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Studies on the Allelopathy of Triterpene Sapogenin from *Pistacia chinesis* Bunge to *Oncomelania hupensis*

Shangguang Xia[1], Yong Zhang[1,2]* and Wanxian Wang[3]

[1]Anhui Academy of Forestry, Hefei 230022, China.
[2]School of Environment and Energy Engineering, Anhui University of Architecture, Hefei 230022, China.
[3]Faculty of Life Science, Hubei University, Wuhan 430062, China.

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In this study, we analyzed allelopathic effects of *Pistacia chinesis* Bunge on *Oncomelania hupensis* through triterpene sapogenin, a potential molluscicide. The snails were exposed to six different concentrations (0, 2, 5, 10, 20, and 40 mg/L) of triterpene sapogenin and under five periods (24 h, 48 h, 72 h, 96 h, and 120 h). The mortality of snails was positively correlated with the concentration of triterpene sapogenin and exposure time. The liver and intestine observation through transmission electron microscope displayed that 10 mg/L triterpene sapogenin caused apparent damages to the structure of *O. hupensis* liver. The esterase (EST) isozyme activity of *O. hupensis* treated by 10 mg/L triterpene sapogenins was higher than that of control between 24-48 h, then decreased after 72 h. It implicates that the extracted triterpene sapogenin from *Pistacia chinesis* Bunge is promising for controlling the snail. Meanwhile, it provides the foundation for constructing plant community of *Pistacia chinesis* to control *O. hupensis*.

Key words: *Pistacia chinesis* Bunge, *Oncomelania hupensis*, Triterpene sapogenin, Molluscicidal activity.

There are more than two million people infected with schistosomiasis in the world. Most of them are from Africa, Asia, and tropical America where schistomiasis is endemic. Since snail (*Oncomelania hupensis*) is the only intermediate host of *Schistosoma japonica*, killing snail is an efficient way to control schistosomiasis[1–5]. Sodium pentachlorophenate and niclosamide are restricted in China due to their high toxicity to non-target organisms, inconvenience to apply and expensive cost[6,7]. On the other hand, the snail-elimination project through physical method is giant and resources intensive. We have observed that a number of plant-derived molluscicides, such as *Radix phytolaccae*, *Brassica napus and Cassia tora Ginkgo biloba* powder, *Nerium indicum* bark powder, and some binary combinations are potent molluscicides against the harmful snail[8-11].

Pistacia chinesis Bunge widely distributes in China as woody oil plants, which are suitable for growing on marginal soils. They have been considered to be ideal plants for second generation biofuel. *P. chinensis* can grow in temperate, subtropical, tropical regions and can be well adapted to harsh conditions and poor quality soils. Meanwhile *P. chinensis* has some comprehensive advantages in molluscicidal yield[12].

* To whom all correspondence should be addressed.
Tel.:86-18255124596; Fax:86-551-63828122;
E-mail: zhangy@ahjzu.edu.cn

Previous studies showed the active component, triterpene sapogenins extracted from *P. chinensis* is anti-bacterial, anti-tumor[13], and lethal to snail[14,15]. In our study, the molluscicidal activity and allelopathic mechanism of triterpene sapogenin extracted from *P. chinensis* on snails are evaluated.

MATERIALS AND METHODS

Materials

P. chinensis were collected locally and identified by Anhui Academy of Forestry. Adult snails *O. hupensis* (9-11mm in length) were collected from Dongting Lake, Hunan province, China. The snails were kept in the laboratory at 22 °C for one month before being used in experiments.

Triterpene sapogenin extraction from *P. chinensis*

Triterpene sapogenin extracted from the fresh leaves of *P. chinensis* were used in experiments. Cleaned fresh leaves were meshed by grinder and immersed in 70%-75% ethanol [1:10 ratio (*w*/*v*)] at 70 °C for 12 h and filtered three times. The filtrate was de-estered by ether, then extracted by *N*-butanol and concentrated to get raw triterpene sapogenin. The raw crystal was dissolved in dechlorinated water and passed through D type-column with large holes resin absorbent washed by 70% ethanol. In order to get pure crystal of triterpene sapogenin, the collected washing liquid was decolored by silica gel and dried through evaporating ethanol. 100 g dry *P. chinensis* leaves were found to contain 101.32 mg triterpene sapogenin.

Molluscicidal assay

Six concentrations of triterpene sapogenin from *P. chinensis* (0, 2, 5, 10, 20, 40 mg/L) were applied in molluscidal assay. Experiments were performed in 23 °C. 100 snails were put into five nylon mesh bags (mesh size 2 mm) and immersed into 2000 mL dechlorinated water solution of different triterpene sapogenin concentration. After 24 h, 48 h, 72 h, 96 h and 120 h, mortality were checked respectively. No response to a needle probe under dissecting microscope was the evidence of snail death. The data were subject to the probity analysis and the LD_{50} values were calculated. SPSS program (SPSS 13.0) was used for all statistical analysis. Two-way ANOVA was used to examine the effects of treatment on all parameters.

Sample preparation for transmission electron microscope

Snails were treated with 10 mg/L triterpene sapogenin from *P. chinensis*. After 12 h, 24 h and 48 h, the livers and intestines were peel off and cut to 1 mm^3 block carefully under optical microscope. After fixed, washed, and dehydrated as described above, the blocks were also dehydrated twice by 100% acetone. The selected sections were sequentially immersed with cyclooxygenresin 812: acetone (1:1) for 30 min, and then embeded for 1 h and solidified (37 °C 24 h and 60 °C 48 h). The block was cut into slices by a LKBV cutter. The dyed slices were examined by H-600 electron microscope (Hitachi, Japan).

Esterase (EST) Isozyme Electrophoresis Assay

50 snails were dealed with lower LD_{50} concentration. Dead snails were removed to avoid contamination of aquarium water during the experiment. After 12 h, 24 h and 48 h exposure period, several living snails were chosen randomly for EST assay. The chosen snails were crushed to get soft tissues. 0.2 mol/L phosphatic buffer solution (pH 7.4) was added at 1 drop/snail. The ice-based homogenates were centrifugated at 8000 rpm for 10 min on 1 °C. Bromophenol blue as indicator was added to the supernatant. 10 ¼L of each sample were analyzed by Polycrylamide gel electrophoresis (PAGE) with a Hoefer Vertical Electrophoresis System SE 600. The separating gel consisted of 7.5% Acrylamide/Bisacrylamide and 0.04 M Tris-HCL pH 8.8. The stacking gel consisted of 3.75% Acrylamide/Bisacrylamide) and 0.01 M Tris-HCL pH 6.8. The electrophoresis buffer was Tris-Glycine buffer pH 8.3.

RESULTS AND DISCUSSION

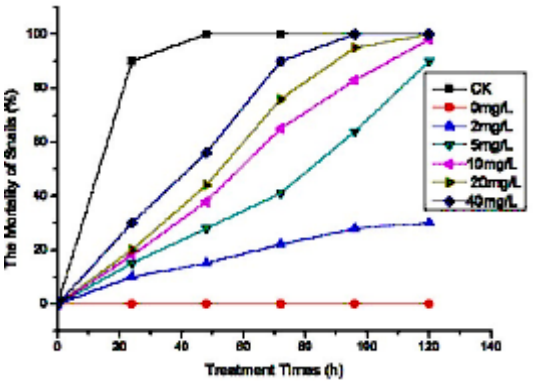
Molluscicidal Activity

The results showed that both the concentration of triterpenoid saponin and the time of treatment significantly influenced the mortality of *P. chinensis* (Tab. 1; all, $p < 0.01$). After 3 or 5 days' treatment, triterpenoid saponin higher than 20 mg/L killed snails completely, which is in accordance with the effects of 1 mg/L niclosamide treated for 1 or 2 days (Fig. 1). Furthermore, allelopathic effects of *P. chinensis* on snails via triterpenoid saponin depended on exposure time and concentration of triterpenoid saponin. The

Table 1. Variance analysis of killing effect of triterpene sapogenin from *P. chinensis* to *O.hupensis*

Source	Type III Sum of Squares	df	Mean square	F	$F_{0.01}$
Concentration(C)	45274.327	5	9054.865	146.254**	8.507
Time (T)	22408.154	4	5602.038	87.633**	10.184
C×T	8754	20	437.7	11.264**	5.255
Error	1764	60	29.4		
Corrected Total	78200.481	89			

** represents statically significant at $P < 0.01$



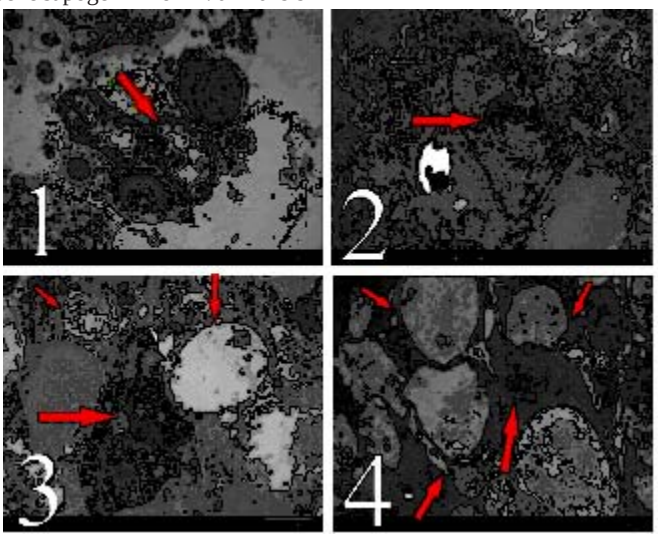
ck, treated by 1 mg/L niclosamidum. The others were treated by H_2O, 2 mg/L, 5 mg/L, 10 mg/L, 20 mg/L, 40 mg/L of triterpene sapogenin from *P. chinensis*, respectively.

Fig. 1. The mortality of Snails after treated by different concentrations of triterpene sapogenin from *P. chinensis*

probit analysis results showed that the LD_{50} of triterpenoid saponin from *P. chinensis* by immersion for 2 d, 3 d, 4 d, 5 d were 38.64, 29.18, 15.34, 7.26 mg/L, respectively.

***O. hupensis* liver and intestine observation**

After treated with 10 mg/L triterpene sapogenin, both liver and intestine of *O. hupensis* showed evident destructions in cell structure under observation from scanning electron microscope (Fig. 2). After snails were treated for 24 h, we found out that the nuclear was swollen up, nucleolus decomposed, nucleoplasm reduced. rough endoplasmic reticulum (rER) destroyed and vesiculated. The amount of mitochomdrias in liver cells were higher than those in normal ones. The damages were more severe than before after 48 h treatment. rER were almost destroyed and became many small saccules. Some nucleus and



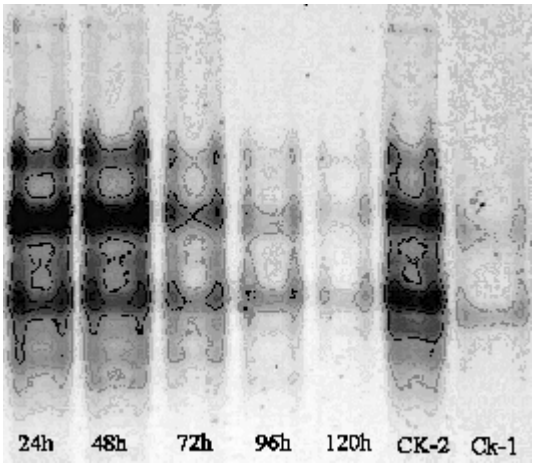
1: The normal liver cells of *O.hupensis* (Bar: 1900 nm). 2: The liver cells of the *O.hupensi* under 12 h triterpene sapogenin from *P. chinesis* treatment (Bar: 1900 nm). 3: The liver cells of the *O.hupensi* under 24 h triterpene sapogenin from *P. chinesis* treatment (Bar = 1900 nm). 4. The liver cells of the *O.hupensi* under 48 h triterpene sapogenin from *P. chinesis* treatment (Bar: 6000 nm).

Fig. 2. The *O. hupensis* liver and intestine observation through transmission electron microscope

mitochondrias were ruptured. After being treated for 24 h, part microvilli arrange of intestines was out of order and fallen. The area around intestines swelled as well. After treated for 48 h, most microvilli arrange became out of order and fallen. The area around intestines swelled deeply. Massive vacuoles became visible. Some nucleolus were dissociated. Cell organelles in cytoplast appeared vacuoles with different sizes, and their configurations were hard to be distinguished. Cell membranes were imperfect and damaged seriously.

EST electrophoresis Assay

In the early stage (24 h and 48 h treatment) of toxicosis, enzyme activity of treated samples was higher than that in control. Novel PAGE bands were displayed. In middle and late stages (72-120 h treatment) of toxicosis, the activity of esterase (EST) isozyme treated with 10 mg/L triterpene sapogenin was lower than control. The enzyme bands disappeared totally (Fig. 3).



ck-1: sample treated by 1 mg/L niclosamidum. ck-2: sample treated by H_2O. The other samples were treated by 10 mg/L triterpene sapogenins from *P. chinesis* for 24 h, 48 h, 72 h, 96 h and 120 h, respectively.

Fig. 3. EST isozyme profiles under 10 mg/L triterpene sapogenin from *P. chinesis* treatment

The results from this study revealed the allelopathic effects of *P. chinesis* on *O. hupensis* via triterpene sapogenin, an important allelopathic substance extracted from *P. chinesis*. According to the molluscicidal activity, triterpene sapogenin showed significant killing effect to snails. The mortality rate of *O. hupensis* positively correlated with both triterpene sapogenin concentration and treating time. The eliminating effects of triterpene sapogenin on snails were in accordance with the standard of effectively killing snails[16].

In terms of the microscopic field for the morphologic pathology of *O. hupensis*, the results showed that plant molluscicides can evidently damage the structure of soft tissue, liver of snails through allelopathic effects. Snail liver is not only an important apparatus in charge of detoxification but also liable to toxicant. It is sensitive to alterations in physiological environment[17,18]. For example, triterpene sapogenin is able to damage the frame of endoplasmic reticulum in liver cell and reduce activities of detoxification enzymes. Thereby it causes snail death[19]. On the other hand, other organs, such as head, antenna, foot and intestine, determine resistance of *O. hupensis* to allelopathic substance as well. Allelopathic effects function through destroying the skin structure, the disintegration of microvilli, life-sustaining plasma important electrolyte leakage, or influences on digestion and absorption function. These effects speed up the infiltration of triterpenoid saponin and eventually kill the snails. A serious of pathological changes in the cell ultrastructure and enzymes activity of snail caused by triterpenoid saponin in this study accorded with the damages from Purple Streptomyces[20]. In summary, the results from this study showed strong allelopathic evidences of *N. indicum* on *O. hupensis* and provided the foundation for constructing plant community with strong allelopathy to kill *O. hupensis*.

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