



Effects of *Areca catechu* Linn. extract on motility of *Opisthorchis viverrini*

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Abstract

In this study, the effects of crude extract from *Areca catechu* L. against the motility of newly-excysted juveniles (NEJs) *O. viverrini* on relative motility (RM) assay, Eggs per gram of feces and worm recovery were studied. The liver flukes were divided into 2 experiment groups. Group 1 was control group (untreated), and group 2 was incubated in RPMI-1640 medium containing 200 µg/ml, 1 mg/ml, 5 mg/ml and 10 mg/ml of crude extract. After *in vitro* experiments, the RM values were evaluated after 0, 30, 60, 90, 120, 150 and 180 minutes of incubation, using scoring under the stereomicroscopy. The results showed at 0 minute (before treatment), NEJs of *O. viverrini* has an active. The crude extract of *A. catechu* L. at all concentrations showed rapid decrease of motility at 30 minutes incubation. The initial reduction of motility occurred at 30 minutes and more reduction at 60 minutes in *A. catechu* L extract at the concentration of 200 µg/ml, 1 mg/ml and 5 mg/ml. No motile parasites were observed when they were incubated with 10 mg/ml of crude extract for 150 and 180 minutes. Fecal egg outputs and worm burden form infected Syrian golden hamsters were significantly decreased in *A. catechu* L. extract treated group when compared to control group (infected with no treated). Results from the present study suggested that *A. catechu* L. extract has the effects of inhibition of the liver fluke movement. Therefore, *A. catechu* L. may be further developed into herbals treatment for treating parasitic diseases.

Keywords: *Opisthorchis viverrini*, *A. catechu* L., motility, anthelmintic herb

1. Introduction

Opisthorchiasis caused by *Opisthorchis viverrini* (*O. viverrini*) infected is still a major public health problem for people in Thailand especially in northeast, including the Lao People's democratic Republic (PDR), Cambodia and Vietnam (Jongsuksuntigul & Imsomboon, 2003). In Thailand, this well-known food-borne trematode is widely endemic mainly in northeastern regions, particularly in Khon Kaen Province (Humans, 1994). Infection occurs when people who, for cultural and personal reasons, eating uncooked food and raw fish that contaminated with infective stage (metacercariae) of *O. viverrini*. After ingestion, the metacercariae excystation in the duodenum, newly-excysted juveniles migrate from the duodenum and develop where they become adults in the biliary system. The pathogenic effects of the parasite cause acute and chronic bile duct infection. There is currently clear evidence that the infection for a long time can induce to cholangiocarcinoma (CCA) (Sripa et al, 2007; Sripa & Pairojkul, 2008). The drug that is effective in treatment of liver fluke is praziquantel which is reported to cause side effects such as nausea and vomiting (Jaoko, Muchemi, & Oguya, 1996). It also causes inflammation if used frequently in cases of recurrent infections.

Areca catechu Linn. (*A. catechu* L.), in Arecaceae family, is an important herbal medicine used in China for the treatment of many diseases, especially parasitic diseases, digestive function disorders, and depression. The major constituents are alkaloids, tannins, flavones, triterpenes, steroids, and fatty acids. Moreover, alkaloids has been reported to be the main and active component of *A. catechu* L. (Peng et al, 2015). These compounds have many pharmacological activities including several bioactivities including anti-parasitic effect (Tian et al, 2002), digestive effect (Li, Fan, Lv, Wei, & Hu, 2013; Xi-jun, 2009), anti-depressive effect (Dar & Khatoon, 2000), anti-oxidant effect (Bhandare, Kshirsagar, Vyawahare, Hadambar, & Thorve, 2010), anti-bacterial effect (Boniface, Verma, Cheema, Darokar, & Pal, 2014), anti-inflammatory



and analgesic effects (Khan et al, 2011). Due to the various properties of *A. catechu* L., researcher is interested to study extract from *A. catechu* L. in order to study the effects of anti on *O. viverrini* activity.

2. Objectives

To study effects of *A. catechu* Linn extract on motility of *Opisthorchis viverrini*

3. Materials and Methods

3.1 Preparation of Metacercariae

The metacercariae of *O. viverrini* were obtained from naturally infected cyprinoid fish captured. The fish were digested with artificial gastric juice, a solution of 0.25% pepsin A and 1.5% HCl and incubate at 37°C for an hour. The digested mixture was strained through a set of four sieves with the mesh size of 1100, 350, 140 and 250 µm, respectively. Then the pellet on the last sieve was sedimented in normal saline solution (NSS) in sedimentation jar until the supernatant was clear. *O. viverrini* metacercariae were collected and counted under dissecting microscope and kept in NSS at 4°C until for used.

3.2 Effects of *A. catechu* L. on motility of newly-excysted juveniles (NEJs)

Thirty metacercariae added into 96-well plates were excysted with 0.25% trypsin, and the NEJs were separated from the empty cyst walls. NEJs were incubated in RPMI-1640 medium containing various doses of the crude extract serial concentrations at 200 µg, 1, 5, and 10 mg/ml. The worms were incubated at 37°C. After 30, 60, 90, 120, 150 and 180 minute of incubation time, observations were made for the time taken to paralyze and / or death of individual worm up to three hours of test period by examination under a stereomicroscope.

Motility scores were assigned by using the following criteria: 2 = movement of the whole body, 1 = movement of only part of the body, 0 = immobile and dead. The efficacies of the tested drugs against NEJs were calculated as the relative motility (RM) value using the formula listed below (Lorsuwannarat, Saowakon, Ramasoota, Wanichanon, & Sobhon, 2013). A small RM value indicated stronger drug activity, and when all flukes died this value was 0

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

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

n = motility score, N = number of flukes with the score of “n”

The control group where all the parasites scored 2 had the RM value of 100, and the smaller RM values indicated stronger drug activity.

3.3 Infection hamster model

Female Syrian golden hamsters, aged 6-8 weeks, obtained from the Animal Unit, Faculty of Medicine, Khon Kaen University were used in this experiment. The hamsters were divided into 2 groups (4 hamsters/group). Each animal was infected with 50 metacercariae in 1 ml NSS by intragastric intubation under light anesthesia with isoflurane and then put into group. Group 1 was control group, untreated; group 2 were fed with crude extract. At day 90 post-infection, all animals were euthanized with isoflurane in a fume hood. *O. viverrini* adults were collected from the livers and worm recovery calculated. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Khon Kaen University, based on the Ethic of Animal Experimentation of National Research Council of Thailand (no. IACUC-KKU-65/61).



3.4 Eggs per gram of feces

To determine the indirect reproductive organ development, feces from hamsters after infected 3 weeks were collected for detection of eggs per gram of feces. Modified formalin technique was performed for the quantitative *O. viverrini* egg count (Songsri et al, 2016). One pellet of feces from the rectum was weighed, fixed, and mixed thoroughly with 1,000 μ l of 10% formalin. The solution (100 μ l) was smeared with 1% iodine solution on a glass slide, and the number of *O. viverrini* eggs was counted under a stereomicroscope. The procedure was repeated 3 times, and the results were averaged. The number of eggs per gram was calculated as follows:

$$\text{Eggs per gram (EPG)} = \frac{\text{Number of Opisthorchis eggs} \times 1,000}{\text{Feces weight (g)} \times 100}$$

3.5 Worm recovery

Whole liver of infected Syrian hamster in saline were individually dissected for finding and counting adult worms.

4. Results and Discussion

4.1 Motility score of newly-excysted juveniles (NEJs)

The metacercariae of *O. viverrini* were collected from infected cyprinoid fish. Metacercariae were excysted (Figure 1), and NEJs were separated from the empty cyst walls (Figure 2). All NEJs in the control group showed active movement throughout the experimental period (RM = 100). The tested NEJs were incubated in the crude extract various concentrations. The results shown that *A. catechu* L. extract at all concentrations showed rapid decrease of motility at 30 minutes incubation. All NEJs exposed to 200 μ g/ml of the crude extract for 180 minutes, which results in a slight decrease in motility. The initial reduction of motility decreased RM value occurred at 30 minutes in crude extract at the concentration of 1 mg/ml and the more decrease at 60 min, then RM value declined gradually from 60 minutes until 150 min incubation, then RM value was the lowest stable between 150 and 180 min. In 5 mg/ml of the *A. catechu* L. extract, the NEJs exhibited gradual reduction of RM value at 30 minutes, and the more decrease at 60 minutes, then RM value declined gradually from 60 minutes until 180 minutes incubation. NEJs incubated in crude extract at the concentration of 10 mg/ml, exhibited reduced motility at a more rapid rate than the previous dose, as the RM value dropped rapidly from the start point, and the parasites became completely immobile and killed at 150 minutes as shown in Figure 3. Our study shows the same result as *Areca catechu*, *Tamarindus indica* Linn, *Daucus carota* L., *Nigella sativa* Linn, *Ocimum tenuiflorum* that can inhibit the spontaneous motility i.e. Paralysis of Earthworms (Goswami et al, 2016).

4.2 Eggs per gram of feces

In the average egg per gram of feces from the hamster model in treated group with *A. catechu* L., the worms were mature and began to produce eggs after 2 weeks post-infection. The eggs were found in faeces after 4 weeks. The results shown that the number of eggs in the treated group was significantly less than the control group. Moreover, the number of eggs were dramatic dropped when feeding *A. catechu* L. on day 51, 57-69 (Figure 4). In our study, the *A. catechu* L. may be disrupted muscle function of *O. viverrini* because muscle contractions of *O. viverrini* is required for reproduction and egg laying.

4.3 Comparative gross pathology of liver

Observation of the gross pathology of the livers revealed few differences between the control groups (*O. viverrini* infection) and treated group (*O. viverrini* infection and treated with *A. catechu* L.). In control group, liver surfaces were smooth and shiny, with slightly opaque common bile ducts and straw-colored in gallbladder and common bile duct that may be caused by inflammatory reactions surrounding the worms at bile duct (Figure 5A). In the *A. catechu* L. treated group, liver surface, common bile duct and common bile duct gallbladders were more translucent and healthier than control group (without treatment) (Figure 5B). The



severe pathogenic liver lesion in gross specimen was not clearly found in both groups because gross inflammatory lesion should be observed in long term infection and it depends on the number of worms. However, the histopathology observation should be additional in further study (Wonkchalee et al, 2012).

4.4 Comparative worm burden

The comparative average worm burden between control and treated group showed that worm recovery in treated group were significantly less than control group. The result from the hamster infected with 50 metacercaria of *O. viverrini* for 2 months showed that the average of adult worms obtained from group of *O. viverrini* infections were about 22 ± 3.77 , while the average of adult worms in *O. viverrini* infection and treated with *A. catechu* L. group were 11 ± 4.51 . The infectivity rate of decrease adult number (% reduction) was 50%. All adult worms were most obtained from the common bile duct, gallbladder, and hepatic bile ducts (Table 1).



Figure 1 Photographs of *O. viverrini* mature metacercaria



Figure 2 Photographs of *O. viverrini* newly-excysted juveniles

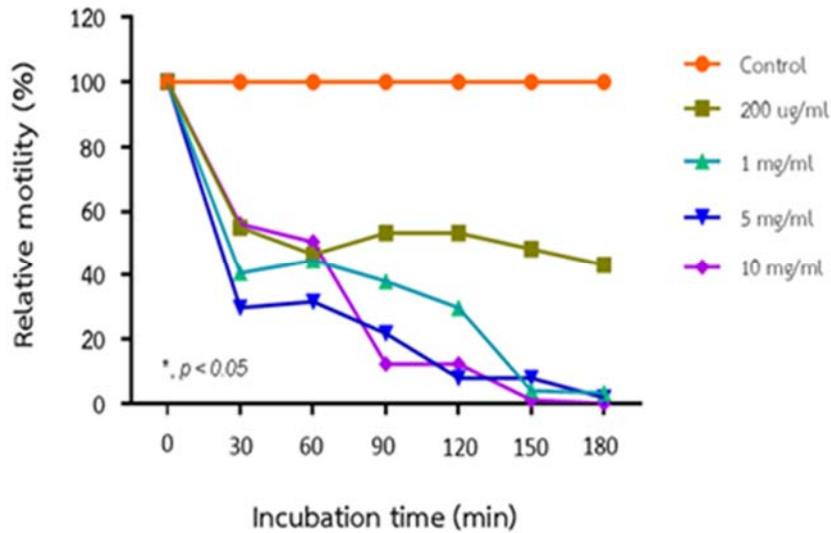


Figure 3 Relative motility (RM) values of the control and the experimental of NEJs treated with crude extract of *A. catechu* L. at various concentrations and times

Table 1 Effects of *A. catechu* L. on worm burden and worm reduction

Groups	Worm burden (mean \pm SD)	Worm reduction (%)
Control	22 \pm 3.77	-
<i>A. catechu</i> L.	11 \pm 4.51	50

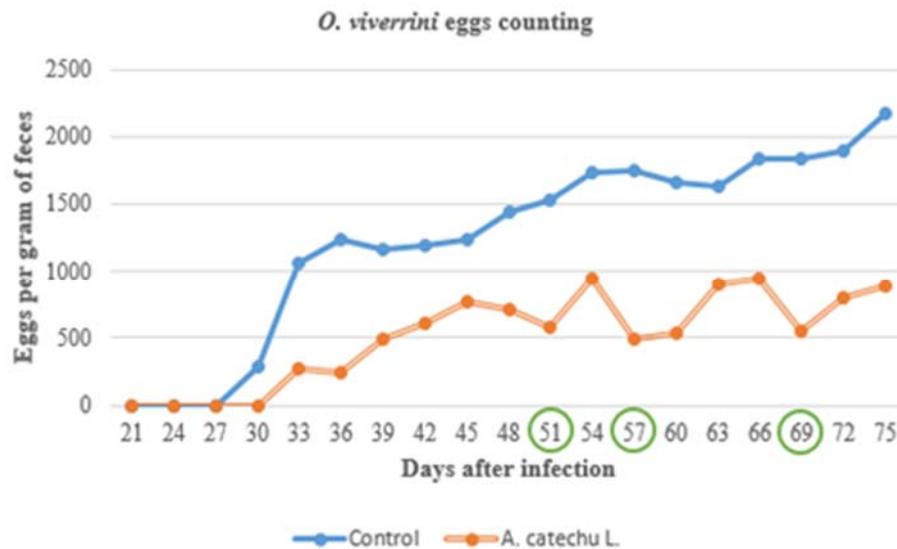


Figure 4 Average no. of eggs per gram of feces collected

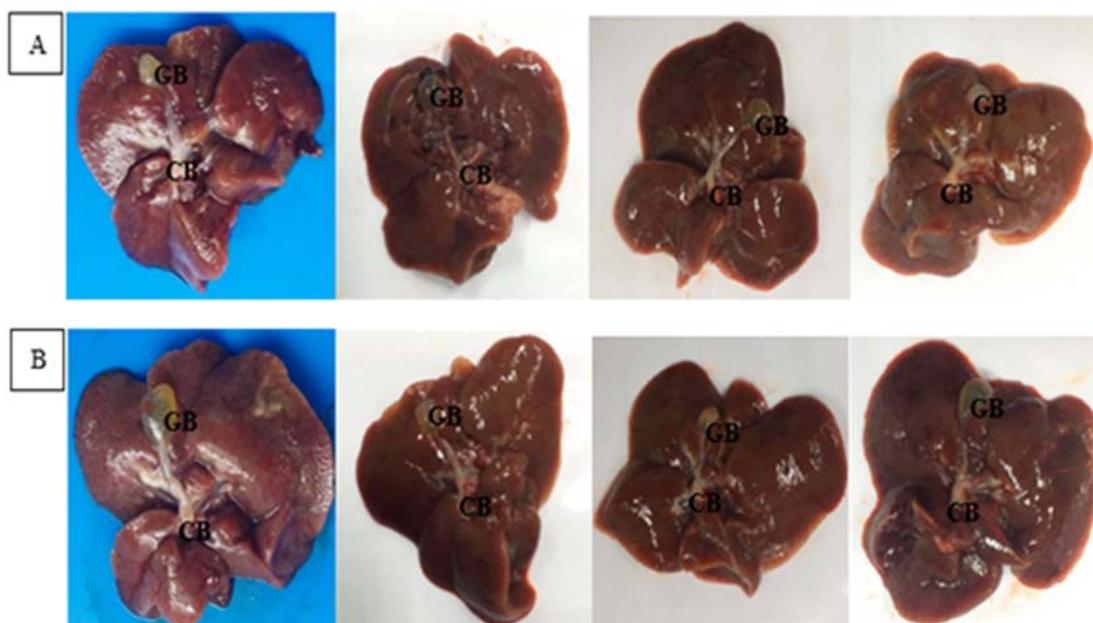


Figure 5 Gross appearance of the color and surface liver in the group of *O. viverrini* infection (A), *O. viverrini* infection treat with *A. catechu* L. (B), GB gallbladder, CB common bile duct

5. Conclusion

The result of this study may conclude that *A. catechu* L. extract has the effects of inhibition to the muscle function of the liver fluke due to decrease muscle contraction. Consequently, *A. catechu* L. extract can decrease movement, locomotion, laying eggs, migration of *O. viverrini*. Muscle function is the most essential activity of worm survival. Subsequently, the disruption of muscle contraction is a cause of death in worm. Therefore, *A. catechu* L. should be further developed into herbal treatment for treating parasitic diseases.

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7. References

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