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Evaluation of Total condensed tannin content and anthelmintic activities of organic extracts of four Bangladeshi plants on *Tubifex tubifex* worm using i*n vitro* method

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ABSTRACT

To explore the therapeutic effects of methanol extract of roots of *Curculigo recurvata* W. T. Aiton (Satipata), leaf of *Amorphophallus bulbifer* (Roxb.) Bl. (Olkachu), leaf of *Phyllanthus sikkimensis* Muell. Arg. (Dalpata) and whole plant of *Thunbergia grandiflora* Roxb. (Nillata) in anthelmintic (*in vitro*) and to determine their total condensed tannin content. Roots of *C. recurvata* , leaf of *A. bulbifer*, leaf of *P. sikkimensis* and whole plant of *T. grandiflora* was extracted with pure methanol, which are tested for anthelmintic activity on aquarium worm *Tubifex tubifex* by using three concentrations viz., 2.5, 5 and 10 mg/ml of each. Total condensed tannin content determined based on the procedure of Oyedemi *et al* (2010). Among the four extract, *P. sikkimensis* exhibited strong anthelmintic activity *in vitro*. Where it paralyzed (6.48±0.09 min) and produced death (12.26±0.65 min) of the *Tubifex tubifex* at 10 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 well to moderate at all, but *P. sikkimensis* (45.14±0.5mg catechin/g) contained highest among them. For both of experiment, activity found as follows, *P. sikkimensis* > *A. bulbifer* > *T. grandiflora* > *C. recurvata*. These findings suggest that *P. sikkimensis* and *A. bulbifer* among the four plants may be potential source for the development of new anthelmintic and condensed tannin may one of such phytochemical, which exhibit anthelmintic activity.

Keywords: *A*nthelmintic, *P. sikkimensis, A. bulbifer, Tubifex tubifex*, condensed tannin

INTRODUCTION

Phytomedicines have been used for the treatment of serious diseases throughout the world before the advent of modern clinical drugs. The use of medicinal plants still plays an important role to cover the basic health needs in the developing countries. Several top selling drugs of modern times such as Quinine, Artemisinine, Shikonin, etc. are obtained from plant [1]. Most of the Phytochemical, secondary metabolites of plants, are physiologically active [2]. The plants are known as to provide a reach source of botanical, 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 [5]. 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 [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] 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.

Condensed tannins (proanthocyanidins) are polymers formed by the condensation of flavans. They do not contain sugar residues. They are called proanthocyanidins as they yield anthocyanidins when depolymerized under oxidative conditions. Different types of condensed tannins exist, such as the procyanidins, propelargonidins, prodelphinidins, profisetinidins, proguibourtinidins or prorobinetidins, formed from flavonoids structures corresponding to the related anthocyanins. Tannins of tropical woods tend to be of a catechin nature rather than of the gallic type present in temperate woods. Commercial sources of condensed tannins are plants such as quebracho wood (*Schinopsis lorentzii*), mimosa bark (*Acacia mollissima*), grapes seeds (*Vitis vinifera*), pine barks and spruce barks.[11, 12] Pycnogenol is a trademark for a French maritime pine bark extract. Condensed tannins are formed in tannosomes, specialized organelles, in Tracheophytes i.e vascular plants.

Curculigo recurvata W. T. Aiton (Satipata), *Amorphophallus bulbifer* (Roxb.) Bl. (Olkachu), *Phyllanthus sikkimensis* Muell. Arg. (Dalpata) and *Thunbergia grandiflora* Roxb. (Nillata) are native to Bangladesh. They are used as traditional medicines for cardiac diseases and blood purification. *C. recurvata* traditionally used as antidote, to stop bleeding. Leaves, tuber and whole plant of *A. bulbifer* are used as anti-inflammatory, analgesic and antibacterial [13, 14]. Leaves of *Phyllanthus sikkimensis* locally used to induce diarrhea. Leaves and stem of *Thunbergia grandiflora* used as a poultice in stomach complaints and to treat eye diseases in Chittagong Hill Tracts. The aim of the present study was to identify the anthelmintic activity and total tannin content of methanol extract of *Curculigo recurvata*, *Amorphophallus bulbifer*, *Phyllanthus sikkimensis* and *Thunbergia grandiflora*.

METHOD AND MATERIALS

Plant collection & identification: Root of *Curculigo recurvata*, leaf of *Amorphophallus bulbifer*, leaf of *Phyllanthus sikkimensis* and whole plant of *Thunbergia grandiflora* were collected from different parts of Chittagong region, Bangladesh. The plants were identified by Dr. Shaikh Bokhtear Uddin, Taxonomist and Associate Professor, Department of Botany, University of Chittagong.

Extract preparation: Each of the plant materials was dried and ground (Moulinex Blender AK-241, Moulinex, France) into powder (40-80mesh, 700 g) and soaked for 7 days with 2–3 days interval in 3.0 L of methanol at room temperature (23 ± 0.5°C). Filtrate obtained through cheesecloth and Whatman filter paper No. 1 was concentrated under reduced pressure at the temperature below 50°C using rotary evaporator (RE 200, Sterling, UK). The extracts (yield 3.8–5.2% W/W) were all placed in air tight glass tube. A 100 mg each of the extracts was suspended in 10 ml distilled water and the suspension was shaken vigorously on a vortex mixer and used for anthelmintic assay. The concentration (1mg/ml) prepared for condensed tannin content determination.

Chemicals: All chemicals used were of analytical reagent grade. Methanol and hydrochloric acid were purchased from Merck, Germany. Levamisole was purchased from ACI Limited, Bangladesh. Vanillin was purchased from Sigma Chemicals Co. (P.O. Box 14508, St. Louis, MO 63178 USA). Catechin was purchased from BDH Chemicals (BDH Chemicals Ltd. Poole, England).

Total condensed tannins: Condensed tannins (proanthocyanidin) was determined based on the procedure of Oyedemi *et al*.**[24]** 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.

***In-vitro* Anthelmintic Assay:** The anthelmintic activity of methanol extract of *Curculigo recurvata*, *Amorphophallus bulbifer*, *Phyllanthus sikkimensis* and *Thunbergia grandiflora* were carried out as per the procedure of Ajaiyeoba et al. [16] 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.[17-19] 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 different fractions (2.5, 5 and 10 mg/ml) in double distilled water [20, 21] 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. [22] 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 [23].

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

Total condensed tannin content: The total phenol contents of the extracts are shown in Table 1. The total condensed tannin content was higher at methanol extract of *Phyllanthus sikkimensis* leaves, which was 45.14±0.5mg catechin/g. *Amorphophallus bulbifer* and *Thunbergia grandiflora* showed condensed tannin content as 27.57±1.18 and 14.95±0.265mg catechin/g. But *Curculigo recurvata* possessed lower content of condensed tannin, 6.31±0.61mg catechin/g. So condensed tannin content at different extracts are as follows,

P. sikkimensis > *A. bulbifer* > *T. grandiflora* > *C. recurvata*

Table 1: Contents of condensed tannin (expressed as mg catechin/g dry weight) in *C. recurvata, A. bulbifer, P. sikkimensis* and *T. grandiflora*.

Sample	Total Proanthocyanidin(mg catechin/g)
Curculigo recurvata	6.31 ± 0.61^{b}
Amorphophallus bulbifer	27.57 ± 1.18^{a}
Phyllanthus sikkimensis	**45.14 ± 0.5^{b}**
Thunbergia grandiflora	14.95 ± 0.26^{a}

Values are mean ± SEM, (n = 3). Bold text indicates the highest tannin content of methanol extract of *Phyllanthus sikkimensis*. The different superscripted (a, b) values have significantly different ($^{a}P < 0.05$, $^{b}P < 0.001$) from the other sample in same column.

***In vitro* anthelmintic activity:** Results of study were recorded as shown in Table 2 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 extracts produced paralytic effect much earlier and the time to death was shorter for all worms. From the above study it was seen that the methanol extract showed dose dependent anthelmintic activity as compared to a standard drug levamisole. Different treatment showed different anthelmintic activity. But methanol extract of *P. sikkimensis* showed highest anthelmintic activity. Where it paralyzed (6.48±0.09 min) and produced death (12.26±0.65 min) of the *Tubifex tubifex* at 10 mg/ml dose near the value of the standard (3.3±0.38 min and 6.5±0.76 min) at 1 mg/ml. *C. recurvata* showed the lowest anthelmintic activity. It's paralyzing and death time of *Tubifex tubifex* is 30.74±1.74 min and 86.16±1.23 min at dose 2.5mg/ml. So the anthelmintic activities of different extracts are as follows,

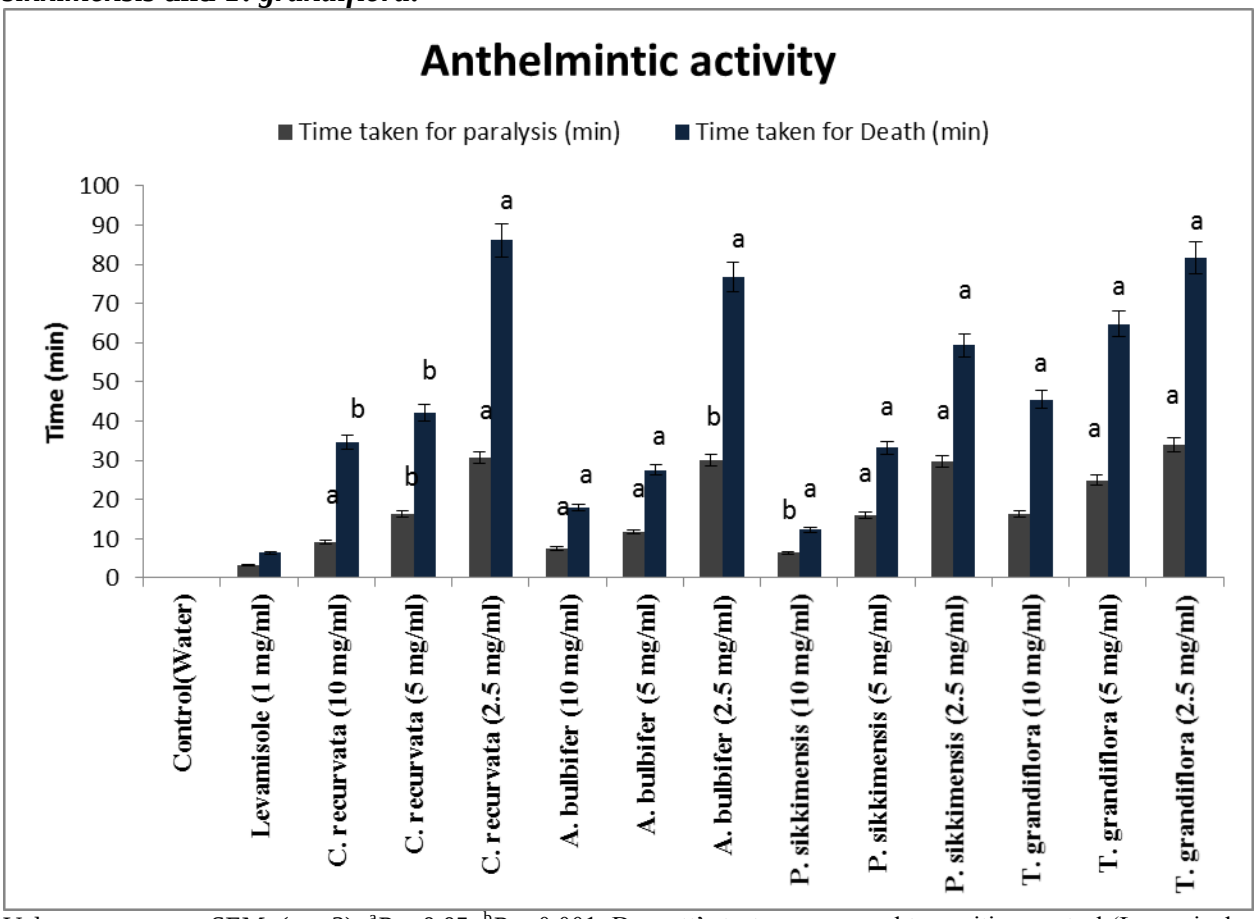
P. sikkimensis > *A. bulbifer* > *T. grandiflora* > *C. recurvata*

Table 2: Anthelmintic activity of methanol extract of *C. recurvata*, *A. bulbifer*, *P. sikkimensis* and *T. grandiflora*.

Treatment	Time taken for paralysis (min)	Time taken for Death (min)
Control(Water)	0	0.00
Levamisole (1 mg/ml)	3.30±0.38	6.50±0.76
***C. recurvata* (10 mg/ml)**	9.05±0.27[a]	34.59±0.24[b]
***C. recurvata* (5 mg/ml)**	16.29±0.1[b]	42.25±0.10[b]
***C. recurvata* (2.5 mg/ml)**	30.74±1.74[a]	86.16±1.23[a]
***A. bulbifer* (10 mg/ml)**	7.53±0.32[a]	18.01±0.78[a]
***A. bulbifer* (5 mg/ml)**	11.74±0.24[a]	27.53±0.55[a]
***A. bulbifer* (2.5 mg/ml)**	29.98±0.38[b]	76.83±1.38[a]
***P. sikkimensis* (10 mg/ml)**	**6.48±0.09**[b]	**12.26±0.65**[a]
***P. sikkimensis* (5 mg/ml)**	16.06±0.68[a]	33.17±1.15[a]
***P. sikkimensis* (2.5 mg/ml)**	29.67±0.95[a]	59.37±1.99[a]
***T. grandiflora* (10 mg/ml)**	16.35±1.68	45.53±1.86[a]
***T. grandiflora* (5 mg/ml)**	24.98±0.75[a]	64.78±2.28[a]
***T. grandiflora* (2.5 mg/ml)**	34.02±1.33[a]	81.58±2.25[a]

Values are mean ± SEM, (n = 3); [a]$P < 0.05$, [b]$P < 0.001$, Dennett's test as compared to positive control (Levamisole, 1 mg/ml). Statistical representation of the effective paralysis and dead time by *C. recurvata*, *A. bulbifer*, *P. sikkimensis* and *T. grandiflora* methanol extract, positive anthelmintic control (Levamisole ,1 mg/ml) processed by paired t-test analysis (Dennett's test). Bold text indicates the highest anthelmintic activity of methanol extract of *P. sikkimensis*. Data were processed by paired t-test analysis by using SPSS for windows, version 16.0.

Figure 1: Anthelmintic activity of methanol extract of *C. recurvata*, *A. bulbifer*, *P. sikkimensis* and *T. grandiflora*.



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DISCUSSION

Anthelmintic are the drugs that expel out parasitic worms (helminthes) from the body by either causing paralysis or by directly killing them by damaging its cuticle, leading to partial digestion or rejection by immune mechanisms [25]. 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, [26] 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[27]. Some synthetic phenol anthelmintics e.g. niclosamide, oxyclozanide and bithionol are shown effects to interfere with energy generation in antihelminth parasites by uncoupling oxidative phosphorylation and phosphorylation[28]. 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.

Continued reliance on mass drug administration with a limited number of synthetic anthelmintics has the potential to place heavy selection pressure on drug-resistant parasites, and widespread anthelmintic drug resistance is already a serious problem in many livestock production systems. The use of natural dietary compounds has the potential to be a

complementary control option which may reduce this reliance on drug treatment, and slow the development of resistance. Here we have carried out a comprehensive *in vitro* assessment of the effects of condensed tannin rich extracts on one of the most prevalent worm of *Tubifex tubifex*.

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 [30-32]. 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 main threat to the use of solely chemical drugs is the rapid development of resistance to any anthelmintic drug in worm populations after commercialization [33] and the spread of anthelmintic resistance within worm populations [34].

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. [39], which were more effective against larvae than adults. This can also be explained by the difference 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. [40, 41].

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. But 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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Competing interests: The authors declare that they have no competing interests.

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