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Anthelmintic and α-amylase Inhibition Effects of Ethanol Extract and Its Different Fractions of *Rhaphidophora Glauca* (Wall.) Schott Leaves

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Abstract

Objective: To investigate the therapeutic effects of ethanol extract and its fractions of *Rhaphidophora glauca* leaves in anthelmintic (*in vitro*) and α-amylase inhibitory activity (*in vitro*).

Methods: Leaves of *Rhaphidophora glauca* was extracted with pure ethanol and fractioned with chloroform (CHFRG), petroleum ether (PEFRG), n-hexane (NHFRG) and ethyl acetate (EAFRG), which are tested for anthelmintic activity on aquarium worm *Tubifex tubifex* by using three concentrations viz., 5, 10 and 20 mg/ml of each. α-amylase inhibitory activity estimated by modified starch iodine protocol.

Result: Among the EERG and various fractions, PEFRG exhibited strong anthelmintic activity in vitro. Where it paralyzed (4.54±0.39 min) and produced death (8.37±0.86 min) 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. EERG and its all fractions showed good α-amylase inhibitory activity, but PEFRG [IC50=1.757±0.025mg/ml] showed highest activity among them as compared to Acarbose.

Conclusion: These findings suggest that the plant may be a potential source for the development of new anthelmintic and α-amylase inhibitory agent.

Keywords: *Rhaphidophora glauca*, *in vitro*, anthelmintic, *Tubifex tubifex*, α-amylase.

I. Introduction

The growing gratitude of the burden imposed by human helminthiasis has led to the implementation of large-scale control and elimination programmes. Helminthiasis still considered as the major cause of ill health of number of peoples throughout the world especially peoples from deprived communities of undeveloped countries with poorer sanitary and health facilities, because it is mostly caused and sprayed through to environmental contamination and transmission. [1, 2] This parasitic infection is also responsible for increasing the mortality and morbidity day by day all over the world. The worms which are associated with problem of ill health include the trematodes (flukes), cestodes (tapeworms), *Tubifex tubifex* and intestinal nematodes (roundworms).

Tubifex tubifex is a cosmopolitan naidid annelid sensu [3] representing one of the major components of the benthic fauna in freshwater communities [4]. Also present in polluted waters, T. tubifex is widely used in laboratories for ecotoxicology research [5] and as a model organism for the study of annelid development [6]. *Tubifex tubifex* is characterized by considerable variability in its morphological features [7] and by a mixed reproductive strategy, with parthenogenesis [8], self-fertilization [9], and biparental reproduction through cross-mating [10].

So to it is recommended to develop new alternatives which can treat helminthiasis completely with giving assurance about the safety in significant manner. In the recent years, the importance of Herbal drugs in the field of Medicine has greatly increased because of their assurance about safety and complete cure. Therefore the demand for the herbal formulation is also increasing accordingly.

Amylases are a class of hydrolases widely distributed in microbes, plants and animals. They can specifically cleave the O-glycosidic bonds in starch, glycogen and several oligosaccharides [11]. α-amylases and related amylolytic enzymes are among the most important enzymes and of great significance in the present day biotechnology. They could be potentially useful in the semi synthetic chemistry for the formation of oligosaccharides by transglycosylation [12]. The spectrum of amylase application has widened in many other fields, such as clinical, medicinal and analytical chemistry; as well as their widespread application in starch saccharification and in the textile, food, paper and pharmaceutical industries [13-16]. In plant, amylases also play a significant role in seed germination and maturation and are instrumental in starch digestion in animals resulting

in the formation of sugars, which are subsequently used for various metabolic activities [17]. Amylases from different sources have been studied in great depth. For example, in germinating cereal grains, α-amylases are the most abundant starch-degrading enzymes. The enzymes are secreted by aleuronic cells into the starchy endosperm where they degrade the starch grains [18].

Rhaphidophora glauca (Wall.) Schott (family: Araceae), an aroid liane native to the subtropical and warm temperate regions of the eastern Himalaya. Which is also distributed In Nepal through NE India to Bangladesh and Myanmar and N Thailand to N Laos and Vietnam.

The aim of the present study was to identify the anthelmintic activity and α-amylase inhibition of ethanolic extract and its different fractions of leaves of *Rhaphidophora glauca*.

II. Method And Materials

1.1. Plant collection & identification

Leaves of *Rhaphidophora glauca* (Accession No. 1314 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 Bokhtear Uddin, Associate Professor, Department of Botany, University of Chittagong, Chittagong-4331, Bangladesh.

2.2. 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 leaves (900 g) of *Rhaphidophora glauca* was soaked in 1.3 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 (50 g) of the concentrated ethanol extract (EERG) was fractioned by the modified Kupchan partitioning method [19, 20] into chloroform, CHFRG (13 g), n-hexane, NHFRG (13 g), ethyl acetate, EAFRG (12 g) and petroleum ether, PEFRG (17 g).

2.3. Chemicals

All chemicals used were of analytical reagent grade. Ethanol, chloroform, pet ether, ethyl acetate and n-hexane were purchased from Merck, Germany. Normal saline solution was purchased from Beximco Infusion Ltd. Levamisole was purchased from ACI Limited, Bangladesh. α-amylase was purchased from Sigma-Aldrich (Sigma-Aldrich Co., USA). Starch, iodine was purchased from Fluka (Fluka chemie GmbH, CH-9471 Buchs). Shimatdzu Biospec 1601 UV visible spectrophotometer (Shimatdzu, Japan) was used to measure the absorbance.

2.4. In-vitro Anthelmintic Assay

The anthelmintic activity of ethanolic extract and its fractions of leaves of *Rhaphidophora glauca* were carried out as per the procedure of Ajaiyeoba et al. [21] 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 [22, 23, 24]. The worms were collected from the local market of Chittagong, average size of worms 2-2.5 cm. were taking study. The standard drug Levamisole (positive control) and three different concentrations of ethanol extract and its fractions (5, 10 and 20 mg/ml) in double distilled water [25, 26] were prepared freshly and used for the study of anthelmintic activity. One group was composed of water and it was considered as negative 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 [27]. 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 [28].

2.5. In vitro α-amylase inhibitory activity

This study was performed by a modified starch iodine protocol as Hossain et al [29] with minor modification. In short, 1 mL of plant extract or standard of different concentration (2, 1, 0.5 mg/mL) was taken in prelabeled test tubes. The reaction mixture contained 20 µl of α-amylase solution (10 mg/ml), phosphate buffer (0.02 M, pH 7.0) with 0.006 M NaCl (0.4 ml) was added to each test tube and incubated for 10 min at 37°C. After the incubation 200 µl of 1% starch solution was added to each test tube and the mixture was re-incubated for 1 h at 37 °C. Then 200 µl of 1% iodine solution was added to each test tube and after that, 10 mL distilled water was added. Absorbance of the mixture was taken at 565 nm. Sample, substrate and α-amylase

blank were undertaken under the same conditions. Each experiment was done in triplicate. IC50 value was calculated by using regression analysis.

$$\% \text{ α-amylase inhibition} = \left[1 - \frac{(SA - SBB) - SMB}{AAB}\right] \times 100$$

SA=Sample absorbance, SMB=Sample blank, SBB=Substrate blank, AAB=α-amylase blank

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 and Student's t test was performed between IC50 values for α-amylase inhibition assay. Regression analysis was performed to calculate IC50 values. 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.

III. Results

3.1. In vitro anthelmintic activity

Results of study were recorded as shown in table-1 and figure-1 as in the form of time required to get 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 methanolic extract showed dose dependent anthelmintic activity as compared to a standard drug levamisole. Different treatment showed different anthelmintic activity. But petroleum ether fraction of ethanol extract of *R. glauca* showed highest anthelmintic activity. Where it paralyzed (4.54±0.39 min) and produced death (8.37±0.86 min) of the *Tubifex tubifex* at 20 mg/ml dose near the value of the standard (3.3±0.38 min and 6.5±0.76 min) at 1 mg/ml. EAFRG showed the lowest anthelmintic activity. It's paralyzing and death time of *Tubifex tubifex* is 22.09±0.28 min and 62.85±1.17 min at dose 5mg/ml. So the anthelmintic activities of ethanol and its fractions of *R. glauca* leaves are as follows,

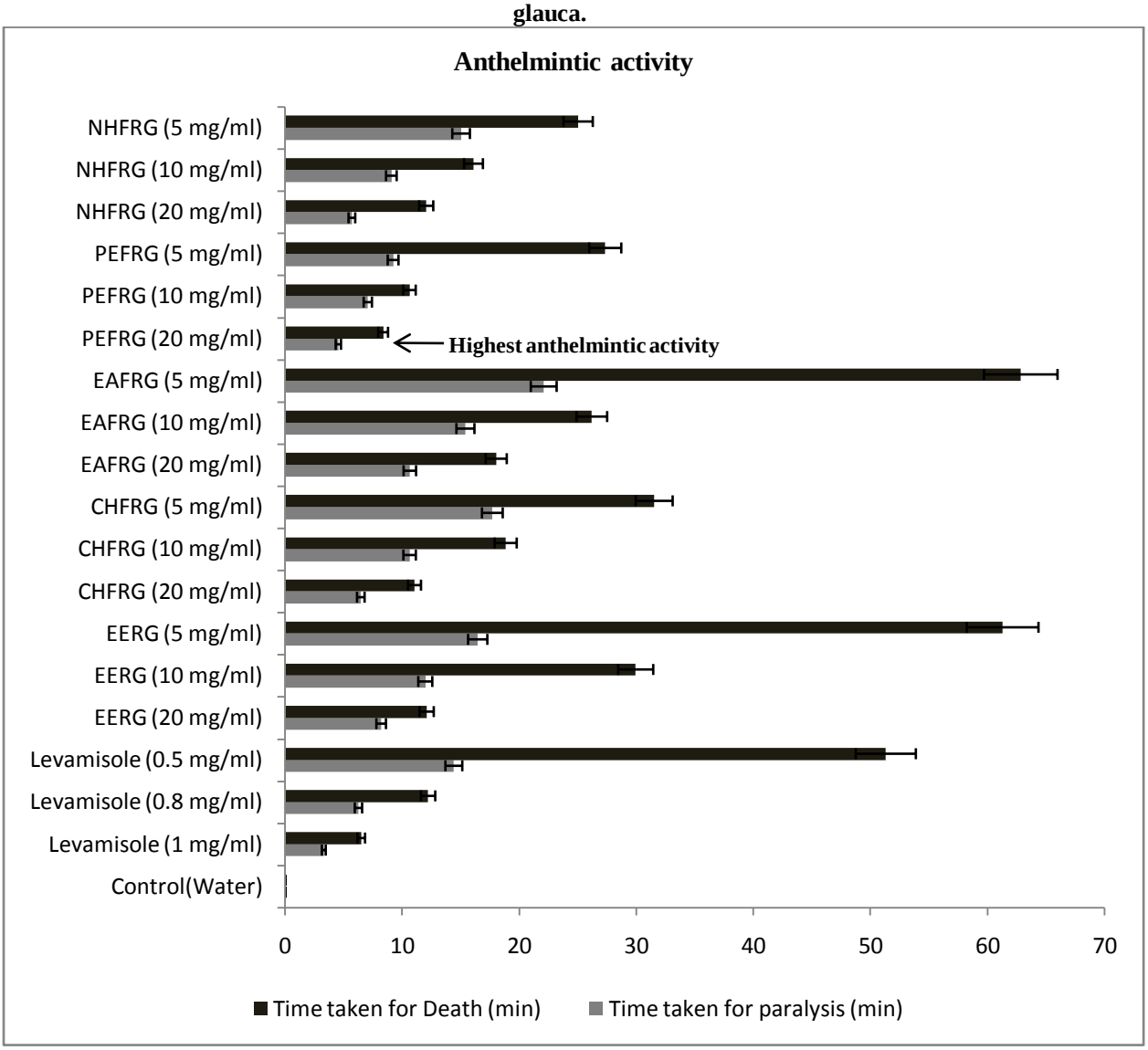
PEFRG>CHFRG>NHFRG>EERG>EAFRG

Table 1: Anthelmintic activity of ethanolic extract and its different fractions of leaves of *Rhaphidophora glauca.*

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**
EERG (20 mg/ml)	8.2±0.54*	12.09±0.6*
EERG (10 mg/ml)	11.97±0.44*	29.95±1.3*
EERG (5 mg/ml)	16.45±0.85*	61.3±1.43*
CHFRG (20 mg/ml)	6.46±0.21**	11.05±0.31*
CHFRG (10 mg/ml)	10.63±0.23**	18.84±0.32*
CHFRG (5 mg/ml)	17.7±0.44*	31.53±0.55*
EAFRG (20 mg/ml)	10.65±0.48*	18.03±0.97*
EAFRG (10 mg/ml)	15.4±0.07**	26.2±0.07**
EAFRG (5 mg/ml)	22.09±0.28**	62.85±1.17
PEFRG (20 mg/ml)	**4.54±0.39***	**8.37±0.86***
PEFRG (10 mg/ml)	7.06±0.57	10.62±0.74
PEFRG (5 mg/ml)	9.22±0.52*	27.35±1.08*
NHFRG (20 mg/ml)	5.7±0.33	12.05±0.27*
NHFRG (10 mg/ml)	9.07±0.27*	16.09±0.29*
NHFRG (5 mg/ml)	15.03±0.4*	25.03±1.03*

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 *Rhaphidophora glauca* 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 pet ether fraction of ethanol extract of *Rhaphidophora glauca* (PEFRG). Data were processed by paired t-test analysis by using SPSS for windows, version 16.0.

Figure 1: Anthelmintic activity of ethanol extract and its different fractions of leaves of Rhaphidophora glauca.



Petroleum ether fraction of ethanol extract of *Rhaphidophora glauca* (PEFRG) leaves showed highest anthelmintic activity, which indicated by arrow mark in this graph.

3.2. In vitro α-amylase inhibitory activity

Ethanol extract and all fractions showed good α-amylase inhibition. PEFRG showed highest IC50 value (1.757±0.025) mg/mL; P<0.05, whereas standard Acarbose showed (0.912±0.015) mg/mL; P<0.05. Ethanol extract and its fractions significantly inhibited α-amylase activity in a dose dependent manner like Acarbose. Therefore we can conclude that this leaves extract have good α-amylase inhibitory activity. All results are shown in Table 2. The α-amylase inhibitory activities of ethanol and its fractions of *R. glauca* leaves are as follows,

PEFRG>CHFRG> EERG>EAFRG>NHFRG

Table 2: IC_{50} values (mg/mL) for ethanol extract and its different fractions of leaves of *Rhaphidophora glauca* and Acarbose in α-amylase inhibitory assay.

Extract/Standard	Concentrations in mg/mL with (% Inhibition)			IC_{50} value mg/mL
EERG	0.50(22.17±0.098)	1.00(34.54±0.356)	2.00(52.78±0.401)	1.839±0.016**
CHFRG	0.50(23.17±0.637)	1.00(35.94±0.733)	2.00(53.72±0.309)	1.786±0.021**
EAFRG	0.50(22.51±0.979)	1.00(34.81±0.712)	2.00(51.05±0.249)	1.912±0.005**
PEFRG	**0.50(24.84±0.924)**	**1.00(36.47±0.284)**	**2.00(54.38±0.656)**	**1.757±0.025***
NHFRG	0.50(21.84±0.667)	1.00(33.47±0.379)	2.00(48.05±0.249)	2.079±0.018**
Acarbose	0.25(15.89±1.117)	0.50(26.05±0.438)	1.00(55.38±0.888)	0.912±0.015*

Values are the mean of triplicate experiments and represented as mean±SEM (n=3); *P < 0.05, **P < 0.001. Student's t test was performed to analyze this data set. Bold text indicates the highest α-amylase inhibition of pet ether fraction of ethanol extract of Rhaphidophora glauca (PEFRG). Data were processed by student't' test analysis by GraphPad Prism, version 6.0.

IV. Discussion

One-third of the human population of the world is at risk of helminthes infection, and infection is also very common in animals. Regrettably, there are still no effective vaccines for controlling these infections, so that both treatment and prophylaxis rely on anthelmintic drugs. The continued use of these drugs has given rise to concerns over levels of resistance and promoted the search for new knowledge and understanding that might slow its progress. The cholinergic anthelmintics, which include levamisole and pyrantel, are an important group of anthelmintics and like other major anthelmintics (ivermectin and emodepside) target parasite ion channels. Levamisole selectively opens a restricted subgroup of nematode acetylcholine receptor (AChR) ion channels in nematode nerve and muscle. Opening of AChR channels produces depolarization [30], entry of calcium through the opened channels, and an increase in sarcoplasmic calcium, producing spastic muscle contraction [31]; the parasite is then unable to maintain its location (often in the intestine) and is then swept away, effecting the cure. Interest in this class of anthelmintic has increased recently because application of new methods has demonstrated the presence of diverse receptor subtypes and different cholinergic anthelmintic subtype selectivities. It has also allowed better mechanistic explanations of resistance and the development of exciting novel compounds such as monepantel and derquantel. This review describes new knowledge and insights that have increased our understanding of the biology of these receptors.

Alpha-amylase is a prominent enzyme found in the pancreatic juice and saliva which breaks down large insoluble starch molecules into absorbable molecules [32]. Inhibitors of α-amylase delay the breaking down of carbohydrates in the small intestine and diminish the postprandial blood glucose excursion [33].

In this study, the effect of *Rhaphidophora glauca* leaves extract and its fractions on the activities of α-amylase was evaluated. The plant extract and its all fractions showed potent inhibition of α-amylase activity. This result is in agreement with previous reports which indicated that excessive inhibition of pancreatic α-amylase could result in the abnormal bacterial fermentation of undigested carbohydrates in the colon and therefore mild α-amylase inhibition activity is desirable [34]. This plant inhibits α-amylase competitively. This suggests that the active components in the extract compete with the substrate for binding to the active site of the enzyme thereby preventing the breaking down of oligosaccharides to disaccharides [35].

V. Conclusion

The results stated above showed that the ethanol extract and its fractions of *Rhaphidophora glauca* possessed significant anthelmintic effects. Also it has well α-amylase inhibitory activity. All of these effects of *Rhaphidophora glauca* evidenced that it could be a very good source of natural medicines on standard formulation. However, further study is needed to isolate the active principle(s) in this plant which is responsible for this activity.

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Competing interests

The authors declare that they have no competing interests.

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