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Phytochemical screening and study of cytotoxic, thrombolytic and anthelmintic activity of extract of *Glycosmis pentaphylla* (Retz) leaves

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Abstract
The study aimed for phytochemical screening and to evaluate the cytotoxic, thrombolytic and anthelmintic activities of methanol extract of the leaves of *Glycosmis pentaphylla* (Retz.). The cytotoxic activity of crude extract was determined using brine shrimp lethality bioassay and LC_{50} values of the sample was 11.246 ± 0.65 µg/ml whereas for standard vincristine sulphate it was 8.50 ± 0.16µg/ml, act as a positive control. The extract showed 17.17 ±3.42% clot lytic activity as compared to standard streptokinase's (30.45% ± 2.67%). The anthelmintic activity was done by using *Tubifex tubifex* with three concentrations *viz.*,2.5, 5 and 10 mg/ml of the extract was studied which was mainly thoughtful about the finding of time of paralysis and time of death of the worms. The gradual increase in a dose exhibited a gradual increase in the activity. The extract exhibited significant anthelmintic activity at highest concentration of 10 mg/ml as compared with levamisole (1 mg/ml) was performed as standard reference and distilled water as control.

Keywords: *Glycosmis pentaphylla*, methanol extract, cytotoxic activity, thrombolytic activity, anthelmintic activity

Introduction
Plant-based foods contain bioactive compounds, which provide desirable health benefits beyond basic nutrition. Epidemiological evidence trust that consumption of a diet rich in vegetables and fruits has positive implications for human health. The World Health Organization reported that 80% of the world populations rely on indigenous medicine and that the most of traditional therapies involve the use of plant extracts or of their active constituents (WHO, 1993) [39] and over 25% of modern medicines that are commonly used worldwide contains compounds extracted from medicinal plants(Robbers *et al*.,1996) [28]. In Bangladesh there is abundant of medicinal plants and ninety percent of the medicinal plants are from wild source (Ghani, 1998; SEDF & IC, 2003) [15].
During recent decades, there has been an uprising demand for finding newer and safer chemotherapeutic agents. Cancer is the third leading cause of death worldwide, only preceded by cardiovascular disease, infectious and also a parasitic disease (Mathers *et al*., 2001) [18]. Extracts of medicinal plants are believed to contain a wide spectrum of polyphenolic, flavonoids, alkaloids, terpenoids and saponin compounds, which might have therapeutic properties and hinder cancer formation (Dia & Mumper, 2010) [11]. Over 60% of current cytotoxic agents have been formed from natural sources containing plants, pelagian organisms and microorganisms, either directly or through chemical synthesis based upon natural lead components (Newman *et al*., 2003; Cragg *et al*., 2005) [23, 7]. Therefore, natural products have wide application in cancer chemotherapy (Cragg *et al*., 2005) [7].
Cardiovascular disease caused by blood clot (thrombus) formation is one among the top severe diseases which are uprising with an alarming rate in the current years. Homeostasis maintains the integrity of the circulatory system after damaging of the vascular channel. Thrombus development is a difficult event in the arterial diseases associated with myocardial infarction, anoxia, hypertension, stroke, a decrease of the total blood supply to the liver (Bekker *et al*., 2009) and venous thromboembolic disorders that account for a considerable number of deaths worldwide (Furie, 2008) [14].

Remarkable works have been made towards the discovery and the development of natural constituents from various plant and animal sources which have antiplatelet, anticoagulant, antithrombotic and thrombolytic activity. Thrombolytic agents are used to dissolving clot and in the management of thrombosis in patients (Watson *et al*., 2002) [37] Thrombolytic compounds such as tissue plasminogen activator (t-PA), Urokinase (UK), streptokinase (SK) etc, are used worldwide for the treatment but their use is associated with hyper risk of haemorrhage, anaphylactic reaction and lacks specificity (Anwar *et al*., 2011) [1]. Because of the blames in the existing thrombolytic agents, a number of research works are underway to improve the variants of these drugs for their better effective nature.

Helminthic infestations are now being recognized as cause of chronic ill health and slackness amongst the children. World Health Organization calculated two billion people transited with helminths and it was also calculated that 100% of all age group of school going children are at high risk of morbidity (WHO, 2010) [38]. The major phyla of helminths are nematodes (round worms) which are soil-transmitted helminths that mostly cause the intestinal infection, filarial worms cause the Onchocerciasis and lymphatic filariasis, while Platyhelminthes (flatworms) also known as trematodes like schistosomes and cestodes causes cysticercosis (De Silva *et al*., 2003; Steinmann *et al*., 2006) [9, 32]. Current estimates believe that over half of the world population is infected with intestinal helminths, such as *Ascaris*, hook worms, *Trichuris*, *Enterobius*, *Strongyloides*, and tapeworms, and that most of these transited people live in outlying rural areas in the developing countries (De Silva *et al*., 2003) [9]. In case of other animals also gastrointestinal parasites cause infections that diminish the animal survival, growth rates and reproductive performance. Aberration from nematodes is common with diabetes and lung cancer (Bethony, 2006) [5]. The helminths parasites mainly subsist in human body in intestinal tract, but they are also found in tissue, as their larvae migrate towards them (Tripathi, 2003) [34]. 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 (Mali and Mehta, 2008) [16]. Henceforth it is important to look for different strategies against gastrointestinal nematodes, which have led to the proposal of screening medicinal plants for their anthelmintic activity.

Glycosmis pentaphylla (Retz.) DC, commonly called as orange berry, belongs to the family Rutaceae, is a shrub or small (1.5–5.0 m) tree widely distributed in Bangladesh. It is reported to contain arborinine, glycozolicine, 3- formyl carbazole, glycosinine, mupamine, varbazole, 3-
methyl carbazole, glycolone, glycozolidol, glycozolinine, glycophymoline, glycophymine, glycomide, glycozoline, noracronycine, des-N-methylacrocynine and des-N-methylnoracronycine. *Glycosmis pentaphylla* has also been found to have antioxidant, galactagogue, immune stimulant, larvicidal activity, antipyretic and hepatoprotective activities (Raju and Rao, 2010) [27]. Juice of leaves is used in treat fever, liver problems and as a vermifuge, while leaves are considered good antidote for eczema and other skin troubles (Muthukrishnan *et al*., 1999) [22]. In traditional Indian medicine *Glycosmis pentaphylla* is used to treat diarrhoea, coughs, rheumatism, anaemia, and jaundice (Valkenburg and Bunyapraphatsara, 2001) [35]. The present study was undertaken to inquire the cytotoxic, thrombolytic and anthelmintic activity of leave extract of this plant.

Materials and Methods

Chemicals

Lyophilised streptokinase vial (1 500 000 IU) was purchased from Square Pharmaceuticals Ltd, Bangladesh. Methanol was purchased from Merck, Germany. Normal saline solution was purchased from Beximco Infusion Ltd. Levamisole was purchased from ACI Limited, Bangladesh. All chemicals used were analytical reagent grade.

Plant Materials

Fresh leave of *G. pentaphylla* for this study were collected from the local area of Chittagong, Bangladesh and were authenticated by Dr. Sheikh Bokhtear Uddin, Associate Professor, Department of Botany, University of Chittagong, Chittagong-4331, Bangladesh.

Preparation of Crude Extract

The collected leave were dried for a period of 2 weeks under shade and ground. The ground leave (750 gm) were soaked in proper amount of methanol for one week at room temperature with shaking and stirring. The sediments were filtered and the filtrates were dried at 40 °C in water bath. The solvent was completely shifted by filtering with Whatman number 1 filter paper. The solvent was evaporated under reduced pressure at room temperature to yield semisolid. The extract was then preserved in a refrigerator till further use.

Phytochemical Screening

Phytochemical screening of the crude methanol extract of *G. pentaphylla* leave was carried out using standard phytochemical methods described by Muanda, 2010 [21].

Brine Shrimp Lethality Assay

The assay was fulfilled in accordance with the principle and protocol formerly described by Meyer *et al*., with slight modifications. Here simple zoological organism (*Artemia salina*) was used as a convenient monitor for the screening. Dried cysts of *Artemia salina* were collected from an aquarium shop (Chittagong, Bangladesh) and hatched in artificial seawater (3.8% NaCl solution) for 48 h to mature shrimp called nauplii. After hatching, active naupli free from egg shells were taken from brighter portion of the hatching chamber and used for the assay.

The test sample (extract) were prepared by mixing them into the DMSO (not more than 50 µL in 5 mL solution) plus ocean water (3.8% NaCl in water) to attain the concentrations of 10, 25, 50, 100, 200, 300, 500 and 800 µg/ ml. A vial with 50 µL DMSO diluted to 5 mL was used as a control. Vincristine sulphate was used as positive control. Then after 24 hours, the number of survival of nauplii was calculated and percentage of mortality was determined using the equation:

% mortality = (no. of dead nauplii/ initial no. of live nauplli) x 100.

Statistical method of probit analysis (Finney, 1971) [13] was used to calculate LC_{50}. Criterion of toxicity for fractions was established according to (Déciga-Campos *et al*., 2007) [8]. LC_{50} values > 1000 µg/mL (non-toxic), ≥ 500 ≤ 1000 µg/mL (weak toxicity) and < 500 µg/mL (toxic).

Thrombolytic Test

This test was done according to the pocess described by Prasad *et al*., 2006 [24]. In the commercially available lyophilised streptokinase vial (1 500 000 IU), 5 mL sterile distilled water was added and then mixed properly. This suspension was used as stock solution from which appropriate dilution was made.5 ml of venous blood was taken from the healthy volunteers (n=10) without the history of oral contraceptive or anticoagulant treatment and was distributed (0.5 mL/tube) to each 10 previously weighed sterile micro centrifuge tube and incubated at 37 °C for 45 min to form the clot. After this clot formation, serum was completely withdrawn without disturbing the clot and each tube having clot was again weighed to find out the clot weight. A total volume of 100 µL of methanol extract (10 mg/ mL) was mixed to each micro centrifuge tube containing pre weighed clot. As a positive control, 100 µL of streptokinase and as a negative control 100 µL of distilled water were differently added to the control tube marked. All the tubes were then incubated at 37 °C for 90 min and audited for clot lysis. After the incubation, fluid released was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis.

***In-vitro* Anthelmintic Assay**

The anthelmintic activity of methanolic extract of leave of *G. pentaphylla* was carried out as per the procedure of Ajaiyeoba *et al*., 2001; [2] with some minor modifications. The aquarium worm *Tubifex tubifex* were used in the current study because it has bodily similarity and belongs to the same group of intestinal worm i.e. annelida (Verma *et al*., 2013; Dutta *et al*., 2012; Rajagopal *et al*., 2013) [36, 12, 26]. The worm were culled from the local market of Chattogram, average size of worms 2-2.5 cm. were taking study. The standard drug levamisole and three different concentrations of methanol extracts (2.5, 5 and 10 mg/ml) in double distilled water (Satish *et al*., 2009; Deore *et al*, 2009) [29, 10] were prepared freshly and used for the study of anthelmintic action. 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. Death was again 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 (Mongla and Yadav, 2005) [20].

Result

The lethality of the crude extract of leave of *G. pentaphylla* to brine shrimp was determined on *Artemia salina* after 24 hr of exposure the samples, the negative control DMSO and sea water and vincristine sulphate used as standard. This technique was employed for the determination of general toxic property of the plant extract. The LC_{50} value (Figure 1) of the extract was 11.246 ± 0.65 µg/mL and that for standard vincristine sulphate was 8.50± 0.16 µg /ml. No mortality was found in the control group, using DMSO and ocean water. The plant extract showed moderate clot lysis activity (17.17 ±3.42%) as compared to standard streptokinase's clot lysis (30.45% ± 2.67%) activity (Figure 2). Results of study were recorded as shown in table-1 as in the form of time needed to get sequent attacks of paralysis and at the end time required for complete death of parasite. From the observations made, higher concentration of extract produced paralytic effect much earlier and the time to death was brief for all worms. From this study it was seen that the methanolic extract showed dose dependent antihelmintic activity as compared to a standard drug levamisole. The extract showed paralyzing time of *Tubifex tubifex* with the dose of 2.5, 5 and 10 mg/ml were found to be 18.20 ± 2.185, 5.54 ± 1.484 and 3.08 ± 0.322minutes respectively. In the meantime levamisole at a dose of 0.5, 0.8 and 1 mg/ml causes paralysis in the above helminth in 14.41 ± 2.643, 6.26 ± 1.261 and 3.30 ± 0.645 minutes respectively. The mean death time of *Tubifex tubifex* with the extract at dose of 2.5, 5 and 10 mg/ml were found to be 31.10 ± 3.206, 21.19 ± 3.712 and 5.20± 1.149 minutes respectively and the standard levamisole at a dose of 0.5, 0.8 and 1 mg/ ml causes death in the above helminth in 51.32 ± 4.825, 12.21 ± 2.512 and 6.50 ± 1.314 minutes respectively. No paralysis or death was observed in case of control (water). The phytochemical screening indicates qualitative presence of alkaloid, glycosides, saponins, steroids, flavonoids, tannins and resin (Table1).

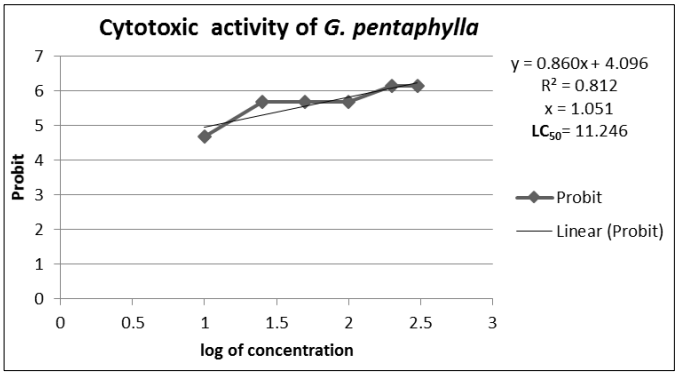

Fig 1: Toxicity assay of *G. pentaphylla* in brine shrimp. The results are manifested as mean ±SEM of three measurements.

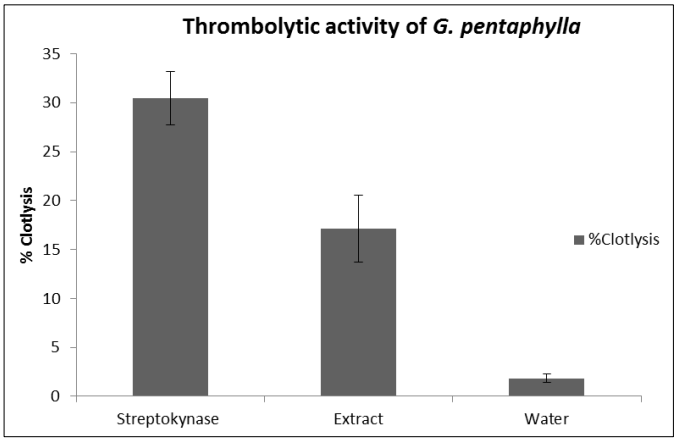

Fig 2: The clot lysis activity of *G. pentaphylla* extract and streptokinase. All results are mean ± SEM of three consecutive experiments.

Table 1: Phytochemical constituents identified in the plant extracts of *G. pentaphylla*.

Phytochemical constituents	Results	Phytochemical constituents	Results
Alkaloids	+	Flavonoids	+
Terpenoids	-	Tannins	+
Glycosides	+	Resin	+
Saponins	+	Anthraquinones	-
Steroids	+	Reducing sugar	-

+: indicate presence; -: indicate absence.

Statistical Analysis

All the results gained by *in vitro* experiment were manifested as mean (±SEM) of three measurements followed by Dunnet's test where $P<0.05$ was considered as statistically significant.

Table 2: Anthelmintic activity of *G. pentaphylla*.

Test Sample	Concentration mg/ml	Time taken for paralysis (min)	Time taken for Death (min)
G. pentaphylla	10	3.08 ± 0.322	5.20 ± 1.149
	5	5.54 ± 1.484	21.19 ± 3.712
	2.5	18.20 ± 2.185	31.10 ± 3.206
Standard *(Levamisole)*	1	3.30 ± 0.645	6.50 ± 1.314
	0.8	6.26 ± 1.261	12.21 ± 2.512
	0.5	14.41 ± 2.643	51.32 ± 4.825
Control (water)		--	--

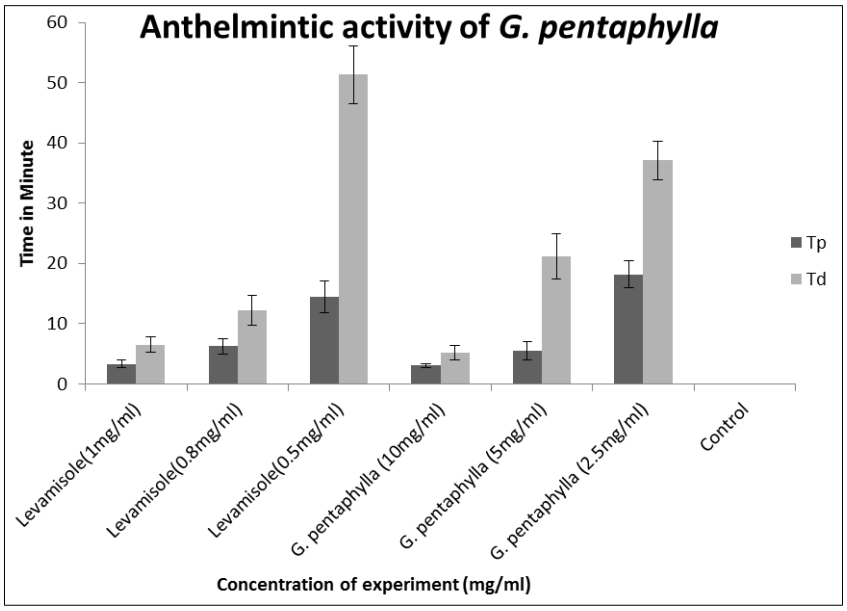

Fig 3: The anthelmintic activity of *G. pentaphylla* extract and levamisole. All results are mean ± SEM of three consecutive experiments

Discussion

Ideally, any compound useful in the treatment of cancer should not be toxic to the normal cell. However, in reality, anticancer agents are often toxic to normal cells, particularly towards rapidly growing cells (Priest man, 2008) [25]. It is necessary to test this extract to assess its potency and also versus various cancer cell lines as well as normal cell line to justify the potential to further investigate this plant for anticancer activity.

Most thrombolytic agents work by activating the enzyme plasminogen, which clears the cross-linked fibrin mesh responsible for clot formation. This makes the clot soluble and subject to further proteolysis by other enzymes, and restores blood flow over obturated blood vessels. Thus thrombolytic agents are necessary for the treatment of different illness like myocardial infarction, thromboembolic strokes, deep vein thrombosis and peripheral embolism, to clear a blocked artery and avoid permanent damage to the perfused tissue (e.g. myocardium, brain, and leg).

Anthelmintics are the drugs that expel out parasitic worms (helminths) from the body by either causing paralysis or by directly killing them (Chaturvedi *et al*., 2009) [6] by damaging its cuticle, leading to partial digestion or rejection by immune mechanisms (Thorn *et al*., 2009). Levamisole works as a nicotinic acetylcholine receptor agonist that causes extensive stimulation of the parasitic worm muscles, leading to paralysis. The literature have been indicated that the presence of flavonoids, tannins and polyphenolic compounds show anthelmintic activity (Shrestha *et al*., 2009) [31], 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 (Athanasiadow *et al*., 2001) [3]. 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 (Martin, 1997) [17].

Conclusion

The study concludes that the plant under study has found to possess good cytotoxic, moderate thrombolytic and momentous anthelmintic activity in dose dependent manner. The plant might have potential to be exhibited 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 enhance the potency.

Conflict of Interests: The authors declare that there is no conflict of interest regarding the publication of this paper

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