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RESEARCH ARTICLE

Anthelmintic activity and pathophysiological effect of anthelmintic drugs against carcinogenic liver fluke, *Opisthorchis viverrini*

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ABSTRACT

Human liver fluke, *Opisthorchis viverrini* poses a significant risk for development of cholangiocarcinoma (CCA) in Thailand, primarily attributed to consumption of undercooked cyprinoid fishes. The current use of anthelmintic drug treatment such as praziquantel (PZQ), as the main therapeutic agent against *O. viverrini*. There is a need to explore the efficacy of alternative anthelmintic drugs for *O. viverrini* treatment. This study aimed to assess the efficacy of anthelmintic drugs, which are commonly use in endemic areas of Southeast Asian countries; PZQ, albendazole (AL), niclosamide (NI), and mebendazole (ME) at concentrations of 600, 400, 500, and 500 mg/ml. The study included a negative and positive control group treated with roswell park memorial institute (RPMI) and PZQ. Reactive oxygen species (ROS) levels, indicative of oxidative stress, were quantified using 2',7'-dichlorofluorescein diacetate staining. Morphological changes were observed using scanning electron microscopy. Furthermore, motility assessments were conducted at various time points (0, 5, 30 minutes, 1, 3, 6, 12, and 24 hours), calculating relative motility (RM) and survival index (SI). The results revealed a significant increase of ROS levels with the intensity and corrected total worm fluorescence (CTWF) mostly observed in order of PZQ, followed by NI, ME, and AL, respectively. Morphological damage was presented the tegumental swelling, papillae changes, and disruption of microvilli (Mv), particularly in the group treated with the most effective anthelmintics PZQ, NI, ME, and AL, while negative control group did not exhibit such alterations. Also, the most efficacy for suppressing the motility of adult worms were displayed in PZQ treatment group, followed by NI, ME, and AL, respectively. Overall, first novel findings suggest that apart from NI, ME, and AL demonstrate potential as alternative therapeutic options for *O. viverrini* infection. Furthermore, animal model is needed to investigate the efficacy of NI, ME, and AL compare with standard treatment.

Keywords: *Opisthorchis viverrini*; anthelmintic drugs; scanning electron microscope; reactive oxygen species; motility.

INTRODUCTION

The liver fluke, *Opisthorchis viverrini*, remains a significant public health issue in Thailand, particularly in the northeastern and northern regions, where the incidence of cholangiocarcinoma (CCA) is highest. *O. viverrini* causes opisthorchiasis and is classified as a Group 1 biological carcinogen for humans, dogs, and cats (IARC, 2024). Infection occurs through the consumption of raw or undercooked cyprinoid fish containing infective stage, metacercaria. A maximum prevalence of *O. viverrini* was 5.74% among the population in Cambodia to examine by formalin ethyl-acetate concentration technique (FECT) (La *et al*., 2022). Furthermore, polymerase chain reaction (PCR) analysis of DNA samples revealed a positivity rate of 6.89% (La *et al*., 2023). Comparatively, the infection rate of *O. viverrini* in Lao PDR was found to be 54.8% (Saiyachak *et al*., 2016), while an epidemiologic survey in lower Myanmar reported a prevalence of 0.7% (Sohn *et al*., 2019), and central Vietnam is identified as an endemic area for opisthorchiasis with a human infection rate of 11.4% (Dao *et al*., 2016).

Additionally, the first documented case of *O. viverrini* infection in Thailand dates back a century ago, and it continues to be a community level problem. In 2021, over 1.6 million individuals were infected with *O. viverrini* (Wattanawong *et al*., 2021), resulting in healthcare expenses and lost wages totaling approximately 120 million dollars annually. *O. viverrini* infection in Thailand communities through proactive surveillance from 2010 to 2015.

Seventeen community surveys were conducted, predominantly in the northeastern region. Out of the 7 surveys conducted, infection rates exceeded 20%, with the highest recorded at 45.7%. Infections were most prevalent in individuals aged 35 and above, males, and agricultural laborers. Despite a potential decline in the national prevalence, research indicates that *O. viverrini* infection rates persist at high levels in the northeastern region. Therefore, prioritizing the population residing in the northeastern region of Thailand is crucial for screening infections and modifying dietary behaviors (Kaewpitoon *et al*., 2015).

Controlling post-infection diseases caused by liver fluke currently involves chemical treatment, primarily utilizing 75 mg/kg of praziquantel (PZQ) (CDC, 2020a) and PZQ serves as the primary pharmaceutical intervention for the treatment of Opisthorchiasis, operating by inducing spastic spasms in parasites through the facilitation of calcium ion influx. However, reports suggest adverse effects such as pulmonary hemorrhage in individuals treated with PZQ (Flisser & Mclaren, 1989) and associated with a notable correlation to the development of cholangiocarcinoma (CCA), while concerns regarding emerging drug resistance have been raised (Fallon & Doenhoff, 1994; Cioli & Pica-Mattoccia, 2003; Luvira *et al*., 2018). The persistence of apprehensions surrounding PZQ's side effects as highlighted, underscores the imperative to explore alternative drug remedies (Erko *et al*., 2012).

Other recommended medications by the world health organization (WHO) include albendazole (AL) 400 mg for *Ascaris lumbricoides* treatment and mebendazole (ME) 500 mg for *Trichuris trichiura* (WHO, 2020). They demonstrated high efficacy for preventing *A. lumbricoides* infections, with cure rates exceeding 96% and egg reduction rates exceeding 99.8%. However, ME is less effective against *T. trichiura*, with a cure rate of 34.7% and an egg reduction rate of 92.3%, compared with AL rates of 13.9% and 63.4%, respectively (Legesse *et al.*, 2002). Additionally, two grams (g) of niclosamide (NI) have been used to treat *Taenia solium* infections (WHO, 2021). These medications have proven effective in treating various helminthic infections. Many reports focusing on specific side effects, it appears that adverse reactions are predominantly mild and transient, occurring primarily within hospital settings in India. An incident of adverse events following AL administration to children (aged 2-19 years) resulting in 25 hospital admissions due to AL-related adverse events was reported. Among these cases, 92% were female, with an average age of 14 years (Agrawal *et al.*, 2017). The symptoms ranged from mild to severe but were transient in nature, with overall improvement observed following hospital treatment (Agrawal *et al.*, 2017).

A large scale is studied on mass drug administration (MDA) of NI for Taeniasis in Peru, utilizing a 2 g dose of NI administered in 3 rounds for 72 hours observation periods to assess adverse events, reported a total of 1,418 adverse events, all of which were predominantly mild with no severe manifestations (Haby *et al.*, 2020). Additionally, in France, nine cases of adverse effects related to NI usage were documented, including gastrointestinal disturbances (abdominal pain, nausea), headache, polymorphic erythema, sweating, and an anaphylactoid reaction leading to hospitalization following a single dose administration. The use of NI for echinococcosis led to the occurrence of non-threatening erythematous rashes (Haby *et al.*, 2020). The most reported side effects associated with ME use are gastrointestinal disturbances, including abdominal discomfort and nausea (LiverTox, 2012). Toxicity related to mebendazole is typically limited to gastrointestinal upset. However, severe adverse effects have been reported in patients receiving higher doses or prolonged treatment durations (Wilson & Rausch, 1982).

Anthelminthic drugs as above mentions were the commonly used in the population at risk in the endemic areas, the efficacy of ME 500 mg in school-aged children across Brazil, Cambodia, Cameroon, Ethiopia, Tanzania, and Vietnam are compared with AL in a dose of 400 mg. The cure rates of *A. lumbricoides*, hookworm, and *T. trichiura* were reported as 98.0%, 80.6%, and 62.7% for ME, and 99.9%, 96.2%, and 64.5% for AL, respectively (Levecke *et al.*, 2014). Furthermore, the efficacy of AL was assessed through randomized trials among the population in Lao PDR, which infected with *T. trichiura*, and revealed the decreasing of infection rate at 13.4% and eggs reduction rate at 79.6% (Keller *et al*., 2021). Additionally, a study investigated the efficacy and PZQ in treating neurocysticercosis (NCC) caused by *T. solium* in Vietnam. Sixty patients were treated, and post-treatment clinical improvement was observed in 75% of cases, with 90% showing clear improvement in MRI results (Thang *et al*., 2022). However, NI, AL, and ME are effective to any helminths, but lack of evident of *O. viverrini*. Therefore, the primary objective of this study is to assess the efficacy of existing anthelmintic drugs through *in vitro* evaluation of *O. viverrini* adult worms.

MATERIALS AND METHODS

Ethical statement

The ethical approval for this study was obtained from the committee of the institute of research and development at Suranaree University of Technology (SUT), Thailand. Approval was granted for both animal ethics (Ethical clearance No. SUT-IACUC-0013/2023) and bio ethics (Ethical clearance No. SUT-IBC-008-2023).

***O. viverrini* adult worms preparation**

O. viverrini metacercariae were procured from naturally infected cyprinid fish in an endemic region of northeastern Thailand. Fresh cyprinid fishes were digested with a 0.25% pepsin-hydrochloric acid solution, followed by incubation at 37°C for 2 hours. Consequently, the solution was filtrated using a 0.85% normal saline solution in a sedimentation jar. *O. viverrini* metacercariae were identified by parasitologist based on their morphology (Vajrasthira *et al*., 1961) and carried out under a stereomicroscope.

The male syrian golden hamsters, aged 6-8 weeks, were orally infected with 50 metacercariae through intragastric intubation. The development of *O. viverrini* adult worms in the liver bile ducts was monitored over a period of 3 months. Subsequently, the hamsters infected with *O. viverrini* were euthanized to collect adult worms from liver bile ducts, and the obtained parasites were examined under a stereomicroscope to confirm their identity as *O. viverrini*, and they were allocated to be incubated with each anthelminthic drug.

Anthelminthic drugs preparation

Positive control drug, praziquantel (PZQ) (HK Pharmaceutical, Bangkok, Thailand) was utilized at a concentration of 600 mg/ml from a finely ground tablet (CDC, 2020a). Subsequently, drug powder was mixed with 1 ml of roswell park memorial institute 1640 (RPMI-1640) medium (Thermo Fisher Scientific, Massachusetts, USA). Other anthelminthic drugs, albendazole (AL) (The Medicpharma, Samut Sakhon, Thailand) was employed at a concentration of 400 mg/ml (CDC, 2019), wherein a tablet of AL, with a concentration of 200 mg, was utilized to achieve the desired concentration of 400 mg and used of four tablets in 2 ml of RPMI-1640, resulting in a concentration of 800 mg/2 ml or 400 mg/ml. A concentration of 500 mg/ml was employed for niclosamide (NI) (Krungdheb Pharmacy, Bangkok, Thailand) (CDC, 2020b) and mebendazole (ME)

(Thai Nakorn Patana, Nonthaburi, Thailand) treated group (CDC, 2019), utilizing a tablet each, finely ground and then mixed with 1 ml of RPMI-1640. Additionally, each group supplemented their preparations with 100 µg/ml of Penicillin-Streptomycin (Pen-Strep) antibiotic (Cytiva, Pascing, Austria).

O. viverrini adult worms exposure with anthelmintic drugs

O. viverrini adult worms were selected and divided into five groups (RPMI-1640 served as the negative control group, PZQ was positive control group, and groups of other anthelminthic drugs were AL, NI, and ME) and used 10 worms/group. Group 1 was treated with RPMI-1640, group 2 with PZQ at a concentration of 600 mg/ml, group 3 with AL at a concentration of 400 mg/ml, group 4 with NI and group 5 with ME at a concentration of 500 mg/ml of each drug. Ten adult worms were allocated to each group and placed in individual wells of a 24-well plate, with five adult worms/well. The wells were filled with 300 µl of diverse conditions of each anthelmintic drug. Subsequently, the parasites were incubated under varying time intervals (0, 5, 30 minutes, 1, 3, 6, 12, and 24 hours) under a controlled temperature at 37°C.

Pathophysiological effect of anthelminthic drugs

Measurement of stress generation

The induction of stress was initiated through anthelminthic drugs, commencing the cellular response assessment after a 6-hours of incubation period. This incubation period facilitated the establishment balance between the defense against free radicals and reactive oxygen species (ROS). Consequently, *O. viverrini* adult worms were thoroughly washed with distilled water (DW) multiple times. Succeeding the washing process, the adult worms were stained with 30 mM of fluorogenic dye, 2',7'-Dichlorodihydrofluorescein diacetate (H2DCFDA) (Med Chem Express®, New Jersey, USA), and incubated in dark room at 37°C for 30 minutes. After that, excess dye was removed by rinsing the liver flukes with DW. The stained liver flukes were mounted on sterile slides for fluorescence imaging using inverted fluorescence microscope (Nikon, Tokyo, Japan) at 488 and 525 nm of maximum excitation and emission spectra, respectively.

The process of determining cellular fluorescence levels from the images involved several steps by analyzing through ImageJ software (https://imagej.net/ij/download.html). Firstly, the desired images were selected using the drawing/selection tools. Consequently, the image menu was accessed, followed by selecting color and split channels. The measurement parameters were set by choosing set measurements and selecting area, integrated density (IntDen) and mean grey value. Finally, the measurements were obtained by clicking "Measure" in the analyze menu (El-Sharkawey, 2016). The measurement values were used to calculate the corrected total worm fluorescence (CTWF) according to the outlined formular (El-Sharkawey, 2016).

$$\text{CTWF} = \text{Integrated density} - (\text{Area of selected cell} \times \text{fluorescence of background reading}) \quad (1)$$

Morphological study by scanning electron microscope (SEM)

The morphological alterations of *O. viverrini* adult worms after treatment for 12 hour were conducted using SEM. Consequently, each group of adult worms were washed for triple times with DW, followed by fixation using a glutaraldehyde fixative solution for overnight at 4°C. After post-fixation, the samples were immersed in a 1% osmium tetroxide solution in 0.1 M phosphate-buffered saline (PBS) (pH 7.2) for an hour, followed by two rinses with DW. The specimens were dehydrated using a series of graded acetone solutions (30, 50, 70, 90, 95, and 100% alcohol) twice. Following dehydration, the specimens were subjected to critical point drying using a critical point dryer (Leica CPD 300® Vienna, Austria) and examined under a SEM (FESEM/Carl Zeiss Auriga® Dresden, Germany) with an applied electrical potential of 3 kilovolts.

Anthelmintic drugs activity on *O. viverrini* adult worms

Motility assay

The locomotion was conducted from examination of adult worm under a stereo microscope, with scoring based on the criteria; 3 = full locomotion, 2 = partial locomotion, 1 = no locomotion, but still viable, and 0 = deceased (Jiraungkoorskul *et al*., 2005; Jeyathilakan *et al*., 2012). The relative motion (RM) value was calculated based on the locomotion scores throughout the entirety of each experimental trial for all groups, particularly emphasizing the negative control group, which comprised specimens scoring 3 across all parameters, indicating RM = 100. Consequently, a decrease in RM value was observed in the experimental group treated with anthelminthic drugs. This reduction is calculated utilizing the formula detailed below (*n*= motion score, N= number of parasites with a score of motion) (Kiuchi *et al*., 1987; Lorsuwannarat *et al*., 2013). Furthermore, the survival index (SI) was computed to ascertain the proportion of viable worms at a designated time post-treatment. Adult worms manifesting a motility score of 0 were categorized as deceased, while those attaining scores of 3, 2, and 1 were still alive. The SI was determined utilizing the formula outlined below (Kiuchi *et al*., 1987; Lorsuwannarat *et al*., 2013).

$$\text{Motility index (MI)} = \frac{\sum n\text{N}}{\text{N}} \quad (2)$$

$$\%\text{Relative motility (RM)} = \frac{\text{MI test} \times 100}{\text{MI negative control}} \quad (3)$$

$$\%\text{Survival index (SI)} = \frac{\text{number of live worm (each group)} \times 100}{\text{Total worm (each group)}} \quad (4)$$

Viability assessment

After 24 hours incubation period, the adult worms were stained with 0.4% Trypan blue stain (Thermo Fisher Scientific, Massachusetts, USA) at room temperature for 3 minutes. Then, any surplus staining was removed by rinsing with 1xPBS, and the viability of the parasites was assessed using a light microscope.

Statical analysis

The RM and SI values were subjected to analysis utilizing their respective formulas. Mean scores and standard deviations of motility were computed for each group. The data analysis was conducted by SPSS version 20.0 for windows (SPSS Inc., Chicago, USA), with a one-way analysis of variance (ANOVA) performed across five groups [negative control, positive control (PZQ), and three anthehelminthic drugs treated groups (AL, NI, and ME)] to assess and compare the mean motility scores. The statistical significance was determined by a *P*-value < 0.05.

RESULTS

Stress generation levels

The ROS was observed in all groups after a 6-hours incubation period. The presence of ROS was determined using the H2DCFDA fluorescent dye, facilitating the analysis of ROS levels. The minimum ROS production was identified in the negative control group (Figure 1a, Figure 1f, and Figure 1k) in the anterior, central, and posterior regions of *O. viverrini* adult worm. Conversely, the maximum ROS production was observed on the treated adult worm with PZQ 600 mg/ml (Figure 1b, Figure 1g, and Figure 1l), NI 500 mg/ml (Figure 1d, Figure 1i, and Figure 1n), and ME 500 mg/ml (Figure 1e, Figure 1j, and Figure 1o) groups, predominantly around the oral sucker, along the lateral and posterior regions of the parasite. In contrast, the treated group with AL 400 mg/ml group exhibited relatively indistinct levels of ROS in the fully mature *O. viverrini* adult worm (Figure 1c, Figure 1h, and Figure 1m).

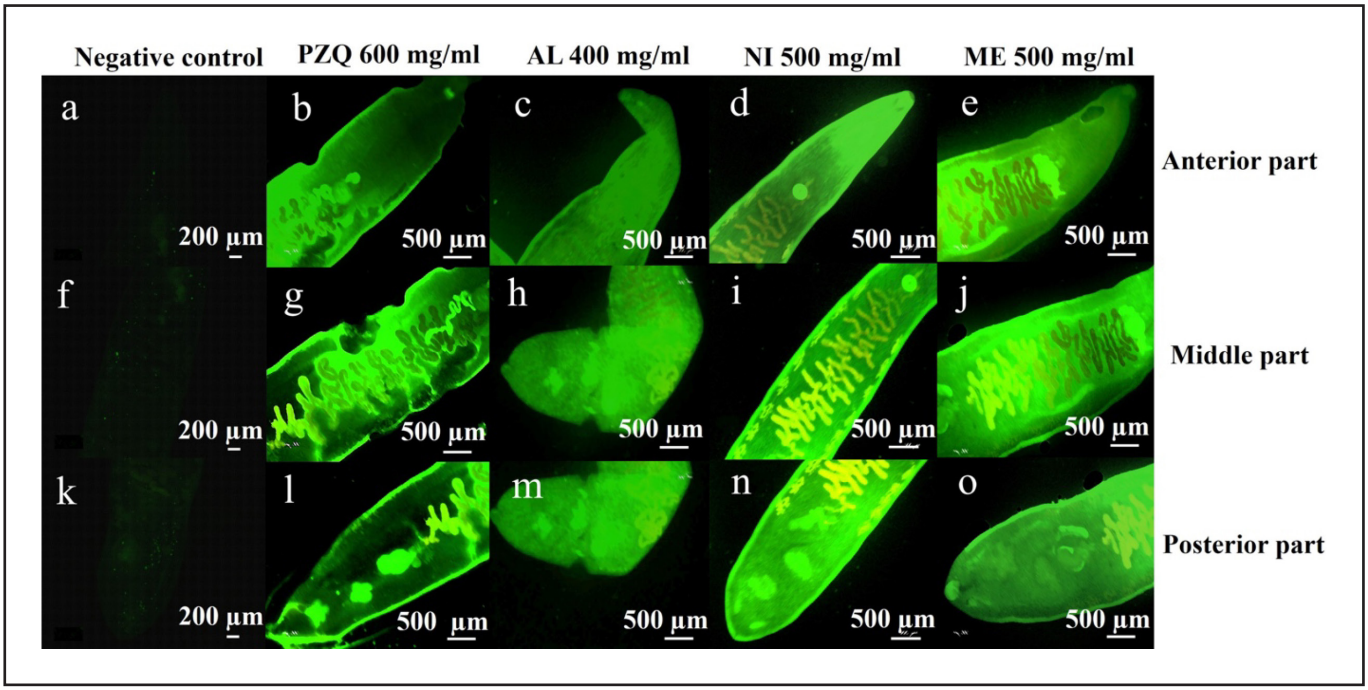

Figure 1. ROS generation in treated *O. viverrini* adult worms with various anthelminthic drugs after 6-hours exposure period. (a-e) Depicted the anterior part, (f-j) illustrated the middle part, and (k-o) displayed the posterior part. Scale bar 200 and 500 µm.

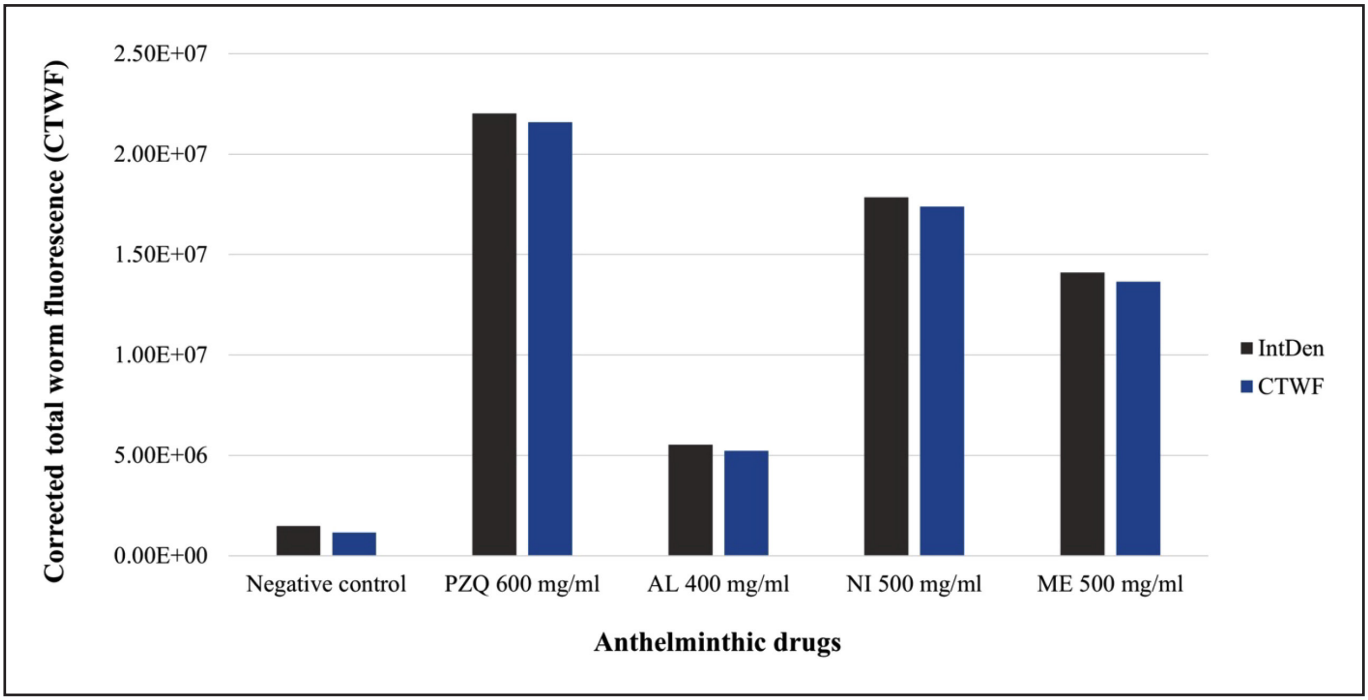

Figure 2. IntDen and CTWF levels revealed that negative control group was lower than in the tested group with anthelminthic drugs. IntDen and CTWF were observed to be higher in the respective order for PZQ, NI, ME, and AL, respectively.

IntDen was measured by ImageJ software, the negative control group was represented lowest IntDen and CTWF values of 1,484,518 and 1,155,026.03 respectively. In comparison, the tested adult worms with PZQ 600 mg/ml, NI 500 mg/ml, ME 500 mg/ml, and AL 400 mg/ml, their IntDen and CTWF values were higher, with values of 22,024,925, 17,843,045, 14,106,799, and 5,529,631, and 21,598,473.2, 17,381,721.4, 13,649,973.8, and 5,226,170.13, respectively (Figure 2).

Surface morphology changes

Opisthorchis viverrini adult worm of negative control group exhibited a smooth surface around the oral sucker (Figure 3a) and ventral sucker (Figure 3f), a well dispersed arrangement of papillae in the periphery (Figure 3k), this group also displayed normal surface features without damage. In comparison, the treated group with PZQ at a concentration of 600 mg/ml showed an irregular and uniformly textured surface. Papillae were dispersed around the oral

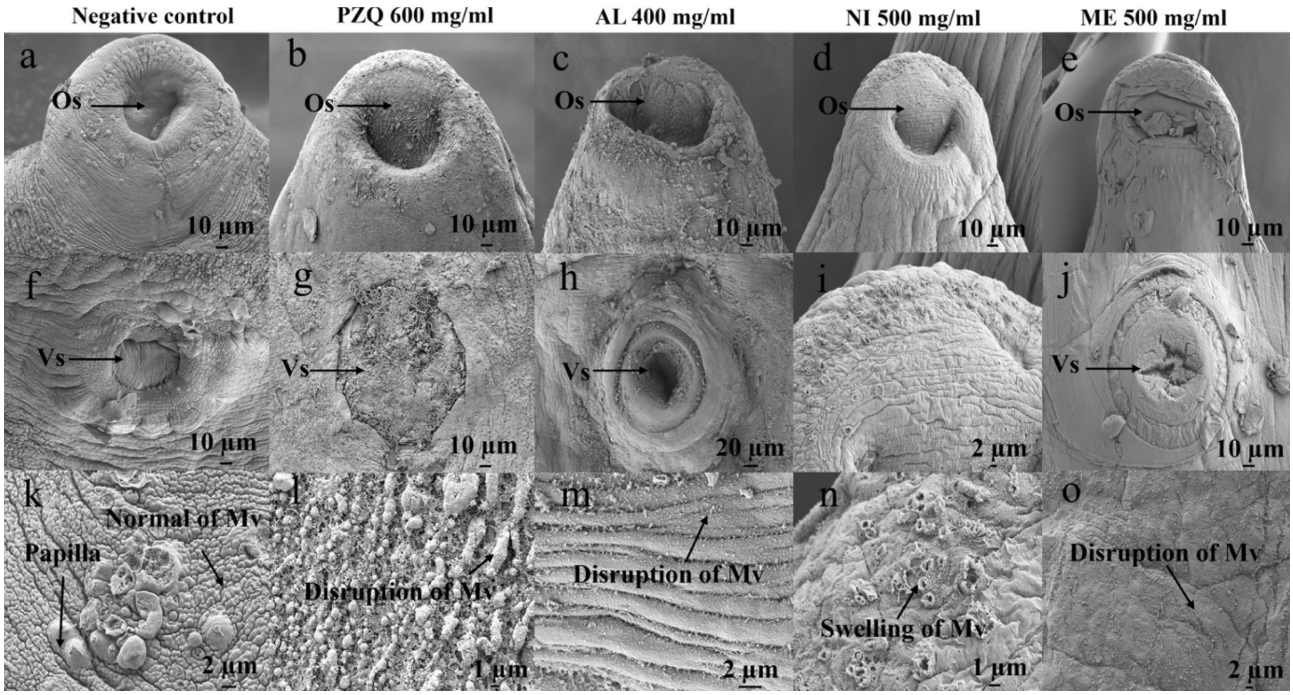

Figure 3. Ultrastructure of treated *O. viverrini* adult worms with each condition. (a-e) Depict the area of the oral sucker (Os). (h and j) The area of the ventral sucker (Vs). (k-o) Illustrate the surface features of parasite that represented the disruption of microvilli (Mv). (a, f, and k) Negative control group, (b, g, and l) PZQ 600 mg/ml, (c, h, and m) AL 400 mg/ml, (d, i, and n) NI 500 mg/ml, and (e, j, and o) ME 500 mg/ml. Scale bar 1, 2, 10, and 20 µm.

and ventral sucker regions (Figure 3b and g), and the fluke exhibited elongation, evident damage by disruption of microvilli (Mv) (Figure 3l). Consequently, the tested flukes with AL at a concentration of 400 mg/ml displayed an irregular and uneven surface. Severe exfoliation occurred in the oral sucker region and the surface surrounding it (Figure 3c). There was also pronounced swelling around the ventral sucker (Figure 3h) and notable surface peeling (Figure 3m). The treated group with NI at a concentration of 500 mg/ml, the flukes exhibited a consistently uneven surface. Severe damage occurred around the oral sucker (Figure 3d) and ventral sucker (Figure 3i), with significant swelling and cracking of papillae on the fluke's surface (Figure 3n). Conclusively, in the treated group with ME at a concentration of 500 mg/ml, the surface of the fluke showed marked swelling and intense exfoliation around the oral sucker (Figure 3e). Additionally, there was significant swelling around the ventral sucker (Figure 3j) and indicating pronounced surface peeling (Figure 3o). A comparative analysis between the negative control group and the treated groups with anthelmintic drugs revealed distinct effects on the surface of the *O. viverrini* adult worms. Specifically, PZQ and NI induced clear elongation of the fluke's surface and damage of microvilli, while AL and ME caused noticeable swelling around the oral and ventral suckers.

Motility and viability tests of *O. viverrini* adult worm

The RM and % motility of adult worms was evident in the negative control group (RM = 100), while the treated groups with anthelmintic drugs showed a gradual reduction in motility. Specifically, PZQ, AL, NI, and ME groups, motility decreased steadily within 24 hours, with values of 0, 48.66, 0, and 0%, respectively. However, after 1 hour of anthelmintic drug exposure, a decline in motility was observed in each drug group, with RM values of 54.25, 42.88, 38.59, and 28.60%, respectively. As time progressed, after 12 hours, the parasites began to exhibit reduced motility, with values of 0, 48.02, 0, and 9.96%, respectively. This indicates that anthelmintic drugs PZQ and NI were more effective in promptly halting parasite motility compared with AL and ME. All parasites ceased movement except for those treated with AL after treated for 24 hours, which showed a decreasing trend, but still maintained some level of motility (Figure 4).

The SI and overall survival over the entire testing period were assessed for adult worms in both the negative control group and the groups subjected to different types of anthelminthic drugs. Notably, PZQ exhibited the most significant reduction in SI values at 6, 12, and 24 hours, registering 80, 0, and 0%, respectively. Following closely was NI, with SI values of 90, 0, and 0% at the same time intervals. Meanwhile, ME demonstrated SI values of 50% at 3, 6, 12 hours and 0% at 24 hours, respectively. In contrast, all specimens in the AL group survived the entire testing duration, indicated by 100% of SI (Figure 5).

A one-way ANOVA was employed and indicated significant differences between the negative control group and the experimental group treated with PZQ 600 mg/ml and NI 500 mg/ml ($P< 0.05$), respectively. The observations revealed no statistically significant differences between PZQ 600 mg/ml, AL 400 mg/ml, and NI 500 mg/ml, as well as ME 500 mg/ml ($P > 0.05$). Anthelminthic drugs possessed the capacity to eliminate liver flukes. The observed movement in the AL group was indicated a comparatively slower mortality rate in comparison to the other drugs (Figure 6).

Upon concluding the 24 hours the motility testing period (RM = 0 and SI = 0), confirmation of viability was carried out through trypan blue staining (Figure 7). This staining method revealed the viable of *O. viverrini* adult worms in the negative control group (Figure 7a), whereas treated groups with PZQ, NI, and ME showed stained on adult worms, indicative of parasite mortality (Figure 7b, d, and e). Notably, the AL-treated group (Figure 7c) exhibited partial staining, suggesting the presence of adult worms living.

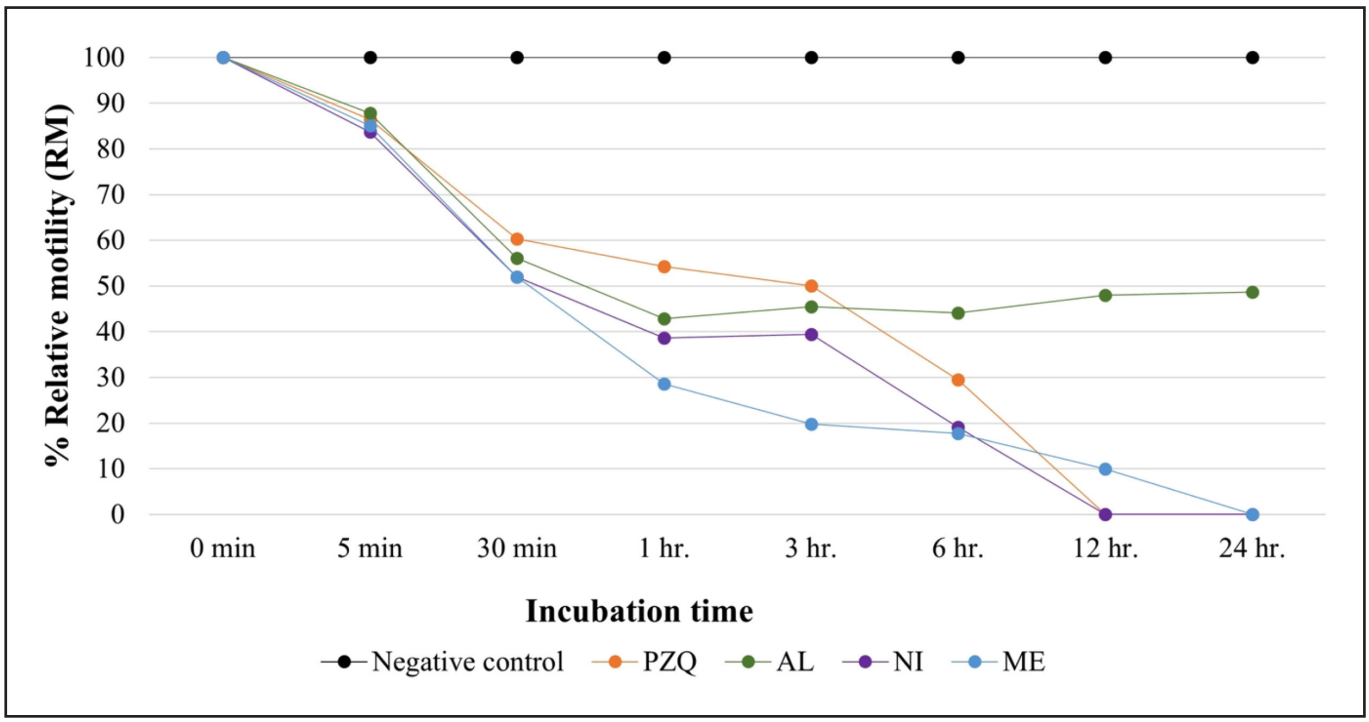

Figure 4. The RM of parasites was assessed after exposure with each anthelminthic drugs at different time points 0, 5, 30 minutes, 1, 3, 6, 12, and 24 hours, respectively.

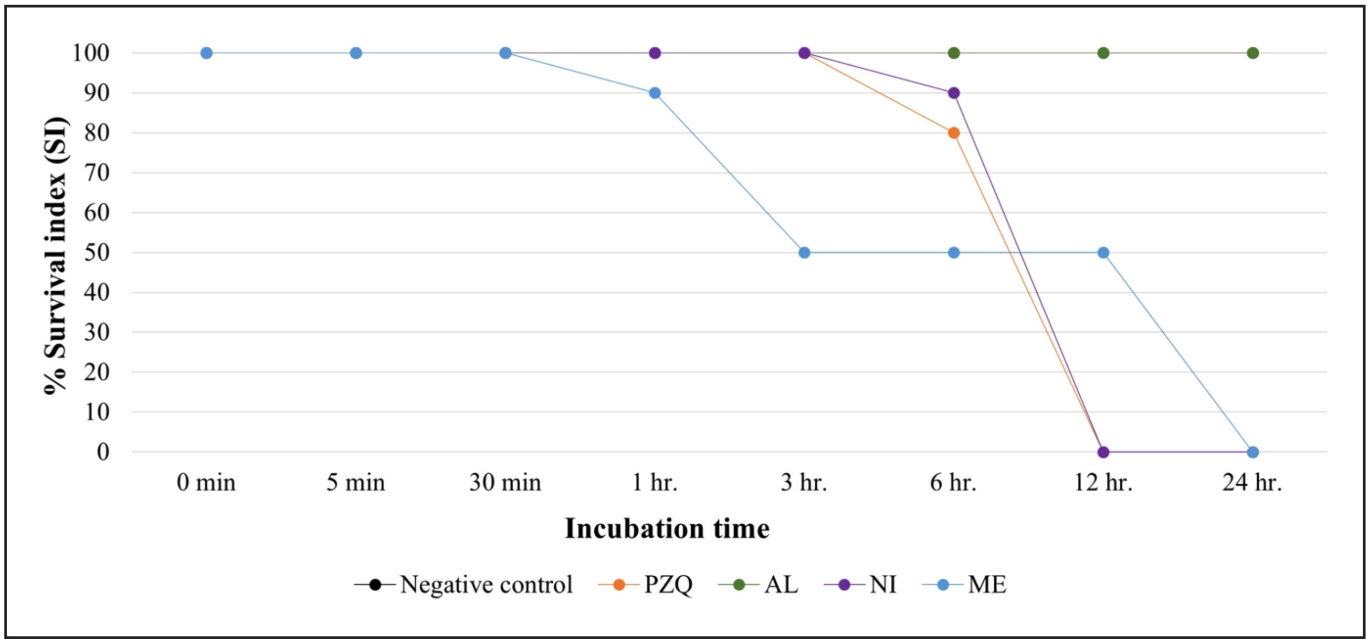

Figure 5. The SI of the parasite was assessed after testing with each type of anthelminthic drugs at time intervals 0, 5, 30 minutes, 1, 3, 6, 12, and 24 hours.

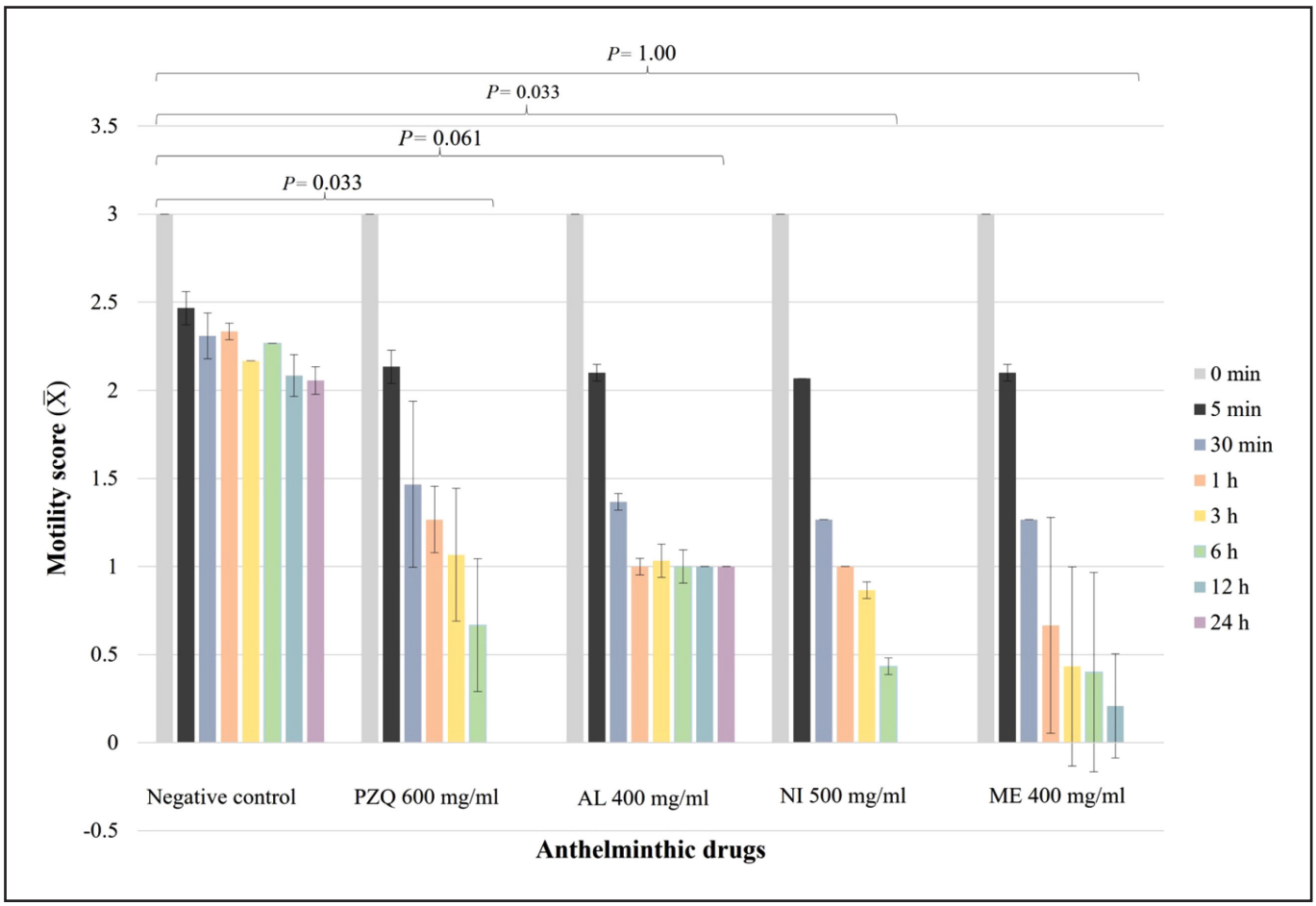

Figure 6. The motility scores depict statistically significant differences between the negative control group and the experimental group treated with anthelminthic drugs. Conversely, no statistically significant differences were observed between PZQ 600 mg/ml and AL 400 mg/ml, NI 500 mg/ml, and ME 500 mg/ml.

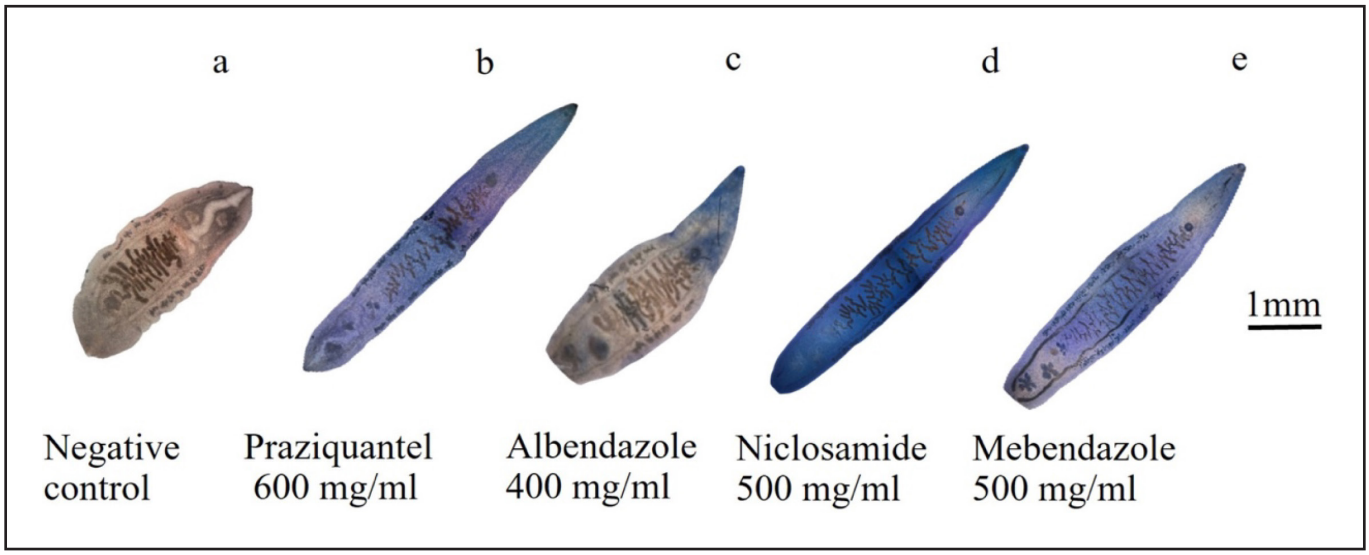

Figure 7. Viability of *O. viverrini* adult worms were assessed through trypan blue staining. (a) negative control group did not exhibit discernible staining, (b) PZQ 600 mg/ml with comprehensive staining throughout the body, (c) AL 400 mg/ml with partial staining limited to the anterior region, (d) NI 500 mg/ml with intense and widespread staining of the entire parasite, particularly in the anterior and posterior regions, and (e) ME 400 mg/ml with comprehensive staining across the entire body. Scale bar 1 mm.

DISCUSSION

The present study elucidated the anthelmintic activity and pathophysiological effect of anthelmintic drugs on *O. viverrini* adult worms. The analysis of ROS levels revealed minimal ROS production in the negative control group, predominantly in the anterior, middle, and posterior regions of the *O. viverrini* adult worms. Conversely, the groups treated with PZQ, NI, and ME exhibited the highest ROS levels, primarily around the oral sucker, lateral sides, and posterior regions of the parasites, respectively. In contrast, AL demonstrated indistinct ROS levels in parasite. Generally, ROS production resulting from stress is associated with the generation of highly reactive molecules such as superoxide anions, hydrogen peroxide, and hydroxyl radicals within cells or living organisms in response to various stressors. These stress-inducing factors encompass physical, chemical, environmental, or biological agents capable of disrupting the balance of cellular ROS.

Under stressful conditions, excessive ROS production may lead to oxidative stress, linked to the loss of proteins, lipids, and DNA, affecting cell functionality (Finkel, 2011). The intricate process of ROS generation induced by stress is closely tied to numerous cellular pathways, playing a crucial role in cellular processes such as signal transduction and immune system responses, although outcomes can be either beneficial or detrimental (Schieber & Chandel, 2014). However, stress induced by anthelminthic drugs has not been explicitly studied, and a comparative analysis of stress induction by AL and ML over time in mice, revealed interesting insights (Locatelli *et al*., 2004). The examining blood and liver samples, demonstrating that both AL and ME triggered oxidative stress through the generation of thiobarbituric acid reactive species and a reduction in antioxidant defenses. Furthermore, AL exhibited robust ROS and reactive nitrogen species (RNS) generation, while ME displayed lower values, with transient effects on ROS production. Researcher suggests that ME could be a preferable treatment option for parasitic infections, as it induces lower oxidative stress reactions in the host (Locatelli *et al.*, 2004). Additionally, AL induced stress was evident through increased ROS production upon exposure to the drug, causing more extensive damage to various biomolecules, such as proteins, lipids, and DNA to suggest that AL led to greater DNA damage compared to other biomolecules, emphasizing its potential harmful effects (Martínez-Espinosa *et al.*, 2015).

The study investigated the morphological changes on the tegumental surface of *O. viverrini* adult worms. The negative control group exhibited normal tegumental features characterized by a smooth surface without desquamation. The oral and ventral suckers displayed typical morphological characteristics without any damage. Papillae in the oral sucker region exhibited normal features consistent with the general description of *O. viverrini* provided by previous reports (Scholz *et al*., 1992; Apinhasmit *et al*., 1993). *O. viverrini* adults worm demonstrated well defined papillae on the tegumental surface, resembling buttons, consistent with the general morphological description (Scholz *et al*., 1992; Apinhasmit *et al*., 1993). The tegumental surface exhibited button-like structures or small protuberances that were relatively uniform in shape and size. Additionally, the body had randomly scattered papillae, and the oral sucker was located at the anterior end, with the ventral sucker featuring nearly circular-shaped oral openings. Both oral and ventral suckers were surrounded by numerous papillae, and the ventral sucker was positioned near the opening of the genital atrium. Furthermore, the tegumental surface of *O. viverrini* adult worm was covered with microvilli, with three types of tubercles dispersed throughout. Numerous openings of tegumental gland cell openings were observed in the surrounding areas of the oral sucker and the ventral sucker, with an abundance of cells around the openings in the genital atrium region (Apinhasmit *et al*., 1993). The group of parasites subjected with anthelmintic drugs, specifically PZQ at a concentration of 600 mg/ml, exhibited a consistently uneven and rough surface, with papillae buttons distributed around the oral sucker and ventral sucker, along with elongation of the parasite's surface indicative of visible damage. These observations align with a previous study (Lorsuwannarat *et al*., 2013), which investigated the anti-parasitic properties of plumbagin in laboratory tubes against *Schistosoma mansoni* compared with PZQ. SEM revealed distinct changes in the tegument of *S. mansoni*, characterized by swelling and the beginning of tegument detachment into large pieces from the surface (Flisser & McLaren, 1989). The impact of PZQ on the morphological changes of the parasite's tegument, affecting membrane permeability by increasing calcium influx, resulting in severe structural and functional damage to the tegument, ultimately leading to muscular spasms and paralysis, ultimately resulting in the death of the parasite (Becker *et al.*, 1980). The efficacy of PZQ and *Carica papaya* seed extracts against *Hymenolepis nana* infection in mice, wherein treated mice exhibited significant swelling and blistering of *H. nana* scolex under compound microscopy, with parasites dissolving and disappearing within 2 hours post-treatment, accompanied by contraction of the scolex and visible suction cup inflation in parasites (Abou Shady *et al*., 2014). Furthermore, the morphological characteristics of tested adult parasites with NI at 6-hours showed significant changes, with a consistently uneven surface around the oral sucker, severe damage, and bursting of papillae on the parasites' surface, consistent with the previous study (Kumchoo *et al.*, 2007). Additionally, the effects of NI on the tegumental surface of *Haplorchis taichui*, using SEM, demonstrated swelling and hemorrhage of the tegument in the abdominal and dorsal regions after 6-hours, with increased bleeding and surface erosion in both abdominal and dorsal regions, and in some instances, damage to the hooks around the periphery (Kumchoo *et al.*, 2007). Also, high concentrations of NI exhibited inhibitory properties against *H. taichui* (Kumchoo *et al.*, 2007).

Parasites exhibited continuous motility and sustained survival throughout the duration of the experimental period. Conversely, the group subjected to PZQ treatment displayed a rapid decline in RM and SI within the initial 3-6 hours, leading to cessation of motility or mortality within 12 hours, marking the endpoint where complete immobilization was observed within 24 hours. These findings are congruent with investigation on the anthelmintic properties of plumbagin in experimental tubes against *S. mansoni*, wherein the plumbagin-treated group exhibited a significantly faster reduction in RM compared with the PZQ treated group (Lorsuwannarat *et al*., 2013). Furthermore, PZQ demonstrated superior efficacy in treating helminthic infections, followed by ME and AL, aligning with previous study comparative study on the efficacy of various anthelmintics against *Clonorchis sinensis* infected mice, with PZQ exhibiting the highest efficacy at 98.6%, followed by ME at 95.2%, and albendazole at 91.9% (Xiao *et al.*, 2011). Additionally, the efficacy of NI decreased in motility within 1-6 hours and complete mortality within 12 hours, although the motility of NI treated parasites has not been studied extensively. Additionally, researcher investigated the efficacy of NI in experimental tubes and *in vivo* against *Toxoplasma gondii* RH infection in BALB/c mice, demonstrating that NI resistance against *T. gondii* in the mouse body resulted in 20%, 40%, and 50% survival rates in positive control groups compared with 160, 200, and 240 mg/kg dosages in the treatment groups, respectively, highlighting the inhibitory effects of NI against *T. gondii* (Zhang *et al.*, 2019).

This research contributes valuable information regarding the anthelmintic potential of alternative drugs against *O. viverrini*, particularly in PZQ, the standard treatment, reveal the highly efficacy for carcinogenic liver flukes. Accordingly, anthelmintic drugs indicated a significant decrease liver flukes motility, mostly observed in the order of NI, followed by ME, and AL, respectively. However, the effectiveness of AL was evident, albeit accompanied by a comparatively slower mortality rate in comparison to the other anthelmintic drugs. This first novel opening avenues for further research and the development of improved therapeutic

strategies against this carcinogenic liver fluke. Further investigation necessitates *in vitro* studies involving a substantial quantity of liver flukes. Additionally, it is imperative to conduct experiments on animals infected with liver flukes and subsequently treated with each of NI, ME, and AL, comparing the outcomes with those treated with PZQ. Although, PZQ stands as the established standard treatment for *O. viverrini* infection, alternative treatments such as NI, ME, and AL may potentially provide comparable efficacy, thus presenting equivalent options to the standard treatment.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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