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In vitro evaluation of antiparasitic activity of oyster mushroom (*Pleurotus ostreatus*) protein hydrolysates against *Haemonchus contortus* larvae

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Abstract
Gastrointestinal parasitism has been the major cause for economic loss in small ruminant production. *Haemonchus contortus*, the stomach worm of sheep and goats has been incriminated for severe economic loss due to anemia, deceased growth rate, infertility and death in severe infections. Anthelmintic drugs have been used with variable rate of success but the treatment failure occurred due to anthelmintic resistance. It is the need of the hour to search for an alternate, sustainable and economical stategy for the treatment and control of *H. contortus.* Varios plants have been screened for anthelmintic potential using *in vitro* and *in vivo* models. The oyster mushroom, *Pleurotus ostreatus* has been explored for its various bioactive compounds proven for antitumour, antioxiadant and immunomodulatory properties. The present study explored the *in vitro* anthelmintic potential of mushroom enzyme hydrolysates on the *H. contortus* larval development. The mushroom protein hydrolysates (MPH) were prepared from trypsin, papain and pepsin. The MPHs were used to evaluate their *in vitro* anthelmintic activity using larval development assay. The results revealed that papain mushroom protein hydrolysate showed 100 percent mortality of *H. contortus* larvae compared to pepsin and trypsin mushroom hydrolysates. It is concluded that the oyster mushroom can be the potential alternate to anthelmintic drugs for the control of *H. contortus*.

Keywords: *Pleurotus ostreatus*, *Haemonchus contortus*, *in vitro* Antiparasitic activity, anthelmintic resistance, larval development assay

Introduction
The abomasal nematode parasite of small ruminants, *Haemonchus contortus* is an economically important nematode causing loss to the farmers due to anemia, death, weight loss and decreased fertility and fecundity. The anthelmintic drugs have been used for treating the clinical parasitism and also for prophylaxis. These practices resulted in increasing resistance to current anthelmintic drugs besides the increased residues in meat and high cost of production [1, 2]. This has encouraged use of alternate strategies and parasite control methods which ultimately lead to enhanced productivity with a minimum consumer and environment associated problems.

The selection and combination of strategies for an effective management program for *H. contortus* including vaccines, bioactive forages, pasture/grazing management, behavioural management, natural immunity, FAMACHA, *Refugia* and strategic drenching, mineral/vitamin supplementation, copper oxide wire particles, breeding and selection/selecting resistant and resilient individuals, biological control and anthelmintic drugs according to the unique requirement of individual farm have been attempted [1].

The alternate and sustainable methods of controlling this nematode with extracts of medicinal plants are being tested as an alternative strategy to control *H. contortus* in the recent past. Various plants species naturally selected by sheep while grazing were assessed *in vitro* for activity against *H. contortus* and they have shown variable protection levels against *H. contortus* [3]. Mushroom probiotic-based study on gastrointestinal parasites has also been attempted in the recent past with encouraging results [4].

Pleurotus ostreatus is widely cultivated around the world and is reported to contain bioactive compounds that can protect health due to their antioxidant properties [5, 6, 7, 8], immune-nutritional recovery [9], antitumor, anti-inflammatory and cytotoxic effects [10, 11, 12]. Most of the previous works were relative to the nutritional evaluation for mushroom proteins, and few

current studies focus on the bioactive natural component of low molecular weight peptides with multi-action bioactivity hydrolyzed by enzymes from the *P. ostreatus* proteins or other edible mushrooms. The present study has been carried out to evaluate the *in vitro* antiparasitic effect of Oyster mushroom protein hydrolysates on the larvae of the nematode parasite, *H. contortus*.

Materials and Methods

Collection of oyster mushroom

Pleurotus ostreatus (Oyster Mushroom) was purchased from EFGC Biological farms, Chennai, Tamil Nadu, India.

Isolation of total protein from Oyster mushroom

The oyster mushroom were sliced, crushed and ground to make a homogenate with posphate buffer saline solution (pH 7.6) and the contents were transformed into a centrifuge tubes and centrifuged at 8000 rpm for 20 minutes. The supernantant solution was aliquated in tubes and stored at 4 ºC. Total protein isolated from Oyster mushroom was estimated by Biuret method using bovine serum albumin as a standard [13].

Preparation of mushroom protein hydrolysates (MPHs)

Protein isolated from Oyster mushroom was hydrolysed by using Trypsin, Pepsin and papain. Enzyme and substrate were used at a ratio of 1:5. Hydrolysis was carried out at 37 ºC for 4 hours at pH 7.0 for trypsin and Papain and at pH 2.0 for pepsin [14]. This mushroom protein hydrolysate was used for anti-parasitic activity studies.

Haemonchus contortus larvae culture

Abomasum along with its contents were collected from sheep slaughtered at Chennai abattoir and transported to the laboratory. Live *H. contortus* worms were collected from stomach contents and were washed thrice in tap water. Female worms were separated based on morphology and kept in normal saline and incubated at 37 ºC for two hours [3]. Eggs laid by the worms were collected and mixed with the sterilized dung material. Then it was filled in a container till half of its capacity and the mouth of the container was covered with muslin cloth and kept in dark place for five days. On sixth day muslin cloth was removed and the container was filled with normal saline and kept upside down in a petri dish in slanting position. After one hour, the fluid collected in the petridish from the container was examined for the presence of L3 larvae of *H. contortus* [15]. Collected L3 larvae were made in a dilution of 30 larvae/30 µl.

In vitro evaluation of antiparasitic effects of enzyme hydrolysates of Oyster mushroom

Trypsin, pepsin and papain enzyme hydrolysates of Oyster mushroom protein were added at 2ml/well in six well sterile plates in duplicates at three concentrations *viz.* 10 mg/ml, 25 mg/ml and 50 mg/ml. In each well 30 µl normal saline containing 30 numbers of *H. contortus* larvae was added and kept under room temperature and the larval motility was examined at hourly intervals for 48 hours [16].

Results

In pepsin enzyme hydrolysate of Oyster mushroom after one hour of incubation, there was 10% larvae with normal motility and 80% (24/30) larvae with sluggish motility was noticed. Whereas, 10 % of larvae were not affected and they did not showing active motility. This pattern was observed in all three concentrations of larvae. There was no change in the observations after 2, 4 and 12 hours of incubation in 10 mg/ml and 25 mg/ml concentrations (Fig.1). All larvae were found non motile and dead at 24 hr of incubation at 25 mg/ml and 50 mg/ml concentrations.

In Trypsin enzyme hydrolysate, 99% (29/30) of larvae were immotile after one hour of incubation whereas, 1% larvae were showing active motility. After two hours of incubation 20% larvae were showing sluggish motility, remaining 80% larvae were nonmotile. All larvae were non motile and dead 12 hours after incubation (Fig. 2). At 24 hr observation revealed 100 per cent lrval mortality in all the three concentrations.

Papin enzyme hydrolysate showed very good antiparasitic activity against the larvae compared to Pepsin and Trypsin mushroom protein hydrolysates. In papain hydrolysate, 100 % larval mortality was observed after one hour of incubation (Fig. 3). It persisted up to 24 hours of incubation. Same observations were recorded in all the three papain mushroom protein hyrolysate concentrations.

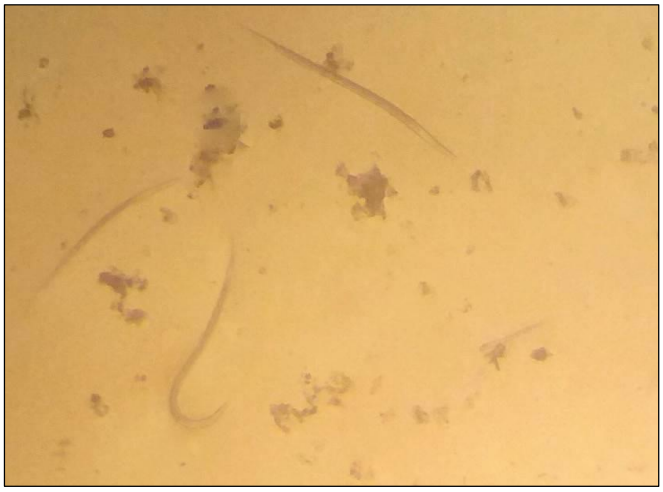

Fig 1: *H. contortus* larvae showing 20% motility in Pepsin Mushroom protein hydrolysate

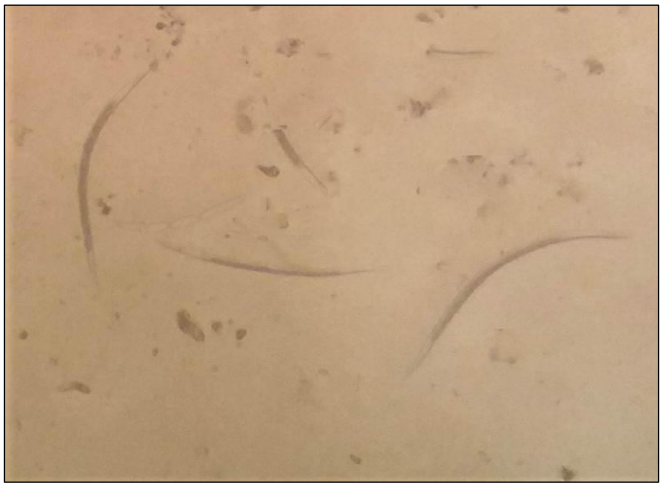

Fig 2: *H. contortus* larvae showing sluggish motility in Trypsin Mushroom protein hydrolysate

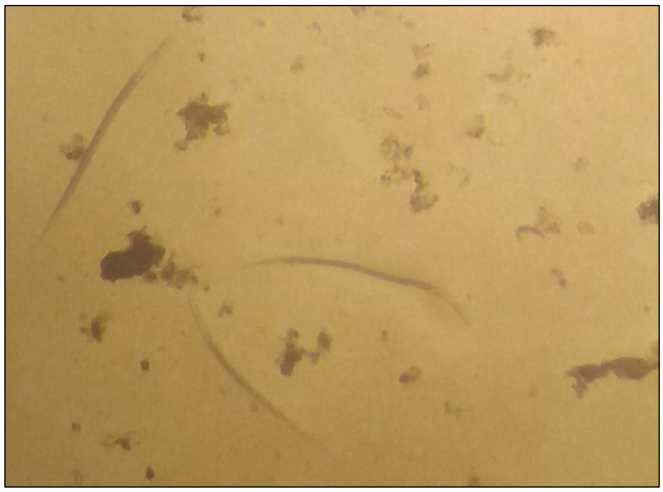

Fig 3: *H. contortus* larvae showing 100% motility in Papain Mushroom protein hydrolysate

Discussion

The research on edible mushrooms have been increasing in the recent past for its beneficial effects such as antioxidant, immunmodulatory, anticancer and antiparasitic effects. The *P. ostreatus* has been evaluated for antinematodal effects against the parasites of palnts and animals.

Fibrinolytic protease activity was detected from a crude extract of the fruit body of *Pleurotus ostreatus* using the fibrin plate method. The enzyme cleaved fibrin, β and γ chain of human fibrinogen. The presence of Zn^{2+} was detected by mass spectral analysis as 0.77 mol of Zn^{2+} per mol protease and the authors reported that the enzyme is likely to be a Zn^{2+} metalloprotease [17].

Anthelmintic activity of crude extract of *Pleurotus florida* was investigated against *Ascaridia galli* revealed paralysis adult nematode worm [18]. The nematocidal effect of *P. ostreatus* aqueous extract was evaluated against *Haemonchus contortus* eggs and infective larvae (L3) *in vitro* and also in articially infected gerbils (*Meriones unguiculatus*) [16] with 100% inhibition of egg hatching at 2.24 mg/ml and larvicidal effect at 50 mg/ml protein concentration. The chemical analyses indicated that constituents of aquous extracts were tridecanoic, tetradecanoic, linolelaidic, 9,15-octadecadienoic and oxalic acids.

The crude hydroalcoholic extractsand fractions of edible mushroom, *Pleurotus djamor* were assessed against *H. contortus* eggs and L3 Larvae. The extracts showed 98.7% larva; mortality at 320 mg/ml concentration. Five major compounds identified were four fatty acids:

i) Pentadecanoic.
ii) Hexadecanoic.
iii) Octadecadienoic.
iv) Octadecanoic acid and one terpene identified as *β*-sitosterol [19].

The hydroalcoholic extract of *Neolentinus ponderosus*, *P. ostreatus*, *P. eryngii* and extracts of mycelium, basidiomata and spent substrate of *P. ostreatus* were evaluated *in vitro* aganinst eggs and L_3 larvae of *H. contortus* at a concentrations ranging from 2 mg/ml to 200 mg/ml showing upto 97% larval mortality [15, 20, 21]. *Pleurotus* spp. reported to produce secondary metabolites with antiparasitic activities from different parts such as basidiomata, mycelia and degraded substrates [22] with a maximum effectiveness from mycelium hydroalhocolic extract against eggs and larvae of *H. contortus* [16].

In this study pepsin, trypsin and papain enzymes were user for hydrolysis of mushroomprotein. The mushroomprotein hyrolytaes were used at three different concentration *viz.* 10mg/ml, 25 mg/ml and 50 mg/ml against the larvae of *H. contortus*. The results revelaed 100% mortality in papain mushroom protein hydrolysate as early as one hour of incubation at 50 mg/ml followed by 25 mg/ml concentration. Trypsin mushroom protein hydrolysate showed 100% reduction in motility at 50 mg/ml concentration by 12hrs of incubation and 100% mortality at 24 hours. Pesin mushroom protein hydrolysate showed 100 % inhibition of motility and mortality at 24 hr of incubation only in 25 mg/ml and 50 mg/ml concentrations.

Recent studies revealed that the processing conditions are critically relevant to selectively recover high-valuable bioactive compounds in a sustainable way [23]. Presence of a wide range of phenolic compounds *viz.*, gallic, *trans*-cinnamic, caffeic, ferulic, aspartic and vanillic acid and flavonoid (quercetin) compounds in both unhydroyzed mushroom extracts and mushroom protein hydrolysates (MPHs) were identified through HPLC [24]. Recent study revealed fractionation of various extracts proved to separate active biomolecules active against egg and larvae of *H. contortus* such as trehalose, polyols, galactitol, D-mannitol, D-glucitol, myoinositol, adipic acid, stearic acid, squalene and *β*-sitosterol [25].

The present study concluded that the mushroom protein hydolysates obtained through pepsin, trypsin and papain enzymes hydrolysis revealed antiparasitic effect against *H. contortus*. The papain hydrolysate showed comparatively faster and potent activity against *H. contortus* larvae by 100% mortality of the larvae compared to pepsin and typsin mushroom protein hydrolysates. Further reasearches are warranted to explore the compositions of the hydolysates and also *in vivo* evaluation of antiparasitic activity of these mushroom protein hydrolyate in small ruminats.

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