Research Article

The *In vitro* Effect of a Crude Extract from *Artocarpus lakoocha* Roxb on *Paramphistomum cervi*

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Abstract

The present study was carried out to evaluate the anthelminthic activity of the extract from *Artocarpus lakoocha* Roxb (Traditional name: "Puag-Haad" (PH)) against rumen fluke, *Paramphistomum cervi*. The parasites were incubated in M199 medium containing either extract of PH or albendazole (ABZ) at 250, 500, 750, 1,000, 2,000 µg/ml, for 3, 6, 12 and 24 h. The efficacy of the extracts or ABZ was assessed by using relative motility (RM) assay which was determined on the basis of comparing the loss of spontaneous movement and/or death of the trematodes between treated and control group. The trematodes were further observed under scanning electron microscopy (SEM). PH expressed the potential in killing the trematode and the maximum efficacy was exhibited at 2,000 µg/ml which was higher and had significantly different RM and SI values than ABZ ($p<0.05$). Through observations by the SEM it was found that the PH also caused more damage on the tegument of *P. cervi* than ABZ, while the sequence of tegumental alterations was similar, i.e. comprising of swelling, blebbing, which subsequently eroded the tegumental syncytium. The severity and rapidity of the damages were directly related to concentration of the crude extract and time of incubation. The results obtained in the present SEM-based study established the anthelmintic activity of PH against *P. cervi*.

***Key Words*:** *Artocarpus lakoocha*; Paramphistomosis; Anthelmintic drug; Organic farming

Introduction

Parasitic infections of ruminants as a result of grazing are considered one of the greatest challenges to animal health in organic production systems. Paramphistomosis is primarily gastrointestinal parasitic disease of ruminants worldwide (e. g., Asia, the Americas, Europe, Africa and Oceania). It is caused by digenetic trematodes that belong to the family Paramphistomatidae. The juvenile stage of this parasite inhabits the small intestine and abomasum, and then move to the rumen in the adult stage (Eduardo, 1982; Sanabria, 2008). Massive infections of immature paramphistomes in the

small intestine can cause acute gastroenteritis characterized by catarrhal and hemorrhagic enteritis and provoke significant loss with decreasing production or even death of the animal, particularly in young animals (Rieu et al., 2007). In Thailand, several species of paramphistomes have been recorded, and the most prominent is *Paramphistomum cervi* (Prasitirat et al., 1997; Panyarachun et al., 2010).

Only pasture management is not a sufficient means to control the outbreak of parasite infection in all situations. The use of veterinary chemicals (anthelminthics) is available in organic farming, but it is limited and on a curative basis. There is also mounting pressure in conventional livestock systems to reduce reliance on anthelminthics because of the increasing prevalence of drug-resistant worms and because of human consumption of chemical residues. The alternative therapies based on phytotherapy or homeopathic preparations are largely recommended in organic farming. In Thailand, "Puag-Haad" (PH), a dried brown aqueous extract which is derived from the process of boiling the heartwood chips of *Artocarpus lakoocha* Roxb. has been traditionally used as an anti-helminthic drug against taeniasis by local people in Thailand and Laos (Charoenlarp et al., 1989; Maneechai et al., 2009; Salguero, 2003). Recently, the efficacy of PH against trematodes has been reported. Wongsawad et al., (2005) demonstrated that PH was effective against an intestinal fluke, *Haplorchis taichui*. In 2008, Soawakon et al. demonstrated that PH was effective against a ruminant fluke, *Fasciola gigantica*. The efficacy of PH against taeniasis and other intestinal and liver fluke as stated above has made it an appealing candidate for controlling other parasites. The extract of this plants has not been tested against *paramphistomum* spp. If found effective, this novel compound would provide an alternative to the current paramphistomicidal drug.

Hence the aim of this study is to 1) determine the paramphistomicidal efficacy of the aqueous extract from Thai medicinal plant: *Artocarpus lakoocha Roxb* on *P. cervi* and 2) investigate its effects on the tegument of the parasites using scanning electron microscope (SEM).

Materials and Methods

Parasite

Adult *P. cervi* were collected in 0.9% phosphate-buffered saline (PBS; pH 7-7.2) from the rumen of infected cattle killed for consumption at the local slaughter houses in Phetchaburi Province and identified according to published morphological criteria (Soulsby, 1987), the worms were fixed in alcohol-formal-acetic acid (AFA), stained with Carmine, differentiated in acid-alcohol, dehydrated in ascending concentrations of ethanol, cleared in xylene and whole-mounted in a permount (Saowakon et al., 2013). After washing with saline several times healthy flukes showing normal appearance and good motility were selected and, kept in culture medium 199 (M-5017, Sigma, USA, Lot No. 077K83001) containing antibiotics (penicillin 50 IU/ml; streptomycin 50 mg/ml) until the incubation experiment began.

Plant extract

The crude extract of *A. lakoocha*, traditionally named Puag-Haad (PH), was bought from Lanna-Chiangmai Herbal Drugstore. According to traditional knowledge, the preparation of PH crude extract was obtained by boiling the wood chips of *A. lakoocha* in water. The foam, forming on the surface of the

boiling-water containing *A. lakoocha*, was continuously harvested and was subsequently dried under sunlight. The end product was a crude brown powder of PH which was used for further experiments.

Bioassay

Two hundred-eighty adult flukes were randomly assigned to eleven groups (40 flukes per group): group 1, the parasites were incubated in M199 medium containing 0.1% (v/v) DMSO and antibiotics (penicillin 50 IU/ml, streptomycin 50 µg/ml, gentamycin 30 IU/ml); groups 2, 3, 4, 5 and 6 the parasites were incubated in the same medium containing albendazole (ABZ) (Medicpharma, Bangkok, Thailand, Lot No. MZ76807) at a concentration 250, 500, 750, 1,000 and 2,000 µg/ml, respectively. Flukes in groups 7-11 were incubated in the medium containing the crude extract of *A. lakoocha* at 250, 500, 750, 1,000 and 2,000 µg/ml, respectively. The parasites in all groups were incubated in the culture medium in an incubator aerated with 5% CO_2 at 37 °C. After 3, 6, 12, and 24 h incubation, motility, survival, and tegument alterations were assessed by examination under the Olympus SZ-ST stereomicroscope (Tokyo, Japan). The experiment was repeated in three replicates.

Assays for the drug's activities

Motility criteria

Motility scores were assigned by using the following criteria: 3 = movement of the whole body, 2 = movement of only parts of the body, 1 = immobile but not dead and unstained with the vital dye (1% (w/v) methylene blue in 0.85% NaCl solution), and 0 = immobile and stained with the vital dye. The efficacies of the tested drugs against *P. cervi* 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.

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

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

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

Survival index (SI)

Survival index (the percentage of live flukes) was determined at each point of time during the incubation. The flukes with motility score of 0 (immobile and stained with the vital dye) were counted as dead, and those with other scores (3, 2, 1) were counted as still alive. Survival index was calculated using the formula listed below, and the survival index of 0 indicated that all flukes were killed.

$$\text{Survival index} = (\text{Number of live flukes} / \text{Number of all flukes}) \times 100$$

Specimen preparation for scanning electron microscopic (SEM) observation

The worms incubated in the M199 culture medium, ABZ or PH at 250, 500, 750, 1,000 and 2,000 µg/ml were collected and fixed in 2.5% glutaraldehyde-phosphate buffer (0.1 mol/l, pH 7.4) at 4 °C for 24 h and post-fixed in 1% osmium tetroxide for 1 h. They were dehydrated through a graded series of ethanol, dried in a Hitachi HCP-2 critical point dryer using liquid carbon dioxide as a transitional medium. After drying, they were mounted on aluminum stubs and coated with platinum and palladium in an ion-sputtering apparatus, Hitachi E-102, set at 10–15 mA for 6 min. They were examined and photographed in a Hitachi scanning electron microscope S-2500 (Hitachi High-Technologies, Hitachi-Naka City, Japan), operating at 15 kV.

Isolation of Standard trans-2, 3', 4, 5'-tetrahydroxystilbene (THS)

THS isolated from the traditional drug PH was subjected to column chromatography [Silica gel 60 (Merck, 0.040-0.063 mm), 4.5 cm i.d. × 15 cm] eluted with hexane/ethyl acetate/acetone (47.5: 47.5: 5.0 v/v/v) to provided 2.46 g of light-yellow solid. Further purification by a column chromatography [Silica gel 60 (Merck, 0.040-0.063 mm), 2.2 cm i.d. × 15 cm] and eluted with hexane/ethyl acetate/acetone (47.5: 47.5: 5.0 v/v/v) to obtained 102 mg of purified light-yellow crystalline THS.

The purity of the isolated THS was confirmed by melting point, high performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) [Silica gel 60 F_{254} plates (Merck), 2 cm × 5 cm]. The developing solvent was hexane/ethyl acetate/acetone (2:2:1 v/v/v). The spots were detected by UV irradiation (256 and 365 nm) and by heating after spraying with 1% $CeSO_4$ in 10% aqueous H_2SO_4. The structure of isolated THS was confirmed by UV and NMR spectral data. ^{1}H-NMR (300 MHz) and ^{13}C-NMR (75 MHz) spectra were recorded on a Brucker Avance using TMS as an internal standards at 25°C.

High Performance Liquid Chromatography (HPLC) Analysis of the THS in Puag-Haad

Standard solutions with an accurate concentration of 0.16 mg/ml were prepared by dissolving standard THS (16.1 mg) in 95% methanol (100 ml). Further dilution into 4 standard solutions (0.08, 0.04, 0.02 and 0.01 mg/ml) by serial dilution were prepared. Sample solutions were prepared by dissolving Puag-Haad (1.0 mg) in 10 ml 95% methanol. Chromatographic conditions were as follows with a Thermo Scientific HYPERSIL ODS-2 column (250 × 4.6 mm, 5 μm): flow rate 1.0 ml/min; mobile phase was composed of solvent A (water) and solvent B (0.78% acetic acid in 50% methanol) in gradient system: initially 30% B linear gradient to 30% B in 10 min, then linear gradient to 100 % B in 15 min, hold at 100 % B for 3 min, then linear gradient to 30 % B in 5 min, and then hold at 30% B for 5 min. Detection was conducted at 254 nm and quantification was based on the integrated peak areas with reference to external standard.

Statistical analysis

Comparisons of anthelminthic effects between groups, was performed with one-way analysis of variance by applying Duncan's test for multiple comparisons with the level of significant difference set at p-value <0.05.

Results

Relative motility (RM) and survival index (SI) values of the parasites treated with albendazole (ABZ) and crude extract of *A. lakoocha* (PH)

All worms incubated in control medium were alive and remained active by showing whole body movement throughout the study period of 24 h (RM = 100, SI = 100).

The worm in albendazole-treated group at all concentration were slowly decreased in motility during 3-12 h (RM: 80-90), then the RM were sharply decreased at 24 h (RM: 20-40) (Figure 1A). Only albendazole-treated group at concentration 2,000 mg/ml, exhibited SI lowers than 50 at 24 h (Figure 1B).

The worm incubated with PH at concentration of 250-750 mg/ml showed slowly decreasing motility at 3 h until the end of the experiment (RM: 20-40) (Figure 1A). The worm incubated with PH at concentration of 1,000 and 2,000 mg/ml, exhibited more gradual reduction in motility at 6 h of incubation.

At 24 h, the RM values of 1,000 and 2,000 mg/ml PH-treated group were 48.10 and 2.56, respectively (Figure 1A). Only 2,000 mg/ml PH-treated group showed sharp reduction of SI at 6 h and drop to 6.67 at the end of the experiment (SI=6.66) (Figure 1B).

When comparing the anthelmintic effects between crude extract of PH and ABZ at the same concentration at 24 h, PH at 2,000 mg/ml showed higher and significantly different RM and SI values than PZQ ($p < 0.05$).

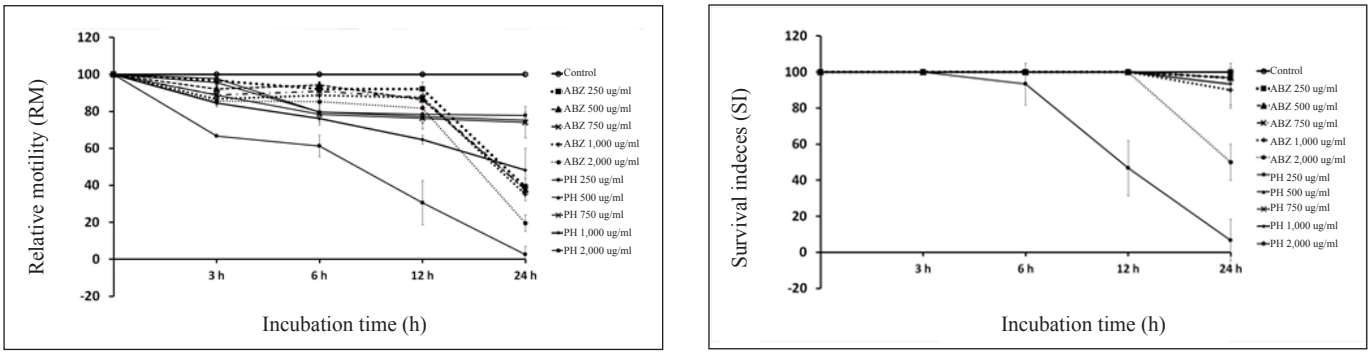

Figure 1 (A) Relative motility (RM) and (B) survival indeces (SI) values of the control and the experimental worms treated with albendazole (ABZ) and crude extract from *A. lakoocha* or Puag-Haad (PH) at various concentrations and durations. Each point in graph represents the response from 10 flukes (3 replicates). Data are expressed as mean ± standard deviation (SD).

Scanning electron microscopic observations

Control group

The surface topography of the tegument of untreated *P. cervi* showed normal appearance throughout 24 h of incubation in Medium-199 containing 0.1% DMSO. The pear-shaped body of *P. cervi* was convex dorsally and slightly concave ventrally (Figure 2A). The oral sucker (os) is placed at the anterior end, and the genital pore (gp) is positioned ventrally at the middle of the anterior third of the body. The posterior sucker (ps) or acetabulum is located sub-terminal at the posterior tip (Figure 2A-E). The topography of tegument is composed of transversed majorfolds (fo) separated with major grooves (gr) (Figure 2C). The transversed major folds on the anterior third of the body have numerous cluster of dome-shaped papillae (pa) arranged in rows (Figure 2F). At the posterior end, the acetabulum has a large thick muscular rim. The morphology in this area shows smoother with fewer larger major folds and deeper groove (Figure 2E). The dorsal part exhibit similar topography as the ventral surface, but they have less folds and grooves than ventral surface as described by Panyarachun et al. (2010).

Effect of albendazole

At 3 h incubation time, the general topography of ABZ-treated flukes appeared similar as in the control group at 24 h post incubation. (Figure 3A). However, when observed at higher magnification, the tegumental surface has marked by deep furrows between the major folds (Figure 3B). During 6-12 h incubation, the swelling of tegument surface was found on both anterior and posterior parts of the fluke body (Figure 3C-D). At 24 hincubation time,the degrees of tegumental alterations were more severe on the ventral surface when observed under high magnification comparison to the dorsal surface. There was erosion of affected area around ge-nital pore (Figure 3E). Minor damage was observed in the acetabulum (Figure 3F).

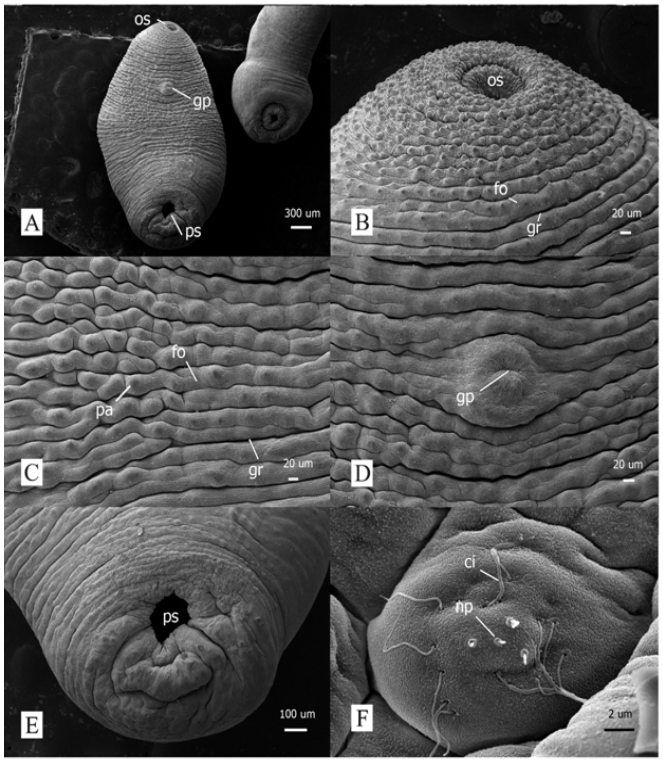

Figure 2 Scanning electron micrograph of *P. cerviincubated* in medium M199 containing 0.1% DMSO for 24 h. A)The oral sucker (os) is placed at the anterior end, and the genital pore (gp) is positioned ventrally at the anterior one-third of the body. The posterior sucker (ps) or acetabulum is located sub-terminal at the posterior tip. B-C) The topography of tegument is composed of transversed major folds (fo) separated with major grooves (gr) D) The genital pore is placed on the middle third of the ventral of the body. E) At the posterior end, the acetabulum has a large thick muscular rim. The morphology in this area shows smoother with fewer larger major F) The transverse major folds on the anterior third of the body have numerous cluster of dome-shaped papillae (pa) arranged in rows.

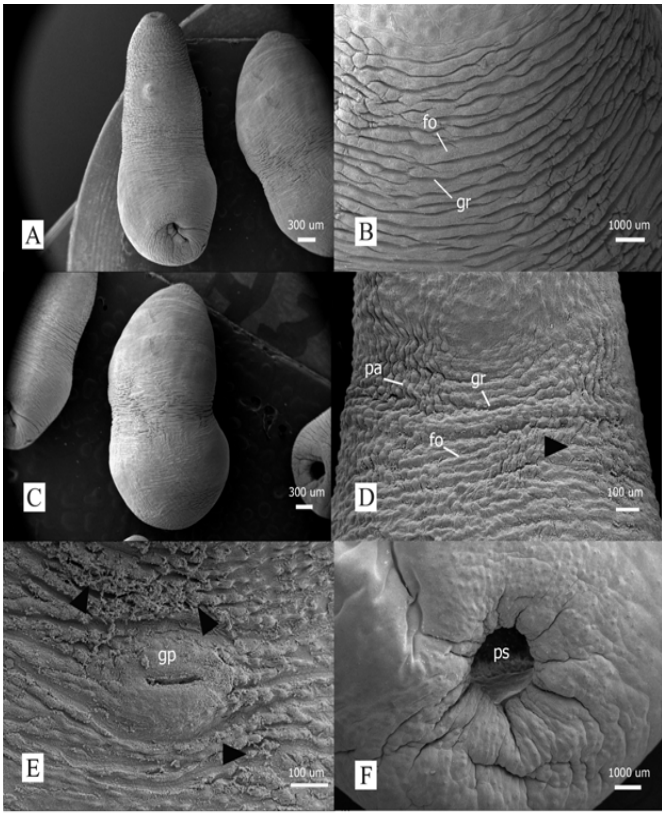

Figure 3 A) At 3 h incubation with 250 µg/ml of ABZ, the general topography of flukes appeared normal morphology. B) At higher magnification, the one third of the ventral surface showing major folds (fo) alternated with major grooves (gr). C) After 6 h incubation with 250 µg/ml of ABZ, the swelling of dorsal surface was found on both anterior and posterior parts of the fluke body. D) At 24 h incubation with 750 µg/ml of ABZ, the tegument on the ventral showed severe alterations. The deformity of papillae (pa) and erosion of tegument (arrowhead) was observed. E) At 24 h incubation with 2000 µg/ml of ABZ, the erosion (arrowheads) around the genital pore was observed. F) At 24 h incubation with 2000 µg/ml of ABZ, minor deformity on the tegument was observed at posterior sucker.

Effect of crude extract of *A. lakoocha*

The alteration of the tegument induced by PH treatment followed the same consequence for all dosages, but there was a difference in degree of severity depending on concentrations and incubation period. During 3-12 h-incubation period, the early sign of change was the swelling of the surface which started in small areas and then spreaded over the whole body surface (Figure 4A-C). At 24 h, the formation of blebs was observed on the surface that later were disrupted, as present around the oral sucker which blebs were formed on top of papillae (Figure 4D-E). The focal erosionswere formed and large areas of tegument were sloughed from the surface, resulting in the peeling of the tegument syncytium and exposed the basal laminar (Figure 4F).

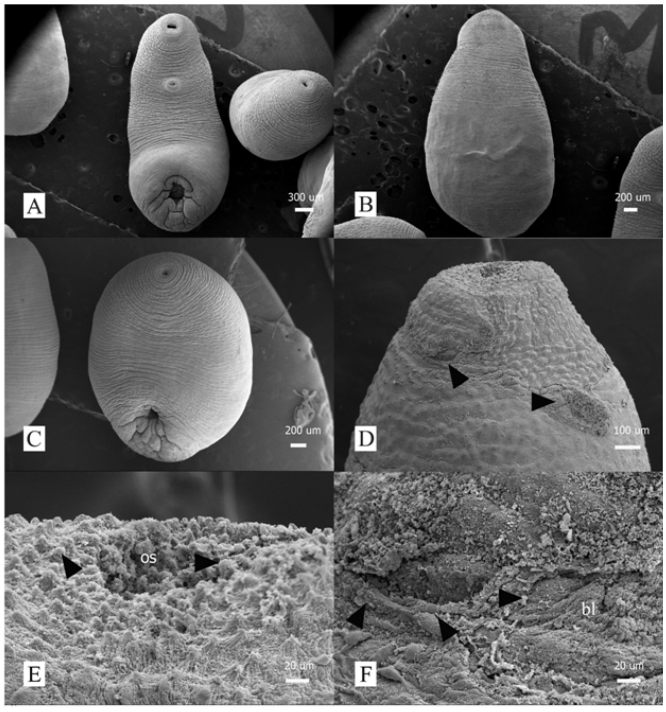

Figure 4 The alteration of the tegument induced by PH treatment followed the same consequence for all dosages, but difference in degree of severity depending on concentrations and incubation period. A-B) At 6 h incubation with 250 µg/ml of PH, the early sign of change was swelling which was found both on the ventral and dorsal surface. C) At 12 h incubation with 250 µg/ml of PH, the whole worm body was swelling. D) At 24 h incubation with 2000 µg/ml of PH, focal erosions (arrowhead) were observed on the anterior part of the worms. E) At 24 h incubation with 2000 µg/ml of PH, the formation of blebs was observed on the surface that later were disrupted, as present around the oral sucker (os) which blebs were formed on top of papillae. F) The focal erosions were formed and large areas of tegument were sloughed from the surface, resulting in the peeling of the tegument syncytium (arrowhead) and exposed the basal laminar (bl).

Analysis of the oxyresveratrol in the Puag-Haad

The purity of purified standard THS was confirmed by the HPLC (Figure 5A). The structure of purified standard oxyresveratrol was elucidated by the NMR as the 2, 3', 4, 5'-tetrahydroxystilbene which spectroscopic data were melting point (mp = 199-201°C, $mp_{Lit.}$ = 201°C [1(UV λ_{max} (MeOH) nm (log ε): 325 (4.241), (log ε)$_{Lit}$: 328 (4.329) (Mongkolsuk et al.,

1975). The ^{1}H- and ^{13}C-NMR spectral data with those of authentic sample (Kanchanapoom et al., 2002; Zhang et al., 2008). ^{1}H-NMR (CD_3COCD_3) δ: 6.25 (1H, *t*, J= 2.1 Hz), 6.34 (1H, *dd*, J=8.4, 2.4 Hz), 6.42 (1H, *d*, J = 2.4Hz), 6.53 (2H, *d*, J = 2.1 Hz), 6.88 (1H, *d*, J = 16.5 Hz), 7.32 (1H, *d*, J = 16.5 Hz), 7.38 (1H, *d*, J = 8.4 Hz), 8.33 (3H, *s*, D_2O exchangeable), 8.62 (1H, *s*, D_2O exchangeable), ^{13}C-NMR (CD_3COCD_3) δ: 102.3, 103.6, 105.2, 108.5, 117.2, 124.3, 126.2, 128.3, 141.6, 156.7, 158.9, 159.3

The HPLC chromatogram of THS in PH is shown in Figure 5B. Quantification was based on the integrated peak areas with reference to external standard (Figure 5A). The retention time of THS was 20.9 minutes and the naturally occurring amount of THS in PH was found to be at 72.6±1.8%.

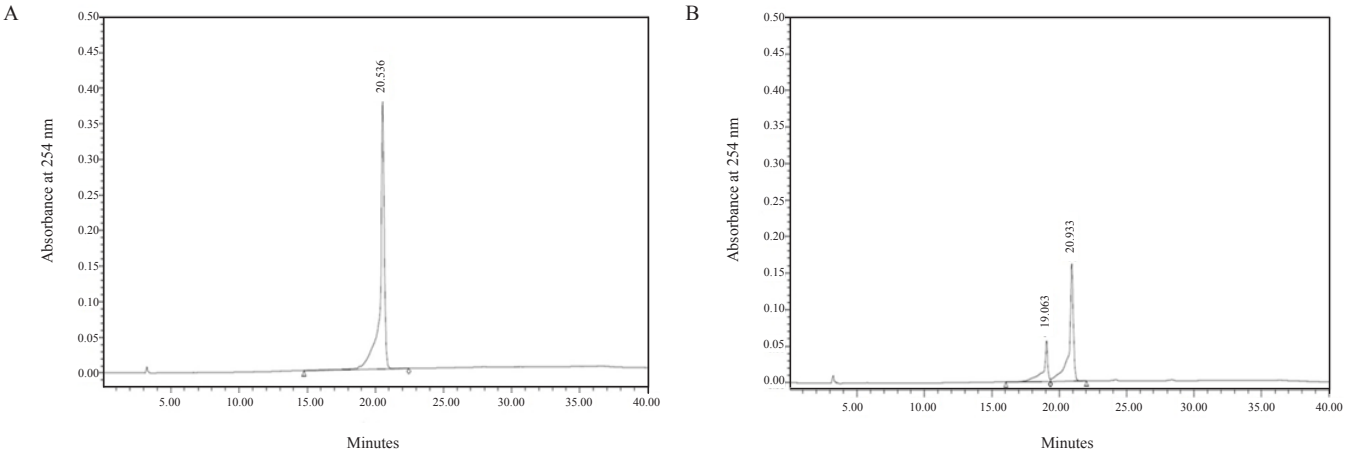

Figure 5 HPLC chromatogram of sample A) purify oxyresveratrol for external standard (0.161 mg/ml) B) Puag-Haad (0.101 mg/ml). The detection was conducted at 254 nm and quantification was based on the integrated peak areas with reference to external standard. The retention time of oxyresveratrol was 20.9 min.

Discussion

The application of medicinal plants for therapeutic purpose has a long history, and compounds derived from these plants have made a big impact on the pharmaceutical industry (Newman et al., 2003; Newman and Cragg, 2007). PH is one of those plants that is traditionally used in Thailand and Lao with little scientific support on its efficacy. Previous research demonstrated that the major bioactive constituent responsible for the anthelminthic activity in the heartwood of this plant is trans-2, 4, 3', 5'-tetrahydroxystilbene (THS) (Likhitwitayawuid et al., 2006; Mongkolsuk et al., 1957; Poopyruchpong et al., 1978). To our knowledge, this is the first report on the anthelminthic effect against *P. cervi* by "Puag-Haad", Thai medicinal crude extract from *A. lakoocha*,

In this study, the anthelminthic effectiveness of the PH has been evaluated on the basis of reduction in motility and/or death of the *P. cervi*. Both albendazole and PH showed the activity against *P. cervi* in dose and time dependent fashion ($p<0.05$). We demonstrated that PH was effective to paralyze and kill the trematodes. PH atdose 2000 ug/mlwas the most efficient and significantly more effective in killing the worm than albendazole at the same concentration. The distinct damage on the tegument of treated-parasite was observed under scanning electron microscope. Previous studies showed that PH was found to be effective against *F. gigantica*, *H. taichui* and *Schistosoma mansoni* (Saowakon, 2009; Wongsawad et al., 2005; Preyavichyapugdee, 2016), and the major targetorgan that was highly affected is the tegumentwhich is

similar to our result. The tegument of trematodes bears an important function for the survival of the parasite. It plays crucial role in host-parasite interface, maintenance of homeostasis like osmo-regulation, performing all the vital activities such as protection, absorption and secretion (Halton, 2004). The alteration of the tegument was found in both PH and ABZ-treated parasite which consisted of swelling, blebbing and sloughing of the tegument.

The earliest sign of change on the tegument of worm treated with crude extract was swelling. This could be elicited by the osmotic imbalance which may be due to Na^+ influx into the syncytium. Fasciolicidal drugs which are phenolic compounds such as Nitroxynil, hexachlorophane or oxyclozanide caused uncoupling of oxidative phosphorylation followed by Na^+ influxand alteration of the tegument morphology. HPLC analysis showed that the major compound of the PH extract is the phenolic compound, THS (PubChem CID: 5281717), which corresponded to the previous reports (Mongkolsuk et al., 1957; Poopyruchpong et al., 1978). So it is possible that THS may interfere with oxidative phosphorylation in the cell with the same mechanism of killing the worm as those phenolic compounds of fasciolicide.

In contrast, albendazole (ABZ) is a microtubule-targeting anthelminticfrom benzimidazole group of compounds (Ehteda et al., 2013). ABZ induces degenerative alterations in the tegument of the trematode by binding to the colchicine-sensitive site of tubulin, thus stopping the polymerization of microtubules. As a consequent, the carrying of secretory granules from the tegument cell bodies to the tegument is obstructed. This could lead to advanced damage of the tegumental surface, especially the replacement of the rapidly turned-over surface membrane. This leads to weakening and disrupting of the tegument (Stitt and Fairweather, 1993). Besides, the loss of the cytoplasmic microtubules leads to impaired uptake of glucose by the trematode. The worm is then unable to maintain energy production, which leads to its immobilization and eventual death (Vignaduzzo et al., 2015).

Conclusion

In conclusion, the present study substantiated the utilization of *A. lakoocha* as anthelminthic in traditional medicine practiced by indigenous people of Thailand and Laos (Salguiro, 2003). Previously reports demonstrated that, crude extract of PH showed anthelminthic activity against *F. gigantica*, *H. taichui* and *S. mansoni* (Saowakon, 2009; Wongsawad et al., 2005; Preyavichyapugdee, 2016). And the anthelminthic activity of crude extract against *P. cervi* in this study expressed the disruption of the tegument of the parasite in a time-dependent manner. Thus, traditional use of crude extract of PH in helminthic infestation was established scientifically. The study might possibly help to developing herbal-based anthelmintic. Further studies should also examine the mechanisms of actions of THS, its cytotoxicities and activities against other paramphistomum species and also evaluation of its in vivo effects in animal models is also required.

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