Research Report

The *in vitro* effect of crude extract from *Artocarpus lakoocha* Roxb, *Murraya paniculata* Linn and *Tamarindus indica* on *Paramphistomum cervi*

By

Mananya Preyavichyapugdee

A Research is Sponsored by Phetchaburi Rajabhat University

2009

รายงานวิจัยฉบับสมบูรณ์

การศึกษาศักยภาพของสารสกัดจากพืชสมุนไพรมะหาด แก้ว และมะขาม ต่อการตายของพยาธิใบไม้ในกระเพาะ *Paramphistomum cervi*

โดย

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Abstract

The present study was carried out to evaluate the anthelminthic activity of the extract from *Artocarpus lakoocha* Roxb, *Murraya paniculata* Linn and *Tamarindus indica* against rumen fluke, *Paramphistomum cervi*. The parasites were incubated in M199 medium containing either extract from *A. lakoocha*, *M. paniculata* and *Tamarindus indica* 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 determined on the basis of comparison with 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). There were no paramphistomicidal effect of crude extract from *M. paniculata* Linn and *T. indica* against *P. cervi*. Only the crude extract from *A. lakoocha* was expressed the potential in killing the trematode and the maximum efficacy was exhibited at 2,000 mg/ml which was higher than ABZ and significantly different in both RM and SI values ($p<0.05$). According to the SEM analysis the PH 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 were erosion of the tegumental syncytium. The severity and rapidity of the damages were directly related to concentration of the crude extracts and time of incubation. The results obtained in the present SEM-based study established the anthelmintic activity of PH against *P. cervi*.

Keywords: *Artocarpus lakoocha*, *Murraya paniculata* Linn, *Tamarindus indica*, *Paramphistomum cervi*

CONTENTS

CHAPTER 1

INTRODUCTION

Paramphistomosis is a gastrointestinal parasitic disease of domestic and wild ruminants, worldwide (e.g., Asia, the Americas, Europe, Africa and Oceania). It was caused by digenetic trematode that belongse to the family Paramphistomatidae. In the former the presence of paramphistomes infection, regarded to be relatively harmless in cattle. The juvenile stage of this parasite inhabits in the small intestine and abomasums, and then move to the rumen in the stage of adult (Eduardo, 1982; Sanabria, 2008). Massive infections of immature paramphistomes in small intestine can cause acute gastroenteritis characterized by catarrhal and hemorrhagic enteritis and provoke significant loss with decreasing in production or even death of animal, particularly in young animals (Rieu et al., 2007).

In some areas of india, the Republic of South Africa and Australia, the mortality of cattle has reached 80-90% in sheep and cattle (Boray, 1959; Soulsby, 1987). In Argentina, there has been an increasing in recording of cases, mainly in the northeast and central-east of Argentina (Bulman et al., 2002; Raccioppi et al., 1995; Sanchez et al., 2005). In Thailand, several species of paramphistomes have been recorded, and the most prominent one is *Paramphistomum cervi* (Prasitirat et al., 1997; Panyarachun et al., 2010).

Various fasciolicidal compounds have been used for treatment of paramphistomiasis (Panyarachun, 2010). However, the drug resistance and concern about toxicity derived from drug residues in animal products and environment have grown rapidly and encompasses a vision of a more sustainable approach to animal production. The anthelminthic drug from natural resource is an alternative approach to control the parasites. Plants have been found to contain several bioactive substances and are promoted for treating various illnesses throughout history for their medicinal properties. The application of medicinal plants are not only focused on curing human diseases but also applied in ethnoveterinary practice and animal health management. In Thailand, “Puag-Haad” (PH) is 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 anthelmintic 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 2009 , Soawakon and her coworker demonstrated that PH was effective against a ruminant fluke, *F. 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. Wongsawad et al. (2005) demonstrated that the aqueous extract of *M. paniculata* has killing effect on the trematode, *Haplorchis taichui* (*in vitro*). The study under scanning electron microscope showed that the tegumental surface of the dead worms showed blebing, rupturing and loss of spines. Husain and Anwar (1975) also showed that crude extract from seed of *T. indica* had decreased larval hatching of round worm, *Meloidogyne incognita*. However, the extract of these plants has not been yet tested against *paramphistomum* spp. If it is effective, this novel compound will provide an alternative to the current paramphistomicidal drug.

OBJECTIVES

1. To determine the paramphistomicidal efficacy of the aqueous extract from Thai medicinal plant: *Artocarpus lakoocha* Roxb, *Murraya paniculata* Linn and *Tamarindus indica* on *Paramphistomum cervi*.

2. To investigate its effects on the tegument of the parasites using scanning electron microscope (SEM).

CHAPTER 2

LITERATURE REVIEW

2.1. Paramphistomosis (Sanabria and Romero, 2008)

Paramphistomosis is one of the common parasites that found in domestic and wild ruminants caused by trematoda belong to the family Paramphistomidae. Gupta (1993) reported that the infection of immature paramphistomes was found in the small intestines of immunologically incompetent hosts., from where they move to the rumen to the immature flukes are responsible for destroying the mucosal walls of the alimentary tract on their way to finally lodge as adult trematodes in rumen. The occurrence of paramphistomosis in an area is involving or dependeing on a number of factors influence by hosts, parasitic agents, transmission process and environmental effects. In much the same way as *Fasciola hepatica*, these digeneans require snail in its life cycle to complete the first stage of the life cycle. Although their pathogenicity is still controversial, there are worldwide descriptions of sporadic outbreaks reporting deaths or undetermined losses with or without clinical symptoms.

2.1.1. Taxonomy

At present the order Echinostomida (included in subclass Digenea) contains the superfamily Paramphistomoidea (Olson et al., 2003) which includes the families Paramphistomidae and Gastrothylacidae, containing the majority of the known paramphistomes of ruminants. Paramphistomidae is divided into two subfamilies, whereby the subfamily Paramphistominae is represented by the genera *Paramphistomum*, *Cotylophoron*, *Calicophoron*, *Explanatum*, *Gigantocotyle* and *Ugandocycle*. The family Gastrothylacidae contains the genera *Fischoederius*, *Carmyerius*, *Gastrothylax* and *Velazquezotrema*. Not in the Paramphistomoidea, the Balanorchiidae (with *Balanorchis anastrophusas* the only species present) is located within the superfamily Cladorchoidea.

2.1.2. Life cycle development

Paramphistomum have an indirect life cycle with freshwater snails as the intermediate hosts, e.g. of the genus Bulinus, Planorbis, etc. Within the

intermediate host (IH): Eggs are shed in the intestine and eliminated with the faeces of the definite host. About 2 weeks later miracidia hatch out of the eggs., The production of miracidia can be delayed by alteration of environmental conditions such as lower temperatures.

The miracidia swim in the water until they find a suitable snail. When sensing the soft outer layer of a snail, they attach and burrow into the tissue. The miracidia transform to mature sporocysts in about 11 days, and release rediae, which have a pharynx and ceca, and a great phagocytic capacity. They can also form secondary new generations of young rediae, and these in turn may produce more generations of the same phase all leading to the following phase of cercariae, or alternatively, develop to cercariae directly.

The cercariae emerge from their rediae and undergo a maturation period of approximately 10 days, within the snail. These cercariae have pigmentation and 2 eye spots. (Durie 1951, 1956; Yamaguti, 1958; Sey, 1991). After emergence, they encyst on subaquatic vegetation, to which they adhere and begin to develop a cysts leading to metacercaria, constituting the infective phase for ruminants when ingested. The process of cyst formation takes about 20 minutes (Horak, 1971), and the resulting metacercaria are able to survive for at least 29 days if a humid environmental temperature persists (Horak, 1962).

When the metacercaria are ingested and reaches the anterior part of small intestine the immature flukes are excysted. The juvenile worms remain attached to the intestine wall feeding cellular detritus. Once they have developed, they migrate towards the rumen, where the parasites will reach the adult stage, remaining there and living on ruminal fluid.

2.1.3. Pathology and pathogenesis

The pathogenicity depends on the individual vulnerability, species susceptibility, as well as on the number of the ingested metacercaria. Young animals are more susceptible. Adult cattle and sheep may produce clinical or subclinical conditions after the ingestion of high doses of metacercariae (Boray, 1959; Horak, 1971). Young parasites adhere in the small intestine by their acetabulum, producing a “sucker effect” The effect is a result of detachment of the acetabulum in act to move

forward in migration, and multiplied by the number of parasites present. As a result, they leave injured areas with exposition to vascular strata and the loss of electrolytes and proteins producing anorexia, diarrhoea, and loss of corporal condition. Protein loss generates a generalized oedema (hydrothorax, hydropericardium, ascites, lung oedema) (Sanabria and Romero, 2008).

2.1.4. Diagnosis

Diagnosis relies on a combination of post mortem findings, history of the outbreak, clinical signs observed in the animal, and response to drenching. Identify eggs in faeces by filtration technique with sieves and sedimentation (Happich and Boray, 1969) is the most accurate method. Eggs of paramphistomum must be differentiated from those of *Fasciola hepatica* which are similar in shap but slightly larger (160 – 180 μ), and transparent in aspect. Lugol's solution is used to distinguish the differences between eggs two species. The animals with acute symptoms without previous exposure may not find the eggs in the fecae. The prevalence of the cases should be related to the geographical characteristics of the area where the necessary intermediate host snail species are present.

2.1.5. Control

Although there are practical limitations, the following measures provide some control of stomach fluke: Drain affected areas, Fence off affected areas, Provide alternative water sources and Treat stock with drenches effective against stomach flukes. Under this perspective, as an integrated control measure, the distribution of grazing paddocks is established according to their geographical features, combined with the anthelmintic therapy and the study of other epidemiological variables such as the infection prevalence in the intermediate host. On farms where this is possible, herd rotation can be carried out according to the prepatency and the possibilities of egg contamination. Adult animals are less susceptible, so they could be grazed in infected paddocks and then moved to higher pastures when the prepatent period has been completed. The need of treatment must be carefully evaluated, as outbreaks with pathogenic signs are unusual, and the only presence of adults in the rumen in necropsies is no evidence of a disease outbreak.

2.2. *Artocarpus lakoocha* Roxb.

Artocarpus lakoocha Roxb trees of the Moraceae family. It is commonly called as Monkey jack. *Artocarpus lakoocha* Roxb. is found in Bangladesh, Bhutan, Cambodia, India, Laos, Malaysia, Myanmar, Nepal, Sri Lanka and Thailand. A large deciduous tree reaching 15-18m in height. The ripe fruit is sour sweet, tonic to liver. The seed are good purgative for childrens (Pandey and Bhatnagar, 2009). It has many pharmacological activities such as anti-inflammatory, antiviral, anticancer and anti-HIV (Kirtikar and Basu, 2007).

2.2.1 Taxonomical classification

Kingdom: Plantae – Plants
Subkingdom: Tracheobionta – Vascular plants
Superdivision: Spermatophyta – Seed plants
Division: Magnoliophyta – Flowering plants
Class: Magnoliopsida – Dicotyledons
Subclass: Hamamelididae
Order: Urticales
Family: Moraceae – Mulberry family
Genus: *Artocarpus*
Species: *Artocarpus lakoocha* Roxb.

2.2.2. Local names

Burmese (myankdok); English (monkey jack); Hindi (lakuch, dhau, dephal, badhal); Malay (tampang); Nepali (badahar,arhar); Thai (lokhat); Trade name (lakuch)

2.2.3. Morphological characters

Artocarpus lakoocha is a medium to large deciduous tree with a spreading crown. The bark is grey with milky latex. Leaves alternate, 10-25 cm long, elliptical, pointed and leathery. Flowers unisexual-male and female flowers on the same tree. Male flowers are yellow-orange while the female are reddish. Fruit is a syncarp, irregularly rounded, green when young, turning yellow at the time of

maturity, later brown. The diameter is typically 5-10 cm while fruit weights 200-350 g. The number of seeds/fruit varies accordingly, but typically there are 10-30 per fruit. Seeds irregular and vary in size like the fruits. At maturity, most seeds are about one cm long, more or less flattened and pointed at the embryo end, the seed-coat is thin and white. The seeds contain sticky white latex.

2.2.4. Chemical constituents

The *Artocarpus* species are rich in phenolic compounds including flavonoids, stilbenoids and arylbenzofurons. The major chemical constituent is trans-2,4,3',5'-tetrahydroxystilbene (THS) (Mongkolsuk et al., 1957)

2.2.5. Traditional uses

Heyne (1987) and Perry (1980) reported that many members of the genus *Artocarpus* have also been used as traditional folk medicine for the treatment of inflammation, malarial fever and to treat the ulcers, abscess and diarrhea. The edible *Artocarpus lakocha* fruits pulp is believed to acts as a tonic for the liver. The raw fruits and male flowers spikes (acidic and astringent) are utilized in pickles and chutney. The crude aquest extract called Puag-Haad in Thailand is a product prepared by boiling the wood chips of *Artocarpus lakoocha* Roxb. and then evaporating water away. This preparation has been used as a traditional anthelmintic drug for treatment of tapeworm infection in Thailand (Charoenlarp et al., 1989; Salguero, 2003).

2.3. *Murraya paniculata* Linn. (Gill et al., 2014)

Murraya paniculata Linn. (*Murraya exotica* L.) Belongs to the family Rutaceae, and is generaly known as orange jasmine. It is distributed in tropical Asia from India and Srilanka to Myanmar (Burma), southern China and Taiwan, Thailand, and east words throughout the Malesian region to northeastern Australia and Caledonia.. Many parts of this tree have been used in folk medicine to treat dysentery. Many different phytochemicals constituent were extracted from *Murraya paniculata*. The extract revealed the presence of alkaloids, flavonoids, phenolic compounds, carbohydrate, proteins & amino acids and; while fixed oil, saponins and mucilage were absent.

2.3.1. Taxonomical classification

Kingdom: Plantae

Division: Tracheophyta

Class: Magnoliopsida

Order: Sapindales

Family: Rutaceae

Genus: Murraya

Species: *Murraya Paniculata* (Linn.) Jack

2.3.2. Local name

Thai (Kaew) Indonesia (Kemuning) china (moon tangerine) India (kamini) Brazilian Portuguese (murta de cheiro, jasmim laranja)

2.3.3. Morphological characters (Little and woodbury, 1974; Liogier, 1988)

It is an evergreen shrub or occasionally a small tree, usually 2 to 3 m in height but reaching 7.5 m and 13 cm in stem diameter. The leaves are alternately arranged along the stems and borne on stalks (i.e. petioles). These leaves (6-11.5 cm long) are once-compound (i.e. pinnate) with 3-9 leaflets. The glossy leaflets (1.5-7 cm long and 1.2-3 cm wide) are narrowly oval (i.e. narrow-elliptic) to somewhat egg-shaped in outline (i.e. ovate or obovate). They have entire margins, wedge-shaped (i.e. cuneate) bases, and pointed tips (i.e. acuminate apices). Stem bark is gray, becoming fissured and rough. Its younger stems are green and hairless (i.e. glabrous) or covered in tiny hairs (i.e. minutely pubescent). Older stems become woody and brown or grey in colour. They can reach up to 13 cm across and may eventually become fissured and rough. The fragrant flowers are borne in clusters, containing up to eight flowers, at the tips of the branches or in the upper leaf forks (i.e. terminal or upper axillary cymes). Each flower has five green sepals and five white petals (10-18 mm long) that are curved backwards (i.e. recurved). They also have ten stamens and an ovary topped with a style and a globular (i.e. capitate) stigma. Flowering occurs irregularly throughout the year, often in response to rain, but is most common from late winter through to late spring. Shiny, red elliptic fruits about 1 cm long develop. One or two light green seeds are embedded in the bitter, watery pulp. The seeds are tear-drop

shaped, rounded or flattened on one side depending on whether there are one or two seeds per fruit.

2.3.4. Chemical constituents

Many chemical constituents like flavanoids, alkaloids, coumarins, essential oils etc are obtained from different parts of *Murraya paniculata* parts like leaves, fruit, roots, stem, and flowers.

2.3.5. Traditional uses

In Indonesia, Decoction of dried material were used for gas pains, pain due to sprain and contusions, rheumatic bone pain and poisonous snake bites. Leaves and root bark used for rheumatism, cough, and hysteria. Used for abscesses, cellulites, tapeworm disease, rheumatic fever, coughs, giddiness, hysteria, thirst, and burning of the skin. Infusion used for herpes of the stomach, and the sediment applied externally. In Yi medicine in China, used for common colds, fever, cough, sore throat, influenza. In the Gujarat region of India, used toregulate fertility. In Singapore, leaves are ingredient of a tonic given for irregularities in the regenerative organs of young women. In China, plant is widely used for stomachaches, toothaches, rheumatism, paralysis, and diabetes.

2.4. ***Tamarindus indica*** (Isha and Milind, 2012)

Tamarind belonging to family Caesalpiniaceae is scientifically known as *Tamarindus indica* Linn. a perennial evergreen tree with a spreading crown; feathery evergreen foliage and fragrant flowers. It grows widely in the tropical and subtropical regions, yielding hard yellowish wood and long pods with edible chocolate-colored acidic pulp. It is cultivated almost in all the states of the country, except in Himalayas and Western dry regions (Rao et al., 1999). Tamarind is not only a food item but its pulp, leaves, and bark also has medical applications (Dagar et al., 1995).

2.4.1. Taxonomical classification

Kingdom: Plantae

Subkingdom: Tracheobionta

Super division: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Rosidae

Superorder: Rosanae

Order: Fabales

Family: Fabaceae

Subfamily: Caesalpinioideae

Genus: *Tamarindus* L.

Species: *Tamarindus indica* L.

2.4.2. Local name

Afrikaans (Tamarinde); Arabic (Aradeib); Burmese (Ma gyi); Chinese (Da ma lin) English (Tamarind); Indonesian (Asam jawa) Thai (Ma khaam)

2.4.3. Morphological characters (Isha and Milind, 2012)

Tamarind is a large, slow growing, long living evergreen tree with a trunk of diameter up to 1.5-2m, which can grow 20-30m high. The bark of Tamarind tree is brown-gray colored. It can tolerate diversity of soils like loam, sandy, clay soil, but well drained slightly acidic soil is best for its growth. Leaves are elliptical ovular, alternate, pinnate with reticulate venation and is a mass of bright green, dense foliage with feathery appearance. Leaves are 7.5-15 cm in length, each having 10 to 20 pairs of oblong leaflets (1.25-2.5 cm) and 5-6 mm wide. The inconspicuous, inch-wide, five-petal flowers are borne in small racemes and are yellow with red streaks. The flower buds are pink due to the outer color of the 4 sepals, which are shed, when the flower opens. The fruits are usually between 5 to 14 cm in length and approximately 2 cm wide. It is an indehiscent legume, with a hard, brown shell called as pods. Along with the new branches, there is abundant growth of irregularly curved pods. On maturation of pod, the flesh becomes brown or reddish brown and is filled with somewhat juicy, acidulous pulp. As the fruits ripe fully, the shells are brittle and

easily broken. The pulp has a pleasing sweet/sour flavor along with high content of acid and sugar. It is also rich in vitamin B and high in calcium content. The pulp dehydrates to a sticky paste, enclosed by a few coarse strands of fiber. The pods may contain 1 to 12 large, flat, glossy brown, obviate seeds embedded in the brown, edible pulp. There are wide differences in fruit size and flavor in seedling trees. Asian types have longer pods with 6 - 12 seeds, while the African and West Indian types have shorter pods, containing only 3 - 6 seeds.

2.4.4. Chemical constituents

Phytochemical investigations carried out on *T. indica* revealed the presence of many active constituents, such as phenolic compounds, cardiac glycosides, mallic acid, tartaric acid, uronic acid, mucilage, pectin, arabinose, xylose, galactose and glucose (Coutino-Rodriguez et al., 2001)

2.4.5. Traditional uses (Havinga et al., 2010)

Laxative and constipation reliever: Ripe and unripe fruit pulp, mixed with milk, honey or lemon juice, due to high amount of maleic acid, tartaric acid and potassium acid tartarate

Wound healer: Leaves and bark, due to high amount of tannin, applied externally on the spot as a decoction or as powder

Antipyretic: Fresh fruit

Antimalarial agent: Tamarind fruit pulp and leaves

Antidiarrheal and Antidysentry agent: Fruit pulp with lemon or milk, leaf juice

Antiasthmatic and Antitussive: Bark and Leaves

Antileprotic: tamarind fruit

Anti scurvy: Vitamin C deficiency causes scurvy, so this fruit supplements Vitamin C

Anti-inflammatory: Leaves and Bark, due to high tannin content

Astringent: Bark, due to tannin

Anthelmintic (Bark, seed)

Antibacterial (Fruit)

Antidiabetic (Leaves)

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

- 0.9% Phosphate-buffered saline (PBS; pH, 7-7.2)
- Alcohol-formal-acetic acid (AFA),
- Ethanol
- Xylene
- Medium 199 (M-5017, Sigma, USA, Lot No. 077K83001)
- Antibiotics (penicillin 50 IU/ml; streptomycin 50mg/ml)
- Crude extract of *A. lakoocha* (Puag-Haad: PH)
- Fresh leaf (200 g) of *M. paniculata* Linn.
- Seed of *T. indica*
- Albendazole (ABZ)
- Methylene blue
- 2.5% Glutaraldehyde-phosphate buffer
- 1% Osmium tetroxide

3.1.2 Instruments

- Olympus SZ-ST stereomicroscope
- CO_2 incubator
- Scanning electron microscope S-2500 (Hitachi High-Technologies, Hitachi-Naka City, Japan),

3.2 Methods

3.2.1 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, the 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 50mg/ml) until incubation experiment began.

3.2.2 Plant extracts

The crude extract of *A. lakoocha*, traditionally named Puag-Haad (PH), was bought from Lanna-Chiangmai Herbal Drugstore. Acccording 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.

The fresh leaf (200 g) of *M. paniculata* Linn. was blended in water (1,000 ml) mechanically using commercial electrical stainless steel blender. The supernatant of crude extract was collected after centrifuged at 5000 rpm. The extract was concentrated under reduced pressure of 22–26 mm Hg at 45°C, and the residue obtained was stored at 4°C for further experiments.

The seed (2 kg) of *T. indica* were roasted and removed the seed coat. The white pulp (endosperm) were collected and blended in water (1,000 ml) mechanically using commercial electrical stainless steel blender. The supernatant of crude extract was collected after centrifuged at 5000 rpm. The extract was concentrated under reduced pressure at 22–26 mm Hg at 45°C, and the residue obtained was stored at 4°C. for further experiments.

3.2.3 Bioassay

Eight-hundred-forty 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

of 250, 500, 750, 1,000 and 2,000 µg/ml, respectivey. 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, respectivey. The parasites in all groups were incubated in the culture medium in an incubator, and 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 three replicates.

3.2.4 Assays for the drug's activities

3.2.4.1 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 adult *F. gigantica* were calculated as the relative motility (RM) value using the equations (1) and (2) as listed below (Kiuchi et al., 1987). A small RM value indicated stronger drug activity, and when all flukes died this value was 0.

$$\text{Motility index (MI)} = \sum Nn / N \qquad (1)$$

$$\text{RM value} = \text{MI test} \times 100 / \text{MI control} \qquad (2)$$

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

3.2.4.2 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 equation (3), and the survival index of 0 indicated that all flukes were killed.

$$\text{Survival index} = (\text{Number of live flukes} / \text{Number of all flukes}) * 100 \qquad (3)$$

3.2.4.3 Specimen preparation for scanning electron microscopic (SEM) observation

The worms incubated in the M199 culture medium, PZQ 175 µg/ml, PH at 250, 500 and 750 µg/ml were collected and fixed in 2.5% glutaraldehyde-phosphate buffer (0.1mol/L, pH 7.4) at 4 °C for 24 h and post-fixed in 1% osmium tetroxide for 1h. 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 paladium in an ion-sputtering apparatus, Hitachi E-102, set at 10–15mA 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.

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

THS isolated from the traditional drug 'Puag-Haad', a dried aqueous extract prepared from heartwood of *A. lakoocha*. Puag-Haad (5 g) 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 provide 2.46 g of light-yellow solid. Further purification by a column chromatography [Silica gel 60 (Merk, 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.

3.2.6 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 of 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.

3.2.7 Statistical analysis

Comparisons of anthelmintic 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.

CHAPTER 4

RESULTS

4.1 RM and SI values of the parasites treated with albendazole (ABZ) and crude extract of *A. lakoocha* (PH), *M. paniculata* Linn and *T. indica*

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) (Fig 1A). Only albendazole-treated group at concentration of 2,000 mg/ml, exhibited SI lowers than 50 at 24 h (Fig 1B).

The worm incubated with PH at concentration of 250-750 mg/ml showed slowly decreasing in motility since 3 h until the end of the experiment (RM: 20-40) (Fig 1A). The worm incubated with PH at concentration of 1,000 and 2,000 mg/ml, exhibited more gradually reduction in motility since 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 at 24 h, respectively (Fig 1A). Only 2,000 mg/ml PH-treated group showed sharply reduction of SI since 6 h and dropped to 6.67 at the end of the experiment (SI=6.66) (Fig 1B).

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

The worms incubated with crude extract from *M. paniculata* Linn and *T. indica* expressed good motility and was similar to the control group. The worms from these two treatment groups were slightly decreased the motility at 24 h (RM: 98-99) and all of the worm survived until the end of the experiment (SI: 100). (Fig 2-3).

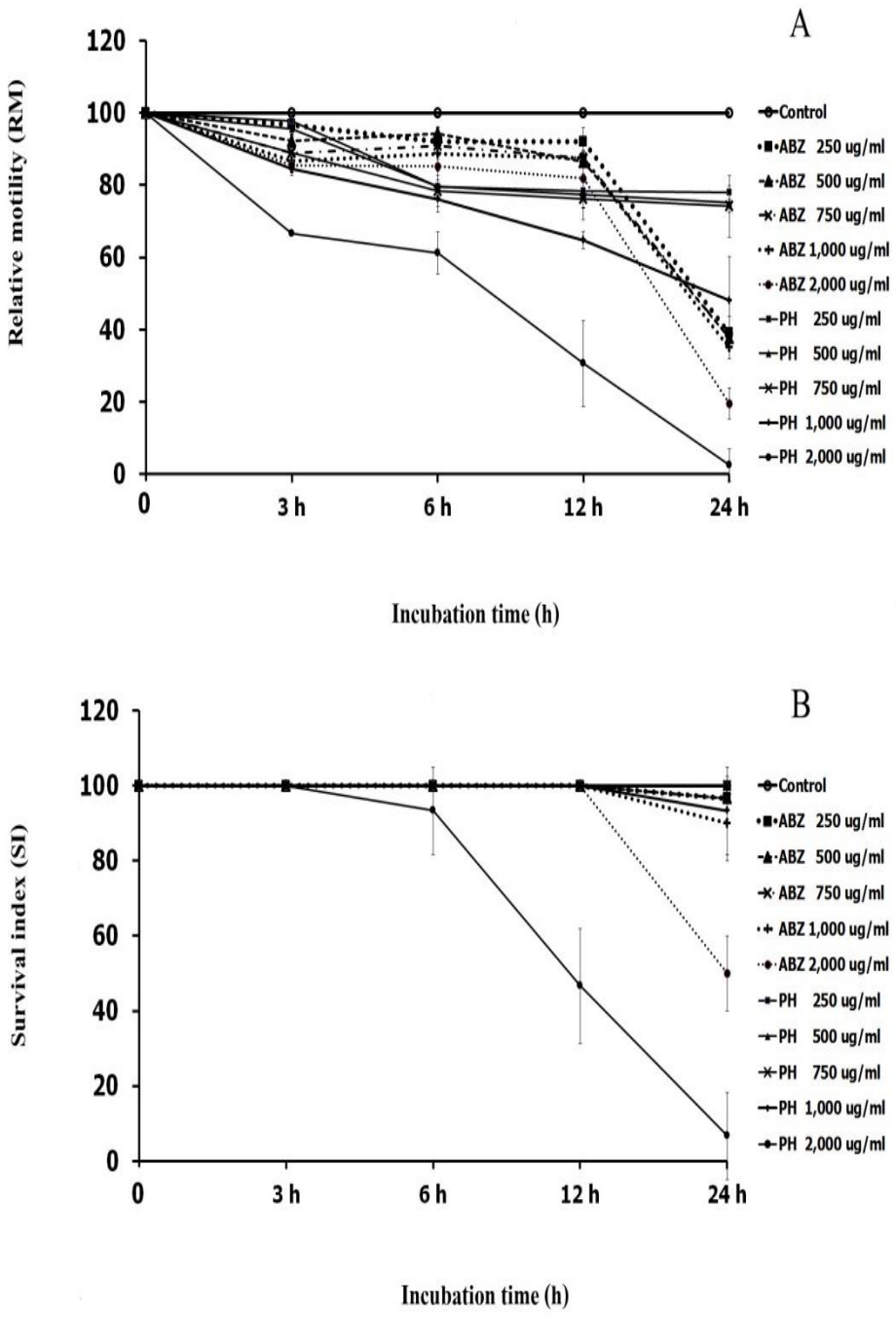

Fig. 1 (A) Relative motility (RM) and (B) survival index (SI) values of the control and the experimental worms treated with Albendazole (ABZ) and crude extract from *A. lakoocha*- Puag-Had (PH) at various concentrations and durations. Each point in graph represents the response from 10 flukes (3 replicates).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

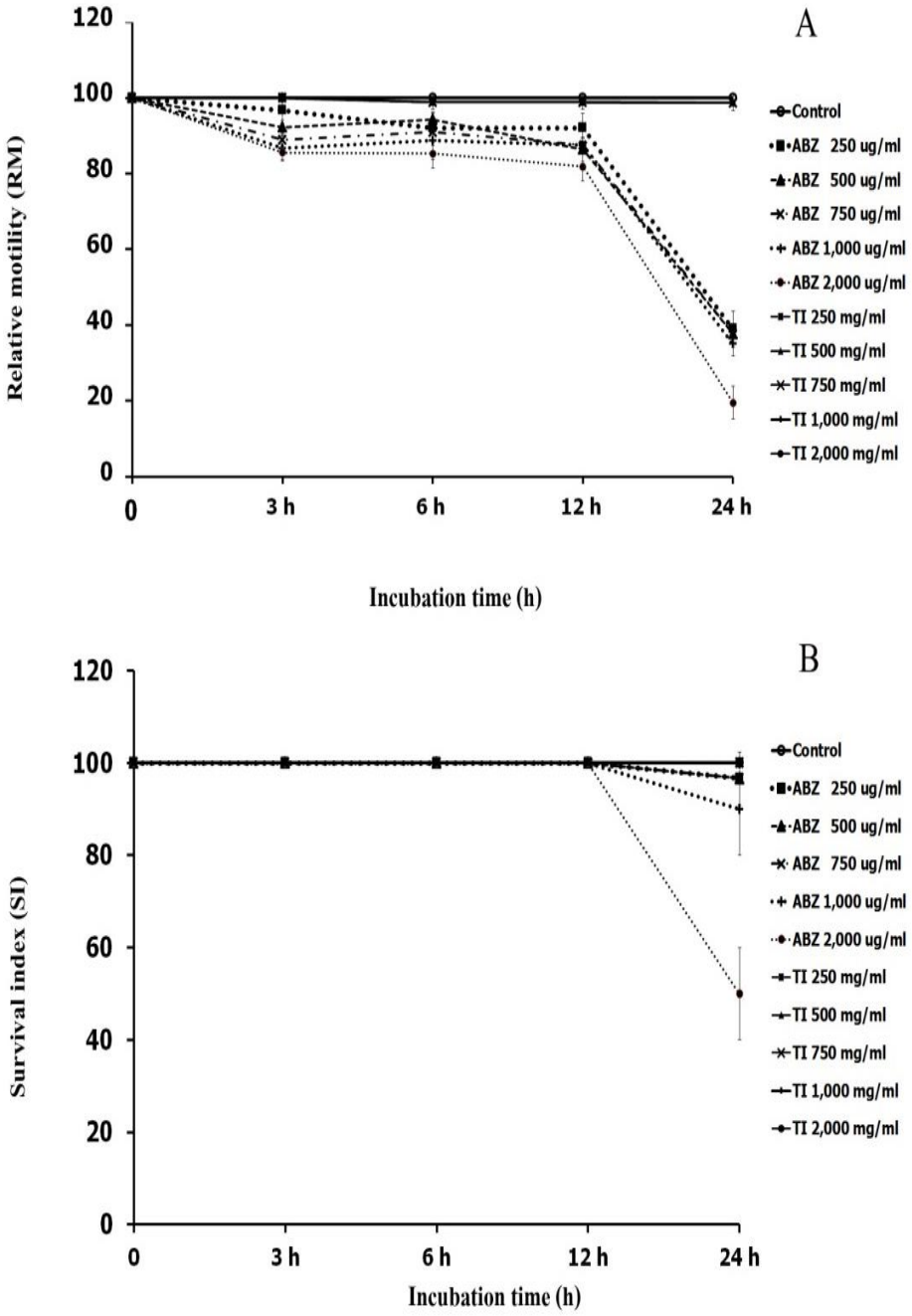

Fig. 2 (A) Relative motility (RM) and (B) survival index (SI) values of the control and the experimental worms treated with Albendazole (ABZ) and crude extract from *T. indica* (TI) at various concentrations and durations. Each point in graph represents the response from 10 flukes (3 replicates). Data are expressed as mean ± standard deviation (SD).

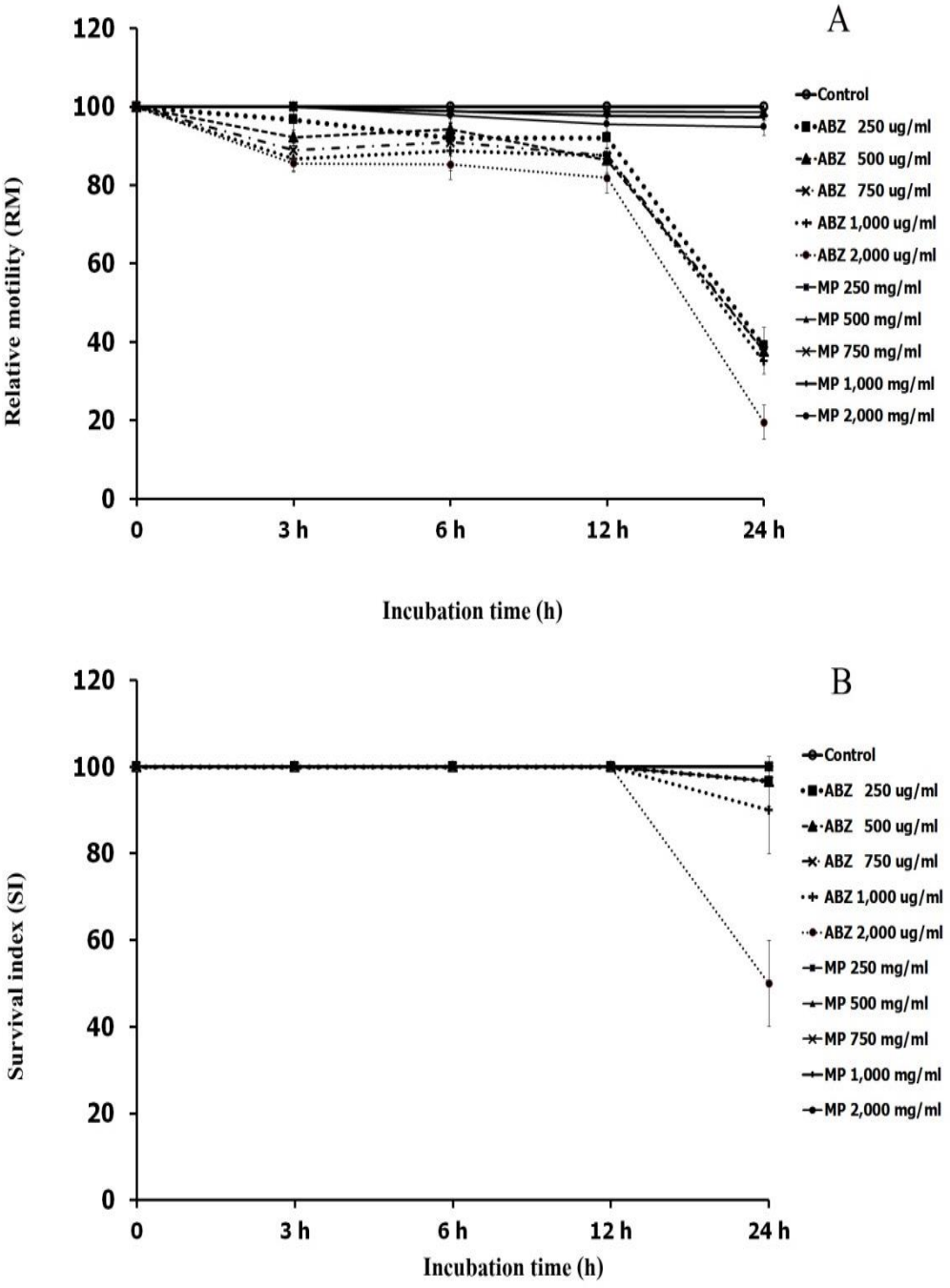

Fig. 3 (A) Relative motility (RM) and (B) survival index (SI) values or survival indies of the control and the experimental worms treated with Albendazole (ABZ) and crude extract from *M. paniculata* (MP) at various concentrations and durations. Each point in graph represents the response from 10 flukes (3 replicates). Data are expressed as mean± standard deviation (SD).

4.2 Scanning electron microscopic observations

4.2.1 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 concaves ventrally (Fig. 4A). 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 (Fig. 4A-E). The topography of tegument is composed of transversed major folds (fo) separated with major grooves (gr) (Fig. 4C). 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 (Fig. 4E). The transverse major folds on the anterior third of the body have numerous cluster of dome-shaped papillae (pa) arranged in rows (Fig. 4F). 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).

4.2.2 Effect of ABZ.

At 3 h incubation with albendazole, the general topography of ABZ-treated flukes appeared similar to the control group at 24 h post incubation. (Fig. 5A). However, when observed at higher magnification, the tegumental surface has marked by deep furrows between the major folds (Fig. 5B). During 6-12 h incubation, the swelling of tegument surface was found on both anterior and posterior parts of the fluke body (Fig. 5C-D). At 24 h incubation, the degrees of tegumental alterations were more severe on the ventral surface when observed under high magnification in comparison to the dorsal surface. There was erosion of affected area around genital pore (Fig. 5E). Minor damage was observed in the acetabulum (Fig. 5F).

4.2.3 Effect of crude extract of *A. lakoocha.*

The alteration of the tegument of the worm induced by PH treatment followed the same consequence for all dosages, but difference in degree of severity depending on concentrations and incubation period. During 3-6 h-incubation period,

the early sign of change was swelling which was found both on the ventral and on dorsal surface (Fig. 6A-B). At 12 h, the whole worm body was swelling (Fig. 6C). 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 (Fig. 6D-E). The focal erosions were formed and large areas of tegument were sloughed from the surface, resulting in the peeling of the tegument syncytium and exposed the basal laminar (Fig. 4F).

4.3 Analysis of the oxyresveratrol in the Puag-Haad

The purity of purified standard THS was confirmed by the HPLC (Fig 7A). The structure of purified standard oxyresvertrol 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 ($log\varepsilon$): 325 (4.241), $(log\varepsilon)_{Lit}$: 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 Puag-Haad is shown in Fig. 7B. Quantification was based on the integrated peak areas with reference to external standard (Fig 7A). The retention time of THS was 20.9 minutes and the naturally occurring amount of THS in Puag-Haad was found to be at 72.6±1.8%.

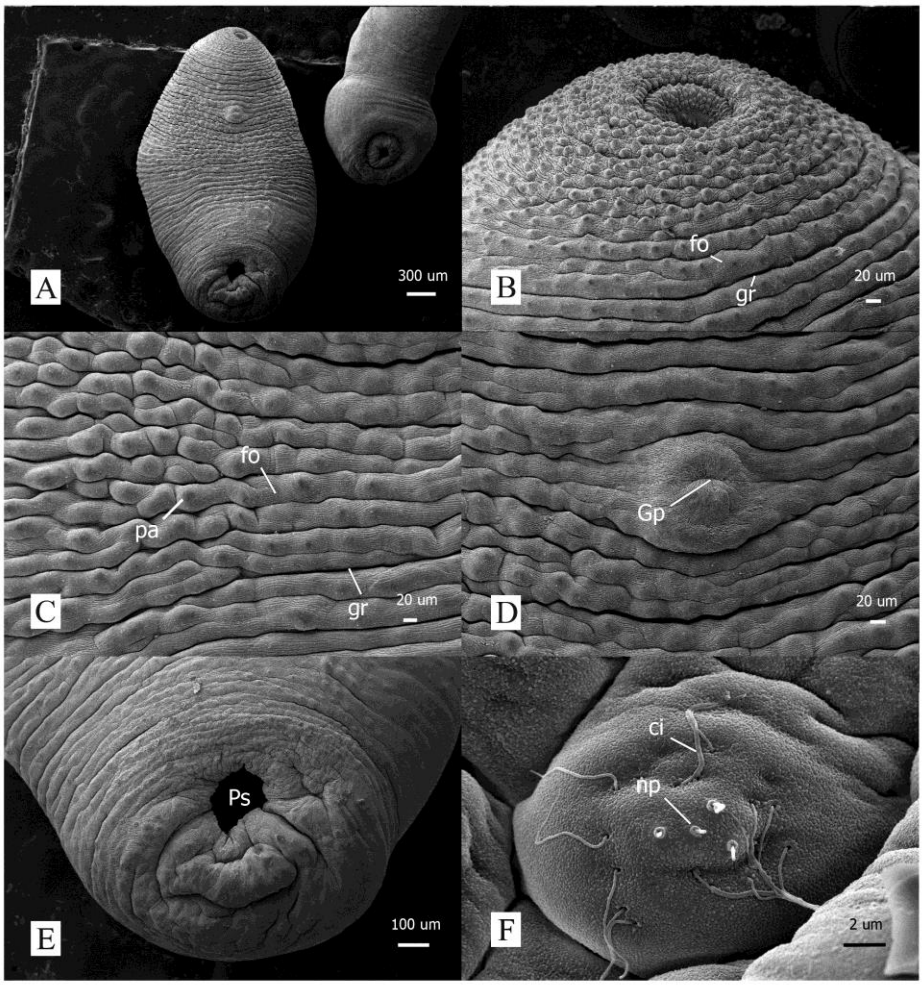

Fig. 4 Scanning electron micrograph of *P. cervi* incubated in medium M199 containing 0.1% DMSO for 24 h. A) The oral sucker (os) is placed at the anterior end, and the genital pore (gp) is positioned ventrally at the anterior one-third of the body. The posterior sucker (ps) or acetabulum is located 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

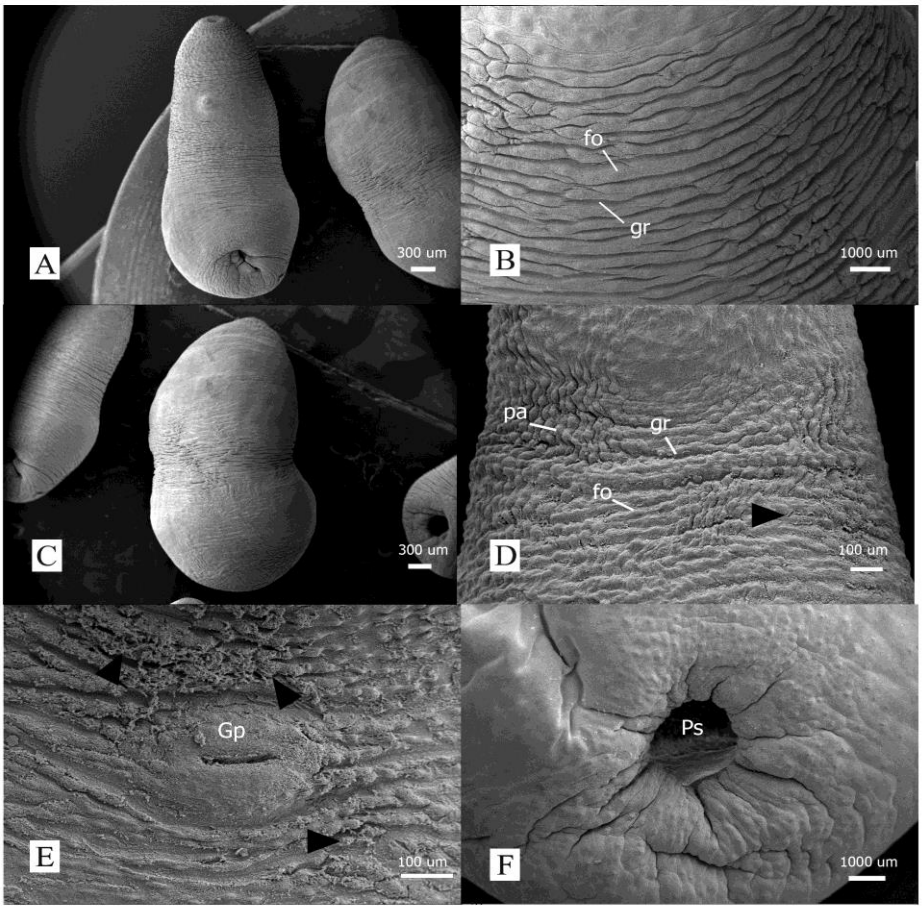

Fig. 5 A) At 3 h incubation with 250 µg/ml of ABZ, the general topography of flukes appeared similar to the control group at 24 h post incubation. B) At higher magnification, the one third of the ventral surface showing major folds (fo) alternated with major grooves (Gr). C) After 6 h incubation with 250 µg/ml of ABZ, the swelling of tegument 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 more severe alterations than in the dorsal surface. The deformity of papillae (pa) and erosion of tegument (arrowhead) was observed. E) At 24 h incubation incubation with 2000 µg/ml of ABZ, the erosion (arrowheads) around the genital pore was observed. F) At 24 h incubation incubation with 2000 µg/ml of ABZ, minor deformity on the tegument was observed at the acetabulum.

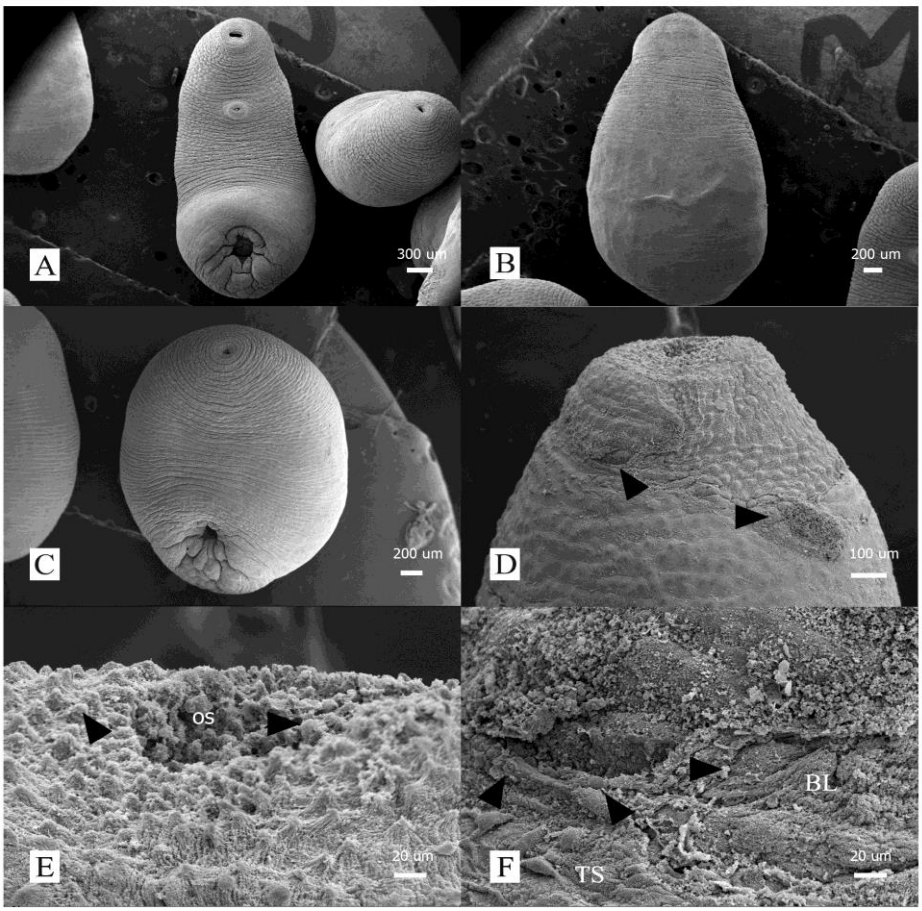

Fig. 6 The alteration of the tegument induced by PH treatment followed the same consequence for all dosages, but difference in degree of severity depending on concentrations and incubation period. A-B) At 6 h incubation with 250 µg/ml of PH, the early sign of change was swelling which was found both 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 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 and exposed the basal laminar.

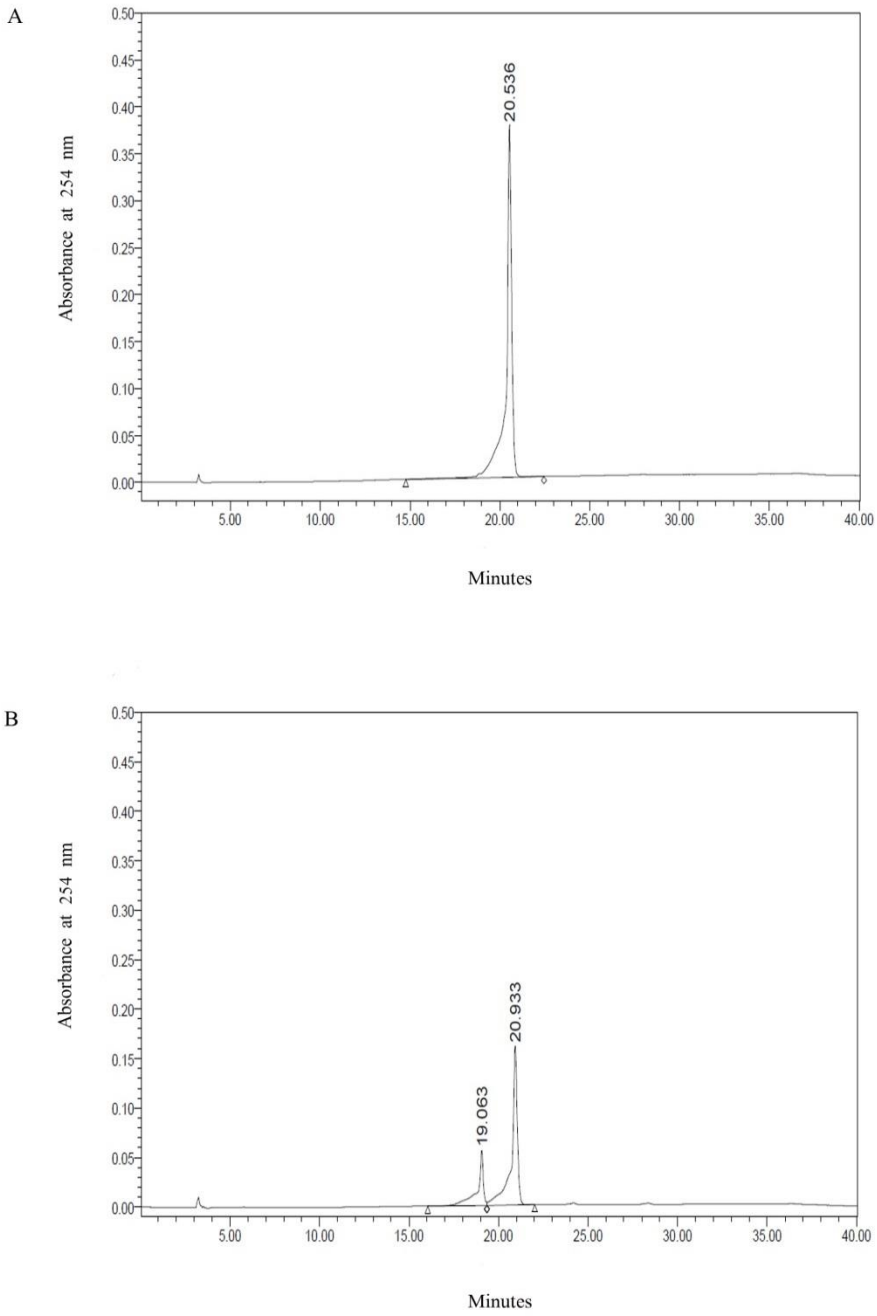

Fig. 7 HPLC Chromatogram of sample (A) purify oxyresveratrol for external standard (0.161 mg/ml) (B) Puag-Haad (0.101 mg/ml. The detection was conducted at 254 nm and quantification was based on the integrated peak areas with reference to external standard. The retention time of oxyresveratrol was 20.9 min.

CHAPTER 5

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). In this study there was no anthelmintic effect of crude extract from *M. paniculata* Linn and *T. indica*. Only PH expressed the killing effect on the worms in dose and time dependence.

PH is one of those plants that used empirically in Thailand and Lao, with little scientific support on their efficiency. Previous researches were demonstrated that the major bioactive constituents responsible for the anthelmintic 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 anthelmintic effect against *P. cervi* by "Puag-Haad", Thai medicinal crude extract from *A. lakoocha***.**

In this study, the anthelmintic 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 *paramphistomum* in dose and time dependence ($p<0.05$). We demonstrated that PH was effective to paralyze and kill the trematodes. PH at dose 2000 µg/ml was 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 effective against *F. gigantica*, *H. taichui* and *S. mansoni* (Saowakon, 2009; Wongsawad et al., 2005; Preyavichyapugdee, 2016), and the major target organ that was highly affected is the tegument which was 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 crude extract- treated-worm was swelling. This could be elicited by the osmotic imbalance which might be due to Na^+ influx into the syncytium. Fasiolicidal drugs which were phenolic compounds such as Nitroxynil, hexachlorophane or oxyclozanide caused uncoupling of oxidative phosphorylation followed by Na^+ influx and alteration of the tegument morphology. HPLC analysis showed that the major compound of the PH extract was THS (PubChem CID: 5281717), which was phenolic and corresponded to the previous reports (Mongkolsuk et al, 1957; Poopyruchpong et al, 1978). So it is possible that THS might interfere oxidative phosphorylation in the cell with the same mechanism in killing the worm as those phenolic compounds of Fasciolicide.

In contrast, Albendazole (ABZ) is a microtubule-targeting anthelmintic from benzimidazole group of compounds (Ehteda et al., 2013). ABZ induce degenerative alterations in the tegument of the trematode by binding to the colchicine-sensitive site of tubulin, thus inactivate the polymerization of microtubules. As a consequent, the carrying of secretory granules from the tegument cell bodies to the tegument is obstructed. This could conduct to advanced damage of the tegumental surface, especially the replacement of the rapidly turn-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 led to its immobilization and eventual death (Vignaduzzo et al., 2015).

CHAPTER 6

CONCLUSION

In conclusion, the present study substantiated the utilization of *A. lakoocha* as anthelmintic in traditional medicine practiced by indigenous people of Thailand and Laos (Salguiro, 2003). Besides, the crude extract of PH is not only potent anthelmintic activity against *F. gigantica*, *H. taichui* and *S. mansoni* (Saowakon, 2009; Wongsawad et al., 2005, Preyavichyapugdee, 2016), but also effective against *P. cervi*, causing significant disruption of the tegument of the parasite in a time-dependent manner. Thus, traditional use of crude extract of PH in helmintic infestation was established scientifically. The study might possibly help up 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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