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Fasciola gigantica: Anthelmintic effect of the aqueous extract of *Artocarpus lakoocha*

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ABSTRACT

The effect of the crude extract of *Artocarpus lakoocha* (70% composition is 2,4,3′,5′- tetrahydroxystilbene -THS) on adult *Fasciola gigantica* was evaluated after incubating the parasites in M-199 medium containing 250, 500, 750 and 1000 μg/ml of the crude extract, or triclabendazole (TCZ) at the concentrations of 80 and 175 μg/ml as the positive control, for 3, 6, 12 and 24 h, using relative motility (RM) assay and observation by scanning electron microscope (SEM). Decreased contraction and motility were first observed after 3 h incubation with TCZ at the concentration 80 and 175 μg/ml. TCZ markedly reduced the parasite's motility at the concentration of 175 μg/ml at 6 h, and killed the worms after 12 h exposure. The crude extract of *A. lakoocha* at all concentrations reduced the parasite's motility similar to TCZ at 3 h incubation. In 250 and 500 μg/ml of the crude extract, the values were decreased from 3 to 12 h, then they were stable between 12 and 24 h and reduced to the level approximately 30–40% of the control. At 750 and 1000 μg/ml concentrations the crude extract rapidly reduced the RM values from the start to 12 h and killed the parasites between 12 and 24 h incubation. The crude extract also inhibited the larval migration by 75% and 100% at the concentrations of 250–500 and 750–1000 μg/ml, respectively. TCZ and the crude extract caused sequentially changes in the tegument including swelling, followed by blebbings that later ruptured, leading to the erosion and desquamation of the tegument syncytium. As the result, lesion was formed which exposed the basal lamina. The damage appeared more severe on the dorsal than the ventral surface, and earlier on the anterior part and lateral margins when compared to the posterior part. The severity and rapidity of the damages were enhanced with increasing concentration of the crude extract. Hence, the crude extract of *A. lakoocha*, may exert its fasciolicidal effect against adult *F. gigantica* by initially causing the tegumental damage.

1. Introduction

Fasciolosis, caused by the liver fluke of *Fasciola* spp., is a serious disease that causes substantial production and economic losses in sheep, cattle and other ruminants in many countries (Fairweather and Boray, 1999; Rivera et al., 2004). As it is a zoonotic disease, human may also be infected; thus it is considered to be a serious public health problem as well (Wiwanitkit et al., 2002). It has been estimated that 2.4–17 million people are infected and more than 90 million people are at risk (Keiser et al., 2005). The infection has caused loss worldwide estimated at US$ 2000 million per annum (McManus and Dalton, 2006; Spithill and Dalton, 1998). In Thailand, the annual loss, has been estimated around 350–400 million baht, mainly due to weight and meat loss, reduction in dairy product and fertility of the animals (Sukhapesan et al., 1990). At present, effective vaccines are not yet available (López-Abán et al., 2007; Sexton et al., 1990; Sobhon et al., 1998; Wedrychowicz et al., 2007), therefore, anthelmintic drugs are the main method employed for controlling the fluke infection. Triclabendazole (TCZ) is the drug of choice and the most effective flukicide against both juvenile and adult flukes, and it has been used to control fasciolosis since 1983 (Keiser et al., 2007a). However, the resistance to this drug has emerged and may pose a serious problem as no other effective drug is available (Fairweather, 2005; Keiser et al., 2007b). Thus new anthelmintics are urgently needed. In this report we have investigated a novel anthelmintic effect of the extract from *Artocarpus lakoocha* Roxb. a medicinal herb commonly used by indigenous people in Thailand and Laos as anthelmintics (Charoenlarp et al., 1981, 1989). The brown powder (called Puag-Haad in Thai) is a product of the aqueous extraction of *A. lakoocha* Roxb. prepared by boiling the wood chips and then evaporating water

Abbreviations: DMSO, dimethyl sulphoxide; THS, 2,4,3′,5′-tetrahydroxystilbene.

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away. This preparation has been used as a traditional anthelmintic drug for treatment of tapeworm infection in Thailand. Seventy percent of the crude aqueous extract of *A. lakoocha* is composed of a phenolic compound, trans-2,4,3′,5′-tetrahydroxystilbene (THS) (Likhitwitayawuid et al., 2006; Mongkolsuk et al., 1957; Poopyruchpong et al., 1978). Recent studies reported that THS exhibited antiherpetic, anti-HIV (Likhitwitayawuid et al., 2005), anti-inflammatory (Chung et al., 2003), anti-oxidation (Lorenz et al., 2003), and anti-apoptotic activities, and it also showed neuroprotective action (Andrabi et al., 2004). Furthermore, the chemical structure of THS is similar to that of halogenated-phenolic fasciolocides. Hence, it is possible that THS could act as a drug for the treatment of liver fluke infection in cattle and human, as it was shown in a previous *in vitro* study that the crude extract of *A. lakoocha* could inhibit motility and caused morphological changes in the tegument of the fish trematode, *Haplorchis taichui* (Wongsawad et al., 2005). Hence, the aims are to investigate the effects of the crude extract of *A. lakoocha* on motility, tegumental surface changes, and the killing of adult *F. gigantica* following the *in vitro* incubation, and on migration of the newly excysted juvenile (NEJ).

2. Materials and methods

2.1. Parasites

Adult liver flukes were collected from the bile duct and gall bladder of cattle which were killed at a local slaughter house in Phatumthani province, Thailand. They were washed several times with 0.85% NaCl solution. Only intact and actively mobile worms were used immediately for this study.

2.2. Drugs

The crude extract of *A. lakoocha* was prepared in the Department of Chemistry, Faculty of Science, Mahidol University, Bangkok, Thailand (lot no. VR-11817). The concentration used in this study was based on the report by Wongsawad et al. (2005), which demonstrated that the lowest concentration of the crude extract that could killed the adult *H. taichui in vitro* was 250 μg/ml. The stock solution was prepared by dissolving 6 g powder of the crude extract in ten milliliters of dimethyl sulphoxide (DMSO). The M-199 medium (Sigma) containing antibiotics (penicillin 50 IU/ml, streptomycin 50 μg/ml, gentamycin 30 IU/ml), was mixed with the stock solution so that the final concentrations of the crude extract in the medium were at 250, 500, 750, and 1000 μg/ml for use in this experiment. A commercial anthelmintic, triclabendazole (TCZ) (Fasinex® 10%, Ciba Geigy) was dissolved in the medium at concentrations of 80 and 175 μg/ml and used as the positive controls. The latter concentration of TCZ was equivalent to 175 μg/ml of THS in the crude extract at the concentration 250 μg/ml, as THS makes up 70% of the crude extract (Mongkolsuk et al., 1957; Poopyruchpong et al., 1978). The negative control was carried out by incubating the worms in the culture medium containing 0.1% (v/v) DMSO and antibiotics similar to those listed above.

2.3. Assay methods for drug's activities

2.3.1. Motility assay of adult fluke

Two hundred and eighty adult flukes were randomly assigned to seven groups (40 flukes per group): group 1 was the negative control and groups 2–3 were treated with TCZ as the positive controls. Flukes in groups 4–7 were incubated in M-199 culture medium containing various doses of the crude extract as mentioned above. The worms were incubated for 24 h in an incubator with 5% CO_2 at 37 °C. After 3, 6, 12, and 24 h incubation time (10 parasites per each observation time), motility was assessed by examination under a stereomicroscope, and the tegumental alterations were observed under light (LM) and scanning electron (SEM) microscopes.

Motility scores were assigned by using the following criteria: 3 = movement of the whole body, 2 = movement of only part of the body, 1 = immobile but not dead and unstained with the vital dye – 1% methylene blue in 0.85% NaCl solution, and 0 = immobile and stained with the vital dye. The efficacies of the tested drugs against adult *F. gigantica* were calculated as the relative motility (RM) value using the formula listed below (Kiuchi et al., 1987). A small RM value indicated stronger drug activity, and when all flukes died this value was 0.

$$\text{Motility index (MI)} = \frac{\sum nN}{N}$$

$$\text{RM value} = \frac{\text{MI test} \times 100}{\text{MI control}}$$

n = motility score, N = number of flukes with the score of n.

2.3.2. Larval migration inhibition assay

The metacercariae of *F. gigantica* were collected from infected snails, *Lymnaea rubiginosa*. Metacercariae were excysted, and the newly excysted juveniles (NEJs) were separated from the empty cyst walls, unexcysted metacercariae and debris by transferring to the excystment tower placed within the 24-well plate and incubated at 37 °C as previously described (Wilson et al., 1998). The active NEJs migrated through the membrane of the excystment tower fitted in the well of the microplate. After NEJs migrated through the membrane of the excystment tower, they were collected in a fresh M-199 medium containing the antibiotics as mentioned earlier. The 400 active NEJs were added to each 15 ml test-tube containing 1 ml medium and the crude extract of *A. lakoocha* at the concentration 250, 500, 750 and 1000 μg/ml, or TCZ at the concentration 175 μg/ml. The NEJs incubated in the culture medium containing 0.1% (v/v) DMSO and antibiotics were used as the negative control. The NEJs were incubated at 37 °C for 2 h. Then, each sample was transferred to the excystment tower fitted within 24-well plate. Any air trapped between the tower and plate was removed by tapping the plate. The plates were covered with lids and were incubated at room temperature for overnight. Then, the excystment towers were removed, and NEJs that passed through the membrane of the excystment tower into each well were counted and the percents of migration inhibition were calculated according to the formula shown below, and each experiment was repeated three times.

$$\%\ \text{larval migration inhibition} = \frac{(\text{number of controlled NEJ} - \text{number of tested NEJ}) \times 100}{\text{number of controlled NEJ}}$$

Number of controlled NEJ = number of NEJs in control solution without the drugs that migrated through the excystment tower into the collecting well.

Number of tested NEJ = number of NEJs in drug-treated group that migrated through the excystment tower into the collecting well.

2.3.3. Observations of tegumental changes by LM using semithin sections, and SEM

For LM and SEM studies another two hundred and eighty adult flukes were divided into seven groups and treated similarly as mentioned in Section 2.3.1. At 3, 6, 12, 24 h, 5 flukes were taken