THE EFFECTS OF CRUDE EXTRACT OF *PLUMBAGO INDICA* ROOT ON LIVER FLUKE, *OPISTHORCHIS VIVERRINI*

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ผลของสารสกัดหยาบจากรากเจตมูลเพลิงแดง ต่อพยาธิใบไม้ตับ *Opisthorchis viverrini*

นายญาณวรุตม์ คำชื่น

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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Opisthorchis viverrini เป็นพยาธิใบไม้ตับของมนุษย์ในตระกูล Opisthorchiidae ซึ่งเป็นสาเหตุของ Opisthorchiasis ซึ่งในปัจจุบันยังไม่มีวัคซีนที่ใช้ในการรักษาการติดเชื้อจากปรสิตในกลุ่มดังกล่าวโดยตรง Praziquantel (PZQ) เป็นยาแผนปัจจุบันที่ได้รับการแนะนำว่ามีประสิทธิภาพมากที่สุดในการรักษาการติดเชื้อจากพยาธิดังกล่าว Plumbagin (PB) เป็นสารประกอบธรรมชาติที่สกัดได้จากรากของพืชในสกุล Plumbago สารประกอบนี้ใช้เป็นยาแผนโบราณสำหรับการรักษาโรคต่าง ๆ รวมถึงการติดเชื้อพยาธิ วัตถุประสงค์ของการศึกษาครั้งนี้เพื่อศึกษาผลของสารสกัดหยาบจากรากเจตมูลเพลิงแดง (*Plumbago indica*) ต่อพยาธิใบไม้ตับ *Opisthorchis viverrini* โดยการประเมินจากค่า relative motility (RM) และ survival index (SI) ต่อตัวอ่อนพยาธิในระยะ newly excysted juveniles (NEJs) และ อายุ 4 สัปดาห์ นอกจากนี้ยังทำการตรวจสอบการเปลี่ยนแปลงผิวของพยาธิและโครงสร้างที่เกี่ยวข้องด้วยกล้องจุลทรรศน์แบบใช้แสง (LM) กล้องจุลทรรศน์อิเลคตรอนแบบส่องกราด (SEM) และกล้องจุลทรรศน์อิเลคตรอนแบบส่องผ่าน (TEM) เพื่อศึกษาถึงเป้าหมายการการออกฤทธิ์ของยา ส่วนประสิทธิภาพของยาเมื่อใช้ในสิ่งมีชีวิตและผลข้างเคียงที่อาจเกิดขึ้นนั้นศึกษาโดยการวัดค่า worm recovery การตรวจเอนไซม์ตับและตัวบ่งชี้ทางจุลพยาธิวิทยาเนื้อเยื่อ

ผลการศึกษาพบว่า PB ออกฤทธิ์ในการฆ่า NEJs ร้อยละ100 ที่คล้ายกับ PZQ ที่ความเข้มข้น 1 ไมโครกรัม/มิลลิลิตร ภายใน 24 ชั่วโมง ขณะที่สารสกัดหยาบจากรากเจตมูลเพลิงแดง (cPI) แสดงผลใกล้เคียงกับ PZQ เมื่อใช้ cPI ที่ความเข้มข้น 10 ไมโครกรัม/มิลลิลิตร ที่ทำให้พยาธิตายถึงร้อยละ 93 ขณะที่การศึกษาในตัวอ่อนพยาธิอายุ 4 สัปดาห์พบว่า ตัวอ่อนไม่สามารถเคลื่อนที่ได้อย่างสมบูรณ์เมื่อบ่มด้วย PZQ ที่ 1 ไมโครกรัม/มิลลิลิตร แต่ไม่ตายอย่างสมบูรณ์ภายใน 24 ชั่วโมงในขณะที่ PB และ cPI สามารถฆ่าพยาธิได้สูงถึงร้อยละ 70 และ 30 ตามลำดับ โดย PZQ และ PB ที่ความเข้มข้น 10 ไมโครกรัม/มิลลิลิตรสามารถฆ่าพยาธิร้อยละ 100 และ 96 ในขณะที่ cPI ที่ความเข้มข้นเดียวกันนี้สามารถฆ่าพยาธิได้เพียงแค่ร้อยละ 74 เมื่อทำการศึกษาผ่านกล้อง SEM พบว่า PZQ PB และ cPI สามารถทำให้เกิดการเปลี่ยนแปลงบนผิวของพยาธิที่คล้ายกัน แต่มีความรุนแรงของการเปลี่ยนแปลงที่แตกต่างกัน ประกอบด้วยการบวมของผิว เกิดตุ่มน้ำ ผิวแตกประทุ การเกิดรอยถลอกและการหลุดลอกของชั้นผิว เมื่อทำการศึกษาด้วย TEM พบว่าผลที่ได้มีความ

สอดคล้องกับผลของ SEM โดยพบการถูกทำลายของชั้นผิว การสลายตัวของไมโทคอนเดรียและการย่อยสลายเม็ดแกรนูล ซึ่งปรากฏในกลุ่มการรักษา PZQ PB และ cPI เมื่อทำการศึกษาในสัตว์ทดลองพบว่า ค่า worm recovery ของกลุ่มที่ได้รับ cPI มีค่าแตกต่างจากกลุ่มที่ไม่ได้รับการรักษาอย่างมีนัยสำคัญ และ cPI จะมีประสิทธิภาพมากขึ้นเมื่อเพิ่มค่าความเข้มข้นมีค่าไม่แตกต่างจากเท่ากับ PB ที่ความเข้มข้นเดียวกัน สำหรับการประเมินระดับเอนไซม์ในตับ (ALT และ AST) ที่ 24 ชั่วโมงและ 7 วันหลังจากได้รับ cPI พบว่าพารามิเตอร์ทั้งสองมีผลไม่มีความแตกต่างจากกลุ่มควบคุมที่ไม่ได้รับ cPI และเมื่อทำการทดสอบความเป็นพิษในสัตว์ทดลองพบว่าไม่มีการเปลี่ยนแปลงพฤติกรรมและการตายของสัตว์ทดลองเกิดขึ้นหลังจากได้รับ cPI ที่ความเข้มข้นสูงสุด (3000 มิลลิกรัม/กิโลกรัม) และผลจากการตรวจสอบการเปลี่ยนแปลงทางจุลพยาธิวิทยาชี้ให้เห็นว่าเกิดพยาธิสภาพขึ้นที่ตับและไตที่ 24 ชั่วโมงและพยาธิสภาพดังกล่าวได้ฟื้นตัวดีขึ้นในเวลา 7 วันหลังจากได้รับ cPI จากการศึกษานี้บ่งชี้ให้เห็นว่าสารสกัดหยาบจากรากเจตมูลเพลิงแดงนี้มีประสิทธิภาพเพียงพอและสามารถพัฒนาต่อไปเพื่อใช้เป็นยาในการรักษาผู้ป่วยที่มีการติดเชื้อพยาธิใบไม้ตับกลุ่มนี้ และหากไม่ได้รับการรักษาการติดเชื้อนี้มีโอกาสที่จะพัฒนาไปเป็นมะเร็งท่อน้ำดีต่อไปได้ในอนาคต

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YANWARUT CAMCHUEN : THE EFFECTS OF CRUDE EXTRACT OF *PLUMBAGO INDICA* ROOT ON LIVER FLUKE, *OPISTHORCHIS VIVERRINI.* THESIS ADVISOR : ASST. PROF. NARUWAN SAOWAKON, Ph.D. 157 PP.

PLUMBAGO INDICA/ PLUMBAGIN/ *OPISTHORCHIS VIVERRINI*/ TOXICITY/ TEGUMENTAL CHANGES

Opisthorchis viverrini is the human liver flukes in the family Opisthorchiidae that causes the opisthorchiasis. Nowadays, the parasitic vaccine is not yet available. Praziquantel (PZQ) is an effective drug recommended for treatment of opisthorchiasis. Plumbagin (PB) is a natural compound extractable from the root of many plants, especially in the *Plumbago* genus plant. This compound is used as a traditional ayurvedic medicine for treatment of several ailments including antiparasitic activity. This study aimed to investigate the possible anthelmintic effects of crude extract of *P. indica* root (cPI) against *O. viverrini* by estimating relative motility (RM) and survival index (SI) on newly excysted juveniles (NEJs) and 4-week-old flukes. The alterations of tegumental and associated structures after drug treatment were also investigated by light (LM), scanning electron (SEM), and transmission electron microscopy (TEM) to elucidate the target of action of cPI. The therapeutic effect of cPI in animal model and its adverse effect were studies by detecting the percent of worm recovery, liver enzyme test, and histopathological changes

The results showed that within 24 h of incubation, PB at 1 μg/ml killed 100% of NEJs which similar to 1 μg/ml PZQ while cPI showed an anthelmintic effect

similar as PZQ at 1 µg/ml when we used cPI at 10 µg/ml that cause of worm dead to 93%. In the 4-week old flukes, the complete immobility of flukes was found when incubated with PZQ at 1 µg/ml but flukes were not completely dead within 24 h while PB and cPI killed flukes up to 70% and 30%, respectively. At 10 µg/ml, PZQ and PB could kill 4-week-old flukes up to 100% and 96% while cPI showed only 74% of worm killing. When observed by SEM, the PZQ, PB, and cPI caused progressive severe rupture tegument, bleb surface, and erosion formation. TEM study corresponded with SEM result, the bleb formation in the tegumental syncytium and mitochondria and secretory granule degradation appeared in PZQ, PB, and cPI treatment groups. The numbers of fluke recovery in cPI showed significantly lower than the untreated group and cPI exhibited more effective when the increasing concentration and effect of cPI were equal to PB as shown by higher percent of worm protection. Liver enzymatic levels (ALT and AST) were evaluated at 24 h and 7 days following oral administration. Both parameters showed similar percent to the control group. The toxicity test revealed no alterations in behavior and mortality in hamsters after receiving a high dose of 3000 mg/kg cPI. Liver and kidney exhibited histopathological changes at 24 h and recovery in 7 days post-administration. Overall, further investigation of cPI as natural medicine targeting cholangiocarcinoma and/or positive *O. viverrini* infection in human clinical trials should be useful.

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LIST OF ABBREVIATIONS

µg/ml	=	Microgram per milliliter
µl	=	Microliter
µm	=	Micrometer
g	=	Gravitational acceleration
ANOVA	=	Analysis of variance
DI water	=	Distilled water
DMSO	=	Dimethylsulfoxide
g	=	Gram
h	=	Hour
IC_{50}	=	Median inhibition concentration
kDa	=	Kilodalton
kg	=	Kilogram
L	=	Liter
min	=	Minute
mg	=	Milligram
mg/ml	=	Milligram per milliliter
ml	=	Milliliter
nm	=	Nanometer
PBS	=	Phosphate buffered saline
rpm	=	Revolution per minute

LIST OF ABBREVIATIONS (Continued)

RPMI 1640	=	Roswell Park Memorial Institute number 1640
SD	=	Standard deviation
v/v	=	volume by volume
PZQ	=	Praziquantel
PB	=	Plumbagin
cPI	=	Ethanolic crude extract from *Plumgo indica* root
NEJs	=	Newly excysted juvenile
RM	=	Relative motility
SI	=	Survival index
Os	=	Oral sucker
Vs	=	Ventral sucker
T	=	Tegument layer
Vc	=	Vacuole
Bl	=	Blebbing
St	=	Swollen tegument
Pa	=	Papillae
Sp	=	Spines
Ep	=	Excreatory pore
B	=	Basal lamina
V	=	Vitelline gland
In	=	Intestine

LIST OF ABBREVIATIONS (Continued)

SEM	=	Scanning elctron microscope
TEM	=	Transmission electron microscope
op	=	Outer membrane
ip	=	Inner membrane
CMu	=	Circular muscle
LMu	=	Longitudinal muscle
BM	=	Basement membrane
Mi	=	Mitochondria
dg	=	Dense granule
lg	=	Light granule
Gly	=	Glycocalyx
AST	=	Aspartate aminotransferase
ALT	=	Alanine aminotransferase
BUN	=	Blood urea nitrogen
Cr	=	Creatinine

CHAPTER I

INTRODUCTION

1.1 General introduction

Food-borne trematodiasis is classified as one of the major groups of the neglected tropical diseases (NTDs) worldwide with more than 40 million people infected and 750 million people at risk infection. The trematodes infections in humans also are reported more than 100 species, including *Opisthorchis viverrini*, *Clonorchis sinensis*, *Fasciola* spp., *Paragonimus* spp. and intestinal flukes. The human liver flukes infection has remained an extensive public health problem in many areas of the world especially in developing countries (Sripa et al., 2010). Although, the trematode infection usually occurs in many parts of the world, particularly in Southeast Asia (Graczyk and Fried, 2007). *Opisthorchis viverrini*, *O. felineus* and *C. sinensis* are the human liver flukes in the family Opisthorchiidae that cause the opisthorchiasis and clonorchiasis, respectively. *Opisthorchis felineus*, *O. viverrini*, and *C. sinensis* are prevalent in Siberia and East Europe, Southeast, and East Asia, respectively. In the People's Republic of China (P.R. China), Korea and North Vietnam are extensively widespread of trematode infection by *C. sinensis*, whereas *O. viverrini* is endemic in Southeast Asia, including Thailand, Lao People's Democratic Republic (Lao PDR), Cambodia and central, south Vietnam, respectively (IARC, 2012).

Current reports of clonorchiasis, paragonimiasis, fascioliasis, and opisthorchiasis worldwide have shown as many as 35, 20.7, 2.4-17 and 1.2-10 million infected people, respectively. Recent reports suggested that over 15 million *C. sinensis*-infected human in P.R. China alone, and another 8-10 million individuals infected with *O. viverrini* in Thailand and Lao PDR (Jongsuksuntigul and Imsomboon, 2003; Andrews et al., 2008) and more than 600 million people in Asia, are at risk of combined infection with *C. sinensis* and *O. viverrini* (Keiser and Utzinger, 2009). The infections are associated with several hepatobiliary diseases including hepatomegaly, cholangitis, and fibrosis of the periportal system, cholecystitis, and gallstones. They are the major problem of bile duct cancer, are called cholangiocarcinoma (CCA).

Infections of *O. viverrini* and *C. sinensis* have been classified as Group 1 carcinogens-metazoan parasites by the International Agency for Research on Cancer, World Health Organization (WHO) (IARC, 1994; Bouvard et al., 2009), which is associated with the induction of epithelial bile duct cancer, CCA. Endemic infection areas of *O. viverrini* are in the lower Mekong area including Lao PDR, Cambodia, Vietnam and Thailand (Jongsuksuntigul and Imsomboon, 2003; IARC, 2011). Recently, epidemiological studies have signified the worldwide increase in both incidence and mortality of cholangiocarcinoma over the past 2 to 3 decades (Sawanyawisuth et al., 2012). In Thailand, the incidence of cholangiocarcinoma is the highest in the world especially in Northeast Thailand, Khon Kaen province, and it is one of the top 5 diseases of highest mortality (Sripa and Pairojkul, 2008).

Normally, people in Southeast Asia live around natural water resources such as ponds and rivers, where have numerous species of freshwater fishes. So, fish is a basic food for poor people in rural areas. There are many supporting reasons for the

high prevalence of *O. viverrini* infections, including lack of sanitation knowledge, poor concern about the trematode infections because of their asymptomatic clinical presentation in the early infection (Sripa et al., 2010). People acquire the infection by consumption of raw or undercooked cyprinid (freshwater) fish, fermented or partially freshwater cyprinid fish, the second intermediate hosts, containing the infective metacercariae stage. The local traditional dishes of fermented, raw or partially cooked cyprinid fish (namely *Koi-pla*, *Pla-som*, *Jom-planoi*, *Pla-ra* etc.) are favorite meals of rural people in the North and the Northeast of Thailand and Laos' people in the Greater Mekong subregion (Grundy-Warr et al., 2012; Onsurathum et al., 2016). Therefore, the high prevalence of *O. viverrini* infection has still occurred in those groups. Mostly prevalence rate is found in the northeastern, followed by the northern and central regions but it is very lightly prevalence in the southern region (Sithithaworn et al., 2012). The infection rate of *O. viverrini* in cyprinid fish varies depending on the species and localities where they inhabit. High infection rates have been reported in many species of native fish in the family Cyprinidae such as *Puntius brevis* (92%), *Esomus metallicus* (75%), *Dangila lineata* (69.6%), *Cyclocheilichthys repasson* (58.5%), *Mystacoleucus marginatus* (50%), *Hampala dispar* (44.4-72.7%), *Cyclocheilichthys armatus* (43.1-100%), *Henicorhynchus lineatus* (42.9%), *Puntioplites falcifer* (33.3%), *Osteochilus waandersii* (30.5%), and *Puntioplites proctzysron* (26.8%) (Manivong et al., 2009; Touch et al., 2013). The *O. viverrini* metacercariae in fish were most commonly found the highest density in the head region, followed by the caudal fin and muscles of fins, and the lowest in the anal fin (Tesana et al., 1985). The contaminated metacercariae food is exposed to digestive enzymes in the stomach and intestines that induces newly excysted juveniles (NEJs)

in the duodenum, and migrate and live in the bile duct until they are mature liver flukes. The adult fluke can survive for more than 20 years in humans, which explains the persistent infection of long duration (Sripa et al., 2011).

There are many methods currently used for reducing and controlling the prevalence rates of this liver fluke including improved sanitation and health education and snail elimination management. Nowadays, the parasitic vaccine is not yet available. Hence, treatments of infected hosts with anthelmintic drugs are the most effective treatment in current use. Although praziquantel (PZQ) is the effective drug recommend for treatment of opisthorchiasis, the short term PZQ treatment in the hamster model showed the indirect side effects of PZQ. Pinlaor and colleagues (2008) reported that PZQ induced the inflammatory cell activities by increasing expression of inducible nitric oxide synthase (iNOS), nuclear factor-κB (NF-κB) in *O. viverrini* infected hamsters. Moreover, the prolonged drug treatment in the infected host can induce drug resistance in the parasites. For these reasons, there is an urgent need to find new effective compounds. Investigations on indigenous medical plants for their anthelmintic activities may contribute to the development of alternative folk medicine and novel drugs.

Plumbagin (PB, 5-hydroxy-2-methyl-1-4-naphthoquinone) is a natural compound extractable from the root of many plants, especially in *Plumbago* genus plant. This compound is used as a traditional ayurvedic medicine for the treatment of several ailments including diarrhea, skin disease, antimicrobial, anticancer, and anti-inflammation (Krishnaswany and Puruchothaman, 1980; Paiva et al., 2003). Recently, the antiparasitic activities of PB have been investigated by previous studies which showed that PB had the strongest activity against *Caenorhabditis.elegnas*

(Atjanasuppat et al., 2009), *Paraphistomum cervi* (Saowakon et al., 2013), *Schistosoma mansoni*, and *Fasciola gigantica* (Lorsuwannarat et al., 2013) Moreover, PB affects the motility and hatching of *Haemonchus contortus, Ascaris suum* (Fletterer and Fleming, 1991). From their reports, PB inhibited motility and induced tegumental changes of liver flukes. At present, nobody has been studied the potential development of crude extract of *P. indica* root as an anthelminthic drug. The hypothesis of this study is a crude extract of *P. indica* root may possess the anthelmintic effect against *O. viverrini*. Therefore, this study investigated the possible anthelmintic effects of crude extract of *P. indica* against *O. viverrini* and explore the side effect of this compound on a mammalian model to ensure its property. The crude extract of *P. indica* root may be developed as the alternative anthelmintic drug for the treatment of opisthorchiasis in the future. Moreover, it may reduce the therapeutic cost in poor patients, increase the herb-market values, and support the growth of agricultural farming.

1.2 Research objectives

The overall objectives were to investigate the anthelmintic effects of crude extract of *P. indica* root against *O. viverrini* and evaluate the side effects of the extract on a mammalian model to ensure its harmless anthelmintic property.

The specific purposes of this study were as follows:

1. To study the effects of crude extract of *P. indica* root on newly excysted juveniles (NEJs) and 4-week-old of *O. viverrini* for relative motility (RM) and survival index (SI). Both stages of *O. viverrini* were compared under a scanning electron microscope (SEM) and transmission electron microscope (TEM).

2. To evaluate the protective effect against *O. viverrini* of crude extract from *P. indica* root, PB and PZQ in the hamster model.

3. To study the toxicity of crude extract of *P. indica* root on a hamster model.

1.3 Scope and limitations of the study

The metacercariae were collected from cyprinid fish in the Mekong area (at Khammouan Province in Lao and Mukdahan Province in Thailand). The active metacercariae were identified by the special technician in the Department of Parasitology, Faculty of Medicine, Khon Kaen University. Hamsters in this study were purchased from Animal Care Unit of Faculty of Medicine, Khon Kaen University. This experiment focused on the anti-parasitic activities of the *P. indica* root crude extract and its toxicity in both female and male Golden Syrian hamsters. The anti-parasitic activities of the extract were limited to *in vitro* effect on NEJs and 4-week-old of *O. viverrini*. The protective effect of the extract against *O. viverrini* was investigated *in vivo* using the hamster model.

1.4 Research Hypothesis

1. The crude extract of *P. indica* root might have the anthelmintic effect on infected stages of *O. viverrini.*

2. The crude extract of *P. indica* root could act like PB by changing the tegument of *O. viverrini*.

3. The crude extract might have low toxicity in the hamster model.

CHAPTER II

LITERATURE REVIEWS

2.1 Taxonomy, general structure, and biology of *Opisthorchis viverrini*

Opisthorchis viverrini (*O. viverrini*) is belonging to the Opisthorchiidae family, genus *Opisthorchis.* The life-cycle of *O. viverrini* is well established (Figure 2.1), it is greatly complicated by the genetic variation found in different habitats, with specific genotypes related to habitat types or intermediate host species in different wetlands (Sithithaworn et al., 2012). The definitive host of *O. viverrini* is humans, and reservoir hosts are piscivorous mammals such as cats and dogs. The adult worms produce eggs, which pass into the bile duct of the host and reach the exterior following defecation. Embryonated eggs are developing inside the host. Then, the first intermediate snail host of the genus *Bithynia* (Figure 2.1) digests these eggs. In the snail hosts, they exhibit several developmental and multi-stages (miracidia, sporocyst, redia, and cercaria). After 5 weeks, the final stage of *O. viverrini* in the snail host is called cercaria that subsequently emerges from snail to the outside environment. Then, cercariae penetrate between the scales of freshwater cyprinid fish belonging to Cyprinidae family (Figure 2.1) which are the second intermediate host.

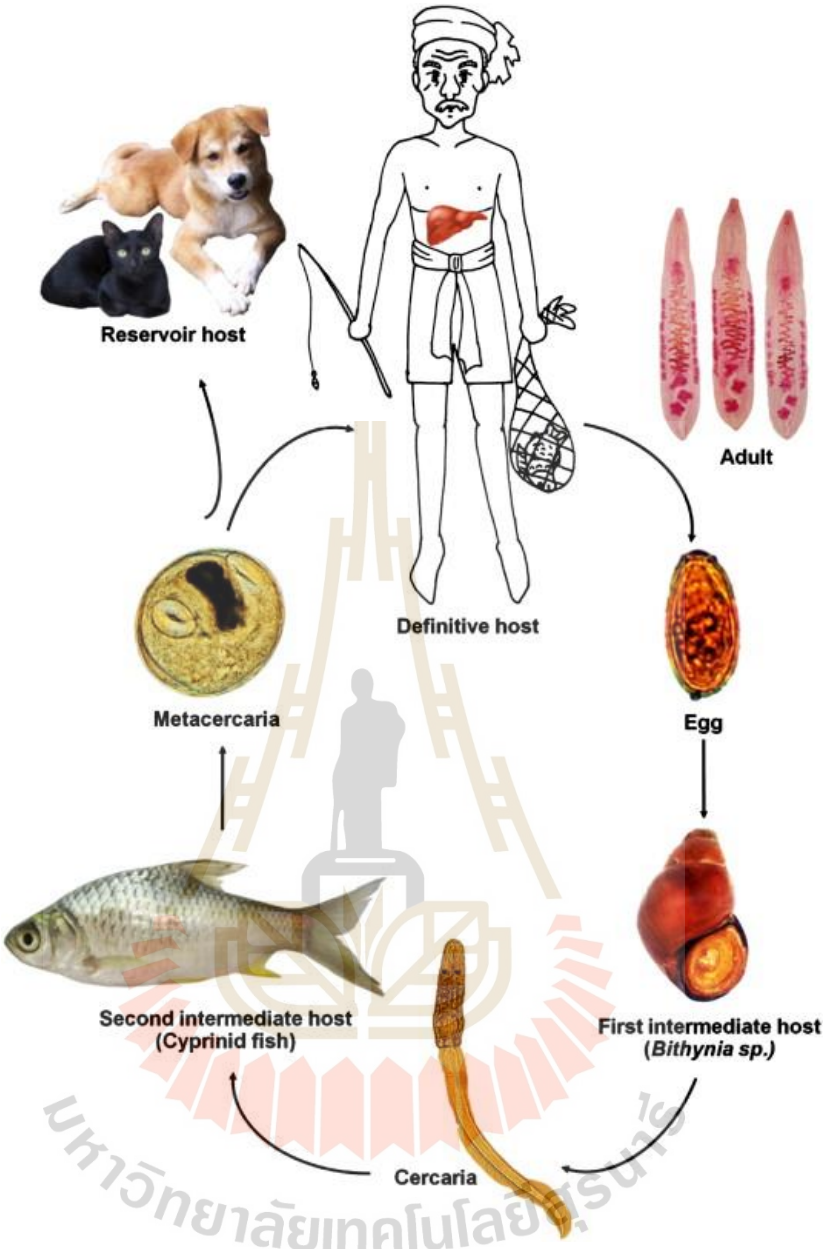


Figure 2.1 Life cycle of *Opisthorchis viverrini* (Petney et al., 2012)**.**

After a few days, cercariae encyst in the fish's muscle becomes metacercariae. Humans can acquire the infection by ingesting raw or inadequately cooked infected fish such as in the common name *Pla-som, Koi-Pla* (Figure 2.2). The digestive

enzymes in the stomach and intestines induce the metacercaria excystment in the duodenum, become newly excysted juveniles (NEJs) which migrate through the ampulla of Vater into the bile duct, where they mature into adult worms in 2 weeks. The adult fluke inhabits the biliary tract, generally localize within the intrahepatic bile ducts. The adult fluke can survive in humans for more than 20 years, which explains the prolonged persistent. Locations of endemic infection reflect the geographic distribution of the essential intermediate host snails (Sripa et al., 2011).

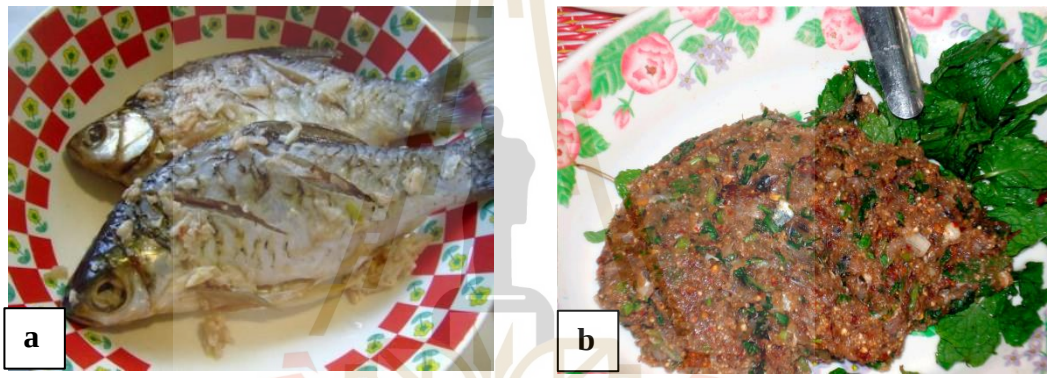


Figure 2.2 Photographs of raw and fermented fish dishes, the sources of *O. viverrini* infection, (a) Pla-Som, (b) Koi-Pla (Sripa et al., 2011).

Meesabye (1985) reported that the adult *O. viverrini* has the body like as leaf, the anterior part found the oral and ventral suckers. At high magnification by scanning electron microscope, the surface appears studded with numerous stubby microvilli which are closely packed together. Among microvilli, there are sensory bulbs or papillae which are distributed throughout the surface. At the dorsal surface of worm, this surface appeared smoother with fewer papillae and the microvilli have shorter and more widely-spaced than that of the ventral surface.

Apinhasmit and Sobhon (1996) reported that adult *O. viverrini* has a corrugated surface of the tegument and numerous sensory papillae located along the lateral border.

2.2 Host immune response to *Opisthorchis viverrini* infection

When the newly excysted juveniles of *O. viverrini* migrate from the duodenum into the lumen of the bile duct, the parasites do not invade surrounding tissue or come into intimate contact with the lymphoid tissue except in severe infection (Sun et al., 1969). The metabolic products or perhaps soluble antigenic structural components shed from the parasite surface may diffuse through the epithelial lining of the bile duct into the surrounding tissue, where they may induce an immune response (Sun and Gibson, 1969; Flavell et al., 1980). The antibody was also noted in the patients with opisthorchiasis and it was reported that the percentage of serum positive in severe infection was significantly higher than those with mild infection. Serum from infected hamsters contained components; possibly specific immunoglobulins capable of reacting with an adult, juvenile, excretory-secretory (ES) and egg antigens (Flavell, 1981; Sirisinha et al., 1983; Sripa and Kaewkes, 2000b). Antibody response to both somatic and ES antigens was detected in the sera from the second week of infection onward and the peak response was noted at the end of the second month (Sirisinha et al., 1983a). Sripa and Kaewkes (2000) reported that the antibody responses in hamsters were first detected at 7–14 days after infection. During early infection, hamsters infected with 50 or 100 metacercariae showed higher antibody levels than that of 25 metacercariae. After that, the antibody level has rapid increase to be constant at approximately 2 months (Sripa and Kaewkes, 2000b). Secretory IgA

antibody reacted with egg and somatic antigens also appeared in the bile of infected hamsters (Sirisinha et al., 1983b). Similarly, IgG, IgA and IgE antibodies in serum and bile have been reported in patients with opisthorchiasis (Srivatanakul et al., 1985; Wongratanacheewin and Sirisinha, 1987; Wongratanacheewin et al., 1988a, 1988b; Akai et al., 1995) as well as in animals and patients infected with *C. sinensis*, a related liver fluke (Sun and Gibson, 1969a; Yen et al., 1992; Wu et al., 1993; Yong et al., 1993, 1999; Lin et al., 1995). There was also a significant increase in antibody in the bile and serum of *O. viverrini*-infected patients. Analysis of bile and serum from individual patients showed that while IgG was predominant in the serum of all patients, IgA was present at approximately the same level as IgG or higher in the bile. IgE antibody could be detected in many of the bile samples (Wongratanacheewin et al., 1988a). The IgG level to the somatic antigen was most markedly raised in disease cases compared with normal subjects and was closely associated with gall bladder size and dysfunction (Haswell-Elkins et al., 1991). Furthermore, they also correlated significantly with *O. viverrini* egg count (Elkins et al., 1991). The antibodies to both *O. viverrini* and *C. sinensis* were decreased after treatment (Thammapalerd et al., 1988; Wongratanacheewin et al., 1988b). Serum antibody levels correlated with liver ultrasonography findings that including cholangiocarcinoma (Akai et al., 1994). In the hamster, the parasite antigens were observed in the epithelial lining of intrahepatic and extrahepatic bile ducts as early as day 3 after infection. The antigens were not observed in the small bile ducts of the liver, which are not normally inhabited by flukes (Sripa and Kaewkes, 2000a). Altogether, those reports indicated that sufficient antigens were produced by the parasites even during the early stage of infection to stimulate the host immune system. In contrast to the extensive study on human

immune response, the study on cell-mediated response is limited. In 1978, Bhamarapravati and colleagues demonstrated that at the early stage of infection, there was an acute inflammatory reaction occurred involving the bile duct and the portal area where the juvenile flukes resided. As the flukes developed into adults, there was a granulomatous response to adult flukes and eggs. After 6 weeks of infection, epithelioid granuloma with multinucleated giant cell infiltration was observed. The inflammatory granuloma was also surrounded by the precipitation of eosinophils, neutrophils, mononuclear cells, and macrophages. It was quite possible that eosinophils precipitated surrounding the parasites and eggs, represented immune complexes and that the epithelioid granuloma was related to cell-mediated immune reactivity. Similar results were noted in patients with clonorchiasis (Sun, 1980). These patients exhibited egg-induced granuloma, both in the gall bladder and in the liver portal area (Sun, 1969, 1980). The pathological changes in hamsters occurred not only in the gall bladder where the parasites resided but also in the extrahepatic bile duct which was more severe (Sripa and Kaewkes, 2000a). Although both cellular and humoral immunity against *O. viverrini* infection were reported, the antigenic recognition molecules of T and B cells were studied and found to be striking differences (Wongratanacheewin et al., 1991). The scientific evidence presented above clearly showed that such as the superficial infection by the fluke could stimulate both systemic humoral and cell-mediated immune responses during the course of infection, the possible significances of these immune responses to protective immunity have presently unknown. It has been noted that some patients from the endemic area may harbor several thousand worms (Bunnag et al., 1981).

2.3 Cholangiocarcinoma

Cholangiocarcinoma is an inclusive term for adenocarcinoma of the biliary tree, which encompasses a site of origin within intrahepatic, perihilar and extrahepatic bile ducts (Wyatt, 2011). Recent epidemiological studies have signified the worldwide increase in both incidence and mortality of cholangiocarcinoma over the past 2 to 3 decades (Sawanyawisuth et al., 2012). Epidemiological studies have determined that the incidence of cholangiocarcinoma correlates strongly with the prevalence of *O. viverrini* infection. Cholangiocarcinoma is the predominant type of liver cancer in Thailand (Figure 2.3).

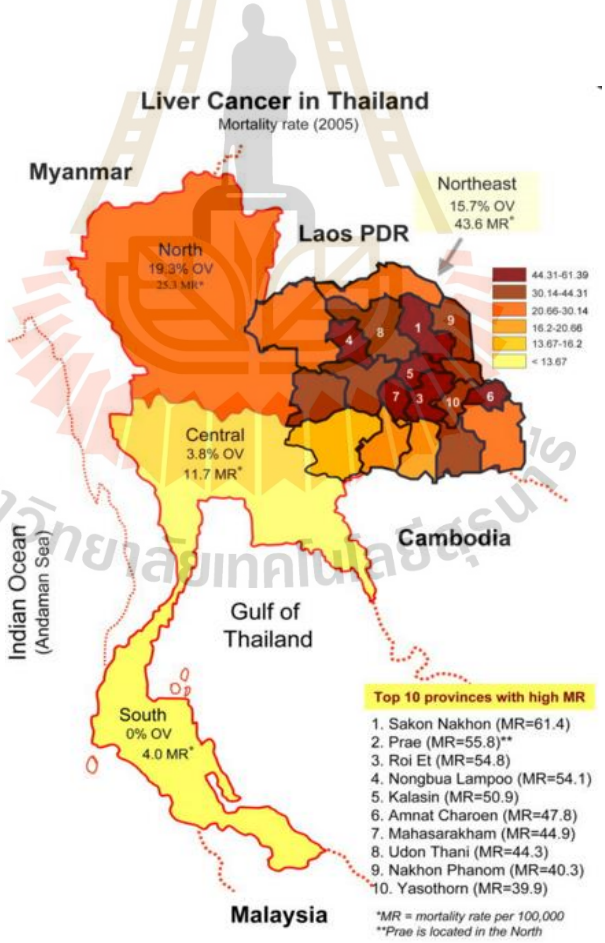


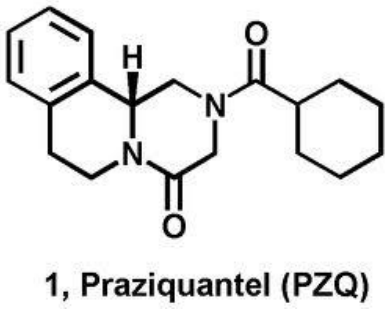
Figure 2.3 Endemic of liver cancer in Thailand (Sripa et al., 2008).

The high incidence of cholangiocarcinoma is found in Southeast Asia, especially in the Northeast of Thailand, where *O. viverrini* infection is a well-known risk factor (Sripa et al., 2007). In contrast, the Western countries, cholangiocarcinoma arise from the intrahepatic ducts 5-10% and the extrahepatic ducts 90–95% while in Southeast countries especially Thailand and Lao PDR showed intrahepatic cholangiocarcinoma (40%) and extrahepatic duct (60%); most of the cases identified in Khon Kaen Province were intrahepatic type (Sripa and Pairojkul, 2008). The epidemiology studies and animal model studies indicated that the *O. viverrini* infection associated with cholangiocarcinoma development (Thamavit et al., 1978). The risk factors for cholangiocarcinoma in areas where liver flukes are not endemic in Thailand include primary sclerosing cholangitis, hepatolithiasis, and choledochal cysts (Sripa and Pairojkul, 2008). These factors share long-standing inflammation and chronic injury of the biliary epithelium as a common determinant (Sripa et al., 2011). Nowadays, the treatment of *O. viverrini* infection uses praziquantel. Praziquantel (PZQ) is the first choice of trematode infection treatment that can be used in both humans and animals.

2.4 Praziquantel

Praziquantel (PZQ) is a white to the nearly white crystalline powder of bitter taste (Figure 2.4). The compound is stable under normal conditions and melts at 136-140°C with decomposition. The active substance of PZQ is hygroscopic. Praziquantel is easily soluble in chloroform and dimethyl sulfoxide (DMSO), soluble in ethanol and very slightly soluble in water. Praziquantel is a wide broad-spectrum drug of treatment against trematodes and cestodes infection in humans. The advantages of

PZQ are highly effective in a single oral dose for the treatment of *O. viverrini* infection and safety for pregnancy and children. The efficacy of PZQ showed high curing from chronic infection by more than 90%. Praziquantel causes severe spasms and paralysis of nematode's muscle by increasing cell permeability to Ca^{2+}. The rapid influx of Ca^{2+} results in prolonged continuous muscle contraction (tetanic contraction and makes them immobile or paralysis). After liver flukes paralysis, they detach from the host's tissues. In addition, PZQ can activate the host immune response against the trematode. Previous studies reported that the PZQ was very effective against *O. viverrini* (Bunnag et al., 1981; Tawatsin et al., 1983; Saowakontha et al., 1993). In addition, the researchers found that after treatment with PZQ, the hepatobiliary fibrosis of the infected *O. viverrini* patients was reduced due to tissue re-absorption (Pinlaor et al., 2009). However, short term exposure to PZQ induces the inflammatory cell activities by increasing iNOS and oxidative stress. In addition, other adverse side effects of PZQ are generally transient stomachache, headache, skin rash, dizziness, and nausea but not found a mutagenic activity. Praziquantel is absorbed in the gastrointestinal tract and then is found in the blood plasma to 80% after 1-3 h of oral administration, and the half-life of PZQ is about 0.8-1.5 h. Praziquantel is metabolized in the liver and is excreted via urine.



1, Praziquantel (PZQ)

Figure 2.4 Praziquantel structure ($C_{19}H_{24}N_2O_2$) (https://www.pharmawiki.ch).

2.5 Plumbagin

Plumbagin (PB) is a yellow pigment quinonoid compound, the chemical formula is $C_{11}H_8O_3$ (Figure 2.5), and its molecular weight is 188.18. Plumbagin is an extractable compound from the roots of many plants in the *Plumbaginaceae*, *Droseraceae*, and *Ebenceae* families, especially in *Plumbago* species such as *P. indica* (*P. rosea* in India), *P. zylanica*, and *P. capensis*. *Plumbago indica* is one of the most extractable in the commercial product.

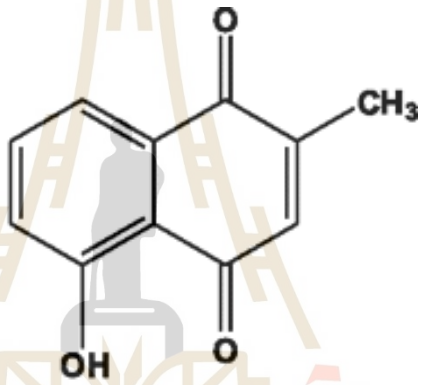


Figure 2.5 Plumbagin structure ($C_{11}H_8O_3$) (https://www.extrasynthese.com).

Plumbago indica tree is a native mainly to India and can be found anywhere in tropical countries including India and Southeast Asia. It is an important medicinal plant that is distributed widely in the North and South of Thailand. It is perennial shrub that goes up to a height of 2 to 4 feet. Leaves of this plant can be measured as 3 inches long and 1.5 inches wide. The flower stalk is 4-12 cm long that produces red flowers in clusters. The fruit is long and slender containing long seeds. The roots are long like fingers and are aromatic in taste (Figure 2.6, insets 2 and b).

Figure 2.6 *Plumbago indica* (*P. rosea*) plant is composed of part of leaves (1, a), root morphology (2, b) and flower characteristic (3, c) (https://medthai.com).

Previous researchers recorded that the *P. indica* root was composed of flavonoids, benzenoid, quinoid, and carbohydrate. The ethanol extract had been used for several gastrointestinal problems including diarrhea, dyspepsia, hemorrhoids and anti flatulence (Krishnaswamy et al., 1980; Kini et al., 1997). In additional, PB has been reported to possess wild a range of biomedical activities such as antioxidant, antimicrobial (Paiwa, 2003), anti-inflammatory, antifungal, and antineoplastic actions and reducing the liver enzymes function. These biomedical activities arise mainly from its ability to generate reactive oxygen species (ROS), achieve a redox reaction

with glutathione (GSH) resulting in the oxidation of GSH to glutathione persulfide (GSSH), and chelate trace metals in the biological system due to its C-5 hydroxyl group in the PB structure. Although the pharmacokinetic information on the metabolism, distribution, and excretion of PB in humans has been not yet, there is some information on the oral bioavailability of PB in the rat model. Twenty four hours after oral administration, PB reaches the maximum concentration in plasma within 1-2.5 h. The half-life of the PB is approximately 1.3-15 h; the main portion of the drug metabolites is excreted in urine and 49% of excretion occurs by feces (Padhye et al., 2010).

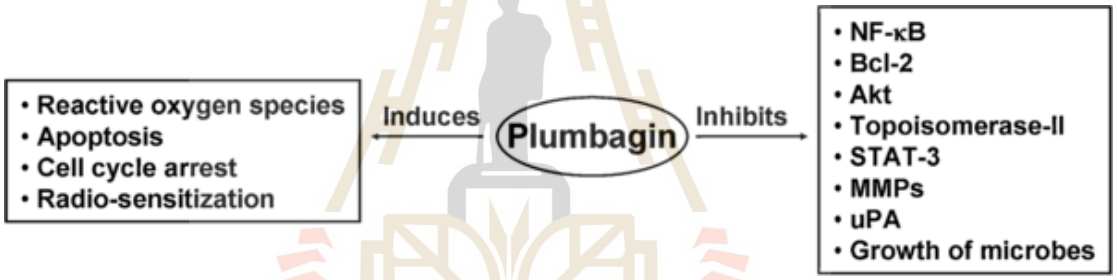


Figure 2.7 Summary of molecular biological effects of plumbagin (Padhye et al., 2010).

The toxic effect of PB is very limited, the folk physician used the root of *P. indica* for abortifacient effect, and induce anti-estrogenic with the prolonged estrous cycle in female Wistar rats (Sandeep et al., 2011), thus raising questions of its safety in pregnancy and the fetus. In addition, PB may affect the male reproductive system as well. Recently, anti-parasitic effect of PB had been investigated which exhibited that PB showed the strongest activity against *Caenorhabditis elegans* (IC_{50} at 9.71 µg/ml) and *Paramphistomum epiclitum* (IC_{50} at 130 µg/ml) (Atjanasuppat et al., 2009), by inhibiting the motility (Lorsuwannarat et al., 2013; Saowakon et al., 2013)

and changing the tegumental surface of liver flukes (Lorsuwannarat et al., 2014), respectively. The PB also inhibited the hatching rate of nematodes (Fetterer and Fleming, 1991). Solomon and colleagues (1993) reported that crude extract from *P. indica* root containing PB decreased the weights of liver, spleen, testis, uterus, and thymus gland in mice. The structure of PB or 5-hydroxy-2-methyl-1, 4-naphthoquinone is similar to atovaquone (hydroxyl-1, 4-naphthoquinone) which is a drug-using for treating pneumonia, pneumocystis, toxoplasmosis, and malaria. Atovaquone acts as a specific inhibitor of the electron transport system in the mitochondria resulting in decreasing ATP production and may result in parasite death (Basco et al., 1994, Siregar et al., 2015) (Figure 2.8).

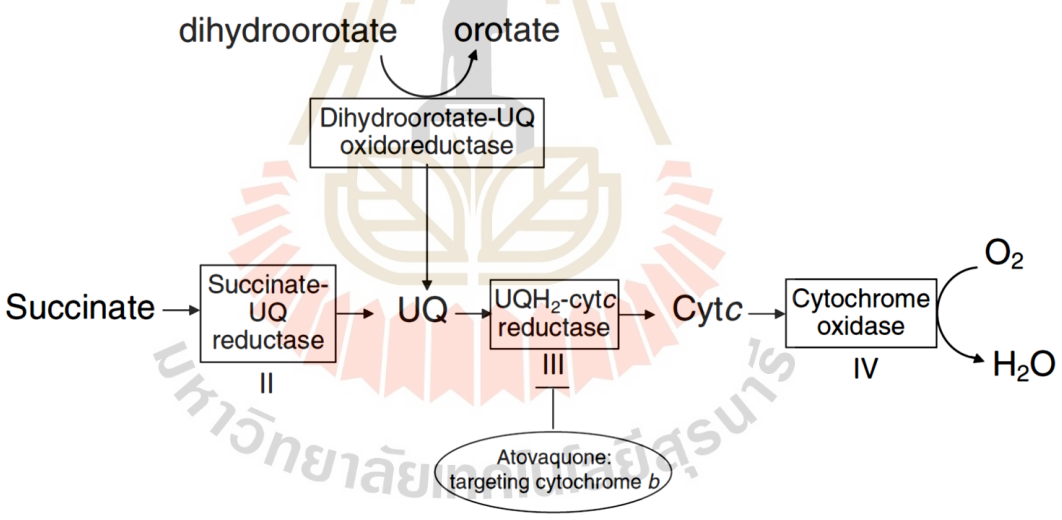


Figure 2.8 Role of the mitochondrial respiratory chain in the parasite. (Siregar et al., 2015).

CHAPTER III

EXPERIMENT

3.1 Materials

3.1.1 Plant

Roots of *P. indica* were collected and purchased from Chiang Mai Province, Thailand. The characteristics of *P. indica* trees, roots and flowers were identified and authenticated by Dr. Santi Wattana, Institute of Biology, Scientific Botany of Suranaree University of Technology (SUT voucher; N. Saowakon 01, SUT Botanical Garden, Suranaree University of Technology, Nakhon Ratchasima, Thailand).

3.1.2 Animal

Six-weeks old male and female Golden Syrian hamsters were used in this experiment. These animals were purchased from Laboratory Animal, Faculty of Medicine, Khon Kaen University, Thailand. Hamsters weighing 80-100 g were acclimatized for 7 days prior to the study. They were housed in stainless steel cages with free access to normal diet and water *ad libitum*. The room temperature was maintained at 25±2 °C, relative humidity 45-50% and a 12 h daylight/dark cycle. In this study, all experimental procedures were approved by the Committee of Animal Care, Suranaree University of Technology, Thailand (SUT approach; 4/2558).

3.1.3 Parasite

The metacercariae of *Opisthorchis viverrini* were obtained from naturally infected cyprinid fish that were captured from freshwater reservoirs in endemic areas of Thailand (Nakhonpanom and Mookdaharn Provinces, Thailand).

3.2 Methods

3.2.1 Preparation of ethanolic *Plumbago indica* root crude extract

The roots of *P. indica* were air-dried, cut into small pieces, blended into a powder by using an electric blender. The powder was sieved through a 250 µm mesh to obtain the resultant fine powder. The ethanolic *P. indica* root crude extract (cPI) was prepared by soaking 100 g of *P. indica* root powder in 1000 ml of 95% ethanol at room temperature for 7 days with an orbital shaker at 250 rpm (Innova 42/42R, New Brunswick Scientific, France). The macerate was filtered and concentrated with a rotary evaporator (Rotavapor R-210/215, BUCHI, Switzerland) under reduced pressure. Then, the extracts were lyophilized into powder by a lyophilizer (Freezone, LABCONCO Co, MO) at -40°C for 3 days. The yield of cPI extract powder was calculated.

$$\% \text{ Yield (g/100 g of dry plant material)} = (W1 \times 100) / W2$$

W1 was the weight of the extract after lyophilization. W2 was the weight of the dry plant material.

The cPI extract powder was stored in a refrigerator at -20°C until use. The cPI powder was weighed and dissolved in 0.1% Dimethyl sulfoxide (DMSO) to obtain the required concentrations.

3.2.2 Determination of plumbagin in cPI by using high-performance liquid chromatography (HPLC) technique.

Slight modifications from Nayak and colleagues (2015) were performed for the chromatographic analysis using high-pressure liquid chromatography (HPLC) with an Agilent 1100 series HPLC System (Agilent, Satan Clara, CA). Standard plumbagin and methanol (HPLC grade) were purchased from Sigma-Aldrich (Darmstadt, Germany) and Merck Millipore, Germany, respectively. A stock standard plumbagin solution (Sigma-Aldrich, Darmstadt, Germany) was freshly prepared in HPLC grade methanol at 100 μg/ml before each analysis. The cPI powder was re-dissolved in methanol (HPLC grade, Merck Millipore, Germany) in order to obtain a sample concentration of 100 μg/ml for HPLC analysis. In this study, the methanol was used as a blank solution while plumbagin solutions were used as a standard. The mobile phase was methanol (HPLC grade, Merck Millipore, Germany). The mobile phase was filtered under vacuum conditions through a 0.22 μm membrane filter (Merck Millipore, Germany) and degassed by sonication before use. The column was equilibrated with the mobile phase for an hour and then pumped at the rate of 0.5 ml/min. For calibration, standard plumbagin solutions were prepared at a concentration range of 0.01-5 μg/ml in methanol. A stock standard solution of plumbagin was filtered through a 0.22 μm membrane filter. A volume of 10 μl was then injected into HPLC column in each time. The concentrations of standard solution (1 and 5 ppm) were injected in triplicates and the average detector response was then measured. Chromatographic analysis was carried out by C18 reversed-phase column (4.0 × 250 mm, Agilent Technology Co, CA). The chromatographic system was composed of system software (Agilent Technology Co, CA). Plumbagin, cPI and

combine plumbagin and cPI were measured in triplicates and detection was done at 254 nm. The chromatographic peaks were confirmed by comparing their retention time and the UV spectra of the reference standard. The entire experiment was carried out at ambient temperature.

3.2.3 Evaluation for the toxicity of cPI in hamsters

3.2.3.1 Oral administration

The toxicological study was divided into two parts, acute and subacute stages based on OECD (Organization for Economic Cooperation and Development, number 423) guidelines and Solomon and colleagues (1993). After acclimatization, sixty hamsters were randomized and divided into 5 groups with 12 hamsters in each group with 6 females and 6 males. *In vivo* experiments were repeated twice. For the acute toxicity test, the hamsters were fasted for 12 h overnight, and they were administered as follows: Group 1 received 0.1% DMSO in distilled water as a vehicle control group by oral gavage for both sexes, while groups 2-5 were orally administered with cPI (cPI was dissolved in 0.1% DMSO in distilled water) at 100, 400, 1000, or 3000 mg/kg concentrations. The hamster behaviors were continuously observed for 1 h, 6 h, 24 h and daily thereafter. According to the OECD 423 guidelines (OECD/OCDE, 2001), following the toxicity signs and symptoms were examined: mortality, salivation, lethargy, diarrhea, chill or tremor and convulsion. We were also attentive to changes in body weight, respiratory, circulatory, posture, and locomotion (central nervous system), skin and fur, eyes and mucous membranes evaluation, as well as to alterations in the behavior patterns. In the subacute toxicity test, thirty hamsters were divided into 5 groups as in the acute toxicity, four groups receiving different cPI concentrations (100, 400, 1000, and 3000 mg/kg) and the vehicle control group

treated with 0.1% DMSO dissolved in distilled water. The experiment followed the OECD 423 guidelines and thus was performed twice. The dosages were decided according to previous studies by Solomon and colleagues (1993). The animals are given daily oral administration with gavage for 7 days, then they were observed for physical and behavioral changes as recommended by the OECD 423 (cited at item 2.5). At the end of the experiment, the animals were fasted overnight, anesthetized and sacrificed by cardiac puncture. Blood samples were collected and allowed to clot in tubes without anticoagulant. Then the serum was separated by centrifugation at 1,398 x g for 15 min by using Hettich U320R centrifuge. Subsequently, serum was evaluated of biochemical parameters. The visceral organs (heart, lung, liver, kidney, spleen, ovary, uterus, testis and epididymis) were collected and weighed and then fixed in 10% neutral-buffered formalin fixative solution for histopathological examination.

The formula of the liver index :

$$\text{Liver index} = \frac{\text{Liver weight (g)}}{\text{Body weight (g)}}$$

3.2.3.2 Evaluation of liver and renal function

A semi-automatic analyzer (Ortho Clinical Diagnosis: VITROS 5600), and commercially available test kits (Ortho Clinical Diagnosis, New Jersey and Erba Diagnostics Manheim, Miami) were used for the evaluation of aspartate aminotransferase (AST: Vitros Chemistry Products Kit 165 5281), alanine aminotransferase (ALT: Kit 843 3815), creatinine (CRE: Erba kit BLT00020), and blood urea nitrogen (BUN: Erba kit BLT00061) at Clinical Laboratory of Suranaree

University of Technology Hospital. The serum of vehicle control group was used as the baseline of the liver enzyme function.

3.2.3.3 Histopathological examination

Conventional histopathological examination was performed using hematoxylin and eosin (H&E) staining. Tissue samplings were prepared by paraffin sectioning. Tissue samples were washed twice in 70% ethanol with 10 min/wash to remove the fixative solution. Then, they were dehydrated in a serial-graded ethanol solution, cleared with xylene, infiltrated and embedded in a paraffin block. After that, paraffin blocks were sectioned at 5 µm in thickness and stained with H&E, followed by dehydration, clearing, and mounting. Photographs of five sections of each tissue sampling were observed under light microscope (80i, Nikon, Japan) at 400x magnification.

3.2.4 Preparation of *Opisthorchis viverrini* in newly excysted juveniles and 4-week-old phases.

3.2.4.1 Newly excysted juveniles (NEJs) of O. viverrini

The infected fish were blended and digested by 0.1% pepsin (BDH Laboratory Supplies, Poole, England) with 0.1% hydrochloric acid at 37°C for 1 hour. After washing with normal saline in a sedimentary jar, the metacercariae were sediment, collected and identified under a dissecting stereo-microscope following the method described by Pinlaor and colleagues (2004). Briefly, the metacercariae of *O. viverrini* is embedded in a double-walled cyst surrounded by a thick layer of fish muscle. It lies longitudinally between the muscle bundles. Most cysts are oval in shape. The cyst walls are thin, outer wall being about 3–8 µm thick and the inner wall

so thin that it can be recognized only after the worm has escaped. The average size of the encysted metacercaria is 201 × 167 μm. After penetration into the fish skin, the cercaria requires at least 21 days to be an infective metacercaria. The body of metacercaria is folded within the cyst bringing the oral sucker close to the posterior end of the worm. The mature larva moves vigorously at room temperature. At rest, the characteristic of excretory bladder appears as an oval area containing a dense mass of dark granules and the brownish-yellow pigments scattered throughout the body. The oral and ventral suckers are clearly seen. The average size of excysted metacercariae is 558 × 145 μm with morphologically similar to the encysted larva described above (Vajrasthira et al., 1961). The active metacercariae in cyst were collected for use in subsequent *in vitro* and *in vivo* studies.

Metacercariae were excysted to newly excysted juveniles (NEJs) with 0.2% Trypsin in Tyrode's solution in 30 minutes. The active NEJs were collected.

3.2.4.2 4-week-old O. viverrini

Fifty metacercariae were infected in 6-week old hamsters. After 4 weeks for infection, hamsters were sacrificed and collected for 4-week-old worms from the liver and bile duct.

3.2.5 *In vitro* study for anthelmintic effects of cPI on *Opisthorchis viverrini*.

3.2.5.1 Evaluation for relative motility (RM) and survival index (SI) on newly excysted juveniles (NEJs) of O. viverrini

The ten active NEJs were collected and incubated at 37°C with praziquantel (PZQ), plumbagin (PB) or ethanolic *P. indica* root crude extract (cPI) at various concentrations. The final concentrations of PZQ, PB, and cPI used in this study were

0 (control), 0.01, 0.1, 1 and 10 µg/ml. NEJs were observed and scored for survival index (SI) and relative motility (RM) at 3, 6, 12 and 24 h after incubation by using stereomicroscope (Kiuchi et al., 1987). And finally, the tissue samples were fixed in paraformaldehyde for scanning electron microscopy.

The formula of SI :

$$\text{SI value} = \frac{\text{Live worms in each group}}{\text{Total worms in each group}}$$

The formula of RM :

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

The formula of motility index (MI) :

$$\text{MI value} = \frac{\sum nN}{N}$$

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

Motility score for NEJs was assigned by using the following criteria;

2 = movement of the body

1 = immobile but not dead (unstained with the 1 % methylene blue)

0 = immobile and dead (stained with 1 % methylene blue)

3.2.5.2 Evaluation for relative motility (RM) and survival index (SI) on 4-week-old O. viverrini

The ten adult worms were incubated with PZQ, PB or cPI at the concentrations of 0 (control), 0.01, 0.1, 1, and 10 µg/ml. Worms were observed and scored for survival index (SI) and relative motility (RM) at 3, 6, 12 and 24 h after incubation by using stereomicroscope. All samples were fixed with fixative solutions for scanning and transmission electron microscopy.

The formula of SI :

$$\text{SI value} = \frac{\text{Live worms in each group}}{\text{Total worms in each group}}$$

The formula of RM :

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

The formula of MI :

$$\text{MI value} = \frac{\sum nN}{N}$$

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

Motility score for adult worms was assigned by the following criteria;

3 = movement of the whole body

2 = movement of only part of the body

1 = immobile but not dead (unstained with the 1% methylene blue)

0 = immobile and dead (stained with 1% methylene blue)

3.2.6 *In vivo* study for anthelmintic effect of cPI on *O. viverrini*

Six-week-old male and female hamsters were infected with 50 metacercariae. Then 3 weeks after infection, hamsters were divided into 9 groups of 5 each. The first control group was untreated, the second vehicle group was treated with 0.1% dimethyl sulfoxide (DMSO) in distilled water, the third negative control group was treated with distilled water, and group 4-9 were treated with PZQ, PB and cPI at two doses of 10 and 100 mg/kg body weight, for each group. After daily treatment for 7 days, all hamsters were anesthetized by intraperitoneal injection with 40 mg/kg body weight of pentobarbital sodium and sacrificed by cardiac puncture and collected for blood, liver,

and spleen. Worms were collected from liver and bile duct, counted and evaluated for percent of worm recovery or percent protection using the formula below :

$$\% \text{ Protection} = \frac{(\text{Number of Worms}_{\text{ in control group}} - \text{Number of Worms}_{\text{ in treated group}}) \times 100}{\text{Number of Worms}_{\text{ in control group}}}$$

3.2.6.1 Scanning electron microscopy

After the experiment, the NEJs and adult worm samples were fixed in 2.5% glutaraldehyde (Electron Microscopy Science, Hatfield, England) and 2% paraformaldehyde in 0.1 M phosphate buffer solution (PBS), pH 7.4 for overnight at 4°C. Samples were washed 3 times in cool PBS, and posted-fixed in 1% osmium tetroxide (OsO_4) in 0.1 M PBS, pH 7.4 at 4°C for 2 h. Then, they were washed in cool distilled water for 3 times, dehydrated through ascending concentrations of ethanol (70, 80, 90, 95% and absolute ethanol), dried in a critical point drying machine (Semdri-PVT-3B, Tousimis, USA), mounted on stubs, coated with gold in an ion-sputtering machine (JFC-1100E, JEOL, Japan) set at 10 mA for 10 min. Specimens were observed and photographed in a scanning electron microscope (JSM-6010LV, JEOL, Japan).

3.2.6.2 Transmission electron microscopy

After collecting from the liver, 4-week old worms were chopped into 3 parts; upper, middle and lower, and fixed in Karnovsky's fixative at 4°C for 4 h. Specimens were washed 3 times with 0.1 M PBS, pH 7.4 and post-fixed in 1% OsO_4 at 4°C for 2 h. Then, they were washed in cool distilled water for 3 times, dehydrated through ascending concentrations of ethanol (70, 80, 90, 95% and absolute ethanol) at 4°C for

30 min in each step, and infiltrated with propylene oxide for 12 h. Subsequently, specimens were placed into a mixture of propylene oxide and Araldite-502 resin at the ratios of 2:1 for 1 h and 1:2 for overnight, respectively. Finally, specimens were infiltrated with pure Araldite-502 resin at room temperature for 12 h and polymerized at 45°C and 60^{o}C in an oven for 48 h in each step. Ultrathin sections were cut at 60-90 nm thick using glass knives and collected on 300 mesh copper grids, doubly stained with 1% uranyl acetate and lead citrate for 30 min in each step. The sections were view at 75 kV and photographed with JEOL transmission electron microscope.

3.3 Statistical Analysis

Results were reported as means ± SD and processed by using SPSS13.0 software. The data were analyzed using Student's t-test and by one-way analysis of variance (ANOVA), followed by Tukey's-b test. The differences between groups were considered statistically significant at $p<0.05$.

CHAPTER IV

RESULTS

4.1 The ethanolic *Plumbago indica* root crude extract (cPI) preparation

4.1.1 Extraction yield

The ethanolic *P. indica* root crude extract (cPI) was prepared by soaking *P. indica* root powder in 95% ethanol (1:10; m/v) for 7 days. The macerate was filtered, concentrated with a rotary evaporator, and lyophilized into powder by a lyophilizer. The percentage yield of cPI extract powder was 9.20% of the total extract (from root powder 1600 g).

4.1.2 Analysis of plumbagin in the ethanolic *P. indica* root crude extract by using HPLC technique

The validation method was based on the standard method (Nayak et al., 2015). Identification and quantification of plumbagin in the ethanol *P. indica* root extracts were performed on the basis of the retention time (RT) and peak area of plumbagin authentic standards. The naphthoquinones or plumbagin was used as a standard marker for quantitative analysis of its content in the crude extract of *P. indica* roots. This was performed with a reverse-phase HPLC system using optimized conditions. The methanol is used as a blank. Several ratios of methanol and DI-water were

examined as mobile phases, and the optimum ratio of methanol to DI-water was determined to be 0:100 (v/v).

The result showed specificity for plumbagin, there was peak interference in the early retention time of plumbagin standards whereas the chromatograms of the blanks or methanol (Figure 4.1A) had no interference. The retention time of plumbagin in the *P. indica* crude extract correlated to those of the plumbagin standards, in which the retention times of the standards plumbagin in methanol were 5.313 and 5.309 min at 1 ppm and 5 ppm, respectively (Figure 4.1B and 4.1C). The retention time of ethanolic *P. indica* crude extract at 50 ppm was 5.309 min (plumbagin contain 3.01 ppm) (Figure 4.1D) which was similar to the plumbagin standard, but its peak showed less than the standard. There was peak interference in early retention time as the plumbagin standard. Therefore, we applied the standard plumbagin at 1 ppm with the *P. indica* crude extract at 12.5 ppm. We got a similar result as 50 ppm of *P. indica* crude extract (plumbagin contain 1.79 ppm) (Figure 4.1E).

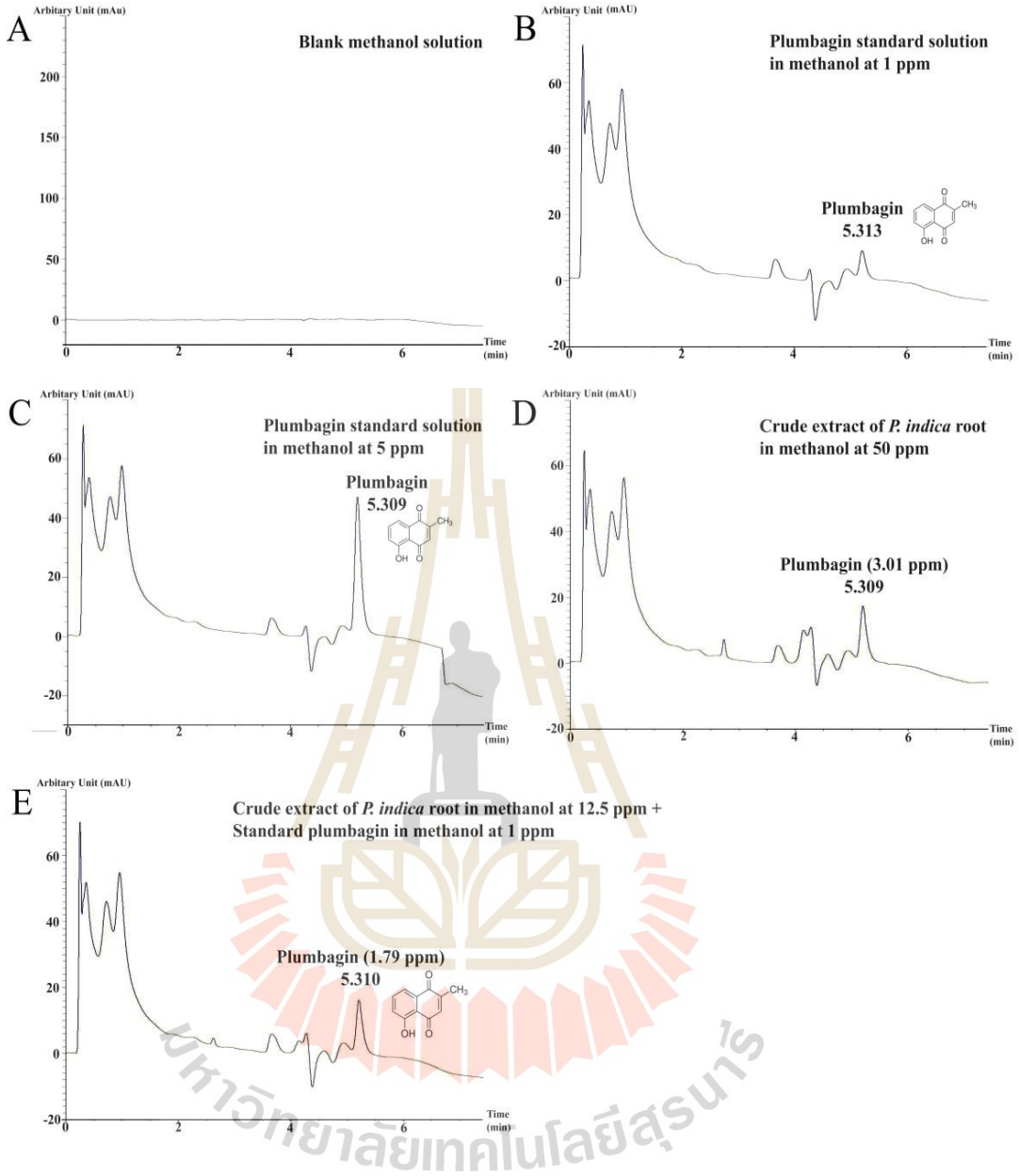


Figure 4.1 The specificity validation for the HPLC analytical method for quantification of plumbagin standard in methanol and methanolic *P. indica* root crude extract. (A) Blank methanol solution. (B) Plumbagin standard solutions at 1 ppm and (C) 5 ppm. (D) The crude extract of *P. indica* root at 50 ppm. (E) Combination mixture between plumbagin at 1 ppm + crude extract of *P. indica* root at 12.5 ppm.

4.2 To study the toxicity of cPI on a hamster model

4.2.1 Mortality study

After 24 h oral administration, the acute toxicity test, following the OECD guideline (OECD/OCDE, 2001) and Solomon and colleagues (Solomon et al., 1993), showed that all groups treated with cPI did not show any signs of morbidity or mortality. Although the mean value of animal bodyweights in cPI 3000 mg/kg (105.00±14.72 g) was higher than the control group and other cPI treated groups, there was no significant difference in body weight between treated and control group (96.25±18.87 g). The internal organ weights of cPI treated groups did not show significant changes relative to that of the control group except for the liver and kidney weight of 3000 mg/kg treated group (6.05±0.49 g and 1.14±0.05 g, respectively) which was significantly increased as shown in Table 4.3. The liver index value from the liver weight per bodyweight indicated the liver enlargement from the pathological changes. The liver index in 3000 mg/kg treated group (0.055±0.002) was significantly different from the control group (0.030±0.002), and the treatement groups. A similar result was also found in kidney weight. The kidney weight of 3000 mg/kg cPI treatment group was significantly increased 1.2 fold (1.14±0.05 g) compared to vehicle control group (0.88±0.10 g). During the acute period, the weight of heart and lung gradually increased in treated cPI groups, but there was not significantly different from the control group. Results from reproductive organ weights of females and males were shown in Table 4.1. Compare within cPI treated groups showed that female reproductive organ weights of cPI treated animals at 400 mg/kg (0.55±0.08 g) were significantly higher than other cPI treated groups whereas it showed no statistical changes when compared with a control group (0.38±0.01 g). In contrast, the

testis weight was not significantly different when compared vehicle control with all cPI treated groups.

In subacute toxicity study, animals were administered with cPI at the concentrations of 100, 400, 1000 or 3000 mg/kg daily through 7 days. All treated animals did not show any pathological changes and no death was observed. As shown in Table. 4.1, there was no significant difference in body weight gain between the control group and all treated groups (Table 4.1). The internal organ weights of cPI groups were significantly increased in the liver, heart, and lung when compared with controls. The liver weight of animals treated with cPI at 3000 mg/kg (5.16±0.59 g) was statistically significant when compared with a control group (3.21±0.18 g) and other treated groups, the value of the liver index of cPI 3000 mg/kg (0.047±0.005) was also significantly increased related to the control group (0.032±0.001) whereas it was not significantly different from 400 mg/kg treatment group (0.039±0.004 g). The liver weight was correlated with the liver index. The values of liver index in some treated groups (400 and 3000 mg/kg) were also significantly increased when compared with control. The weight of heart and lung of treated groups gradually increased and depended on progressive dosages of cPI. The lung weight 3000 mg/kg cPI treatment group (1.05±0.41 g) was statistical significance from control (0.66±0.05 g) and other treated groups, but the heart weight was different from control (0.39±0.04 g) and 100 mg/kg cPI treated group (0.39±0.01 g). Moreover, the female reproductive organs weight of treated hamster with cPI 3000 mg/kg (0.39±0.07 g) displayed higher than the control (0.24±0.02 g) and other treated groups whereas the male reproductive organ weight was not different.

Table 4.1 Body weight and internal organs weight of hamsters after treatment with cPI at 24 h and 7 days.

				cPI treatment (mg/kg bw)							
		Control		100		400		1000		3000	
		1D	7D	1D	7D	1D	7D	1D	7D	1D	7D
Body weight	**Before**	100.00±9.12	96.25±18.87	87.50±15.00	86.25±13.77	88.75±10.31	85.00±10.00	93.75±11.09	97.00±8.12	87.50±6.45	105.00±14.72
	After	96.25±18.87	100.00±7.07	86.25±13.77	97.50±2.89	85.00±10.00	88.75±16.52	97.00±8.12	97.50±5.00	105.00±14.72	111.25±10.31
Liver		2.91±0.14	3.21±0.18	3.26±0.22	3.33±0.14	3.34±0.22	3.25±0.24	3.17±0.10	3.65±0.24	6.05±0.49*#	5.16±0.59*#
Liver index		0.030±0.002	0.032±0.001	0.038±0.001	0.035±0.001	0.038±0.001	0.039±0.004*	0.034±0.002	0.036±0.002	0.055±0.002*#	0.047±0.005*#
Kidney		0.88±0.10	1.01±0.08	0.94±0.09	1.02±0.09	0.94±0.09	0.98±0.17	0.91±0.03	1.02±0.07	1.14±0.05*#	1.19±0.11*#
Heart		0.35±0.04	0.39±0.04	0.37±0.05	0.39±0.01	0.37±0.04	0.42±0.04	0.37±0.04	0.42±0.03	0.44±0.04*#	0.49±0.04*#
Lung		0.65±0.04	0.66±0.05	0.60±0.10	0.67±0.06	0.68±0.15	0.68±0.09	0.67±0.04	0.67±0.06	0.72±0.09	1.05±0.41*#
Spleen		0.15±0.02	0.16±0.02	0.18±0.03	0.15±0.01	0.17±0.05	0.19±0.06	0.16±0.02	0.19±0.01	0.21±0.04	0.20±0.06
Uterus and Ovary		0.38±0.01	0.24±0.02	0.42±0.13	0.29±0.03	0.55±0.08*#	0.27±0.10	0.21±0.02	0.13±0.01	0.28±0.02	0.39±0.07#
Testis		4.08±0.06	4.36±0.01	3.47±0.27	5.33±0.20	4.23±0.52	5.08±0.59	4.48±0.30	5.42±0.69	3.68±1.11	4.09±0.05

Data are expressed as mean ± SE (n=6). One-Way ANOVA followed by Tukey's-b test (n=6). The values were considered to significantly difference at $p<0.05$, * different from the control group, # different within treated groups at the same time.

4.2.2 Biochemical blood analysis

The biochemical parameters are used to evaluate possible toxic effects of the acute and subacute tested within the organism and in the possible target organs. The biochemical blood analyses were performed in both sex and the results were analyzed for liver and kidney function tests as shown in Table 4.2. The levels of liver enzyme AST, ALT and AST/ALT ratio of cPI treated groups at 24 h and 7 days did not reveal any significant difference when compared to the control group. In this study, we observed that a subacute assessment with cPI 3000 mg/kg caused a reduction in the activity of AST and ALT. The kidney function test, BUN, creatinine and BUN/creatinine ratio were no statistical differences when compared to the control group.

Table 4.2 Biochemical parameters of hamsters after treatment with cPI at 24 h and 7 days.

	Control		cPI treatment (mg/kg bw)							
			100		400		1000		3000	
	24 h	7 days	24 h	7 days	24 h	7 days	24 h	7 days	24 h	7 days
AST (U/L)	82.75±44.49	118.75±77.26	128.50±21.05	123.75±54.58	75.50±14.57	123.00±50.02	83.25±19.62	114.25±30.02	87.00±7.35	84.00±29.22
ALT (U/L)	60.25±13.74	67.00±21.86	70.50±32.13	73.50±53.57	62.75±23.21	78.50±30.29	50.25±12.28	59.75±12.63	69.00±24.91	56.75±15.90
AST/ALT	1.32±0.56	2.15±1.74	2.05±0.69	1.90±0.51	1.35±0.58	1.73±1.01	1.69±0.31	1.94±0.51	1.52±0.96	1.63±0.79
BUN (mg/dl)	0.53±0.26	0.53±0.00	0.85±0.76	0.53±0.46	0.53±0.75	0.89±0.56	0.44±0.56	2.22±2.52	0.84±0.81	4.45±6.31
Cr (mg/dl)	10.93±5.45	3.60±0.57	1.36±1.12*	2.93±2.44	8.00±6.29	2.93±4.41	3.47±3.23	4.53±2.31	3.47±2.36	2.40±2.77
BUN/Cr	0.08±0.08	0.15±0.02	0.72±0.45	0.28±0.20	0.04±0.06	0.23±0.10	0.12±0.07	0.68±0.81	0.32±0.36	4.26±8.24

AST=Aspartate aminotransferase, ALT=Alanine aminotransferase, BUN=Blood urea nitrogen, Cr=Creatinine.

Data are expressed as mean ± SE (n=6). One-Way ANOVA followed by Tukey's-b test (n=6). The values were considered to significantly difference at $p<0.05$, * different from the control group, # different within treated groups at the same time.

4.2.3 Histopathological changes

There were no gross morphological changes or any pathological abnormality in all treatment groups relative to the control. The results of histopathological changes were related to visceral organ weights. The histopathological changes of visceral organs were assessed in animals exposed to different doses of cPI (100, 400, 1000 and 3000 mg/kg) in acute and subacute studies. As shown in Figure 4.2-4.5, in control group, the hepatic lobule consisted of hepatocytes lying in radial direction pattern by opening into the central veins as normally, and hepatocytes were polyhedral in shape. The hepatic morphology of cPI treated groups did not exhibit any alteration except cPI treated with 3000 mg/kg. The acute hepatic necrosis was found at the centrilobular area of the hepatic lobules in cPI 3000 mg/kg treated group at 24 h after treatment. In addition, there were nuclear and cytoplasmic changes surrounding the central vein, red blood cell congestion, cellular hypertrophy and vacuolization (Figure 4.2). Although more spread of hepatic lobular changes was observed in the hepatic area, the overall morphological changes of the liver at day 7 were a better improvement than 24 h. The hepatic lobules were similar to hepatic lobules in the vehicle control group, but the blood congestion was still been observed.

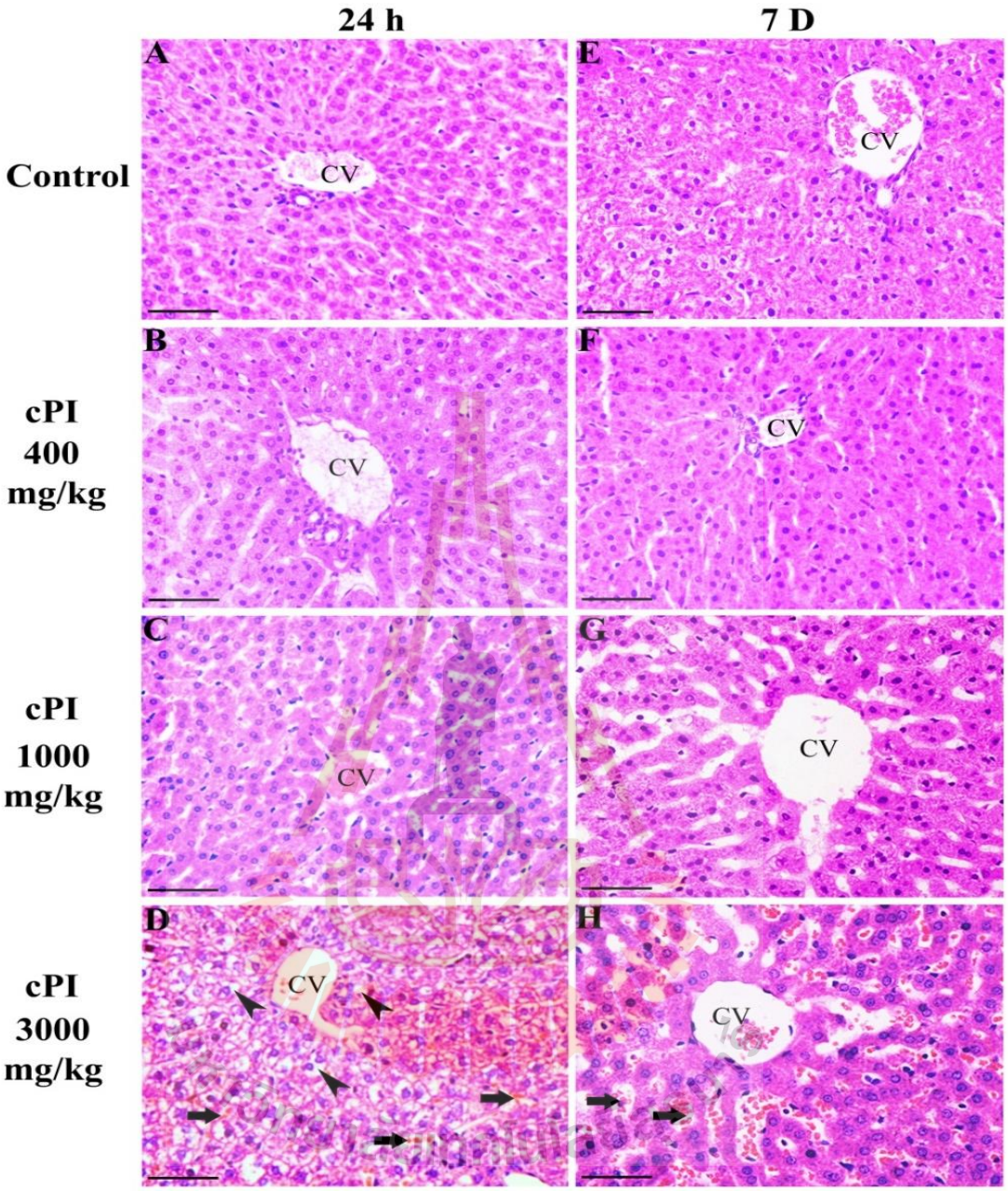


Figure 4.2 Histopathological photomicrographs of liver sections (H&E stained) from the control (A) and treated-groups (B-D) in 24 h (acute stage). Histopathological photomicrographs of liver section from the control (E) and treated-groups (F-H) in 7 days (subacute stage). No pathological changes present in the hepatic lobule except highest cPI oral administration (3000 mg/kg). Size bars for comparison are 10 µm. CV=Central vein, arrow head=Cytoplasmic vacuole, arrow= Red blood cell**.**

The microstructure of the kidney consisted of glomeruli and tubular compartments as shown in Figure 4.3. After 24 h oral administration, the sampling renal tissues did not present the damages after cPI treatment at the concentration of 100 to 400 mg/kg and have no morphological changes (data not shown). Histopathological changes were found only in the renal tissue that was treated with cPI at concentrations 1000 and 3000 mg/kg. Epithelial lining in proximal tubules was swelling and hypertrophy that made the absence of luminal space, and angiectasis in glomerulus was observed (Figure 4.3). Moreover, the red blood cell congestion in the renal interstitial space had been observed in the acute stage. Surprising, renal changes observed 7 days post cPI 3000 mg/kg, treatment was recovered 24 h later; the renal tubular epithelial cells recovered to normally arranged renal tubular epithelial structure, and luminal space was present in the proximal tubules. However, the angiectasis in the glomerulus and interstitial spaces were still present in some areas of the renal cortex. Though the heart and lung weight of the 3000 mg/kg cPI treated group was significantly higher than the control group, but there was no evidence of histopathological changes from those organs. Some lung samplings showed red blood cell accumulation in the alveolar duct and sac (data not shown).

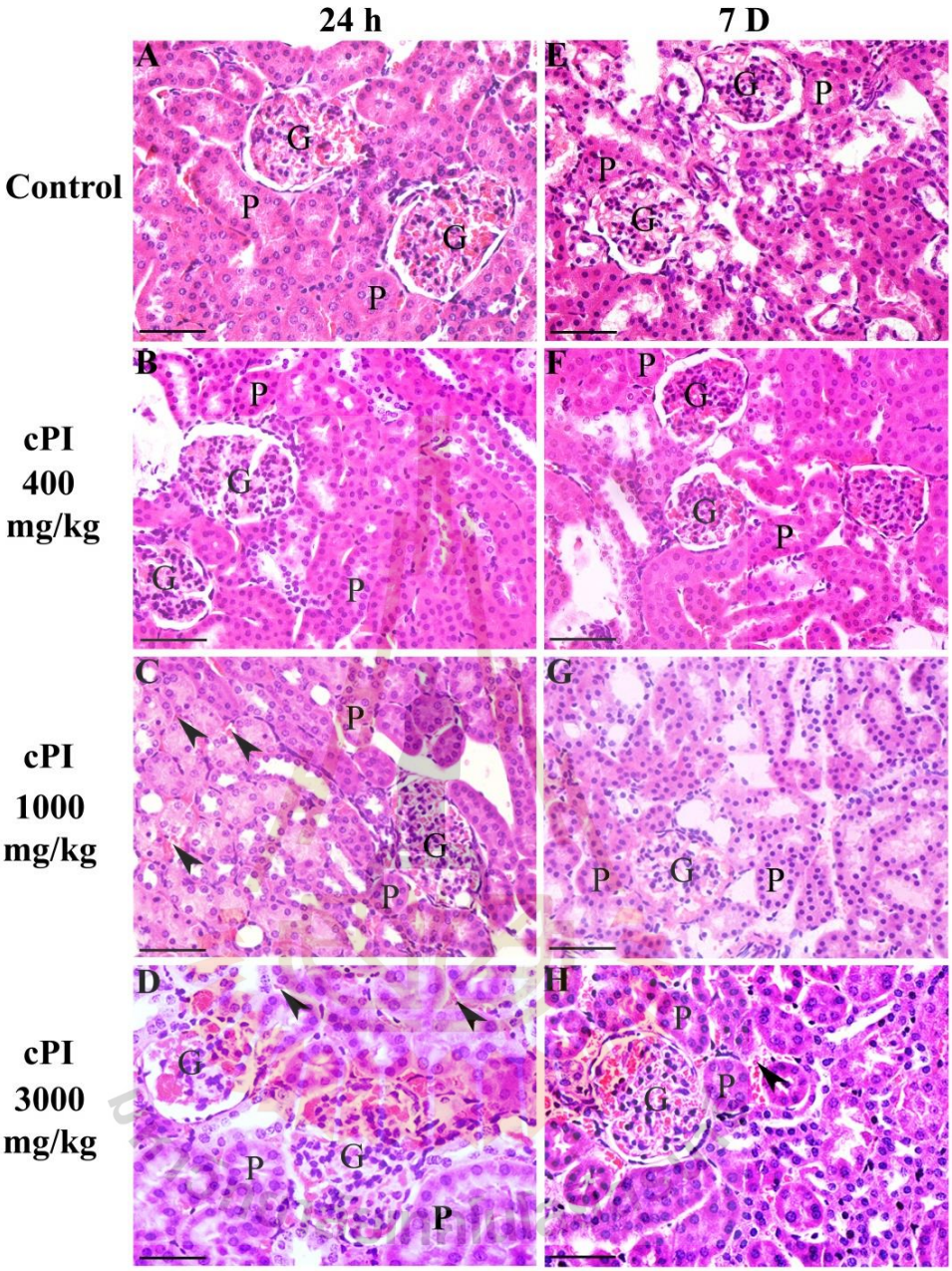


Figure 4.3 Histopathological photomicrographs of kidney sections (H&E stained) from the control (A) and treated-groups (B-D) in 24 h (acute stage). Histopathological photomicrographs of kidney section from the control (E) and treated-groups (F-H) in 7 days (subacute stage). The proximal tubules present swelling and red blood cell congestion in the interstitial space. Size bars for comparison are 10 µm. G=Glomerulus, P=Proximal convoluted tubule, arrow head=Red blood cell.

In the testis of the vehicle control group, there were several spermatic stages; spermatogonium, pachytene spermatid, round spermatid, and elongate spermatid (Figure 4.4). The lesion in the testis of cPI treated groups of 100 to 3000 mg/kg no exhibited morphological changes in acute and subacute stages. The female reproductive organs were composed of ovary and uterus, the primordial ovum, primary and secondary ovum lied on the ovarian epithelium (Figure 4.5). No histopathological changes in the ovary were found in all cPI treated groups. The epithelial lining in the endometrium layer of ovary in all cPI treated groups was similar to the vehicle control group.

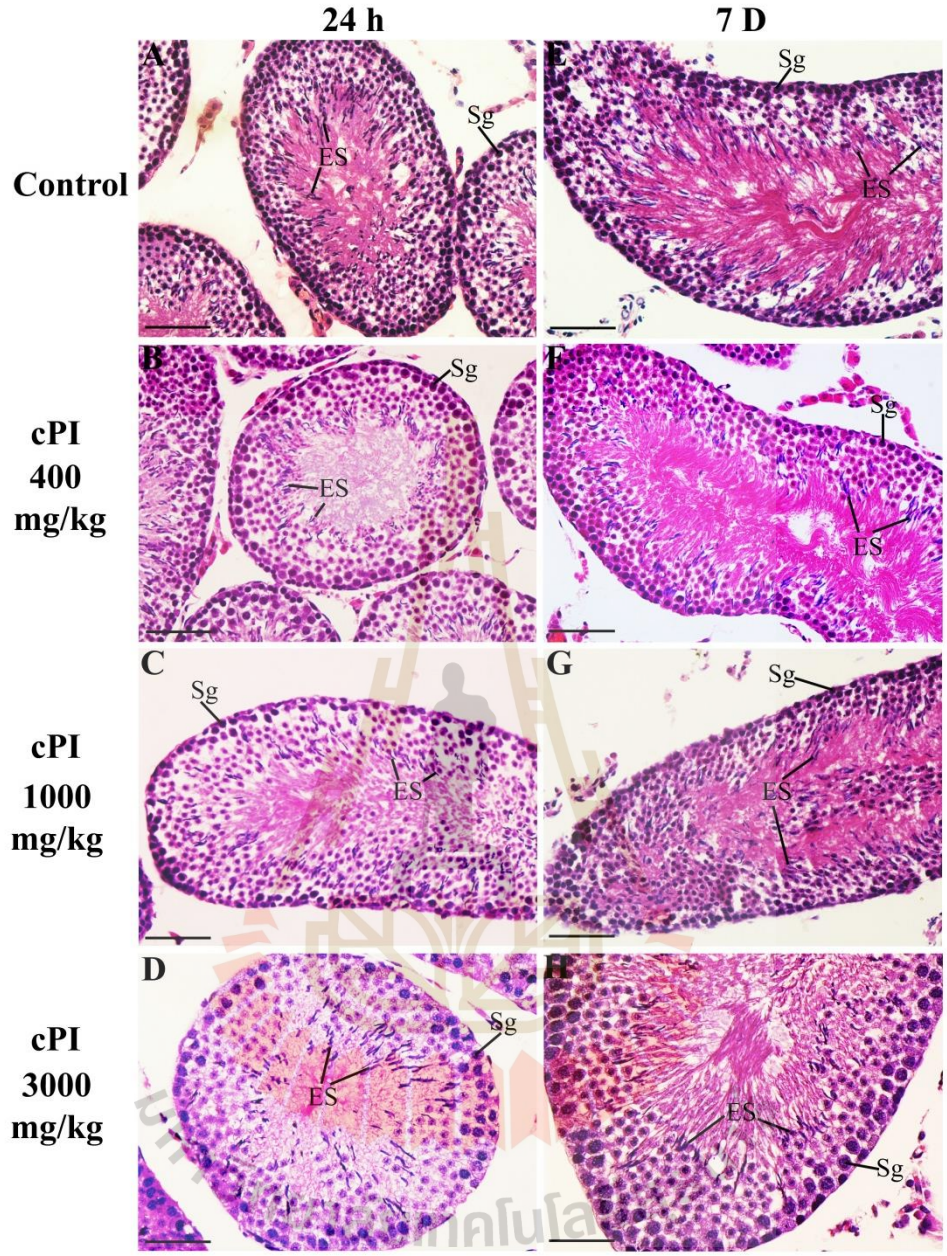


Figure 4.4 Histopathological photomicrographs of seminiferous tubule sections (H&E stained) from the control (A) and treated-groups (B-D) in 24 h (acute stage). Histopathological photomicrographs of seminiferous tubule section from the control (E) and treated-groups (F-H) in 7 days (subacute stage). No pathological changes present in all cPI treatment groups. Size bars for comparison are 10 µm. Sg=Spermatogonium, ES=Elongate spermatid.

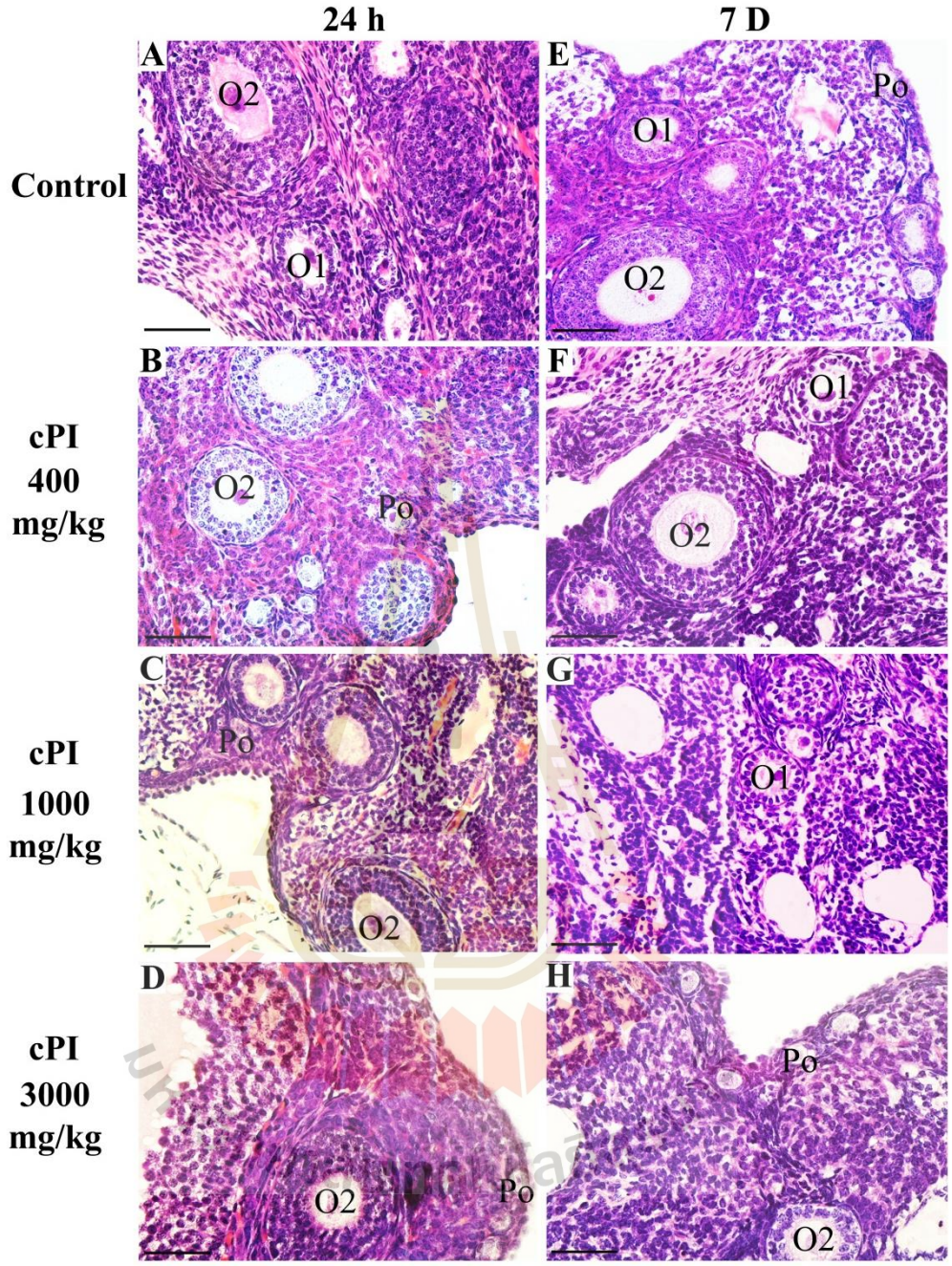


Figure 4.5 Histopathological photomicrographs of ovary sections (H&E stained) from the control (A) and treated-groups (B-D) in 24 h (acute stage). Histopathological photomicrographs of an ovary section from the control (E) and treated-groups (F-H) in 7 days (subacute stage). No pathological changes present in all cPI treatment groups. Size bars for comparison are 10 µm. Po=Primordial ovum, O1=Primary ovum, O2=Secondary ovum.

4.3 Relative motility (RM) and survival index (SI) values of the liver flukes treated with PZQ, PB, and cPI

4.3.1 Negative control and vehicle control groups of NEJs

The results of relative motility (RM) and survival index (SI) values of NEJs stage of *O. viverrini* was shown in Figure 4.6-4.9. NEJs in the negative control group showed freely active movement in the whole body and they were alive throughout the experiment of period. While vehicle control incubated in media contained 0.1% DMSO, they showed few reducing of motility since 3 h throughout 24 h but SI values were 100. However, it was not significantly different when compared with other incubation times of negative control.

4.3.2 Relative motility and survival index values of treated NEJs groups

At the concentration of 0.01 µg/ml

The RM values of PZQ-treatment group were 16% reduction ((RM=84) at 3h and reducing more 21% (RM= 63) at 6 h which SI value at 3 h was 88% and was 76% at 6h. At 12 h, the RM value was less than 50% (RM=33) but SI value of worms has been still more than 50% (SI=60). At the end of the experiment, both RM and SI values were lower than 50% (RM=22, SI=38) as shown in Figure 4.6A and 4.6B.

PB-treatment group, the PB did not involve on RM and SI values of worms when compared with control groups at 3 h (RM=93, SI=100) but it involved on the motility and survival of worms which was similar with PZQ at 6 h (RM=65, SI=78). Although the effect of PB on RM value showed lower than PZQ, the SI value of PB showed no significant difference from PZQ at 12 (RM=44, SI=62) and 24 h (RM=30, SI=33), respectively (Figure 4.6A and 4.6B).

cPI-treatment group, the cPI did not have an effect on both RM and SI values of worms when compared with control groups at 3 h (RM=97, SI=100). At 6 h, RM and SI values were significantly differenced when compared with control groups but the effect of cPI (RM=85, SI=91) showed lower than PZQ (RM=63, SI=76) and PB-treatment groups (RM=65, SI=78), respectively. Effect of cPI reduced worm motility less than 50% but it exhibited lower than PZQ. Interesting, the efficiency of cPI presented nearly with PB at 12 h (RM=46, SI=65) and 24 h (RM=37, SI=51) (Figure 4.6A and 4.6B).

From those results, we implied that the concentration of PZQ, PB, and cPI at 0.01 µg/ml was not optimized for NEJs killing.

At the concentration of 0.1 µg/ml

PZQ-treatment group, the efficiency of PZQ showed continuously decreasing in RM and SI values more than 0.01 µg/ml at 3 h (RM=74, SI=82) and 6 h (RM=44, SI=62). At 12 h, PZQ had 87% reduction of RM and 76% reduction of SI (RM=13, SI=24). At the end of the experiment, NEJs were not completely dead at 24 h (RM=6, SI=12) (Figure 4.7A and 4.7B).

PB-treatment group, the efficiency of PB on RM and SI values was increased more when increasing concentration and when compared with control groups. The results were similar to PZQ at 3 h (RM=78, SI=88). During 6 – 12 h incubation, the results showed that PB had motility effect on NEJs significantly lower than PZQ (RM=54 vs RM=44 and RM=24 vs RM= 13), respectively. On another hand, the SI values at 6 and 12 h were not different from PZQ (SI=68 vs SI=62 and SI=29 vs SI=24). At 24 h, the effect of PB on RM and SI values was not significantly different from PZQ, were 11 in RM and 19 in SI values. (Figure 4.7A and 4.7B).

cPI-treatment group, the RM value of flukes at 3 h was 80% but there was involving in SI value (SI=98) when compared with control groups. At 6 h, they were 29% reduction of RM (RM=71) and 19% reduction of SI (SI=81). The effect of cPI-treatment group continuously involved the RM value but the result showed significant difference with PZQ (RM=26 and RM =18) at 12 h and 24 h, respectively. The effect of cPI on RM values showed similar to PB at 12 h and 24 h but SI values of cPI showed higher than PB (SI=40 vs SI= 29 and SI=29 vs SI=19). When comparing the efficiency between PZQ, PB, and cPI on the SI values, the PZQ-treatment group was the best for against NEJs, PB was second ordinary, and the cPI-treatment group was the third ordinary (Figure 4.7A and 4.7B).

As those results of PZQ, PB, and cPI-treatment groups at 0.1 µg/ml could not be used to kill NEJs completely at the end of the experiment

At the concentration of 1 µg/ml

PZQ-treatment group at 1 µg/ml, we have been observed the reducing motility than 0.1 µg/ml since 3-12 h incubation time, and the reducing of RM and SI values had been exhibited (RM=48, SI=79 at 3h, RM=25, SI=33 at 6 h and RM=2, SI=4 at 12 h, respectively). At 24 h, RM and SI values were 99% reduction (RM=1, SI=1) (Figure 4.8A and 4.8B).

PB-treatment group, the effect of PB on RM values showed significant difference with PZQ at 3 and 6 h incubation times (RM=59 and RM=39) while the SI values were significantly different with PZQ at only 6 h (SI=52). Interesting at 12 h and 24 h incubation times, the PB showed similarly to PZQ against NEJs (RM=2, SI=5 vs RM =2, SI=4 at 12h and RM=1, SI=1 vs RM=1, SI=1 at 24h) (Figure 4.8A and 4.8B).

cPI-treatment group, the efficiency of cPI on RM and SI values were significantly lower than both PZQ and PB-treatment groups throughout the experiment excepted at 3 h, the SI value of the cPI-treatment group was 16% reduction (SI=84) at 3 h (Figure 4.8A and 4.8B).

All of those results indicated the concentration 1 µg/ml of PZQ and PB showed 99% NEJs killing whereas the percent of killing in cPI was 83% (SI=17) at 24 h.

At the concentration of 10 µg/ml

The result of PZQ-treatment group exhibited the best effect for against the motility and surviving abilities of NEJs, the NEJs were completely dead at 12 h (RM=0, SI=0) as shown in Figure 4.9A and 4.9B. Although the effect of PB on NEJs had no significantly different when compared with PZQ at 12 h (RM=2, SI=4), and PB-treatment group showed RM and SI values higher than PZQ until 24 h incubation time (RM=0, SI=0). Interesting, PB killed worms as absolutely dead at the same concentration of PZQ at the end of the experiment. For the cPI-treatment group, there was 87% reduction of RM and 82% reduction of SI (RM=13, SI=18) at 12 h incubation time. Finally, the results at 24 h showed that RM and SI values of NEJs-treated with cPI was not significantly different when compared with both PZQ and PB-treatments (RM=6, SI=7) and the effect of cPI could not kill completely dead of NEJs at 24 h. The result in this experiment implied that PZQ was the best anthelmintic drugs for NEJs killing, PB and cPI were also potent but PB exhibited weaker effect than PZQ and cPI was the least effective. PZQ and PB killed NEJs 100% while cPI showed the highest reduction of 93%.

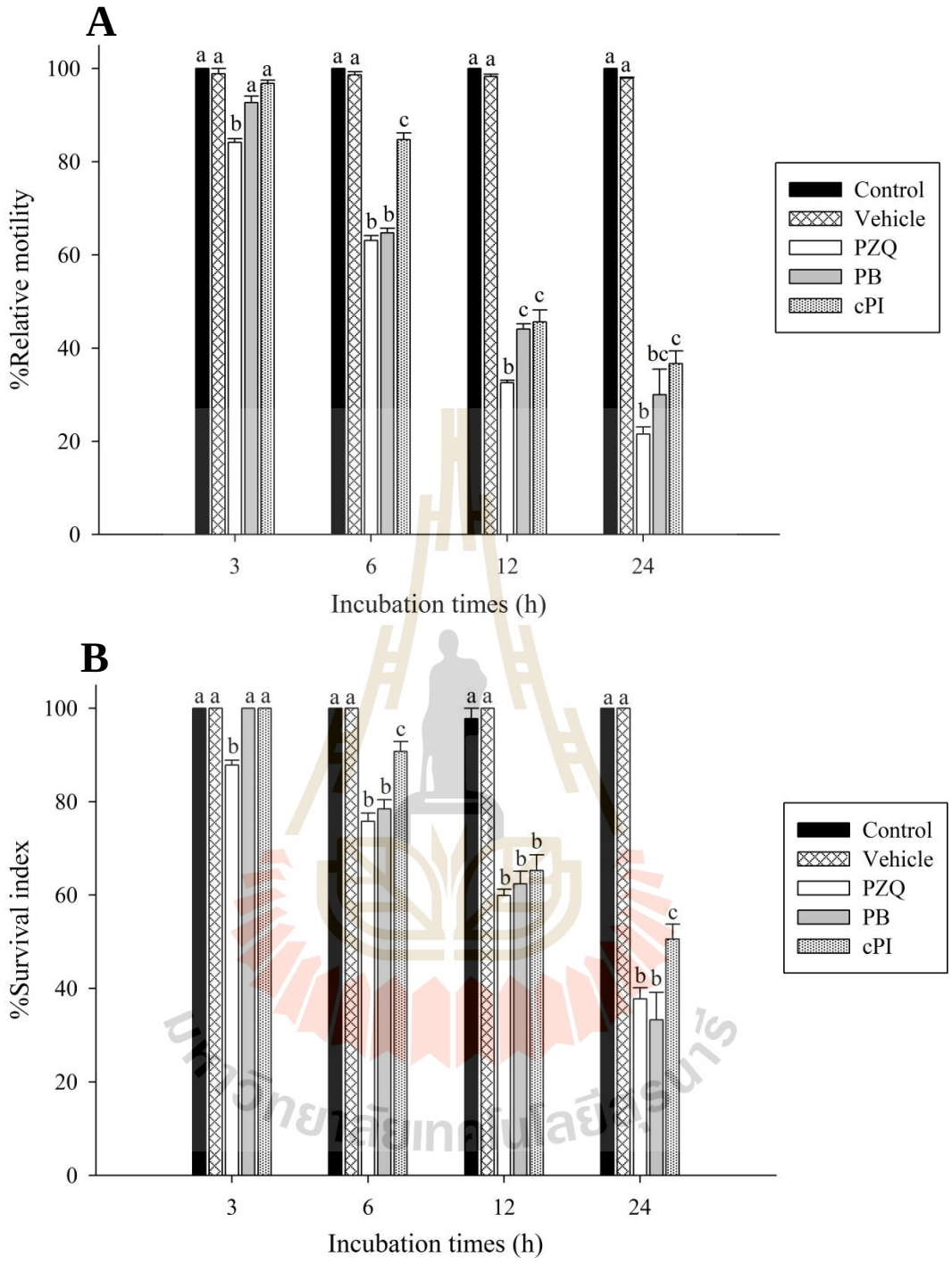


Figure 4.6 Percentage of relative motility value (RM) (A) and survival index (SI) (B) of *Opisthorchis viverrini* in newly excysted juvenile (NEJ) stage treated with PZQ, PB, and cPI at 0.01 µg/ml at 3 - 24 h incubation times. Significantly difference at $p<0.05$ between groups at the same incubation time was indicated by "a, b, c, and d".

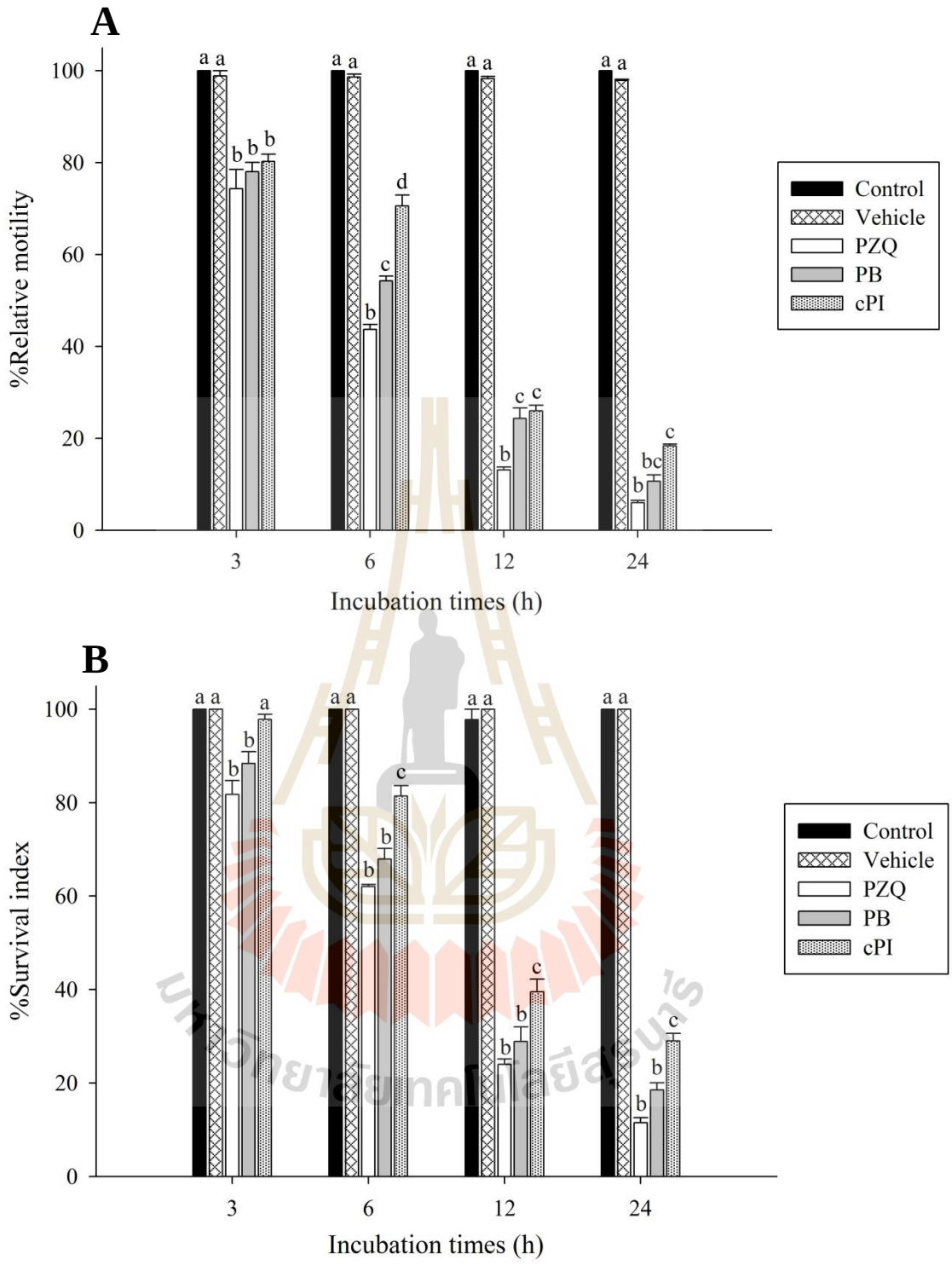


Figure 4.7 Percentage of relative motility value (RM) (A) and survival index (SI) (B) of *Opisthorchis viverrini* in newly excysted juvenile (NEJ) stage treated with PZQ, PB, and cPI at 0.1 µg/ml at 3 - 24 h incubation times. Significantly difference at $p<0.05$ between groups at the same incubation time was indicated by "a, b, c, and d".

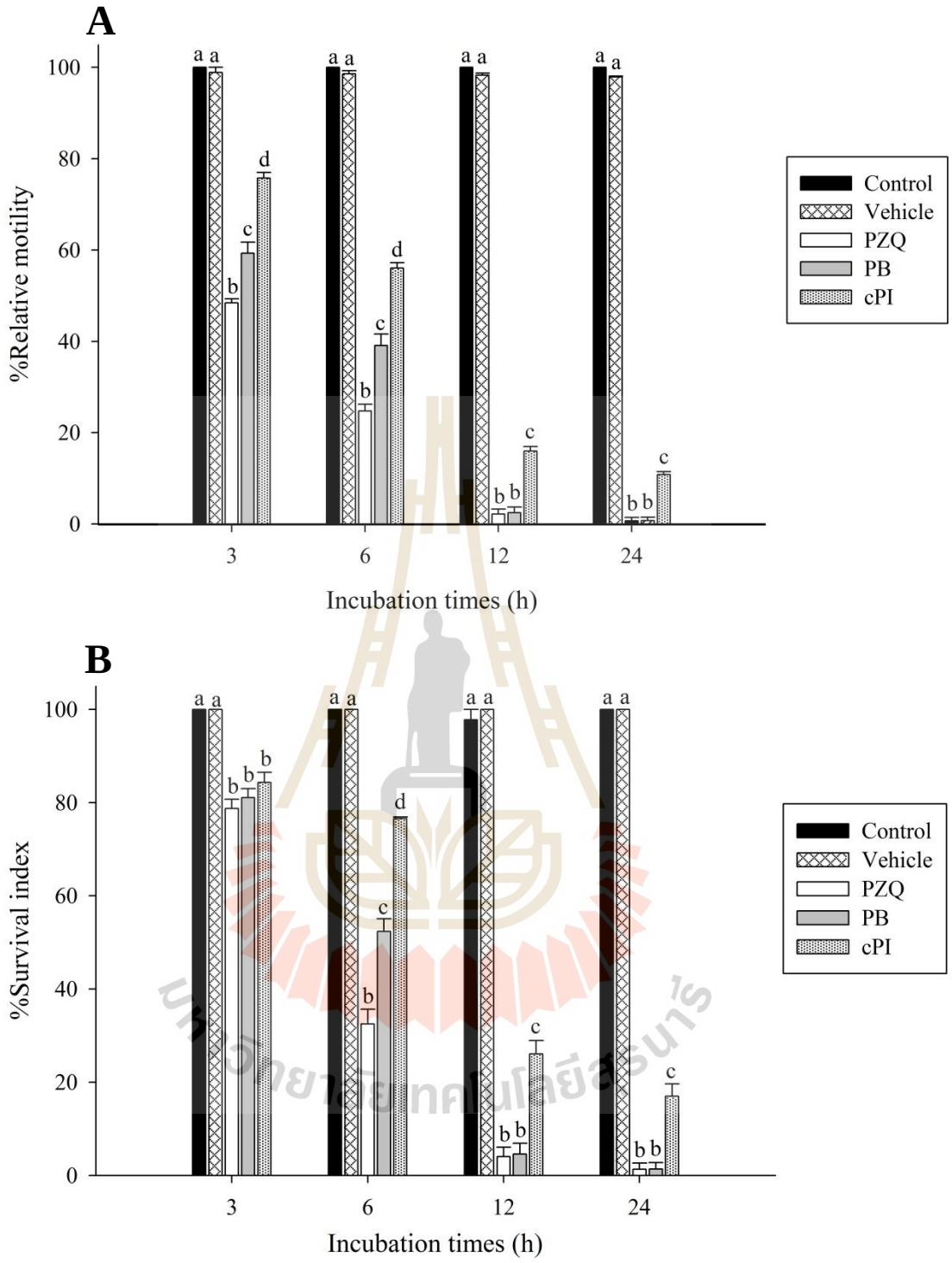


Figure 4.8 Percentage of relative motility value (RM) (A) and survival index (SI) (B) of *Opisthorchis viverrini* in newly excysted juvenile (NEJ) stage treated with PZQ, PB, and cPI at 1 µg/ml at 3 - 24 h incubation times. Significantly difference at $p<0.05$ between groups at the same incubation time was indicated by "a, b, c, and d".

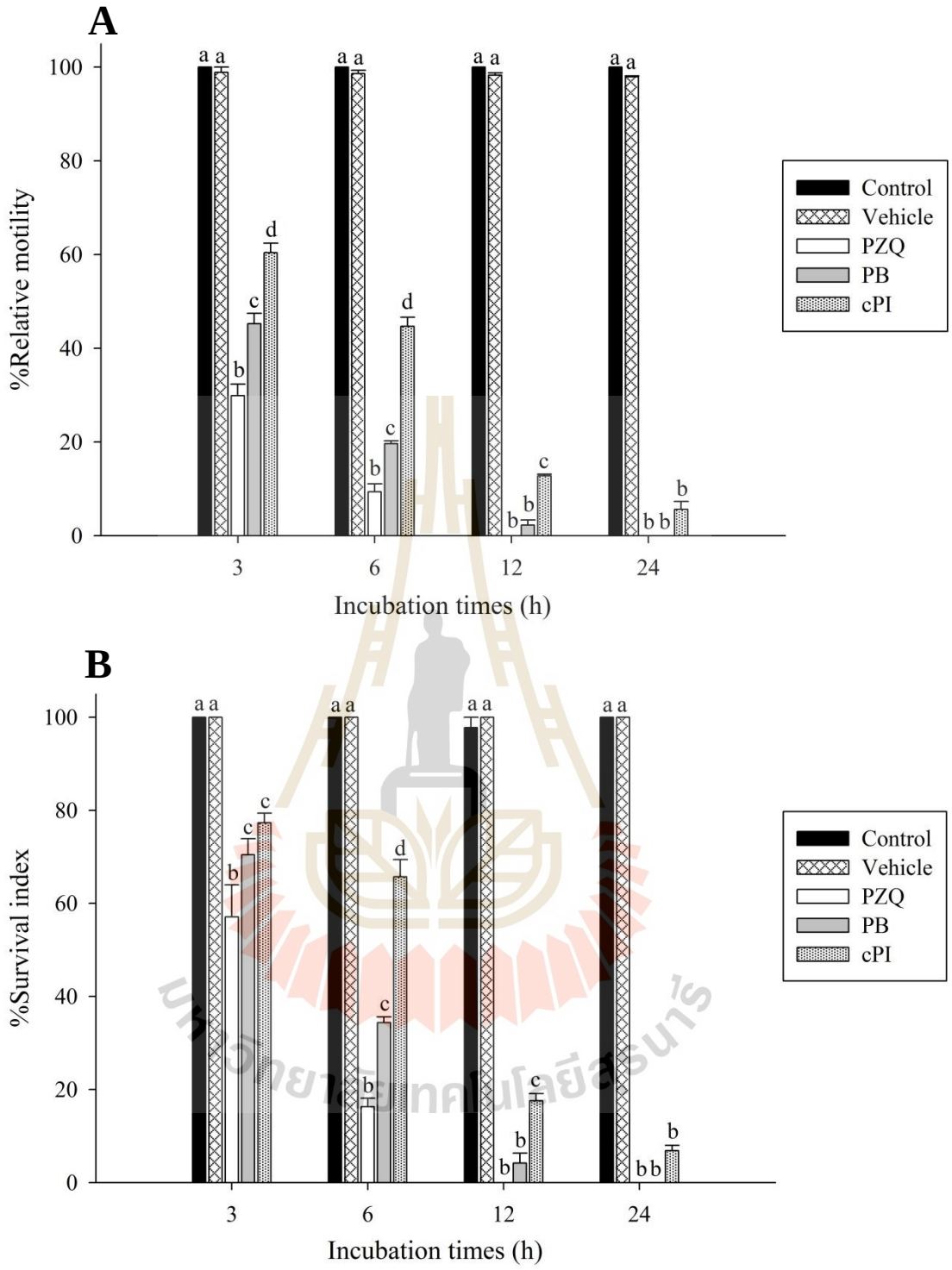


Figure 4.9 Percentage of relative motility value (RM) (A) and survival index (SI) (B) of *Opisthorchis viverrini* in newly excysted juvenile (NEJ) stage treated with PZQ, PB, and cPI at 10 µg/ml at 3 - 24 h incubation times. Significantly difference at $p<0.05$ between groups at the same incubation time was indicated by "a, b, c, and d".

4.3.3 Negative control and vehicle control groups of 4-week old worms

The results of relative motility (RM) and survival index (SI) values of the 4-week-old stage of *O. viverrini* were shown in Figure 4.10-4.13. Worms in the negative control and vehicle control groups showed active motion and they were alive throughout the incubation time except at 12 h. The worms of both control groups were dropped of RM and SI values at 12 h. However, it was not significantly different when compared with other incubation times of negative control.

4.3.4 Relative motility and survival index values of 4-weeks old treated fluke groups

At the concentration of 0.01 µg/ml

PZQ-treatment group, the reducing of RM values of 4-weeks flukes were found since 3-6 h incubation time when compared with both control and vehicle groups. But the SI values in the PZQ-treatment group at 3 (RM=70, SI=97) and 6 h (RM=66, SI=97) were not significantly different. At 12 h, PZQ-treatment group was 50 % reduction of RM value (RM=50) and showed a reduced of 63% RM (RM=37) at 24 h. The SI values of PZQ-treatment group were 33% and 39% reduction at 12 and 24 h, respectively (SI=67 and SI=61) as shown in Figure 4.10A and 4.10B.

PB-treatment group, the effect of PB-treatment group on RM value were nearly reduced to PZQ at 3 – 12 h (RM=81 vs RM=70, RM=74 vs RM=66 and RM=62 vs RM=50). SI values of PB-treatment showed a significant difference with PZQ only at 12h (SI=93 vs SI=67). When compare between 12 h and 24 h, PB-treatment group showed reducing more 20% and 21 % on RM and SI values, respectively (RM=42, SI=72). The efficiency of PB on RM and SI values were not significantly different when compared with PZQ at 24h (Figure 4.10A and 4.10B).

When compared the cPI-treatment group with both control and vehicle groups at 3 h, cPI did not act against worm motility, and the RM value was 97 while SI value was 100. Although RM values of cPI-treatment group were 90 at 6 h and 73 at 12 h whereas SI values were 100 and 96 at the same incubation times. The cPI-treatment group exhibited weaker effect than both PZQ and PB-treatment groups throughout 3-12h. At 24 h, cPI treatment group showed 39% RM reduction (RM=61) and the effect of cPI on RM was less than PZQ and PB-treatment groups. The SI value of cPI-treatment group showed no significant difference when compared with PB. But SI value of cPI was lower than PZQ at the 24 h (SI=78) (Figure 4.10A and 4.10B).

Those results indicated that all of the drugs (PZQ, PB, and cPI) at concentration of 0.01 µg/ml were unsuitable for killing worms on 4-week-old.

At the concentration of 0.1 µg/ml

The reducing of RM and SI values of PZQ-treatment groups were found at all incubation times, RM value was 31% reduction (RM=69) at 3h and was 37% reduction (RM=63) at 6h. But SI values were not a difference between 3 and 6 h (SI=97 and SI=96). After 12 h, flukes showed slowly down movement, RM value was 31% and SI value was 58%. Finally, PZQ -treatment group showed 78% and 53% reduction on RM and SI values at 24 h (RM=22 and SI=47) (Figure 4.11A and 4.11B).

The reducing RM value of PB-treatment group had been found at 3 h when compared with the control groups. Then, the RM value was reduced more 22% that value was near to PZQ-treatment group (RM=78 vs RM=69). Flukes in PB and PZQ-treatment groups at 6 h could not be killed because SI values of PB and PZQ were 98 and 96, respectively. Although the RM value in PB-treatment group had been

continuously decreased at 12 h (RM=35), it was not significantly different when compared with PZQ (RM= 31). The SI value of PB treatment group was 23% reduction (SI=77) that was less effective than PZQ (SI=58). For 24 h, RM values of PB group showed reducing more 4% of 12 h (RM=31) and its effect was lower than PZQ. The effect of PB on SI value was not significantly different when compared with PZQ at the same incubation time (SI=57) (Figure 4.11A and 4.11B).

At the 3 h, the RM and SI values of worms in the cPI-treatment group were similar to control groups they were 95 in RM and 100 in SI. Then, the RM value was decreased to 89% while SI value was 98 at 6 h. At 12 h, the rapidly decreasing of RM value became 64 and SI value was 86. In addition, the efficiency of cPI was a significant difference only PZQ but no significant difference in the PB. The result of RM in cPI-treatment group between 12 and 24h did not differ (RM = 64 vs RM=56). In contrast, the SI values between 12 and 24 h showed a significant difference (SI= 86 vs SI= 66). When compare cPI with PZQ and PB, the effect on SI value of cPI treatment was lower than PZQ but it was similar to PB. SI value was 47 in PZQ and was 57 in PB-treatment groups (Figure 4.11A and 4.11B).

The result of summation in 4-weeks-old flukes treated with 0.1 µg/ml of drugs showed that the best worm killing was PZQ, next was PB and the last one was cPI, the percent of killing values at 24 h were 53%, 43%, and 34%, respectively. It implied that all drugs in this concentration were not chosen for the killing of 4-weeks-old *O. viverrini*.

At the concentration of 1 µg/ml

PZQ-treatment group, the effect of PZQ involved 48% reduction (RM=52) at 3h and more to 60% reduction (RM=39) at 6h when comparing with control groups.

Even though the RM values were less than 60%, the SI values of flukes treated with PZQ were 97% and 94% at 3 h and 6h, respectively. After 12 h incubation, the RM and SI values were 97% and 92% reduction, and finally, the effect of PZQ could kill 4-week-old to 99% (RM=1, SI=1) as shown in Figure 4.12A and 4.12B.

PB-treatment group, the effect on RM value of PB showed lower than PZQ but the SI value did not differ with PZQ at 3h by the RM value was 67 while SI value was 92. Then at 6 h incubation, the PB treatment group had been continuously reduced their motility (RM=60) but both RM and SI values were the insignificant difference when compared with PZQ (SI=89 vs 94). After 12 h, the percent of RM reduction in PB was 70% (RM=30) and SI was 38% reduction (SI=62) that showed lower effect than PZQ. At 24 h, PB could inhibit the motility to 88% and 70% reduction on RM and SI values similarly with PZQ at the same time, respectively (RM=12, SI=30) (Figure 4.12A and 4.12B).

cPI-treatment group, cPI could not kill 4-week old by evaluating from RM and SI values until 6 h (RM=92, SI=96). At 6 h, flukes in the cPI treated group showed slow down movement but it did not involve in SI value (RM=84, SI=98). Next, they had more inhibited movement and showed paralysis but some of them have been alive (RM=59, SI=75). For 24 h incubation time, the effect of cPI displayed to inhibit movement to 54% and made the parasitic dead to 39% (RM=46, SI=61) (Figure 4.12A and 4.12B).

For those results, we assumed that at 1 µg/ml concentration of PZQ, PB, and cPI inhibited the motility effect of 4-week-old at 24 h as 99%, 70%, and 39%, respectively. But at this concentration was unsuitable for killing 4-week old worms.

At the concentration of 10 µg/ml

PZQ-treatment groups showed more 50% reduction of RM at 3 h, but it had not affected in SI values (RM=49, SI=98) and its effect on RM and SI showed a clear difference at 6 h when compared with control groups (RM=27, SI=77). Surprisingly, flukes in PZQ treatment group were immobilized and paralysis when we shook their incubation discs at 12 h (RM=1, SI=3)., and finally all of them were completely dead at 24 h (RM=0, SI=0) (Figure 4.13A and 4.13B).

PB-treatment groups, the effect of PB on the motility of worms has exhibited since 3 h incubation time by RM values were decreased to 63% and 33% at 3 h and 6 h, respectively. But it did not involve to make them dead by SI values were 92% and 84% at the same duration. The effect of PB showed lower than PZQ in the early 6 h incubation, but PB showed the progressive reduction of RM value when flukes exposed until 12 h (RM=19, SI=52) and 24 h (RM=4) and the effect of PB on RM and SI values was similar to PZQ at 24 h (SI=4) (Figure 4.13A and 4.13B).

cPI-treatment groups, the effect of cPI on the motility showed a significant difference when compared with the control groups at early 3 h incubation whereas its effect was lower than PZQ and PB along with the experiment. It showed a 15% reduction of RM while most of all flukes were living (RM=85, SI=94). After 6 h, cPI began to kill parasites by the decreasing of SI value which was a significant difference from control groups, and cPI acted on SI value similarly as PZQ and PB at 6h (RM=76, SI=85). During 12 to 24 h incubation times, the RM and SI values in cPI treatment group were continuously decreasing by its effect presented similar to only PB (RM=23, SI=57) but not PZQ. However, the effects of all of cPI, PZQ, and PB on motility were not significantly different at 24 h (RM=9). Although the effect of cPI on

SI value was reached 74% reduction (SI=26) at 24 h, its effect was lower than PZQ and PB (Figure 4.13A and 4.13B).

From those results of this experiment indicated that at 10 µg/ml of PZQ, PB, and cPI had a killing effect on 4-week-old after 24 h incubation of *O. viverrini* as 100%, 96%, and 74%, respectively.

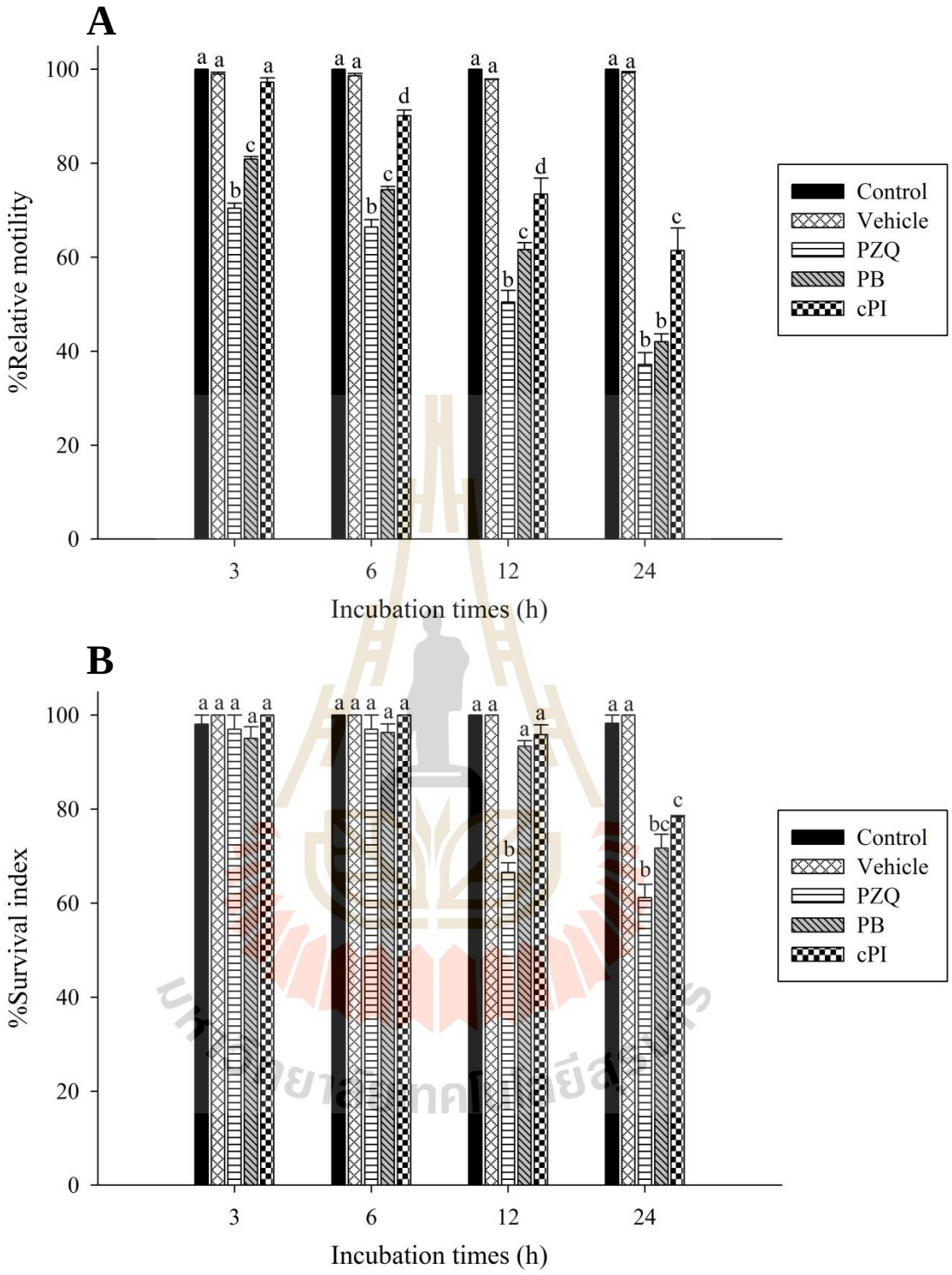


Figure 4.10 Percentage of relative motility value (RM) (A) and survival index (SI) (B) of *Opisthorchis viverrini* in a 4-week-old stage treated with PZQ, PB, and cPI at 0.01 µg/ml at 3 - 24 h incubation times. Significantly difference at $p<0.05$ between groups was indicated by "a, b, c, and d".

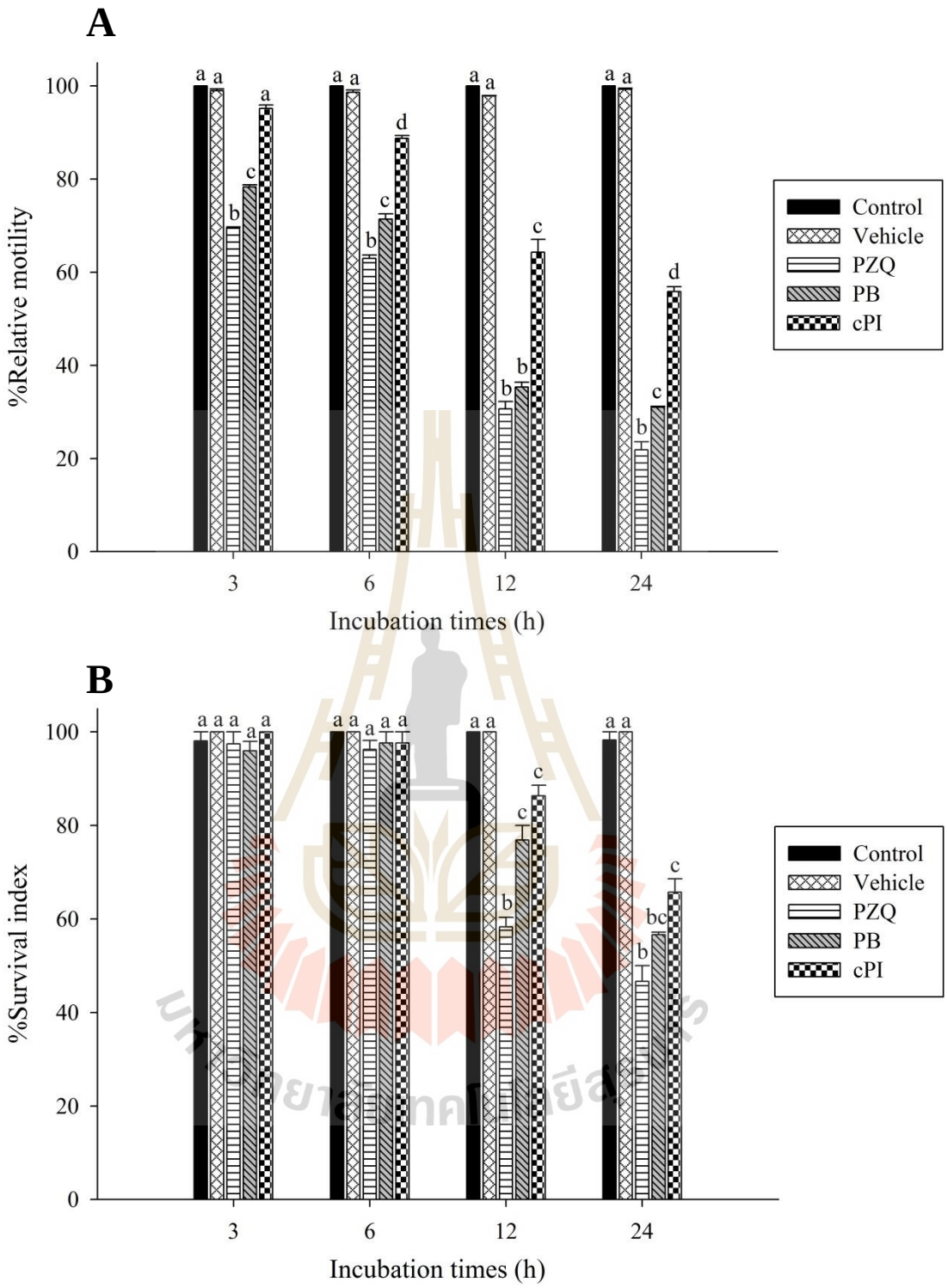


Figure 4.11 Percentage of relative motility value (RM) (A) and survival index (SI) (B) of *Opisthorchis viverrini* in a 4-week-old stage treated with PZQ, PB, and cPI at 0.1 µg/ml at 3 - 24 h incubation times. Significantly difference at $p<0.05$ between groups was indicated by "a, b, c, and d".

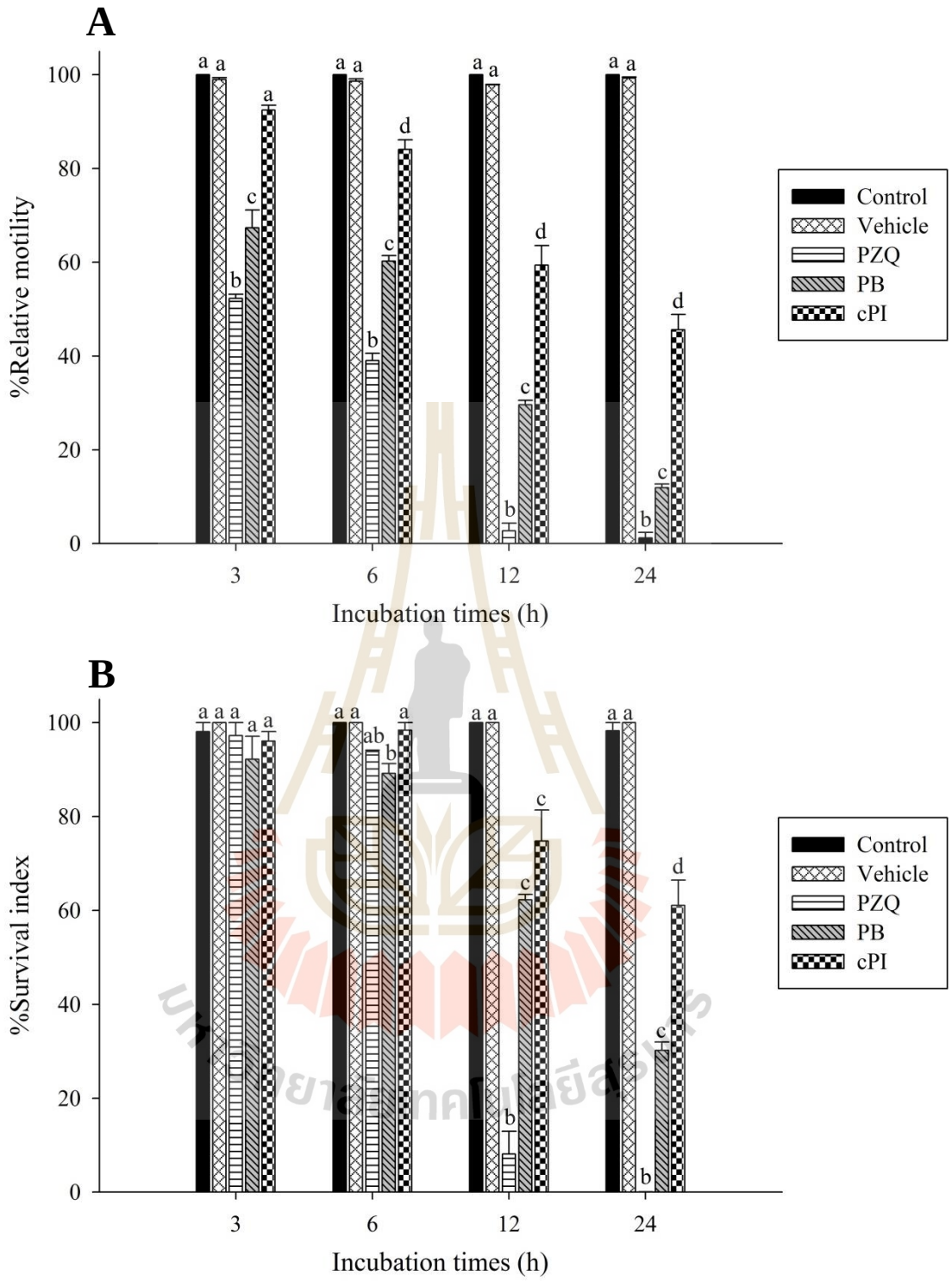


Figure 4.12 Percentage of relative motility value (RM) (A) and survival index (SI) (B) of *Opisthorchis viverrini* in a 4-week-old stage treated with PZQ, PB, and cPI at 1 µg/ml at 3 - 24 h incubation times. Significantly difference at $p<0.05$ between groups was indicated by "a, b, c, and d".

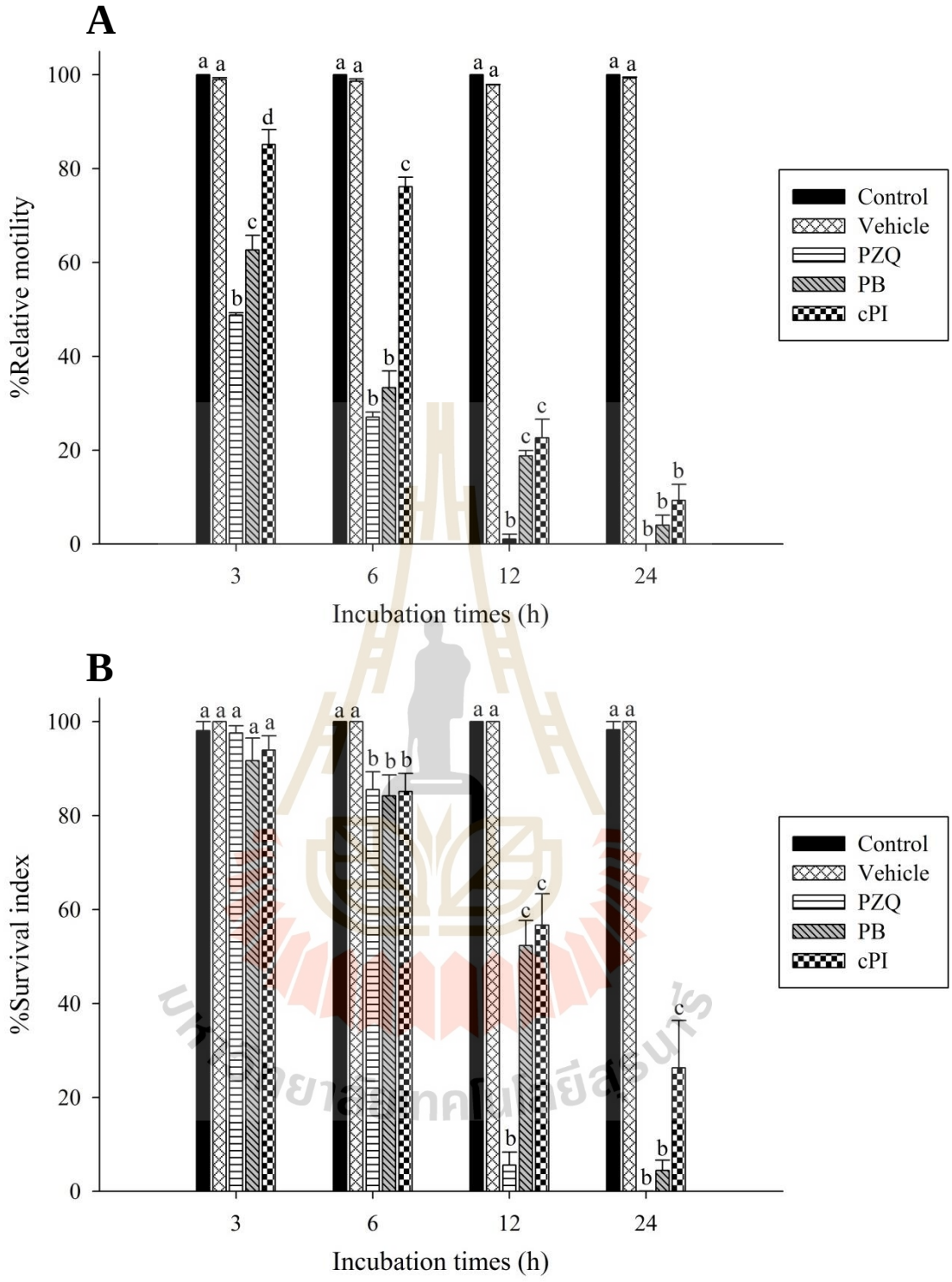


Figure 4.13 Percentage of relative motility value (RM) (A) and survival index (SI) (B) of *Opisthorchis viverrini* in a 4-week-old stage treated with PZQ, PB, and cPI at 10 µg/ml at 3 - 24 h incubation times. Significantly difference at $p<0.05$ between groups was indicated by "a, b, c, and d".

4.3.5 Comparison of Stage-related sensitivities to the drugs and comparison of the anthelmintic effects of praziquantel (PZQ), plumbagin (PB) and crude extract of *P. indica* (cPI)

The relative sensitivities of the NEJs and 4-week-old juveniles to PZQ, PB, and cPI were compared by evaluating the cumulative RM and SI values of the two stages from all time points (Figure 4.14 - 4.16).

When NEJs incubated in 0.01µg/ml PZQ (Figure 4.14A-B and Figure 4.15A-B), the NEJs showed 78% reduction of RM values and 62% of flukes were dead (RM=22, SI=38), while in 4-week-old flukes showed 63% reduction of RM values and only 39% of flukes were dead (RM=37SI=61). In the 0.1 µg/ml PZQ, NEJs showed 94% reduction of RM values and 88% of flukes were dead (RM=6 SI=12), while in 4-week-old flukes showed 78% reduction of RM values and only 53% of flukes still alive (RM=22, SI=47). In the 1 and 10 µg/ml PZQ, NEJs showed 99, 100% reduction of RM values and 99, 100% of flukes were dead (RM=1, SI=1 and RM=0, SI=0), while in 4-week-old flukes showed 99, 100% reduction of RM values and completely dead in 100% of flukes (RM=1, SI=0 and RM=0, SI=0),

In 0.01µg/ml PB treatment group (Figure 4.14A-B and Figure 4.16A-B), the NEJs flukes showed 70% reduction of RM values and 67% of them were dead (RM=30, SI=33), while in 4-week-old worms showed 58% reduction of RM values and 28% of flukes were dead (RM=42, SI=72). In the 0.1 µg/ml PB, NEJs showed 89% reduction of RM values and 81% of flukes were dead (RM=11, SI=19), while in 4-week-old flukes showed 69% reduction of RM values and only 57 of flukes still alive (RM=31, SI=57). In the 1 and 10 µg/ml PB, NEJs showed 99, 100% reduction of RM values and 99, 100% of flukes were dead (RM=1 SI=1 and RM=0, SI=0),

while in 4-week-old flukes showed 88, 96% reduction of RM values and 70, 96% of flukes were dead (RM=12, SI=30, and RM=4, SI=4).

PZQ caused insignificantly lower RM values than PB in the NEJs stage and the 4-week-old flukes in all concentrations except at 1 µg/ml, while there was no significant difference between the two stages at 0.01,0.1, and 10 µg/ml (p>0.05). In contrast, in the PZQ treatment, NEJs showed significantly lower RM values than 4 week-old flukes only at 0.1 µg/ml, and it was a significant difference in SI values of the two groups.

In 0.01µg/ml cPI treatment group (Figure 4.15A-B and Figure 4.16A-B), the NEJs flukes showed 63% reduction of RM values and 49% of them were dead (RM=37, SI=51), while in 4-week-old worms showed 39% reduction of RM values and 22% of flukes were dead (RM=61, SI=78). In the 0.1 µg/ml cPI, NEJs showed 82% reduction of RM values and 71% of flukes were dead (RM=18, SI=29), while in 4-week-old flukes showed 44% reduction of RM values and only 66% of flukes still alive (RM=56, SI=66). In the 1 and 10 µg/ml cPI, NEJs showed 89, 94% reduction of RM values and 83, 93% of flukes were dead (RM=11, SI=17, and RM=6, SI=7), while in 4-week-old flukes showed 54, 91% reduction of RM values and 39, 74% of flukes were dead (RM=46, SI=61 and RM=9, SI=26).

PZQ caused significantly lower RM and SI values than cPI in the NEJs and the 4-week-old stages in all concentrations except at 10 µg/ml, while there was no significant difference between the two stages at 10 µg/ml (p>0.05). Although in the PZQ treatment, NEJs showed not a significant difference in RM values than 4 week-old flukes at 10 µg/ml, and but there was a significant difference in SI values of the two groups.

PB caused significantly lower RM values than cPI only in the 4-week-old flukes in all concentrations except at 10 µg/ml, and there were a significant difference lower SI values than PB between the two stages in all concentrations (p<0.05). In contrast, in the PB treatment, NEJs showed no significantly lower RM values than 4 week-old flukes in all concentrations except at 10 µg/ml, and there was a significant difference in SI values of the two groups.

The comparison of the anthelmintic effects between PZQ, PB, and cPI at each concentration was done by pooling RM and SI values form all time points (Figure 4.14 – 4.16). For the NEJs, PZQ treatment group resulted in significantly lower RM and SI values than negative control and PB and cPI treatment groups in all concentrations except at 10 µg/ml (p<0.05). For 4-week-old worms, PZQ treatment results in significantly lower RM and SI values than the negative control and PB and cPI treatment groups in all concentrations except at 10 µg/ml (p<0.05). All of drugs in this experiment inhibited motility and killed worm in NEJs better than 4-week-old flukes. PZQ was the best for inhibiting motility and kill worms in NEJs and 4-week-old stages and killing effects than PB in both NEJs and 4-week-old stages of worms. PB was a second and cPI showed the lowest effect on motility and worm killing.

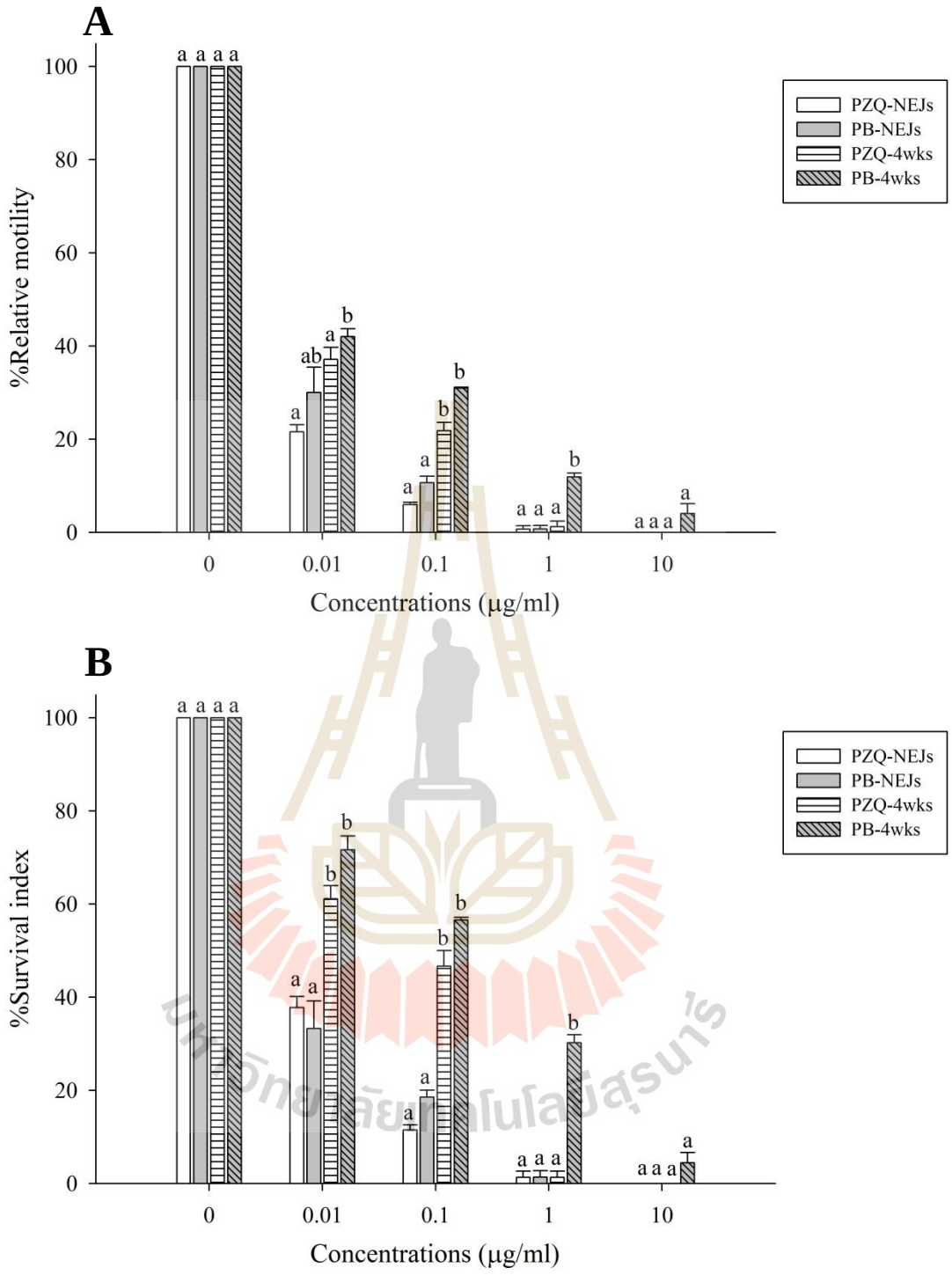


Figure 4.14 Percentage of relative motility value (RM) (A) and survival index (SI) (B) of *Opisthorchis viverrini* in the newly excysted juvenile (NEJs) stage and 4-week-old stage treated with PZQ and PB at various concentrations at 24 h. Significantly difference at $p<0.05$ between groups was indicated by "a, b, and c".

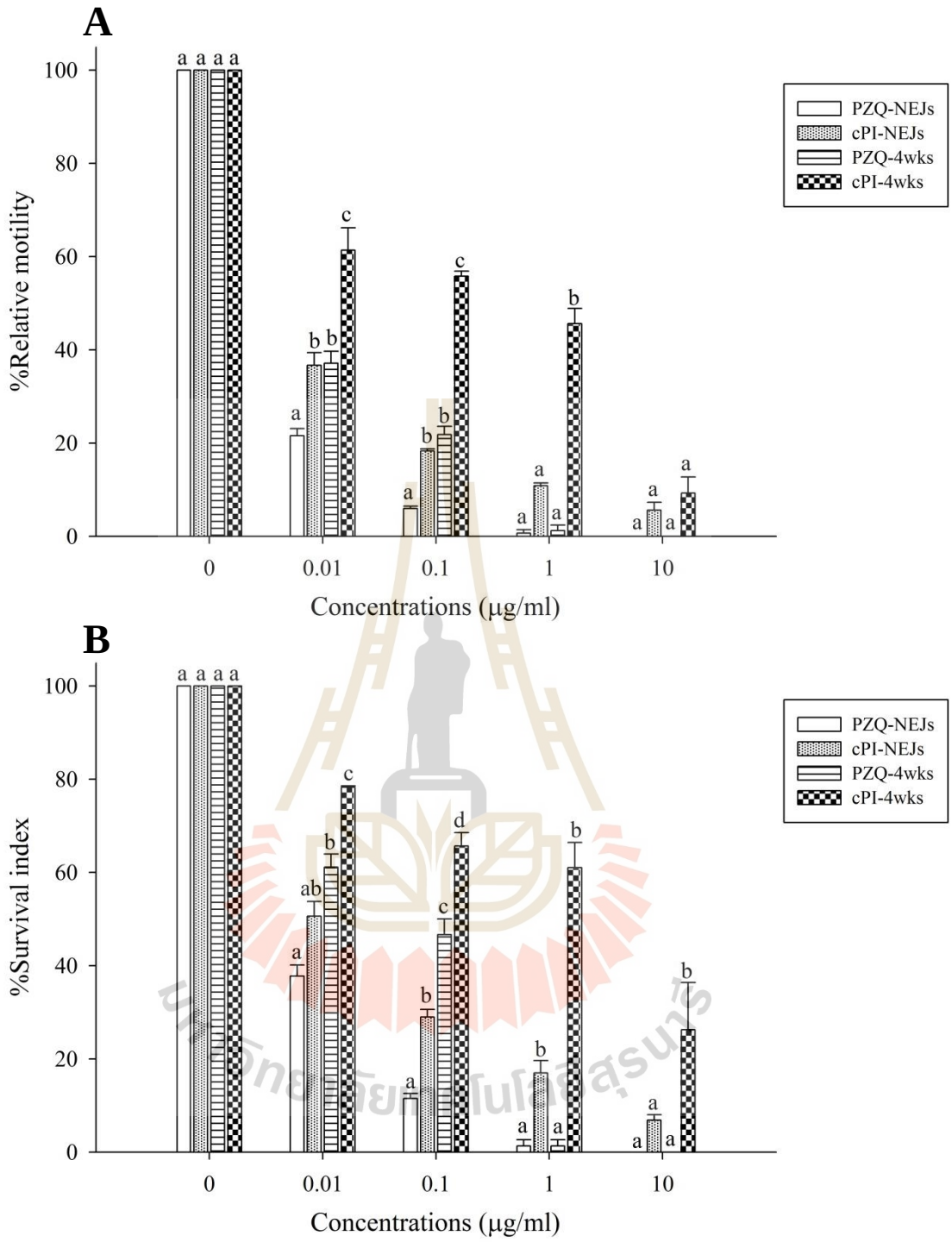


Figure 4.15 Percentage of relative motility value (RM) (A) and survival index (SI) (B) of *Opisthorchis viverrini* in the newly excysted juvenile (NEJs) stage and 4-week-old stage treated with PZQ and cPI at various concentrations at 24 h. Significantly difference at $p<0.05$ between groups was indicated by "a, b, and c".

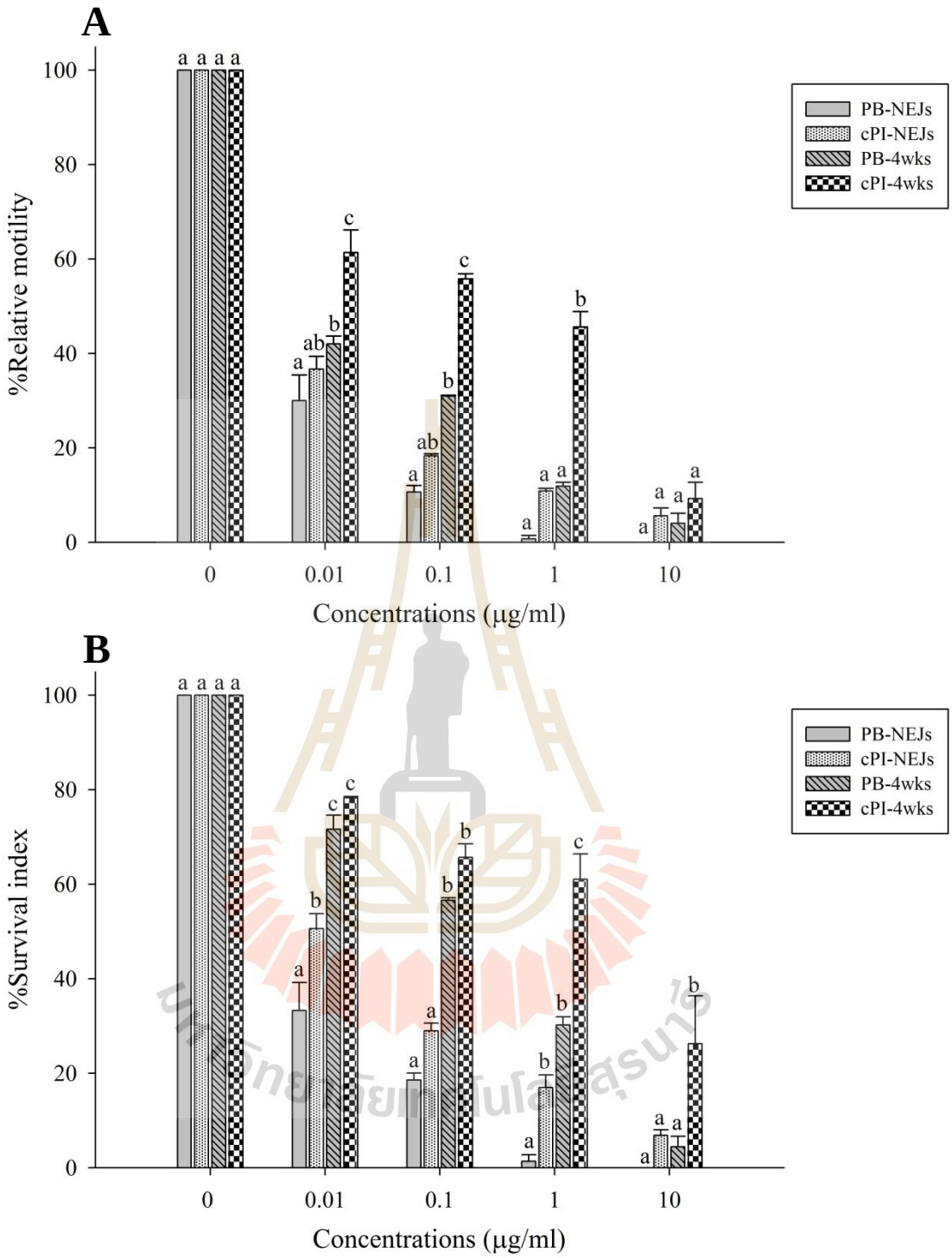


Figure 4.16 Percentage of relative motility value (RM) (A) and survival index (SI) (B) of *Opisthorchis viverrini* in the newly excysted juvenile (NEJs) stage and 4-week-old stage treated with PB and cPI at various concentrations at 24 h. Significantly difference at $p<0.05$ between groups was indicated by "a, b, and c"

4.4 Evaluation of the therapeutic effect of PZQ, PB, and cPI in infected hamsters by using worm recovery and percent of protection

When comparing the percent of worm recovery or percentage of protection in hamsters infected with metacercaria of *O. viverrini*, it revealed that the percent of protection depended on the concentration of the tested compounds as shown in Figure 4.17. At the concentration of 10 mg/kg body weight, the most potent drug was PZQ with the percent protection ranging from 56.53% to 88.67% (mean±SD: 72.6±16.07%), the second was PB with the protective percentage of 48.16% to 84.54% (mean±SD: 67.85±19), and the least potent of the worm protection was cPI (45.24±19.39%). The protective percentage of PZQ, PB, and cPI of the infected hamster was not different. The protective percentage of PB was similar to PZQ. Even though the trend of worm protection of cPI was lower than PZQ and PB, the statistical analysis was not significantly different ($p>0.05$).

At 100 mg/kg body weight, the results showed the trend on the percent of protection in each compound exhibited the same pattern as at the concentration of 10 mg/kg as shown in Figure 4.17. PZQ was the best (91.07±4.92%), PB was second (72.62±8.94%), and cPI was the lowest of percent of protection (70.24±5.83%), respectively. Surprisingly, the effect of the cPI treatment group showed nearly to PB treatment group when increasing the concentration. When comparing the effect between PZQ, PB and cPI did not show the significantly different ($p>0.05$). Those results implied that the therapeutic effect of the cPI could be the same as PB when we using at 100 mg/kg in the hamster model and against more 70% of worm recovery.

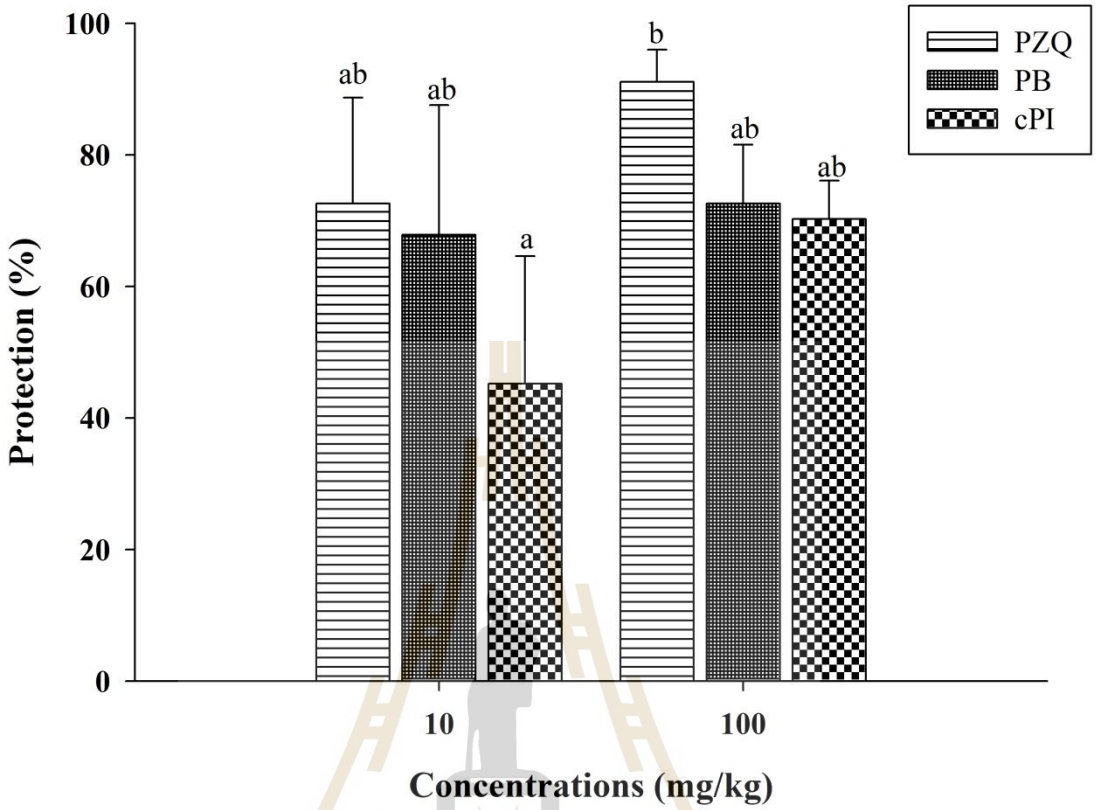


Figure 4.17 Protective percentage of PZQ, PB, and cPI treatment groups at the concentration 10 and 100 mg/kg body weight after oral administrated for 7 days. Significantly difference at $p<0.05$ between groups were indicated by "a and b".

4.5 Microphotograph of tegumental changes of *Opisthorchis viverrini* from scanning electron microscopic technique

4.5.1 Effect of drugs (PZQ, PB, and cPI) on the morphology of flukes by stereomicroscope

The morphological changes of flukes on both dorsal and ventral surfaces after drug treatment were evaluated by using stereomicroscope (Figure 4.18). The 4-week-old *O. viverrini* treated with PZQ presented the elongated of body and extensive

staining of vital dye while flukes treated with PB showed deep blue staining on the head, vitelline gland and bladder region (Figure 4.18F) the body color of flukes without stained with vital dye exhibited brown and were deeper in the higher concentration. In the cPI treated group showed the positive staining less than PB on head and tail regions, and also was found at the reproductive organs.

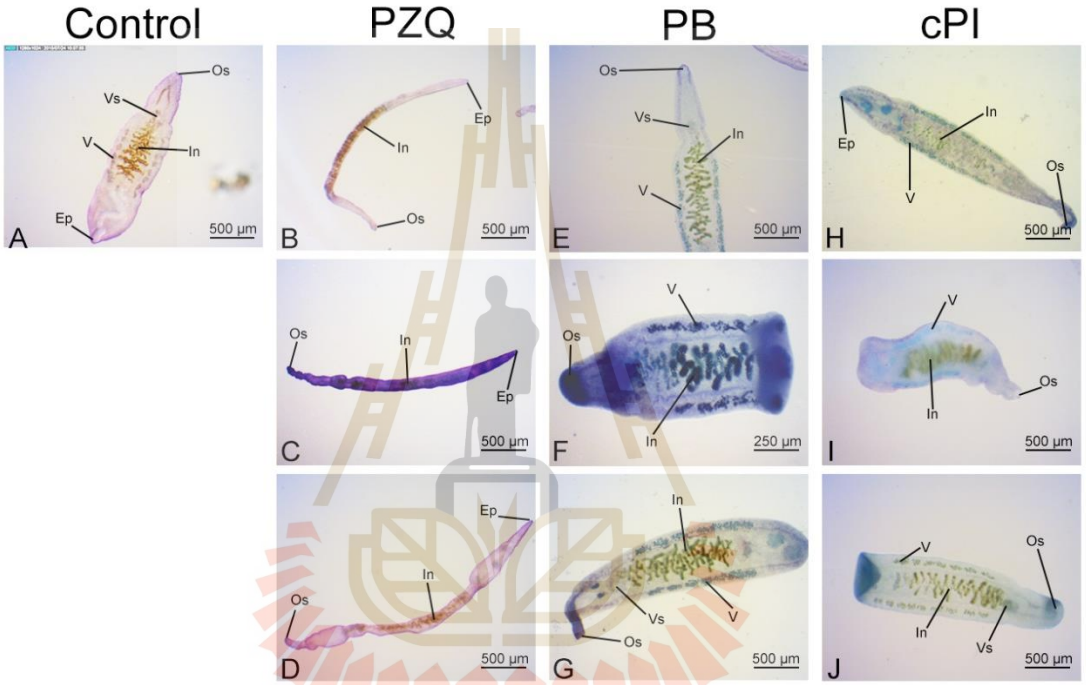


Figure 4.18 Four-week-old of *Opisthorchis viverrini* : (A) Non-treated flukes, (B-D) 10 µg/ml PZQ treated fluke, (E-G) 10 µg/ml PB-treated flukes, and (H-J) 10 µg/ml cPI-treated flukes at 24 h incubation. Oral sucker (Os), ventral sucker (Vs), vitelline gland (V), intestine (In), and excretory pore (Ep).

4.5.2 Effect of drugs on the tegument and associated structures as observed by the light microscope technique (LM)

In the negative control group flukes that were incubated in the RPMI-1640 medium containing 0.1% DMSO, the tegumental syncytium interacted with the

plasma membrane with embedding spines located above the muscular layers and tegumental cell bodies. The lesion did not present in the subtegumental layer and intestinal cecum. In the treated groups, PZQ showed the smooth tegumental syncytium and the lesion in the subtegumental area also expressed. In contrast, the tegumental syncytium in the cPI treatment group showed lesion on the ventral surface more than dorsal surface and damage of subtegumental surface also were presented (Figure 4.19).

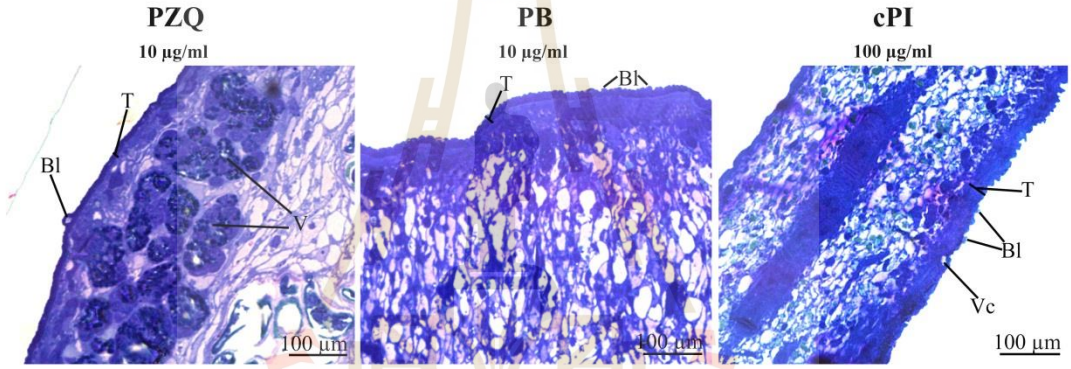


Figure 4.19 Light micrographs of the semithin section of a 4-week-old of *Opisthorchis viverrini* : (A) 10 µg/ml PZQ treated fluke, (B) 10 µg/ml PB-treated flukes, and (C) 100 µg/ml cPI-treated flukes at 12 h incubation. Tegumental layer (T), tegumental blebbing (Bl), vacuole (Vc), and vitelline gland (V).

4.5.3 Effect of drugs on the tegumental surface and associated structures as observed by SEM in different stages

4.5.3.1 NEJs stage

4.5.3.1.1 Control group

The tegumental surface architecture of newly excysted juvenile flukes from the control group showed normal morphology (Figure 4.20). The ventral surface showed clearance of the oral sucker, ventral sucker, and excretory pore. Papillae lie on the lateral part of flukes (Figure 4.20A). The spines are sharp and well arrangement (Figure 4.20B and 4.20D). The oral cone showing numerous papillae. Moreover, inside area of oral sucker shown papillae distribute around there (Figure 4.20C)

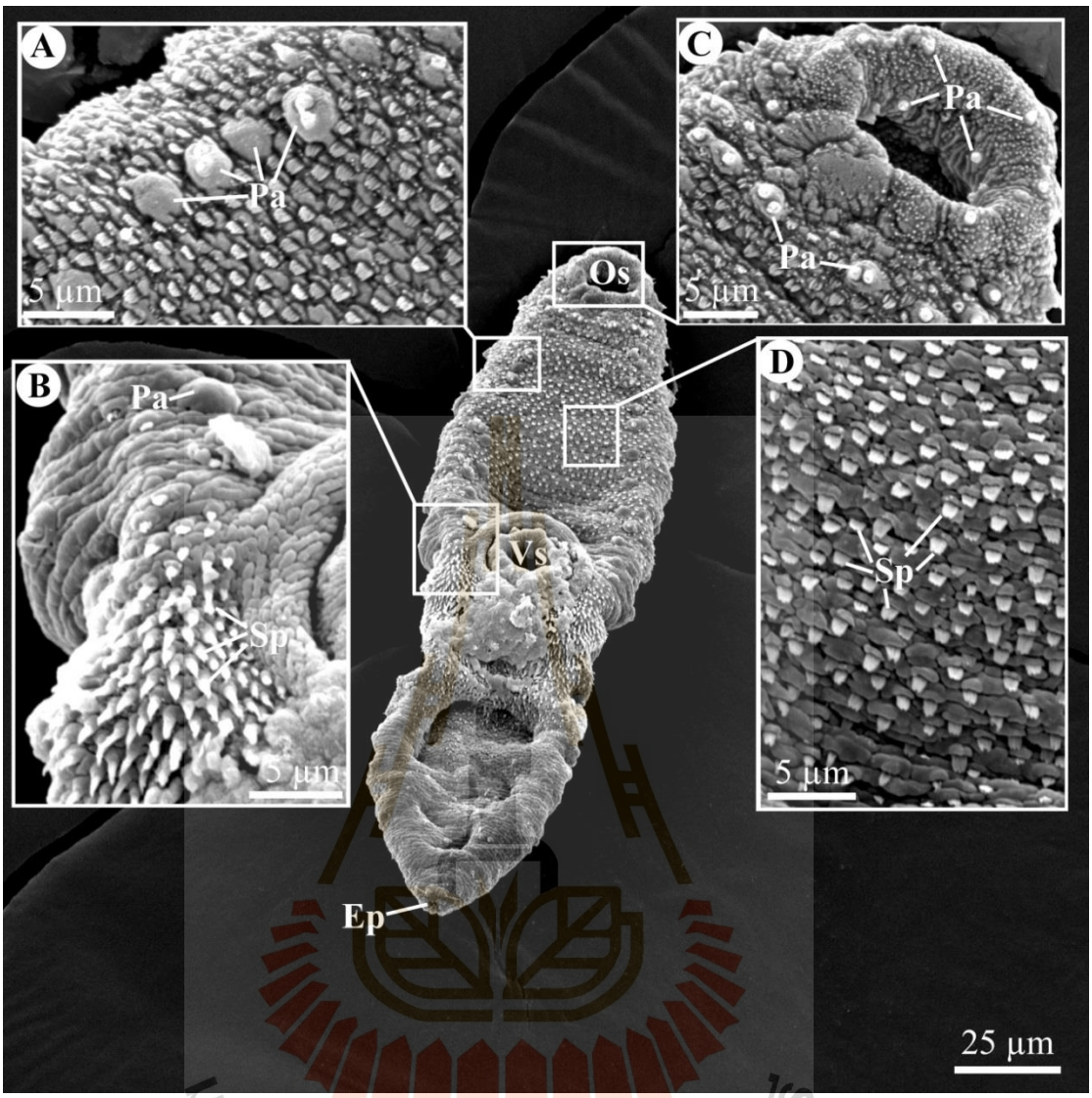


Figure 4.20 Scanning electron micrographs (SEM) of the tegumental surface of newly excysted juvenile control *Opisthorchis viverrini*. The low magnification image of fluke showing oral sucker (Os), ventral sucker (Vs) and excretory pore (Ep). (A) The ventral surface of the fluke showing papillae (Pa). (B and D) The 2 types of spines (Sp) are located on the lateral surface of the body and anterior mid-body, respectively. (C) The oral cone region at higher magnification showing papillae and spines.

4.5.3.1.2 PZQ-treatment group

Three hours incubation time

The flukes are showing no different from the control group. Their morphological are similar with no treatment flukes; oral sucker, ventral sucker, papillae, spines, and others are no different (data is not shown).

Six hours incubation time

At the concentration of 0.01 and 0.1 µg/ml, oral sucker, ventral sucker, papillae, and spines are a similar pattern as control fluke (Figure 4.21A and 4.21B). Some area of the body surface, the spines are loss normal morphology but the swollen tegumental occur (Figure 4.21C-II), papillae are well morphology (Figure 4.21C-II and III) in 1 µg/ml treatment group. At 10 µg/ml concentration, the morphological changes no displayed at oral sucker (Figure 4.21D-I) and ventral sucker (Figure 4.21D-II). Blebbing of tegument occurred at the ventral surface around anterior mid-body (Figure 4.21D-III).

Twelve hours incubation time

At concentration 0.01 µg/ml the results showed that the dorsal surface around anterior mid-body showing blebbing (Figure 4.22A-I). The ventral surface is found in spine size reduction (Figure 4.22-II). Numerous blebbing occurs around the oral cone (Figure 4.22A-III). At 0.1 µg/ml of concentration, the morphological changes are more severe, ventral surface is showing numerous blebbing at anterior mid-body (Figure 4.22B-I and V) and pores occur at posterior mid-body (Figure 4.22B-III). The papillae and spines did not change in morphology (Figure 4.22B-II and IV). At 1 µg/ml of PZQ, the low magnification image of whole fluke showed the extended body. Tegumental swollen and blebbing are more appearances in ventral surface

(Figure 4.22C-I). Papillae and spines still no changes (Figure 4.22C-II and III). At high dose treatment-group (10 µg/ml), the low magnification image showing the tegumental disruption is appearing on whole fluke body. The oral cone is loss spines and papillae (Figure 4.22D-I). Large pores appear on mid-body (Figure 4.22D-II). Tegumental distortion occurs in the anterior part of the body (Figure 4.22D-III).

Twenty-four hours incubation time

At the lowest concentration (0.01 µg/ml), the low magnification image showing the body contraction of fluke. Spines are no changes around the oral cone (Figure 4.23A-I). Clearance tegumental blebbing occurs on ventral and dorsal surface at anterior mid-body (Figure 4.23A-II). The spines lie below ventral sucker are no changes in morphology (Figure 4.23A-III). Increase the concentration up to 0.1 µg/ml, the low magnification image showing tapering effects. The blebbing formation appeared on ventral surface anterior mid-body and dorsal surface posterior mid-body (Figure 4.23B-I and II). Papillae and spines still normal form (Figure 4.23B-III). At the concentration of 1 µg/ml, the tapering effects occur at low magnification. The oral sucker is distorted. Ventral sucker with papillae and spines still no changes (Figure 4.23C-I and II). Numerous blebbing appear at ventral surface anterior mid-body (Figure 4.23C-III). At 10 µg/ml concentration, the low magnification image showing the tegumental disruption appear on flukes. The muscular layer occurred on some part of the body and blebbing still occurred (Figure 4.23D-I).

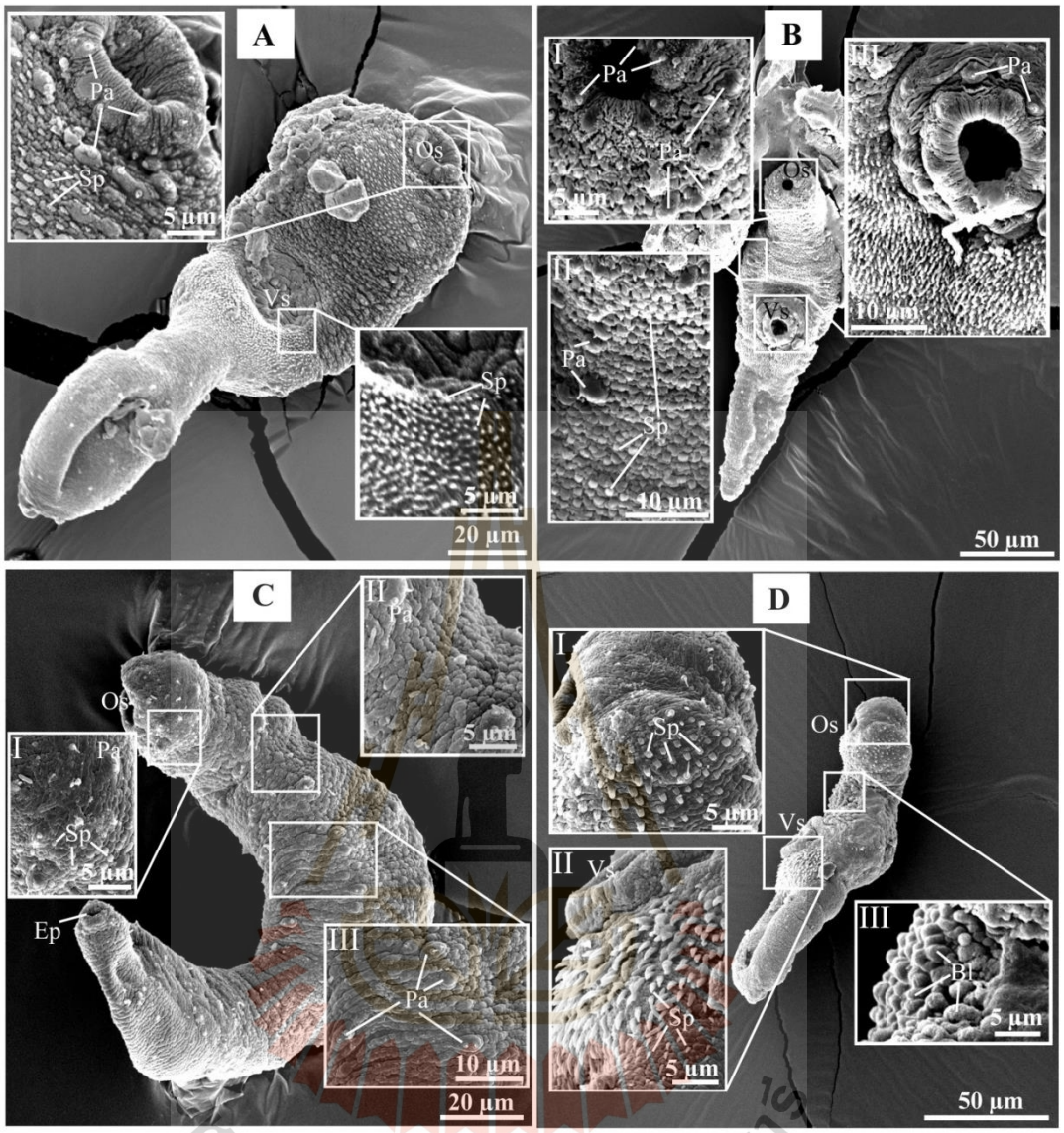


Figure 4.21 Scanning electron micrographs (SEM) of the tegumental surface of newly excysted juvenile *Opisthorchis viverrini* 6 h post-treatment *in vitro* with 0.01, 0.1, 1, and 10 µg/ml PZQ (A, B, C, and D), respectively; oral sucker (Os), ventral sucker (Vs), papillae (Pa), spines (Sp) blebbing of tegument (Bl), excretory pore (Ep).

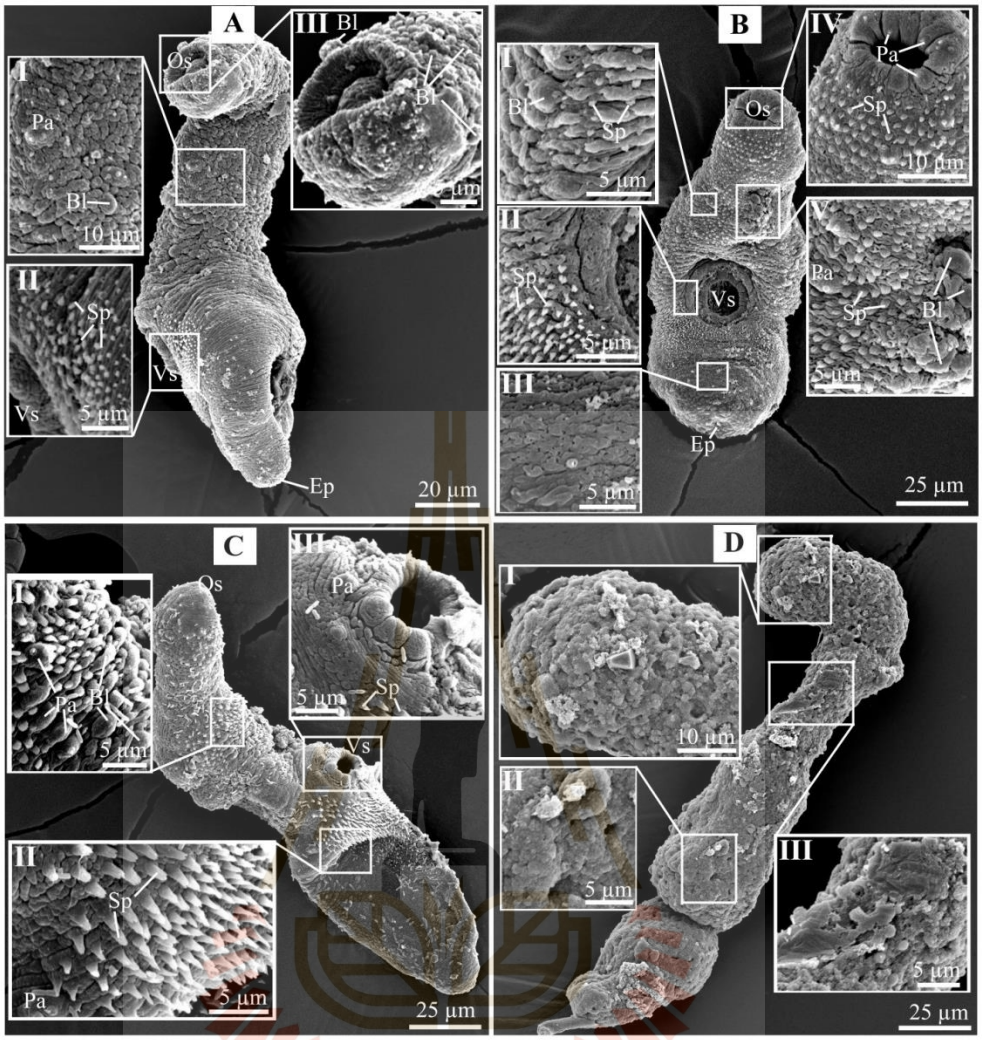


Figure 4.22 Scanning electron micrographs (SEM) of the tegumental surface of newly excysted juvenile *Opisthorchis viverrini* 12 h post-treatment *in vitro* with 0.01, 0.1, 1, and 10 µg/ml PZQ (A, B, C, and D), respectively; oral sucker (Os), ventral sucker (Vs), papillae (Pa), spines (Sp) blebbing of tegument (Bl), excretory pore (Ep).

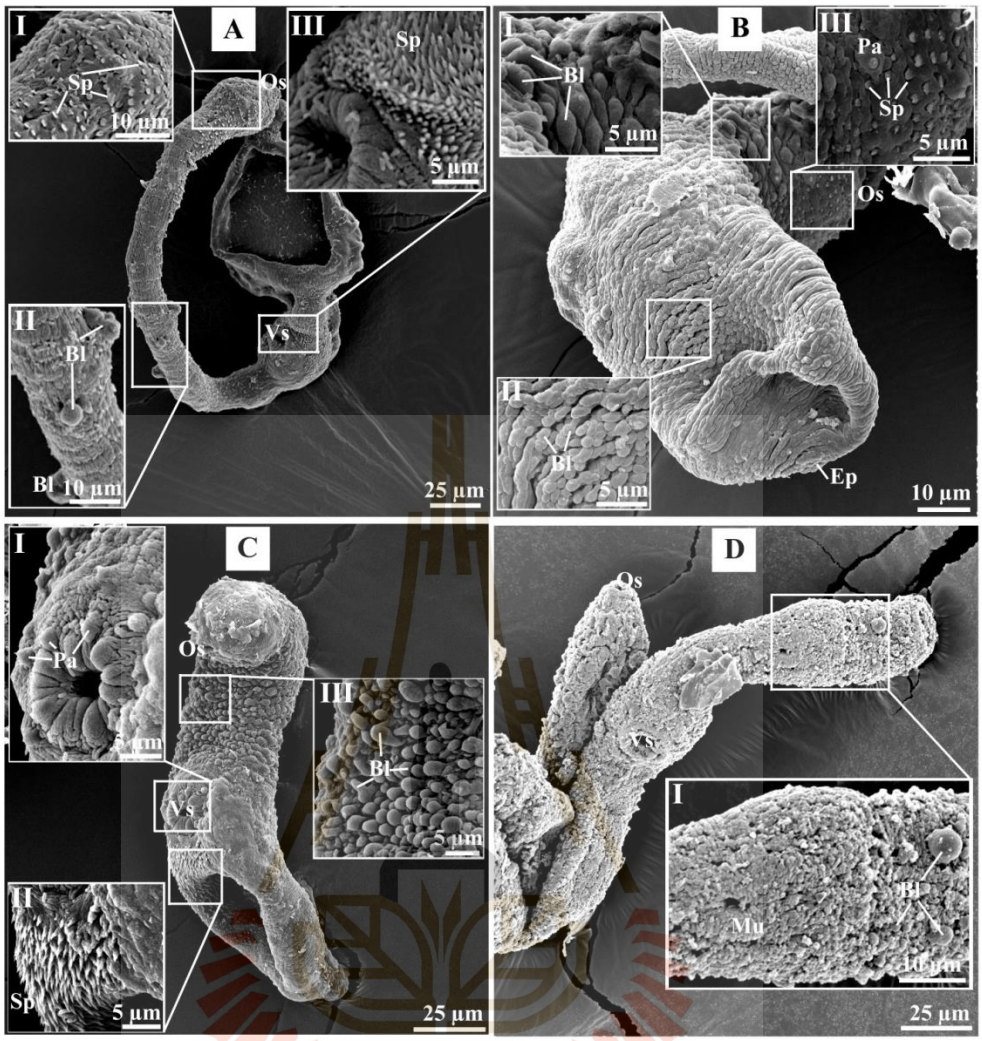


Figure 4.23 Scanning electron micrographs (SEM) of the tegumental surface of newly excysted juvenile *Opisthorchis viverrini* 24 h post-treatment *in vitro* with 0.01, 0.1, 1, and 10 µg/ml PZQ (A, B, C, and D), respectively; oral sucker (Os), ventral sucker (Vs), papillae (Pa), spines (Sp) blebbing of tegument (Bl), excretory pore (Ep), muscular layer (Mu).

4.5.3.1.3 PB-treatment group

Three hours incubation time

All of there were no morphological changes of treated flukes compared control group; oral sucker, ventral sucker, papillae, spines, and others were not different (data was not shown).

Six hours incubation time

At 0.01 µg/ml concentration, the tapering effects appeared at the low magnification image. There were no morphological changes in papillae and spines (Figure 4.24A-I and II). No morphological changes were observed in parts of flukes treated with 0.1 and 1 µg/ml of PB (Figure 4.24B-I, II, III and Figure 4.24C-I, II, III). At 10 µg/ml treatment-group, the papillae located at the lateral body area displayed abnormal morphology (Figure 4.24D-I). Spines lie on the ventral surface posterior mid-body, were short in length (Figure 4.24D-II), whereas the oral sucker was normal round in shape and papillae around oral cone were a good form (Figure 4.24D-III).

Twelve hours incubation time

At 0.01 µg/ml of PB, the contraction of the body appeared on anterior mid-body and the oral sucker is deformed. Whereas the papillae and spines did not change in morphology (Figure 4.25A-I and II). At 0.1 µg/ml, the body was warped, and advanced morphological changes. The spines lie lateral body was short and flattened (Figure 4.25B-I). Papillae located around the inside of the oral sucker were exploded (Figure 4.25B-II). Tegumental babbling appeared on the dorsal surface (Figure 4.25B-III). At the concentration of 1 µg/ml, the tegumental damage progressed to severe level. The flattening occurred at the mid-region of the body while some region of the tegument was completely sheared off and the underlying musculature was exposed

(Figure 4.25C-I). Blebbing still appeared on the whole body of fluke (Figure 4.25C-II). At the concentration of 10 µg/ml, the body of fluke was extended and tegumental disruption occurred on the whole body. The tegumental blebbing appeared in common part of body (Figure 4.25D-I and II). The muscular layer appeared after the shearing off the tegument was complete (Figure 4.25D-III).

Twenty-four hours incubation time

The tegumental changes of flukes are advance damage at the 0.01 µg/ml concentration of PB (Figure 4.26). Microblebling appeared on the ventral and dorsal surfaces but spines were still normal shape (Figure 4.26A-I, II, and III). At the ventral surface, posterior mid-body showed a large baggy part of the fluke body (Figure 4.26A-II). At 0.1 µg/ml PB, the swollen tegument was commonly found on ventral surface (Figure 4.26B-I). The oral cone showed normal form of papillae and spines (Figure 4.26B-II). For 1 µg/ml concentration, the whole body revealed severe tegumental damage in whole body. The tegument was completely sheared off and the underlying musculature was exposed (Figure 26C-I and III). In oral sucker, there was a complete loss of normal texture (Figure 26C-II). Most interesting, the flukes treated with 10 µg/ml PB showed the worst damage to the tegument. The deformities were revealed on the body; oral and ventral sucker disruption (Figure 4.26D-I), syncytium disintegration (Figure 4.26D-II), swollen tegument and exposed the basal lamina (Figure 4.26D-III).

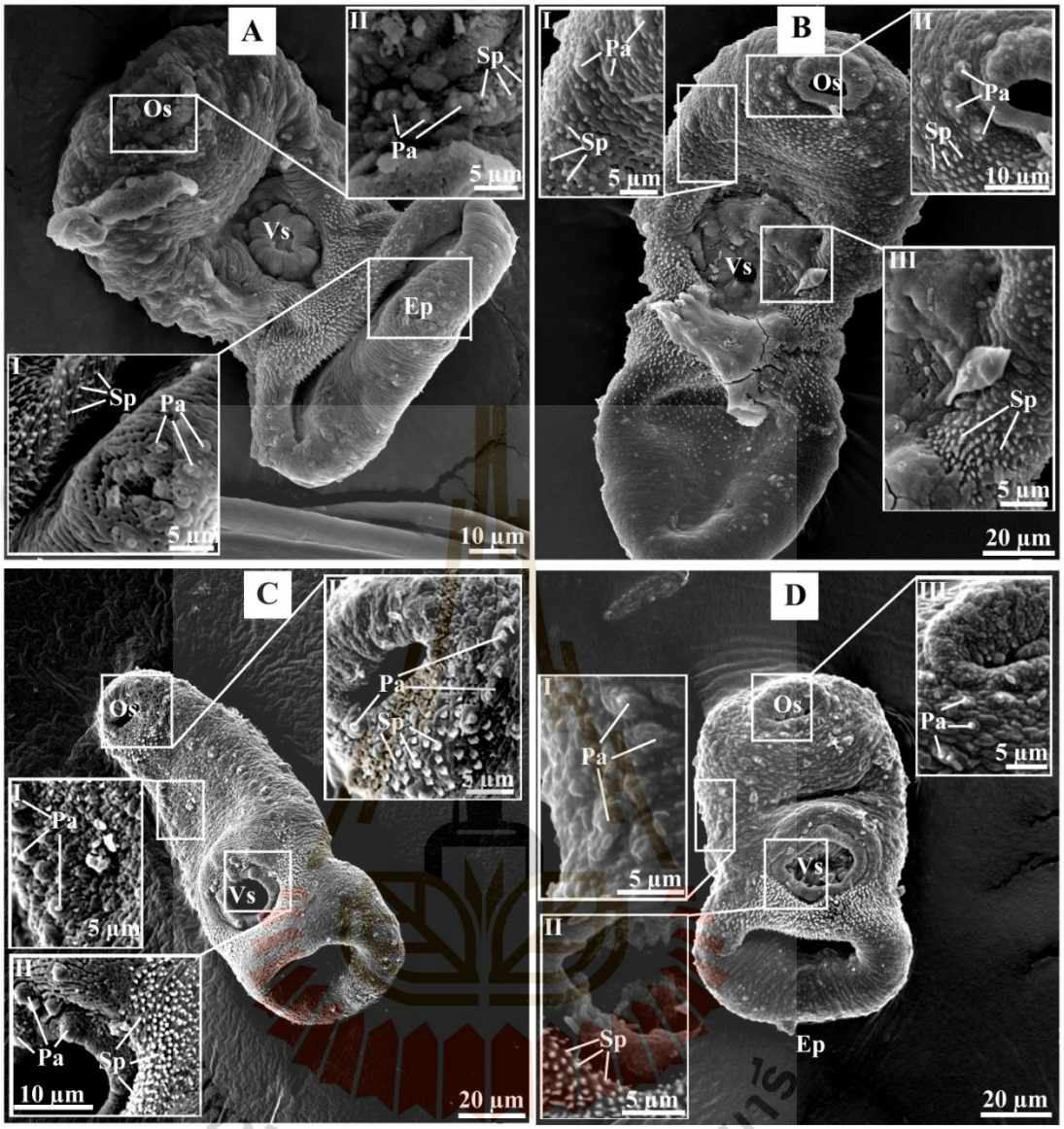


Figure 4.24 Scanning electron micrographs (SEM) of the tegumental surface of newly excysted juvenile *Opisthorchis viverrini* 6 h post-treatment *in vitro* with 0.01, 0.1, 1, and 10 µg/ml PB (A, B, C, and D), respectively; oral sucker (Os), ventral sucker (Vs), papillae (Pa), spines (Sp) blebbing of tegument (Bl), excretory pore (Ep).

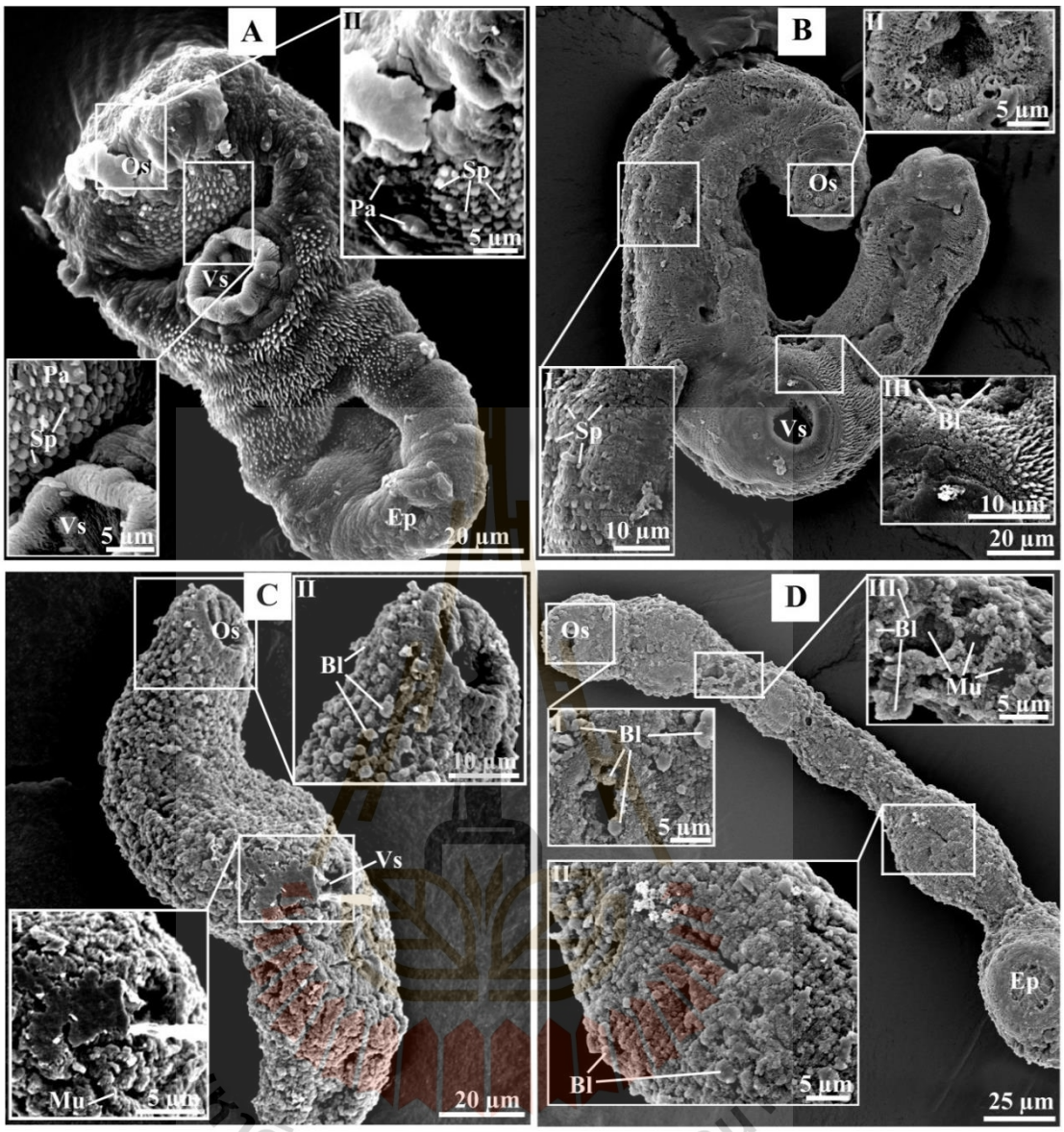


Figure 4.25 Scanning electron micrographs (SEM) of the tegumental surface of newly excysted juvenile *Opisthorchis viverrini* 12 h post-treatment *in vitro* with 0.01, 0.1, 1, and 10 µg/ml PB (A, B, C, and D), respectively ; oral sucker (Os), ventral sucker (Vs), papillae (Pa), spines (Sp) blebbing of tegument (Bl), excretory pore (Ep), muscular layer (Mu).

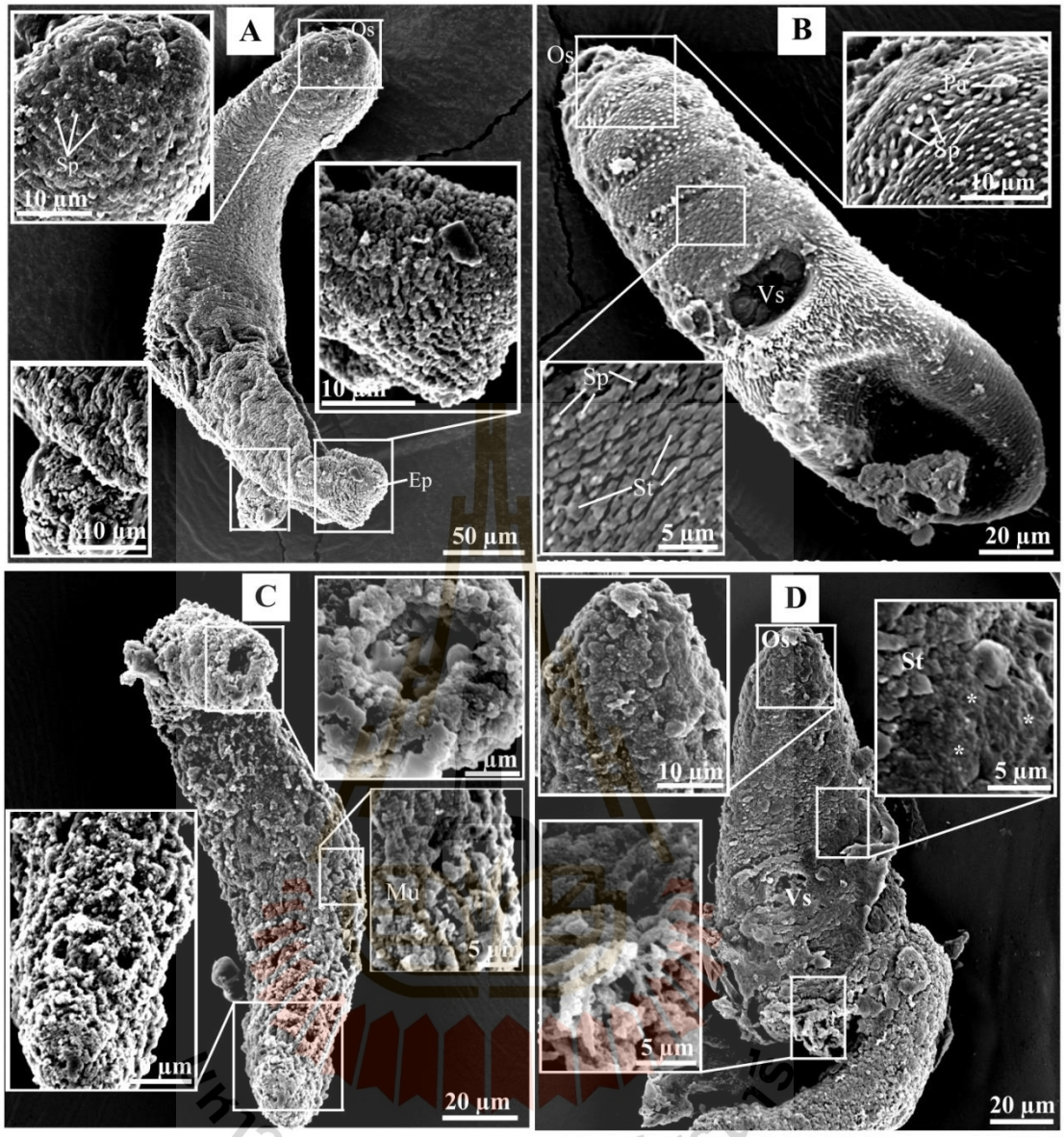


Figure 4.26 Scanning electron micrographs (SEM) of the tegumental surface of newly excysted juvenile *Opisthorchis viverrini* 24 h post-treatment *in vitro* with 0.01, 0.1, 1, and 10 µg/ml PB (A, B, C, and D), respectively; oral sucker (Os), ventral sucker (Vs), papillae (Pa), spines (Sp) blebbing of tegument (Bl), excretory pore (Ep), swollen tegument (St), basal lamina represent by asterisk.

4.5.3.1.4 cPI-treatment group

Three hours incubation time

At 3 h incubation, there were no morphological changes in cPI-treated flukes compared to the controls. Oral sucker, ventral sucker, papillae, spines, and others were not different (data was not shown).

Six hours incubation time

At 0.01 µg/ml of cPI, the treated flukes still showed normal tegumental surface (Figure 4.27A-I and II). The morphological changes appeared at a concentration of 0.1 µg/ml (Figure 4.27B). The ventrolateral surface of anterior mid-body showed the appearance of swollen tegument and micro blebbing (Figure 4.27B-I) which were not found in posterior region (Figure 4.27B-II). Moreover, an occurrence of tegumental blebbing was observed in the oral cone and dorsal surface of 1 µg/ml treated flukes (Figure 4.27C-I and II). More advance damage was observed in 10 µg/ml treated group, the most tegumental changes appeared in the dorsal surface (Figure 4.27D). Spines are deformed and flattened but still appeared around oral cone (Figure 4.27D-I). Blebbing and shearing off the tegument to expose the basal lamina were found on mid-region of body (Figure 4.27D-II).

Twelve hours incubation time

The flukes still showed the normal morphology after 0.01 µg/ml treatment of cPI (Figure 4.28A-I and II). Papillae and spines were still in normal form in the 0.1 µg/ml exposed group (Figure 4.28B-I). In addition, there was an appearance of tegumental wrinkling on the dorsal-lateral surface of the posterior mid-body of flukes (Figure 4.28B-II). Swollen tegument occurred on the ventral-lateral surface of anterior mid-body (Figure 4.28C-II) but papillae and spines still kept their normal form

(Figure 4.28C-I, II and III). At 10 µg/ml, the flukes were extended and tegument was disrupted in the whole body (Figure 4.28D). Tegumental blebbing was commonly found on the body (Figure 4.28D-I). Numerous blebbing occurred inside and outside regions of oral sucker (Figure 4.28D-II).

Twenty-four hours incubation time

The morphological changes occurred in the group treated with 0.01 µg/ml of cPI. The body of flukes was warped and tegumental wrinkles appeared on dorsal surface (Figure 4.29A-II) but papillae and spines were still normal (Figure 4.29A-I and II). Whereas increasing the concentration to 0.1 µg/ml, the advance damage most appeared on dorsal surface (Figure 4.29B). The oral cone still kept the normal form of papillae (Figure 4.29B-I). The lateral region displayed basal lamina from tegumental shearing off (Figure 4.29B-II). Swollen tegument appeared on lateral surface (Figure 4.29B-III). Severe damage was observed in group treated with 1 µg/ml of cPI (Figure 4.29C). Papillae and spines on anterior mid-body were still kept in the normal form but the tegumental surface around them changed to abnormal appearance with swollen tegument (Figure 4.29C-I). Spine deformation and numerous blebbing occurred on posterior mid-body (Figure 4.29C-II and III). The fluke body was flattened and extended in 10 µg/ml treatment group (Figure 4.29D). The oral cone around the oral sucker showed tegumental disruption and numerous blebbing (Figure 4.29D-I). Microblebbing and tegumental wrinkling showed on posterior mid-body region (Figure 4.29D-II). Moreover, tegumental blebbing revealed inside and outside of ventral sucker (Figure 4.29D-III).

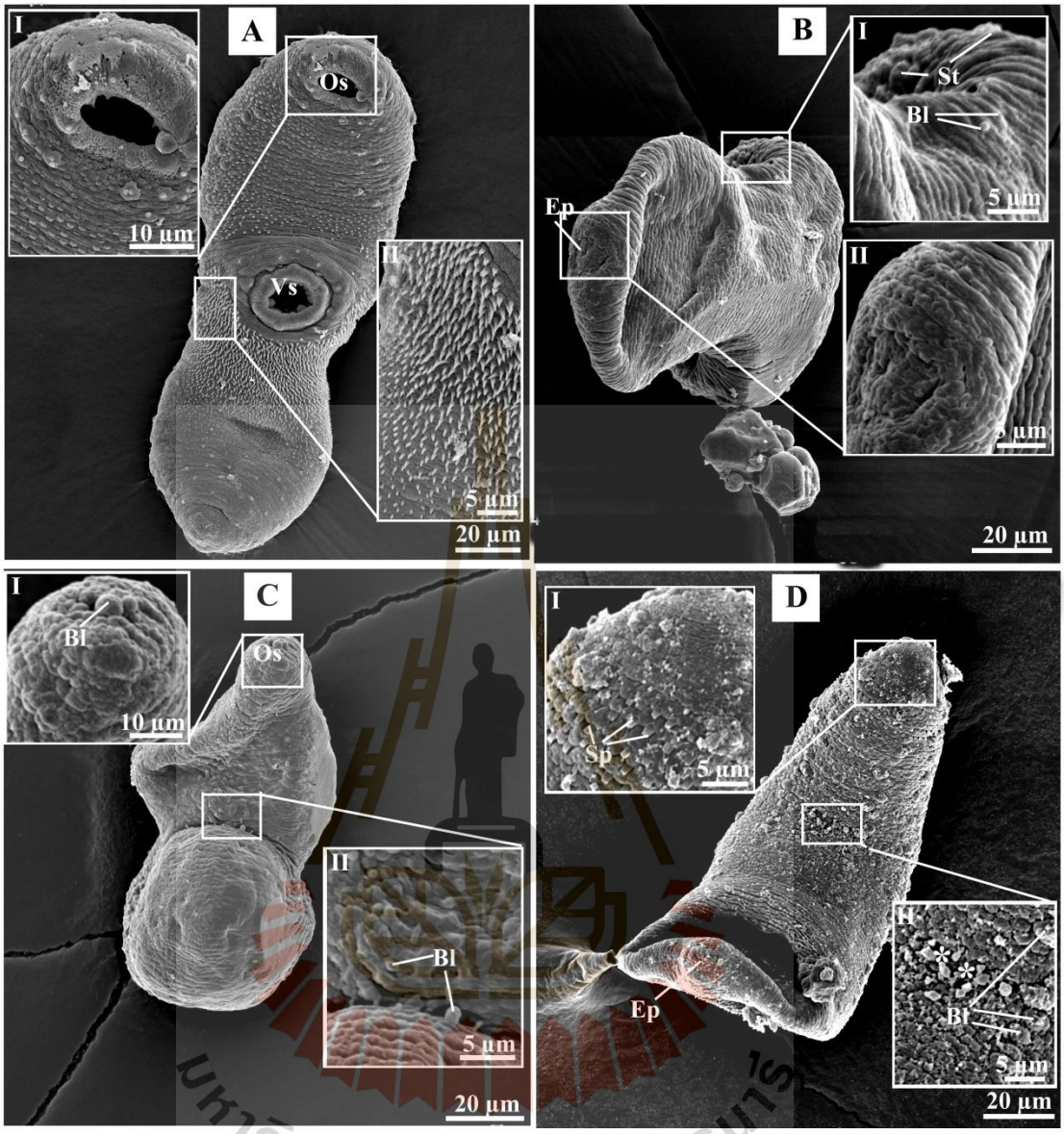


Figure 4.27 Scanning electron micrographs (SEM) of the tegumental surface of newly excysted juvenile *Opisthorchis viverrini* 6 h post-treatment *in vitro* with 0.01, 0.1, 1, and 10 µg/ml cPI (A, B, C, and D), respectively; oral sucker (Os), ventral sucker (Vs), papillae (Pa), spines (Sp), blebbing of tegument (Bl), excretory pore (Ep), swollen tegument (St), basal lamina represent by asterisk.

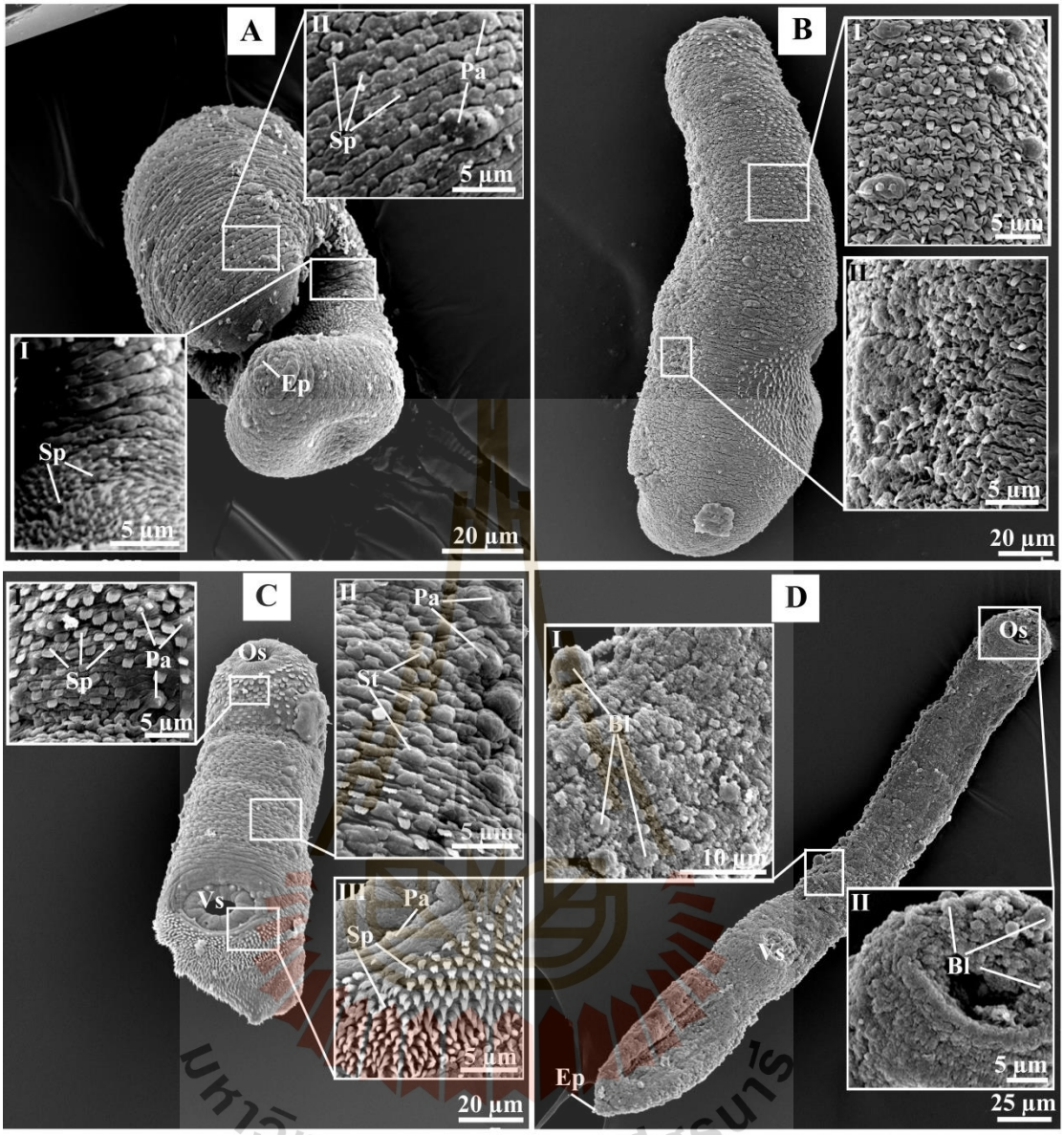


Figure 4.28 Scanning electron micrographs (SEM) of the tegumental surface of newly excysted juvenile *Opisthorchis viverrini* 12 h post-treatment *in vitro* with 0.01, 0.1, 1, and 10 µg/ml cPI (A, B, C, and D), respectively; oral sucker (Os), ventral sucker (Vs), papillae (Pa), spines (Sp), blebbing of tegument (Bl), excretory pore (Ep), swollen tegument (St).

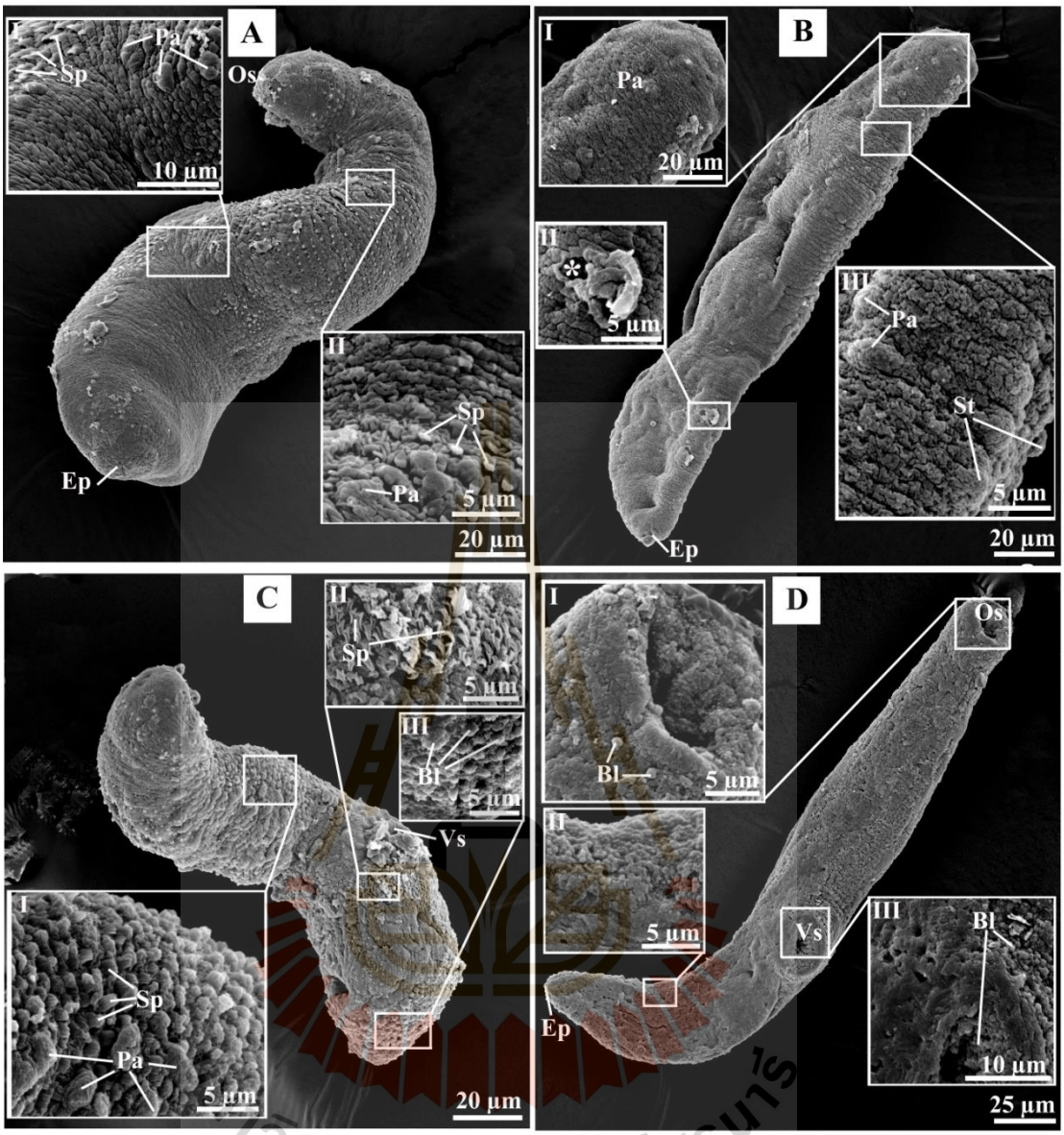


Figure 4.29 Scanning electron micrographs (SEM) of the tegumental surface of newly excysted juvenile *Opisthorchis viverrini* 24 h post-treatment *in vitro* with 0.01, 0.1, 1, and 10 µg/ml cPI (A, B, C, and D), respectively; oral sucker (Os), ventral sucker (Vs), papillae (Pa), spines (Sp), blebbing of tegument (Bl), excretory pore (Ep), swollen tegument (St), basal lamina represent by asterisk.

Table 4.3 Summaries of the tegumental alterations in NEJs of *O.viverrini* as observed by scanning electron microscope (SEM) after treatment with various drugs.

Treatment	Doses (µg/ml)	Incubation times (h)	Ventral surface		Dorsal surface	
			Anterior	Posterior	Anterior	Posterior
0.1% DMSO (Control)		24	-	-	-	-
PZQ	0.01	3	-	-	-	-
		6	-	-	-	-
		12	#+	-	+	-
		24	+	+	+	+
	0.1	3	-	-	-	-
		6	-	-	-	-
		12	+	+	+	+
		24	+	+	+	+
	1	3	-	-	-	-
		6	-	-	#+	#+
		12	+	+	+	+
		24	+0	+	+	-
	10	3	-	-	-	-
		6	+	+	-	-
		12	#*0	+	0	-
		24	+0	+0	0	0
PB	0.01	3	-	-	-	-
		6	-	-	-	-
		12	0	-	-	-
		24	+	+	+	+
	0.1	3	-	-	-	-
		6	-	-	-	-
		12	#*	-	+	+
		24	*	0	+	+
	1	3	-	-	-	-
		6	-	-	-	-
		12	+0	+	+	+
		24	0	0	0	0
	10	3	-	-	-	-
		6	#*	#*	-	-
		12	0	0	0	0
		24	0	0	0	0
cPI	0.01	3	-	-	-	-
		6	-	-	-	-
		12	-	-	-	-
		24	-	-	+	+
	0.1	3	-	-	-	-
		6	+	-	+	-
		12	-	-	-	-
		24	-	-	+0	+0
	1	3	-	-	-	-
		6	-	-	+	+
		12	+	-	+	-
		24	-	+	#	#+
	10	3	-	-	-	-
		6	#	-	#0	-
		12	+0	+0	+0	+0
		24	+0	+0	+0	+0

- Normal tegument

\# Spine deformation

* Papillae deformation

\+ Tegumental blebbing and/or tegumental swelling

0 Tegumental disruption and/or tegumental destroyed

4.5.3.2 4-weeks-old stage

4.5.3.2.1 Control group

The tegumental surface architecture of 4-week-old flukes from the control group showed normal morphology (Figure 4.30). The ventral surface showed clearance of the oral sucker, ventral sucker. Papillae lied on the oral cone (Figure 4.30-I) and lateral part of flukes (Figure 4.30-II). The oral cone showed numerous papillae (Figure 4.30-III). Moreover, the spines were not shown.

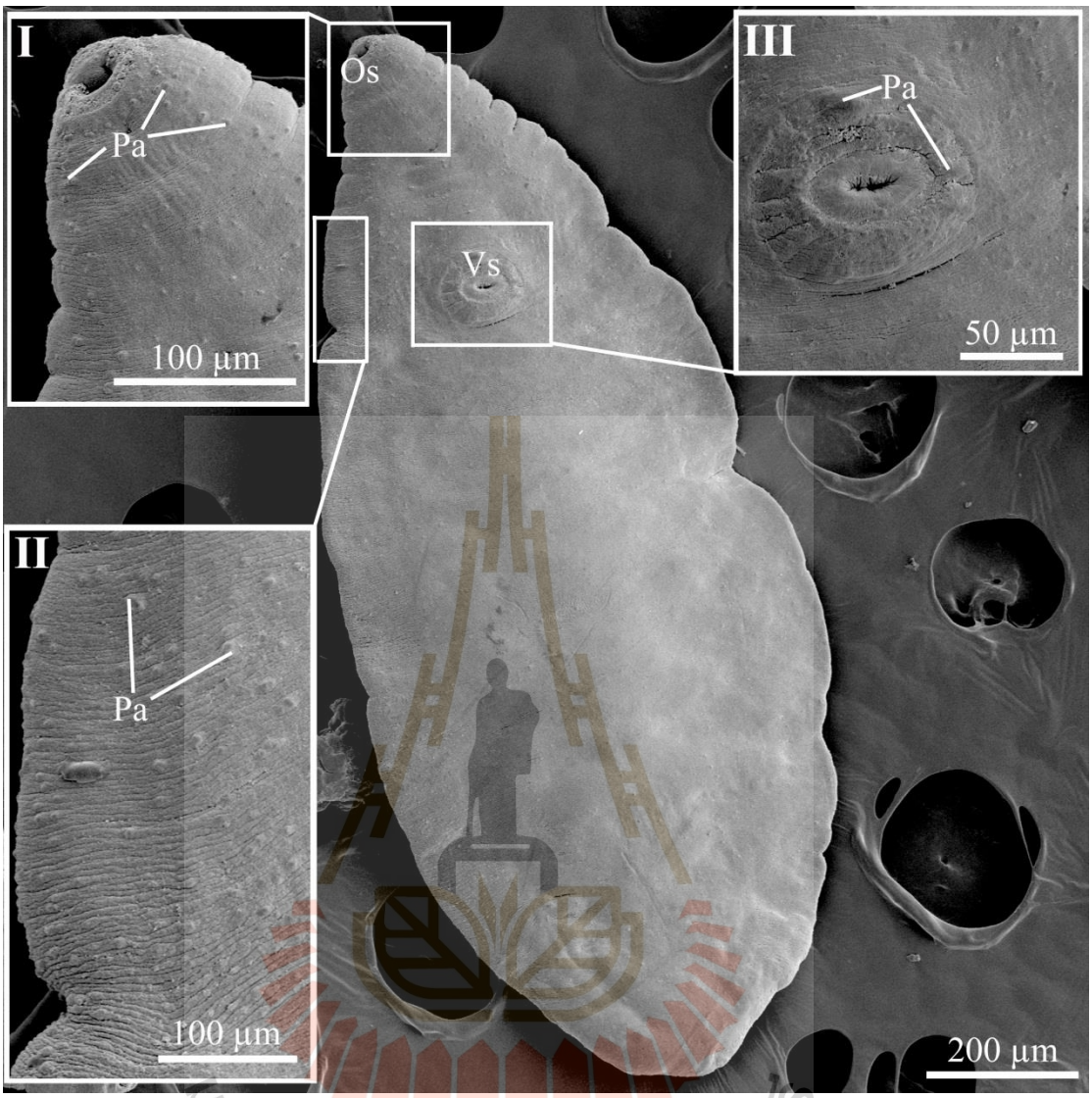


Figure 4.30 Scanning electron micrographs (SEM) of the tegumental surface of 4-week old control *Opisthorchis viverrini*. The low magnification image of fluke showing the fine ventral surface, oral sucker (Os), and ventral sucker (Vs). (I) The ventral surface of the fluke showing papillae (Pa) around the oral cone, (II) the papillae located common on lateral area, and (III) the papillae locate around ventral sucker.

4.5.3.2.2 PZQ-treatment group

Three hours incubation time

The morphology of 3 h PZQ treated flukes was similar to the control group. However, some areas of them showed distorted spines (data not shown).

Six hours incubation time

The morphological changes occurred at 6 h the post incubation. At 0.01 µg/ml of PZQ, the body of flukes was extended at low magnification image and the oral cone showed normal formation of papillae (Figure 4.31A-I). Blebbing appeared inside area of ventral sucker (Figure 4.31A-II). The extended body still appeared at 0.1 µg/ml concentration but the morphological changes were similar to group treated with 0.01 µg/ml concentration (Figure 4.31B). In addition, the numerous pores on tegument appeared in some part of flukes (Figure 4.31B-I) and ventral sucker occurred in uncommon part when compared with normal flukes (Figure 4.31B-II). The flukes treated with 1 µg/ml concentration showed body contraction with distorted oral and ventral suckers (Figure 4.31C). Microblebbing occurred on whole body and tegumental disruption led to exposed basal lamina on dorsal surface of posterior mid-body (Figure 4.31C-II). At concentration of 10 µg/ml of PZQ, the tegumental damages were similar 1 µg/ml treated group. Tegumental blebbing was the most damage appearance of the oral cone and ventral surface (Figure 4.31D-I and II).

Twelve hours incubation time

The contraction of the whole body of flukes occurred in all treatments, severe damage level depended on the concentration in each treatment group. 0.01 µg/ml of PZQ induced similar morphological changes to the treatment group at 6 h after

incubation at the same concentration. The oral and ventral suckers were still normal (Figure 4.32A-I and III). In addition, the blebbing appeared in a large amount around the lateral area (Figure 4.32A-II). Numerous blebbing and pores appeared on the oral cone and ventral sucker in flukes treated with 0.1 µg/ml concentration (Figure 4.32B-I and II). Microblebbing could be found on lower area of ventral sucker (Figure 4.32B-III). Increasing the concentration up to 1 µg/ml, the fluke body was more bent (Figure 4.32C). The pores and blebbing were common display on the whole body (Figure 4.32C-I and II). At the highest concentration, the dorsal surface of oral cone showed numerous pores but papillae were still occurred in normal form (Figure 4.32D-I). The tegumental swelling is more damage occurred in this group (Figure 4.32D-II). The ventral sucker was narrowed with the nearly cleared sucker cavity (Figure 4.32D-III).

Twenty-four hours incubation time

This incubation time showed the tegumental changes at several levels, it depended on concentration. At 0.01 µg/ml of PZQ, the blebbing and pores appeared in a wide area; oral sucker throughout the excretory pore (Figure 4.33A-I and II). The muscle contraction dominantly occurred in anterior mid-body region (Figure 4.33A-III). So, the oral and ventral suckers were still kept in their normal form. Similar to 0.01 µg/ml treated group, the oral sucker showed normal morphology with papillae but the oral cavity was not opened (Figure 4.33B-I). The posterior mid-body displayed shearing off to expose the underlying musculature (Figure 4.33B-II). Around the neck or upper area from ventral sucker showed the blebbing and muscular layer (Figure 4.33B-III). The flukes treated with 1 µg/ml concentration showed advance damage on tegument (Figure 4.33C). Oral cone showed the numerous pores (Figure 4.33C-I) and blebbing appeared in round-shape in ventral surface (Figure

4.33C-II). The burst-bleb showed in a lateral surface of the body (Figure 4.33C-III). The severe damage was shown in 10 µg/ml treatment group, the oral sucker showing papillae and the oral surface was completely destroyed and tegumental shearing off to display the basal lamina (Figure 4.33D-I). Basal lamina was stripped off to expose parenchyma in various areas of the body (Figure 4.33D-II, III, and IV). The complete tegumental disruption occurred in dorsal surface (Figure 4.33D-III, IV).

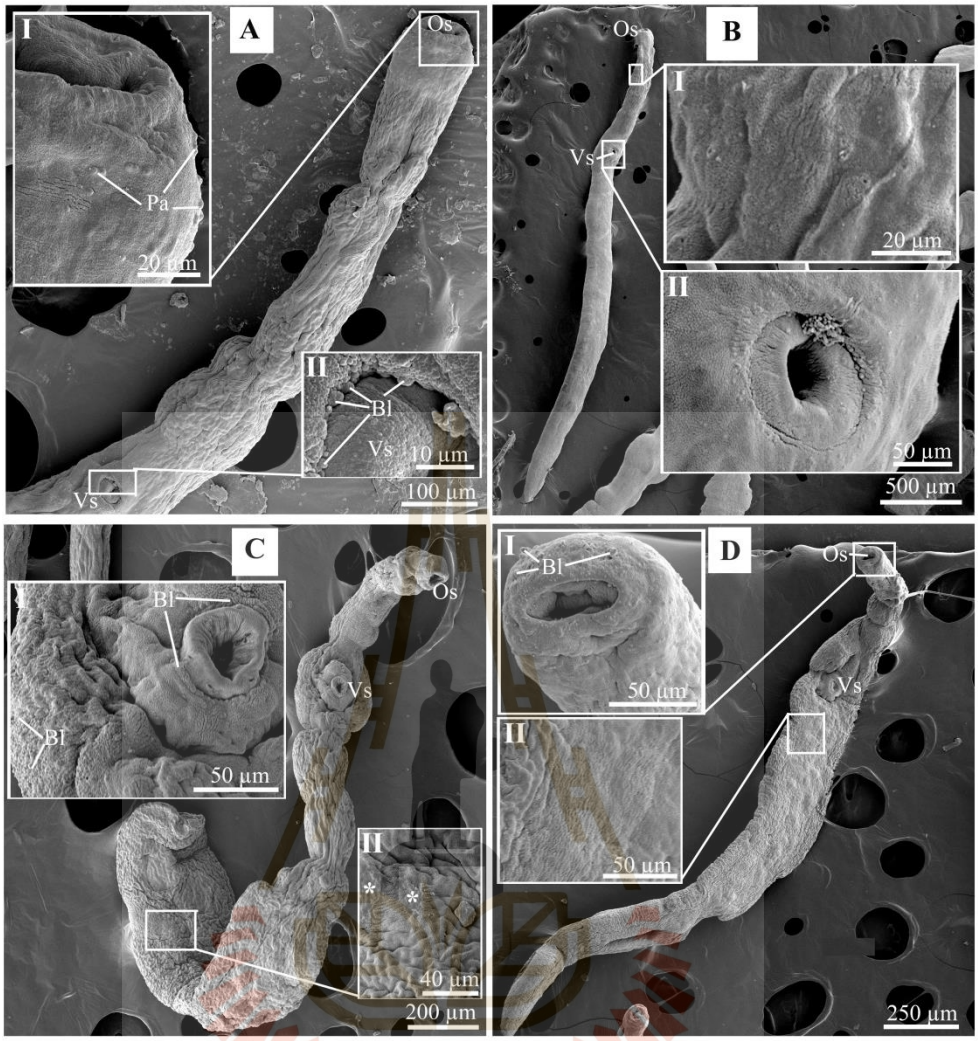


Figure 4.31 Scanning electron micrographs (SEM) of the tegumental surface of 4-week old *Opisthorchis viverrini* 6 h post-treatment *in vitro* with 0.01, 0.1, 1, and 10 µg/ml PZQ (A, B, C, and D), respectively; oral sucker (Os), ventral sucker (Vs), papillae (Pa), blebbing of tegument (Bl), basal lamina represent by asterisk.

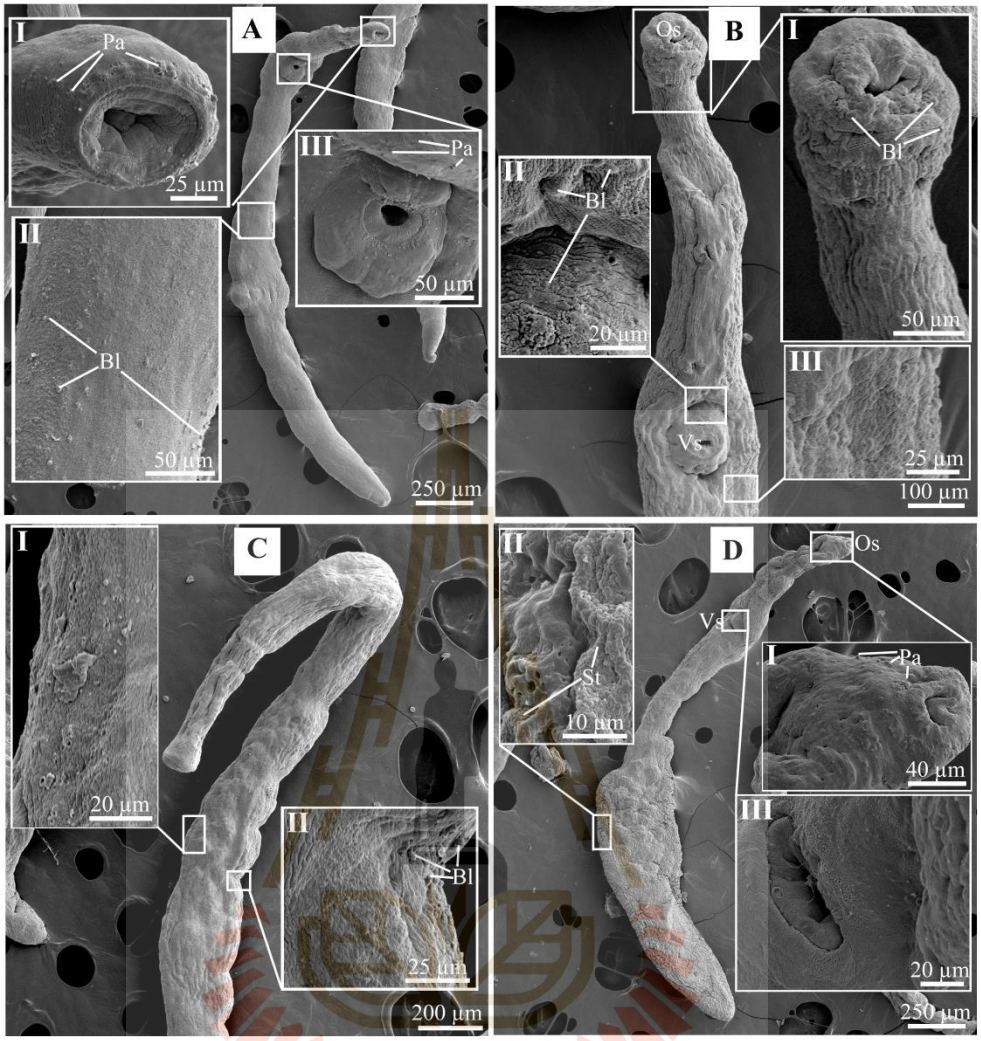


Figure 4.32 Scanning electron micrographs (SEM) of the tegumental surface of 4-week old *Opisthorchis viverrini* 12 h post-treatment *in vitro* with 0.01, 0.1, 1, and 10 µg/ml PZQ (A, B, C, and D), respectively; oral sucker (Os), ventral sucker (Vs), papillae (Pa), blebbing of tegument (Bl), swollen tegument (St).

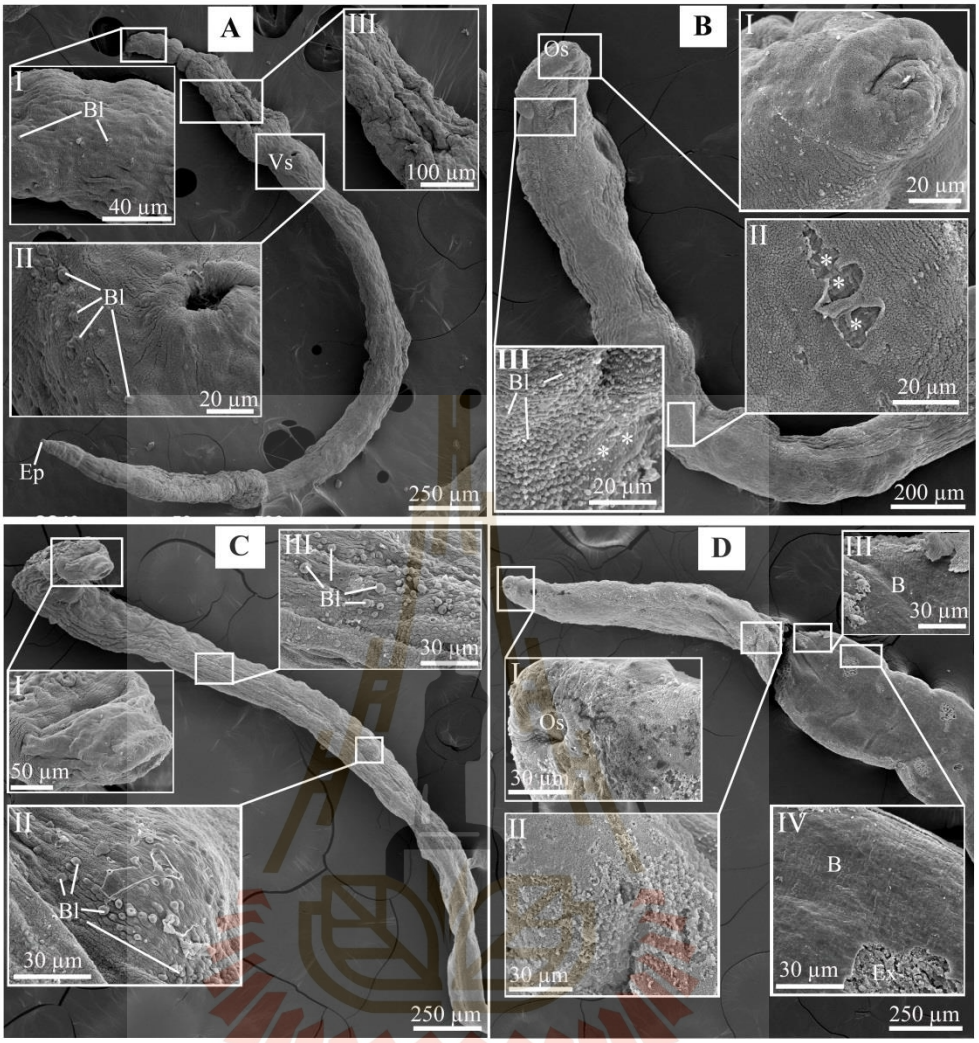


Figure 4.33 Scanning electron micrographs (SEM) of the tegumental surface of 4-week old *Opisthorchis viverrini* 24 h post-treatment *in vitro* with 0.01, 0.1, 1, and 10 µg/ml PZQ (A, B, C, and D), respectively; oral sucker (Os), ventral sucker (Vs), papillae (Pa), spines (Sp), blebbing of tegument (Bl), excretory pore (Ep), basal lamina (B), and tegumental disruption represent by asterisk.

4.5.3.2.3 PB-treatment group

Three hours incubation time

3 h of PB-treated flukes showed morphology similar to the normal flukes. However, some areas showed distorted spines and a few scaly skins, the most area showed no morphology alteration (data not shown).

Six hours incubation time

The apparent tegumental changes occurred on this incubation time throughout the experiment at 24 h. 0.01 µg/ml PB treated flukes displayed wrinkling-flatten in shape in the whole body (Figure 4.34A). The oral cone area was smooth tegument with no papillae but the oral sucker was in a normal form (Figure 4.34A-I). The ventral surface of anterior mid-body appeared widespread micro blebbing tegument (Figure 4.34A-II). The swollen tegument and blebbing occurred in ventral surface anterior of mid-body region (Figure 4.34B-I). Whereas the dorsal surface, basal lamina displayed on the overall surface (Figure 4.34B-II). The tapering effect appeared on flukes treated with 1 µg/ml concentration (Figure 4.34C). Oral sucker showed destructed tegument and blebbing appeared around the oral cone (Figure 4.34C-I) and ventral sucker area (Figure 4.34C-II). At 10 µg/ml concentration, the tapering effect occurred in similar to 1 µg/ml treated group. The ventral surface was wrinkling in oral cone and mid-body regions (Figure 4.34D). Tegumental blebbing appeared in ventral surface both of anterior and posterior regions as round-shape. In addition, tegumental had been stripped off to expose basal lamina beneath in lateral region and numerous pores were at a similar area (Figure 4.34D-I, II).

Twelve hours incubation time

Tapering effects were observed in 0.01 µg/ml of PB treated group and tegumental disruption found in the apical-posterior of dorsal surface (Figure 4.35A). Tegumental wrinkling and micro blebbing occurred in lateral regions. Tegumental disruption was found in oral sucker (Figure 4.35A-I). Tegumental pores were displayed over on ventral surface of posterior mid-body (Figure 4.35A-II). The wrinkling body also occurred in 0.1 µg/ml treatment group (Figure 4.35B). Microblebbing was found over the ventral surface including ventral sucker but papillae showed normal formation (Figure 4.35B-I and II). Tapering effect and wrinkling body were present in 1 µg/ml treatment group (Figure 4.35C). The oral sucker showed no more papillae in the oral cavity and oral cone (Figure 4.35C-1). The varies size of blebbing was showing overall the ventral and dorsal surface (Figure 4.35C-I and II). The distorted and wrinkling of body were present in whole body except in dorsal mid-body region (Figure 4.35D). Numerous tegumental blebbing and pores appeared in both ventral and dorsal surfaces especially around ventral sucker (Figure 4.35D-I). The tegumental disruption was more severe damage around the oral cone (Figure 4.35D-II).

Twenty-four hours incubation time

In this incubation time, the morphological changes were similar in pattern, severe level depended on concentration. At 0.01 µg/ml of PB treatment, the fluke showed a flattened body and distorted oral cone (Figure 4.36A). The area below the oral cone showed blebbing in various sizes (Figure 4.36A-I). The ventral surface of posterior mid-body exhibited extensive blebbing with often burst and numerous pores (Figure 4.36A-II). Tegumental disruption was displayed in posterior-lateral regions

(Figure 4.36A-III). Tegumental pores and blebbing were present around ventral sucker (Figure 4.36A-IV). The tapering effect appeared in anterior and posterior parts of fluke treated with 0.1 µg/ml of PB (Figure 4.36B). The ventral surface showed severe damage, basal lamina with micro blebbing occurred in anterior mid-body (Figure 4.36B-I). The posterior region at the same surface showed burst-blebbing in mid-body and tegumental disruption was found in lateral surface of the body (Figure 4.36B-II and III). The treatment of 1 µg/ml caused the shearing off of tegument to expose basal lamina in widespread area on dorsal surface (Figure 4.36C-I, II and III). Microblebbing appeared in dorsal surface anterior mid-body region close to oral cone (Figure 4.36C-IV). The worst damage occurred to the tegument in the group treated with 10 µg/ml (Figure 4.36D). The flukes were complete loss of normal body texture, the oral cone and neck showed tegumental disruption on all areas (Figure 4.36D-I and II). Whereas the dorsal surface of posterior region exhibited basal lamina with burst-blebbing tegument in the overall (Figure 4.36D-III).

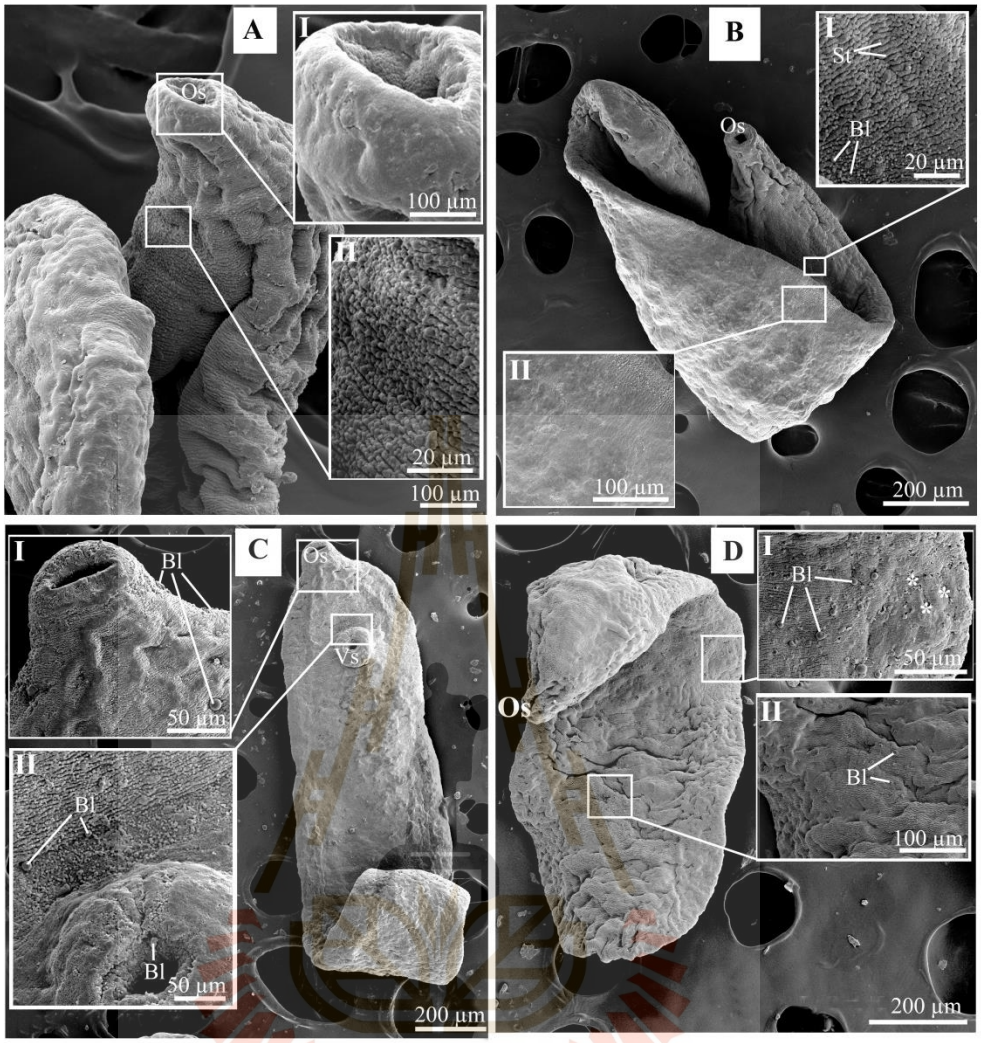


Figure 4.34 Scanning electron micrographs (SEM) of the tegumental surface of 4-week old *Opisthorchis viverrini* 6 h post-treatment *in vitro* with 0.01, 0.1, 1, and 10 µg/ml PB (A, B, C, and D), respectively; oral sucker (Os), ventral sucker (Vs), blebbing of tegument (Bl), swollen tegument (St), basal lamina (B), tegumental disruption represent by asterisk.

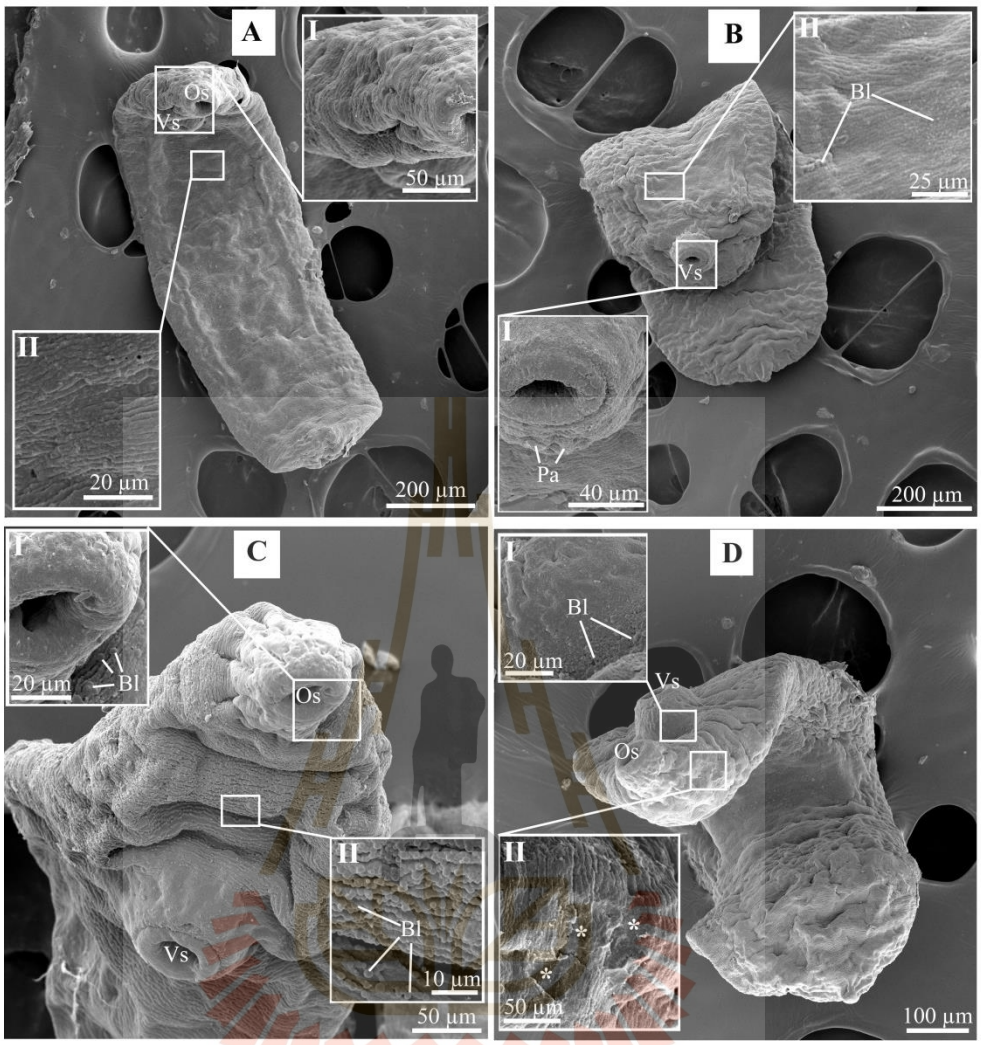


Figure 4.35 Scanning electron micrographs (SEM) of the tegumental surface of 4-week old *Opisthorchis viverrini* 12 h post-treatment *in vitro* with 0.01, 0.1, 1, and 10 µg/ml PB (A, B, C, and D), respectively; oral sucker (Os), ventral sucker (Vs), papillae (Pa), blebbing of tegument (Bl), tegumental disruption represent by asterisk.

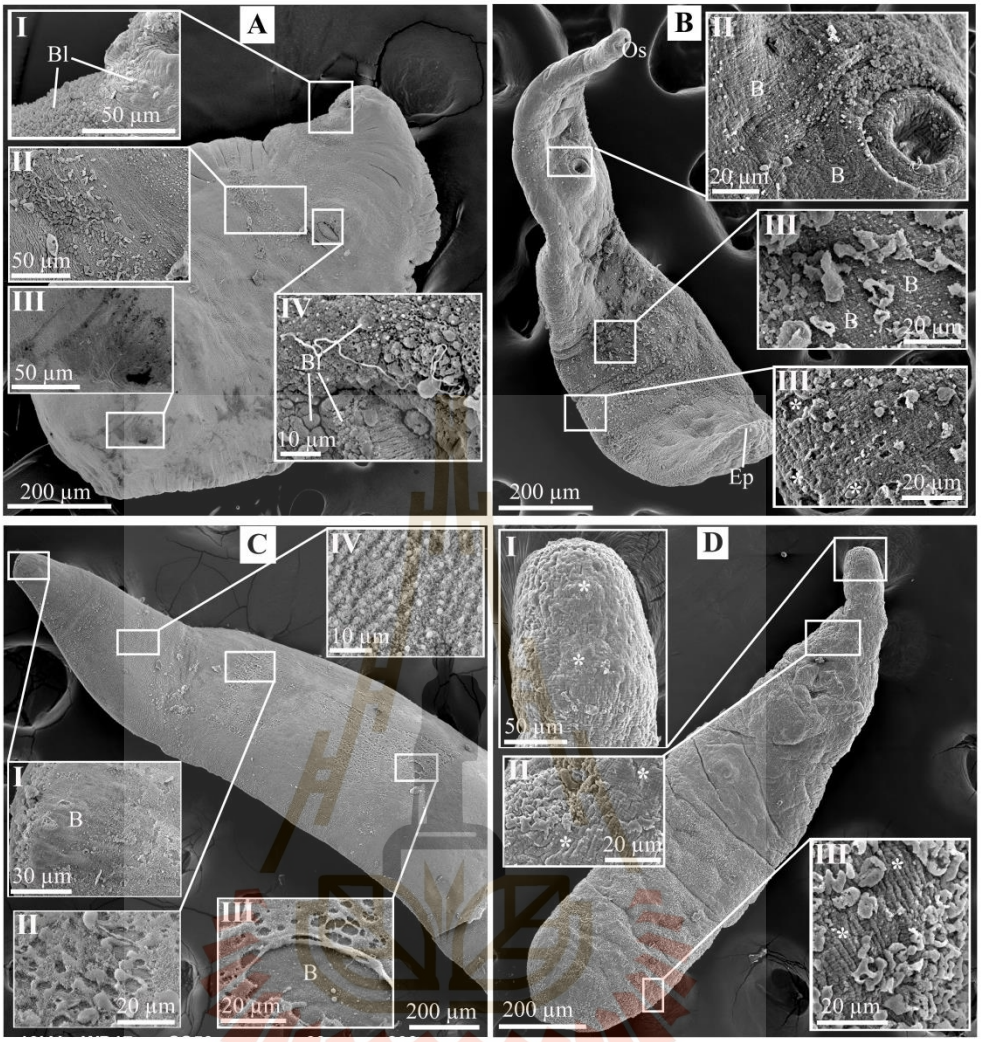


Figure 4.36 Scanning electron micrographs (SEM) of the tegumental surface of 4-week old *Opisthorchis viverrini* 24 h post-treatment *in vitro* with 0.01, 0.1, 1, and 10 µg/ml PB (A, B, C, and D), respectively; oral sucker (Os), ventral sucker (Vs), papillae (Pa), blebbing of tegument (Bl), basal lamina (B), tegumental disruption represent by asterisk.

4.5.3.2.4 cPI-treatment group

Three hours incubation time

3 h incubation of flukes showed morphology closely to the normal flukes. Some area showed distorted spines and a few scaly skins, the tapering effect appeared in some area but most other areas showed no changes in morphology (data not shown).

Six hours incubation time

In the group treated with the lowest concentration of 0.01 µg/ml of cPI, the syncytium appeared disintegrated in anterior mid-body (Figure 4.37A-I). The scaly skin was the only morphological change occurred in the dorsal surface (Figure 4.37A-II). More advance damage occurred in 0.1 µg/ml treatment group, the posterior part has tapering effects (Figure 4.37B). The oral sucker showed the round shape and the papillae around oral cone were in a normal form (Figure 4.37B-I). Microblebbing appeared in ventral surface of anterior mid-body region (Figure 4.37B-II). Blebbing was commonly found in oral cavity and ventral surface, anterior mid-body appeared more blebbing than posterior part (Figure 4.37C-I and II). The highest concentration of 10 µg/ml cPI induced the wrinkling in the lateral part (Figure 4.37D). The tegumental surface below the oral cone appeared basal lamina combined with blebbing (Figure 4.37D-I). The blebbing increasing in number in ventral surface posterior mid-body, burst-blebbing was also found in this area (Figure 4.37D-II).

Twelve hours incubation time

There was no morphology alteration in 0.01 µg/ml cPI treatment at 12 h the post incubation. The ventral and oral suckers were showing in normal shape (Figure 4.38A). The papillae around the ventral and oral suckers were in normal number and

formation (Figure 4.38A-I and II). For other flukes showed blebbing in the ventral surface of posterior mid-body region (data was not shown). At the treatment group at 0.1 µg/ml, the tapering effect was found in the posterior part (Figure 4.38B). Oral sucker was fine tegumental formation and papillae appeared on oral cone was in normal form (Figure 4.38B-I). Around the ventral sucker, micro blebbing appeared in widespread area but the papillae were still normal in morphology (Figure 4.38B-II). The advance damage was revealed in flukes treated with 1 µg/ml of cPI, the aberrant part was shown in lateral area (Figure 4.38C). The numerous blebbing occurred in ventral surface of anterior mid-body (Figure 4.38C-I) and became increasing to the lateral side of the body (Figure 4.38C-II). The number of papillae reduced on oral cone but still maintained in normal form in flukes treated with a concentration of 10 µg/ml cPI (Figure 4.38D-I). The tegumental disruption displayed in the lateral part and tegumental blebbing appeared over the ventral surface (Figure 4.38D-II).

Twenty-four hours incubation time

This incubation time displayed tegumental changes in various patterns. At the group treated with 0.01 µg/ml concentration, the posterior part wrinkling and tegumental blebbing combined with pores were shown in the dorsal surface (Figure 4.39A-I). In progression damage, the tegumental disruption, exposed basal lamina, and common aberrant appearance in the dorsal surface of mid-body and the posterior parts were displayed (Figure 4.39A-II and III). At the concentration of 0.1 µg/ml, the tapering effect was displayed in the anterior part (Figure 4.39B). The oral sucker was completely destroyed and papillae disappeared (Figure 4.39B-I). Interestingly, the eggs and sperms were shaded out from the ventral sucker cavity, micro blebbing still occurred in ventral surface (Figure 4.39B-II). The ventral-lateral regions showed

micro blebbing and tegumental disruption (Figure 4.39B-III). For 1 µg/ml treated group, the flukes showed severe damage especially on dorsal surface (Figure 4.39C). The ventral sucker was completely destroyed and numerous blebbing appeared on the oral surface and oral cone. The oral cavity was absolutely closed (Figure 4.39C-I). Shearing off the tegument appeared on dorsal-lateral surface to exhibit the parenchyma (Figure 4.39C-II). The advance damages on tegument were present in group treated with 10 µg/ml, the whole body was flattened and tapering effect in anterior part appeared (Figure 4.39D). Oral sucker was completely destroyed on tegument and loss of normal shape, disruption of tegument and blebbing appeared overall on oral cone (Figure 4.39D-I). The tegumental changes on a ventral surface showed blebbing and exposed basal lamina. Loss of texture of oral sucker was present on ventral surface with numerous pores (Figure 4.39D-II and III).

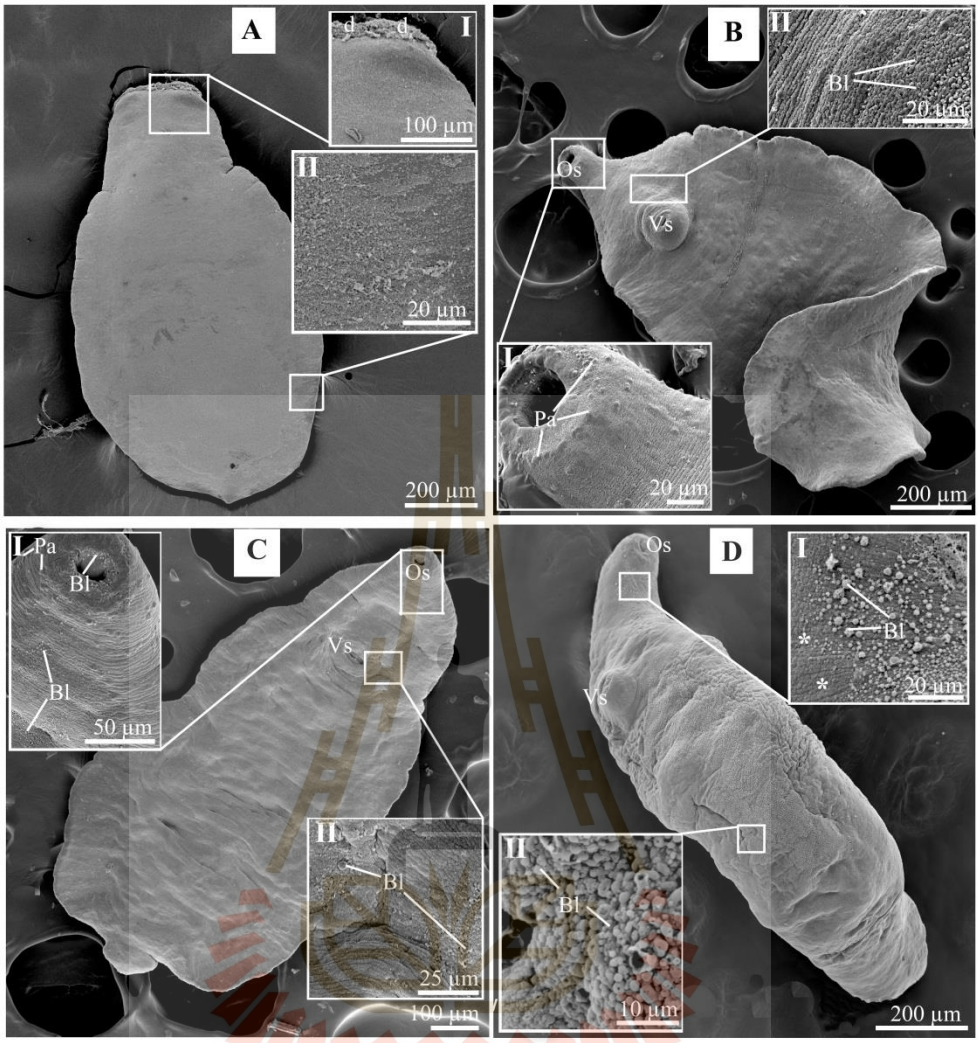


Figure 4.37 Scanning electron micrographs (SEM) of the tegumental surface of 4-week old *Opisthorchis viverrini* 6 h post-treatment *in vitro* with 0.01, 0.1, 1, and 10 µg/ml cPI (A, B, C, and D), respectively; oral sucker (Os), ventral sucker (Vs), papillae (Pa), , blebbing of tegument (Bl), and disintegrate syncytium (d).

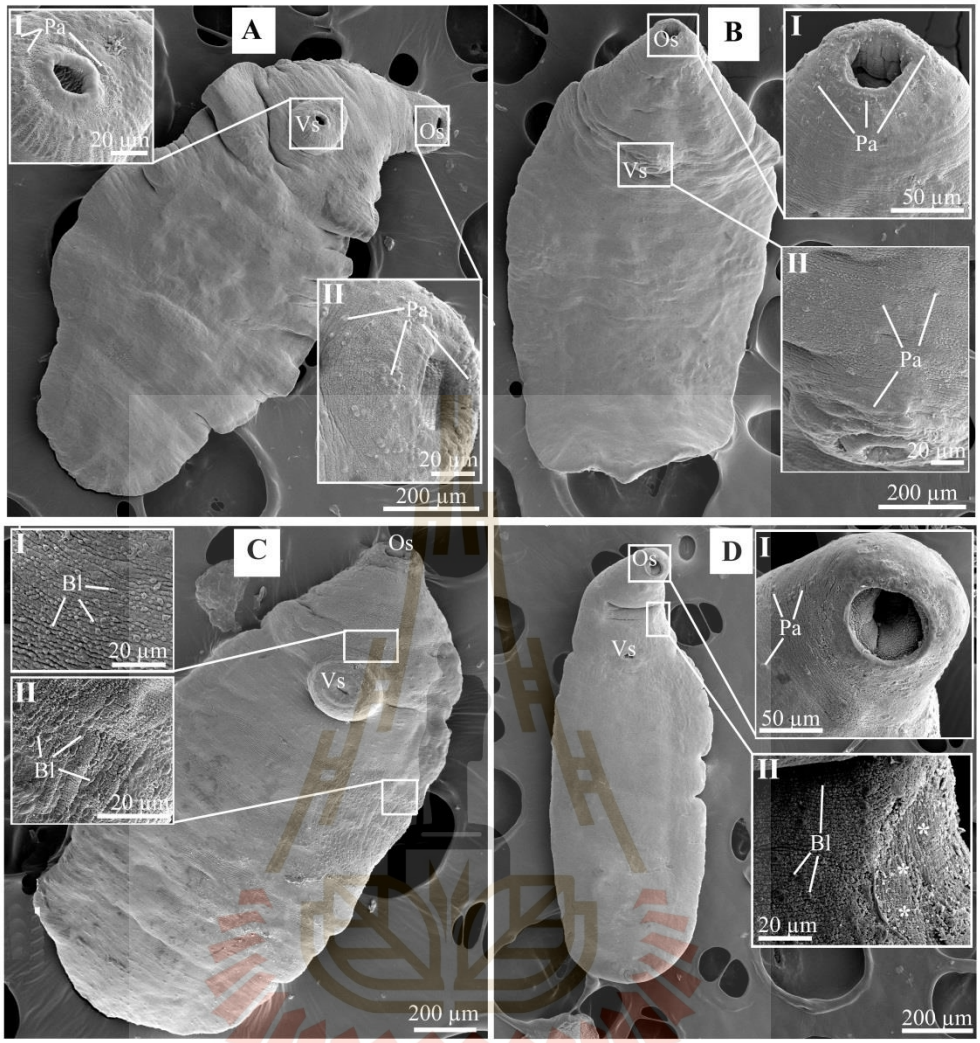


Figure 4.38 Scanning electron micrographs (SEM) of the tegumental surface of 4-week old *Opisthorchis viverrini* 12 h post-treatment *in vitro* with 0.01, 0.1, 1, and 10 µg/ml cPI (A, B, C, and D), respectively; oral sucker (Os), ventral sucker (Vs), papillae (Pa), blebbing of tegument (Bl), basal lamina represent by asterisk.

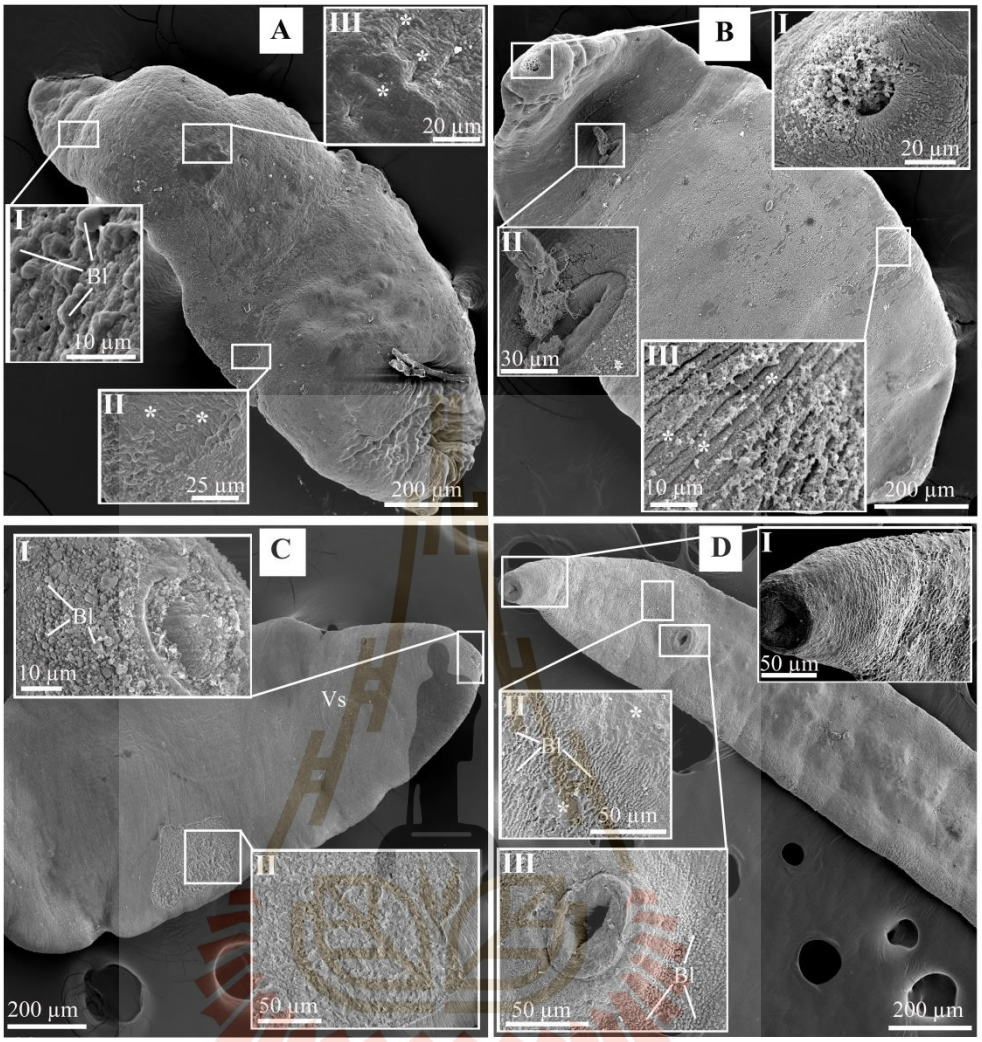


Figure 4.39 Scanning electron micrographs (SEM) of the tegumental surface of 4-week old *Opisthorchis viverrini* 24 h post-treatment *in vitro* with 0.01, 0.1, 1, and 10 µg/ml cPI (A, B, C, and D), respectively; oral sucker (Os), ventral sucker (Vs), papillae (Pa), blebbing of tegument (Bl), basal lamina represent by asterisk.

Table 4.4 Summaries of the tegumental alterations in 4-week-old of *O.viverrini* as observed by scanning electron microscope (SEM) after treatment with various drugs.

Treatment	Doses (µg/ml)	Incubation times (h)	Ventral surface		Dorsal surface	
			Anterior	Posterior	Anterior	Posterior
0.1% DMSO (Control)		24	-	-	-	-
PZQ	0.01	3	-	-	-	-
		6	+	-	+	-
		12	+	+	+	+
		24	+	+	+	+
	0.1	3	-	-	-	-
		6	+	-	+	-
		12	+	+	+	+
		24	0	0	+	+0
	1	3	-	-	-	-
		6	0	+	0	+
		12	+0	+	+	+0
		24	+0	+0	+0	+0
	10	3	-	-	-	-
		6	+0	+	+0	+
		12	+0	+	+	+0
		24	+0	+0	+0	+0
PB	0.01	3	-	-	-	-
		6	+	-	+	-
		12	+	+	+	+
		24	0	+	0	+
	0.1	3	-	-	-	-
		6	-	0	-	0
		12	+	+	+	+
		24	+0	+	+	+
	1	3	-	-	-	-
		6	+0	-	+	-
		12	+	+	+	+0
		24	+0	+0	+	+
	10	3	-	-	-	-
		6	+	+0	+	+
		12	+0	+	+0	+
		24	+0	+0	+0	+0
cPI	0.01	3	-	-	-	-
		6	-	-	-	-
		12	+	+	-	-
		24	-	+	+	+
	0.1	3	-	-	-	-
		6	+	+	-	-
		12	+	+	+	-
		24	*+	*+	*+	*+
	1	3	-	-	-	-
		6	+	+0	+	+
		12	+	+	+	+
		24	0	0	+	+0
	10	3	-	-	-	-
		6	+	+0	+	+0
		12	+	+0	+	+0
		24	+0	+0	+	+0

- Normal tegument

Spine deformation

* Papillae deformation

+ Tegumental blebbing and/or tegumental swelling

0 Tegumental disruption and/or tegumental destroyed

4.6 Ultrastructure of the tegument of 4-week old *Opisthorchis viverrini* from transmission electron microscopic technique

4.6.1 Vehicle control group

The ultrastructure of the tegument and associated structure of 4-week-old fluke in the vehicle control group incubated in Medium-RPMI-1640 containing 0.1% DMSO showed normal morphology (Figure 4.40) at all incubation times. After the end of the experiment (24 h), the tegumental syncytium showed numerous ridges, pits with spines and microvilli on the apical surface and was coated with a thin layer of glycocalyx. The tegumental syncytium was divided into 2 parts; the outer and inner plasma membranes by using contents and location. The apical zone of the outer plasma membrane exhibited the numerous mitochondria and tegumental granules. The tegumental cells were underneath the basal lamina, had numerous cytoplasmic processes that extended upward to the inner plasma membrane and fused with tegumental syncytium. We also found the invagination between the inner plasma membrane folded in a long parallel-structure of the basal cytoplasm, which was called basal infolds. Spines were anchored within basal lamina. The basal lamina was supported by the outer circular and inner longitudinal muscular layers (Figure 4.40).

The parenchymal cells located below the muscular bundles. The granules in the cytoplasmic syncytium of the tegument were classified into 2 types based on the criteria of shape and density of their contents as described by Apinhasmit and colleagues (2000). They were dense and light granules. The content of the dense granules showed highly electron-dense and located on the outer plasma membrane, whereas that of the light granules showed filamentous vesicle and located on the lower half of inner plasma membrane. In addition, the dense and light granules were divided into 2 subtypes by content; dense spherical, dense discoid, light spherical and light discoid granules, respectively.

4.6.2 PZQ, PB, and cPI treatment groups

In these previous results of SEM and motility observation, we found the tegumental changes at 12h after flukes treated with PZQ at 10 μg/ml, therefore we designed to evaluate and compare the pathological changes of flukes treated groups by TEM. The ultrastructure changes occurred in fluke-treated groups after 12 h incubation (Figure 4.41). In the PZQ-treated group at 10 μg/ml, the apical surface of tegumental syncytium was uncovered with glycocalyx, and electron-dense was presented at the top of ridges in the outer plasma membrane (Figure 4.41A and B). There was also the disruption of the outer plasma membrane. There were few numbers of dark and light granules and swollen mitochondria in the syncytium compared to the control flukes. Microtrabecular network depolymerization and organelles damaged appeared throughout the tegument layer. The dense granules migrated upward to the apical surface while most of the light granules showed depolymerization and necrotic disruption. The apical layer of the syncytium exhibited prominently swollen tegument (Figure 4.41C). The many various sizes of membrane-

bound vacuoles were also present in the cytoplasmic syncytium and circular muscular layer (Figure 4.41A and C). In the subtegmental layer, mitochondria were severely swollen, the musculatures were broken and showed depolymerization of myofilament.

In the PB-treated group at 10 μg/ml, the pathological changes of tegument showed many blebbing formations on the apical surface without the glycocalyx coating. The accumulation of light granules displayed in the outer plasma membrane while a few numbers of dense granules showed in the tegumental ridge (Figure 4.41A and B). Some areas of the cephalic part of the fluke showed a huge disruption of blebs with the necrotic tegumental component (Figure 4.41C). No pathological changes were shown in the muscular layers.

In the cPI-treated group at 100 μg/ml, the damages of tegument at cephalic head showed various sizes of bleb formations and disrupted blebs in the tegumental syncytium and also numerous granules accumulation was found in the tegumental syncytium of the cephalic and body regions of the fluke (Figure 4.41G-I). In some area of tegumental syncytium was uncoated with the glycocalyx, so the outer plasma membrane did not see the trilaminar layer. Both light and dark granules migrated upward to the top of the tegumental surface but most light granules were degraded. The electron-dense was also present on the ridges. In the subtegmental region, the circular muscle bundle showed necrotic with large membrane-bound vacuoles. The location of the blebbing formation in the cPI-treated group showed a similar pattern to the PB-treated group whereas the vacuolization between the circular muscle bundles presented similar pattern to PZQ.

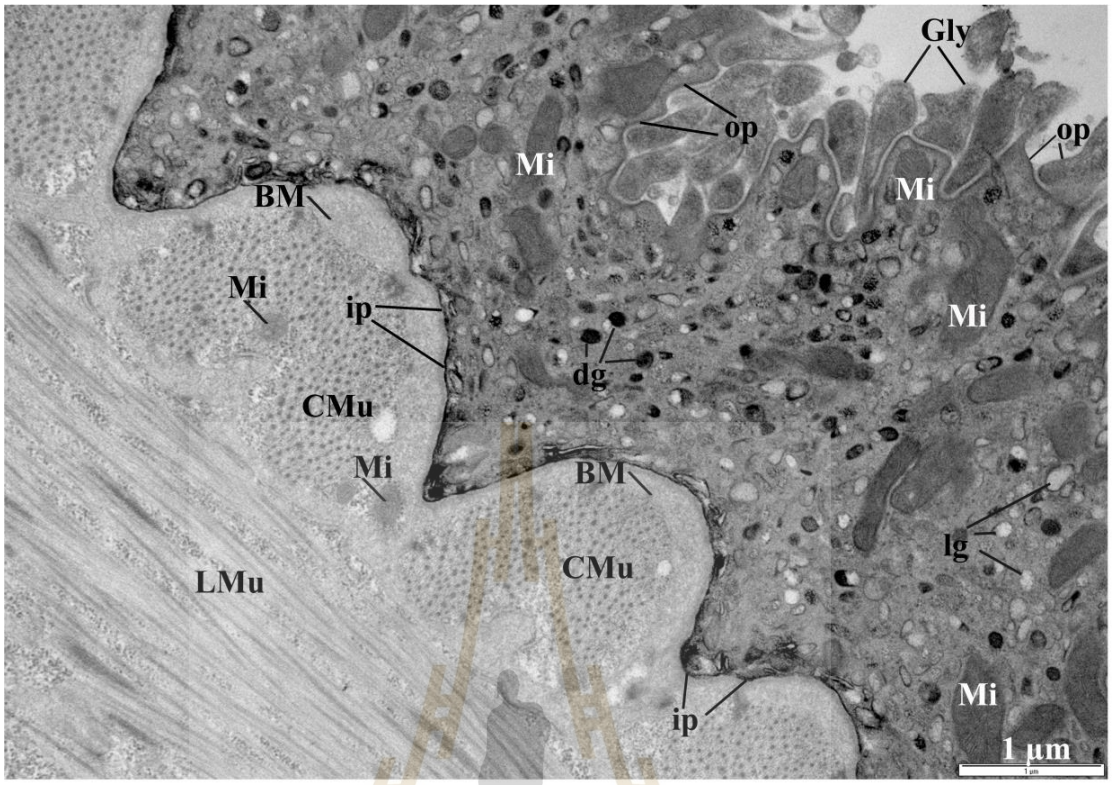


Figure 4.40 TEM micrograph of the tegument of the 4-week old flukes. The tegument was covered by the outer (op) and inner (ip) plasma membranes. The outer plasma membrane was covered by glycocalyx (Gly). The tegument contained 2 types of granules including dense (dg) and light (lg) granules. The tegument based on the basement membrane (BM) and dispersed from the circular (CMu) and longitudinal (LMu) muscles. Mitochondria (Mi) was commonly found in the tegument and muscular layer.

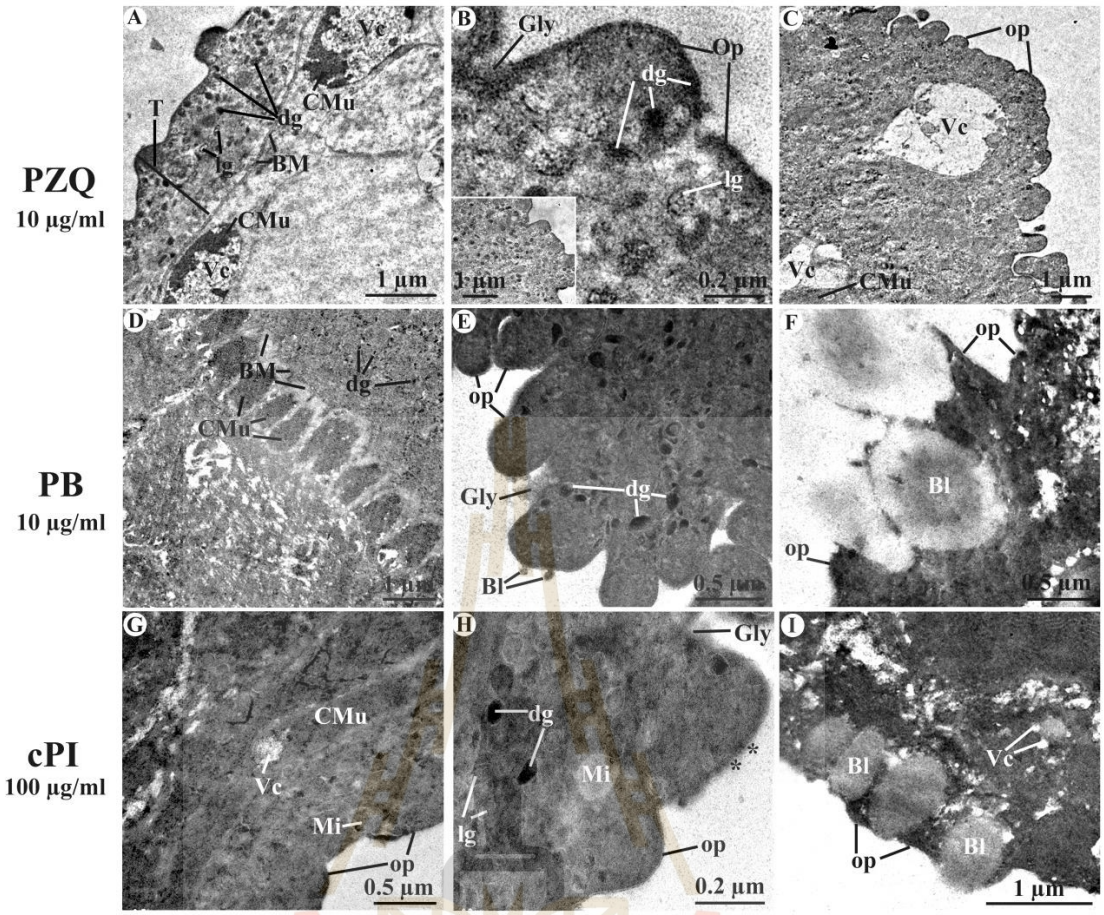


Figure 4.41 TEM micrographs of the tegument of the 4-week old flukes treated with PZQ (A-C), PB (D-F), and cPI (G-I). (A-C) Tegument of flukes treated with PZQ showing the electron-dense at the ridge without glycocalyx (Gly), and dense (dg) and light granule (lg) deformations. In the cytoplasmic syncytium and circular muscle layer (CMu) showed the large vacuoles (Vc). (D-I) Tegument showed glycocalyx disappearance with the blebbing (Bl) formation in PB and cPI treatment groups, vacuoles were present in between muscle bundles. Trilamonar layer was deformation on the outer plasma membrane (*). Mi=Mitochondria, BM=Basement membrane, op=outer plasma membrane.

CHAPTER V

DISCUSSION

5.1 To investigate the *in vitro* anthelmintic effects of plumbagin, crude extract of *Plumbago indica* root on the motility and survival of newly excysted and 4-week-old juveniles of *O. viverrini*, and the stage-related sensitivities to the drug

The potential of natural products for anthelmintic drugs was evaluated *in vitro* using nematode, trematode and cestodes models. Plumbagin, derived from Plumbagineae and Droseraceae families, was used in treating tuberculosis, dyspepsia, skin infection, antibacterial, and antifungal activities (Krishnaswamy and Purushothaman, 1980; Srinivas et al., 2004; Dzoyem et al., 2007; Sharma et al.,2009; Padhye et al., 2010). In previous studies, the pure plumbagin extract from *Plumbago indica* root was studied for the anthelmintic activities against *Parasphistomum epiclitum, Caenorhabditis elegans, Fasciola gigantica, Schistosoma mansoni,* and *P. cervi* (Atijanasuppat et al., 2009; Lorsuwannarat et al., 2013 and 2014; Saowakon et al., 2013). Therefore, we hypothesize that PB may also possess an anthelmintic effect against some species of trematodes such as *O. viverrini*. We investigated whether PB and crude extract from *P. indica* root (cPI) possess any anti-helminthic activity against different infected stages of *O. viverrini*. We compared the effect of PB and cPI on immature and 4-week-old of *O.viverrini* which is the infective stage leading to the development of the pathology in the infected animals.

To evaluate the efficacy of the PB and cPI as anthelmintic drugs in comparison with PZQ, an available commercial anthelminthic drug. The sensitivity of NEJs and 4-week-old juvenile flukes to the drugs was assessed by using motility and survival scores in exposed flukes. A smaller RM value indicates susceptible to anthelmintic drugs. This is the first time, that PB was reported to kill NEJs in 100% within 24 h and 1 µg/ml possesses similar as 1 µg/ml PZQ while the concentration of cPI has to be 10 µg/ml. In the 4-week-old flukes, the complete immobility (100% of motility inhibition) of flukes was found when incubated with 1 µg/ml PZQ but flukes were not completely dead within 24 h as PB only killed flukes up to 70%. In contrast, cPI was ineffective in worm killing (30%) at the same incubation time. At 10 µg/ml, PZQ and PB could kill flukes up to 100% and 96% of 4-week-old while cPI showed only 74% of worm killing. Similarly, the previous studies reported that 100% of parasite dead was obtained in PZQ treatment against adult *O. viverrini* at 10 µg/ml (Apinhasmit and Sobhon, 1996) and the killing concentration of PB on both sex of *S. mansoni* had to be more than 100 µg/ml (Lorsuwannarat et al., 2013). NEJs and 4-week-old of *F. gigantica* were dead after treated with PB at 10 and 100 µg/ml, respectively (Lorsuwannarat et al., 2014). Differences in effective dose of PB might relate to differences in the size of target parasites. *Fasciola gigantica* is bigger in size than *O.viverrini*, therefore, the effective dose should be higher than targeting *O. viverrini*. Interesting, the concentrations of PB that used to kill the two stages of flukes were similar to PZQ at the same incubation time, and the killing percentage was not so different. It implied that PB was almost as potent as PZQ in killing on NEJs and 4-week-old *O. viverrini*. Plumbagin is a derivative of naphthoquinone compound, whose structure is the 5-hydroxy-2-methyl-1,4-naphthoquinone. The

quinone compound can induce oxidative stress by generating oxygen free radicals that are toxic to parasites. Oxidative stress can cause damages to membranes, proteins and also DNA. Nakornchai (1993) reported that PB increased the rate of superoxide anion production and enhanced lipid peroxidation in *Plasmodium falciparum* culture. We suggested that the therapeutic dose for killing worms in the different stages should be 10 µg/ml. We also focused on anthelmintic effect of the cPI in this project. At present, there has been no report on the exact mode of action of cPI. The previous study reported that the compounds in the root of *P. indica* are flavonoids, benzenoid, quinoid, and carbohydrate. The major compound in *P. indica* root is the naphthoquinone which is consists of plumbagin, 3-chloroplumbagin, 6-hydroxyplumbagin, plumbaginol (Padhye et al., 2010). Therefore, the effect of cPI might be lower than PB because there are other components in a root. Furthermore, our work evaluated the different sensitivities of *O. viverrini* NEJs and 4-week-old flukes to PB and cPI, the NEJs stage is more susceptible to PZQ, PB, and cPI than the 4-week-old juveniles. These results are similar to previous studies of TZQ treatment on different stages of *F. gigantica* and PZQ treatment on 1-week-old and adult stages of *O. viverrini* (Bennett and Kohler, 1987; Apinhasmit and Sobhon, 1996; Duthaler et al., 2010; Lorsuwannarat et al., 2014). The studies also indicate that juveniles were more sensitive to commercial drugs than adults. Differences in susceptibility between the stages could be a reflection of their structural difference. The tegumental surface of NEJs never exposed to antigens, therefore, tegumental cells are never facilitated to produce the secretory granules and release on the tegumental surface. In contrast, the elder juveniles are exposed to antigens and protect themselves by increasing production of secretory granules. Then, the granules are released and covered on the

tegumental surface that is called glycocalyx, therefore, this is a reason for found a turnover rate of flukes in elder fluke *F. hepatica* (Bennett and Threadgold, 1973; Bennett and Kohler, 1987). The lower turnover rate and lower capability in the repair of its surface membrane, explain the less resistance of NEJs than elder juvenile and adult stages in response to chemical agents such as anthelminthic drugs and PB.

5.2 The effect of anthelmintic drugs on the tegument and associated structures of the *O. viverrini* by using electron microscopic techniques

Scanning electron microscopy (SEM) was used for evaluation of surface changes in flukes resulting from drug exposure by localization of damaged area and severity of drug testing. The study by transmission electron microscopy (TEM) is more focused on internal changes resulting from drug treatment (Stitt and Fairweather, 1994; Meaney et al., 2003 and 2004). When observed by SEM, PZQ caused progressive severe rupture of the tegumental surface of *O. viverrini* with longer exposure periods *in vitro*. PZQ has a rapid and severe effect on juvenile *O.viverrini* more than the 4-week-old stage. The sequences of pathological changes observed in the tegument were initially composed of the swelling of the tegumental folds and ridges whose initial appearance looks like spines were sunk into the tegument especially on the ventral surface of head region. Subsequently,there was the multi-foci of bleb formations at the surface which was later ruptured resulting in erosion formation in some area of tegumental surface, then more lesions and more distribute sloughing off the tegument and spines which were barely visible in some area following 24 h incubation. Other PB and cPI showed the damage in the same

pattern but differ in severity damages. The tegument is slough off and showed the associated tegument structure such as muscular layers when fluke were incubated with 1 and 10 µg/ml of PB and cPI since early 12 h whereas the tegumental surface of flukes treated with cPI present multi-foci bleb and severe erosion with lesion in the whole body and finally free tegument surface at the end of experiment. The severity of pathological damages became progressively more severe with longer period of exposure was also concentration-dependent time. The alterations of tegument observed in this study are similar to several previous studies such as the studies of adult *F. gigantica* exposed with artesunate (Tansatit et al., 2012), adult *F. gigantica* treated with crude extracts of *Artocarpus lakoocha* (Saowakon et al., 2009) and *T. catappa* (Anuracpreeda et al., 2017), adult *Paramphistomum cervi* incubated with plumbagin (Saowakon et al., 2013), adult *Fischoederius cobboldi* exposed with ethanol extract of *T. catapa* (Anuracpreeda et al., 2016) and several Thai medical plants on *Carmyerius spatiosus* (Minsakorn et al., 2019).

Moreover, feeding occurred in drug incubation and movement. Therefore, parasites may be ingested in the digestive tract and in the bladder. Gut contents were moved in and out of the midline of fluke quite readily, while the periphery parts were incompletely flush off. Therefore, we suggested that both lateral margin damages by drugs were due to an accumulation of drug in the peripheral region of the gut. On the other hand, the cephalic cone region of flukes always showed the tegumental erosion at the oral sucker, implying that they intake this drug by oral route, and the swelling of tegument is possibly due to the disruption of the ion pumps known to be present on the apical plasma membrane (Bricker et al., 1983; Skuce et al., 1987; Jiraungkoorskul et al., 2006). It was reported that the uncoupler of oxidative phosphorylation induces

the loss of ATP (McKinstry et al., 2003 and 2007). Decreased production of ATP caused by ion pump activity, leading to the influx of water and consequent swelling of the syncytium. In addition, damage to the CNS of fluke located on the cephalic region may affect the worm motility. As a result, flukes became less active and eventually became immobile after prolonged incubation.

The changes observed in the tegumental syncytium in our TEM study corresponded with those described at the SEM study. We chose to observe the ultrastructural changes with SEM and relative motility values. In this study, we found that the bleb formation in the tegumental syncytium and associated tegumental structure deformation such as mitochondria and secretory granule degradation without the glycocalyx coating. The tegument is one of the primary targets of several drugs because of its direct exposure to the chemical and antigens. Tegumental syncytium disruption and deformation implied that drugs might penetrate deeper tissues, causing severe damages to the fluke. This damage would interfere with the physiological process associated with the tegument: osmoregulation, nutrient uptake, secretion and immunoprotection (Halton, 2004). Normally, the trematode tegumental syncytium transport chemical and water by using ion gradient and diffusion (Threadgold and Brennan, 1978). The movement, release, and production of tegumental secretory bodies have been shown to be energy-dependent processes (Hana, 1980). Since the integrity of the apical plasma membrane is dependent on the continual turnover of secretory granules, any increased production in their numbers would be an important factor in drug action. In addition, uncouplers are known to cause the release of Ca^{+} ions from mitochondria, the effect was explained in terms of Ca^{+} movements within the mitochondria of the worm and finally mitochondria necrosis occurs.

The basal plasma membrane is invaginated to form long,parallel-structures known as basal infolds. The mitochondria and secretory granules located in those membrane-infolds at the apical plasma membrane. The Na^+/K^+ATPase localized in the apical plasma membrane and the basal infoldings, which is indicative of the presence of ion-pumps on the tegument membrane (Threadgold and Brennan, 1978). It seems likely that the initial swelling of the tegument is due to an osmotic effect of the drugs brought about by a change in the permeability of the apical plasma membrane. Thus, the large influx of Na^+ ions might overcome the capacity of the Na+/K+-ATPase-driven ion pump situated along the apical plasma membrane (Threadgold and Brennan, 1978), and the Na^+ ions could penetrate deeper into the syncytium and being pumped into the basal infolds. The lumen of the infolds would become hypertonic with respect to the surrounding cytoplasm, and water would be drawn into the infolds from the cytoplasm, forcing them to swell and eventually detached from the basal lamina as seen in TEM after prolonged incubation with the drugs.

In addition, the associated subtegumental structures that are the muscular layers also showed autophagic vacuoles between muscle bundles and swollen mitochondria. Those are indicated that tegument is destroyed and allowed the chemical influx inside of the parasite although osmoregulation inside of parasite tries to fix its problem but not enough so the lesion presented in subtegumental area. There are similar results as the previous studies in crude extract of *Artocarpus lakoocha* on *F.gigantica* (Saowakon et al., 2009) and drug response *in vitro* studies.

Although, the exact mode of action of PB is unknown, the helminths living in a host by using anaerobic oxygen for cellular respiration by using NADH-fumarate

reductase and using rhodoquinone instead of ubiquinone (CoQ). Glycolysis is the main pathway, and oxidation phosphorylation is an important process for generating ATP. Generally, the adult fluke always used anaerobic glycolytic pathway, phosphoenolpyruvate carboxykinase (PEPCK)-succinate pathway (Figure 5.1), and ATP can be generated under the low oxygen conditions. NADPH-fumarate reductase is a terminal step of the phosphoenolpyruvate carboxykinase-succinate pathway, which is found in many anaerobic organisms. The composition and linear sequential order of the respiratory components of NADPH-fumarate reductase have been elucidated with mitochondria from the parasite nematode, *Ascaris suum* (Omura et al., 2001; Kita et al., 2003). Electrons from NADPH are accepted by rhodoquinone through complex I and then transferred to fumarate through complex II. The anaerobic electron transport couples site I phosphorylation in complex I by translocating protons across the inner mitochondrial membrane, providing ATP even in the absence of oxygen. The NADH-dependent anaerobic respiration is not present in the host mammals, it is considered to be a promising target for anthelmintic drugs.

Although the anthelmintic activity of PB has been still unknown, its chemical structure can react in the electron receptor in the electron transport chain (Kita et al., 2003; Srinivas et al., 2004). Therefore, it implies that the PB opisthorchiasicidal effect is linked to is able to compete with CoQ in electron transport chain of fluke. Almost TEM lesion showed at mitochondria damages that supported that PB and cPI would interrupt the electron transport chain in the *O. viverrini*.

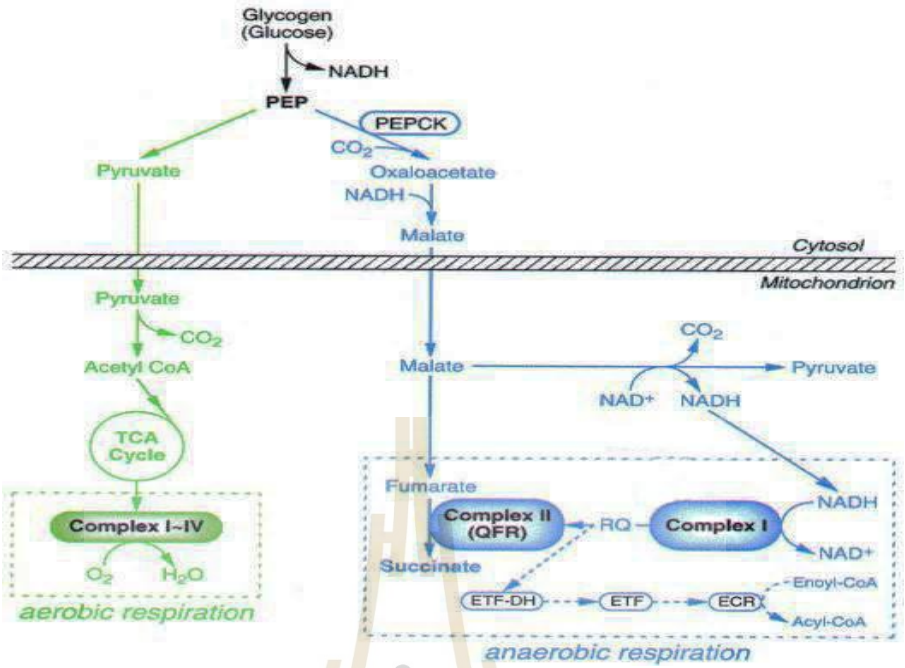


Figure 5.1 The schematic diagram showing the NADH-fumarate reductase system in anaerobic respiration of adult A. suum mitochondria as possible inhibition target site of PB. In the phosphoenolpyruvate carboxykinase (PEPCK) - succinate pathway, phosphoenolpyruvate (PEP) produced by a glycolytic process is carboxylated to form oxaloacetate and is then reduced to malate. The cytosolic malate is transported into the mitochondria, where it is first reduced to fumarate, and finally to succinate by the rhodoquinol-fumarate reductase activity of complex II. The terminal step is catalyzed by the NADH-fumarate reductase system comprising complex I, rhodoquinone (RQ), and complex II. Aerobic and anaerobic respiratory chains are boxed in dash lines (Modified from Omura et al., 2001).

5.3 To evaluate the protective effect against *O. viverrini* in comparison to crude extract from *P. indica* root, PB and PZQ in the hamster model

After the *in vitro* study, we wondered whether the *in vitro* studies are related to *in vivo* study. Hence, we proved the hypothesis that all of those drugs should be cure the parasitic infection by evaluating the number of worm recovery after oral administration. The results showed that the percent of worm recovery in PZQ treated group was 72% while PB was 68% and cPI was 45% at 10 mg/kg body weight. We compared with the previous study of fluke burden in *F.gigantica* showed only 63% protection at the same concentration (in Lorsuwannarat thesis, unpublished). The effect of PB showed insignificant difference lower than PZQ but its effect is better than the previous studies that used 100 mg/kg *Garcinia mangostana* pericarp (mangosteen hulls) which showed 36% of *O. viverrini* worm recovery (Aukkanimart et al., 2015), 400 mg/kg tribendimidine with 77.2% worm protection in *O. felineus* (Pakharukova et al., 2019). At 10 mg/kg body weight of cPI treatment had no effect on infected hamsters. Therefore, we evaluated the effect of drug by increasing concentration in 10 folds, 100 mg/kg body weight, and the results showed that the best effective drug is PZQ with 91% of protection, and the second is PB, 72% and the least effective one is cPI, 70% with the up more dose 100 mg/kg body weight. In contrast, the average of worm recovery in the untreated group is 28±8.69. Surprising, the effect of cPI on worm recovery is better and was almost equal to PB. It seems to be that the volume of plumbagin in the crude extract is enough to against parasitic infection. On another hand, there is only a little information about biotransformation,

metabolism, and absorption of plumbagin in humans has been reported. The previous study reported the plumbagin was detected in the plasma samples of Wistar rats collected within 1-48 h after the oral administration but was not detected in the samples collected after oral administration 72 h. Plumbagin was found in the urine samples collected from day 1 to day 4 after dosing in Wistar rats but was not detected on day 5. PB was found in the urine 4 h post-oral administration. The pharmacokinetic profile of plumbagin following a single oral dose of 100 mg/kg body weight suggests the delayed absorption and short residence time (median values of time to maximal concentration and elimination half-life in plasma = 9.63 and 5.0 h, respectively) (Sumsakul et al., 2016). The pharmacokinetic profile of crude extract of *P. indica* has not been reported yet. However, We hypothesized that its mechanism should be mimic with PB. We suggested that cPI is another choice for treatment of *O. viverrini* infection by realizing the possible side effect and the period of treatment time. Furthermore, cPI should be further studied in the clinical trial in the human with cholangiocarcinoma and/or positive *O.viverrini*.

5.4 To study the toxicity of crude extract of *P. indica* root on a hamster model

Knowledge of traditional medicinal plant toxic side effects is limited and thus their applications are potentially dangerous. In order to examine the safety of *P. indica* root extract, we performed the toxicological and histological assessments of cPI in Golden Syrian Hamsters. Previous studies reported that *P. indica* root contains flavonoids, benzenoid, quinoid, and carbohydrates. The ethanolic extract of *P. Indicia*

root has been used to alleviate gastrointestinal problems, including diarrhea, dyspepsia, hemorrhoids, and anti-flatulence (Krishnaswamy and Purushothaman, 1980; Kini et al., 1997). Naphthoquinones compounds found in *P. indica* root comprises of plumbagin, 3-chloroplumbagin, 6-hydroxyplumbagin, and plumbaginol. Plumbagin possesses wide a range of biomedical activities such as antioxidant (Padhye et al., 2010), antimicrobial (Paiva et al., 2003), anti-inflammatory (Anuthakoengkun and Itharat, 2014; Makchuchit et al., 2017), antifungal, antineoplastic actions, and reducing the liver enzyme function (Krishnaswamy and Purushothaman, 1980; Paiva et al., 2003). Although the pharmacokinetic information on the metabolism, distribution, and excretion of plumbagin have not been available yet, there is some information on its oral bioavailability the rat model. After 24 h oral administration, plumbagin reaches the maximum concentration in plasma within 1-2.5 h. The half-life of plumbagin is approximately 1.3-15 h, and the main portion of the drug metabolites is excreted in urine (51%) and in feces (49%) (Padhye et al., 2010). Solomon et al. (1993) reported that the crude extract from *P. indica* root dissolved in 30% PEG 400 intraperitoneally injected into mice showed LD_{50} at 239.88 mg/kg while oral administration to rats showed LD_{50} at 1148.15 mg/kg. They also reported the mortality of mice after oral administration of crude root extract of *P. indica* at the concentration of 1,000 mg/kg was 20%. In the acute toxicity test of our study, hamsters given oral administration of cPI at 100, 400, 1,000 and 3,000 mg/kg were still alive at the end of the experiments and no behavior changes were observed from 24 h and 7 days. Regarding to OECD 423 guidelines, if all animals survived after their scheduled termination, the extract is deemed to have low toxicity and should be classified in category 5, with a LD_{50} estimated between 2,000-5,000 mg/kg. In the

subacute toxicity test, animals receiving the oral administration of cPI at similar doses of acute stage (100, 400, 1,000 and 3,000 mg/kg), did not reveal alterations in behavior, body weight, and mortality. However, the internal organ weights were significantly increased than the vehicle control group. The internal organ weights showed progressive increases, but no histopathological changes were observed in all cPI treated groups, except in 3,000 mg/kg of cPI-treated group that showed significant increases in weights of liver, kidney, heart, and lung. Examinations for histopathological changes also confirmed the results from organ weights. The liver and kidney showed severe damage at 24 h (acute stage) and recovery in the subacute stage.

A main function of the liver is biotransformation and detoxification of exogenous chemical compounds; therefore, it is one of the major organs that are adversely affected by toxic compounds. Hepatic necrosis and angiectasis in the hepatic lobules in the cPI treatment may have been the cause of the liver weight increase. Moreover, the hepatic sinusoid may lose the vascular permeability, therefore white blood cells and red blood cells are able to migrate through the interstitial space, leading to red blood cell congestion, white blood cell infiltration, extravasation of blood, necrosis, and fibrosis in the organ (Silva-Cunha et al., 2009). When the liver is overloaded, chemical toxin accumulates in the bloodstream that is able to induce acute renal injury. Thus, the glomeruli and proximal tubular swelling and angiectasis in glomerulus were found in the renal cortex leading to damage of renal tissue as indicated by “acute glomerulonephritis” (Silva-Cunha et al., 2009, Tain et al., 2015).

Normally, the hepato-cellular toxicity is evaluated by measuring levels of enzymatic markers (AST and ALT levels) for identifying liver damage (Calil

Brondani et al., 2017). There were no significant differences among treatment groups and the control in the acute toxicity test. Therefore, hepatoxicity likely did not occur in cPI treatments. Moreover, the levels of ALT and AST in the groups treated with cPI at 1,000 and 3,000 mg/kg showed a reduction in comparison to the control group. Perhaps the animals recovered as the histopathological observation in the subacute stage showed no negative effects. The liver function remained within the physiological limits which imply no toxicity at these doses. Other studies reported similar findings regarding the hepatoprotective effect of plants (Calil Brondani et al., 2017; Lima et al., 2017). Urea and creatinine are markers for kidney damages. There was no evidence of cPI inducing nephrotoxicity since the values of BUN appeared within normal ranges. Thus, cPI did not cause kidney dysfunction because the value was still within the physiological limits (Lima et al., 2017). From these findings, we suggest that scientists should be aware of the doses per safety and side effect of *P. indica* when using this plant in an infected animal model in a future study.

CHAPTER VI

CONCLUSION

6.1 The anthelmintic effect of plumbagin and crude extract of *Plumbago indica* root on the motility and survival of newly excysted and 4-week old juveniles of *O. viverrini*, and the stage-related sensitivities to the drug

The effect of PB and cPI on the NEJs and 4-week old stages of *O. viverrini* were observed by incubating the flukes *in vitro* in RPMI-1640 medium containing drug at concentrations of 0.01, 0.1, 1, and 10 µg/ml for 3, 6, 12 and 24 h. The damage was evaluated by observation of the mobility of the whole organism as well as of differential dye uptake by nonmotile or damaged parasites. In this study, the effect of PB and cPI was evaluated in terms of the relative mobility value (RM) and survival index (SI) value. The results showed that all NEJs and 4-week-old flukes were immobile (RM=0) and died (SI=0) when incubated in 1 and 10 µg/ml of PB at 12 and 24 h. In contrast, NEJs treated with cPI at 1 µg/ml stopped moving but they were nearly dead (RM= and SI=17) when increasing dosage became to 10 µg/ml, cPI could kill to 93% of NEJs. For 4-week-old flukes treated with cPI, only 10 µg/ml could inhibit the movement and induced 74% dead worm.

When comparing stage-specific sensitivities to the drugs, NEJS were more sensitive to PZQ, PB and cPI treatment than 4-week-old flukes as shown in smaller RM and SI values at the same concentrations.

6.2 Histopathological Changes of the 4-week-old *O. viverrini* after treatment with PZQ, PB, and cPI by using light (LM), scanning electron (SEM) and transmission electron microscopy (TEM)

6.2.1 Light microscope observations

Under a stereomicroscope, we saw the brown color on the tegument of flukes that were incubated in medium containing cPI or PB (unstained with vital dyes) while flukes treated in PZQ did not change their tegumental color. The deeper brown color on the tegument was present with increased concentrations. All drugs in this experiment caused extensive bleb formation, bleb disruption and pieces of tegument slough off in the medium. PZQ caused spasm and spinal contraction of body of flukes.

When the semithin sections of flukes were investigated by bright field light microscope, 4-week-old of *O.viverrini*, PZQ, and PB caused blebbing, tegumental disruption and without spines in some area of the tegument. The tegument in PB groups showed damage in the subtegumental area as well.

6.2.2 SEM observations

In the NEJs stage, the tegumental changes were clearly observed at 6 h incubation until 24 h, damages were mostly seen in both sides of the head region and anterior half of the whole worm. In contrast in 4-week-old *O. viverrini*, there were distributed the damage in the large area of worm especially on ventral half of fluke. In

the present study, regional differences in response to drug action were observed; the ventral surface tend to be more severely disrupted than the dorsal surface, and the head region of fluke was more disrupted than the middle and tail parts. The pattern and sequence of changes were similar in PZQ, PB and cPI treatments, each change displays earlier and more severe in the PB and cPI than PZQ treated flukes. The severity of tegumental damages increased for each drug when incubated with higher concentration and longer incubation times.

6.2.3 TEM observations

The ultrastructural changes were observed in the tegumental syncytium and neuromuscular compartment. The open bodies and both secretory granules flew up to the apical plasma membrane, and swollen mitochondria were found in the syncytium. The vacuolation phenomenon was found between muscle bundles in the early 12 h observation and increased severity involvement in the higher dose when investigated at the same time. Tegumental cells showed swollen mitochondria and granular endoplasmic reticulum, and an autophagic necrotic sign was also observed in the cytoplasm of the neuron.

6.3 The *in vivo* anthelmintic effects of crude extract of *Plumbago indica* against *O.viverrini* and its possible adverse effect in hamsters

The therapeutic effect of cPI in hamsters and adverse effect on host tissues were evaluated by estimating the percent of worm reduction (recovery) or percent of protection, liver enzyme levels, and histopathological changes.

6.3.1 Worm recovery

The number of flukes from the liver after all treatment groups were counted and calculated the percentage of worm recovery. The numbers of flukes in PZQ- and PB-treated groups were fewer than cPI and the untreated group as control at the concentration 10 mg/kg body. However, numbers of fluke recovery in cPI showed significantly lower than untreated group. In the higher concentration (100 mg/kg body weight), the percent protection showed higher than low dose, and cPI exhibited more effective and be equal to PB as shown by higher percent of worm protection.

6.3.2 Liver enzymatic levels

Liver enzymatic levels were evaluated at 24 h and 7 days following oral administration. The AST level expressed at a higher level at 24 h and lower level than untreated group, but they were still in the normal range. The ALT level showed lower level in the acute and subacute periods of time. For body clearance, creatinine and BUN were indicated the kidney function. All of both parameters showed similar results to the control group.

6.3.3 The adverse effects on liver and kidney weight and morphological changes in liver and kidney

There are adverse effects on liver and kidney weight and morphological changes in the liver and kidney in early 24 h and morphological changes in both liver and kidney have better improvement in subacute stage. However, the morphological structure of other organs including uterus, ovary, testis, and lung showed normal appearance as the untreated group. We did not focus on the estrous cycle in female

hamster because we designed the toxicity test for 7 days that is the limitation of this study.

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APPENDICES

APPENDIX

THE PREPARATIONS OF REAGENTS

Phosphate Buffer saline pH 7.4

Chemicals

Sodium chloride (NaCl)	8	g
Potassium chloride (KCl)	0.2	g
Disodium hydrogen phosphate (Na_2HPO_4)	1.44	g
Potassium dihydrogen Phosphate (KH_2PO_4)	0.24	g
Distilled water	1	L

Preparation

Add chemical compounds one by one into 800 ml of distilled water. Adjust the pH to 7.4 with HCl. Add distilled water to a total volume of 1 liter. Sterilization by autoclaving (20 min, 121°C, liquid cycle). Store at room temperature.

Hematoxylin

- Hematoxylin crystals 4 gm
- 95% Alcohol 200 ml
- Ammonium or potassium alum 6 gm
- Distilled water 200 ml
- Glycerin 200 ml
- Glacial acetic acid 20 ml

Eosin

- **1% Stock alcoholic Eosin**
  - Eosin Y water soluble 1 g
  - Distilled water 20 ml
  - 95% Alcohol 80 ml
- **Working eosin solution**
  - Eosin stock solution 50 ml
  - 80% Alcohol 150 ml
  - Glacial acetic acid 7.5 ml

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1. Camchuen, Y., Saowakon, N. (2015). Evalationof the formaldehyde exposure during gross anatomy laboratory. The 41st Congress on Science and Technology of Thailand (STT41). 6th to 8th November. Suranaree University of Technology, Nakhon Ratchasima, Thailand.
2. Camchuen, Y., Saowakon, N. (2017). Toxicity effect of the ethanolic root extract from *Plumbago indica* no Golden Syrian hamsters. 6th International Graduate Research Conference (6th IGRC 2017). 9th to 11th February. The Empress Hotel Chaing Mai, Chaing Mai, Thailand.