Effect of arecoline, aqueous and methanolic areca nut extracts on rumen fluke

N Puyathorn1, C Sukonpan1, P Toungsuwan2, W Narakornwit1, T Phaechamud1*

1Faculty of Pharmacy, 2Faculty of Education, Silpakorn University, Nakhon-Pathom, Thailand. 73000.

*Corresponding author: phaechamud_t@su.ac.th

Abstract Paramphistomosis is a parasitic disease that causes a reduction of livestock production and economic losses worldwide. In order to avoid side effect and chemical residue from usage of chemical drugs, medicinal plant is an alternative drug for treatment. Areca nut (Areaca catechu) which one of the domestic plants in Southeast Asia had antihelmintic activity. In this study, arecoline, aqueous and methanolic extracts of areca nut prepared in solutions were tested for antihelmintic activity against rumen flukes (Paramphistomum sp.). The parasites were incubated in a culture media (RPMI-1640, pH 8) at 39°C in a carbon dioxide-incubator containing 0.01, 0.1 and 1 mg/mL of the tested compounds for 15, 30 min, 1 h and 24 h. The results showed that the methanolic and aqueous crude extracts from seeds of Areaca catechu and arecoline HBr at concentration of 1 mg/mL solutions could destroy the tegument (surface membrane) of the flukes and killed them all at initial time. Thus, these areca nut extracts exhibited the potential alternative medicine with antihelmintic activity against rumen flukes.

1. Introduction Paramphistomosis is an infectious disease caused by rumen fluke in Paramphistomum sp. This disease is one of a serious disease occurred in buffaloes, cattle, goats and sheep worldwide [1]. This parasitosis has been found in domestic and wild ruminants caused by trematode. In the early stage of infection, young flukes are located in the small intestine and abomasums; subsequently they move to the rumen to finally lodge as adult trematodes [2,3]. The parasites caused the infected animals to diminish their appetite and delay the growth from malnutrition and death finally if untreated. This leads to a reduced livestock production and an economic loss [4]. Excessive usage of chemical drug will increase a production cost and leave some chemical residues in the animal products. To avoid these effects, herbal medicine is interested [5]. Areca catechu Linn or areca palm is an endemic plant in tropical Pacific and Southeast Asia. Its seeds that called areca nut or betel nut or “MAK” in Thai (Figure 1A and B) had been chewed along with betel leaf and also used in Ayurvedic medicine since ancient times [6]. One of major active alkaloids found in areca nut is arecoline (methyl-1, 2, 5, 6-tetrahydro-1-methyl-nicotinate) (Figure 1C) which has various pharmacological activities [7]. One of the interesting activities is antihelmintic activity. Arecoline HBr is a white crystalline powder or needle-shaped crystal with a melting point of 169-171°C, odorless and bitter in taste, soluble in water and alcohol [8]. Arecoline (Figure 1C) is used as veterinary medicine as antihelmintic compound to diminish parasites [7,8]. It was found that betel nut extract was able to control the liver fluke of goats. The aim of this study was to investigate the effect of aqueous and methanolic crude extracts from areca nut as well as arecoline HBr on mobility and modification of tegument of rumen flukes.
2. Experimental

2.1 Collection and selection of rumen fluke
The adult rumen flukes were selected and treated before use as following procedure. Live rumen flukes (Paramphistomum sp.) were directly collected from the rumens of infected cattle from a local/domestic slaughter house in Nakhon Pathom, Thailand. The collected flukes were cleaned with 0.85 % NaCl solution for several times prior to immerse in the culture media (RPMI-1640, pH 8) and subsequently incubated at 39°C in a carbon dioxide-incubator (CB150/210, BINDER, England).

2.2 Preparation of crude extracts of areca seed
Fresh ripe areca nuts from local orchard in Nakhon Pathom, Thailand were collected and its voucher specimen has been deposited at the Department of Pharmacognosy, Silpakorn University, Nakhon Pathom. The nuts were cleaned and removed all fibrous husk to get the seed. The nut and meat part inside the husk are shown in Figure 1A, B. The seeds were chopped into small pieces and dried in a hot air-blowing oven at 50°C. The dried samples were subsequently powdered by passing through 30 mesh stainless screen of cutting mill and kept at 4°C. Methanol extract and water extract of areca seed were prepared by immersing the seed powder in methanol and distilled water, respectively, in the ratio of the powder to solvent 1:2 for 24 h. The solvents were removed under the vacuum until dry. The crude extracts were storage in dry and cool place prior to test.

Arecoline HBr (Figure 1C) was purchased from Sigma chemical, USA.

2.3 Antihelmintic activity of crude extracts of areca seed and arecoline
Antihelmintic activity was tested by two studies: movement behavior and modification of tegumental surface after exposing to the extracts at different designed times. About 50 adult flukes were placed into 10 petri dishes. One plate was assigned as a control group that did not added any sample solutions and the rest were experimental group that separately treated the flukes with the aqueous and methanolic extracts of areca seed and arecoline HBr at the concentrations of 0, 0.01, 0.1, 1.0, and 10 mg/mL, respectively. Movement behavior and physical appearance of the flukes in each plate were visually observed and recorded at 15, 30, 60 min, 12 h and 24 h, respectively. After finished the final observation, the flukes from the control group and the experiment groups were taken and soaked with Bouin’s solution for fixing prior to further preparing paraffin embedded specimens. At the end of the embedded process, the flukes in the paraffin block were cut by rotary microtome to get 6 µm-thick specimens. After paraffin removing and rehydration, the specimen sections were submitted to Harris’s hematoxylin staining and further observed tegumental modification of the flukes under a stereoscope (Miotic SMZ-171 Series, Hong Kong).

3. Results and discussion
The percentage yields of aqueous and methanolic crude extracts of areca nut were 23.35% and 23.41%, respectively. After incubated the flukes in media containing the extracts and arecoline HBr with various concentrations, the changing of movement behavior and physical appearances of the flukes were observed at assigned time (as shown in Table 1). At 1 mg/mL concentration, two crude extracts and
arecoline HBr could kill all flukes in the plates. All parasites stopped moving immediately after adding the test solutions and their bodies were swelling after left them in the extract solutions for 24 h (Figure 2 C). At 0.1 mg/mL concentration, they showed a slightly different result after 24 h of incubation. All parasites stopped moving immediately after adding the test solutions but, at 24 h-incubation, about 10.7% and 14.5% of parasites in the plates containing aqueous extract and methanol extract, respectively, started moving slowly. At 0.01 mg/mL concentration, arecoline HBr showed better result in stopping parasite mobility. Arecoline HBr could halt the moving for longer than the extracts did since this substance has been reported as anthelmintic compound [7,8]. Only 5% of parasites recovered and started moving slowly after incubated them with 0.01 mg/mL of arecoline for 24 h while all of parasites incubated with the extracts started moving again at 15 min (Table 1). The results from the regressive hematoxylin staining cross-section of the fluke demonstrated that the parasites were killed from the tegument destruction [9,10]. Comparing to a smooth and thick tegument (tg) in control group in Figure 3, damage tegument’s surface as in Figure 4 was observed in the fluke treated with 1 mg/mL aqueous areca nut extract. According to the chemical composition study by gas chromatography/mass spectrometry (GC-MS), areca nut had volatile methyl esters (arecoline and guvacoline) and six chemically related alkaloids (methyl nicotinate, ethyl nicotinate, nicotine, davidine and ammine) [7]. The chemicals in aqueous and methanolic areca nut extracts might contain these compounds especially arecoline and influence on rumen fluke mobility with tegumental modification. The insecticide and lavicidal activities of *Areca catechu* seed have been reported previously [11,12]. The high tannin content of its aqueous and methanolic extracts as 42.41% and 47.72% with total phenolic of 42.06 ± 2.00 and 45.12 ± 2.04 g/100g crude extract as gallic acid, respectively [12], could be related with tegument modification of rumen fluke from its ability for protein precipitation.

### Table 1 Effects of aqueous and methanolic crude extracts and arecoline of areca nut and arecoline on physical appearance and movement behaviour of rumen fluke.

| Tested compounds | Physical appearance and movement behavior of rumen fluke |
|------------------|---------------------------------------------------------|
|                  | Aqueous extract                                         |
|                  | Methanolic extract                                      |
|                  | Arecoline                                               |
| Time             | 1 mg/mL | 0.1 mg/mL | 0.01 mg/mL | 1 mg/mL | 0.1 mg/mL | 0.01 mg/mL | 1 mg/mL | 0.1 mg/mL | 0.01 mg/mL |
| 15 min           | No move (100%)                                         | No move (100%) | Shrink but still move (100%) | No move (100%) | No move (100%) | Shrink but still move (100%) | No move (100%) | No move (100%) | No move (100%) |
| 30 min           | No move (100%)                                         | No move (100%) | Move (100%) | No move (100%) | No move (100%) | No move (100%) | Move (100%) | No move (100%) | No move (100%) |
| 1 h              | No move (100%)                                         | No move (100%) | Move (100%) | No move (100%) | No move (100%) | Move (100%) | No move (100%) | No move (100%) | No move (100%) |
| 24 h             | No move and swell (100%)                               | Slowly move (10.07%) | Move (100%) | No move and swell (100%) | Slowly move (14.5%) | Move (100%) | No move (100%) | No move (100%) | Slowly move (5.0%) |

*data in the parentheses are the percentage of worms with the above appearance

### 4. Conclusions

Methanolic and aqueous crude extracts from seeds of *Areca catechu* could destroy the tegument of the flukes and killed them; therefore, these areca nut extracts showed the potential anthelmintic activity against rumen flukes. The arecoline and phenolic content especially tannin in these extracts should be active compounds promoting tegument modification of rumen flukes. Nevertheless, their adverse reactions and side effects on cattle should be monitored and considered.
**Figure 2.** Physical appearance and fluke mobility (A. Mixed rumen flukes in cattle gut; B. Rumen flukes in control group had pear-shape and moved around.; C. Rumen flukes treated with 1 mg/mL water extract had swollen body and stopped moving).

**Figure 3.** Smooth and thick tegument (tg) and gut (g) in a rumen fluke of control group.

**Figure 4.** Destroyed tegument (tg) found in rumen fluke treated with 1 mg/mL aqueous areca nut extract.

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