Osmoregulatory and tegumental ultrastructural damages to protoscoleces of hydatid cysts *Echinococcus granulosus* induced by fungal endophytes

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Abstract Characteristic ultrastructural changes were observed when protoscoleces of hydatid cysts *Echinococcus granulosus* was treated with extract of endophytic fungi *Eupenicillium* and *Chaetomium* sp. isolated from *Azadirachta indica* and *Piper longum* plants respectively. A sharp decrease in viability of protoscoleces was observed after 6 h of incubation with fungal extracts. The ultrastructural changes included rostellum disorganization, loss of hooks and shedding of the microtriches of scolex region. The formation of digitiform projections on tegument layer which, increased in size as prolong incubation with extract and get burst, leading to an osmoregulatory damage into tegumental layers of parasite. This osmoregulatory damages caused the loss of turgidity due to leakage of cell contents, which might be the major cause of the mortality in treated parasites. It is remarkable, since very similar type of ultrastructural changes were observed with some pyrazinoisoquinoline derivatives, as praziquantel. Our initial results indicate that extract of endophytic *Eupenicillium*

and *Chaetomium* spp. are having significant anti-cestodal activity and have selective activity on tegument layer. Further chemical prospection is required through rigorous bioassay guided fractionation coupled with robust high resolution mass spectrometric analysis to get final stereostructures responsible for the parasitocidal activity. This initial strain selection outcome will serve a platform for isolation and characterization of new drug lead that can be useful in tailoring novel, safe and effective anthelmintics.

Keywords Endophytic fungi · *Eupenicillium* sp · Hydatid cysts · SEM · Anti-cestodal activity

Introduction

Endophytic fungi are ubiquitously present in the internal tissues of higher plants usually with no symptom (Porrás-Alfaro and Bayman 2011; Verma et al. 2009a). They are highly diverse and relatively underexplored group of microorganisms associated with different plants. Endophytic fungi now have started attracting scientists as they are good source of bioactive secondary metabolites with an array of biological activities. They could sometimes produce bioactive compounds analogous to that of their host. Many host specific molecules have been discovered so far such as taxol, piperine, azadirachtin, hypericin etc. (Verma et al. 2011; Kusari et al. 2012; Zhou et al. 2011). However, the mechanism is still not known, it was assumed that the related genes which control the synthesis of bioactive compounds might be transferred between the endophytic fungi and host during long time of symbiosis. Although the HGT (horizontal gene transfer) theory advocates the transfer of genetic elements between host and endophytes, it is still controversial. These endophytic fungi can easily

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be harvested for production of novel bioactive molecules against a plethora of ailments. Bio-prospection of microbial endophytes seems to be the need of the time. The substances produced by endophytic fungi originate from different biosynthetic pathways which includes isoprenoid, polyketide, amino acids etc. These molecules have diverse structural groups such as terpenoids, steroids, xanthenes, quinones, phenols, isocoumarins, benzopyranones, tetralones, cytochalasins and enniatins (Tan and Zou 2001; Guo et al. 2008; Guantilaka 2006; Verma et al. 2009b). These compounds have promising activities like antibiotics, antioxidant, immunomodulators, anti-cancer and anti-parasitic compounds (Chandra 2012; Verma et al. 2009a).

We are particularly interested in analyzing endophytes for their potential against major neglected tropical diseases (NTDs). Despite increasing interest in endophytic research, very few attempts have been made to find out anti-parasitic agents from fungal endophytes. Although, chemotherapy is one of the non invasive way available for symptomatic cysts but there are many severe side effects (Table 1) of

these synthetic drugs (McManus et al. 2003), but concern remains high for asymptomatic cysts. Increasing side effects and appearance of resistance to the synthetic anthelmintics are added further complications in cure of NTDs. Thus there is an urgent call for exploration of natural alternatives from medicinal plants and their associated endophytes utilizing traditional knowledge base available in Ayurvedic medical treatment systems. There are several reports on protoscolicidal activity of crude products of different medicinal plants (Table 2) against *Echinococcus granulosus*, but no extensive survey has been done for the associated endophytic microbes for the same. Few of the recent reports provides insight about the role of endophytic microbes and their bioactive prototype molecules for developing drugs to combat various NTDs (Rosa et al. 2011), but there is need for rapid screening and strain development program against NTDs.

In the present study, therefore, we have investigated battery of endophytic fungal strains isolated from *Azadirachta indica* A. Juss., and *Piper longum* L. plants and

Table 1 Some synthetic drugs available for treatment of cystis echinococcosis and their associated side effects

Scolicidal agents	Treatment time	Effectiveness	Side-effects	References
Cetrimide (0.5–1 %)	10 min	100 %	Methemoglobinemia peritonitis, convulsions and coma	Puryan et al. (2005)
Albendazole sulphoxide (100 µg/ml)	15 min	100 %	Elevate liver enzyme function hard solubility	Adas et al. (2009)
Nitazoxanide (10 µg/ml)	24 h	50 %	Cellular autolysis	Walker et al. (2004)
Flubendazole (10 µg/ml)	72 h	100 %	Dyspepsia and sleepiness	Elissondo et al. (2006)
Hypertonic Saline (20 %)	15 min	100 %	Hypernatremia Intracranial bleeding Myelinolysis	Erzurumlu et al. (1998)
Silver nitrate (20 %)	20 min	100 %	Toxic on absorption Through cysts wall, pleura and Peritoneal membrane	Caglar et al. (2008)

Table 2 Some herbal plant extracts having scolicidal activity against *Echinococcus granulosus* and the principal compounds identified

Plants/herbs	Principal components	Effective dose	References
<i>Zataria multiflora</i>	Carvacrol and thymol	10–25 mg/ml	Moazeni and Roozitalab (2012)
<i>Satureja khuzistanica</i>	Limonene, terpinole thymol, ocimene, β-cymene	3–5 mg/ml	Moazeni et al. (2012)
<i>Salvia officinalis</i>	Thymol and menthol	50 mg/ml	Yones et al. (2011)
<i>Thymus vulgaris</i>			
<i>Alium sativum</i>	Miscellaneous	100 mg/ml	Sadjjadi et al. (2008)
<i>Dendrosicyos socotrana</i>	Cucurbitacins sterol, ketosteroids and terpenoids	5,000	Barzinji et al. (2009)
<i>Jatropha unicostata</i>		1,000 µg/ml	
<i>Artemisia annua</i>	Artemisinin, artesunate	10–40 µM	Spicher et al. (2008)
<i>Trachyspermum ammi</i>	Thymol, γ-terpinene, p-cymene	10 mg/ml	Moazeni et al. (2012)
<i>Mentha piperita</i>	Methone, piperitone myrcene, menthofuran	10, 5, 1 µg/ml	Maggiore et al. (2011)
<i>Mentha pulegium</i>			

characterized the strains of endophytic *Eupenicillium* sp. and *Chaetomium* sp. with significant parasitocidal activities against hydatid cysts *E. granulosus*. This is a first report of its kind that deals with thorough screening of fungal endophytes for anti-cestodal potential against hydatid cysts.

Materials and methods

Plant samples

Random samples of *A. indica* A. Juss. and *P. longum* L. were collected from across the campus of Banaras Hindu University, Varanasi (25.5° N 82.9° E). Fresh plant samples of healthy and symptomless leaves, stems and roots were collected. The cut ends were wrapped with parafilm™ before they were placed in sterile polythene bags. The samples were then sent for further isolation procedures in the Laboratory for Gastrointestinal Infections and Molecular Diagnosis, Department of Microbiology, Banaras Hindu University, India.

Isolation and preliminary screening of endophytes

Surface treatment was performed by sequential dipping into 70 % ethanol for 1 min, 5 % NaOCl for 5 min, 96 % ethanol for 0.5 min, followed by three rinses in sterile distilled water as previously established method (Verma et al. 2007, 2009a, 2011). All tissues were then placed onto separate petri dishes (60 mm) containing potato dextrose agar (PDA, Himedia, Mumbai) supplemented with 250 mg l⁻¹ oxytetracycline hydrochloride (Terramycin, Pfizer) for isolating endophytic fungi. All petri dishes were sealed with sterile Parafilm™ to protect them from any contamination during repeated handling while examining endophytes, and from desiccation. The plates were incubated at 26 ± 2 °C in enclosed translucent white cover plastic boxes in a BOD cum humidity incubator for ~25 days. Fungi that grew from the tissue fragments were subcultured onto fresh PDA plates. After isolation of endophytic microbes from the selected host plants, a thorough preliminary screening of their anti-cestodal activity was performed with about 43 strains belonging to 13 representative genera of fungi, along with 11 sterile (*mycelia sterilia*) unidentified strains. Initially all these strains were liquid cultured on shake flask PD broth (Himedia, India) at 200 rpm for ~21 days. After fermentation the liquid spent were extracted with equal volume of ethyl acetate (EtOAc) twice and condensed *in-vacuo* in a rotary evaporator. The dried extracts were re-dissolved in 10 ml of normal saline and used for anti-cestodal activity against hydatid cysts of *E. granulosus*.

Collection of cysts

Hydatid cysts of *E. granulosus* were collected aseptically from infected liver and lungs of cattle slaughtered in an abattoir located in Varanasi City, India. The intact cysts were immediately placed in an icebox and transported to Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi India. Cysts were washed 3 times in sterile phosphate buffered saline (0.9 % PBS, pH 7.2) and surface was disinfected with 70 % ethanol to remove any surface contaminations. The vesicle fluid containing protoscoleces was separated from the meta-cestode tissue and host adventitia by using sterile syringe. After removing vesicle fluid the cyst was cut open and entire germinal layer was removed. Repeated washings provide protoscoleces rich fluid adheres to the germinal layer.

Preparation and culture of protoscoleces

Hydatid fluid containing protoscoleces were allowed to settle completely into 15 ml falcon tubes without centrifugation at room temperature. Protoscoleces thus obtained were washed in Hanks balanced salt solution (HBSS) and were maintained in a sterile preservative solution RPMI-1640. This preservative medium was not amended by any antibiotic or antifungal drugs as this might interfere with the survival of the protoscoleces. Viability of the protoscoleces were assessed using the eosin red exclusion technique, prior to any experiments, briefly 0.01 ml solution of pooled protoscoleces was transferred over a cavity slide and mixed with 0.01 ml of 0.1 % aq. eosin red stain and was observed with light microscope (Nikon eclipse E100 research microscope) after 30 min incubation at 37 °C. Unstained protoscoleces were considered as viable while stained protoscoleces were considered as non-viable (Smyth and Barrett 1980). When the percentage of viable protoscoleces in the sediment was 95 % or more, they were considered to be appropriate for further experiments.

Anti-cestodal activity

In this study partially purified extracts of nine randomly selected endophytic fungi were evaluated for parasitocidal activity against protoscoleces of *E. granulosus*. Extracts of selected Endophytes in the volume of 5 ml at the concentration of 100 mg/ml was placed in a test tube, and 100 µl protoscoleces-rich sediment was added to the tube and mixed gently. The tube was then left at room temperature for 8 h at 37 °C. The supernatant of the solution was then pipetted avoiding settled protoscoleces. Then 2 ml of 0.1 % aq. eosin red stain was added to the remaining settled protoscoleces, mixed gently and incubated at 37 °C for

10 min. After incubation the supernatant was discarded, and protoscoleces sand was washed with 0.9 % PBS several times to remove the excess stain. The remaining settled protoscoleces were then placed on a glass cavity slide and examined microscopically for viability. The percentages of stained dead protoscoleces were determined by counting non-viable unstained protoscoleces. Protoscoleces suspended in 0.9 % PBS with no exposure to any extract were considered as untreated controls, and others treated with praziquantel® (1 µg/ml) were considered positive control in each experiment. The experiments were performed in triplicate to ensure the reproducibility.

Study of ultrastructural changes by SEM

The treated protoscoleces were subjected to scanning electron microscopy (SEM). For SEM analysis, a drop containing 100 µl of treated protoscoleces were fixed in 2.5 % glutaraldehyde for overnight. The fixed protoscoleces were then washed again by 0.1 M sodium cacodylate buffer pH 7.4 at 4 °C and left for 3 h. The settled protoscoleces were then dehydrated by sequential dipping into alcohol from 30 to 90 % followed by a dip into absolute alcohol. The dehydrated protoscoleces were sputter-coated with gold under sputtering device and observed under a Zeiss EVO-LS-10 scanning electron microscope. The images obtained were processed using imaging software SmartSEM® VS10.

Results

A number of endophytic fungi were isolated and documented from the selected hosts, among which some dominant strains were *Pestalotiopsis*, *Phomopsis*, *Nigrospora*, *Eupenicillium*, *Chaetomium*, *Periconia*, *Fusarium*, *Colletotrichum* etc. In our

preliminary effort, we concentrated to find out fungal strains having promising activity against hydatid cysts. A total of about 43 strains belonging to 13 representative genera of fungi, along with 11 sterile (*mycelia sterilia*) unidentified strains were screened for their anti-cestodal activity. Nine out of 43 strain extracts were found to have activity against hydatid cysts (Table 3). Significant reduction in the viability of protoscoleces was observed with all nine tested strains as compared to the PBS control, the extract treated protoscoleces lost their viability and turgidity within 5–6 h in all cases. Most significant loss of viability was observed with *Eupenicillium* sp. isolated from Neem plant (*A. indica* A. Juss), where the 70–85 % viability loss occurred within first 6 h. Another strain of *Chaetomium* sp. from pepper plant also had significant activity against protoscoleces, where highest viability loss was observed in 7 h. Figure 1 represent the preliminary viability test using eosin red exclusion test, protoscoleces at their initial phases in preservation medium were mostly invaginated, highly turgid and have rapid characteristic muscular movement. All control cysts appeared turgid with no observable collapse of the germinal layer (Fig. 1a), while the dead protoscoleces after treatment with *Eupenicillium* and *Chaetomium* sp. extract shows more than 70–85 % viability loss during the same incubation period (Fig. 1b). The above mentioned two strains were found to be the most active strains as compared to PBS control and standard scolicidal drug praziquantel (Fig. 2). These strains show significant loss in viability of protoscoleces and can be a strong candidate for chemo-prospecting. Based on these preliminary results, we focused on to ultrastructural damage by scanning electron microscopy (SEM). Before SEM analysis, we performed light microscopy and observed remarkable tegumental damages associated with significant anatomical alterations. The extract of endophytic *Eupenicillium* sp. caused distinguishing balloon like

Table 3 Selected endophytic strains from the two hosts that have significant activity against protoscoleces of *Echinococcus granulosus*

Plant/Host	Fungal endophytes	Anti-parasitic activity (% viability in hours)							
		1	2	3	4	5	6	7	8
<i>Azadirachta indica</i> A. Juss. (Neem or Indian lilac)	<i>Colletotrichum</i> sp.	97.8	83.4	79.1	64.7	55.6	39.8	20.4	10.3
	<i>Phomopsis</i> sp.	98.2	92.0	84.4	69.3	54.1	39.6	19.5	12.9
	<i>Nigrospora</i> sp.	98.6	91.1	84.5	68.4	56.3	39.6	22.8	9.6
	<i>Eupenicillium</i> sp.	85.4	61.6	40.5	28.3	15.0	0.0	0.0	0.0
	<i>Fusarium</i> sp.	97.6	89.2	77.4	69.1	54.2	21.9	9.7	0.0
	<i>Chaetomium</i> sp.	96.8	88.4	73.6	61.8	50.2	21.6	7.9	0.0
<i>Piper longum</i> L. (Pepper plant)	<i>Periconia</i> sp.	95.5	81.8	74.7	66.3	30.3	23.5	10.1	0.0
	<i>Chaetomium</i> sp.	97.4	76.2	55.6	38.6	23.0	9.1	0.0	0.0
	<i>Pestalotiopsis</i> sp.	96.3	86.8	74.2	69.9	49.4	24.8	5.1	0.0
PBS control		99.6	98.5	99.5	99.1	98.3	98.0	97.3	96.6
Praziquantel (1 µg/ml)		76.4	35.1	13.5	6.3	1.8	0.0	0.0	0.0

Fig. 1 The treated protoscoleces with extract of *Eupenicillium* sp. under light microscope **a** the live invaginated protoscoleces remains unstained without treatment. **b** The dead stained protoscoleces after 4 h incubation with extract

Fig. 2 The survival curve of protoscoleces after treatment with extract of endophytic *Eupenicillium* and *Chaetomium* sp. a sharp decrease in the viability can be observed as compared to control

(digitiform) projections appeared from the tegumental layers leading to loss of viability of protoscoleces (Fig. 3), while none among the other strains display this type of activity, however they also showed remarkable tegumental damage. Apart from these characteristic changes, the other associated degenerative changes observed were rostellor disorganization, loss of hooks and shedding of microtriches

Fig. 3 The tegumental digitiform extensions observed at the outer tegument of protoscoleces treated by fungal (*Eupenicillium*) extract

of the scolex region (SR). To get a better understanding of the extent of damage occur due to the treatment of fungal extract, we perform the scanning electron microscopy. The SEM analysis of extract treated protoscoleces showed remarkable damages to the scolex region (SR), including severe contractions in soma region and disorganization of the rostellor cone when compared with control. A complete degeneration of the soma region was observed by loss of turgidity and the local leakage of the cellular contents due to severe tegumental layer damage (Fig. 4a). The rostellor region (RR), which is responsible for the attachment of parasite in host intestinal wall, was also damaged due to shedding and loosening of hooks (H) and microtriches (Fig. 4b). Figure 4c represent the level of tegumental damage (marked by arrow), besides complete loss of suckers in soma region, this is significant. The extract treatment causes severe damage to the rostellor cone in particular since many hooks were shed off from the treated parasite, Fig. 4d–f (see inset) represent the unique hook structure of *E. granulosus*, along with the projection of several bleb and microtriches at the soma region.

Discussion

Many traditional systems of medicines have several crude formulations having promising effects on these parasites, but unfortunately none of these formulations has been characterized till date. Thus there is an urgent need for chemoprospection of new herbal remedies of plant and microbial origin for safe and sustainable anti-cestodal compounds. Looking to the promising scope of the fungal endophytes as new horizon to discover new chemistry, we have for the first time evaluated fungal endophytes from neem and pepper plants (*A. indica* A. Juss., and *P. longum* L. plants respectively) for anti-cestodal metabolites. An endophytic *Eupenicillium* sp. has been found potentially active against *E. granulosus* as compared with other tested strains. The associated anatomical and ultrastructural changes

Fig. 4 The ultrastructural damages observed with scanning electron microscopy when treated with endophytic *Eupenicillium* extract from neem tree. **a** The protoscoleces showing complete disorganization of *SR* scolex, and *BR* body region, **b** the disorganization of *H* hooks at the *RR* rostellum region, **c** the single evaginated protoscoleces showing damage to the *SC* suckers at *SR* sucker region, the damage at the surface teguments are also observed marked by *arrow*, **d** the loss of

H hooks and microtriches marked by *arrow*, was also observed at *RR* rostellum region, **e** the rostellum cone disorganization is prominently visible along with significant damage in *SC* Scolex region and suckers, **f** the bursting of the soma region marked by the *arrow* indicate complete loss of tegumental integrity and thus a disturbed osmoregulatory stress leading to loss of viability of protoscoleces

observed by the treatment with extract of *Eupenicillium* sp. shows contraction in soma region, abnormality in sucker areas, formation of blebs on the tegument, rostellum cone disorganization, shedding of hooks and microtriches, very similar to synthetic drugs such as praziquantel (Rodríguez-Caabeiro et al. 1989; Urrea-París et al. 1999, 2000),

benzimidazole (Rodríguez-Caabeiro et al. 1989; Pérez-Serrano et al. 2001), and ivermectin (Casado et al. 1989). Rostellum cone disorganization along with deformities in sucker region and destruction of microtriches are the factors which might interfere with the establishment of parasite with its host for nutrient absorption and defense (Smyth et al.

1966). In some protoscoleces, the presence of digitiform tegumental extensions appeared on the tegument of the soma region (Fig. 3). These extensions after further incubation were ruptured and cause severe morphological alterations leading to the loss in viability. There is possibility that these extensions enlarges with due course of time and burst out leading to the disturbance in osmoregulatory system of protoscoleces. This indicates that the bioactive fraction in our crude extract might have some compound that has activity like pyrazinoisoquinoline derivative and selectively active on tegumental surface. In few of the recent studies, it was observed that praziquantel have substantial effect when used in combination of other scolicalidal agents such as albendazole (Cobo et al. 1998). Therefore, we speculate that our extract together with other synthetic drug/s might be more effective. Additionally, some large vacuoles appeared in the distal cytoplasm of the soma region that caused major leakage of the cytoplasm contents (Fig. 4c, f). Taken together the present study is an attempt to screen out novel natural strains of endophytic fungi that have potential activity against hydatid cysts and can be developed as new alternative therapy for helminthes parasites.

The initial results obtained are quite encouraging since the extract of endophytic *Eupenicillium* sp. and *Chaetomium* sp. showed activity very close to some synthetic scolicalidal drugs and can be further studied for its complete chemical characterization leading to the identification and stereo specific activities. We are currently working with the crude extract for purification and characterization of bioactive fractions of endophytic *Eupenicillium* and *Chaetomium* sp., and expecting some novel chemistry that exerts these activities in its purified form. Additionally more exhaustive evaluation of our fungal extract chemotherapeutic efficiency will be investigated in vivo conditions using animal models in future study.

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