Evaluation of molluscicidal activities of benzo[c]phenanthridine alkaloids from Macleaya cordata (Willd) R. Br. on snail hosts of Schistosoma japonicum

Zhong Ming¹,²,₄, Li Gui-Yin³, Zeng Jian-Guo², Zhang Li¹,₄, Huang Ke-Long¹,* She Jin-Ming¹, Li Xiao² and Wei Wang-Yuan⁴

¹School of Chemistry and Chemical Engineering, Central South University, Changsha 410083, China.
²Hunan Engineer Research Center of Botanical Extract, Changsha 410301, China.
³Hunan Vocational College of Science and Technology, Changsha, Hunan 410118 China.
⁴School of Chemistry and Chemical Engineering, Hunan Institute of Science and Technology, Yueyang 414006, China.

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Three botanical products, including bisulfates of sanguinarine (BSA), bisulfates of chelerythrine (BCHE) and bisulfates of total alkaloids (BTA), were obtained from the fruits of Macleaya cordata (Willd) R. Br. They were evaluated for the molluscicidal activities against snail of Oncomelania hupensis, intermediate host of schistosomiasis. It was shown that the molluscicidal activities of three botanical products were correlated with the presence of benzo[c]phenanthridine alkaloids (sanguinarine and chelerythrine). The three products all exhibited both time- and concentration-dependent toxicity against the snails. The significant LC₅₀ values at 72 h were found to be 0.19 mg/L (BSA), 0.40 mg/L (BTA) and 2.05 mg/L (BCHE), respectively. The results indicated that BTA from M. cordata was a potent molluscicide, since it exhibited high molluscicidal activity and was easy to be produced from the plant with low-cost.

Key words: Macleaya cordata (Willd) R. Br., Oncomelania hupensis, molluscicidal activity, benzo[c]phenanthridine alkaloid.

INTRODUCTION

Schistosomiasis is a major health problem in many developing areas, including Africa, South America, the Caribbean, the Middle East and Asia (Chitsulo et al., 2000). The World Health Organization (WHO) in 1993 year estimated that over 200 million people are infected by Schistosomiasis in 73 countries while about 5% of the world’s populations (500 ~ 600 million) are at risk of being infected (WHO Technical Rep, 1993). Schistosomiasis is still a serious endemic disease in middle and southeast China (WHO, 1983). Snail, Oncomelania hupensis, is the only intermediate host of Schistosoma japonicum, which is the species causing schistosomiasis in China. The most effective method of reducing the risk of schistosomiasis transmission in endemic area is to delink the life cycle of schistosome by killing snails. Chemical molluscicides, such as niclosamide and pentachlorophenate, are found to control snail effectively, but they are expensive and not always convenient to use (Chen et al., 2007). Moreover, the compounds generally have toxicity to water fauna, domestic animals and human beings. Therefore, it is significant to find natural molluscicides which are effective, cheaper and environmental friendly to kill snails.

Macleaya cordata (Willd) R. Br. is a perennial plant of the Papaveraceae family (Zhang et al., 2005). It distributes throughout the middle and southeastern of China and is available in most schistosomiasis-endemic areas of China. In our previous work, the extract (total alkaloids) from the fruits of M. cordata was found to
present significant molluscicidal activity against *O. hupensis* (Zhong et al., 2009). Further studies indicated that the main ingredients in the total alkaloids were benzophenanthrene alkaloids and the molluscicidal activities were correlated with the presence of these substances (Singh et al., 1999). Sanguinarine and chelerythrine are the two main benzophenanthrene alkaloids in the plant. The structure of sanguinarine and chelerythrine are shown in Figure 1. It was also observed that a botanical product, named as bisulfates of total alkaloids (BTA), was obtained from the total alkaloids of *M. cordata* exhibited potent molluscicidal activity. The BTA contains mainly of bisulfate of sanguinarine (BSA) and bisulfate of chelerythrine (BCHE) and can be obtained from the fruits of *M. cordata* (Zhong et al., 2009). However, BTA, BSA and BCHE have been little reported for the molluscicidal activity. Therefore, it is significant to development of new molluscicide with bisulfate of sanguinarine and chelerythrine as main components.

The main objective of this study was to determine the molluscicidal activities of BTA, BSA and BCHE obtained from *M. cordata* against *O. hupensis* according to WHO guidelines (WHO, 1983). Also, the toxicity of BTA to the liver of snails was investigated by transmission electron microscope (TEM) and the mechanism against snail was clarified preliminarily in this paper.

The specimens have been deposited in the herbarium collection of Hunan institute of science and technology, Yueyang, China.

**Extraction and isolation**

Percolation was used to obtain total alkaloids from the fruits of *M. cordata*. Approximately 500 g dried fruits of the herb were packed into a percolator which was a glass column (8 × 100 cm I.D.). After 2 h maceration, an amount of 7.5 L of aqueous sulfuric acid solution (0.1 mol/L, 70°C) flew through the column at 60~65 ml/min. The collected supernatant was added proper volumes of 10% sodium hydroxide solution to adjust pH to 9~10 and then filtrated. Filter residue was dried and dissolved in 600 ml ethanol (95%). Then, the concentrated sulfuric acid was trickled slowly in the ethanol solution and red deposition was emerged. The collected red deposition, which was the BTA comprised mainly of bisulfates of two tertiary benzo[c]phenanthridine alkaloids, was subjected to the isolation of sanguinarine and chelerythrine.

Column chromatography in this experiment was used in the separation process, referring to the methods described by Hu et al. with some modified (HU et al., 1979). A low-pressure glass column (2.5 × 50 cm I.D.) filled with activated silica gel (200~300 mesh) was used. A sample of BTA alkalified by appropriate amount of ammonia water was dissolved in chloroform and loaded onto the column. Then, isocratic elution was performed with benzene/methanol/diethylamine = 40/1/0.3 (v/v) at 2 ml/min. The fractions (A) mainly containing sanguinarine which showed as red ribbon in silica gel column was collected and detected by Dragendorff’s regent. When red ribbon in column chromatography was over, isocratic elution changed into benzene/methanol/diethylamine = 25/1/0.3 (v/v). The fractions (B) with yellow ribbon in column chromatography were obtained, with chelerythrine as the main component. The collected eluents were evaporated with a rotary vacuum evaporator at 45°C. The residues were crystallized repeatedly in acidic ethanol with 0.05 M dilute sulfuric acid. About 2 g crystals of bisulfates of sanguinarine and chelerythrine (namely BSA and BCHE) were obtained and after High-performance liquid chromatography (HPLC) analysis subjected to the molluscicidal activities assay in subsequent processing.

**MATERIALS AND METHODS**

**Plant materials**

Sun-light dried fruits of *M. cordata* were collected from Liuyang, Hunan province, China in November, 2008 and identified in our lab.
HPLC analysis of the extract

The standard substances for sanguinarine and chelerythrine were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The BTA mentioned above was analyzed by HPLC system (Shimadzu Corporation, Kyoto, Japan). The chromatographic conditions used in the analysis procedure were the same as described by Luo et al. (2005). The purity of bisulfates of sanguinarine and chelerythrine was not less 96% according to HPLC analysis based on peak area normalization method. The identity of them was verified by 1HNMR and high performance liquid chromatography-electrospray tandem mass spectrometry method.

Source of snails

Snails, _O. hupensis_, were collected from Junshan, Hunan province, China. They were cultured at 25°C with dechlorinated tap water in laboratory conditions for three days. Adult snails of uniform size (8-10 mm in length) were used as experimental animals.

Molluscicides activity

Molluscicidal activity was evaluated according to WHO guidelines (WHO, 1965). The crystals of bisulphates of the two benz[c]phenanthridine alkaloids and the powder of the BTA were dissolved in third volumetric flasks with distilled water to give a 50 mg/L solution and then transferred to de-chlorinated water to give a serial of diluents (3.125, 1.563, 0.782, 0.395, 0.20 and 0.1 mg/L). Successive dilutions of the test solutions were made in order to find the suitable concentration required to kill snails. Thirty of _O. hupensis_ snails were collected in one nylon net bag and kept in beaker. These bags were immerged in each dilution. Positive control groups were immerged in 50% wet table powder of nicosamide ethanalamine salt solution (2 mg/L) and negative control groups in de-chlorinated water (DCW) with the same conditions. The test animals were exposed continuously to different concentrations of three plant products (Table 1).

After 24, 48 and 72 h of exposure, test snails were washed with de-chlorinated water for 3 times and then kept in de-chlorinated water for another 24 h for recovery before mortality was evaluated. During the recovery period, dead animals were removed to avoid contamination of live animals. Snail mortality was observed by the contraction of the body within the shell, and no response to a needle probe was taken as evidence of death. It was recorded at the interval of 24 h up to 72 h. Experiments were conducted at 25 ± 1°C. Each experiment was replicated three times. Lethal concentration (LC50, LC90) values, upper and lower confidence limits were calculated using SPSS 13 software.

Table 1. LC50 and LC90 (mg/L, 95% CI) of three plant products (BTA, BSA and BCHE) from _M.cordata_ against the snails after 72 h exposure.

<table>
<thead>
<tr>
<th>Exposure period (h)</th>
<th>Plant product</th>
<th>LC50 (mg/L, 95% CI)</th>
<th>LC90 (mg/L, 95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>48</td>
<td>BTA</td>
<td>0.69 (0.59~0.83)</td>
<td>1.39 (1.12~1.94)</td>
</tr>
<tr>
<td></td>
<td>BSA</td>
<td>0.59 (0.49~0.70)</td>
<td>1.38 (1.09~1.95)</td>
</tr>
<tr>
<td></td>
<td>BCHE</td>
<td>2.70 (2.26~3.49)</td>
<td>5.22 (3.89~10.17)</td>
</tr>
<tr>
<td>72</td>
<td>BTA</td>
<td>0.40 (0.32~0.50)</td>
<td>1.24 (0.93~1.87)</td>
</tr>
<tr>
<td></td>
<td>BSA</td>
<td>0.19 (0.15~0.24)</td>
<td>0.54 (0.41~0.84)</td>
</tr>
<tr>
<td></td>
<td>BCHE</td>
<td>2.05 (1.69~2.63)</td>
<td>4.86 (3.54~8.79)</td>
</tr>
</tbody>
</table>

TEM observation of liver cell of snail

To investigate the mechanisms of BTA against _O. hupensis_, the sublethal concentration 3.12 mg/L was applied. After immersing in BTA solution (3.12 mg/L) for 24 h, the agonal snails were selected. Its liver tissues were dissected to prepare conventional electron microscopy specimens and the ultrastructure of liver cell was examined by Hitach JEOL-1230 transmission electron microscope.

RESULTS AND DISCUSSION

Preliminary experiments in our lab exhibited that the studied botanical products with the concentrations over 5 mg/L could promote the growth of some of germs, which parasitized on the shell of _O. hupensis_. It would induce non-linear effect against _O. hupensis_. Therefore, the concentration ranges of three botanical products were set at 0.1~3.13 mg/L in order to obtain their LC50 values against snails. As it is seen from Table 1, the three products exhibited significant molluscicidal activities against _O. hupensis_. BSA showed the highest toxicity on snails with LC50 (0.59 mg/L, 48 h; 0.19 mg/L, 72 h). It is followed by BTA with LC50 (0.69 mg/L, 48 h; 0.40 mg/L, 72 h), which had slightly lower toxicity against snails than BSA. Among them, BCHE exhibited a relatively mild molluscicidal activity with LC50 (2.70 mg/l, 48 h; 2.05 mg/l, 72 h). Figure 2 presented the snail mortalities after exposition to BTA, BSA and BCHE solution at 3.13 mg/L for 24, 48 and 72 h (DCW as blank). It indicated that the exposure time was an important factor to cause a large mortality of snails. As shown in Table 2, the dead number of snails increased with the increase of BTA concentration and exposure time. The LC50 values of the BTA decreased from 0.69 mg/L (48 h) to 0.40 mg/L (72 h) with the increase of exposure time. Moreover, the LC90 values of the BTA decreased from 1.39 mg/L (48 h) to 1.24 mg/L (72 h) as well. It suggested that the product exhibited both time- and concentration-dependent toxicity against the snail. The similar phenomenon could be found for BSA and BCHE from Table 1. The time dependent toxic effect of these botanical products may be due to the uptake of the active compounds which progressively increase in snail bodies with the increase of exposure period (Jaiswal et al., 2008). According to
Singh et al. (1999), molluacidal activities of the three plant products from *M. cordata* were due to the presence of benzo[c]phenanthridine alkaloids, sanguinarine and chelerythrine. The HPLC analysis demonstrated that the contents of BSA and BCHE in BTA were 58.1 and 28.7%, respectively, while others components in BTA were less then 15%. A comparison of the molluscicidal activities of the two purified compounds (BSA and BCHE) clearly demonstrated that the main component with killing snail activity was bisulfate sanguinarine (Table 1). Therefore, the molluacidal activity of BTA with higher BSA content is close to that of BSA with a slightly lower LC$_{50}$ value. As reported by Peeples et al. (1982) and Kaushal et al. (1991), sanguinarine disturbed both membrane of microsome and cytosolic defences in hepatocytes, and destabilized the cytochrome P-450 system. When sanguinarine in the body of snail was metabolized by the cytochrome P-450 system in the inner membrane of liver microsomal, it bound to the enzyme system of cytochrome P-450, leading to irreversible loss of function of the enzyme system and the death of snail. To provide an insight into the mechanism of the action of these plant products in snail body, the liver tissues of an agonal snail, which was immersed 24 h with BTA aqueous solution

Table 2. Concentration-and time-dependent effects of bisulfates of total alkaloids (BTA) on *O. hupensis* after different time exposure under laboratory condition.

<table>
<thead>
<tr>
<th>Concentration (mg/L)</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCW*</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NIC *</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>0.2</td>
<td>0</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>0.39</td>
<td>1</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>0.78</td>
<td>3</td>
<td>13</td>
<td>25</td>
</tr>
<tr>
<td>1.56</td>
<td>3</td>
<td>30</td>
<td>27</td>
</tr>
<tr>
<td>3.13</td>
<td>5</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

* DCW and NIC (2 mg/L) as negative and positive reference substance. The numbers of snails in tested group were 30.

Figure 2. Snail mortality after exposition to BTA, BSA and BCHE at 3.13 mg/L (de-chlorinated water as blank) for 24, 48, 72 h. (A) DCW; (B) BTA; (C) BSA; (D) BCHE.
(3.13 mg/mL), were observed by transmission electron microscope (Figure 3). As is showed in Figure 3, the liver cells were swollen and degenerated (A), some of the mitochondrial and endoplasmic reticulum expanded (B), and some of the cell nuclei atrophied (C) or dissolved (D). It may suggest that the sanguinarine-derived hepatotoxicity was a main factor of snail death. Although, the toxicity of BTA against O. hupensis was lower slightly than that of BSA, BTA may be a potential molluscicides derived from M. cordata. For BTA was a plant product with lower production cost and higher molluscidal activity compared with BSA and BCHE, which were difficult to purify, development of BTA as a potent molluscicide had very good prospects.

Conclusion

BTA from M. cordata may be used as a potent molluscicide because of its low LD₅₀ (0.69 mg/L, 48 h and 1.39 mg/L, 72 h). Compared with BSA and BCHE, BTA was a plant product with cheaply cost and higher toxicity against O. hupensis. It suggested that M. cordata was an important source of botanical molluscicides. The plant product from the herb may be helpful in controlling schistosomiasis, especially in the southeast of China where the schistosomiasis disease is a main endemic. Therefore, the detailed studies should also be carried out on the three botanical products to elucidate both pharmacological effect and the action mechanism of these plant products in snail body. In future, more attention must be paid to the efficient extraction of the alkaloids and the application as botanical molluscicides in rural communities of the third world countries.

ACKNOWLEDGEMENTS

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