Original Article

Laboratory Evaluation of the Molluscicidal Activity of *Pulsatilla Chinensis (Bunge) Regel* Saponins against the Snail *Oncomelania Hupensis**

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Abstract

Objective To observe the toxicity of *Pulsatilla chinensis (Bunge) Regel* saponins (PRS) against *Oncomelania hupensis* (*O. hupensis*).

Methods *O. hupensis* snails were exposed to 40% and 80% of 24 h LC_{50} of PRS for 24 h, and then choline esterase (CHE), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) activities in cephalopodium and liver of snails were determined. Niclosamide (NIC) was used as the reference molluscicide. Zebra fish lethality test was evaluated to non-target aquatic species of PRS.

Results The molluscicidal activity of PRS (LC_{50} at 24 h: 0.48 mg/L) was similar to that of NIC (LC_{50} at 24 h: 0.16 mg/L). Significant alterations about CHE, ALP, and ALT activities both in the cephalopodium and the liver of snails were observed when *O. hupensis* was exposed to 40% and 80% LC_{50} of PRS or NIC for 24 h. PRS and NIC could not affect LDH activity in the cephalopodium and the liver. Lower toxicity to fish of PRS was observed up to the highest concentration tested than NIC.

Conclusion PRS, as compared with the reference molluscicide NIC, is thought to be used for the control of harmful vector snails safely.

Key words: *Oncomelania hupensis*; Molluscicidal activity; Saponin; *Pulsatilla chinensis (Bunge) Regel*; Niclosamide

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INTRODUCTION

Today approximately over 200 million people are infected with schistosome and about 800 million are at risk[1]. This disease of poverty has been proved to be difficult to control for centuries. In China, a national survey in 2007 revealed that the infection rate among residents in Fork Beach endemic area was 1.87% and the infection rate in buffaloes, the main reservoir of *Schistosoma japonicum* for transmission of human schistosomiasis, was 3.80%[2]. Therefore, schistosomiasis is still a serious endemic disease in China now. One of the methods of controlling schistosomiasis in China is to kill the intermediate host, *O. hupensis*. Synthetic molluscicides have been widely used for the effective control of harmful snails[3], but it has now been realized that these cause serious environmental hazards. NIC is the most widely used synthetic molluscicide at present and highly toxic towards nontarget organisms and destroys the ecosystem[4].

Since the 1930s, the molluscicidal properties of

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numerous plant extracts have been studied with a view to developing an accessible and inexpensive technology for appropriate control of the snail vector by local communities[5]. Many Chinese herbal medicines are potential molluscicides, which can be used against the intermediate hosts of schistosome and are easily biodegradable and thus safer to use than their synthetic counterparts[6-8]. There are a few publications concerning elimination of snails with these molluscicides, such as the arecoline (ARE) extracted from the nut of *Areca catechu*. The ARE has a strong molluscicidal effect and a fine inhibiting effect on the climbing-upward activity of snails[9], but it is not available in most schistosome endemic areas of China. *Pulsatilla chinensis (Bunge) Regel* is a botanical with a long history of medical use in China, which exhibits "blood-cooling" and detoxification activities. The roots of *Pulsatilla chinensis (Bunge) Regel* have been widely used in the traditional Chinese medicine for adjunctive treatment of intestinal amebiasis, malaria, vaginal trichomoniasis, bacterial infections and malignant tumor[10]. No studies on the molluscicidal activity of specific compounds or components of *Pulsatilla chinensis (Bunge) Regel* on snails are reported.

The aim of the present study was to conduct laboratory evaluation of molluscicidal activity of *Pulsatilla chinensis (Bunge) Regel* products against the snail *O. hupensis*.

MATERIALS AND METHODS

Test Animals

O. hupensis snails came from the Jiangxi Institute of Parasitic Diseases, Nanchang, China and cultured at 25 °C in laboratory conditions for a week for acclimatization. The snails were fed daily on moist hay during the acclimatization period. Adult snails of almost uniform size (8±0.1 mm shell length and 0.5±0.1 mm shell width) were collected and used for the test. Zebra fish (*Danio rerio*) aged between 6 and 24 months for the lethality test were obtained from an aquarium in Suzhou, China. The study was conducted in accordance with national and institutional guidelines.

Preparation of PRS

Dried *Pulsatilla chinensis (Bunge) Regel* were purchased from a Chinese herbal store in Suzhou City of Jiangsu Province, and were authenticated by Prof. Li Xiaoran at the College of Pharmacy, Soochow University. A voucher specimen (No. 08-02-15-18) has been deposited at the Soochow University.

The dried plant material (2.5 kg) was extracted three times with 70% alcohol under reflux. The solvent was subsequently dried under reduced pressure conditions. The residue was chromatographed over D101 resin column, eluted with water-alcohol gradients combined with a fraction eluted with 60% alcohol and then lyophilized. The resulting powder (PRS, 125 g) obtained was subjected to the following phytochemical and pharmacological studies.

Preparation of NIC

The ethanolamine salt of niclosamide (contain niclosamide 50%) was purchased from Rosen Chemical Co., Ltd., Wujiang, Jiangsu, China.

Test for Molluscicidal Activity

The molluscicidal effects of PRS and NIC against *O. hupensis* were determined by the method of Zhou et al.[11]. The PRS was dissolved in dechlorinated water to 1 000 mL. NIC was diluted by dechlorinated water to 1 000 mL. Snails were collected in absorbent gauze sacks (30 snails per sack) and kept in beakers. Every sack of snails was exposed to one concentration of PRS (8.0, 4.0, 2.0, 1.0, 0.5, and 0.25 mg/L) and NIC (2.0, 1.0, 0.5, 0.25, 0.125, and 0.0625 mg/L) for 24 h at 25 °C. Dechlorinated water exposure was the control. After 72 h recovery period in dechlorinated water, the mortality was checked. No response to a needle probe under dissecting microscope was the evidence of death. Each experiment included three replications.

Preparation of Snail Cephalopodium and Liver

Sixty snails were collected in one absorbent gauze sack and kept in beaker. Then they each were exposed to 40% and 80% LC_{50} of PRS or NIC for 24 h. The dechlorinated water control was performed simultaneously. After exposure, the snails were carefully crushed and their cephalopodium and liver tissues were extracted to homogenize on ice[11]. The homogenates were centrifuged for 20 min at 5 000 g at 4 °C and the supernatant was stored at -70 °C for the enzyme assay. Each experiment was carried out in triplicate[12].

Zebra Fish Lethality Test

The zebra fish toxicity test was conducted by using adult fish of similar length and age, including a negative control (dechlorinated tap water) and a positive control (NIC at 0.5 mg/L) according to the method of Ricardo San Martin et al.[13]. Four fish were used in each aquarium and each experiment was carried out in triplicate. Every 24 h during

96 h, the number of dead fish per aquarium was recorded.

Protein Assay

The protein content of supernatants was determined by using an automatic biochemistry analyzer.

Enzyme Assay

Enzyme activities were expressed as the amount of substrate hydrolyzed or production liberated μmol/min/g protein in the supernatant. CHE and LDH kits were purchased from Nanjing Jiancheng Bioengineering Institute, and the enzyme activities were determined at 520 nm and 440 nm by a semi-automatic biochemical analyzer. ALP and ALT kits were purchased from Japan First Chemicals Co., Ltd. and enzyme activities were determined by a C8000 automatic biochemical analyzer.

Data Analysis

The effects of two molluscicides on *O. hupensis* were expressed by LC_{50}, LC_{90}, and their 95% confidence limit (95% CL). The results of enzyme activities were expressed as Mean±SE of the three replicates by SPSS 13.0. One way ANOVA and LSD were applied to locate significant differences between the treated and the control.

RESULTS

Ingredients of Pulsatilla Chinensis (Bunge) Regel

Dried PRS powder (30 g) was subjected to Si gel column chromatography and eluted with gradient mixtures of chloroform and methanol to afford 6 fractions (Fraction 1-Fraction 6). Repeated CC of Fraction 2 on Si gel (200-300 mesh), with $CHCl_3$/MeOH (80: 20) and Sephadex LH-20, and MeOH, yielded pure Compound 14 (Pulchinenoside A3, 8.2 mg), Compound 15 (3-O-α-L-rhamnopyranosyl-(1→2)- α-L-arabinopyranosyl hederagenin, 15.5 mg). Further purification of Fraction 3 through MPLC on ODS (20-40 μm), by using MeOH-water (70: 30) as eluent, yielded pure Compound 10 (Pulchinenoside B, 93.6 mg), Compound 11 (3-O-α- L-arabinopyranosyl hederagenin 28-O-α-L-rhamnopyranosyl-(1→4)-β-D- glucopyranosyl-(1→6)-β-D-glucopyranosyl ester, 121.7 mg), Compound 12 (23-hydroxyl-3-O-β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L- arabinopyranosyl lup-20 (29)-en-28-oic acid, 83.5 mg) and Compound 13 (3-O-β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl hederagenin, 117.4 mg). Further purification of Fraction 5 through HPLC on ODS (20-40 μm), with a MeOH /H_2O gradient elution (the ratios of MeOH /H_2O ranging from 55: 45 to 65: 35), yielded pure Compound 5 (Pulchinenoside B4, 11.2 g), Compound 6 (3-O-β-D-glucopyranosyl-(1→4)-α-L-arabinopyranosyl hederagenin 28-O-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl ester, 12.6 mg), Compound 7 (3-O-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl hederagenin 28-O-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D- glucopyranosyl ester, 5.86 g), Compound 8 (23-hydroxyl lup-20 (29)-en-28-oic acid 28-O-α-L-rhamnopyranosyl-(1→4) -β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl ester, 9.42 mg) and Compound 9 (hederagenin 28-O-α-L-rhamnopyranosyl-(1→4) -β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl ester, 6.75 mg); Fraction 6 was further subjected to semipreparative HPLC on a Zorbax SB-ODS column and eluted with 50% MeOH at a flow rate of 2.0 mL/min yielded pure Compound 1 (Pulchinenoside E, 17.2 mg), Compound 2 (3-O-α-L-rhamnopyranosyl-(1→2)- [β-D-glucopyranosyl-(1→4)] -α-L-arabinopyranosyl hederagenin 28-O-α-L- rhamnopyranosyl-(1→4) -β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl ester, 135.9 mg), Compound 3 (3-O-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl- (1→4)] -α-L-arabinopyranosyl oleanolic acid 28-O-α-L-rhamnopyranosyl-(1→4) -β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl ester, 13.1 mg) and Compound 4 (3-O-β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl oleanolic acid 28-O-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D- glucopyranosyl ester, 12.4 mg). The structures were identified by comparison of spectrum data with those in literature. These 15 compounds were shown in Figure 1.

Molluscicidal Activity

Molluscicidal activities of PRS and NIC against *O. hupensis* snails were determined by the bioassary. The LC_{50} and LC_{90} for PRS after 24 h exposure are given in Table 1. The activity of the reference molluscicide NIC against *O. hupensis* snails was determined by using the same assay procedure and gave an LC_{50} of 0.16 mg/L and an LC_{90} of 1.18 mg/L. After 24 h exposure in PRS and NIC, PRS showed that Molluscicidal activities of PRS were similar to those of NIC (Table 1).

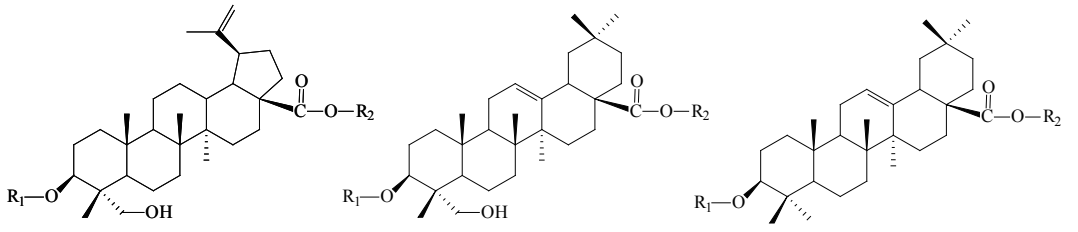

Figure 1. Chemical structures of 15 triterpenoidal saponins from PRS. A: 1. R_1=glc(1→4)rha(1→2)ara, R_2=glc(1→6)glc(1→4)rha; 5. R_1=rha(1→2)ara, R_2=glc(1→6)glc(1→4)rha; 8. R_1=H, R_2=glc(1→6)glc(1→4)rha; 10. R_1=ara, R_2=glc(1→6)glc(1→4)rha; 12. R_1=glc(1→3)rha(1→2)ara, R_2=H; 14. R_1=rha(1→2)ara, R_2=H. B: 2. R_1=rha(1→2)[glc(1→4)]ara, R_2=glc(1→6)glc(1→4)rha; 6. R_1=glc(1→4)ara, R_2=glc(1→6)glc(1→4)rha; 7. R_1=rha(1→2)ara, R_2=glc(1→6)glc(1→4)rha; 9. R_1=H, R_2=glc(1→6)glc(1→4)rha; 11. R_1=ara, R_2=glc(1→6)glc(1→4)rha; 13. R_1=glc(1→3)rha(1→2)ara, R_2=H; 15. R_1=rha(1→2)ara, R_2=H. C: 3. R_1=rha(1→2)[glc(1→4)]ara, R_2=glc(1→6)glc(1→4)rha; 4. R_1=glc(1→3)rha(1→2)ara, R_2=glc(1→6)glc(1→4)rha.

Table 1. Molluscicidal Activity of PRS and NIC on *O. hupensis* Snails after 24 h of Exposure under Laboratory Conditions

PRS		NIC	
Concentration (mg/L)	No. Death	Concentration (mg/L)	No. Death
8.0	30	2.0	30
4.0	29	1.0	27
2.0	25	0.5	20
1.0	19	0.25	16
0.5	15	0.125	14
0.25	11	0.0625	10
Dechlorinated water control	0	Dechlorinated water control	0
LC_{50} (95% CL)	0.48 (0.32~0.66)	LC_{50} (95% CL)	0.16 (0.10~0.22)
LC_{90} (95% CL)	2.64 (1.81~4.88)	LC_{90} (95% CL)	1.18 (0.74~2.61)

Enzyme Activity

Exposure to sublethal doses of 40% and 80% LC_{50} of PRS and NIC for 24 h altered the levels of different enzymes in different body tissues of snails. As shown in tables 2 and 3, PRS and NIC could significantly reduce the activities of CHE, ALP, and ALT in the liver and the cephalopodium as compared to the water control (P<0.05). However, PRS and NIC could not affect the LDH activity in the liver and the cephalopodium.

PRS Effect on Fish

The mortality of fish was assessed by incubation with different concentration of PRS. No impact was observed after fish were exposed at 2, 4, 8, 16 mg/L PRS

Table 2. The Effect of PRS on the Liver Enzyme Activity of *O. hupensis* (u/gprot)

Group	CHE	LDH	ALP	ALT
Water control	0.36±0.021	1.35±0.081	302±5	409±3
PRS 40% LC_{50}	0.24±0.21*	1.16±0.15	184±9*	227±9*
PRS 80% LC_{50}	0.24±0.30*	1.01±0.082	128±9*	184±17*
NIC 40% LC_{50}	0.15±0.015*	1.04±0.038	211±19*	286±7*
NIC 80% LC_{50}	0.087±0.015*	1.08±0.22	131±21*	222±11*

Note. Compared with the water control, *P<0.05.

Table 3. The Effect of PRS on the Cephalopodium Enzyme Activity of *O. hupensis* (u/gprot)

Group	CHE	LDH	ALP	ALT
Water control	5.22±0.09	11.84±0.30	2509±36	1575±35
PRS 40% LC_{50}	4.08±0.14*	11.44±0.24	2329±90*	1463±38*
PRS 80% LC_{50}	3.58±0.60*	11.36±0.12	2049±149*	1190±62*
NIC 40% LC_{50}	3.42±0.40*	11.48±0.17	2121±110*	1380±126*
NIC 80% LC_{50}	3.21±0.48*	11.12±0.11	1789±182*	1256±150*

Note. Compared with the water control, *P<0.05.

for 24, 48, 72, and 96 h, respectively. When fish were exposed to 32 mg/L PRS for 48, 58% fish were dead. Up to 72 h, PRS killed 100% of the fish. In contrast, 0.5 mg/L NIC, the positive control, caused the death of all the fish within 24 h, whereas the fish remained viable in the negative control group.

DISCUSSION

Currently, natural phytochemicals extracted from folk herbal have drawn much attention in complimentary and alternative medicine, and the plant kingdom is considered as a gold mine for developing new molluscicide. In our study, we found that PRS was an effective substance to kill *O. hupensis* snails and the principal active ingredients were triterpenoidal saponins. Although these saponins were not individually isolated compounds, the synergistic molluscicidal effect on snail jointly exerted by the 15 saponins has been researched. 15 individual saponins of PRS came from Fraction 2, 3, 5, 6, respectively. Fraction 1 and 4 mainly contained dissolved solids, and no pure compounds were obtained from them through repeated column chromatography. PRS necessarily contain physiologically active constituents, so further research should concentrate on the isolation of those compounds responsible for the molluscicidal activity. The activity of PRS against *O. hupensis* snails makes it possible to employ a fraction or some fractions derived therefrom, in an integrated molluscicidal management program.

To our knowledge, this is the first report indicating that PRS has a molluscicidal effect about as a molluscicide. Despite the slightly lower efficiency of PRS than the chemical molluscicide NIC, the results suggested a new possibility of using PRS against the snail *O. hupensis*. If the LC_{50} is less than 100 mg/L[14], the plant extracts will be potent molluscicides. In the present study, the LC_{50} of PRS is less than 100 mg/L largely.

It is clear from the result section that treatment with a sublethal concentration of PRS or NIC caused a significant inhibition of the CHE, ALT, and ALP activity in the liver and the cephalopodium of *O. hupensis*. In addition, the enzyme activity was inhibited by increasing concentrations of the molluscicide. Several compounds might affect some vital enzyme activities in different body tissues and lead to the death of snails. CHE which generally exists in the body of vertebrates and invertebrates can hydrolyze the neurotransmitters such as acetylcholine at the nerve synapse. In the absence of such hydrolysis, neurotransmitters accumulate and as a consequence, prolonged electrical activity occurs at nerve endings. Inhibition of the CHE activity is usually regarded as an indicator of organo-phosphorus and carbamate exposure[15]. It has also been shown that ALP plays an important role in the active transport of chemicals across cell membranes[16]. Pilo et al.[17] demonstrated that ALP is associated with protein synthesis. The active sites of ALP[18] contain a serine residue, and therefore, it is possible that the inhibition of ALP observed in this study could be due to the phosphorylation of the active sites of ALP. ALT is a key enzyme of amino acid metabolism that link amino acids to intermediates of pathways involved in energy generation. particularly the TCA cycle. ALT was highly decreased in all of the tissues, confirming the augmentation of stress as a consequence of exposure to PRS. Therefore, it seems that CHE, ALP, and ALT are basic targets of molluscicides. However, PRS and NIC could hardly affect the LDH activity in the cephalopodium or the liver, which forms the center of a delicately balanced equilibrium between catabolism and anabolism of carbohydrates[19].

In the present study, the zebra fish toxicity test was used to assess toxicity to nontarget aquatic species. PRS which contains 15 compounds had lower toxic to zebra fish whereas positive control NIC showed highly toxic to fish at 0.5 mg/mL. NIC is known to be highly toxic to a large number of fish species, with 24 h LC_{50} values ranging from 0.043 to 0.279 mg/L for most of them[20]. It is worthy of note that NIC's high toxicity to fish has been considered a serious drawback of this synthetic molluscicide owing to its impact on aquatic ecosystems and, also because of fish as an important food source in areas where schistosomiasis is endemic[21]. On the other hand, saponins[22] were completely consumed within 10 days which indicates their abilities to degrade in aquatic environments under aerobic conditions. As a result, the use of PRS for snail control in schistosomiasis-infested water bodies is environmentally acceptable.

In conclusion, data from the present study clearly indicate that PRS, in addition to being a rather potent plant molluscicide, is considerably less harmful to nontarget aquatic organisms such as fish than the reference molluscicide NIC. Nevertheless, its use as a natural molluscicide requires further investigation with respect to its active constituents, its acute toxicity, its cutaneous and ocular irritability, as well as its mutagenicity and carcinogenicity. Studies along these lines are presently in progress in our laboratories.

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