https://doi.org/10.26651/2017-42-1-1112 0971-4693/94 Euro 20.00
Allelopathy Journal 42 (1): 145-156 (September, 2017) *International Allelopathy Foundation* 2017
Table: 1, Figs : 4

Preparation and evaluation of camptothecin granules for molluscacidal activity

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(Received in revised form: July 24, 2017)

ABSTRACT

To prevent the schistosomiasis disease is to control the intermediate host (snail, *Oncomelania hupensis* Gredler). We developed and tested an environment-friendly approach to control this snail by preparing the plant molluscacide, camptothecin granules and tested its molluscacidal activity. Aqueous solution (prepared from dissolved granules) of 0.60 g/L concentration of camptothecin showed 100% molluscacidal effect after submerging the *O. hupensis* for 4-5 days. It reduced the glycogen content, total protein and liver alanine aminotransferase activity in the treated snails. Camptothecin proved safe to fish, at 1.0 g/L for 96 h or 0.40 g/L concentration for 30 days. It prevented the schistosomiasis in schistosomiasis epidemic area in China by controlling the snails growth, that carry the worms causing schistosomiasis.

Key words: *Camptotheca acuminata,* camptothecin, granules, *Oncomelania hupensis,* plant, molluscacide, schistosomiasis disease, snail

INTRODUCTION

Schistosomiasis, a water-borne parasitic disease affects nearly 200 million people and poses threat to 600 million in > 76 countries (25). In China about 0.87 million people are infected and 40 million are at risk (2-3). The disease is caused by the parasitic worm *Schistosoma japonica*, whose life cycle depends upon the intermediate snail host *Oncomelania hupensis* (12). The two main methods used to control the schistosomiasis are chemotherapy and transmission reduction via eradication of the intermediate host snails. Efforts to control the intermediate host snail populations play a central role in public health campaigns aimed at reducing human morbidity and mortality associated with schistosomisis (11,26).

Presently, the main molluscicidal methods are physical, chemical and biological control (20,21). Physical control is labour intensive, expensive and does not provide 100% snail control. Chemical control uses molluscacidal drugs, such as Santobrite and Niclosamide. These drugs work well but are toxic to human, livestock and aquatic animals. Biological control, by using plant-based molluscicides extracted from plant materials, has been found to kill *O. hupensis* and/or inhibit it their growth and reproduction while having

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less adverse effects on the environment (15). This approach to eradicate intermediate host snails has been proved to be an important means of schistosomiasis control and lots of plant species have been identified to have anti-molluscasidal activity.

Much research has focussed on the screening of molluscacidal plants, extraction of their active ingredients and use as molluscacides (19). So far > 1,000 plant species have been tested for anti-molluscacidal activity, of these about 50 plants proved effective (16,17), These plants are *Leonurus japonicus* Houtt., *Camptotheca acuminata* Decne., *Cinnamomum camphora, Solanum xanthocarpum* Schrad Wendl. (16,17,28)**.** However, practical use of these plants is very limited.

C. acuminata is deciduous tree with height up to 30 m. Its leaves, bark, root bark and fruit can cure the carbuncle, psoriasis and are effective in treating cancer (28,30)**.** It is active especially in curing acute and chronic leukemia and splenomegaly caused by schistosomiasis. *C. acuminata* is endemic to China, mainly distributed in the south of Qinling Mountains and widely cultivated in schistosomiasis epidemic area. Previous studies have reported that camptothecin in *C. acuminata* is the active ingredient of the molluscacide (28). Camptothecin causes damage to the main body of *O. hupensis* and reduces its liver function. It is also sensitive to the esterase reaction of *O. hupensis*. In the early phase of poisoning, camptothecin stimulates alkaline phosphatase and alanine aminotransferase expression, and detoxification capacity of *O. hupensis* is enhanced due to the increased enzyme activity. Later, the synthesis of the enzyme is inhibited and the enzyme activity decreases dramatically because the drug acts over the physiological threshold (28).

One problem in practical application of camptothecin is that camptothecin powder dissolves rapidly in water and as such is difficult to sink to the bottom of a water body where *O. hupensis* mainly inhabits. This restricts its molluscacidal activity. To overcome this issue, we have synthesized this in a granular form including camptothecin, arabic gum, sugar and flour. These granules sink to the bottom, and camptothecin and sugar is dissolved slowly and forms a concentrated environment around the granules. This attracts *O. hupensis* and kills them. Also, the effect can last for at least one month.

MATERIALS AND METHODS

Source of snails

O. hupensis snails were collected 3 days before the start of the experiment from an experimental site in Gong'an County, Hubei Province, owned by Hubei Academy of Forestry, which authority gave the permission to conduct the study on this site. Field studies did not involve endangered or protected species. The snail experiment was approved by Schistosomiasis Control Office of Hubei Province.

The collected snails were first acclimatized for 72 h to the laboratory conditions and then maintained in the glass aquaria containing dechlorinated tap water at 22-24 °C. The pH, dissolved oxygen, free carbon dioxide and bicarbonate alkalinity were 7.1-7.3, 6.5-7.3 mg/l, 5.2-6.3 mg/l and 102-105 mg/l, respectively. Dead animals were removed to avoid any spoilage of the aquaria water. Non-infected adult snails, 6-10 mm long with 6-9 shells, were selected for the experiment.

Extraction of camptothecin

Leaves of *C. acuminata* were collected in a botanical garden of Hubei Academy of Forestry. Field studies did not involve endangered or protected species. Two kg fresh leaves were cleaned with distilled water and dried in an oven at 75 ^{o}C for 12 h, and then ground into power in a mortar and pestle. The resultant paste was soaked in 2L of 70% ethanol for 24h, after which it was heated for 3 h at 75 ^{o}C. It was then cooled and filtered through 0.45 um membrane with vacuum filtering. The filtrate was concentrated using rotary evaporator (RE52-AAA, Shanghai Jiapeng Technology Co., Ltd.). and the concentrated filtrate was further extracted with chloroform (50 ml) for 24 h and the extract were evaporated and then filered as described above.

The filtrate was mixed with 8 times of the volume of saturated solution of calcium hydroxide with continuous stirring for 30 min. The aqueous extract was filtered, and the pH of the extract was adjusted to 8~9. The extract was then subjected to column chromatography. Briefly, 10 ml extract was loaded on a glass column (10 cm ×25 cm) packed with a microporous resin AB-8, and eluted with 500 ml 90% ethanol (pH : 2~3). The ethanol extract was then concentrated and extracted sequentially with ethyl acetate (30 ml) and then with a mixture of ethyl acetate and petroleum ether (2/3, v/v). The resultant crystalline substance was camptothecin with its identity confirmed by liquid chromatography. From the extraction method described above, 10g camptothecin was obtained from 2000 g fresh leaves of *C. acuminata*.

Preparation of camptothecin plant molluscacide granules

One hundred g camptothecin crystalline powder extracted from leaves was dissolved in 150ml 75% of ethanol. A mixture of sugar, flour and gum Arabic was prepared in a ratio of 4.5:40.8:4.5, and this was added to the camptothecin ethanol solution in a weight/volume ratio of 100: 0.25, followed by addition of 3% (w/v) distilled water. Cylindrical granules (approximate length 0.8-1cm and diameter 0.2-0.4cm) were made and heated at 70-90℃for 15min. After cooling for 10-15 minutes, camptothecin plant molluscacide granules were made, which contained about 0.25% camptothecin.

Molluscicidal effect of camptothecin granules

Six treatments with different concentrations of Camptothecin (0.05 g/L, 0.1 g/L 0.2 g/L, 0.4 g/L, 0.8 g/L and 1 g/L) were prepared by dissolving 200g, 400g, 800g, 1600g, 3200g and 4000g of Camptothecin plant molluscacide granules in 10L deionized and dechlorinated water. Five nylon mesh bags of snails were placed in each glass container and exposed to a specific concentration of camptothecin. Each bag contained 20 snails and each treatment was replicated thrice. Snail mortality was examined at 24, 48, 72, 96 and 120 h after exposure to camptothecin. At each sampling, one bag was randomly selected, transferred to fresh dechlorinated water and maintained for another 24 h. Death of snails in the bag was then determined and confirmed by the lack of reaction to irritation of the foot with a blunt needle to elicit typical withdrawal movement (24).

Deionized and dechlorinated water was used as negative control, and niclosamide (Cayman) **(**0.0000005g/L) used as a positive control. Each test concentration was triplicated and during the exposure and recovery period snails were neither fed nor disturbed.

Bioassay of camptothecin molluscacide granules on snails

The experimental *O. hupensis* snails were treated with three low doses used in the assay of molluscicidal effect, i.e. 0.05 g/L, 0.1 g/L 0.2 g/L for 24h, 48h and 72h respectively. Deionized and dechlorinated water was used as negative control. For each treatment, 50 snails were randomly selected and submerged in beakers containing the test solutions. After exposure for 24 h, 48h or 72h respectively, the snails were washed in dechlorinated water and then left in dechlorinated water. The climbing snails were taken out from the beaker for measurements.

Determination of Glycogen (Gn) content

About 1 g of soft tissues of living snails was sampled and weighed. Then the soft tissue samples were dried in an oven at 40 ^{o}C for 24 h and ground into fine powder. Then 2 ml KOH solution (30%) was added to the test tube with 10 mg of the powder and incubated in a boiling water bath for 20 min and then cooled to room temperature. Finally 10 ml ethanol was added to the tube and the glycogen was obtained by collecting the precipitate after centrifugation at 3000 rpm for 10 min. Glycogen content was analyzed by anthrone-colorimetric method (23).

Determination of Total Protein (TPr) content

The nitrogen content in the powder samples was determined by Kjeldahl nitrogen detection method (32). The total protein content was calculated by multiplying the total % N by 6.25 .

Determination of Liver alanine aminotransferase (ALT) activity

Liver alanine aminotransferase (ALT) activity was measured at 25ºC with 10 snails for each treatment. The snails were killed immediately after treatment and their livers carefully removed under a dissecting microscope. A liver homogenate was prepared in 0.1 ml precooled phosphate buffer (0.2 mol/l, pH 7.1) in an ice bath. After centrifugation at 0-4ºC for 5 min at 8, 000 xg, the supernatant was collected and ALT activity was determined by Guilbault´s spectrophotometric method (10) at 340 nm, using a DBDA full automatic biochemical analyzer (DADE, USA). ALT activity was expressed in international units (U/l).

Toxicity test of *Camptothecin molluscacide* granules to fish

To determine the toxicity of camptothecin molluscicide granules, the acute and chronic toxicity tests were done with the granules on juvenile crucian carps.

Acute toxicity test

Acute toxicity of camptothecin molluscicide granules to crucian carps (*Carassius carassius*) was assessed through a 96-h semi-static exposure test with 24-h renewal (19). The test vessels were plastic buckets **(**20 cm length × 20 cm width × 30 cm height/depth**)** which contained 10 L solution with different camptothecin granule concentration and renewed every 24h. Temperature was set at 25 ± 1°C during the test period. Each group of 20 crucian carps was exposed to the concentration of 0.40 g/L, 0.60 g/L, 0.80 g/L and 1.0 g/L, respectively. Dechlorinated tap water was used as negative control. Each treatment was replicated three times. The fishes were not fed during the experiment and the dead fish were removed immediately. The mortality (%) of each group was recorded at 24, 48, 72 and 96 h, respectively.

Chronic toxicity test

Chronic toxicity of camptothecin molluscicide granules to fish was assessed during a 30-day exposure period with 24-h renewal (9). The test vessels were pools containing 2m^3 of different camptothecin concentration which was renewed every 24 h at ambient temperature **(**22 - 30 ^{o}C**).** Each group of 20 crucian carps was exposed to the concentration of 0.05 g/L, 0.10 g/L, 0.20 g/L, 0.40 g/L, respectively. Dechlorinated tap water was used as negative control. Three replicates were set in this experiment. The fishes were fed with wheat during the experiment, and the dead fish were removed immediately. Body length and weight of test fish were measured after 30 days,.

Calculations and statistic analysis

The mortality of *O. hupensis* snails were calculated as follows:

$$Mortality(\%) = \frac{Snail_{dead}}{Snail_{total}} * 100\%$$

Where $Snail_{dead}$ refers to the number of the dead snails after exposure to camptothecin; $Snail_{total}$ represents the total number of snails at the start of experiment.

Two-way ANOVAs were conducted for the effect of camptothecin concentration and time on mortality of snails, glycogen concentration, protein concentration, and ALT activity. One-way ANOVA was performed for the effects of camptothecin concentration on the body weight and length of crucian carps. Before statistical analysis, data were $\log_{10}$-transformed to meet the assumptions of normality and homogeneity of variance. When ANOVAs were significant, means were compared by least significant difference (LSD) at the 95% confidence level. All the analyses were conducted in SPSS 16.0 (SPSS Inc., USA).

RESULTS AND DISCUSSION

Molluscacidal effect of camptothecin granules

The mortality of snails exposed to different concentrations of the Camptothecin solutions (df=5, F=610, P<0.01) and after different periods of exposure (df=4, F=1529, P<0.01) varied significantly (Table 1). Interaction between concentration and time of exposure also significantly affected snail mortality (df=20, F=68.54, P <0.01). Mortality of snails increased with increasing concentration and exposure time (Table 1). Camptothecin killed approximately all snails after exposure to a concentration of 1 g/L for 24h. Even at the lowest concentration (0.05 g/L), camptothecin solutions killed at least 30% of the Oncomelania after 72 h of exposure, and killed nearly 90% of the after 120 h . After 120h of exposure, all snails died at concentrations above 0.2 g/L. None of the snails treated with deionized and dechlorinated water alone died during the first three days of treatment. Even after 5 days of exposure, only 5% died.

Utilization of plant based chemicals is a promising method of biological control (1,14,18). Plants are known to synthesize chemicals to defend themselves against insects, bacteria, fungi or viruses. The concept of using indigenous plant molluscicides (cultivation, harvesting and extraction) has been well recognized (5, 8, 29). The use of plant materials is culturally acceptable than the use of anti-schistosomal drugs, whose accessibility is limited due to high transport and treatment costs (34).

Table 1. Molluscacidal effects of plant camptothecin.

Compound	Conc. (g/L)	Mortality of *O. hupensis* (%)				
		24h	48h	72h	96h	120h
Water (Control)	0.00	0.0 ± 0.0	0.0 ± 0.0	1.0±0.1	1. 7 ± 0.4	5.0 ± 0.0
Camptothecin	1.00	99. 7 ± 2.4	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
	0.80	30.0 ± 4.1	43.3 ± 2.4	73.3 ± 2.4	100.0 ± 0.0	100.0 ± 0.0
	0.40	25.0 ± 4.1	33.3 ± 2.4	68.3 ± 0.4	100.0 ± 0.0	100.0 ± 0.0
	0.20	20.0 ± 4.1	30.0 ± 4.1	48.3 ± 2.4	80.0 ± 4.1	100.0 ± 0.0
	0.10	11. 7 ± 2.4	26. 7 ± 2.4	43.3 ± 2.4	76.7 ± 2.4	96.7 ± 2.4
	0.05	11. 7±2.4	26. 7±2.4	31. 7±2.4	65.0 ±4.1	91. 7 ± 2.4
Niclosamide	0.0000005	91. 7 ± 2.4	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0

The concentration of camptothecin is calculated based on the content of the granules.

Although studies (16,17,19,28) have reported the effects of plant extracts on *O. hupensis,* practical application is very limited. In this study, we developed an environment-friendly approach to prepare the camptothecin plant molluscacide granules using readily available and inexpensive substances (sugar, flour and Arabic gum) to control the *Oncomelania hupensis*. The environment where snail survive is not stagnant water (4), but flowing water. The applied chemical solution is washed away by the flow, but the granules (mixture of gum, sugar and flour etc.), were not washed away easily. Our results indicate that camptothecin plant molluscacide granules had a strong molluscicidal activity against *O. hupensis*. The molluscacidal effect of *C. acuminata* varied with the concentration and exposure time. The mortality rate of the snails increased with the concentration of camptothecin. High concentrations of 200, 400 and 800 mg/L of camptothecin caused more than 80% of snail deaths while the concentrations of 50 and 100 mg/L showed relatively less efficacy. Longer exposure time was prone to exert higher mortality.

Biochemical changes on Camptothecin plant molluscacide treatment on snails

Snail glycogen concentration was significantly affected by the concentrations of camptothecin ($df=3$, $F=542.73$, $P<0.01$) and different periods of exposure ($df=2$, $F=317.88$, $P<0.01$) (Fig. 1). There was also significant interactive effects between concentrations and exposure period ($df=6$, $F=43.28$, $P<0.01$). Glycogen in treated snails decreased by 29.8%, 35.3% and 45.1% after exposure to 0.05 g/L, 0.1 g/L and 0.2 g/L of camptothecin for 72 h, respectively.

Total protein concentration was also significantly affected by the concentration of camptothecin ($df=3$, $F=178.80$, $P<0.01$) and different periods of exposure ($df=2$, $F=76.07$, $P<0.01$) (Fig. 2). Significant interactive effects were observed between concentration and exposure period ($df=6$, $F=11.32$, $P<0.01$). Total protein in the treated snails was decreased by 7.9%, 16.1% and 24.3% after exposure to 0.05 g/L, 0.1 g/L, and 0.2 g/L of camptothecin solution for 72 h, respectively.

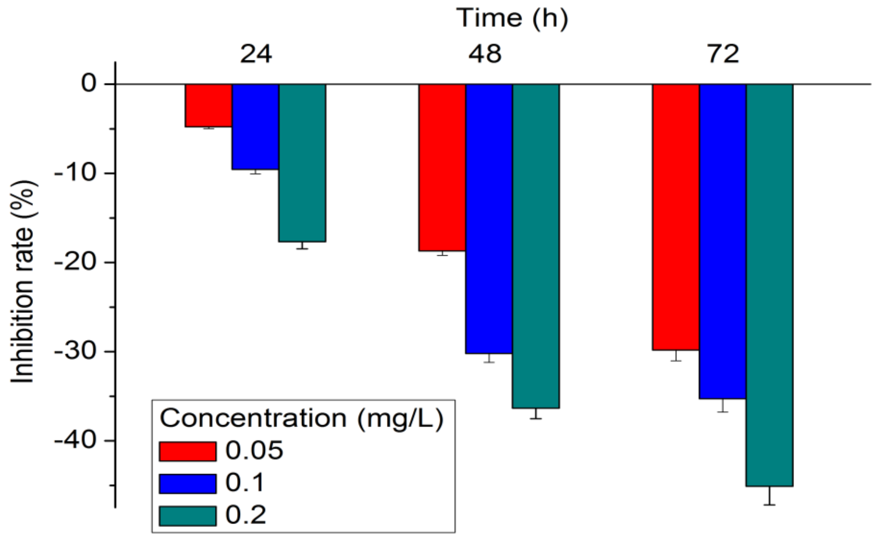

Figure 1. Effects of plant camptothecin on glycogen concentration of snails at 24, 48 and 72 h.
The concentration of camptothecin is calculated based on the content of the granules. Data shows the Inhibition (%) over control.

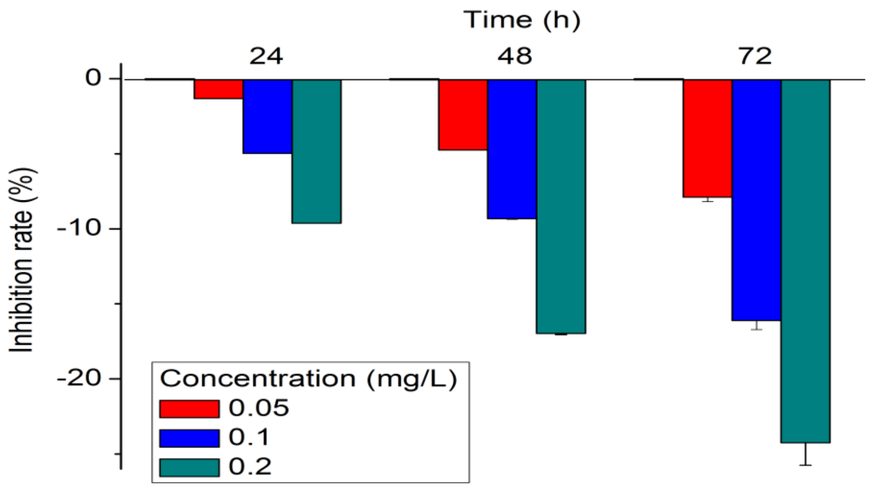

Figure 2. Effects of Camptothecin plant molluscacide on protein concentration of snails at 24, 48 and 72 h.
The concentration of camptothecin is calculated based on the content of the granules. Data shows the Inhibition (%) over control.

Liver ALT activity was significantly affected by the concentration of camptothecin solutions (df=3, F=16.58, P<0.01) and different periods of exposure (df=2, F=142.43, P<0.01) (Fig.3). There were significant interactive effects of concentration and exposure period (df=6, F=57.16, P<0.01). For all three concentration treatments, liver ALT activity increased after 24 h of exposure, compared to the control, but decreased after 72 h of exposure. After 72 h of exposure, liver ALT activity in treated snails were decreased by 38.7%, 38.7% and 56.0% for the concentrations of 0.05 g/L, 0.1 g/L and 0.2 g/L, respectively.

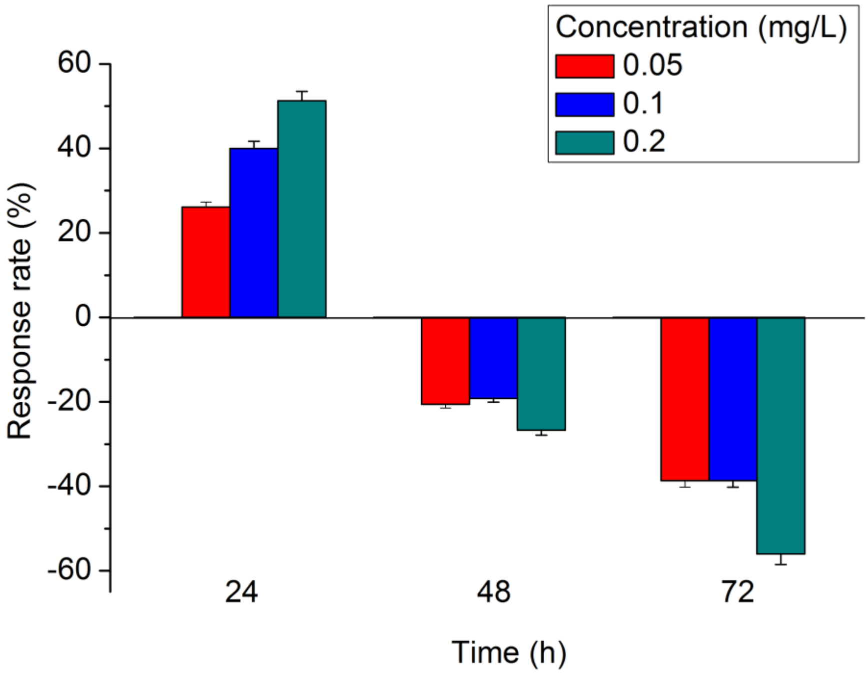

Figure 3. Effects of camptothecin plant molluscacide on liver ALT activity of snails at 24, 48 and 72 h. The concentration of camptothecin is calculated based on the content of the granules. Data shows the response rate (%) over control.

Molluscicidal effects of camptothecin granules were confirmed by physiochemical characteristics. When snails were exposed to camptothecin, their glycogen content decreased. Similarly, the total protein content also decreased to some extent, even though it was less affected than amount of glycogen. Hence, the lethal effects of chemical on snails may be more related to cell metabolism. The mechanism underlying the impact of camptothecin on glycogen metabolism of snails is complicated, but mainly involved the three aspects. (i). It is possible that it induces partial liver cell necrosis by affecting hepatic function, leading to a direct impact on glycogen synthesis. (ii). It perhaps activates or inhibits some enzymes, thus promoting the glycogen decomposition and inhibiting the glycogen synthesis and (iii). it may affect the digestive tract function and cause reduction of glucose uptake, thus inhibiting glycogen synthesis (23, 31). Therefore, further experiments are needed to decipher the molluscicidal mechanism of camptothecin.

Alanine aminotransferase (ALT) plays a critical role in many stressful conditions and reflects liver damage (6). In our study, the ALT activity increased 24 h after exposure to camptothecin but then decreased after 72 h exposure. Since transaminase is necessary in regulating the concentrations of keto amino acids, we presume that the initial increase in ALT activity may be due to the animal`s trial to maintain the amino acid balance (27). The decrease of ALT activity after 72h may be due to destructive damage in the hepatic cells and cardiac tissue (7).

Toxicity analysis of camptothecin on fish

No fish died when exposed to 0.40g/L, 0.60g/L, 0.80g/L, 1.0g/L of camptothecin for 96 h. No significant differences were found in body length (df=4, F=1.01, P=0.41) and weight (df=4, F=0.54, P=0.71) of crucian carps between the control and each of treatment groups (Fig. 4).

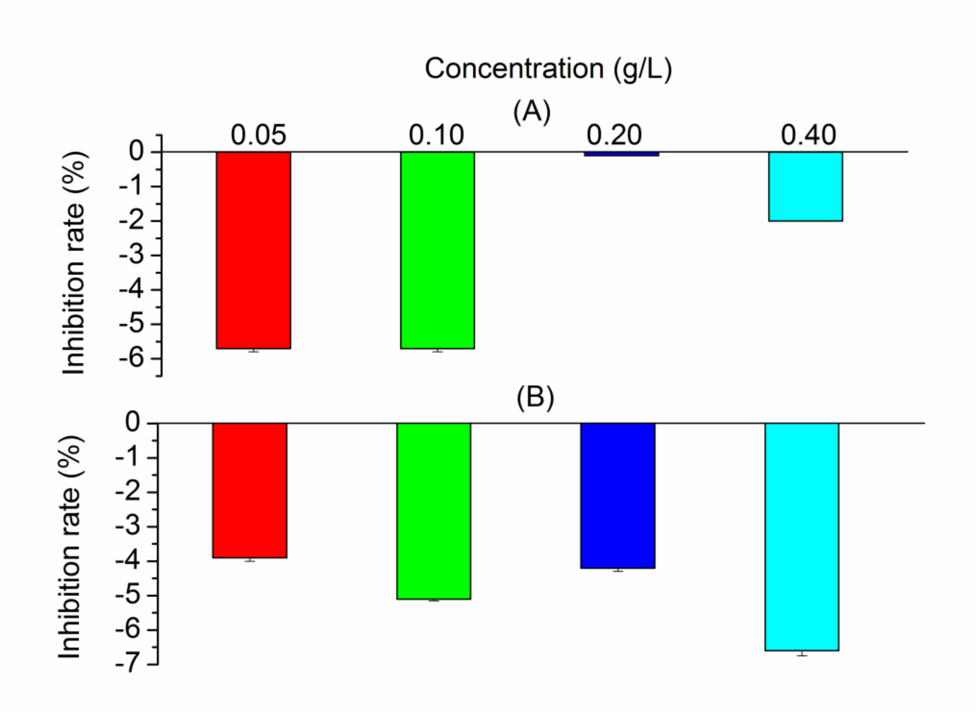

Figure 4. Effects of camptothecin on body weight (A) and length (B) of crucian (Carassius carassius). Body weight and length of crucian was measured at 30 days after application.
The concentration of camptothecin is calculated based on the content of the granules.

Although higher amount of camptothecin was needed than the chemical Niclosamide for preventing schistosomiasis, our results suggested that this will not hinder its practical application. *C. acuminata* is widely cultivated in schistosomiasis epidemic area and plant resource is rich for preparing camptothecin. The content of camptothecin in dry plant tissues can reach up to > 0.07%, which is suitable for mass production (30). Even grown in the field, it can release the active ingredient camptothecin, into the surrounding environment through leaching and rhizosphere secretion and can be poisonous to the snails (33). Also, camptothecin does not harm the environment, suggested by our results that it has no acute or chronic negative effects on fish. This will be a great advantage in application of camptothecin to contrl the *O. hupensist.* Also, the cost of camptothecin from plant materials is lower than the chemical molluscacides but the economic, social and ecological benefits are much higher. At present, wetlands with oncomelania cover about 3.67 billion m^2 in China. It will cost about 615 million RMB (￥) on mollusciciding, if chemical molluscacides (santobrite and niclosamide) are used. But the use of plant based camptothecin will save more than 300 million RMB (￥) annually.

Traditional molluscacide dissolves very rapidly in water thus have low efficacy (13). The molluscacide presented in this study is modified to avoid this problem. The Aribic gum in the camptothecin granules alleviates the release of molluscacide from the granules into water, and therefore greatly extends the lifetime of the molluscacide. Further, oncomelania is easily attracted by sugar and flour added in the granules, which also significantly improves the molluscacidal effect. The plant camptothecin preparation we presented here is a scientific and practical innovation.

ACKNOWLEDGMENTS

This work was supported by the National Scientific and Technological Project of China (No. 2015BAD07B07).

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