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In-vitro scolicidal activity of *Mallotus philippinensis* (Lam.) Muell Arg. fruit glandular hair extract against hydatid cyst *Echinococcus granulosus*

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ABSTRACT

Objective: To investigate new scolicidal agent from natural resources to cope with the side effects associated with synthetic drugs in Echinococcosis. **Methods:** The scolicidal potential of methanolic fruit powder extract (10 and 20 mg/mL) of *Mallotus philippinensis* (*M. philippinensis*) was investigated. Viability of protoscoleces was confirmed by trypan blue exclusion method, where mortality was observed at concentration of 10 and 20 mg/mL in 60 min treatment against *Echinococcus granulosus* (*E. granulosus*), under *in-vitro* conditions with reference to the known standard drug Praziquantel®. **Results:** At concentration 10 and 20 mg/mL, the mortality rate was observed 97% and 99% respectively for 60 min treatment; while up to 93% mortality was observed with 20 mg/mL for only 10 min treatment. The concentration above 20 mg/mL for above 2 h showed 100% mortality, irrespective of further incubation. **Conclusions:** As compared with the standard anti-parasitic drug Praziquantel our extract has significant scolicidal activity with almost no associated side effects.

1. Introduction

'Hydatidosis' or Cystis Echinococcosis (CE) is a chronic zoonosis affecting humans as well as domestic animals[1], caused by the larval stage of a cosmopolitan parasitic cestode *Echinococcus granulosus* (*E. granulosus*)[2,3]. CE is most prevalent in sheep and cattle raising regions like South and Central America, the European and African part of Mediterranean and to some extent the Middle East, Sub-Sharan countries, Russia and china, where it becomes major economic and public health concern[4-6]. *E. granulosus* uses sheep, goats, horses and camels as intermediate hosts, but it is often transmitted to humans after ingestion of infective parasite eggs passed in the faeces from definitive hosts through direct contact or via environmental contamination[7]. These parasitic eggs after ingestion hatched into onchospheres that penetrate intestinal mucosa and migrating through blood stream reaches to the host's viscera and develop into mature larval cysts. The larval cysts are characteristic features of Hydatidosis, that affect mainly liver (50%-70%), but sometimes also infects lungs (20%-30%) leading to the alveolar echinococcosis. Although cysts can be developed in any part of the body but less frequently in spleen, kidney, bones and brains[8-10]. Because of the slow progression of the disease, it might be asymptomatic initially and/or with very little clinical manifestations[11], which further progresses depending on the site of infection and size of cyst[12]. Although in recent years the clinicians and researchers have gathered much experiences translating into new therapeutic approaches for treatment of CE, but there are still too many questions and problems that needs to be address urgently. Surgery is still

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most preferred method for treatment of CE, although there is increasing risk of intra-operative spillage of protoscoleces rich fluid leading to reoccurrence (10% in post operative cases) or secondary echinococcosis[13-15]. Other treatment options are puncture, aspiration, injection of protoscolicidal agent, development of PAIR techniques under guidance of ultrasound, chemotherapy with synthetic anti-helminthes drugs like benzimidazole carbamates derivatives and reliable follow up methods for clinically silent cysts[16]. Chemotherapy is still reliable for symptomatic cysts that are inoperable however there are many side effects of these synthetic drugs of their own[2,16]. Increasing side effects and appearance of resistance to the synthetic anthelmintics, stimulates researchers for exploration of natural alternatives from medicinal plants, utilizing traditional knowledgebase of many Ayurvedic and Unani medical treatment systems. In India alone there are more than 100 medicinal plant products reported to be beneficial in the treatment or control of many parasitic infections during last 5 decades, but they could not be developed into viable drugs for a variety of reasons[17].

Thus due consideration is required for reinvestigation and scientific exploration of these herbal plant products, to find out new anthelmintics alternatives that can overcome the side effects besides being sustainable and environmentally acceptable.

In this present study we are reporting the efficacy of the fruit glandular hair extract of *Mallotus philippinensis* (*M. philippinensis*) for their *in-vitro* protoscolicidal activity against hydatid cysts *E. granulosus*. This plant (commonly called Kamala, Kampillaka, Kapila and locally known as Shendri) is a very common perennial shrub or small tree found throughout the Indian subcontinent, Malaysia and Philippines. *Mallotus* spp. has potential activities as antibacterial[18-20], Anti-pyretic[21], Anti-retroviral[22], Anti-inflammatory[23], Cytotoxic and Antitumor[24], Hepatoprotective[25], Anti-cancer[26,27], Anti-viral[28], Anti-oxidant[29], anti-fertility[30] and many more that can be add into this list. Besides above mentioned activity this plant has been used in various ancient medical treatment systems as potential anti-parasitic and anthelmintics in their various preparations.

It was observed that leaf extract of *M. philippinensis* has anti-filarial activity[31] and Anthelmintic activities[32]. There are scanty reports on protoscolicidal activity of crude product of different medicinal plants against *E. granulosus*, but no published report available on the protoscolicidal activity of the glandular hair extract of *M. phillipinensis* especially against *Echinococcus* spp. Thus the aim of the present work was to determine the in-vitro protoscolicidal potential of methanolic extract of *M. philippinensis* fruit glandular hairs on hydatid cysts *E. granulosus*.

2. Materials and methods

2.1. Plant materials

Fruits of *M. philippinensis* plants were collected from Botanical Garden, Department of Dravyaguna, Institute of Medical Sciences, Banaras Hindu University (25.5° N, 82.9° E; elevation, 79 ft/85 m) India. The plants were identified and authenticated by Prof. R.K. Asthana Department of Botany, Banaras Hindu University India. A reference voucher number RKA/BOT/Sept.10-12 was assigned to the plant samples and preserved in Department of Botany, and also in Department of Microbiology.

2.2. Preparation of extract

The red color glandular hair powder adheres at the surface of shade dried fruits was collected. Approx 100 g of powder was added into 400 mL of methanol and mixed into a separating funnel, the organic fraction was collected and concentrated *in-vacuo* in a rotary evaporator and the residue was dried in desiccators over calcium chloride. Again this residue was defatted with petroleum ether and hexane followed by re-extraction in methanol at room temperature by cold extraction method. The remaining semisolid residue was then dried and stored at room temperature for further experiments.

2.3. Collection of cysts

Hydatid cysts of *E. granulosus* were collected aseptically from infected liver and lungs of cattles slaughtered in an abattoir located in Varanasi City, India. The intact cysts were immediately placed in an ice-box and transported within 3 h to the Enteric laboratory, Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. Hydatid fluid along with protoscoleces was collected as previously described by Smyth *et al*. 1980[33]. Briefly, Cysts were washed several times in sterile phosphate buffered saline (PBS), pH 7.2. Cyst surfaces were sterilized by 70% ethanol and vesicle fluid containing protoscoleces was separated from the metacestode tissue and host adventitia. The fertility of cysts was determined by the presence of free protoscoleces in cystic fluid by microscopic examination of a wet mount drop and characteristic muscular movements.

2.4. Preparation and culture of protoscoleces

Hydatid fluid containing protoscoleces were allowed to settle completely into 15 mL Falcon tubes without centrifugation and it was left to precipitate for an hour to obtain hydatid sand at room temperature. Protoscoleces thus obtained were washed in Hanks balanced salt solution (HBSS) and were maintained in a sterile preservative solution

RPMI-1640. Viability/vitality of the protoscoleces was assessed using the trypan blue exclusion technique, prior to any experiments. A 0.01 mL solution of pooled protoscoleces was transferred over a cavity slide and mixed with 0.01 mL of 0.1% aqueous trypan blue stain and was evaluated by low power microscopy after 5 min. Unstained protoscoleces were considered as viable while stained protoscoleces were considered as non-viable[33]. When 95% or more viable protoscoleces are present in the sediments, it considered to be appropriate for further experiments.

2.5. In-vitro scolicidal activity of extract

In this study, two concentrations of the *M. philippinensis* fruit extract (MPE1 and 2) 10 and 20 mg/mL of were examined for 10, 20, 30 and 60 min treatments. For preparation of *M. philippinensis* extract solution with 10 and 20 mg/mL concentration, 0.1 and 0.2 g of dried extract was dissolved in 10 mL of 0.9% phosphate buffer saline (PBS) at pH 7.3, respectively. Two milliliter of each concentration was placed in a test tube, and a drop of protoscoleces-rich sediment was added to the tube and mixed gently. The tube was then left at room temperature for 10, 20, 30 and 60 min. The supernatant of the solution was then removed with a pipette avoiding settled protoscoleces. Then 2 mL of 0.1% trypan blue stain was added to the remaining settled protoscoleces, mixed gently and incubated at 37 ℃. After incubation the supernatant was discarded, and washed with 0.9% PBS to remove the excess stain. The remaining settled protoscoleces were then smeared on a glass slide, covered with a cover glass and examined microscopically for viability. The percentages of dead protoscoleces were determined by counting protoscoleces. Protoscoleces dissolved in 0.9% PBS with no exposure to *M. philippinensis* extract were considered as control group, and treated with Praziquantel (1 μg/mL) was considered positive control in each experiment. The experiments were performed in triplicate.

2.6. Statistical analysis

The statistical package UNISTAT® was used to test for goodness of fit of the two concentrations towards scolicidal activity at different incubation time. The goodness of fit test was used to test significance between test and control groups. $P<0.05$ were considered to be significant.

3. Results

The fruit glandular hair extracts of *M. philippinensis* was shown significant scolicidal activity against *E. granulosus*, under *in-vitro* conditions with reference to the known standard drug Praziquantel (Figure 1). We have observed 97% to 99% mortality at concentration of 10 and 20 mg/mL in 60min treatments, while 93% mortality was observed with 20 mg/mL for only 10 min treatments (Table 1, Figure 2). Above 20 mg/ml concentration for up to 2 h shows 100% mortality, irrespective to the incubation after 2 h. Based on our preliminary *in-vitro* vitality/viability assessment of protoscoleces of hydatid cysts after treatment with extract of glandular hairs of fruits from *M. philippinensis*, indicate the significant loss in the viability, morphological tegumental alterations including disintegration of protoscoleces. The Figure 3 represent the preliminary viability tested using trypan blue exclusion test, protoscoleces at their initial phases in preservation medium were mostly invaginated, highly turgid and have rapid movements. All control cysts appeared turgid with no observable collapse of the germinal layer (Figure 3a). The treatment of these protoscoleces with

Table 1
Scolicidal effects of *M. phillipinensis* fruit extract on various exposure times.

Concentration	Exposure time (min)	Protoscoleces (Mean±SD)	Dead protoscoleces (Mean±SD)	Mortality rate of extract (%)
MPE1	10	456.00±21.10	363.00±6.97	79.60
(10 mg/mL)	20	728.00±25.20	564.00±31.00	77.47
	30	811.00±20.50	772.00±5.88	95.19
	60	508.00±62.00	494.00±14.00	97.24
MPE2	10	762.00±17.50	713.00±12.90	93.56
(20 mg/mL)	20	913.00±17.70	840.00±5.90	92.00
	30	372.00±7.40	367.00±7.48	98.65
	60	534.00±17.00	530.00±22.60	99.24
PZQ control*	10	370.00±13.70	351.00±7.78	94.86
(1 μg/mL)	20	618.00±16.90	604.00±12.70	97.73
	30	838.00±20.40	830.00±16.70	99.04
	60	598.00±22.30	596.00±9.74	~100.00
0.9% PBS**	10	930.00±26.20	7.66±1.69	11.70
	20	650.00±30.50	8.33±2.49	1.69
	30	475.00±42.10	16.00±5.09	5.02
	60	759.00±26.20	9.66±2.49	1.64

*PZQ- Praziquantel, **PBS- Phosphate buffer saline.

M. philippinensis fruit powder extract, provoked significant loss of motility and turgidity of cysts (Figure 3c), evagination of the protoscoleces were observed after treatment, however all protoscoleces were stained in trypan blue exclusion test, irrespective of evaginated and invaginated (Figure 3c). Very similar types of morphological changes were also observed with Praziquantel (Figure 3b), a known anti-cestodal drug. It was observed that trypan blue exclusion dye possibly have some reactions with the metabolites of the extracts and expresses as somewhat greenish yellow appearance rather than blue, once retained by the non-viable protoscoleces (Figure 3d). The tegumental vacuolization associated with the loss of turgidity of the protoscoleces were the most diagnostic feature that appeared after extract treatment (Figure 4a). Other morphological changes observed were loss of motility, loss of hooks (free hooks can be seen in the preservation medium), and damaged germinal layers (Figure 4b). Additionally the degenerative effect was also observed as loosening of the microtriches and hooks at the scolex region that causes the significant loss in the potential of protoscoleces to attach with host tissues, leading to decrease the infection vigor of the cysts (Figure 4c). It was also observed that the arranged scolex region of the cysts completely disintegrated (Figure 4d). The control live protoscoleces have unique diagnostic features like highly turgid soma and scolex regions, circularly arranged microtriches and hooks, uniform tegumental layer etc. (Figurer 5a). But after treatment with a control drug Praziquantel there are significant changes observed such as loss of turgidity especially with the soma region, and the formation of blebs causes tegumental damages (Figure 5b). Even detrimental changes observed when the protoscoleces were treated with 10 mg/mL of extract, the contraction of soma region, formation of blebs on the tegument and the disorganization of the rosteller region (Figure 5c), moreover with a high dose treatment 20 mg/mL causes tegumental vacuolization leading to the disruption of the tegumental layer and complete collapse of the protoscoleces (Figure 5d).

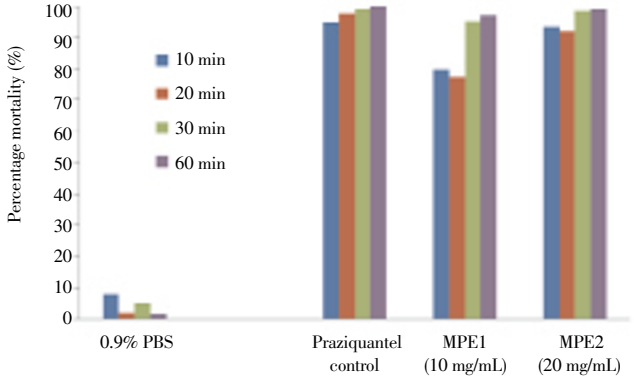

Figure 1. Comparative scolicidal activity of fruit extract with reference to authentic standard drug praziquantel. The two concentrations MPE1 (10 mg/mL) and MPE2 (20 mg/mL) showed promising scolicidal activity.

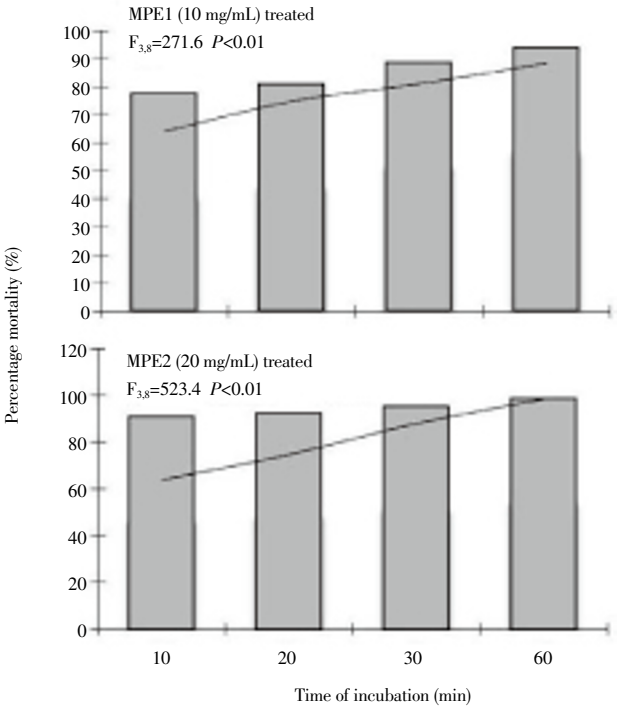

Figure 2. High rate of mortality was observed (97% to 99% respectively) at concentration of 10 and 20 mg/mL for 60 min treatments (P<0.001), while 93% mortality was observed with 20 mg/mL for only 10 min treatments was quite encouraging.

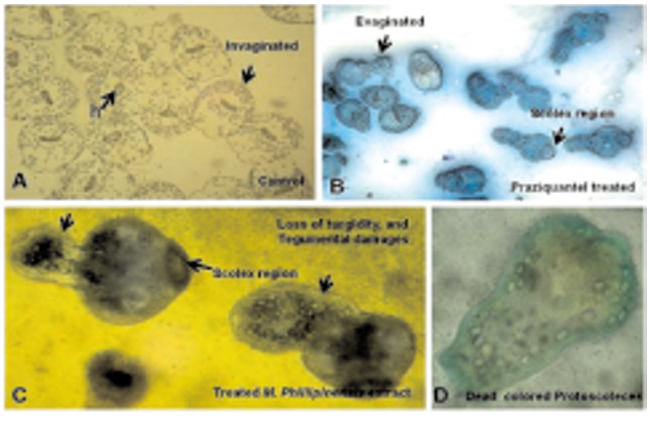

Figure 3. Effect of glandular hair extract on the viability of protoscoleces of *E. granulosus*. (a) Control unstained invaginated protoscoleces of hydatid cyst by wet mount drop (×10). (b) Praziquantel treated (1 μg/mL) positive control hydatid cysts loss their viability and stained blue (trypan blue exclusion). (c, d) Dead stained protoscoleces after treatment with extract of *M. philippinensis* fruit glands. Both the drug control and the extracts showed distinct morphological distortions and degenerative effects such as loss of motility, shedding of hooks and calcareous corpuscles etc.

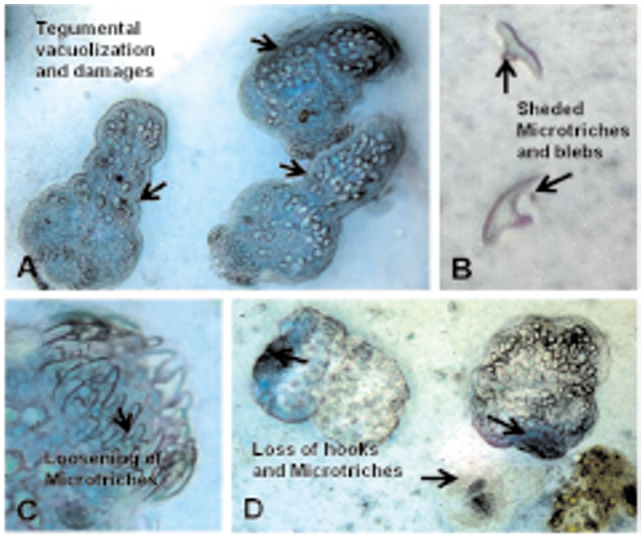

Figure 4. Scolicidal effect of fruit glandular hair extract of *M. philippinensis*. (a) Complete loss of viability of evaginated protoscoleces with an exposure of extract (20 mg/mL) for up to 4–5 h. (b, c) it was observed that loosening of microtriches and hooks and at higher incubation the hooks were leave in to the preservative medium. (d) Disintegration of the calcareous corpuscles and hooks were observed along with loss of turgidity, collapse of cysts after treatment with extract.

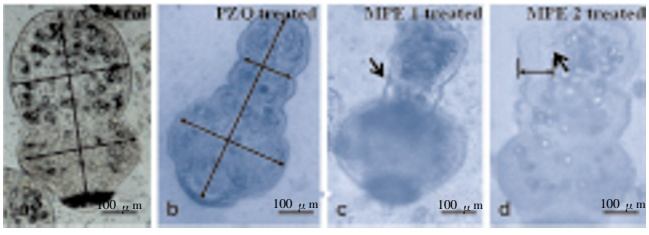

Figure 5. Comparative evaluations of viability assessment and distinguishing morphological changes associated with the fatality of protoscoleces of hydatid cysts. (a) Control live unstained evaginated protoscoleces showing distinct morphological features like cyst turgidity, well defined germinal layers etc. (b) praziquantel (1 μg/mL) treated cyst showed gradual loss of turgidity, and associated damage to the germinal layer. (c) The protoscoleces treated with the extract (MPE1) were also having similar effect as those of praziquantel (d) but at higher dose (MPE 2) the germinal layer disintegrate and causes complete loss of viability of cysts.

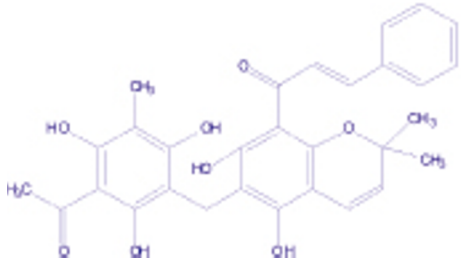

Figure 6. Molecular structure of rottlerin, the main constituent which is partially responsible for scolicidal activity.

4. Discussion

Cystic echinococcosis is omnipresent zoonotic disease, and its treatment is as complicated as its life cycle itself. Unfortunately when symptoms do occur, they are mostly from the cysts in confined spaces like hepatomegaly, jaundice, and palpable peritoneal mass. Under the condition the only diagnosis possible is to surgical removal of cysts, but often there is associated risk of intra-operative spillage of protoscoleces rich fluid leading to reoccurrence of secondary echinococcosis[13-15]. Thus various synthetic anthelmintics are used to prevent the reoccurrence in surgical cases in addition to benzimidazole carbamates as viable options of inoperable symptomatic cysts. However there are serious health risks in human being and are evident with many synthetic drugs like albendazole, nitazoxanide, flubendazole etc. Besides chemicals other alternative scolicidal agents like silver nitrate, hypertonic saline etc. have been reported significant activity but can't be considered as an ideal scolicidal agent since they have too many adverse effects with them[33-35]. Although most of the synthetic drugs have 100% effectiveness but also serious side effects associated with them like Cetrimide (0.5%-1.0%) causes methemoglobinemia, peritonitis, convulsions and sometimes coma[36], albendazole sulphoxide (100 μg/mL) elevate liver enzyme functions and increases solubility[37], nitazoxanide (10 μg/mL) causes cellular autolysis[38], flubendazole (10 μg/mL) causes dyspepsia and sleepiness[39], hypertonic Saline (20%) causes hypernatremia, intracraniel bleeding and myelinolysis[40], silver nitrate (20%) is toxic on absorption through cysts wall, pleura and peritoneal membrane[15,41]. Natural scolicidal agents will be an alternative that can be safe with no adverse associated effects[8,13]. With this hypothesis a large number of medicinal plants having their ethanobotanical history as anthelmintics are used to screen for their scolicidal potential especially against *E. granulosus*, for example *Zataria multiflora*[42], *Satureza khuzistanica*[43], *Salvia officinalis* and *Thymus vulgaris*[44], *Alium sativum*[45], *Dendrosicyos socotrana* and *Jatropha unicostata*[46], *Artemisia annua*[47], *Trachyspermum ammi*[48], Mentha piperita and *M. pulegium*[49]. Additionally it is important to evaluate the dose dependent activity and the sustainability of the effect in light of this fact that many species are becoming resistant. For the first time we are reporting the fruit glandular hair extract of *M. philippinensis* for its scolicidal activity against hydatid cysts *E. granulosus*. Although this plant has been known for its medicinal properties, but most popular activity of this plant is the anti-parasitic specially anti-helminthic reported in the traditional system of medicine[31-32,50]. In our investigations we have established the scolicidal potential of the glandular hair extract of the fruits of this plant, with reference to the standard drug Praziquantel. Although a high dose of extract (20 mg/mL) shows significant scolicidal effect with reference to Praziquantel, but it need to remember that being an natural alternative is has very safe for its

application as scolicidal agent. This plant is known for its chemical diversity as well, since it contains different group of natural compounds mainly di and triterpenoids, steroids, flavonoids, coumarinolignoids, phloroglucinol derivatives and benzopyrans. Among the known compounds rottlerin (5,7-dihydroxy-2,2-dimethyl-6-(2,4,6-trihydroxy-3-methyl-5-acetylbenzyl)-8-cinnamoyl-1,2-chromine), also called mallotoxin, is one of the major constituents of this plant exhibiting various pharmacological activities including anti-helminthic (Figure 6), but latter studies indicate that rottlerin alone is not as effective as together with other fractions of extracts[50]. This clearly indicates that there are some other constituents that may be present in the extract responsible for the anthelmintic activity. Although many other plants are known to have activity against *E. granulosus*[42-49], it was speculated that some major constituents belong to these medicinal plants have potential role in anti-helminthic activity they shown. Additionally these naturally obtained protoscolicidal agents are safe, and have no adverse activities. Thus they provide excellent platform for the screening, isolation and characterization of new generation protoscolicidal agents that are closer towards ideal anti-heminthic agent.

In conclusion, we described herein the potential *in-vitro* scolicidal activity of *M. philippinensis* fruit glandular hair extract for the first time. As compared with the standard drug Praziquantel our extract has significant scolicidal activity, with almost no associated side effects as being natural alternative. Our results shows that there might be some new natural compound that might be responsible for this scolicidal activity, thus currently we are thoroughly investigating into the chemical bioassay guided fractionation of the fruit extract for identification of these active compounds.

Conflict of interest statement

We declare that we have no conflict of interest.

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