Research Article

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Inhibitory activities of ethanolic extracts of two macrofungi against eggs and miracidia of *Fasciola* spp.

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Abstract: Fascioliasis is a disease of livestock which is now recognized as an emerging disease in humans. *Cantharellus cibarius* and *Ganoderma applanatum* are known for their medicinal properties. The use of ethanolic extracts of these macrofungi against the eggs and miracidia of *Fasciola* spp. is a promising method to break the parasite transmission cycle. The aim of the study is to evaluate the inhibitory effects of ethanolic extracts of the mushrooms on eggs and miracidia of *Fasciola* spp. Concentrated eggs and miracidia of *Fasciola* spp. were exposed to different concentrations (1-8 mg/ml) of extracts of *Ganoderma applanatum* (GEE) and *Cantharellus cibarius* (CEE) at different time intervals. GEE showed superior antiparasitic activities when compared to CEE at all concentrations tested. Significant positive correlations were observed between the concentration of GEE and mortality in miracidia ($r=0.980$, $P<0.05$) and CEE and mortality in miracidia ($r=0.968$, $P<0.05$). The study showed that ethanolic extracts of *G. applanatum* and *C. cibarius* have ovicidal and miracidicidal activities. While *G. applanatum* showed excellent activities, activities in *C. cibarius* were moderate. Therefore, these mushroom extracts can be regarded as promising sources of bioactive compounds that could be developed into ovicides and miracidicides.

Keywords: *Fasciola* spp., macrofungi, ovicidal, miracidicidal, inhibitory activities

1 Introduction

Infection by *Fasciola* spp. is endemic both in tropical and temperate regions of the world and causes significant losses in livestock production with liver condemnation and reduced carcass value estimated to exceed US\$3 billion per year [1, 2]. In addition, fascioliasis is now recognized as an emerging human disease with up to 17 million estimated cases worldwide [3]. Climate change, man-made environmental modification, and resistance to anti-fascioliasis mainstay drugs are factors of epidemiological importance in the disease transmission [4, 5]. The life cycle begins when sheep or cattle ingest the encysted cercariae or metacercariae during grazing. The parasite develops into an adult in the bile ducts and lays eggs which are passed into water bodies through feces. The eggs hatch into miracidia which locate fresh water snails belonging to the genus *Lymnaea* for development into sporocysts. Sporocysts develop into rediae which in turn form cercariae. The later stage becomes encysted on aquatic vegetation or other surfaces.

Integrated control measures have been widely advocated as potent management strategies of fascioliasis. These may include controlled grazing, snail control, and chemotherapy. Triclabendazole, due to its efficacy against both immature and adult flukes, is the most currently used fasciolicide. Fasciolicides like albendazole, closantel, clorsulon, and rafoxanide are active against adult *Fasciola* but show impaired activity against young migrating stages [6].

The quest for new drug regimens is necessitated by reports of resistance of flukes to triclabendazole, a widely acclaimed and effective mainstay drug [7, 8]. Anti-fascioliasis medicinal plant research has gained considerable attention in the past years. Among the many herbs which have shown promising effects against *Fasciola gigantica* and *F. hepatica* were *Allium sativum*, *Lawsonia inermis*, *Opuntia ficus*, *Lantana camara*,

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Bocconia frutescens, *Piper auritum*, *Artemisia mexicana*, and *Cajanus cajan* [9,10]. These plants were shown to inhibit adult fluke motility and induce rupturing of internal organs such as the uterus and caeca, especially at higher concentrations [10].

Mushrooms are recognized as food items and contain diverse bioactive ingredients with nutritional and medicinal properties [11, 12]. They are recognized functional foods and can be developed into medicines and nutraceuticals [13]. *Ganoderma applanatum* and *Cantharellus cibarius* are macrofungi species with high medicinal values. These mushrooms show a wide variety of bioactivities including anti-bacterial, anti-tumor, antihypertensive, immunomodulatory, antioxidant, and anti-androgenic properties [14-16].

Very few reports are available on the anti-parasitic potentials of *Cantharellus* and *Ganoderma* macrofungi. While *Cantharellus cibarius* shows promising bioactivity against trypanosome [17], *Ganoderma applanatum* is favored against the oocyst of *Eimeria* species [18]. To further extend the spectrum of anti-parasitic activities of the two mushrooms, the ethanolic extracts of the two macrofungi were tested on the eggs and the immature free swimming miracidia of *Fasciola* spp. This is a novel approach and aims to interrupt the parasite's transmission cycle. The aim of this study therefore was to evaluate the ovicidal and miracidal potentials of two Nigerian edible mushrooms.

2 Materials and methods

2.1 Parasite collection

Gall bladders of cattle naturally infected with *Fasciola* spp. were collected between April and October of 2017 from Bodija Municipal Abattoir, Ibadan, after the animals were slaughtered. The gall bladders collected were transported to the Parasitology Laboratory, Department of Zoology, University of Ibadan, in an ice pack cooler within one hour.

2.2 Collection of *Fasciola* spp. eggs

The bile was aseptically transferred into centrifuge tubes and centrifuged at 2500 rpm for 5 min. The supernatant was removed, and the sediment containing eggs was washed several times using distilled water. Afterwards, the number

of eggs in 2 µl of the egg suspension was examined and recorded. A small quantity (0.5 ml) of the egg suspension was transferred into a glass beaker wrapped with aluminum foil and incubated in a dark cupboard for 14 days at room temperature (26-28°C) for embryonation. The remaining eggs contained in the suspension were transferred to a clean container wrapped with aluminum foil and stored in the refrigerator at 4°C for future use [19].

2.3 Collection and identification of mushrooms

Mushroom fruiting bodies of *Ganoderma applanatum* and *Cantharellus cibarius* were collected from various locations within Kwara State, Oyo State, and Ogun State, Nigeria. The mushroom specimens were identified by a mushroom taxonomist at the Department of Botany, University of Ibadan. The choice of these macrofungi was based on earlier reports of their anti-parasitic activities [17, 18]. So we seek to further extend their spectrum of known activities against the egg and immature stage of *Fasciola* spp.

2.4 Mushroom extract preparation

The mushrooms were air dried, ground into powder using a milling machine, and extracted by maceration in ethanol. About 85 g of each ground mushroom sample was weighed into 700 ml of 95% ethanol in conical flasks. These were covered, shaken every 30 min for 6 h, and then allowed to stand for about 48 h. The extracts were shaken, decanted, and filtered using Whatman No. 1 filter paper. The extraction process was repeated two times with ethanol. The filtrates were combined and evaporated to dryness under reduced pressure at a maximum temperature of 40°C using a rotary evaporator. The crude extracts were stored in the refrigerator prior to use.

2.5 Preparation of mushroom extract suspension

One hundred milligrams (100 mg) of each semisolid extract was weighed and dissolved in 5 ml of 5% DMSO in distilled water to give a stock solution of 20 mg/ml. From the stock solution, final concentrations of 1, 2, 4, 6, and 8 mg/ml were prepared using the simple dilution procedure [19].

2.6 Ovicidal activity of mushroom extracts on ova of *Fasciola* spp.

The ovicidal bioassay activity test was carried out using the *in vitro* *Fasciola* Egg Hatch Test (FEHT) assay described by Alvarez et al. [20], Fairweather et al. [21], and Canevari et al. [22]. The test was carried out in 96-well flat-bottomed plastic plates. Two microliters (2 µl) of egg suspension containing about 100 unembryonated eggs was seeded into each well, and 300 µl of each mushroom extract at varying concentrations (1, 2, 4, 6, and 8 mg/ml) was added to the wells. Albendazole (ABZ; 5 mg/ml) prepared from the dissolution of 25 mg albendazole in 5 ml of 5% DMSO distilled water was used as a positive control. ABZ was used as the positive control because of its superior efficacy against *Fasciola hepatica* eggs compared to other anti-*Fasciola* drugs [20]. The negative control comprised 5% DMSO distilled water. Each concentration and control was tested in triplicate. The eggs were then incubated in a dark cupboard for 24 h. After the 24-h exposure time, the supernatant containing extracts, ABZ, and 5% DMSO distilled water was carefully removed and replaced with 300 µl of distilled water. Following this, the eggs were incubated in darkness for 14 days at room temperature (26–28°C) for embryonation. Then eggs were exposed to incandescent (60 watt bulb) light to stimulate the hatching of miracidia. Hatched and unhatched eggs, including dead eggs, were counted under a microscope at intervals of 30, 60, 90, 120, and 150 min. Ovicidal activity was expressed based on the percentage of eggs that failed to develop and hatch [23]. It is expressed as follows:

$$\text{Ovicidal activity (\%)} = \left(\frac{\text{Number of eggs not hatched}}{\text{Total number of hatched and unhatched eggs}} \right) \times 100$$

2.7 Miracidal activity of mushroom extracts on miracidia of *Fasciola* spp.

The miracidal bioassay test was conducted according to the method described by Obare et al. [24] although with some modifications. *Fasciola* eggs incubated for 14 days in a beaker in darkness were exposed to incandescent (60 watt bulb) light to stimulate miracidial hatching. After an hour, 2 µl of the distilled water containing 20 miracidia was placed in the wells and 300 µl of each mushroom extract at varying concentrations (1, 2, 4, 6, and 8 mg/ml) was added to the wells. Albendazole (5 mg/ml) and 5% DMSO distilled water were the positive and negative controls, respectively. A triplicate was set up for each test group. Each set up was observed under the microscope

at 10 min intervals for a period of one hour (10, 20, 30, 40, 50, and 60 min). Dead or immobile miracidia were enumerated and recorded. Constant motion signified that the miracidia were alive while no motion signified death.

2.8 Statistical analysis

Data were analyzed using SPSS version 18.0. (IBM, Armonk, NY, USA). Analysis of variance (ANOVA) was carried out to test for significance between treatment groups while multiple comparisons between the various treatment groups were done using Tukey's test. The LC₅₀ of the ethanolic mushroom extracts on parasite ova and miracidia was determined by Finney's Probit analysis [25]. $P < 0.05$ was considered statistically significant.

3 Results

The ovicidal activities of ethanolic extracts of *Ganoderma applanatum* (GEE) and *Cantharellus cibarius* (CEE) were concentration dependent ($P < 0.05$). GEE showed superior ovicidal activity when compared to CEE at all of the concentrations tested. GEE tested at 8 mg/ml with 91.3% ovicidal activity was significantly higher than the 68.0% recorded with CEE at the same concentration (Fig. 1).

The miracidal activity of GEE was both concentration and time dependent ($P < 0.05$). A weak miracidal activity (33.3%) was observed at the lowest concentration (1 mg/ml) of GEE within a 60 min maximum exposure time (Table 1). All miracidia (100.0%) were dead within 40 min in a concentration of 6 mg/ml of the mushroom extract and the positive control. A one hundred percent (100.0%) mortality was recorded in 8 mg/ml of the test extract within 30 min of exposure (Table 1). No death was recorded in the negative control groups. A significant positive relationship was observed between the concentration of GEE and mortality ($r = 0.980$; $P < 0.05$), time of exposure, and mortality of the free-swimming larval stage of *Fasciola* spp. ($r = 0.998$; $P < 0.05$).

Generally, CEE showed weaker miracidal activity compared to GEE. Like with GEE, miracidal activity varied significantly with time and concentration of CEE ($P < 0.05$). Miracidal activity varied significantly from a 10.0% mortality in the 1 mg/ml CEE treatment group to a 100.0% mortality in the 8 mg/ml exposed group within 60 min of exposure ($P < 0.05$). All miracidia were observed dead within 50 min of exposure in the 8 mg/ml treatment group and within 40 min of exposure in the 5 mg/ml ABZ positive control group (Table 2). A significant positive

Figure 1.

Table 1. Miracidial activity of *Ganoderma applanatum* ethanolic extract

Conc. (mg/ml)	% Mortality ($\pm$ S.E.)					
	10 min	20 min	30 min	40 min	50 min	60 min
1	0	0	0	1.7 $\pm$ 1.7 ^a	15.0 $\pm$ 2.9 ^b	33.3 $\pm$ 1.7 ^c
2	0	0	3.3 $\pm$ 1.7 ^a	10.0 $\pm$ 2.9 ^b	26.7 $\pm$ 4.4 ^c	45.0 $\pm$ 2.9 ^d
4	11.7 $\pm$ 1.7 ^a	26.7 $\pm$ 4.4 ^{ab}	36.7 $\pm$ 1.7 ^b	55.0 $\pm$ 7.6 ^c	65.0 $\pm$ 5.0 ^{bc}	71.7 $\pm$ 4.4 ^f
6	28.3 $\pm$ 1.7 ^b	58.3 $\pm$ 8.3 ^{bc}	71.7 $\pm$ 7.3 ^c	100.0 $\pm$ 0.0 ^d	100.0 $\pm$ 0.0 ^d	100.0 $\pm$ 0.0 ^d
8	60.0 $\pm$ 5.8 ^{bc}	75.0 $\pm$ 2.9 ^c	100.0 $\pm$ 0.0 ^d	100.0 $\pm$ 0.0 ^d	100.0 $\pm$ 0.0 ^d	100.0 $\pm$ 0.0 ^d
ABZ	65.0 $\pm$ 5.8 ^{bc}	73.3 $\pm$ 6.0 ^c	91.7 $\pm$ 3.3 ^d	100.0 $\pm$ 0.0 ^d	100.0 $\pm$ 0.0 ^d	100.0 $\pm$ 0.0 ^d
5%DMSO	0.0	0.0	0.0	0.0	0.0	0.0

Note: Similar superscripts denote no significant difference while different superscripts denote significant difference. Significant differences were compared across various concentrations and time of miracidia exposure to GEE

Table 2. Miracidial activity of *Cantharellus cibarius* ethanolic extract

Conc. (mg/ml)	% Mortality ($\pm$ S.E.)					
	10 min	20 min	30 min	40 min	50 min	60 min
1	0	0	0	0	3.3 $\pm$ 1.7 ^a	10.0 $\pm$ 2.9 ^a
2	0	0	0	1.7 $\pm$ 1.67 ^a	11.7 $\pm$ 4.4 ^b	21.7 $\pm$ 4.4 ^b
4	0	5.0 $\pm$ 2.9 ^a	16.7 $\pm$ 6.0 ^a	26.7 $\pm$ 6.0 ^b	36.7 $\pm$ 6.0 ^c	45.0 $\pm$ 5.8 ^c
6	13.3 $\pm$ 3.3 ^a	31.7 $\pm$ 3.3 ^b	55.0 $\pm$ 5.0 ^b	58.3 $\pm$ 6.0 ^c	65.0 $\pm$ 7.6 ^d	73.3 $\pm$ 6.0 ^d
8	36.7 $\pm$ 4.4 ^b	55.0 $\pm$ 5.8 ^c	71.6 $\pm$ 6.0 ^c	83.3 $\pm$ 4.4 ^d	100.0 $\pm$ 0.0 ^e	100.0 $\pm$ 0.0 ^e
ABZ	65.0 $\pm$ 5.7 ^c	73.3 $\pm$ 6.0 ^d	91.7 $\pm$ 3.3 ^d	100.0 $\pm$ 0.0 ^e	100.0 $\pm$ 0.0 ^e	100.0 $\pm$ 0.0 ^e
5%DMSO	0.0	0.0	0.0	0.0	0.0	0.0

Note: Similar superscripts denotes no significant differences while different superscripts denotes significant differences. Significant differences compared across concentrations and time of miracidia exposure to CEE.

correlation was observed between the concentration of CEE and mortality in miracidia ($r=0.968$, $P<0.05$), time of exposure, and mortality of miracidia ($r=0.997$, $P<0.05$).

The lethal concentration required to prevent hatching of *Fasciola* eggs by 50% (LC_{50}) decreased with time of exposure in both GEE and CEE. While the ovicidal LC_{50} of GEE was 7.0 mg/ml and 2.1 mg/ml in 10 min and 60 min exposure times, respectively, the ovicidal LC_{50} s 8.6 mg/ml and 3.5 mg/ml were recorded for CEE at the same time intervals (Table 3). Lethal time (LT_{50}), which is the time required to kill 50% of miracidia, was generally higher in the CEE than GEE exposed groups. Highest LT_{50} s of 63.8 min and 83.1 min were recorded in 1 mg/ml GEE and CEE exposed groups, respectively. The highest concentration of 8 mg/ml recorded the lowest LT_{50} values of 0.2 min and 18.8 min for GEE and CEE exposed groups, respectively (Table 4).

4 Discussion

The increase in cases of fasciolicide resistance has necessitated the need for alternative control strategies against *Fasciola* infection. The adult and immature forms of the parasite found in human hosts are the ones often responsible for the problem of drug resistance. This may be largely due to the uncontrolled use of some of

the mainstay anti-*Fasciola* drugs. The targeting of other stages of the parasite not often considered during drug sensitivity assays may offer a promising prospect in drug discovery against *Fasciola* spp. The use of *Fasciola* eggs and miracidia as the target stages for anti-*Fasciola* drug discovery bioassay is a welcome idea owing to these stages' importance in the parasite transmission cycle. To the best of our knowledge, this study was the first to use extracts from mushrooms against these two stages of *Fasciola*. However, studies on ovicidal and miracidal effects of medicinal plants abound. Some of the plants which have been directed against eggs of *Fasciola* included *Nigella sativa* [26], *Zingiber officinale* [19], *Momordica charantia* [27], and *Moringa oleifera* [28]. While many of the aforementioned studies did not consider the mechanisms of ovicidal activities of plant products, the butanol subfraction of *Momordica charantia* was shown to prevent hatching of miracidia through inhibition of blastogenesis [27]. Other efforts have been directed against the snail hosts of trematode infections [28, 29].

Our study showed impressive ovicidal and miracidal activities in *Cantharellus cibarius* and *Ganoderma applanatum*, although the latter is more promising. This study suggests that the two ethanolic mushroom extracts have the potential to inhibit *Fasciola* embryonic development. This inhibitory effect could be the result of embryonic lysis potentiated by the extracts' ability

Table 3. Ovicidal lethal concentration (LC_{50}) of ethanolic extracts of *Ganoderma applanatum* (GEE) and *Cantharellus cibarius* (CEE)

Time (min)	LC_{50} GEE (mg/ml)	Regression	LC_{50} CEE (mg/ml)	Regression
10	7.0	$y=0.828x-0.781$	8.6	$y=0.753x-1.454$
20	6.3	$y=0.917x-0.797$	7.1	$y=0.822x-0.850$
30	4.7	$y=1.076x-0.094$	6.5	$y=0.903x-0.844$
40	3.3	$y=0.927x+1.939$	5.9	$y=0.757x+0.506$
50	2.6	$y=0.775x+2.976$	4.0	$y=0.713x+2.152$
60	2.1	$y=0.682x+3.627$	3.5	$y=0.643x+2.729$

Table 4. Lethal time (LT_{50}) of ethanolic extracts of *Ganoderma applanatum* (GEE) and *Cantharellus cibarius* (CEE)

Conc. (mg/ml)	LT_{50} GEE (min)	Regression	LT_{50} CEE (min)	Regression
1	63.8	$y=0.108x-1.859$	83.1	$y=0.080x-1.661$
2	56.2	$y=0.109x-0.118$	66.5	$y=0.101x-1.722$
4	40.0	$y=0.035x+3.591$	52.7	$y=0.082x+0.687$
6	16.1	$y=0.100x+3.381$	36.3	$y=0.032x+3.825$
8	0.2	$y=0.076x+3.988$	18.8	$y=0.090x+3.314$

to penetrate the parasite egg shell [30, 31]. Similar observations were recorded in nanotized plant product [32]. The penetrating extracts may interfere with the expression of proteins functionally involved in cellular proliferation and cytoskeleton organization during embryogenesis [33]. Disruption of the fine tegument of miracidia and probable interference with protein expression responsible for cellular functions could as well be responsible for the mortality observed in miracidia. Another possibility is the down-regulation of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione-S-transferase (GST) responsible for defense against oxidative damage in the parasite [34]. Mechanistic studies need to be done in order to explore these propositions.

A lower ovicidal LC_{50} with an increase in the time of exposure is very desirable as this suggests an improvement in an extract's activities with time. A similar phenomenon was observed in miracidia LT_{50} values with lower values recorded as the concentration of mushroom extract increased. Other parasites that have been shown to be sensitive to *Cantharellus cibarius* and *Ganoderma applanatum* are *Leishmania donovani* and *Plasmodium falciparum* [35], trypanosomes [17], and *Eimeria* spp. [18]. Some biologically active compounds present in the mushrooms have been suggested to prevent colonization of the intestine by parasites such as *Eimeria* spp. [18] and inhibit locomotion in flagellated parasitic protozoans such as trypanosomes [17]. Although this was not confirmed, an increase in the amount of the bioactive constituents in the ethanolic extracts of *Ganoderma applanatum* compared to *Cantharellus cibarius* could contribute to its superior ovicidal and miracidicidal activities.

5 Conclusions

The present study revealed the promising ovicidal and miracidicidal activities of the ethanolic extracts of *Ganoderma applanatum* and *Cantharellus cibarius* on *Fasciola* spp. eggs and miracidia. When compared to the efficacy of other drugs or extracts from natural sources used by other studies, the ethanolic extract of *Ganoderma applanatum* can be considered to have an excellent ovicidal and miracidicidal potency similar to that of albendazole, which is currently used fasciolicide. On the other hand, the ethanolic extract of *Cantharellus cibarius* can be said to have moderate ovicidal and high miracidicidal potency. Therefore, these mushroom extracts can be regarded as promising sources of bioactive compounds that could be developed into ovicide and miracidicide thereby offering

cheap, effective, less toxic, and environmentally friendly alternatives to synthetic drugs for the control of fascioliasis in endemic areas. It is therefore recommended that isolation of the bioactive compounds responsible for the ovicidal and miracidicidal activities present in the fruiting bodies of the mushrooms be carried out. Follow up *in vivo* controlled studies to ascertain the therapeutic potential of *Ganoderma applanatum* and *Cantharellus cibarius* ethanolic extracts in treating *Fasciola* spp. infection are also recommended.

Conflict of interest: Authors state no conflict of interest

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