 17.50±2.64 mg of GAE/gm of extract. The results of total phenolic contents suggest that the plant may possess very lower antioxidant activity.

Table 2. Determination of total phenolic contents of methanolic extract of *C. latifolium* L. leaves

| Extract | Sl. No. | Absorbance of the sample | Average Absorbance | Total Phenolic Content (mg of GAE / gm) of Extracts |
|--------------------|---------|--------------------------|--------------------|---|
| Methanolic Extract | 1 | 0.144 | 0.142 ±0.005 | 17.50±2.64 |
| | 2 | 0.136 | | |
| | 3 | 0.146 | | |

Data represents mean ± standard deviation (n=3) of duplicate analysis.

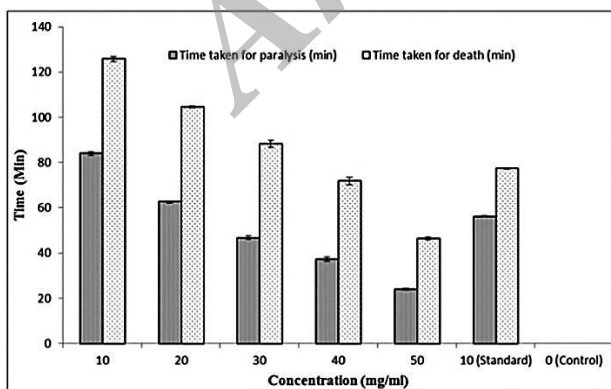


Figure 1. Paralysis and death time of *Pheritima posthuma* for methanolic extract of *Crinum latifolium* L. leaves

Cytotoxic activity

The lethal concentration (LC_{50}) of the test samples after 24 hours was determined by a plot of percentage

of the shrimps died against the logarithm of the sample concentration (toxicant concentration) and the best-fit line was obtained from the curve data by means of regression analysis. The lethality of the extracts to brine shrimps was determined and the results are given in Table 3. Vincristine sulphate (VS) was used as positive control and the LC_{50} value was found as 0.839µg/ml. The LC_{50} value of methanolic extract was found to be 15.652 µg/ml (Table 2).

Table 3. Evaluation of cytotoxic potential of methanolic extracts of *C. latifolium* leaves

| Sample | LC_{50} (µg/ml) | Regression Equation | R^2 |
|----------------------|-------------------|----------------------|-------|
| Vincristine Sulphate | 0.839 | $y = 34.02x + 52.58$ | 0.952 |
| Methanolic Extract | 15.652 | $y = 37.85x + 4.785$ | 0.977 |

Discussion

In the current study all the tests were performed *in vitro*. One of the main advantages of analyzing the biological properties of plant extracts *in vitro* is that, the process is cost effective and includes rapid turnover allowing the screening of plants at large scale.²⁷ Preliminary phytochemical studies on *Crinum latifolium* exposed the presence of glycosides, carbohydrates, alkaloids, tannins, phenols and proteins.¹⁴

Some of these phyto-constituents like alkaloids, tannins, phenols etc. may be accountable to have a significant anthelmintic activity.²⁸ It was reported that, tannins may to interfere with energy generation of worms by uncoupling oxidative phosphorylation or they binds to the free protein of the gastrointestinal tract of the worms and lead to death.²⁹ In another study, alkaloids were reported to cause paralysis of the worms by acting on its central nervous system.³⁰ The prime effect of albendazole is to cause a flaccid paralysis of the worm which results in expulsion of the worm by peristalsis. Albendazole acts to increase chloride ion conductance of worm muscle membrane which produces hyperpolarization and excitability reduction that leads to muscle relaxation and flaccid paralysis of worms.³¹

It is expected that the phytochemicals present in the extract of *Crinum latifolium* may have produced similar effects, causing death of the worms. Therefore, the usual claim of leaves of *Crinum latifolium* as an anthelmintic has been confirmed as the extracts shown significant activity against *Pheritima postuma*.

The present study estimated the total phenolic contents of methanolic extract of *Crinum latifolium* leaves and found that the leaves contained a very lower quantity of phenolic contents. The phenolic compounds may be responsible for the possible antioxidant activity of the plant, because it was proved by Elmastas *et al.*, that hydroxyl groups present in the phenolic contents may have a significant contribution to the antioxidant activity and may play a crucial role in scavenging free radicals.³² Another report also confirmed that, fruit and vegetable phenols and polyphenols, one of the major phytochemicals, showed a wide range of biological activities, which were related to their antioxidant property preventing free radical damage and lipid peroxidation.^{33,34} Thus the phenolic compounds may have role in free radical scavenging ability and antioxidant activity, which is yet to be discovered.

To assess the toxicity of the crude extracts towards the brine shrimp, the brine shrimp lethality bioassay (BSLA) is a routinely and widely used method. This method also indicates the possible toxicity of the extracts on the test materials and several antitumor and pesticidal natural products have been identified using this method.³⁵ Again, we know that plant extracts contains a higher concentration of bioactive

compounds and also several compounds which show cytotoxic activity.³⁶ It was reported that several active compounds such as anthocyanins, saponins, tannins, flavones, and polyphenols etc. are known to be free radical scavenger, reactive species quencher, hydrogen donor, antioxidant enzymes activator, detoxification inducer, normal cell differentiation promoter, tumor production and proliferation cell inhibitor, and apoptosis inducer.^{25,37} So, the bioactive compounds may be accountable for the possible cytotoxicity of the methanolic extract of *Crinum latifolium* leaves, although the exact mechanism of action is yet to be discovered.

Conclusion

The present study deduces that the plant *C. latifolium* can be a good source of novel anthelmintic, antioxidant and cytotoxic agent. So, studies are suggested for the isolation, purification, characterization, and testing of individual compound *in vivo*.

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Conflict of interests

The authors declare that they have no competing interests.

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