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Full Length Research Paper

Molluscicidal effect of sanguinarine against Oncomelania hupensis snails

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A strong molluscicidal effect of sanguinarine against Oncomelania hupensis snails was demonstrated by immersion test in the present study. A positive relationship between doses, immersion time and the mortality of the snails was exhibited, while the optimal dose and immersion time of application towards the field eradication of snails were suggested. The possible mechanisms of effects were explored by determining the changes in liver aminotransferases (ALT, AST) of the snails and it was revealed that the damage of liver cells was the cause of snail death.

Key words: Alanine aminotransferase, liver, Oncomelania hupensis snail, molluscicide, sanguinarine.

INTRODUCTION

Schistosomiasis is a common tropical disease seriously endangering the health of people in large parts of the world (Steinmann et al., 2006; Dai et al., 2008). The World Health Organization (WHO) estimates that about 3 billion people reside in schistosomiasis endemic countries of which more than 200 millions are infected. A third of them are children under 15 years of age (Barbosa, 1995; Steinmann et al., 2006; Yang et al., 2007). In China, Schistosoma japonicum is prevalent with Oncomelania hupensis snail as the only intermediate host (Zhou et al., 2005). Currently, the snail-infested area covers more than 372,263 Hm2, mainly in the lake regions along the Yangtze river, and there are 412,927 infected cases (Hao et al., 2009). Thus, control of the O. hupensis snails is one of most important strategies for interrupting and eliminating Schistosomiasis transmission (Xu et al., 2004), and moluscicidal methods have been the focus of research for nearly half a decade (Zhou et al., 2005; Zhou et al., 2007).

There are several means to eliminate O. hupensis snails, including environmental, physical, biological and pharmacological control measures (Tchounwou et al., 1991; Souza, 1995). To date, artificial chemical agents have routinely been used, since their molluscicidal effects are more durable and easily limited to distribution areas of the snail. The drawbacks are potential toxic effects to humans, livestock, fish and the overall environmental pollution (Andrews et al., 1982; Yang et al., 2008).

Our previous study showed that Eomecon Chionantha Alkaloids, extracted from Eomecon Chionantha Hance, an herbaceous perennial indigenous to China, had an encouraging molluscicidal effect and seems less hazard to non-target organisms, especially to fish (Yang et al., 2003). Sanguinarine, one of the main monomer component (Figure 1) separated from Eomecon Chionantha Hance, had been shown to have antibacterial and insecticidal effects (Dhopeshwarkar et al., 2011; Miao et al., 2011). By means of immersion method, the present studies were designed to determine the optimal molluscicidal effect of sanguinarine.

To further explore the involved working mechanisms, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities of the snail liver were examined.

MATERIALS AND METHODS

Separation of sanguinarine from ECH - Eomecon Chionantha

Hance is a perennial herb, a Papaveraceae of

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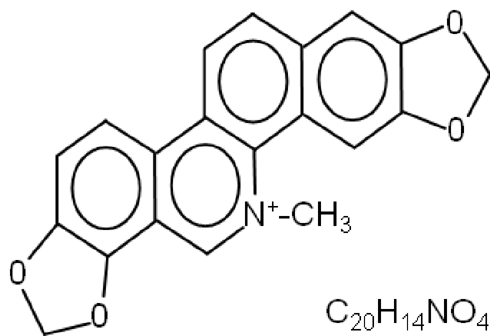

Figure 1. Structure of sanguinarine.

Chelidonium family, established by Hance 1884 (Zhang et al., 1989). This richness in alkaloid of sanguinarine grass was collected from the Renxing hills of Taojiang county, Hunan province, China, in the middle of autumn, November. The rootstalks of the grass were dried and ground into coarse powder, and then extracted by 1% hydrochloric acid aqueous. The extract was absorbed by macroporous adsorption resin, eluted and concentrated by 20, 60 and 90% methanol solution and 100% ethanol, and finally recrystallized to gain sanguinarine. However, 98% purity sanguinarine was prepared for the experiment. The alkaloid of sanguinarine was properly identified by Liu et al. (2005) and the cytotoxity assays with normal cells showed that the compound under the present study was endotoxin free.

Snails

The snails (O. hupensi) were collected from Dongting Lake, Hunan, China, and fed in laboratory for 1-2 days. Adult uninfected snails, 6-10 mm long with 6-9 shell stride, were selected for the experiment.

Molluscicidal test

The test was performed according to WHO "Molluscicides Final Screening Laboratory Methods" of immersion test mode (Webbe, 1961). Sanguinarine was prepared in concentrations 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39 and 0.20 mg/L in water (de-chlorinated water was used throughout the experiment). Each concentration was tested in 4 groups (24, 48, 72 and 96 h immersion times) and each group consisted of 50 snails which were put in a nylon net bag immersed in a 1500 ml solution at 25°C. To test the snail mortality, one bag at a time was removed from each solution after 24, 48, 72 and 96 h respectively, and washed two times with water and stored out of water for 72 h. Same procedures were carried out for snails with water immersion as a control.

The number of dead snails was determined by direct observation of snail movements in water; snails with a closed operculum and/or lack of movement were further tested by the knocking method of Webbe and Lambert (1983), that is, by inspecting movement of the soft body after crushing the shell. Snails without movement in this situation were defined as dead.

The liver enzymes activity test

Same immersion procedures were used for the liver enzymes activity test. Three groups (24, 48 and 72 h immersion times) of 50 snails were assigned for liver ALT, AST measurement at each sanguinarine concentration of 12.5, 6.25 and 3.13 mg/L, and one control group in water without sanguinarine. The snails of each group were exposed for 24, 48 and 72 h, respectively. The snails were sacrificed immediately after 24, 48 and 72 h and their livers carefully dissected out under the dissecting microscope. A liver homogenate was prepared in 0.1 ml pre-cooled phosphate buffer (0.2 mol/L, pH 7.1) in an ice bath. After centrifugation at 0 - 4°C for 5 min at 8000 × g, the upper phase was collected. ALT and AST activities were analysed by Guilbault´s spectrophotometric technique (Guilbault et al., 1976) at 340 nm, using DBDA full automatic biochemical analyser (DADE, USA). The activities were expressed in international units (U/l).

Statistical analysis

Data were analyzed with statistical software SPSS (version 17.0 for Windows, SPSS, Chicago, IL, USA). Unless otherwise noted, all data are presented as mean ± SD. A one-way ANOVA followed by a least significant difference (LSD) post hoc test was used for multi-group and student t-test for two paired group comparison; moreover, $p < 0.05$ was considered as statistically significant.

RESULTS

Molluscicidal effect

A potent molluscicidal effect of sanguinarine against O. hupensis was demonstrated in this study (Table 1). The effects increased with sanguinarine concentrations and exposure times at 25°C immersion solution. The mortalities all reached 90% at the sanguinarine concentrations of 25 mg/L for 48 h, 6.25 mg/L for 72 h and 0.78 mg/L for 96 h, while at 25 mg/L for 72 h and 12.5 mg/L for 96 h, the mortalities attained 100%. Even at the lowest tested immersion time of 0.20 mg/l at 48 h and longer immersion times, the mortalities were clearly different from those of the controls. LC50 was 31.84 ± 12.17 (95% c.i.), 1.42 ± 0.37, 0.33 ± 0.11 and 0.16 ± 0.06 mg/L for 24, 48, 72 and 92 h immersion time, respectively.

Using multiple stepwise linear regression test, the obtained regression equation that describes the found

Table 1. Mortality of snails at different concentrations of sanguinarine and immersion time (%, n = 50/group).

Sanguinarine	Immersion time			
(mg/L)	24 h	48 h	72 h	96 h
50.0	52	96	100	100
25.0	44	90	100	100
12.5	42	76	96	100
6.25	24	76	90	98
3.13	22	68	82	98
1.56	6	52	80	92
0.78	2	44	76	92
0.39	0	34	68	88
0.20	0	10	22	42
Control	0	0	0	0

relation between snail mortality (y), sanguinarine concentration (X1) and exposure time (X2) was $y = 8.68X1 + 20.99X2 - 34.93$, a typical positive relationship was exhibited ($p < 0.01$). According to the variable coefficient linear equations, the optimal molluscicidal effects would be achieved at sanguinarine concentration of 0.78 mg/L and exposure time of 48 h in these laboratory trails.

Changes in liver ALT activity

Remarkable changes in liver alanine aminotransferase (ALT) activity of the snails were revealed after sanguinarine intervention (Figure 2). In the control groups, the ALT activity gradually increased with the immersion time, while there was a bimodal effect in the sanguinarine exposure groups. At shorter exposure times (24 and 48 h) and lower sanguinarine concentration (1.56 mg/L), the ALT activity was significantly higher than that of the controls, reaching 73 ± 8.5 and 91.3 ± 13.1 U/l, compared to 59 ± 4 and 81 ± 10 U/l in controls, respectively ($p < 0.05$). However, after longer exposure periods and higher sanguinarine concentrations, the ALT activity was gradually decreased compared to earlier values and the controls, from 77.7 ± 9 and 62.67 ± 9 U/l after immersion times of 48 and 72 h at the sanguinarine concentrations of 3.13 mg/L, to 50.7 ± 5.5 and 47 ± 3 U/l at the sanguinarine concentrations of 6.25 mg/l, all were significantly different from those of control and group of 1.56 mg/L. The lowest ALT activity gained by 6.25 mg/L of sanguinarine at 72 h immersion time. The early increase and late fall in ALT activity after sanguinarine immersion of the snails was the typical finding in this study.

Changes in liver AST activity

The changes in liver aspartate aminotransferase (AST) activity of the snails presented a similar tendency to the changes of ALT after sanguinarine treatment (Figure 3), although the early increase did not reach statistical significance. Snails after sanguinarine exposure at concentration of 1.56 mg/L, the AST activities were 747 ± 31.8 and 902 ± 43 U/l compared to 707 ± 41 and 873 ± 42 U/l in controls after exposure of 24 and 48 h, respectively. At a concentration of 3.13 mg/L, the AST activity started to decrease after 48 h exposure time but could not reach a significant rate until the sanguinarine concentration was raised to 6.25 mg/L, whereas exposure for 72 h showed that the activities were clearly decreased at concentrations of 3.13 and 6.26 mg/L (642 ± 49.6 and 576 ± 23.9 U/l, respectively). The activities were significantly different from those of the control, which recorded shorter exposure time and lower sanguinarine concentration ($p < 0.01$). The lowest AST activity was obtained by 6.25 mg/L of sanguinarine at 72 h immersion time.

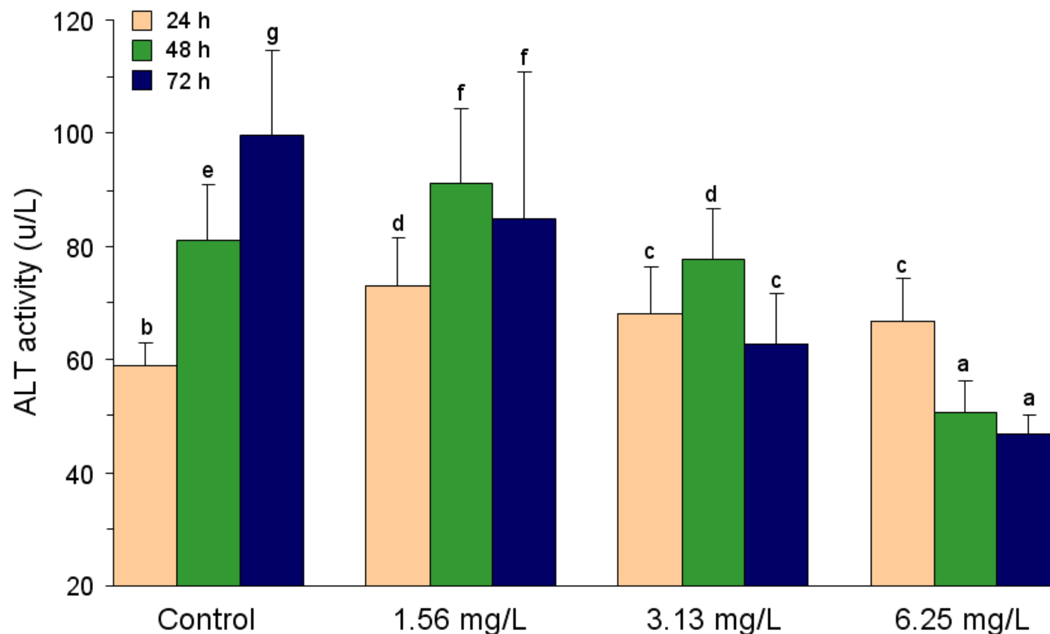

Figure 2. Changes in liver Alanine aminotransferase (ALT) activity of *Oncomelania* snails with different sanguinarine concentrations and immersion times.

Temperature of immersion solution was 25°C. Note that the ALT activity of control animals was gradually increased with immersion time, while dose dependent effect was displayed after higher concentration of sanguinarine exposure. With weaker sanguinarine exposure (1.56 mg/L), the ALT increased more than in the controls while it decreased after stronger (1.56 - 6.25 mg/L) and longer exposures (48 - 72 h). Bars without a common superscript letter differ significantly (Mean ± SD, n = 50/group, p <0.05).

DISCUSSION

A reliable molluscicidal effect of sangunarine was confirmed in present studies. The effect was positively related to sangunarine concentration and immersion time at 25°C. Maximal lethal effect was obtained at 25 mg/L for 72 h and 12.5 mg/L for 96 h, the mortalities reached 100%, whereas the optimal molluscicidal effects were achieved at sangunarine concentration of 0.78 mg/L and exposure time of 48 h.

Except from rootstalks of Eomecon Chionantha Hance, sanguinarine can be also derived from the roots of Sanguinaria Canadensis (Mahady and Beecher, 1994), Chelidonium majus (Vavreckova et al., 1996) and the seeds of the Argemone mexicana (Tandon et al., 1975). The identification of molluscicidal effect in this study broadened the pharmacological function of sangunarine and ensured the active molluscicdal component of Eomecon Chionantha alkaloids, which is great benefit to the development and utilization of Eomecon Chionantha Hance and other sangunarine contained plants. The findings provide practical variables for field mulluscicide trials, for example, the selection of season (water temperature) for spraying the drugs, optimal final concentration of sangunarine to minimize costs and required time for snail-elimination.

Sanguinarine exerts multiple effects within cells, including binding microtubules, blocking the assembly of microtubules, thus inhibiting cells proliferation (Lopus and Panda, 2006); supressing activities of lipoxygenase, acetylcholinesterase, Na+, K+-ATPase, succinate dehydrogenase, NADH dehydrogenase, certain protein kinase and phosphatase. In consequence, it inhibits NF-kB activation, IkB phosphorylation and degradation, thereby altering mitochondrial respiration, reducing ATP production, interfering with cell metabolism and signal transduction, affecting DNA synthesis and gene expression, blocking the cell cycle (Holy et al., 2006, Ahsan et al., 2007). All the pathological alternations above would explain the molluscicidal effect of sanguinarine found in the present study.

ALT and AST are the most important and active transaminase of the O. hupensis snails (Wang and Song, 1990). Other than the effects of ammonia transformation, these two aminotransferases are closely related to the action of Krebs cycle and linked to protein synthesis and glucose metabolism. The enzymes are found mainly in the liver and their changes are the most sensitive indicator of liver cells injury (Kolodziejczyk et al., 2005). Compared with the control, the ALT and AST activities in snails were increased after shorter exposure time at lower sanguinarine concentrations, decreased after

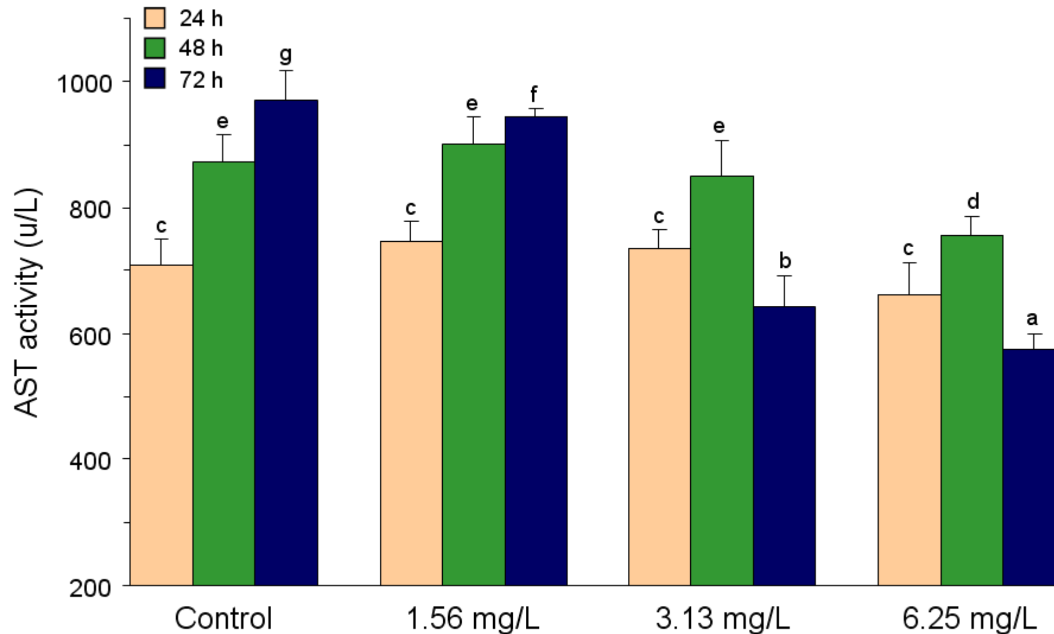

Figure 3. Changes in liver Alanine aminotransferase (AST) activity of *Oncomelania* snails with different sanguinarine concentrations and immersion times.
Temperature of immersion solution was 25°C. Note that the AST activity of control animals was gradually increased with immersion time, while dose dependent effect was displayed after higher concentration of sanguinarine exposure. With weaker sanguinarine exposure (1.56 mg/L), the AST was not different from the controls while it decreased after stronger (3.13 - 6.25 mg/L) and longer exposures (48 - 72 h). Bars without a common superscript letter differ significantly (Mean ± SD, n = 50/group, $p < 0.05$).

longer exposure times at higher sanguinarine concentrations. The results are in agreement with the early studies on Eomecon Chionantha alkaloids molluscicidal effect (Ken et al., 2000; Liu et al., 2005). We have assumed that the early increase was due to enzyme induction in the liver cells, while the later decrease was the result of toxic damage with disordered function or necrosis of the liver cells. Therefore, measurement of ALT and AST activities may reflect the level of liver damage and point to a possible sanguinarine molluscicidal mechanism.

An increase in ALT and AST activities were also present in the control groups of snails. O. hupensis snails are water and land amphibious animals. A long immersion time in water, as in this laboratory situation, would result in hypoxia, manifested as a moderate increase in liver ALT activity. Actually, water immersion of fields is an important means for snail control although eight months are required to reach the aims of eradication of the O. hupensis snails (Xu et al., 2002). In comparison, the present immersion times were short (up to 96 h), presumably far from the lethal time.

The functions of snail liver are breaking down and storing nutrients, secreting of various digestive enzymes and enzymes crucial for various important synthesis and catabolism. It is not only an important detoxifying organ for O. hupensis snail, but also one of the most vulnerable organs to toxins. The morphological studies in our group (Peng et al., 2007) showed tremendous alterations in the liver cells after ECA treatment, for example, oedema and degeneration, accompanied by swollen and dissolved nuclei, enlarged and vacuolated rER, dilated and vesiculated mitochondria with broken crests, indicating a hepatotoxic effect of ECA including sangunare in O. hupensis snails. Such hepatotoxic effects would dramatically lower the detoxifying function of the liver and result in profound enzymatic and metabolic changes that may eventually poison the snails to death.

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