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Effects of Ethanol Extract and its Different Fractions of *Phrynium imbricatum* (Roxb.) Leaves on *In Vitro* Anthelmintic and their Condensed Tannin Content

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Abstract

Background: To identify the therapeutic effects of ethanol extract and its different fractions of *Phrynium imbricatum* leaves in anthelmintic (in vitro) and to determine their total condensed tannin content.

Methods: Leaves of Phrynium imbricatum was extracted with pure ethanol and fractioned with chloroform (CHFPI), petroleum ether (PEFPI), n-hexane (NHFPI) and ethyl acetate (EAFPI), which are tested for anthelmintic activity on aquarium worm Tubifex tubifex by using three concentrations viz., 5, 10 and 20 mg/ml of each. Total condensed tannin content determined based on the procedure of Oyedemi et al.

Results: Among the various crude extract, ethanol extract Phrynium imbricatum (EEPI) exhibited strong anthelmintic activity in vitro. Where it paralyzed (3.69 ± 0.25 min; P<0.001) and produced death (14.28 ± 0.44 min; P<0.001) of the Tubifex tubifex at 20 mg/ml dose near the value of the standard, Levamisole (3.3 ± 0.38 min and 6.5 ± 0.76 min) at 1 mg/ml. The content of condensed tannin good at EEPI and its all fractions, but EEPI (168.44 ± 0.87 mg catechin/g) contained highest among them. For both of experiment, activity found as follows, EEPI>CHFPI>EAFPI>NHFPI>PEFPI.

Conclusion: These findings suggest that the plant may be a potential source for the development of new anthelmintic and condensed tannin may one of such phytochemical, which exhibit anthelmintic activity.

Keywords: *Phrynium imbricatum*; *In vitro*; Anthelmintic; *Tubifex tubifex*; Condensed tannin

Introduction

Plant materials have been utilized for the treatment of serious diseases all through the world before the approach of advanced clinical medications. The utilization of therapeutic plants still assumes an essential part to cover the fundamental wellbeing needs in the developed countries [1]. Most of the Phytochemical, secondary metabolites of plants, are physiologically active [2] and this metabolites are known as to give an achieve wellspring of natural, anthelmintic, antibacterial and insecticides [3]. 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 [4]. The major phyla of helminthes are nematodes (round worms) which are soil transmitted helminthes 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 cyticerosis [5,6]. 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 [6,7]. In case of other animals also gastrointestinal parasites causes infections that diminish the animal survival, growth rates and reproductive performance [8]. Morbidity from nematodes is common with diabetes and lung cancer. The helminths parasites mainly subsist in human body in intestinal tract, but they are also found in tissue, as their larvae migrate towards them [9]. 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 [10]. 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.

In this regard, *Phrynium imbricatum* (Family: Marantaceae) is a rigid herb, which is commonly known as Pitulpata (Bangladesh) [11-13]. Leaves large, oblong, Spikes oblong, bracts oblong with obtuse, minutely toothed tips. Fruits usually 3-seeded. A paste prepared from leaves of *Phrynium imbricatum*, *Blumea clarkei* and an unidentified species (locally called Khedom gas) is applied to affected areas and bandaged for the treatment of fractures (Chakma). Leaves of *P. imbricatum* has activities like antiarthritic and membrane stabilizing [14]. Occurs in the forests of Chittagong, Chittagong Hill Tracts, Cox's Bazar and Sylhet.

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The aim of the present study was to identify the anthelmintic activity and total tannin content of ethanol extract and its fractions of leaves of *Phrynium imbricatum*.

Methods and Materials

Plant collection and identification

Leaves of *Phrynium imbricatum* (Accession No. 1315 CTGUH) were collected from Alu tila, khagrachari, Chittagong, Bangladesh in the month of September 2014 at the last time of its flowering. It is authenticated by Dr. Shaikh BU, Associate Professor, Department of Botany, University of Chittagong, Chittagong, Bangladesh.

Extraction and fractionation

Leaves were cleaned with fresh water and dried for a period of 10 days under shade and then powdered with a mechanical grinder, passing through sieve #40, and stored in a tight container. The powdered of whole plant (850 g) of *Phrynium imbricatum* was soaked in 1.5 L ethanol for 7 days with occasional shaking and stirring and filtered through a cotton plug followed by Whatman filter paper number-1. The extract was then concentrated by using a rotary evaporator at reduced temperature and pressure. A portion (55 g) of the concentrated ethanol extract (EEPI) was fractioned by the modified Kupchan partitioning method [15,16] into chloroform, CHFPI (8 g), *n*-hexane, NHFPI (6 g), ethyl acetate, EAFPI (9 g) and pat ether, PEFPI (14 g).

Chemicals

All chemicals used were of analytical reagent grade. Ethanol, methanol, chloroform, pet ether, ethyl acetate, *n*-hexane and hydrochloric acid were purchased from Merck, Germany. Levamisole was purchased from ACI Limited, Bangladesh. Vanillin was purchased from Sigma Chemicals Co (PO Box 14508, St. Louis, MO 63178 USA). Catechin was purchased from BDH Chemicals (BDH Chemicals Ltd. Poole, England).

In-vitro anthelmintic assay

The anthelmintic activity of ethanol extract and its fractions of leaves of *Phrynium imbricatum* were carried out as per the procedure of Ajaiyeoba *et al.* [17] 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., annelida [18-20]. The worms were collected from the local market of Chittagong, average size of worms 2-2.5 cm. in length were used for the study. The standard drug Levamisole and three different concentrations of EEPI and its different fractions (5, 10 and 20 mg/ml) in double distilled water [21,22] 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 [23]. 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 [24].

Total condensed tannins

Condensed tannins (proanthocyanidin) was determined based on the procedure of Oyedemi *et al.* [25]. To 0.5 ml of 1 mg/ml of the extract solution was added 3 ml of vanillin-methanol (4% v/v) and 1.5 ml of hydrochloric acid was added and vortexed. The mixture was allowed to stand for 15 min at room temperature and the absorbance was measured at 500 nm. Total proanthocyanidin content was evaluated at a concentration of 0.1 mg/ml and expressed as catechin equivalent (mg/g) using the calibration curve equation: $Y=0.5825x$, $R^2=0.9277$, where x is the absorbance and Y is the catechin equivalent.

Statistical Analysis

The data on *in vitro* studies were reported as mean ± S.E.M (n=3). Data were analyzed using one way factorial ANOVA tests using SPSS followed by Dennett's tests on each group except control for anthelmintic. Regression analysis was performed to calculate total tannin content. $P<0.05$ and $P<0.001$ were considered as statistically significant. Statistical program used was Graphpad Prism® (version 6.00; GraphPad Software Inc., San Diego, CA, USA) and Microsoft Excel, 2007, used for graphical presentation.

Results

In vitro anthelmintic activity

Results of study were recorded as shown in Table 1 and Figure 1 as in the form of time required getting consecutive attacks of paralysis and at the end time required for complete death of parasite. From the observations made, higher concentration of extract and fractions produced paralytic effect much earlier and the time to death was shorter for all worms. From the above study it was seen that the ethanol extract showed dose dependent anthelmintic activity as compared to a standard drug Levamisole. Different treatment showed different anthelmintic activity. But ethanol extract of *P. imbricatum* showed highest anthelmintic activity. Where it paralyzed (3.69 ± 0.25 min; $P<0.001$) and produced death (14.28 ± 0.44 min; $P<0.001$) of the *Tubifex tubifex* at 20 mg/ml dose near the value of the standard Levamisole (3.3 ± 0.38 min and 6.5 ± 0.76 min) at 1 mg/ml. EAFPI showed the lowest anthelmintic activity. It's paralyzing and death time of *Tubifex tubifex* is 28.32 ± 0.63 min and 61.45 ± 1.14 min at dose 5 mg/ml. So the anthelmintic activities of ethanol and its fractions of *P. imbricatum* leaves are as follows, EEPI>CHFPI>EAFPI>NHFPI>PEFPI

Ethanol extract of *Phrynium imbricatum* (EEPI) leaves showed highest anthelmintic activity, which indicated by arrow mark in this graph.

Total condensed tannin content

The total phenol contents of the extracts are shown in Table 2. The total condensed tannin content of *Phrynium imbricatum* leaves was higher in plants at ethanol extract, which was 168.44 ± 0.87 mg catechin/g. Fractions of ethanol extract of *Phrynium imbricatum* contain good amounts of condensed tannin, ranging from 152.42 to 114.85 mg catechin/g. So condensed tannin content of ethanol and its fractions of *P. imbricatum* leaves are as follows, EEPI>CHFPI>EAFPI>NHFPI>PEFPI

Discussion

Consumption of high concentrations of condensed tannins (>7% of DM) had a number of detrimental effects on ruminants, such as reduction in food intake, growth inhibition and interference with the morphology and the proteolytic activity of microbes in the rumen, low or moderate concentrations of condensed tannins (<6% of DM) have resulted in the positive effects on. Levamisole works as a nicotinic acetylcholine receptor agonist that causes continued stimulation of the

Treatment	Time taken for paralysis (min)	Time taken for Death (min)
Control(Water)	0	0.00
Levamisole (1 mg/ml)	3.3 ± 0.38	6.5 ± 0.76
EEPI (20 mg/ml)	**3.69 ± 0.25^b**	**14.28 ± 0.44^b**
EEPI (10 mg/ml)	10.37 ± 0.67^a	18.61 ± 0.8^a
EEPI (5 mg/ml)	12.3 ± 0.67^a	23.72 ± 0.92^a
CHFPI (20 mg/ml)	9.15 ± 0.67^b	15.01 ± 0.96
CHFPI (10 mg/ml)	14.92 ± 0.9^a	29.07 ± 1.04^a
CHFPI (5 mg/ml)	21.75 ± 0.87^a	45.9 ± 1.2^a
EAFPI (20 mg/ml)	7.51 ± 0.55	15.29 ± 0.87^a
EAFPI (10 mg/ml)	12.62 ± 0.79^a	23.92 ± 0.81^a
EAFPI (5 mg/ml)	18.35 ± 1.17	39.12 ± 1.24^a
PEFPI (20 mg/ml)	13.69 ± 0.82^b	25.12 ± 1.57^a
PEFPI (10 mg/ml)	18.61 ± 0.53^a	34.62 ± 1.11^a
PEFPI (5 mg/ml)	28.32 ± 0.63^a	61.45 ± 1.14^a
NHFPI (20 mg/ml)	10.61 ± 0.78	19.09 ± 0.97^a
NHFPI (10 mg/ml)	14.01 ± 0.90^a	27.07 ± 0.85^a
NHFPI (5 mg/ml)	22.46 ± 0.58^a	49.11 ± 1.28^a

Values are mean ± SEM (n=3); $^aP<0.05$, $^bP<0.001$, Dennett's test as compared to positive control (Levamisole, 1 mg/ml). Statistical representation of the effective paralysis and dead time by *Phrynium imbricatum* ethanol extract and its fractions, positive anthelmintic control (Levamisole, 1 mg/ml) processed by paired t-test analysis (Dennett's test). Bold text indicates the highest anthelmintic activity of ethanol extract of *Phrynium imbricatum* (EEPI). Data were processed by paired t-test analysis by using SPSS for windows, version 16.0.

Table 1: Anthelmintic activity of ethanol extract and its different fractions of leaves of *Phrynium imbricatum*.

Sample	Total proanthocyanidin (mg catechin/g)
EEPI	**168.44 ± 0.87^b**
CHFPI	152.42 ± 1.02^a
PEFPI	114.85 ± 0.89^b
NHFPI	120.5 ± 1.02^b
EAFPI	136.67 ± 1.47^a

Values are mean ± SEM (n=3). Bold text indicates the highest tannin content of ethanol extract of *Phrynium imbricatum* (EEPI). The different superscripted (a,b) values have significantly different ($^aP<0.05$, $^bP<0.001$) from the other sample in same column.

Table 2: Contents of condensed tannin (expressed as mg catechin/g dry weight) in ethanol extract and its fractions of *Phrynium imbricatum* leaves.

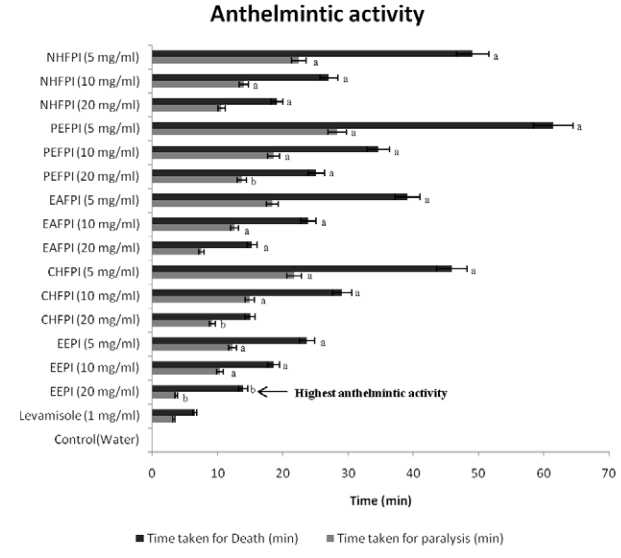

Figure 1: Anthelmintic activity of ethanol extract and its different fractions of leaves of *Phrynium imbricatum*.

parasitic worm muscles, leading to paralysis. The literature have been reported that the presence of flavonoids, tannins and polyphenolic compounds show anthelmintic activity, [26,27] 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 [28]. Some synthetic phenol anthelmintics e.g., niclosamide, oxyclozanide and bithionol are shown effects to interfere with energy generation in antihelminthic parasites by uncoupling oxidative phosphorylation and phosphorylation [29]. Finally, study concludes that the plant under study has found to possess 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.

Similarly, condensed tannins (CTs) have high relevance for livestock production as tannin-rich plants have a long tradition of use not only as forages but also as 'green' control of gastrointestinal nematode infections. Several excellent reviews deal with the various aspects of feeding of small ruminants with forages containing tannin-rich plants or even fodder trees [30-33]. They pointed that bioactive tanniniferous plants represent a valuable option as an alternative to commercial drugs for the control of gastro-intestinal nematodes (GINs) as consumption of these plants has been associated with antiparasitic and anthelmintic effects: reductions in nematode numbers, worm fecundity, and nematode eggs excretion. The principle risk to the utilization of exclusively substance medications is the quick advancement of imperviousness to any anthelmintic medication in worm populaces after commercialization [34] and the spread of anthelmintic resistance within worm populations [35]. Within the last decade a number of studies focused on isolation of condensed tannins and sesquiterpene lactones from various legume forages and plants with the aim to reveal their effects *in vitro* and *in vivo* on various species and developmental stages of nematodes. Differentiated action of condensed tannins on parasite stages was observed by Athanasiadou *et al.* [36,37] which were more effective against larvae than adults. This can also be explained by the distinction between the cuticular components of the pre-parasitic stages (eggs to L3) and the parasitic stages (L4 and adults), as demonstrated by the study of Stepek *et al.* [38,39].

So present studies suggested that condensed tannin really responsible for anthelmintic activity. Because highest condensed tannin containing extract gave highest anthelmintic effect and lowest one gave lowest anthelmintic activity.

Conclusion

Our aim was to determine the anthelmintic activity and condensed tannin content. However, we find out that according to condensed tannin content, extracts giving their anthelmintic activity. This suggested that specific, key processes in the parasite life cycle can be disrupted by condensed tannin. These data encourage further investigations to determine *in vivo* efficacy in animal model. In addition, further mechanistic studies, such as the relationship between the fine structure of condensed tannin molecules and anthelmintic activity, are also a high priority.

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Conflict of Interest

The authors declare that they have no competing interests.

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