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Anthelmintic Effects of Different Extracts of *Hopea odorata* Leaves on *Tubifex tubifex* Worm Using *In vitro* Method and their Condensed Tannin Content

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MSHK and MMH collected the plant leaves and prepared the extracts. Author MSHK designed the study, wrote the protocol, performed the statistical analysis. Author MMH wrote the first draft of the manuscript. Authors MSHK, MMH, TAC, AH and NC performed the experiment. Author AH acted as correspondence. All authors read and approved the final manuscript.

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ABSTRACT

Objective: To investigate the therapeutic effects of different extracts of *Hopea odorata* leaves in anthelmintic (*In vitro*) and to determine their total condensed tannin (proanthocyanidin) content.

Methods: Leaves of *Hopea odorata* was extracted with pure methanol (MEHO), ethanol (EEHO) and water (AEHO), which are tested for anthelmintic activity on aquarium worm *Tubifex tubifex* by using four concentrations viz., 2.5, 5, 10 and 20 mg/ml of each. Total condensed tannin content determined based on the previous procedure of Oyedemi et al. [1].

Results: Among the all extracts, MEHO exhibited strong anthelmintic activity *In vitro*. Where it paralyzed (4.05±0.35 min; $P<0.05$) and produced death (7.5±0.38 min; $P<0.001$) of the *Tubifex*

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tubifex at highest 20 mg/ml dose close to 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 excellent at all extracts, but MEHO (96.99±0.34 mg catechin/g) contained maximum among them. For both of experiment, activity found as follows, MEHO > EEHO > AEHO.
Conclusion: These findings suggest that the plant may be a potential source for the development of new anthelmintic and condensed tannin is a type of phytochemical which may exhibit anthelmintic activity.

Keywords: Hopea odorata; anthelmintic; Tubifex tubifex; Levamisole; *condensed tannin.*

1. INTRODUCTION

The plants are known to provide a rich source of medicinal such as anthelmintic and insecticides [2]. Helminthes are recognized as a one of major problem to farm animal's production throughout tropics. Most of the diseases caused by helminthes are of a chronic and devastating in nature; they probably cause morbidity and greater economic and social deprivation parasites. The parasitic gastroenteritis is caused by mixed infections with several species of stomach and intestinal worms, which results in weakness, loss of appetite and decreased productivity. Chemotherapy is the only treatment and effective tool to cure and control helminth infections, as effective vaccines against them have not been developed so far. Random use of synthetic anthelmintic drugs can lead to resistance of parasites. Herbal drugs have been in use since primeval times for the treatment of parasitic disease in human and could be of value in preventing the development of resistance. Phytochemical such as condensed tannin showed wide range of anthelmintic activity in the previous studies [3,4].

Many plant species contain condensed tannins (CT). Condensed tannins (proanthocyanidins) are polymers formed by the condensation of flavones, which is the polymerisation of monomeric flavan-3-ols. 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 [5].

Hopea odorata belongs to the Dipterocarpaceae family, locally known as Telsur (Bangladesh). The wood of *H. odorata* varies in color from a very pale yellow, or white to brown when first cut and characteristically darkens to a brownish or yellowish-brown color after more or less prolonged exposure to the air. The dammar of this tree is said to have medicinal property used in treating sores and wounds [6]. Phytochemical studies reported that the heartwood of *H. odorata* enclose with certain types of phenolic compounds [7]. These polyphenols are reported to be useful as antioxidants, anticarcinogens, scavengers of free radicals and therefore have implications in the prevention of pathologies such as cancer and cardiovascular disease [8].

The aim of the present studies was to identify the anthelmintic activity and total tannin content of different extracts of leaves of *H. odorata.*

2. MATERIALS AND METHODS

2.1 Plant Material

Fresh leaves of *Hopea odorata* were collected from area of University of Chittagong, Chittagong, Bangladesh in the month of November 2014. It was authenticated by Dr. Shaikh Bokhtear Uddin, Associate Professor, Department of Botany, University of Chittagong, Chittagong-4331, Bangladesh.

2.2 Preparation of Extract

The leaves were dried for a period of 10 days under shade and grounded. The grounded each leaves (400 gm) were soaked in 1.6 L amount of methanol, ethanol and distilled water separately for one week at room temperature with occasional shaking and stirring then the whole mixture was filtered through Whatman paper and the filtrate hence obtained was concentrated using a rotary evaporator (Bibby RE200, Sterlin Ltd, UK) to get a viscous mass. The viscous mass extract kept at room temperature under a ceiling fan to get a dried extract and found MEHO (7.2%), EEHO (6%) & AEHO (8%).

2.3 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. (P.O. Box 14508, St. Louis, MO 63178 USA). Catechin was purchased from BDH Chemicals (BDH Chemicals Ltd. Poole, England).

2.4 *In-vitro* Anthelmintic Assay

The anthelmintic activity of different extracts of leaves of *Hopea odorata* were carried out as per the procedure of Ajaiyeoba et al. [9] 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 [10-12]. 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 four different concentrations of different extracts (2.5, 5, 10 and 20 mg/ml) in double distilled water [13,14] were prepared just before experiment 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. [15] 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 stopped [16].

2.5 Total Condensed Tannins

Condensed tannins (proanthocyanidin) was determined based on the procedure of Oyedemi et al. [1]. 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 condensed tannin 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.

2.6 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 condensed 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.

3. RESULTS

3.1 *In vitro* Anthelmintic Activity

Results of study were recorded as shown in Table 1 and Fig. 1 as in the form of time required getting consecutive attacks of paralysis and at the end time required for complete death of worm. From the observations made, higher concentration of all extracts produced paralytic effect much earlier and the time to death was shorter for all worms. From the above study it was seen that all extracts showed dose dependent anthelmintic activity as compared to a standard drug Levamisole. Different treatment showed different anthelmintic activity. But methanol extract of *Hopea odorata* showed highest anthelmintic activity. Where it paralyzed (4.05±0.35 min; P<0.05) and produced death (7.5±0.38 min; P<0.001) of the *Tubifex tubifex* at highest 20 mg/ml dose, which near the value of the standard (paralysis time, 3.3±0.38 min and produced death, 6.5±0.76 min) at 1 mg/ml dose. AEHO showed the lowest anthelmintic activity. It's paralyzing and death time of *Tubifex tubifex* is 51.22±0.65 min; P<0.05 and 76.78±1.13 min; P<0.05 at dose 2.5 mg/ml. So the anthelmintic activities of different extracts of *Hopea odorata* leaves are as follows,

MEHO > EEHO > AEHO

Table 1. Anthelmintic activity of different extracts of leaves of *Hopea odorata*

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
Levamisole (0.8 mg/ml)	6.26±0.73**	12.21±1.4**
Levamisole (0.5 mg/ml)	14.41±0.95**	51.32±2.78**
MEHO (20 mg/ml)	**4.05±0.35***	**7.5±0.38****
MEHO (10 mg/ml)	6.82±0.39*	11.59±0.45*
MEHO (5 mg/ml)	11.16±0.57*	16.33±0.59*
MEHO (2.5 mg/ml)	17.08±0.68*	28.61±0.76*
EEHO (20 mg/ml)	5.72±0.33	12.31±0.69
EEHO (10 mg/ml)	8.26±0.56	16.38±0.66*
EEHO (5 mg/ml)	13.69±0.30*	20.33±0.42*
EEHO (2.5 mg/ml)	18±0.38*	33.97±1.10*
AEHO (20 mg/ml)	10.23±0.58*	18.45±0.59*
AEHO (10 mg/ml)	20.34±0.57*	44.77±1.17*
AEHO (5 mg/ml)	38.05±0.81*	64.01±0.77**
AEHO (2.5 mg/ml)	51.22±0.65*	76.78±1.13*

*Values are mean ± SEM, (n = 3); *P < 0.05, **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 different extract of Hopea odorata leaves, 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 Hopea odorata (MEHO) at 20 mg/ml dose. Data were processed by paired t-test analysis by using SPSS for windows, version 16.0.*

3.2 Total Condensed Tannin Content

The total phenol contents of the extracts are shown in Table 2. The total condensed tannin content of *Hopea odorata* leaves was higher in plants at methanol extract, which was 96.99±0.34 mg catechin/g. All extracts of *Hopea odorata* contain good amounts of condensed tannin, ranging from 53.88 to 96.99 mg catechin/g. So condensed tannin content of different extracts of *Hopea odorata* leaves are as follows,

MEHO > EEHO > AEHO

Table 2. Contents of condensed tannin (expressed as mg catechin/g dry weight) in different extracts of *Hopea odorata* leaves

Extract	Total proanthocyanidin (mg catechin/g)
MEHO	**96.99±0.34****
EEHO	74.46±0.35**
AEHO	53.88±0.50*

*Values are mean ± SEM, (n = 3). Bold text indicates the highest total condensed tannin content of methanol extract of Hopea odorata (MEHO). The different superscripted (*) values have significantly different (*P < 0.05, **P < 0.001) from the other sample in same column.*

4. DISCUSSION

Drugs work against helminthes by damaging its cuticle, leading to partial digestion or rejection by immune mechanisms [17]. Levamisole works as a nicotinic acetylcholine receptor agonist that causes continued stimulation of the parasitic worm muscles, leading to paralysis. In the previous studies it was reported that the presence of flavonoids, condensed tannins and polyphenolic compounds show anthelmintic activity, [18] 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 [19]. 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 [20]. 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.

Sustained 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 different extracts of *H. odorata* leaves 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 [21-23]. 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 [24] and the spread of anthelmintic resistance within worm populations [25].

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.

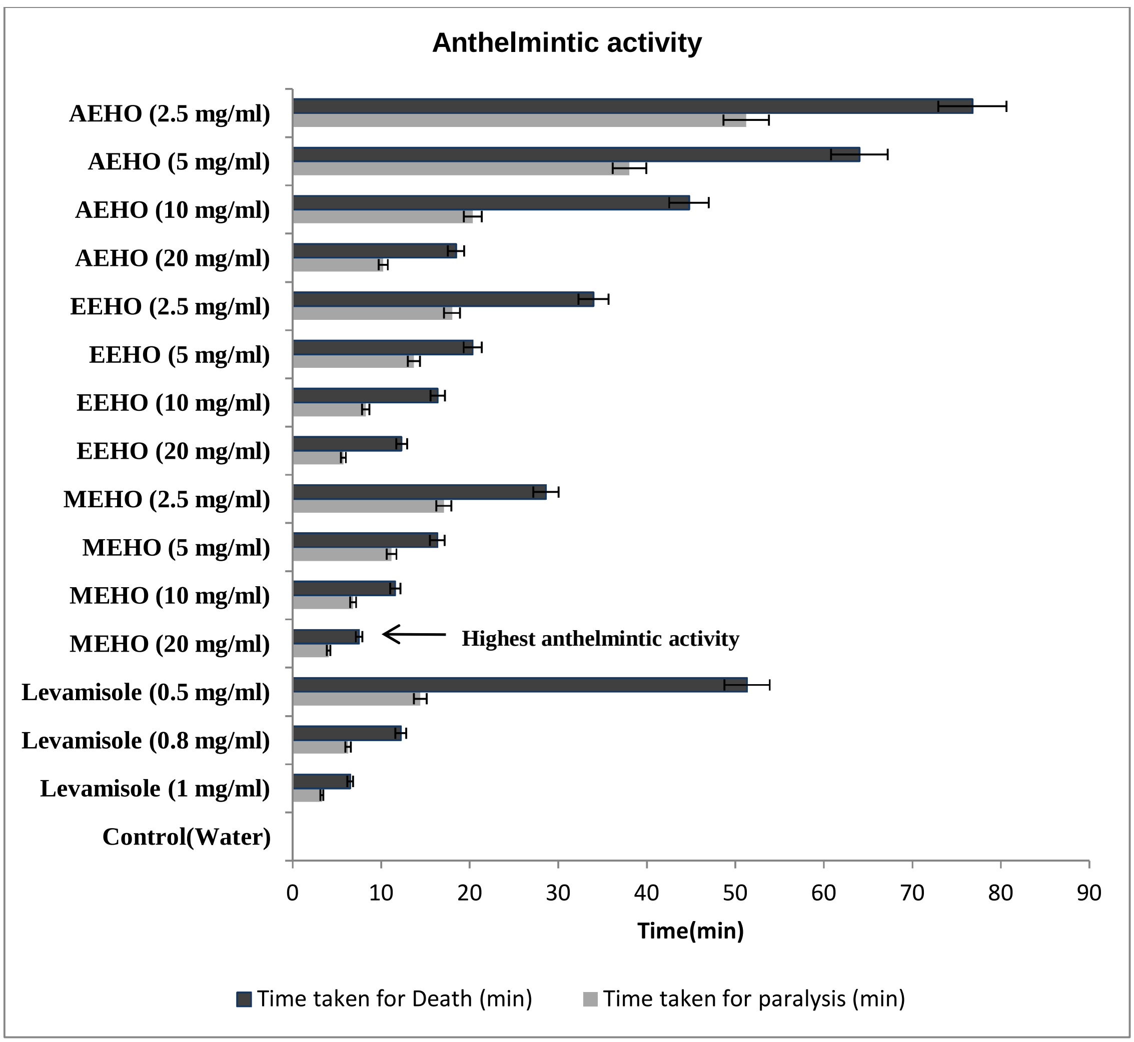

Fig. 1. Anthelmintic activity of different extracts of leaves of *Hopea odorata*

Methanol extract of Hopea odorata (MEHO) leaves at 20 mg/ml dose showed highest anthelmintic activity, which indicated by arrow mark in this graph

5. 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, main chains in the parasite life cycle can be disrupted by condensed tannin. These data support further investigations to determine *In vivo* efficiency in animal model. In addition, further mechanism action should studied, such as the relationship between the fine structure of condensed tannin molecules and anthelmintic activity, are also a high priority.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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