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Histopathological effects of *Camellia oleifera* seed and *Garcinia mangostana* pericarp extracts on *Pomacea canaliculata* snails, an intermediate host for *Angiostrongylus cantonesis*

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Abstract

We examined the histopathological changes in the tissues of *Pomacea canaliculata* snails incubated in crude extract obtained from camellia (*Camellia oleifera*) seed and mangosteen (*Garcinia mangostana*) pericarp, to evaluate the molluscicidal activity of these two plant substances. *P. canaliculata* snails were incubated in various concentrations of each plant extract for 24 hours. As a positive control, another group of snails was incubated in various concentrations of niclosamide solution, a chemical molluscicide. Similar histopathological findings were observed in both experimental and control snails. Both *C. oleifera* seed and *G. mangostana* pericarp extracts showed molluscicidal effects after 24 hours, with 50% and 90% lethal concentrations (LC50 and LC90) of 0.001 and 0.073 g/ml for *C. oleifera*, 0.001 and 0.024 g/ml for *G. mangostana*, and 0.659 and 3.397 ppm for niclosamide. Histopathological changes included alterations in the epithelial lining of the digestive tract, digestive gland, gill and foot. Loss of cilia, degeneration of columnar epithelial cells, and increased mucus vacuoles and cells were observed in the digestive tract and gill, while the digestive gland exhibited an increase in the number of dark granules and basophilic cells and dilation of digestive cells. The muscle cells of the foot had a loss of texture. The present findings indicate that both *C. oleifera* seed and *G. mangostana* pericarp have molluscicidal activity that could be used to control *P. canaliculata*.

Keywords: Camellia oleifera, Garcinia mangostana, Molluscicidal effect, Niclosamide, Opisthorchis viverrini, Pomacea canaliculata

1. Introduction

Pomacea canaliculata, or the golden apple snail, is a freshwater snail that is an invasive pest [1] in many parts of the world, including Southeast Asia. In 1980 the *P. canaliculata* snail was introduced into Taiwan to start an escargot industry [2] and rapidly spread to Hong Kong, southern China, Japan, Korea, the Philippines, Vietnam, Laos, Thailand, Cambodia and Indonesia [3]. *P. canaliculata* live in slow-moving water in lowland swamps, marshes, ditches, ponds, rivers and rice fields [4]. They may even survive months of drought by digging deep into the mud and closing their operculum, surfacing again after renewed flooding [2].

P. canaliculata snails act as an intermediate host for the parasite *Angiostrongylus cantonensis*, or the rat lungworm, which causes angiostrongyliasis by consumption of raw or undercooked *P. canaliculata* snails infected with the parasite. *A. cantonensis* also causes eosinophilic meningoencephalitis in humans [5]. *A. cantonensis* is

frequently found in Asia (i.e., China in 2009), and more recently has been found in North America, the Caribbean and northeastern South America [6].

The parasite migrates and undergoes development to the young adult stage in the central nervous system of mammals. In natural hosts, the parasite travels to the pulmonary arteries, where it reaches sexual maturity. The adult female lays eggs which develop and hatch in the lungs, and the first-stage larvae eventually are eliminated in the feces [7]. In unnatural hosts, such as cattle, pigs, rabbits and humans, the parasite develops in the brain, where it dies, but does not usually travel to the lungs [8].

Chemical molluscicides used to control *P. canaliculata* snails may have toxic effects on other aquatic animals and plants. Therefore, we evaluated *Camellia oleifera* seed and *G. mangostana* pericarp against *P. canaliculata* snails to determine their potential use as mollusicides.

2. Materials and Methods

2.1. Pomacea canaliculata snail collection and preparation

Pomacea canaliculata snails were collected manually by scooping them from a freshwater reservoir in Khon Kaen, northeastern Thailand. The snails were then washed in dechlorinated water and kept in plastic bags for transportation to the laboratory. *P. canaliculata* snails were identified using the standard morphological guidelines [10].

Snails were transferred into individual plastic cups containing dechorinated water, and were examined for parasites by checking for cercarial shedding. The shed cercariae were observed under a stereomicroscope (SZ series; Olympus, Tokyo, Japan). Snails were kept in a 12 hours light–dark cycle. Non-infected P. canaliculata snails was used in this experiment.

2.2. Identification of dead snails

The following criteria were used to identify dead snails [9] : i) sinking to the bottom of the container and completely retracting into the shell; ii) loss of operculum, and showing no movement; iii) failing to retract when probed with a needle; iv) discoloration, and no muscular movement when prodded with a probe.

2.3. Preparation of camellia (C. oleifera) seed extracts solution

Camellia seed (10% saponin) was purchased from Ekyongyut Co. (Samut Sakhon, Thailand). Ten g of camellia seed was dissolved in 100 ml distilled water and then mixed with a magnetic stirrer. This stock solution was used to prepare the following concentrations: 0 (control), 1:10, 1:100, 1:1,000, 1:10,000, 1:100,000 and 1:1,000,000 v/v.

2.4. Preparation of mangosteen (G. mangostana) pericarp extract solution

Samples of fresh mangosteen (*G. mangostana*) pericarp were washed and incubated at 60 °C for 24 hours. Fifty g of dried mangosteen peel in power form was mixed well with 500 ml distilled water using a magnetic stirrer and then filtered through 0.45 µm filter paper. This stock solution was used to prepare the following concentrations: 0 (control), 1:10, 1:100, 1:1,000, 1:10,000, 1:100,000 and 1:1,000,000 v/v.

2.5. Experimental snails

To compare the efficacy of *C. oleifera* seed and *G. mangostana* pericarp extracts, groups of 15 *P. canaliculata* snails were incubated in various concentrations of *C. oleifera* or *G. mangostana* crude extract: 0.1, 0.01, 0.001, 0.0001, 0.00001, 0.000001 and 0 (control) g/ml. The snails were incubated for 1 week; the concentrations that could kill 50% and 90% of the snails (LC50 and LC90) were determined for each concentration after 24 hours exposure. These were compared with the LC50 and LC90 of the standard molluscicide niclosamide at concentrations of 0, 0.015625, 0.03125, 0.0625, 0.125, 0.25, 0.375, 0.5, 0.625, 0.75, 0.875 and 1 ppm. Each concentration was tested in triplicate. LC50 and LC90 were calculated using probit analysis with the program SPSS version 16.0.

2.6. Preparation of tissue sections

Tissue sections were prepared following the method of [13]. In brief, *P. canaliculata* snail tissue was soaked in 10% formaldehyde for 1 week. The snail tissue was then washed with phosphate buffered saline (PBS) three times for an hour each time. Tissue samples were dehydrated with gradually increasing concentrations of alcohol and then cleared with xylene. The tissue was then placed in a 1:1 mixture of xylene:paraffin for an hour at 60 °C and then in paraffin overnight. Paraffin-embedded tissue samples were cut with a microtome into 4 µm sections,

which were placed on individual slides. The slides were incubated at 60 °C for 24 hours and then stored at room temperature.

2.7. Hematoxylin and eosin staining

For the histopathological study, in brief, whole liver tissue was dissected and fixed immediately in 10% buffered formalin for at least 24 hours. Tissue samples were embedded in paraffin; each paraffin block was then cut with a microtome into 4 μm sections. Tissue sections were deparaffinized with xylene, rehydrated through a graded series of ethyl alcohol, and stained with Harris's hematoxylin and eosin. Finally, the tissue sections were dehydrated in a series of ethyl alcohol and mounted on slides with Permount™ mounting medium (Thermo Fisher Scientific, Waltham, MA, USA). Each tissue slide was photographed under a microscope (BX51; Olympus).

3. Results

3.1. Molluscicidal effects of camellia and mangosteen extracts (LC50 and LC90)

To compare the efficacy of camellia (*C. oleifera*) seed and mangosteen (*G. mangostana*) pericarp with the molluscicidal drug niclosamide, *P. canaliculata* snails were incubated in various concentrations of the crude extracts and solution. The LC50 and LC90 after 24 hours were 0.001 and 0.073 g/ml for *C. oleifera*, 0.001 and 0.024 g/ml for *G. mangostana*, and 0.659 and 3.397 ppm for niclosamide.

3.2. Macroscopic findings

The macroscopically visible reactions of *P. canaliculata* snails exposed to crude extracts of C. *oleifera* and *G. mangostana* were similar during the first hour; the snails in both groups were still actively moving. Thereafter, *P. canaliculata* snails only in the *G. mangostana* group showed signs of inactivity, secreted clear mucus, became immobile and died by the end of day 1. After exposure to niclosamide solution at concentrations of 0.5, 0.625, 0.75, 0.875 and 1 ppm, snails retracted into the shell and closed the operculum. After 5 minutes of exposure at 1 ppm, snails fell to the bottom of the container and exhibited no motion. Concentrations greater than 0.75 ppm resulted in survival rates of only 46.67%.

Snails exposed to *C. oleifera* extract had similar reactions to exposure to niclosamide, but at different concentrations. At 0.0001, 0.001, 0.01 and 0.1 g/ml, the snails did not stick to the walls of the container and fell to the bottom. No movement was observed after the first 5 minutes of exposure at 0.01 and 1 g/ml. After 6 hours of exposure at a concentration of 0.001 g/ml, snails showed slower movement, retracted into the shell and closed the operculum for about 30 minutes, and then re-emerged. At a concentration of 0.0001 g/ml, most snails stuck to the container for up to 10 hours. All snails exposed to a concentration of 0.1 g/ml died.

The effects of *G. mangostana* extract at concentrations of 0.0001, 0.001, 0.01 and 0.1 g/ml were similar to those of *C. oleifera* extract. Snails exhibited no movement at different time points, varying from low to high concentrations (after 6 hours, an hour, 30 minutes and 5 minutes of exposure, respectively). Surprisingly, the presence of white mucus on the head-foot and an open operculum were observed only in the group exposed to a *G. mangostana* concentration of 0.1 g/ml.

3.3. Pathological changes after treatment with extracts of C. oleifera seed and G. mangostana pericarp

Head-foot *C. oleifera*, *G. mangostana* and niclosamide groups showed an increased number of mucocyte cells and lipid vacuoles, and atrophy of columnar muscle fibers. Accumulation of pigment cells was observed in the *C. oleifera* group (Figure 1). No pathological changes were observed in the control snails.

Gill Both *C. oleifera*- and *G. mangostana*-treated groups showed dilated interlamellar spaces, enlarged mucocyte cells, loss of heterochromatin and dispersed nuclei, and a reduction in the length and number of cilia (Figure 2).

The tentacle epidermis of control snails showed a regular surface with microvilli. Hypertrophy of cell surfaces and nuclei, and replacement of nuclear cells were observed in the *G. mangostana*-treated group (Figure 3).
Digestive gland All treated groups (*C. oleifera*, *G. mangostana* and niclosamide) exhibited numerous dark granules, accumulation of amebocyte cells, dilation of the digestive gland, and large hemolymphatic spaces when compared with the control snails. Treated groups showed a small n umber of calciferous cells (Figure 4). No pathological changes were observed in the control snails.

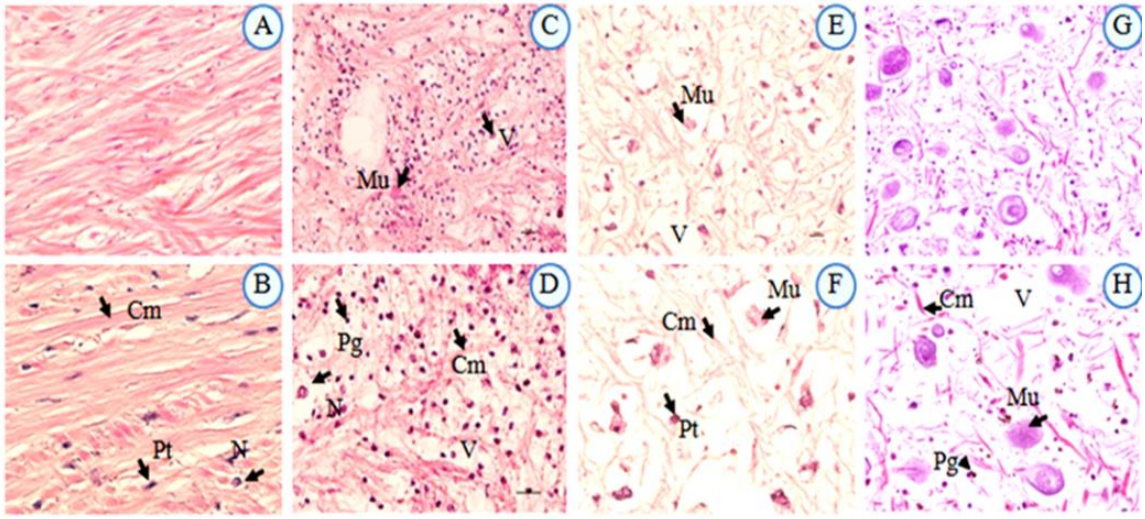

Figure 1 Representative pathology of the *P. canaliculata* tissue: (A; B) control; (C; D) exposed to camellia extracts; (E; F) exposed to mangosteen extracts and (G; H) exposed to niclosamide solution. Pg, pigment cells; Mu, mucocyte cells; Lv, lipid vacuole; Cm, columnar muscle fibers.

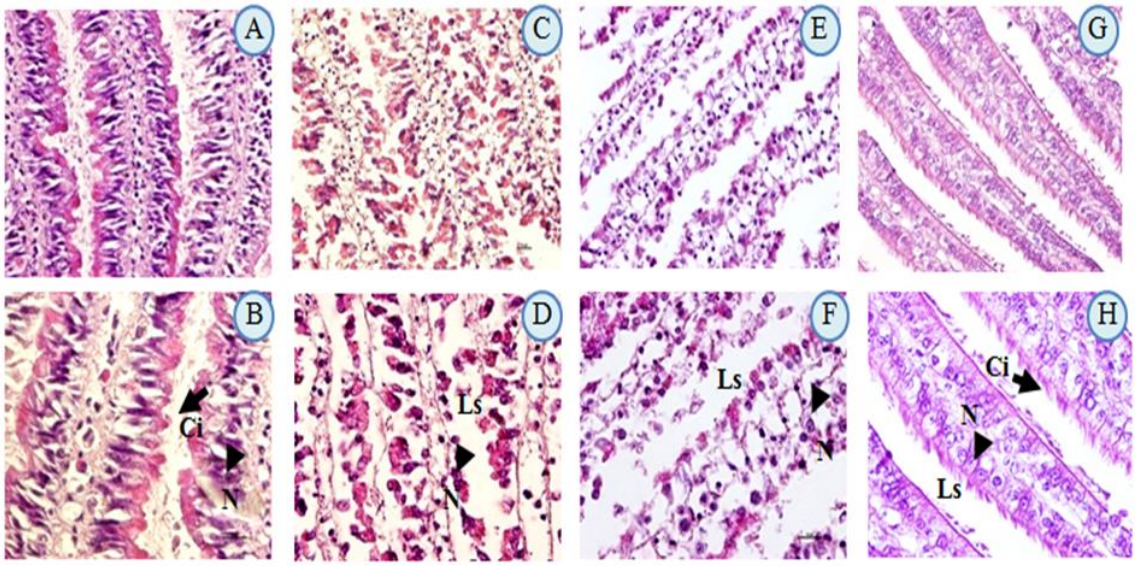

Figure 2 Representative histology of the gill of *P. canaliculata*: (A, B) control; (C, D) exposed to camellia extract; (E, F) exposed to mangosteen extract; and (G, H) exposed to niclosamide solution. N, nuclei with dense heterochromatin; Ci, cilia; m, mucocytes; Ls, lamellar spaces.

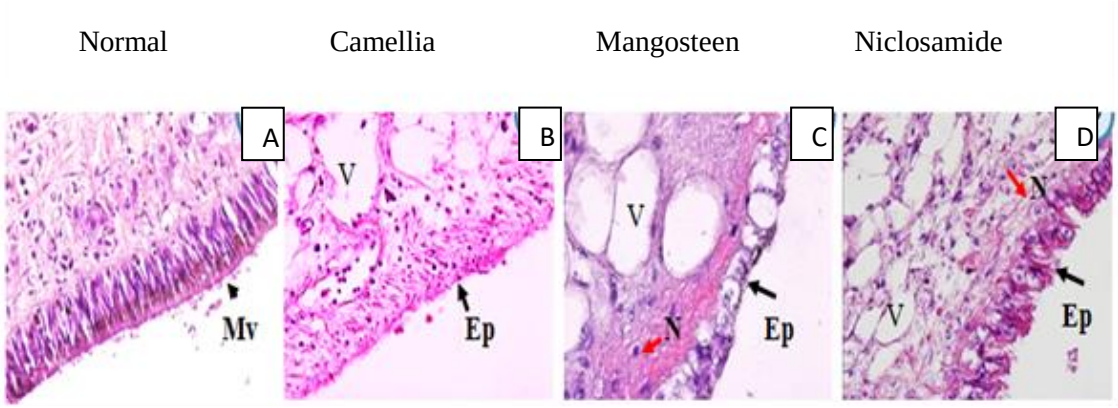

Figure 3 Representative micrographs of the tentacle epidermis of *P. canaliculata*: (A) control; (B) exposed to camellia extract; (C) exposed to mangosteen extract; and (D) exposed to niclosamide solution. Mv, microvilli; Ep, epidermis; N, nuclear cells; V, lysosomal vacuoles.

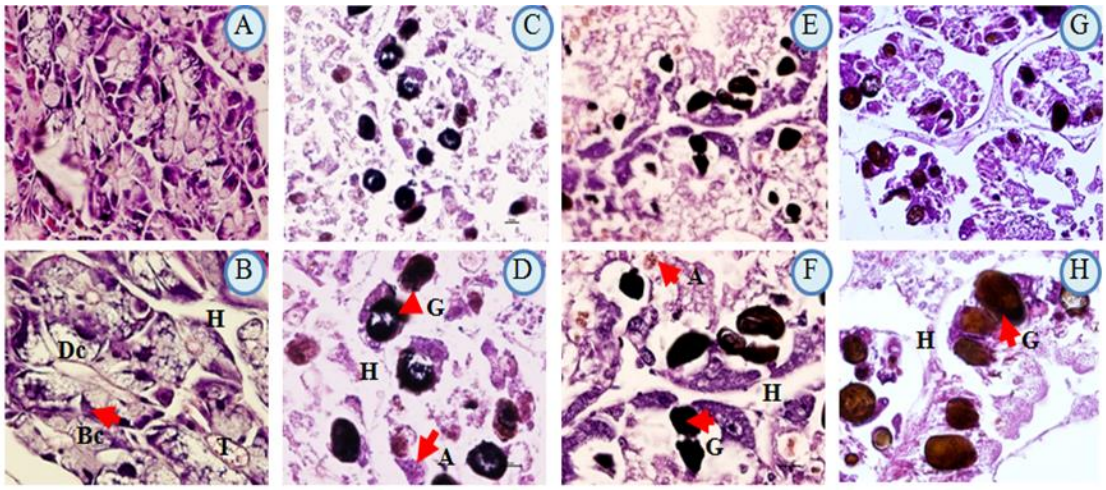

Figure 4 Representative histopathology of the digestive gland of *P. canaliculata*: (A, B) control; (C, D) exposed to camellia extract; (E, F) exposed to mangosteen extract; and (G, H) exposed to niclosamide solution. T, tubules; Bc, basophilic cells; Dc, digestive cells; H, hemolymphatic spaces between the tubules; G, dark granules; A, amebocyte cells.

4. Discussion and Conclusions

Our study shows that *C. oleifera* and *G. mangostana* extracts affect the digestive tract, gill, digestive gland, tentacle epidermis and head-foot of *P. canaliculata*. The gill plays an important role in the transport of respiratory gases, and regulates osmotic and ionic balances [11]. In snails exposed to *C. oleifera* and *G. mangostana* extracts, the gill showed dilated interlamellar spaces, enlarged mucocyte cells, dispersed nuclei and damaged cilia (Figure 2C–F). This is in agreement with results obtained in a previous report by Sawasdee and Kohler [12] who reported the presence of copper and lithium in the ramshorn snail *Marisa cornuarietis*, and Tanhan et al (2011) [13] who found cadmium in Babylonia areolata snails after environmental exposure.

The digestive gland of the gastropod plays a role in the internal defense mechanism [14], detoxification processes, and storage of calcium [15] and glycogen [16]. In treated snails, the digestive gland consisted of tubules composed of digestive and basophil cells. The most common digestive cells were columnar type, with numerous vacuoles containing faintly stained inclusions. The basophil cells occurred singly or in groups and contained basophilic granules of various sizes (Figure 4A, B). In both *C. oleifera* and *G. mangostana* groups there were numerous dark granules, an increase of amebocyte cells, and wider hemolymphatic spaces (Figure 4C–F). This is similar to a previous report that found dark granules in the digestive gland of *P. canaliculata* from polluted sediments [17], and another report which showed that several freshwater and marine mollusks (Babylonia areolata) contained higher levels of heavy metals in the hepatopancreas or digestive gland compared with levels in other tissues [13]. It is well established that the digestive gland is the main target organ for the accumulation and detoxification of heavy metals and other toxicants in gastropod mollusks [18].

The tentacle epidermis of snails exposed to either *C. oleifera* or *G. mangostana* extract showed irregular apical surfaces, hypertrophy of cell surfaces, replacement of nuclear cells, loss of cilia, and enlarged lysosomal vacuoles (Figure 3C,D), which is in agreement with Cengiz et al, [19]. The tentacles of the snail act as a sensory organ to feel around the environment, and also have a light-sensitive patch on them that functions as the “eyes” of the snail. There are also olfactory sense cells on the tentacles.

The foot plays an important role in snail movement, while the mouth is located at the head [20]. The foot tissue contains columnar muscle fibers, connective tissue elements and mucus cells (Figure 1A, B). In the foot tissue of *P. canaliculata* exposed to *C. oleifera* or *G. mangostana* extract, there was an increase in the number of lipid vacuoles and columnar muscle fibers were disrupted, resulting in increased mucus production followed by increased mucus cells (Figure 1C–F). There were also changes in the epidermis, such as an irregular surface and desquamation. Desquamation of parts of the epidermis was also reported in *Marisa cornuarietis* exposed to PtCl2 [18]; this was similar to the effect of the pesticides endosulfan, methyl parathion, quinalphos and Nuvan (DDVP), which caused disorganization of the mantle tissues of the snail *Bellamya dissimilis* [21].

In conclusion, exposure of *P. canaliculata* to crude extract of either *C. oleifera* seed or *G. mangostana* pericarp caused destructive effects in the body tissues, as evidenced by the LC50 and LC90 and histopathological changes. Therefore, both crude extracts are effective molluscicides that may be used in the field or on fish farms to control *P. canaliculata* snails. Further studies are required to determine the mode of action of these plant products and the damage they cause to snail tissues, and also the potential side effects on small organisms as well as other ecotoxicological impacts.

5. Acknowledgements

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