The effect of *Camellia Oleifera* on the reproductive system of *Pomacea canaliculata* Golden apple snail

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Abstract :

The aimed of this study is to explore the effect of *Camellia Oleifera* extract in the reproductive system of *Pomacea canaliculata* that can be help to control the negatives impact of *P. canaliculata*. The Golden apple snail (*Pomacea canaliculata* Lamarck) is a freshwater snail in the family of Ampullariidae. They had been introduced to Thailand in the 1980s and reported as severe rice pests in 1988. The department of agriculture of Thailand recommended using Niclosamide as a chemical molluscicide to control the snails. Natural resource such as *Camellia Oleifera* as one of the molluscicides plant should have a benefit to reduce the quantity of Golden apple snail because price friendly, environmental friendly, and safe for human. *Camellia Oleifera* has the special characteristic for golden apple snail because it's high toxicity, specific performance to the target mollusk, convenience to prepare, and leaving no residuals in the environment. The extract of this plant has been applied for the static aquatic toxicology technique within 48 hours was performed in this experiment. The extraction of *Camellia oleifera* has the effect to the histo-pathology of reproductive organs: Ovary and Testis of *P. Canaliculata.*

Keywords: Plant molluscicide, Golden apple snail, *Camellia Oleifera*, Aquatic toxicology, Patho-histology, and Photo-degradation.

1. Introduction:

Along with the oil palm, the olive, and the coconut, *Camellia oleifera*, a significant member of the Theaceae family, is typically regarded as one of the four primary woody oil trees in the world. Their common name is Tea, Thea, Tea oil, and Cha (in Thai). The original habitat is in East Asia their effective parts is leave, fruit, seed, and root. Their active substances are oleic acid, palmitic acid, linoleic acid, stearic acid, saponin, etc. [1-3] In China, tea oil, which is made from the seeds of *C. oleifera*, has long been valued as premium cooking oil. Tea oil is known as "eastern olive oil" because of its comparable chemical make-up to olive oil. It contains a lot of fatty acids such as linoleic, oleic, palmitic, and stearic. [4-7] The majority of these fatty acids significantly cut the risk of heart disease, boost immunity, lower cholesterol, prevents and treats hypertension, and protect against some malignancies. All parts of *C. oleifera* are valued, and its byproducts are also highly important economically in the fields of agriculture, industry, and medicine, according to earlier studies. In addition to its commercial significance, *C. oleifera* has shown a function in improving ecological habitats, conserving water and soil, and preventing biological fires. China is currently under pressure to raise the percentage of self-sufficiency in the production of cooking oil. In China, where its cultivated area has risen to more than 3.7 million hectares, *C. oleifera* has gradually surpassed other woody oil trees due to its excellent tolerance to drought and the barren soil found in hilly areas. Their toxicity and symptoms are awakening, palpitation, constipation, nerve's stimulant, reduce cholesterol and blood sugar, antioxidant and can be cause of the renal failure. [8-12] The toxicological studies of *C. oleifera* as follows 1) 50 mg/kg, 80 weeks of mice (per oral) caused of liver kidney and intestine injury. 2) 5 g/kg, 14 days of rat (per oral) cause of death 3) the methanol extract of *C. oleifera* at 40 ppm could be molluscicidal effect (Figure 1)

The freshwater snail *Pomacea canaliculata*, a native of South America, is one of the top 100 invasive alien species in the world, according to the International Union for Conservation of Nature and the Invasive Species Specialist Group. Their common name is Golden apple snail, Channel apple snail or Hoy Cherry (in Thai).[13-16] (Figure 2A) The snails were introduced as an aquarium pet or human food to numerous nations in North America, Europe, East Asia, and Southeast Asia after the 1980s. They have a varied diet that includes plants and aquatic

macrophytes, as well as small snails and other aquatic creatures as prey. *P. canaliculata* also exhibits high fertility and rapid growth in invaded wetlands, which are extremely adaptive to adverse environmental circumstances such as low dissolved oxygen concentration, high nutrient content, limited food supply, and temperature. These traits are combined with a lack of effective natural adversaries. Prior research on the biological traits of *P. canaliculata* during various seasons has mostly concentrated on the host's growth, reproduction, and temperature adaptability.[17] In order to improve hatchling survival over the winter, there were fewer eggs per egg mass. In comparison to the winter, *P. canaliculata* hatchling survival was higher in the summer, primarily because of the impact of the outside temperature. In this work, we aimed to explore the effect of extract *C. oleifera* in the reproduction system of *P. canaliculata* that can be help to control the snails.[18-22]

The reproductive system or the gonad of the Golden apple snail looks like a dark gray band attached next to the digestive gland at the end of the body. First, The male reproductive organ or testicular tissues are surrounded by connective tissues called spermatogonium, which is composed of sperm cells and Sertoli cells. The development of sperm starts from spermatocyte to spermatid and spermatozoa respectively. Second, The female reproductive organ or oogonium is ovary that composed of egg cells or ovum and follicle cells. The egg cells are round and have a huge eucentric nucleus, the cytoplasm revealed a purple tone from H&E staining and has some small granules. The follicle cells or niche cells are scattered among the ovum, they function in producing eggshell components. (Figure 2B). [23-24]The chemical molluscicide that used in this experiment is Niclosamide. The fact sheet summarizes the information in the RED document for preregistration case 2455, 2-amino ethanol salt of 2',5'-dichloro-4'-nitro salicylanilide (Niclosamide). Niclosamide was first registered as a pesticide in the U.S. in 1964 by the U.S. Department of Agriculture. Niclosamide was used as 1) a lampricide to control sea lamprey larvae in tributaries to the Great Lakes, the Finger Lakes, and Lake Champlain and 2) a molluscicide to control freshwater snails which carry the vectors for diseases that affect fish and humans. Less than 400 pounds of active ingredient is used each year in lamprey and freshwater snail treatments. Niclosamide has been used as a human and veterinary drug for the treatment of parasites. (Figure 3 and Figure 4).[25-27]

2. Materials and Methods

2.1 All chemicals and reagents used in this research are of analytical grade.

2.2 Collection of and culturing the snails

Adult snails of *P. canaliculata*, ranging in size from 3.5 to 5 cm, will be collected from the rice field habitats located at Amphoe Muang, Khon Kaen. Then the snails will be transferred to the animal's house. After gentle cleaning, 70 snails will be cultured in each tank. The snails will be allowed to acclimate to the laboratory conditions for 4 weeks. The snails will be stopped from being fed for 24 hours before being treated. Snails will be fed soft vegetables such as Chinese cabbage, lettuce, morning glory, etc. The vegetables will be cleaned up before feeding. The snails are fed once every day in the afternoon. The food debris that is not consumed by the animals will be removed from the well every morning. The water will be replaced once every week. After mature snails are acclimatized to the environment, which takes around 6 weeks, they will lay their eggs on the ridge of the container. Normally, they lay eggs at night. After that, eggs will be collected, moved to the plastic net (1 mm mesh), and laid on the 5-liter plastic container filled with water. After 7 days, the snails will hatch, and then they will fall into the water. Snails in this period are very tiny animals, around 0.1 mm in diameter. A few days after hatching, they will start to eat. They will be fed small pieces of soft vegetables.

2.3 *Camellia oleifera* preparation

The seed shells of *Camellia oleifera* were collected from the natural condition at Amphoe Muang, Khon Kaen, with the coordinates from Google Maps (16 26` 22.6500 N and 102 49` 43.4208 E). After collecting the leaves, they were cleaned with DI water and wiped with a soft cloth before being dried in an oven at 60 ^{o}C for 48 hours and ground into powder. The final product has been kept in the desiccator.

2.4 The acute toxicity tests

Golden apple snail will be divided into 3 groups: a control group, a test group, and a positive control group. For the positive control in this research using Niclosamide, ten snails will be intoxicated by being placed in the 10-liter plastic containers filled with 5 liters of each dilution of plant extract at room temperature. Each test will be set up with three replications. A group of controls and a positive control were also tested. After 48 hours, the treated snails will be removed

from the solutions, washed, and transferred to the new containers for another 24 hours. The number of dead and alive snails will be carefully inspected.

2.5 The anatomy of the golden apple snail

Golden apple snails are transferred into 600 ml of DI water after treatment in the various conditions; add 12 g of menthol pellets for anesthetizing the snails, and wait 2 hours until they become unconscious. Shells are carefully removed and stretched out on the paraffin plate with pins. Gonad was represented for the reproductive system, will be removed and transferred to fixatives (10% buffered formalin). The tissues will be left in fixatives for 24 hours.

2.6 Tissue preparation for histological study

Gonads that have been collected have been moved into the freezer for a few hours. A rotary microtome will section the paraffin blocks for 3-5 m. The paraffin ribbons will be floated in a 55–60 ^{o}C water bath. Then, the perfect pieces of ribbon will be selected, attached to the glass slide, labeled, and dried in the 37 ^{o}C incubator for 48 hours. The dried glass slides will be stored in cold and dry conditions or kept in the refrigerator.

Result and Discussion

The water extract of *C. oleifera* showed a sub-lethal concentration of 4.0 ppm, a median concentration of 6.21 ppm, and a lethal concentration of 10 ppm. (Table 1) These records were similar to the report of Chunyapes who reported the absolute lethal concentration of *C. oleifera* to control *P. canaliculata* at 40 ppm within 24 hours in the rice field. And also compatible with, the studies of Charoenkried, who reported the hot water, oil, or alcohol extraction of *Nicotina tabacum, Azadirachta indica, Sapindus emarginatus*, and *Durio zibithinus* can kill Golden apple snails at the 500-2,000 ppm at 72 hours. Consequently, the water extract of *C. oleifera* revealed the highest molluscicidal effect than other plant extracts and easier preparation than alcohol or oil plant extraction. The method of extraction should be simple, uncomplicated including the basic material tools and soluble solvent, probably water extractions.

In accordance with Pereira who reported that the 50 essential oils extracted from 46 plant species were investigated for plant molluscicidal activities, against 2 snails of the genus *Bulinus* and *Biomphalaria*. The oil extract of plant molluscicides that showed the highest toxicity was hydrocarbon monoterpenes and oxygenated monoterpenes. In addition, there were many reports

that showed the molluscicidal effect to control Golden apple snails. The researchers tried to develop traditional medicinal plants such as *Chromolaena odorata*, *Euphorbia tirucalli*, *Thevetia peruviana*, etc. to be plant molluscicides. But some plants showed strong poisonous effects on humans and animals such as *Nerium oleander, Duranta erecta, Erythrophleum succirubrum, Lantana camera*, etc.

The positive control, Niclosamide, showed a sub-lethal concentration of 0.10 ppm, a median concentration of 0.42 ppm, and a lethal concentration of .10 ppm. (Table 1) Compatible with the report of Chunyapes who studied Niclosamide on *P. canaliculata* in the rice field, revealed the LC_{50} at 24 hours as 0.62 ppm. When considering LC_{50} of both experiments showed a slightly different; it might be depended on the conditioning and surrounding of the experimental method. The rice field had other factors to determine the snail's mortality such as temperature, sunlight, soil absorption, approached time, etc. Another chemical that affects *seminole ramshorn* snails (*Planorbella duryi*) approached acute toxicity of methoxychlor exposure within 48 hrs. Showing, the abnormalities of the survival rate, reproduction activity, and locomotion behavior. Synthetic and plant molluscicides were quick and convenient effective methods to control and prevent the spread of Schistosoma snails in many areas. However, the development of molluscicidal plants now a day could be an innovative method for snails and Schistosoma controlling.

Golden apple snails were a dioeciously animal. Their gonad was attached next to the end of the digestive gland. The male reproductive system of *P. canaliculata* was composed of the testis, vas deferens, prostate gland, and penile sheath. (Figure 2B) The histology of testis in the control group was composed of testicular tissue surrounded by connective tissue. Inside spermatogonium were composed of 1) Spermatocyte was the largest cell, the nucleus was huge, eucentric, and strongly H&E staining with a small amount of cytoplasm. 2) Spermatid was a smaller cell, a small nucleus with strong H&E staining. 3) Spermatozoa were the smallest cell and looked like a dark spot with a long tail. The Sertoli cell or the supporting cell was a large cell. The nucleus was an oval shape, usually found at the edge of the tube. (Figure 5A) Rezende described the morphology of testis, spermatozoa, and spermatogenesis in the apple snails *Pomacea dolioides* and *Pomacea diffusa.* Each spermatogonia was surrounded by connective tissue, inside the lumen contained spermatocyte, spermatid, and spermatozoa including Sertoli cell that was located beside the wall.

The histo-pathology of the male reproductive organ in the intoxicated group revealed the tissue alterations as follows: the connective tissue surrounding the testicular tube was lifted and peeled. 1) Spermatocyte cells were increasing in size the nucleus was enlarged and degenerated. 2) Spermatid cells were enlarged and had an increasing amount of cytoplasm. 3) Spermatozoa were unnoticeable changing. The Sertoli cells were increasing in size with the enlarged nucleus. (Figure 5B,C) The spermatocyte, spermatid, and Sertoli cells were increasing in size the nucleus was enlarged and degenerated. These results were compatible with Wangsomnuk who reported the alterations of *Indoplanorbis exutus* testis, there was a lifting of the spermatogonia membrane and the nuclear disintegrating of spermatocyte and spermatid cells. The spermatozoa cells were contracting with shrinkage tails. Rondelaud also reported on the alteration of testicular tissue of *Lymnaea glabra*, there had degenerated nuclei of the different stages of the male reproductive cells with some necrotic areas. The results were similar to Abiona who reported the giant African land snail (*Archachatina marginata*) fed with *Mucuna pruriens*. The results showed the histo-alteration in the ovotestis organ that increased the number of degenerative vacuoles, areas of necrosis, and spermatozoa death. Female reproductive system of *P. canaliculata* was composed of the ovary, oviduct, receptaculumseminis, and albumin gland. The albumin gland in mature female snails showed a large size and red-pink color. (Figure 2B) The histology of the female reproductive organ in the control group was composed of oogonium tissue surrounded by connective tissue. Inside the ovary contained the oocyte or egg cell found around the oviduct. The egg cells were round and large, the nucleus was also large with dark blue staining. The cytoplasm was abundant with contained multiple small granules. The follicle cells were small and situated close to the edge of the ovary. The histo-pathology of the female reproductive organ in the intoxicated group. The abnormalities were similar to the male reproductive system. The connective tissue surrounding the oogonia was lifted and peeled. The oocyte cells were increasing in size with the enlarged and degenerated nucleus. The cytoplasm was enlarged and increased in a number of small granules. The follicle cells had degenerated. (Figure 6B,C) The results were consistent with the experiment of Wangsomnuk that reported the histological changing in the female reproductive tissues *of I. exustus.* There was an increase in the degenerating of the oocytes than normal and the lifting of the oogonium membrane.Also compatible with Rondelaud reported the disintegrating of the nucleus and oocyte cells with multiple necrosis areas of *lymnaea glabra* that intoxicated with the sub-lethal dose of

molluscicide. Additionally with the study of Gao who reported the acute and chronic toxicity of Cu might be the cause of death of pulmonated snails, *Physa acuta*. The acute intoxication of the mature *acuta* snails, with Cu 23.8 ppm within 96 hours could produce polynuclear egg cells, decreased shell length, and reduced the success rate of egg hatching.

4.Conclusion:

Camellia Oleifera that one of the plants that growth in Thailand has been study for the effect on reproductive organ (ovary and testis) of *Pomacea canaliculata* has been study in the research. The structures of the compounds are determined by spectroscopic and spectrometric analyses as well as comparisons with the previously published data. The correlation between the saponins and their molluscicidal activity is also unveiled. The robust molluscicidal activity of these isolated saponins exhibited LC_{50} values ranging from 7.90 to 17.50 µg/mL. Experiments show that at the median lethal concentration for *P canaliculata*, the active ingredients have no significant impact on the aquatic environment. Specifically, the LC_{50} values of the compounds for brine shrimp (*Artemia* sp.) range from 148.55 to 193.22 µg/mL. In addition, the data obtained from the experiment also demonstrate the safety of the *A. armata* root extract. Based on molecular docking, *P. canaliculata* acetylcholinesterase and villin may be considered molecular targets of the *A. armata* saponins isolated and characterized in this work. There is much that is still unknown about the proteomics of *P. canaliculata*, however, and there may be other, yet uncharacterized, protein targets accounting for the molluscicidal activity of *A. armata* components. The obtained results illustrate that the saponin compounds from *A. armata* roots in Vietnam turn out to be potential compounds with excellent efficacy and safety for agricultural applications.

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Author contributions

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Consent for publication, The authors approve the publication of this manuscript.

Competing interests, The authors declare no competing interests

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Figure 1 : *Camellia oleifera*

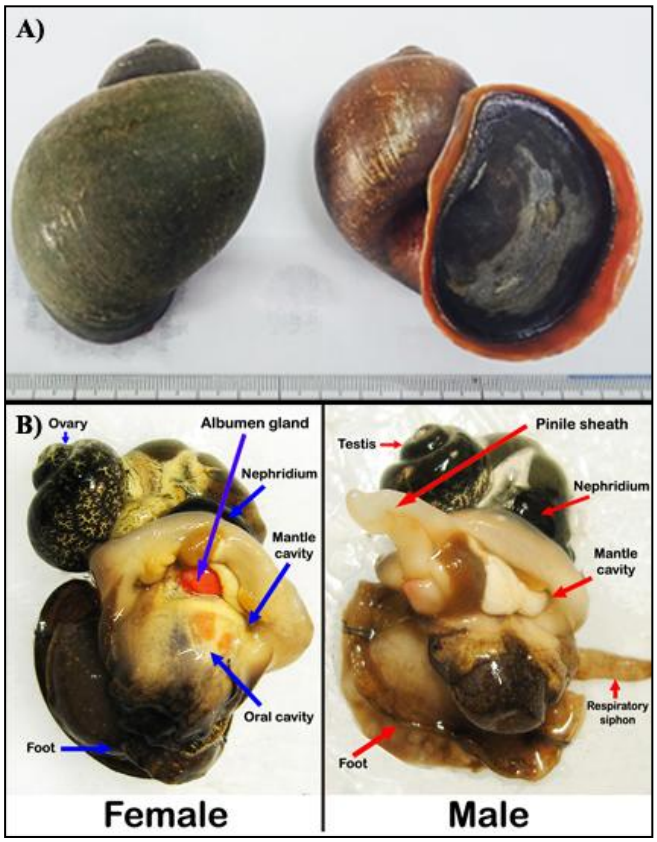

Figure 2 : (A) *P.canaliculata snail* (B) The different reproductive organs of *P.canaliculata* between male and female.

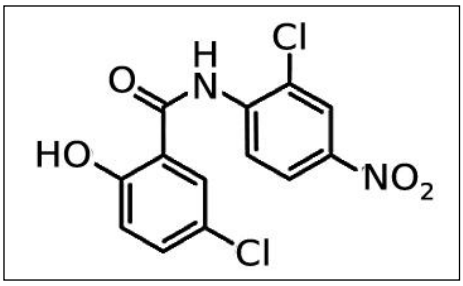

Figure 3 : The chemical structure of Niclosamide

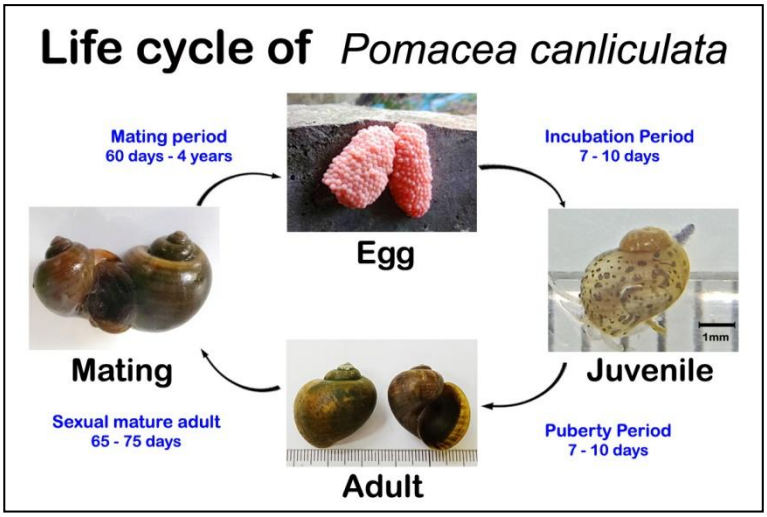

Figure 4 : The life cycle of *Pomacea canaliculata*

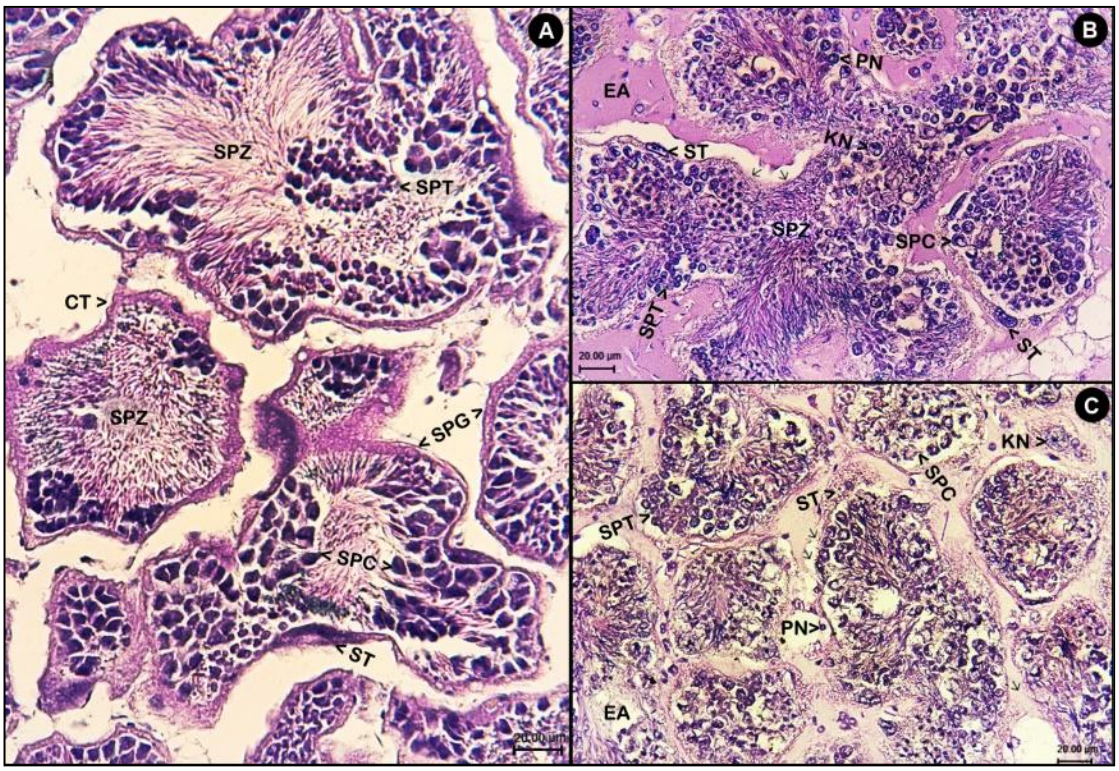

Figure 5 : The image shows high magnification (40X) of the testicular tissue. A) The histology of testis in the control group with H&E staining. B) The histo-alteration of testis in the intoxicated group with H&E staining. C) The histo-alteration of testis in the intoxicated group with PAS staining. SPC = spermatocyte, SPT = spermatid, SPZ = spermatozoa, SPG = spermatogonium, ST = Sertoli cell, CT = connective tissue, PN = Pyknotic nucleus, KN = karyolytic nucleus, EA = eosinophilic area, (←) = cell lifting, V = vacuole

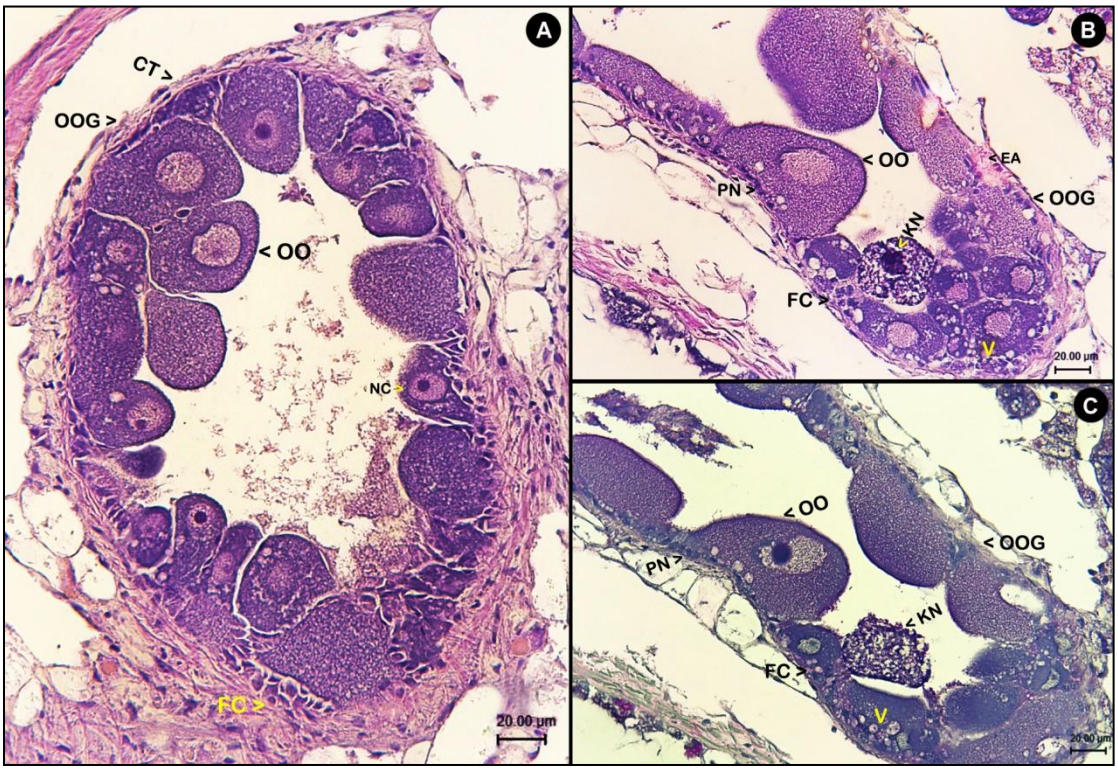

Figure 6 : The image shows high magnification (40X) of the ovary tissue. A) The histology of ovary in the control group with H&E staining. B) The histo-alteration of ovary in the intoxicated group with H&E staining. C) The histo-alteration of ovary in the intoxicated group with PAS staining. OO = oocyte, OOG = oogonium, NC = nucleolus, PN = pyknotic nucleus, KN = Karyolytic nucleus, CT = connective tissue, FC = follicle cell, V = vacuole

Table 1 : The lethal concentration of *Camellia oleifera* and Niclosamide to control Golden apple snails within 48 hours

Molluscicide	**Sub-lethal concentration**	**LC_{10}**	**LC_{50}**	**LC_{90}**	**Absolute-lethal concentration**
Camellia oleifera	4.0	4.75	6.21	8.11	10
Niclosamide	0.10	0.21	0.42	0.79	1.0