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The *in vitro* anthelmintic effects of plumbagin on newly excysted and 4-weeks-old juvenile parasites of *Fasciola gigantica*

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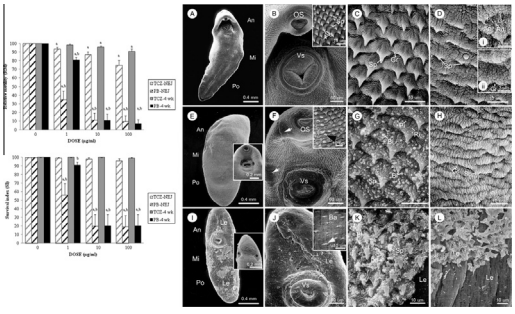
HIGHLIGHTS

- PB showed a greater anthelmintic effect against juveniles of *F. gigantica* than TCZ.
- NEJs appeared to be more affected by PB and TCZ than 4-weeks-old juveniles.
- PB caused greater damage on tegument than TCZ.

GRAPHICAL ABSTRACT



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ABSTRACT

The effect of plumbagin (PB, 5-hydroxy-2-methyl-1,4-naphthoquinone) against newly excysted juveniles (NEJs) and 4-weeks-old immature parasites of *Fasciola gigantica* were compared with triclabendazole (TCZ). The anthelmintic efficacy of 1, 10 and 100 μg/ml of PB or TCZ following incubation *in vitro* for 1–24 h was compared using a combination of relative motility (RM), survival index (SI) and larval migration inhibition (LMI) assays for parasite viability. The RM and SI values of the PB-treated group decreased at a more rapid rate than the TCZ-treated group. For NEJs, the decreased RM values were first observed at 1 h incubation with 1 μg/ml PB, and 90% of flukes were killed at 24 h. In contrast, in TCZ-treated groups a 10-fold higher concentration of TCZ (10 μg/ml) resulted in only 9% dead parasites after 24 h incubation. In 4-weeks-old juvenile parasites, PB reduced the RM value at 10 μg/ml with 100% of flukes dead after 3 h, while TCZ decreased RM values at the concentration of 100 μg/ml but with only 5% of flukes killed at 24 h. NEJs treated with PB exhibited 88%, 99% and 100% of LMIs at the concentrations of 1, 10 and 100 μg/ml, respectively. NEJs incubated with TCZ have an LMI of only 32% at the highest concentration of 100 μg/ml. Similarly PB had a significantly greater killing of immature 4 weeks juvenile stages than TCZ at all concentrations; however, 4-weeks-old juvenile parasites were more resistant to killing by PB or TCZ at all concentrations when compared to NEJs. Further studies were carried out to investigate the alterations of the parasite tegument by scanning electron microscope (SEM). PB caused similar tegumental alterations in 4-weeks-old juveniles as those observed in TCZ treatment but with greater damage at comparative time points, comprising of swelling, blebbing and rupture of the tegument, loss of spines, and eventual erosion, lesion and desquamation of the total tegument. These data indicate that PB had a greater fasciolicidal effect against immature stages of *F. gigantica* parasites than TCZ and warrant further studies for use as a potential new anthelmintic against Fasciola infections.

Abbreviations: DMSO, dimethyl sulphoxide; TCZ, triclabendazole; PB, 5-hydroxy-2-methyl-1,4-naphthoquinone; NEJ, newly excysted juvenile.

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1. Introduction

Fasciolosis is an important global parasitic disease caused by liver flukes of *Fasciola* species. *Fasciola gigantica* is found mainly in tropical countries, where it infects both animals and humans (over 180 million people worldwide are at risk), compromising health and livestock production, causing losses over 3 billion dollars economic loss per year through reduction in weight gain, fertility, meat, milk and wool production of the ruminants (Mas-Coma et al., 2005; Mungube et al., 2006; Spithill et al., 1999; World Health Organization, 2006). At present, anthelmintic drugs are the effective principal method for controlling the fluke infection since effective vaccines are not yet available for the prevention of this disease (McManus and Dalton, 2006; Wedrychowicz et al., 2007). Triclabendazole (TCZ) is the most common drug for controlling this infection in animals and the only drug registered for human use. TCZ is effective against both the adult and the immature larval stages of the parasites through disruption of microtubules of the tegument, thus compromising the integrity of the parasite's tegument (Stitt and Fairweather, 1993). However, resistance to this drug is now rapidly emerging (Duthaler et al., 2010; Moll et al., 2000; Overend and Bowen, 1995), and there are no other compounds which have a satisfactory efficacy against the juvenile migrating stages of the parasites which cause the most severe acute form of the disease and there is an urgent need to find new compounds with efficacy against migrating stages of Fasciola.

Plumbagin (PB, 5-hydroxy-2-methyl-1,4-naphthoquinone) is a natural quinonoid compound extractable from roots of many plants, especially the *Plumbago* species of plants. Evidence suggest that this compound has been used as a traditional medicine for treatments of several ailments (Satyavati et al., 1976 Wuttidhammaved, 1997), and was reported to have antioxidant, anticancer, antimicrobial, and anti-inflammatory activities (Kini et al., 1997; Krishnaswamy and Purushothaman, 1980; Reese et al., 2010; Selma Ribeiro de Paiva et al., 2003; Sharma et al., 2009; Talcott et al., 1985). Recently, we have shown this compound has antihelminthic effects on several parasites *in vitro* including *Schistosoma mansoni* (Lorsuwannarat et al., 2013) and *Paramphistomum cervi* (Saowakon et al., 2013). However, there is no report of the potential fasciolicidal effect of this compound on *Fasciola* spp., and more specifically on immature stages of Fasciola.

In this study, we investigated the *in vitro* anthelmintic effect of PB against NEJ and 4-weeks-old immature juveniles of *F. gigantica* and compared the efficacy against the market leader drug, TCZ, by evaluating viability of the parasites using the relative motility (RM), survival indices (SI) and the percentage of larval migration inhibition (%LMI) assays. We found PB had a superior antihelminthic effect on NEJ and 4 weeks old parasites compared to TCZ when compared at equivalent concentrations. In addition, we investigated the potential mode of action by studying the tegumental surface alterations of the parasites using scanning electron microscopy.

2. Materials and methods

2.1. Parasites

2.1.1. Newly excysted juveniles (NEJ) of F. gigantica

NEJs were obtained from metacercariae of *F. gigantica*. The metacercariae were collected from the snails, *Lymnaea ollula*, infected with miracidia hatched from eggs of adult *F. gigantica* collected from the bile ducts and gall bladders of the cattle and buffaloes at a slaughter house in Pathum Thanee Province, Thailand. The metacercariae were gently brushed from the cellophane paper under stereomicroscope, and then washed with distilled water three times. *In vitro* excystment of metacercariae was performed according to the method of Wilson et al. (1998). The active NEJs migrating through the mesh of the excystment tower into a tissue culture dish (Nunc, Sigma-Aldrich) were collected in M-199 medium (Sigma Co.) at 37 °C to be used in motility, survival and migration inhibition experiments.

2.1.2. 4-Weeks-old juveniles

Juvenile parasites were collected from infected ICR mice 4 weeks post infection. Male and female 6 weeks old mice, weighing approximately 25–40 g, were purchased from National Laboratory Animal Center, Nakhon Pathom Province, Thailand. Metacercariae of *F. gigantica* were collected from infected *Lymnaea ollula* snails. After 1 week of adaptation, each mouse was infected with approximately 30 metacercariae by oral gavage and kept at the Center of Animal Facilities (CAF), Faculty of Science, Mahidol University. Four week after infection, the mice were sacrificed, and the parasites were collected from the minced liver. Then they were washed several times with 0.85% NaCl solution. Only intact and actively mobile flukes were used immediately for the *in vitro* incubation with the drugs. All the animal protocols had been approved by the Animal Ethic Committee, Faculty of Science, Mahidol University.

2.2. Drugs and media

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) was purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). The stock solution of plumbagin (PB) was made by dissolving 100 mg powder PB in 1 ml dimethyl sulphoxide (DMSO) (Sigma Co.). The PB concentrations used in this experiment were based on the study of Lorsuwannarat et al. (2013) and Atjanasuppat et al. (2009), which reported half maximal inhibitory concentrations (IC_{50}) of *Schistosoma mansoni*, *Caenorhabditis elegans* and *Paramphistomum epiclitum in vitro* between 0.95 μg/ml, 9.71 μg/ml and 130 μg/ml, respectively. A commercial anthelmintic triclabendazole (TCZ) (Fasinex® 10%, Novartis) was used as the positive control. The M-199 medium (Sigma Co.) containing antibiotics (penicillin 50 IU/ml, gentamycin 30 IU/ml), was mixed with the drug stock solutions to obtain the required concentrations. The final concentrations of PB and TCZ in the medium were at 1, 10, 100 μg/ml for motility and survival experiments and 0.001, 0.01, 0.1, 1, 10, 100 μg/ml for migration inhibition test. All chemicals used in the experiment were of analytical grade.

2.3. Assessment of drug activities

2.3.1. Relative motility (RM) values and survival indices (SI)

NEJ and 4-weeks-old flukes collected as above were randomly assigned to seven experimental groups in tissue culture dishes (300 and 50 flukes per group for NEJ and 4-weeks-old, respectively): group 1 was the negative control with the parasites incubated in Medium-199 containing 0.1% (v/v) DMSO. Groups 2-4 were positive controls with the parasites incubated in the medium containing TCZ at 1, 10 and 100 μg/ml. Groups 5-7 were experimental groups incubated in the medium containing PB at the same concentrations. The parasites were incubated for 1, 3, 6, 12 and 24 h in an incubator with 5% CO_2 at 37 °C. Each experiment was repeated twice. The motility scores were evaluated by examining the flukes under a stereomicroscope using the criteria modified from a method described by Kiuchi et al. (1987), i.e., 3 = active movement of the whole body, 2 = movement of only some parts of the body, 1 = immobile but not dead, and unstained with a vital dye (1% methylene blue in 0.85% NaCl solution), and 0 = immobile and stained with the vital dye (dead). For NEJs, scores were evaluated using the following criteria, 2 = movement of the body,