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# Mechanisms of Molluscicidal Activity of *Citrus hystrix* against *Pomacea canaliculata*

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## Abstract

*Pomacea canaliculata* (Golden apple snail) is an invasive pest that seriously affects rice cultivation. It causes one of serious agriculture problems in many Asian countries. To control these snail populations, synthetic chemical molluscicides were mainly used. However, these molluscicides may cause long-term disadvantage to the environment and also impact on ecological system. Finding natural substances that kill golden apple snails, therefore, is an alternative way to decrease number of snails and their epidemic. The molluscicidal activity of peels and pulps *Cirus hystrix* (C. hystrix) extracts on *Pomacea canaliculata* were evaluated under controlled laboratory conditions. *C. hystrix* peel methanol extract was toxic to the snail with $LC_{50}$ 71.66 mg/L. Conversely, no snail was found dead in *C. hystrix* pulp methanol extract. The molluscicidal mechanism of *C. hystrix* peel methanol extract on *P. canaliculata* was also evaluated through change of selected biochemical parameters, including acetylcholinesterase activity, glycogen and protein contents. There was no significantly change in any biochemical parameters. These results suggest that methanol extracts from peels of *C. hystrix* had molluscicidal activity which could be utilized as bioherbicide for future weed control. However, the mechanisms of molluscicidal activity were still unclear.

**Keywords:** *Citrus hystrix, Pomacea canaliculata, molluscicidal activity, toxicological mechanism*s

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## 1. Introduction

*Pomacea canaliculata* L, golden apple snail, is an invasive pest which seriously destroyed the wide range of crops of agriculture, especially rice in many Asian countries (Halwart, 1994; Naylor, 1996). Due to high reproductive capacity, quick growth and consumption of young rice seedling, these snails can cause extensive damage to both transplanted and direct-seed rice (Sin, 2003; Joshi *et al*., 2008). In addition, *P. canaliculata* is the vector for the human eosinophilic meningoencephalitis (Joshi *et al.*, 2005). In order to control these pest snails, synthetic molluscicides and insecticides have been widely used, but these are toxic to non-target organisms and may cause serious environmental hazards. Recently, much attention has been given to research on molluscicidal activities of some plants, because they tend to be biodegradable and safe for human and enviro nment (Loh *et al.,* 2011; Li *et al.,* 2012).

There are many classes of flavonoids in citrus fruits. Citrus flavonoids exhibit more biological activities such as antioxidant, antimicrobial and anti-inflammatory (Benavente-Garcia *et al*, 1997; Ram & Singh, 2006). In addition to flavonoids, citrus fruits also contain significant amount of limonoids (Manner, 2007). Limonoids have been reported to possess substantial anticancer, antiviral and insecticide activities (Jayaprakasha *et al.* 1997; Roy & Sara, 2006; Hafeez, 2011). The methanolic extract of leaves of *Citrus hystrix* DC, (kaffir lime) is known to inhibit herpes virus (Chowdhurry *et al*, 2009) and also used as mosquito repellant (Tawtsin *et al*, 2001). Hence, the objective of the present study is to evaluate, in laboratory, the molluscicidal activity of *C. hystrix* from peels and pulps against *P. canaliculata*, and to focus on the mechanism of molluscicidal activities of *C. hystrix*.

## 2. Materials and methods

### 2.1 Preparation of plant extraction

Fresh fruit of *C. hystrix* at the commercial mature stage were gathered from Nakhon Pathom, Thailand. They were cleaned several times with water, separated into pulps (*C. hystrix* residues) and peels. For the preparation of *C. hystrix* residues, fruits were cut into half and squeezed by hand-pressed juice extractor. Each parts of *C. hystrix* were dried at 60°C and crushed into small pieces with electric blender. Dried materials were extracted with 80% methanol (1 g of sample for each 10 ml of solvent) and stirred by shaker at 60 rpm for 72 h at 25°C. Each two extract solutions were concentrated with rotary evaporator and finally evaporated. Each concen trated crude extract was kept at -20°C until use.

### 2.2 Molluscicidal assay

2.2.1 Test animals

Egg masses of snails (*P.canaliculata*) were collected from ponds in Silpakorn University, Nakhon Pathom, Thailand. After hatching, the golden apple snails were collected under laboratory condition. Snails were fed with leaves of morning glory. The active snails, with a shell diameter 3–6 mm, were chosen for the molluscicidal assay.

2.2.2 Molluscicidal activity evaluation

The molluscicidal assay was deter mined through the briefly modified method described by World Health Organization (WHO) (WHO, 1965). The crude extracts were dissolved and diluted with dechlorinated water to obtain the test concentrations. Copper sulfate was used as the positive control. Ten snails were placed in each glass aquarium, containing 50 mL test solution, covered with mosquito net on the top. After indicated time exposure, snails were removed from test solutions to dechlorinated water and kept for 24 h for recovery. The immobile snails were identified as dead snails. During experiment, dead snails were removed to avoid contamination of aquarium water. Each treatment was replicated three times. Snails were deprived of food during the experiments.

**2.3 Acetylcholinesterase (AChE) activity assay**

The activity of AChE was assayed by measuring the formation of yellow color compound produced from thiocholine when reacted with dithiobisnitrobenzoate ion. AChE activity was determined according to the method of Ellman et al, 1961. Briefly, homogenate of snails (50 mg/ mL) was prepared in cold 0.1 M phosphate buffer pH 8.0. The homogenate was centrifuged at 1,000 g for 30 min at 4°C. For 0.1 mL of supernatant, 2.9 mL of phosphate buffer containing 0.1 mL DTNB was added. The absor bance of the mixture was measured at 412 nm.

**2.4 Glycogen assay**

Glycogen was estimated by the anthrone method of Van Der Vies (Van Der Vies, 1954), with minor modification. Briefly, snail homogenate (10 mg/ml) was prepared in cold 5% trichloroacetic acid (TCA). The homogenate were filtered. 2 ml of filtrated and 2 ml 30% KOH were incubated in boiling water for 1 h. After cooling, 1 ml of glacial acetic acid was added to neutralize solution. 2 ml of the mixed solution were added slowly to 4 ml of anthrone reagent in test tube. After mixing, the tube was incubated in boiling water for 10 min and cooled in ice bath. Using glucose as standard, the absorbance of the mixture was measured at 620 nm. Values were presented as mg/g of tissue.

**2.5 Total protein assay**

Total protein assay was measured using commercial BCA protein assay reagent kit according to the manufacturer's protocols. Using bovine serum albumin as standard, total protein was measured at 562 nm. Values were presented as mg/g of tissue.

**2.6 Statistical analysis**

Each treatment of this study is carried out with three replications, and the experiments were repeated three times. All treatments were conducted in a completely randomized design. Data from this study was subjected to one-way ANOVA.

## 3. Results and discussion

**3.1 Molluscicidal activity**

Each peels and pulps methanol extractions of *C. hystrix* were tested for their molluscicidal activity against *P. canaliculata*. Mortality was reported on Probit probabilities and plotted against the log-transformed values of

extract concentrations. For 24 h exposure of *C. hystrix* peel methanol extract, in average from total extract concentration 20 snails were found to be dead. Number of dead snails was dependent on increasing extract concentrations. The $LC_{50}$ values when snails were exposed to *C. hystrix* peel methanol extract was 71.66 mg/L (Figure 1). In contrast to *C. hystrix* peel methanol extract, no snail was found dead in *C. hystrix* pulp methanol extract. Neither higher concentrations of pulp extract (100 – 1000 mg/L) nor more exposure time (48 h) was found to be fatal to the snails. It might be assumed that *C. hystrix* pulp methanol extract did not have molluscicidal activity.

In addition to flavonoids, citrus fruits also contained significant amount of limonoids (Manners, 2007). Compared between peels and residues after juice extraction of *C. hystrix* pulps, the amounts of limonin were different significantly (Chinapongtitiwat *et al*., 2013). Limonin was one of the most prevalent citrus limonoids in all citrus fruit tissue. The amount of limonin varied widely depending on the variety and part of citrus fruit (Ohta & Hasegawa, 1995, Sun *et al*., 2005). Limonin had been reported to possess biological activities, including insecticidal activity (Jayaprakasha *et al.,* 1997, Hafeez, 2011). In the present investigation, no molluscicidal activity in *C. hystrix* pulp methanol extract might be due to lower amount of limonin in this part of citrus fruit.

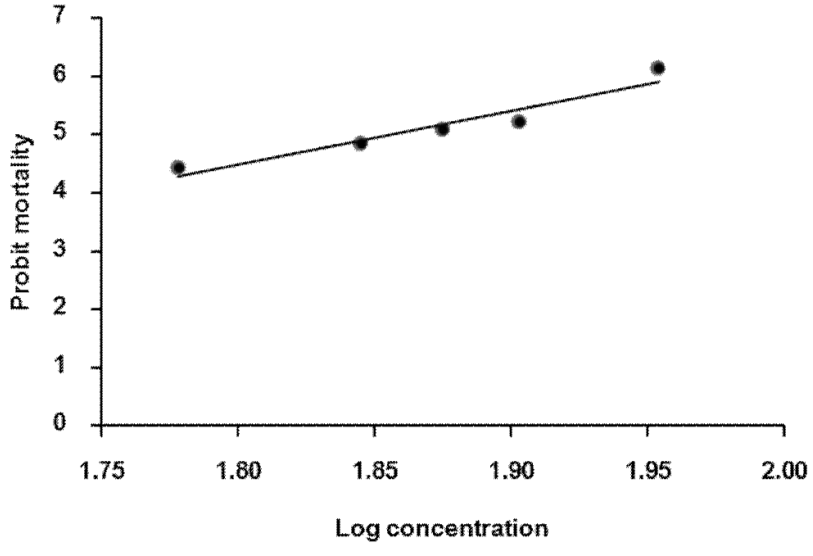


**Figure 1** Probit analysis of mortality of *P.canaliculata* plotted against the logarithms of *C. hystrix* peel methanol extract concentrations. The regression line (y = 9.2417x – 12.146) is used for $LC_{50}$ determination.

### 3.2 Effect of *C. hystrix* peel methanol extract on biochemical analysis

Snails were exposed to sublethal concentrations of *C. hystrix* peel methanol extract. At the end of 24 h of exposure, snails were collected for biochemical analysis. AChE activity, glycogen content and total protein content were evaluated. In this study, there were no significantly change in any biochemical analysis in snail tissues (Figure 2, 3 and 4), although, it was found that molluscicidal activity of *C. hystrix* peel methanol extract was concentration dependent effect.

Recently, molluscicidal mechanisms had been proposed for molluscicidal activities of some plants. *Ginkgo biloba* L. inhibited the gene expression of four mitochondrial enzymes and caused mitochondrial dysfunction (Li *et al*., 2012). *Nerium indicum* decreased AChE activities and decreased glycogen content (Dai *et al*., 2011). *Sapindus mukorossi* and *Terminalia chebula* inhibited AChE, acid and alkaline phosphatase activity in the nervous tissue (Upaphyay *et al*., 2011). In general, the majority of mechanisms of toxicants were determined by these biochemical analyses. AChE was an important enzyme in snails affected by various molluscicidal components of plant such as acetogenin, azadirachtin. Glycogen assay was suitable for measuring stressful levels of pollutants and have been used as indicators of stress (Saravanan *et al*., 2010). Total protein content in tissues could be used to determine physiological changes of organisms (Suryavanshi *et al*., 2009). In this study, however, *C. hystrix* peel methanol extract might change other biochemical or physiological pathways of snail tissues, and the mechanism of action of *C. hystrix* remains to be determined.

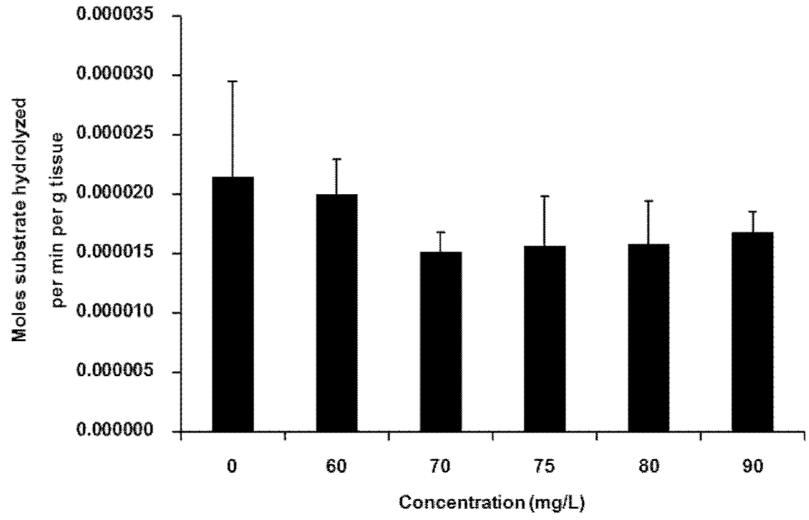


**Figure 2.** Effect of *C. hystrix* peel methanol extract on cholinesterase activity in *P. canaliculata* tissues.

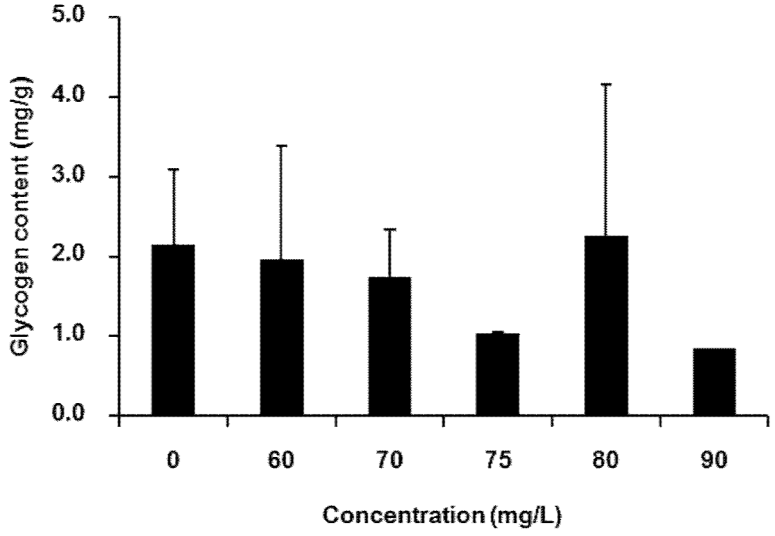


**Figure 3.** Effect of *C. hystrix* peel methanol extract on glycogen content in *P. canaliculata* tissues.

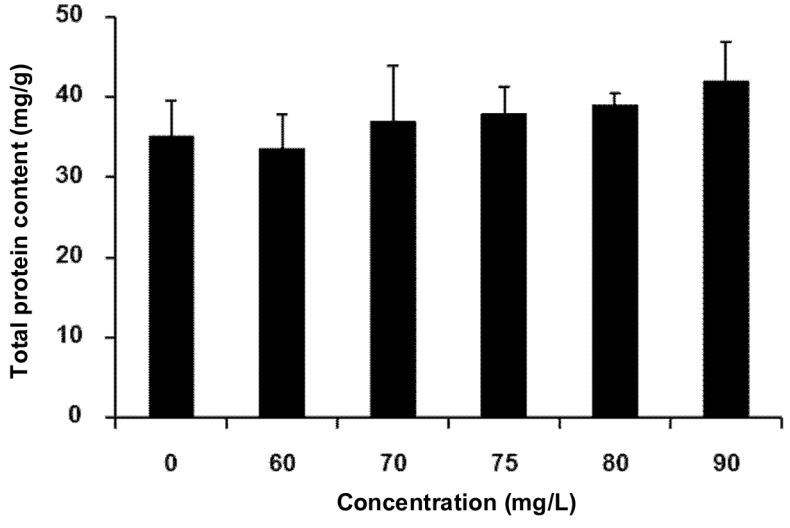


**Figure 4.** Effect of *C. hystrix* peel methanol extract on total protein content in *P. canaliculata* tissues.

## 4. Conclusions

In summary, only *C. hystrix* peel metha nol extract had molluscicidal activity against *P. canaliculata* while pulp methanol extract did not show molluscicidal activity. Its molluscicidal mechanisms might be not related to the alteration of some key enzyme activities, as well as biochemical composition contents, such as glycogen, protein and AChE. Increase in mortality with increasing in exposure time might be due to more uptake of active, toxic compounds in the snail tissues. It might be possible that the active

compounds could change into more toxic forms in the aquarium or in snails by the systemic action of different enzymes. Therefore, other toxic mechanisms as well as molluscicidal activity under field conditions should be studied further.

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