Parasitol Res (2010) 106:1135–1139
DOI 10.1007/s00436-010-1787-0

ORIGINAL PAPER

Effects of ethanol extract of *Artemisia annua* L. against monogenean parasites of *Heterobranchus longifilis*

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Received: 12 January 2010 / Accepted: 19 January 2010 / Published online: 18 February 2010
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Abstract Ethanol extract of *Artemisia annua* was effective in the dislodgement and mortality of monogenean parasites of juvenile *Heterobranchus longifilis* at concentrations ranging from 50 to 200 mg/l. Five hundred 1-week-old juvenile fish were stocked in hapa in earthen pond for 7 days to accumulate parasites. The approximate number of parasites per fish was confirmed by counting the number of parasites attached to body surfaces and the gills with a stereo-microscope before being exposed to the extract under in vivo conditions. The bioactivity of the extract was conducted in plastic Petri dishes with three replications and controls. The results obtained from *A. annua* extract were matched against those produced by pure artemisinin and artesunate powder, respectively, under similar experimental conditions. There was a faster effect of pure artemisinin crystals on the parasites as compared to *A. annua* extract and artesunate. Coagulation of parasite cells was observed with artemisinin treatment, whereas parasites were merely dislodged from their attachment organs and killed some hours later in the same concentration of *A. annua*. There were positive correlations between the number of parasites dislodged/killed and the concentration of *A. annua* extract, artemisinin, and artesunate powder, respectively, as well as the duration of exposure of affected fish to the substances. This led to the conclusion that *A. annua* contains substances that are effective against helminthes parasites of *H. longifilis*.

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Introduction

Artemisia annua L., also known as annual wormwood or Sweet Annie, belongs to the family Asteraceae. It is a highly aromatic herbaceous plant of Asiatic and Eastern European origin, widely dispersed throughout the temperate region (Bailey and Bailey 1976; Simon et al. 1984). It was used by Chinese herbalists since A.D. 341 for the treatment of fevers associated with malaria (Hien and White 1993). Its activity against malarial parasites in primate models was demonstrated in 1971, but the isolation and characterization of the active antimalarial principle, artemisinin, by Chinese scientists were in 1972 (Yeung 1985).

Artemisinin is produced mainly in the leaves and inflorescence of the plant. Its structure has been characterized as a cadinane-type sesquiterpene lactone with an endoperoxide bridge. Artemisinin and its semi-synthetically prepared derivatives including dihydroartemisinin, artesunate, and artemether act as blood schizontocidal agents, which effectively inhibit the late stage ring parasites and trophozoites of *Plasmodium*. They equally affect the early stage of gametophyte development, which reduces further retransmission of the parasites from humans to mosquitoes in areas of low transmission.

Interestingly, within the last decade or so, artemisinin and many other bioactive compounds isolated from *A. annua* have equally displayed unique pharmacological activities against a wide range of bacteria (Bone and Morgan 1992) including *Enterobacter* and *Klebsiella* species, *Streptococcus faecalis*, *Staphylococcus aureus*, *Shigella dysenteriae*, *Escherichia coli*, and *Pneumocystis carinii* (Chen et al.

1994), an opportunistic pathogen which causes pneumonia in AIDS and other immune-compromised patients. Recent studies have also shown that artemisinin has a therapeutic potential against *Toxoplasma gondii* (Jones-Brando et al. 2006), *Trypanosoma*, and *Schistosoma* species (Mishina et al. 2007; Utzinger et al. 2001), which cause toxoplasmosis that is associated with behavioral abnormalities in patients, human trypanosomiasis or "sleeping sickness," and schistosomiasis, respectively, as well as other pathogens responsible for cryptosporidiosis, amoebiasis, giardiasis, leishmaniasis (Ma et al. 2004), and clonorchiasis.

Artemisinin destroys the cells of parasitic organisms through the generation of highly reactive oxygen-based free radicals or electrophilic intermediates, by alkylating and oxidizing proteins and lipids of parasite membranes as well as inactivation of channel proteins (Ridley and Hudson 1998). It has been demonstrated that the effect of artemisinin is equally mediated through disruption of membrane potential by interacting with the electron transport chain in the mitochondrial membrane, resulting in free radical damage and dysfunctional mitochondria (Li et al. 2005).

Monogenean parasites are hermaphroditic worms which inhabit the skin and gills of fish. Two species, Gyrodactylus and Dactylogyrus, have been identified as the major causes of mortality in catfish (*Heterobranchus longifilis*) juveniles under unfavorable culture conditions. While Gyrodactylus are mostly found in the skin and fins, Dactylogyrus are attached to the gill epithelia where they cause serious damage resulting in pathological changes that interfere with gaseous exchange in fish (Obiekezie and Taege 1991). The possession of four eyespots by the gill parasites is a diagnostic feature that distinguishes the two species. Skin and gills of affected host are damaged by the attachment hooks resulting in secondary infection by bacteria and fungi.

It has been demonstrated in earlier studies that supplementing daily rations of poultry with dried pulverized leaves of *A. annua* was found to be effective for the treatment of coccidiosis in chickens (Brisibe et al. 2008; Allen 1997) without any adverse effects. Moreover, dietary supplementation also has the potential to be an effective anthelmintic treatment in small ruminants destined for the meat market (Ferreira et al. 2006; Hart et al. 2007; Turner and Ferreira 2005). Encouraged by these results, the current study was designed to determine the efficacy of extracts of *A. annua* in the dislodgement of monogenean parasites from the skin and gills of *H. longifilis* where they account for 60% to 70% of the mortality found amongst juveniles in Nigeria (Obiekezie and Taege 1991).

The chemotherapeutic agents currently used for the treatment of fish monogenesis include mebendazole, organophosphate, praziquantel, closantel, dichlorvos, formaldehyde, etc. (Szekely and Molnar 1987; Scott 1993; Moser et al. 1986; Hoffman 1983; Lewbart and Gratzek 1990; Gratzek and Blasiola 1992; Chisholm and Whittington 2002). The objective of this study is to find an alternative means of treatment of monogenean disease of cultured fish using an extract of *A. annua* instead of chemical-based substances that may not be friendly to the environment.

Methodology

Freshly harvested *A. annua* leaves and pure artemisinin crystals were provided, courtesy of Molecular Bio/Sciences Limited, 124 M.C.C. Road, Calabar, Cross River State, Nigeria. Artesunate tablets (Bliss Gvs Pharma Ltd, India) were purchased from a local drug store in Calabar, Cross River State, Nigeria.

The leaves were washed thoroughly in running tap water to remove sand and debris. Thereafter, they were dried by spreading under the sun for 3 days and finally in a hot air oven at 60°C for 8 h. The dried leaves were crushed to powder in a mortar and pestle and subjected to Soxhlet extraction with 70% ethanol as the extracting solvent. The solvent was exhausted from the extract with the help of a rotary evaporator. The extract was stored in a refrigerator until required for use.

Preparation of stock and working solutions

The ethanolic extract of *A. annua* was used for the preparation of a stock solution from which the working solution used for the efficacy testing was prepared. The stock solutions were obtained by dissolving 1 g of the extract and the powder in 5 ml of dimethyl sulfoxide (DMSO) and made up to 100 ml with de-ionized water.

Four working solutions (labeled A, B, C, and D that were represented by concentrations of 50, 100, 150, and 200 mg/l, respectively) were prepared from the stock solutions. A preliminary test was carried out to guide in the selection of the concentration of the test solutions.

One-week-old fry of *H. longifilis* obtained by induced breeding were stored in hapa made up of mosquito net of tiny mesh size in an outdoor earthen pond for a period of 1 week. Examination for the accumulation of monogenean parasites was done from the fourth day of stocking.

Efficacy testing

Parasitized fish fry, confirmed with the help of a stereomicroscope, were collected for the test. The parasite burden was confirmed by counting the number of monogenean parasites on the external surfaces of the fish including the gills. This was followed by the exposure of the fish to

different concentrations of *A. annua* extract in plastic Petri dishes. Parasitized fish were also placed in de-ionized water containing 5 ml of DMSO in plastic Petri dishes to serve as control. Fish were exposed for periods ranging from 30 to 180 min in both the test and the control treatments. They were re-examined individually at the expiration of the exposure period for the presence of parasites.

For purposes of comparison, pure artemisinin and artesunate were also tested against the fish parasites under similar experimental conditions. The crystals/powder were dissolved in 5 ml of DMSO and then diluted to the required concentrations (50, 100, 150, and 200 mg/l). The experiments with the substances were replicated three times. The homogeneity of the replicates was checked by Kruskal–Wallis test before the data of the replicates were pooled together.

Toxicity test

Toxicity of the extract of *A. annua* to catfish juveniles was tested for 24, 48, 72, and 96 h, respectively, at higher concentrations (250, 300, 350, 400, and 500 mg/l) to ascertain the safety margin of the substance against the fish host (Table 1). Glass aquaria of 10 l capacity were used, and each tank was filled with 3 l of the test solution and stocked with ten fish under stone aeration for production of dissolve oxygen. The setup was replicated three times with a set of controls under the same experimental conditions. Observation for fish mortality and abnormal swimming behavior was made for 96 h.

Results

Examination of the fish fry at the end of the test periods demonstrated that while some of the fish that previously harbored parasites were found to be free of some of the parasites, others were completely free of the parasites. Comparatively, the number of parasites on the body surfaces of fish in the control was the same throughout the test period.

Table 1 Toxicity test of concentrations (milligrams per liter) of *Artemisia annua* against fish mortality (percent)

Conc. (mg/l)	Fish mortality (%)			
	24h	48h	72h	96h
250	0	10	0	10
300	0	10	10	10
350	10	20	10	10
400	10	10	10	20
500	20	10	20	20

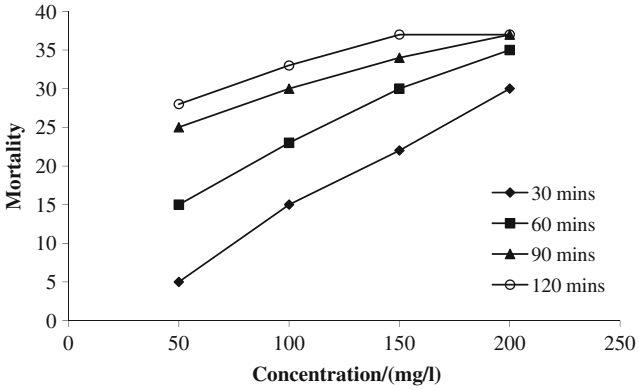

Fig. 1 Parasite mortality against concentrations (milligrams per liter) of *Artemisia annua* at different time intervals

The concentration of *A. annua* in which 50% of the parasites were killed was 100 mg/l within 60 min, and a significant number (about 85%) were killed in 200 mg/l (Fig. 1). In treatment with artemisinin, 50% of the parasites were killed in 70 mg/l after exposure for 30 min, while all parasites were killed with their cells coagulated in 100 mg/l within 60 min of exposure (Fig. 2). Fifty percent parasite mortality was observed in 85 mg/l of artesunate after exposure for 90 min. A mortality rate of about 85% of the parasites occurred when the fish fry were bathed in solution containing 150 mg/l of artesunate for 90 min (Fig. 3). Interestingly, monogenean parasites were all dislodged from their attachment sites before the occurrence of mortality following treatment with artesunate.

It was also observed that the parasite loads were reduced with increasing concentrations of *A. annua* extract as shown in Fig. 1. There was a positive correlation between the number of parasites dislodged from the body surfaces of fish and the time of exposure of fish to the extract. In addition to all of these, an increased agility in the swimming of fish freed from parasites was also observed when compared to their counterparts in the control with all parasites remaining intact.

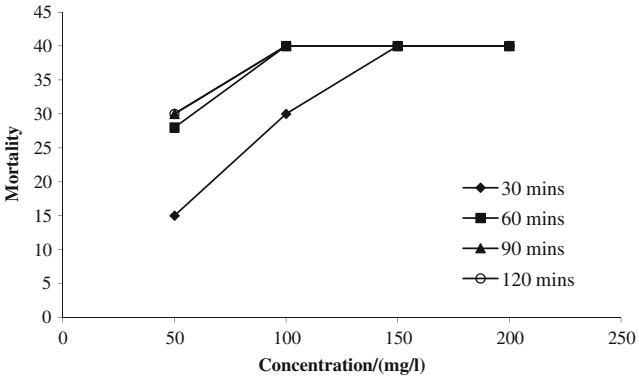

Fig. 2 Parasite mortality against concentrations (milligrams per liter) of artemisinin at different time intervals

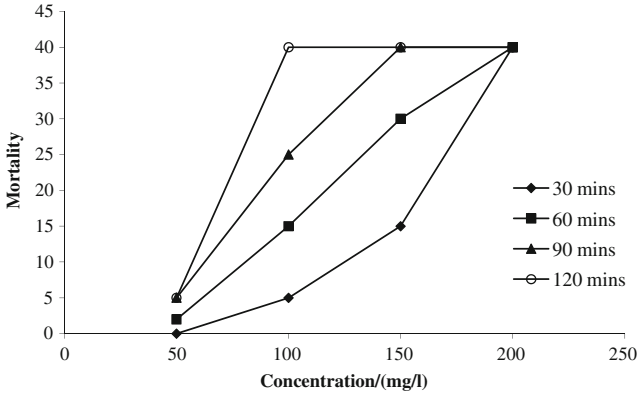

Fig. 3 Parasite mortality against concentrations (milligrams per liter) of artesunate at different time intervals

Results of the toxicity test showed that extract of *A. annua* was well tolerated by *H. longifilis* juveniles. A minimal mortality observation was made throughout the 96-h period of exposure of fish to the extract. A few fish showed weak swimming activity in 350 to 500 mg/l of the test solutions. The highest percentage mortality observed after 96 h in the highest concentration was 20%.

Discussion

The use of artemisinin or any of its semi-synthetically prepared derivatives such as artesunate, dihydroartemisinin, artemether, and arteether or secondary metabolites extracted from the leaves of *A. annua* for the dislodgement of fish ectoparasites has not been reported up until now. The choice of topic for the current study was informed by the fact that the plant contains a beautiful portfolio of secondary metabolites including artemisinin, which has proven to be effective against multi-drug resistant *Plasmodium* strains and other parasitic species in humans, chickens, and small ruminants (Yeung 1985; Jones-Brando et al. 2006; Mishina et al. 2007; Ma et al. 2004; Brisibe et al. 2008; Allen 1997; Ferreira et al. 2006; Hart et al. 2007; Turner and Ferreira 2005).

The results obtained in the current investigation shows that artemisinin, artesunate, and the ethanolic extract of *A. annua* are also effective against monogenean parasites of *H. longifilis.* Their application led to the reduction of the parasite load and even eliminated the parasites completely in some cases within the duration of exposure. During this period, the load of parasites in the control remained the same. So far, several reports have shown the effectiveness of artemisinin, the active substance in *A. annua* against protozoan and helminthic parasites in human, poultry, and small ruminants, respectively (Quinghaosu Antimalarial Coordinating Group 1979; WHO 1981; Yeung 1985; Allen 1997; Brisibe et al. 2008; Ferreira et al. 2006; Hart et al. 2007; Turner and Ferreira 2005). However, this is the first report on its efficacy against fish parasites and perhaps the first against a platyhelminth of fish.

When the parasitized fish fry were exposed to low concentrations of *A. annua* extract for a short period of time (for example, 50 mg/l for 30 min), the extract had little effect on the parasitized fish. Using this same concentration with an increased time of exposure (5 mg/l for 90 min), parasitic load was further reduced, but the parasites were still present. However, increasing the time of exposure further (50 mg/l for 120 min) led to a drastic reduction in the parasitic load to a minimal level. This observation tacitly implies that a low dosage of *A. annua* extract can still be effective for dislodging ectoparasites from fish as long as the duration of exposure to the treatment is increased. This obviously has several advantages over treatments with high dosages using short exposure time.

Of particular interest and significance was the fact that fish exposed to the *A. annua* extracts appeared more active and agile in behavior than their counterparts in the control. This could possibly be explained by the fact that the immune system of these fish may have been boosted as they were free from the effects of the parasites.

Monogenetic trematodes are some of the most threatening parasites of the juvenile catfish in Nigeria and even in developed countries involved in fish importation (Mortensen et al. 2006). Huge economic losses have been reported in situations where appropriate chemotherapeutic agents are not readily employed (Faruk et al. 2004). The commonly used chemical agents for the treatment of this parasite include mebendazole, organophosphate, praziquantel, closantel, dichlorvos, formaldehyde, etc. (Szekely and Molnar 1987; Scott 1993; Moser et al. 1986; Hoffman 1983; Lewbart and Gratzek 1990; Gratzek and Blasiola 1992; Chisholm and Whittington 2002). However, the use of synthetic organic substances in the treatment of diseases in food fish should be discouraged (Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment 1999). Apart from the fear of accumulation in the tissues, their discharges into the aquatic environment may contribute to habitat destruction or degradation. Consequently, there is an urgent need for the development of cheap, nontoxic, and environmentally benign agents for the treatment of such parasite of food fish.

Although the extract of *A. annua* has a wide range of tolerance, administration of the extract in the dislodgement of ectoparasites of fish is done in holding tanks in the hatchery, and the fish are returned to the pond when they have fully recovered from parasites and effects of treatment. Other aquatic organisms do not stand a chance of exposure to the substance.

The extract used in high amount as shown in this study is because it has a wide range of tolerance (therapeutic

index). The dislodgement of fish parasites was effected from 50 mg/l of the extract. It was also observed that within 1 h of exposure at 200 mg/l, 85% of parasites were killed and dislodged from the host without any host mortality. It is also possible to reduce the concentration of extract during treatment but increase the exposure time of fish to the extracts. The reduction in the parasite burden would enhance fast recovery and may pave way for the boosting of host protective immunity (Clark and Dickerson 1997).

Taken together, the results of the present study could lead to the conclusion that the ethanolic extract of *A. annua* leaves has an antiparasitic efficacy against monogenean parasites of cultured Clariidae (*H. longifilis*), possibly due to the medicinal properties of the plant. Consequently, further investigation is recommended on the use of the plant against fish protozoan and other parasites responsible for fish mortalities under culture conditions.

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