

BIOLOGICAL CONTROL OF *HAEMONCHUS CONTORTUS* BY FUNGAL ANTAGONISTS IN SMALL RUMINANTS

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Abstract. *Haemonchus contortus* is a highly pathogenic nematode that causes haemonchosis in ruminants. As *H. contortus* is reported to be resistant to anthelmintic drugs, biological control might be the alternative approach. The study was conducted in Pakistan on different parameters to control *H. contortus* biologically by fungi. *Alternaria alternata*, *Fusarium oxysporum*, *Aspergillus niger*, *Trichoderma harzianum* and *Ochroconis gallopava* were isolated from the feces and abomasum of infected domestic sheep (*Ovis aries*) and goats (*Capra aegagrus hircus*). Different concentrations of the extracted spore-free cultures of these fungi were applied on eggs, larvae and adults of *H. contortus* for eggs hatching inhibition assay, larval mortality assay and adult mortality assay. In eggs hatching inhibition assay, the higher concentrations i.e. 100% and 50% of *A. alternata*, *F. oxysporum*, *A. niger*, *T. harzianum* and *O. gallopava* caused 100% inhibition of eggs. In adult mortality assay, all the culture filtrates caused no mortality in the first and second hour while in the third hour of the post treatment mortality varied with concentrations. In larval mortality assay, higher concentrations i.e. 100% and 50% of *T. harzianum* and *A. niger* caused 100% mortality of L₃ larvae of *H. contortus*. All the extracted spore-free cultures significantly caused eggs hatching inhibition. Higher concentrations of *Trichoderma* and *Aspergillus* significantly caused larval mortality (100%) while adult mortality assays were dose and time dependent and insignificantly caused adult mortality of *H. contortus*.

Keywords: *nematode parasite, haemonchosis, biocontrol, fungal extracts, egg hatch assay, adult mortality assay*

Introduction

Livestock plays an important role in the economy of Pakistan. Agriculture contributes 21% in national gross domestic product (GDP) in which share of livestock is 11.9%. The estimated population of domestic sheep (*Ovis aries*) was 29.1 million while domestic goats (*Capra aegagrus hircus*) were 66.6 million during 2013-14. According to a survey in Pakistan, about 45000 tons of meat was obtained from sheep during the year 2013-2014 (Economic Survey of Pakistan 2013-2014). Small ruminant livestock are highly susceptible to gastrointestinal nematodes (GINs). Among GINs, *Haemonchus contortus*, member of the family Trichostrongylidae (Radostits et al., 2006), is a highly pathogenic nematode parasite that causes haemonchosis in sheep and goat, an infection characterized

by anemia, protein deficiency, bottle jaw and digestive disturbances throughout the world (Getachew et al., 2007; Bowman et al., 2009). It is estimated that an infected sheep with 5000 *H. contortus* worms may lose 250 ml blood/day. Life cycle of *H. contortus* takes about 21 days to complete. *H. contortus*' life cycle is direct i.e. without the involvement of secondary host. Every adult female parasite lays between 5000-10,000 eggs/ day which are passed out in the feces (Hepworth, 2006) as shown in *Figure 1*.

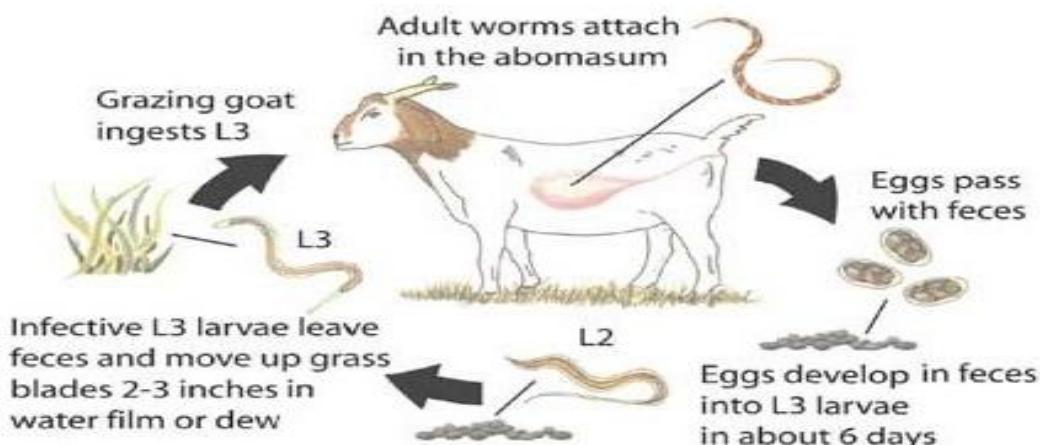


Figure 1. Life cycle of *Haemonchus contortus*. (Courtesy: pinterest.com)

As *H. contortus* is reported to be resistant to anthelmintic drugs (Miller et al., 1987; Sangster, 1999; Jackson and Coop, 2000) biological control might be the alternative approach. Many fungal species have been isolated from nematodes in which *Duddingtonia flagrans* has proven to be the most effective nematode-destroying fungus (Larsen et al., 1997) whose spores are found to survive while passing through the gastro-intestinal tract of grazing livestock (Hertzberg et al., 2001). *Trichoderma*, *Fusarium* and *Acremonium* have ovicidal effect on *Toxocara canis* ova (Maia Filho et al., 2011).

The present study was carried out in the light of previous work done to isolate and identify fungi from the feces of infected sheep and goats to evaluate the fungal filtrates' abilities of suppression the population of *H. contortus* on eggs hatching level, larval developmental level and adult level. These fungal extracts can be used as biological control against this parasite as a spray on grazing pastures or can be fed to animals in the form suspensions or pellets. Moreover, all fungi investigated in this study are of less toxicogenic nature to animals' health so making them more suitable candidates for the purpose of the study.

Materials and methods

This research work carried out in laboratory of the Department of Microbiology, Kohat University of Science and Technology, Kohat, Pakistan.

Sampling

100 fecal samples of rectum from live goats and sheep and 100 abomasal samples from freshly slaughtered goats and sheep were collected from different areas of Kohat-

Pakistan ($33^{\circ}34'47.1''N$ $71^{\circ}26'29.3''E$). The samples were analyzed for the detection of eggs (fecal samples) and worms (abomasa samples) of *H. contortus* as shown in *Table 1*. All the samples were processed in the department of Microbiology, Kohat University of Science and Technology Kohat, Pakistan.

Table 1. Samples collected and analyzed for the detection of eggs and worms of *H. contortus*

Ruminants	Fecal samples	Positive (eggs seen)	Negative (no eggs)	Abomasa	Positive (worms seen)	Negative (no worms)
Goats	50	37	13	50	34	16
Sheep	50	31	19	50	41	9
Total	100	68	32	100	75	25

***Haemonchus contortus* (eggs, adults and larvae)**

Eggs of *H. contortus* were identified and concentrated from fecal samples by simple test tube floatation method (Dryden et al., 2005), Mac Master counting chamber (Soulsby, 1968) and isolation of eggs from feces as described by (Hubert and Kerboeuf, 1992). Adult worms were collected from abomasa based on their typical barber pole appearance of female worms in a clean container as shown in *Figure 2*. Collected worms were washed with distilled water and concentrated in a container having freshly prepared phosphate buffer saline (PBS). Third stage larvae (L_3) were cultivated from the eggs recovered from adult female worms following the protocol of (Amarante, 2011) with slight modification. The L_3 were morphologically differentiated and identified as described by (Van Wyk et al., 2004).



Figure 2. Harvesting worms from abomusam

Fungi (isolation, identification and extraction)

All positive fecal and abomasa samples were processed for the isolation of fungi. One-gram samples' contents were serially diluted and poured on Sabouraud dextrose agar (SDA) media for fungal growth. After 72 h, the grown colonies were sub cultured to isolate pure cultures. For identification, both colony morphology and microscopic characteristics were deeply compared with the identification keys provided by (Navi et al., 1999) and (Larone and Larone, 1987). Among many other isolates, *Alternaria*

alternata, *Fusarium oxysporum*, *Aspergillus niger*, *Trichoderma harzianum* and *Ochroconis gallopava* were the main isolates present in all positive samples. So these fungi were selected for fungal extracts.

For extraction, fungi in broth media were incubated in a shaker incubator for 7 days at 25 °C in dark. After 7 days, they were filtered by common Whatman filter papers and the filtrates were again filtered through Millex HA syringe driven filters (0.45 µm). Different concentration i.e. 100%, 50%, 25%, 12.5% and 6.25% were made from each in phosphate buffer saline (PBS) and distilled water separately. Final filtrates were tested on potato dextrose agar (PDA) and SDA media to confirm the absence of fungal spores.

Egg hatch assay (EHA)

EHA was performed in triplicate following the protocol of (Coles et al., 1992) with slight modification. Isolated eggs were dissolved in 15 ml distilled water with concentration adjusted to 100-200 eggs/75 µl. Then 75 µl egg suspension was added to each well of a 24-welled titration plate having 2.5 ml of each concentration. For positive control, 0.025 mg/ml of Oxfendazole (Glaxo-welcome) was prepared in 0.3% dimethyl sulfoxide (DMSO). Wells having egg suspension and 2.5 ml tap water with no extracts were used as negative control. The plate was incubated at 25 °C for 48 h. After 48 h, one drop of Lugol's oil was added to stop the reaction. Then the plate was observed under 10X of inverted microscope as shown in *Figure 3*. The percent hatching inhibition was observed as:

$$\text{Hatching Inhibition (\%)} = \frac{P_{\text{HI}} \text{Test}}{P_{\text{HI}} \text{Total}} \times 100$$

$P_{\text{HI}} \text{Test}$ = number of unhatched eggs, $P_{\text{HI}} \text{Total}$ = number of unhatched eggs + number of hatched eggs.

Adult mortality assay (AMA)

In AMA, ten worms were treated in triplicate to each filtrate concentration at room temperature. 25 µg/ml of Closantel was used as positive control. Phosphate buffer saline with worms and no fungal extracts were used as negative control. After each one hour of post treatment, all the plates were observed to check the mortality till all worms were dead in negative control. Because adult worms cannot survive without host longer than 9 h. The percent mortality was calculated as:

$$\text{Adult Mortality (\%)} = \frac{P_{\text{AM}} \text{Test}}{P_{\text{AM}} \text{Total}} \times 100$$

$P_{\text{AM}} \text{Test}$ = number of dead worms, $P_{\text{AM}} \text{Total}$ = number of dead worms + number of live worms.

Larval mortality assay (LMA)

One ml of L₃ larval suspension containing 10 larvae were added in triplicate to each well of titration plate having 2.5 ml of fungal filtrates. Negative control well received

only one ml L₃ suspension and 2.5 ml phosphate buffer saline while positive control well received one ml L₃ suspension and 2.5 ml closantel (25 µg/ml). The plate was incubated at room temperature for 3 h. After 3 h, the plate was observed under 10X of inverted microscope. Percent mortality was calculated as:

$$\text{Larval Mortality (\%)} = \frac{P_{\text{LM}} \text{Test}}{P_{\text{LM}} \text{Total}} \times 100$$

P_{LM}Test = number of dead larvae, P_{LM}Total = number of dead larvae + number of live larvae.



Figure 3. *H. contortus*' eggs under inverted microscope

Statistical analysis

Data obtained from bio-assays i.e. EHA, AMA and LMA were analyzed by ONE Way ANOVA and TWO-Way ANOVA through Statistix version 9 and GraphPad Prism version 5 software. The p values <0.05 was considered significant.

Results

Egg hatch assay

After three days' incubation, the effect of different concentration of all fungal filtrates on eggs, were observed as shown in *Figure 4*. Higher concentrations i.e. 100% and 50% of all filtrates caused 100% inhibition while inhibition by lower concentrations varied from specie to specie. There were 100% and no inhibition (0%) in positive and negative control respectively.

Adult mortality assay

All concentrations of fungal filtrates treated with adult worms caused no mortality in the first three hours of post treatment. After three hours, the results were dose and time dependent and caused insignificant percent mortality as shown in *Figure 5*.

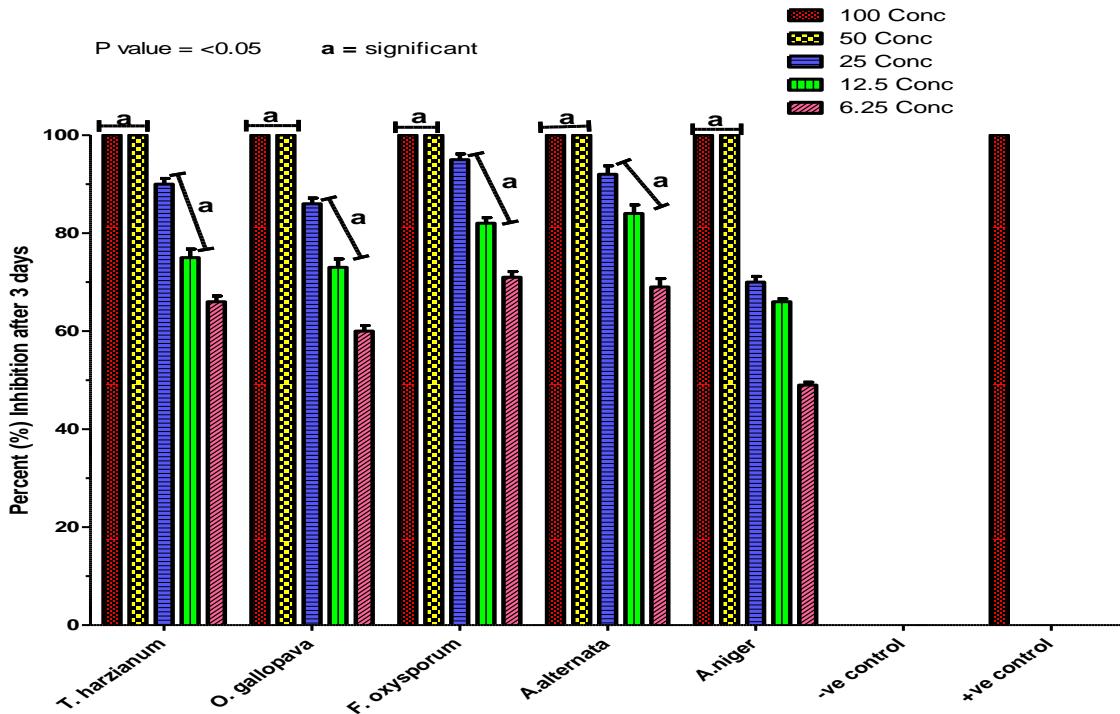
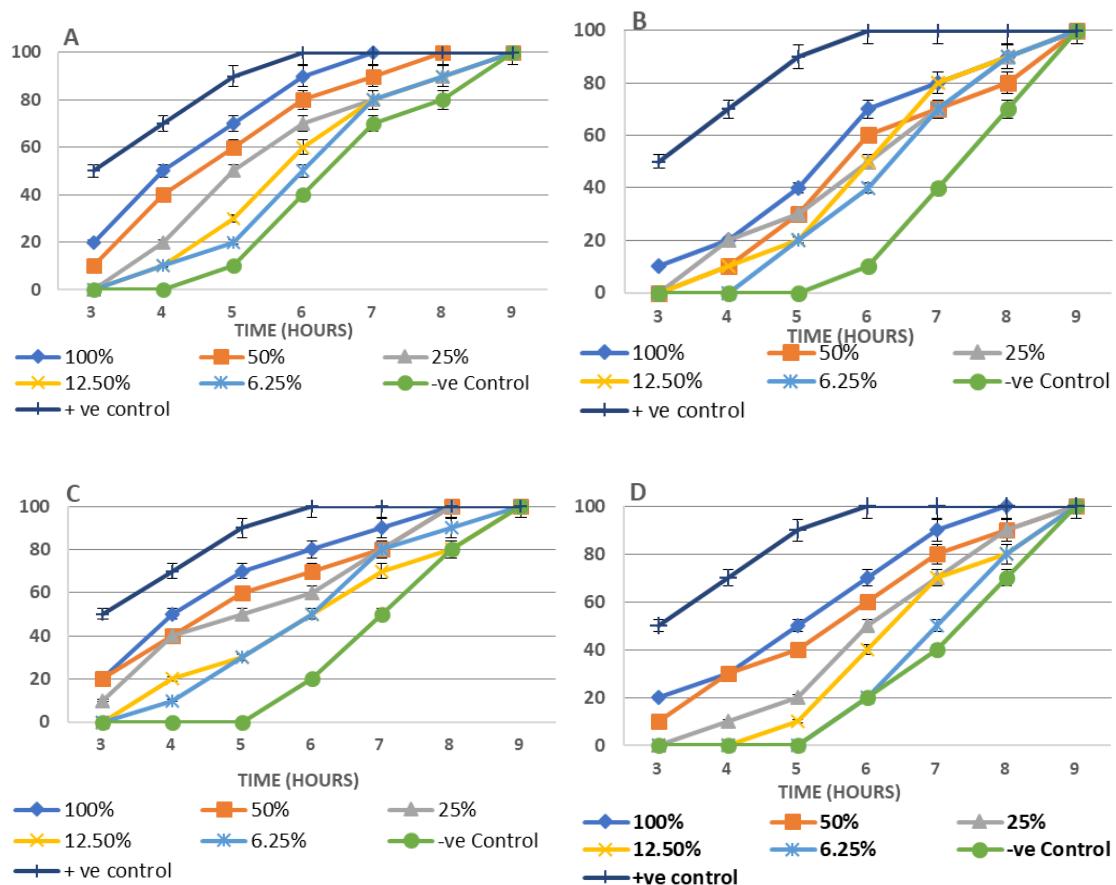


Figure 4. Percent inhibition of eggs hatching via fungal filtrates



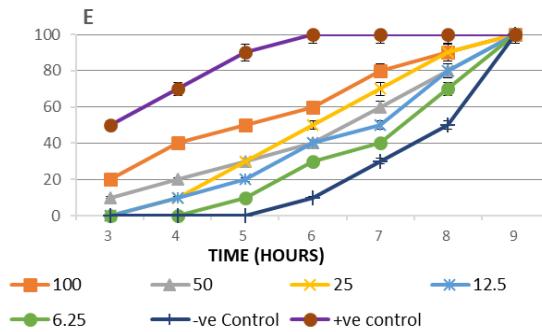


Figure 5. Adult worms' post-exposure against different concentration of fungal extracts. **A.** *F. oxysporum*. **B.** *A. alternata*, **C.** *T. harzianum*, **D.** *A. niger*, **E.** *O. gallopava*

Larval mortality assay

After 3 h post treatment of L₃ larvae with fungal filtrates, higher concentrations i.e. 100% and 50% of the *Trichoderma harzianum* and *Aspergillus niger* caused 100% mortality while 100% concentrations of *F. oxysporum* and *A. alternata* caused 94% and 91% mortality respectively. Mortality caused by lower concentrations varied from species to species. 100% and no mortality in positive and negative control respectively as shown in *Figure 6*.

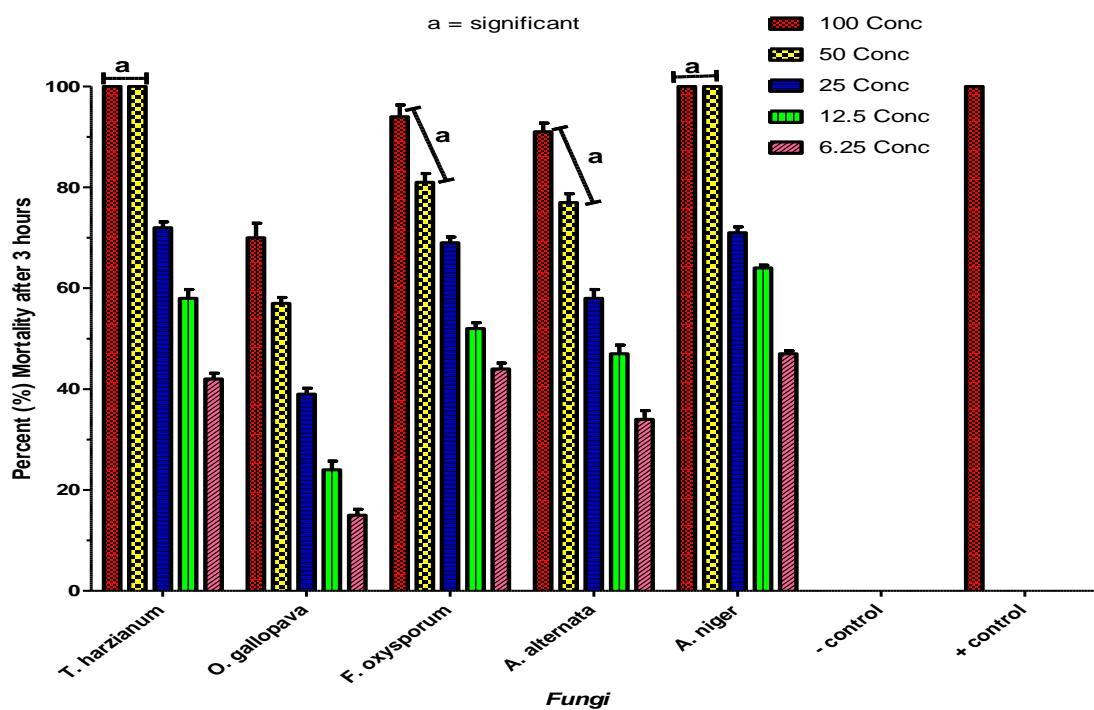


Figure 6. Percent mortality of L₃ larvae after 3 h post treatment

Overall effect on *Haemonchus contortus*

When the averages of all concentrations of each fungal treatment were analyzed by Least Significant Difference (LSD) and All-Pairwise Comparison (APC) tests, the

results showed that all the tested fungi significantly affected all stages of *H. contortus*. Pairwise comparison of fungi showed that all fungi have equal significant effect on eggs of *H. contortus* while the significance of efficacy on adults and larvae varies from fungal to fungal species as shown in *Figure 7*.

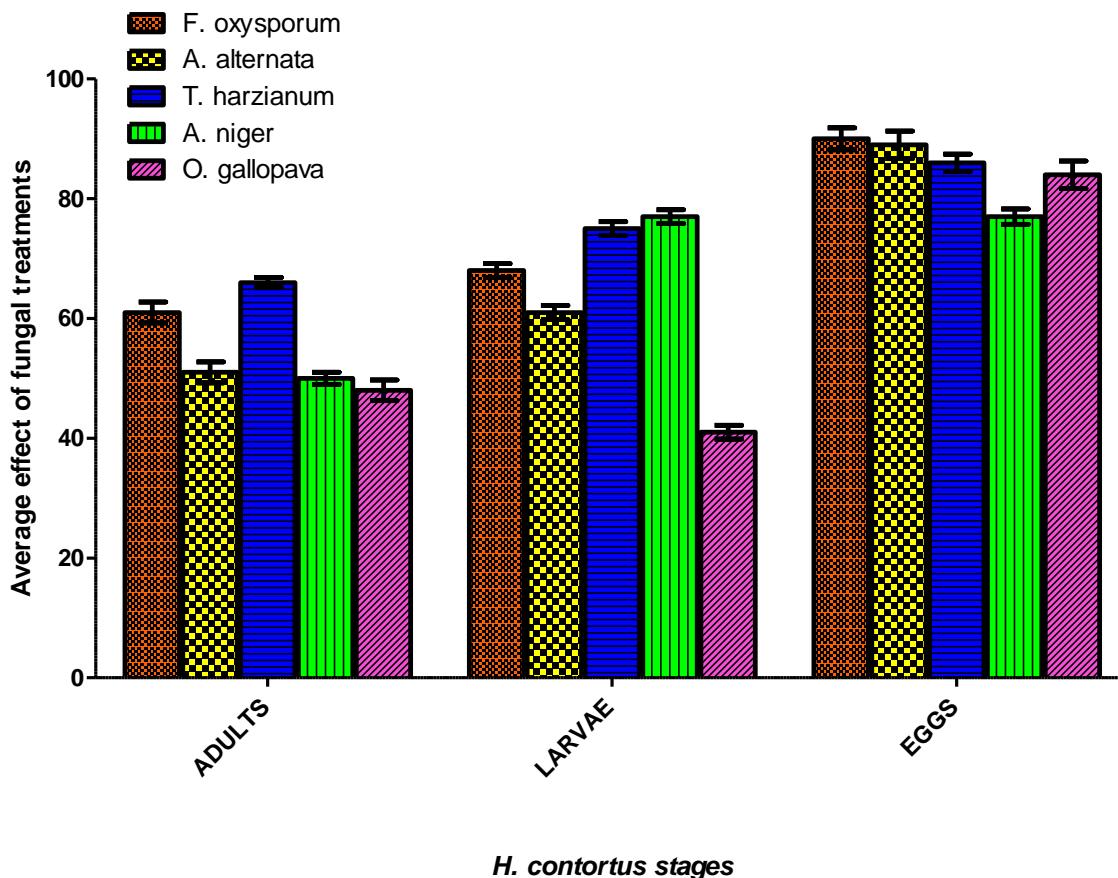


Figure 7. Average effect of fungal treatments on adults, larvae and eggs of *H. contortus*

Discussion

To control *Haemonchus contortus*, common anthelmintics are applied but not enough to erase this parasite completely as it is reported to be resistant to all three broad spectrum families of anthelmintics viz. benzimidazole, imidazothiazole and ivermectin and against drugs with narrow spectrum of activity such as salicylanilides anthelmintics (Kaplan and Vidyashankar, 2012). Keeping in view the importance of this blood sucker, there is need of alternative methods other than common anthelmintics to prevent sheep and goats from this highly dangerous nematode parasite. Biological control is an environment friendly alternative approach towards these nematodes. Among the microorganisms that parasitize on nematodes, fungi hold an important position in continuously eliminating parasitic nematodes (McSorley et al., 2008). About 70 genera and 200 species of fungi have been investigated that they are associated with nematode parasites. Till now, only a few of them are successful biocontrol agents (Nordbring-Hertz et al., 2011).

In the present study, it was tried to investigate the fungal abilities biologically to suppress the *H. contortus* population on eggs hatching, larval and adult levels. Fungi

were isolated from the feces of small ruminants and different concentrations of culture extracts were made from the isolated fungi. The spore-free cultures were applied on eggs, L₃ larvae and adult worms of *H. contortus* to find out the percent inhibition and percent mortality. The results demonstrated that the higher concentrations i.e. 100% and 50% of all the extracted spore-free cultures of *A. alternata*, *F. oxysporum*, *A. niger*, *T. harzianum* and *O. gallopava* showed 100% eggs hatching inhibition of *H. contortus*. For the first time these fungi were applied on eggs of *H. contortus* although in previous studies many other nematophagous and filamentous fungi such as *Arthrobotrys oligospora*, *A. musiformis*, *Cladosporium spp.*, *A. flavus* and *Duddingtonia flagrans* were treated with *H. contortus* eggs and showed similar results (Maia Filho et al., 2011). Also, different plant extracts have been reported previously to affect the biology of the parasitic eggs in the same ratio (Hounzangbe-Adote et al., 2005).

Adult worms of *H. contortus* in this study were treated with different concentrations i.e. 100%, 50%, 25%, 12.5% and 6.25% of the extracted spore-free cultures. The results were dose and time dependent as compared to a similar study conducted on animals grazing on pastures where *D. flagrans* was present which showed 97% efficacy of the fungus against this nematode parasite (Maria et al., 2011). Another study conducted by Chandrawathani and colleagues (Chandrawathani et al., 2003) in which they fed sheep during 24 days with either mineral blocks containing a dose of 500,000 chlamydospores/kg bw/day or pellets with the same fungal dose, and obtained a reduction in adults of *H. contortus* of >95% in the first case and 98% in the second case.

The third stage larvae (L₃) of *H. contortus* in the present study were treated with different concentrations of each extracted spore-free cultures for three hours. After three hours, it was observed that higher concentrations i.e. 100% and 50% of the *Trichoderma harzianum* and *Aspergillus niger* caused 100% mortality in the L₃ larvae while the mortality caused by lower concentrations of extracts varied from species to species. These results resemble a recent study conducted in Iran by Zarrin and colleagues in which they demonstrated that some saprophytic fungi spread in the environment can be effective in reducing the number of third stage larvae of *Trichostrongylidae* family. However, the rate of mortality was different because of the fungal species and isolates. They concluded that there was a considerable decrease in larvae population by *Cladosporium spp.* (98.8%), *Trichoderma spp.* (98.5%), and *Aspergillus spp.* (96.7%) culture. Their results indicated great potential of using appropriate saprophyte fungi for biological control of *H. contortus* larvae in sheep (Zarrin et al., 2015). A similar study conducted on stage 3 larvae of *H. contortus* by Ivonneet al., 2011 investigated that mortality of infective larvae (L₃) by *A. oligospora* was 90.3%, *A. musiformis* was 97%, and *M. thaumasium* was 62.3% (Ivonne et al., 2011). Also, Mendoza De Gives and Vazquez-prats investigated that *A. oligospora*, *Monacrosporium acermatoum* and *A. robusta* caused mortality of the population of *H. contortus* infective L₃ larvae with 93.30%, 95.70% and 10.10%, respectively (Mendoza-De Gives and Vazquez-Prats, 1994).

Conclusion and recommendations

From the study, it is concluded that higher concentrations of all the extracted spore-free culture filtrates from the isolated fungi significantly caused eggs hatching inhibition. Higher concentrations of *Trichoderma harzianum* and *Aspergillus niger* significantly caused larval mortality (100%) while adult mortality assays were dose and

time dependent and insignificantly caused adult mortality of *Haemonchus contortus*. Keeping in view the importance of this problem and encouraging results obtained from the study, there is need to explore the chemistry of the fungal filtrates to find out their active compounds and their mechanism of actions (both *in vitro* and *in vivo*). The effects of these fungi at molecular level like DNA damage and genetic alterations in *H. contortus* need further explorations.

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