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Molluscicidal activity and physiological toxicity of *Macleaya cordata* alkaloids components on snail *Oncomelania hupensis*

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ABSTRACT

In order to search new local plant molluscicides for the control of the vectors of schistosomiasis, leaves of *Macleaya cordata* (Willd) R. Br. were used to extract and separate alkaloid components by thinner acid method and column chromatography, and the molluscicidal effect of alkaloid components against snail *Oncomelania hupensis* was determined by bioassay. The results showed that 7 alkaloid components (AN1-7) were obtained after extracting and separating alkaloids from the leaves of *M. cordata*, where AN2 was found being the most toxic against snail *O. hupensis* with 48 h LC_{50} and LC_{90} values of AN2 of 6.35 mg/L and 121.23 mg/L, respectively. Responses of some critical enzymes to AN2, including activities of Alkaline phosphatase (ALP), Alanine aminotransferase (ALT), Aspartate transaminase (AST), Malic dehydrogenase (MDH) and Succinate dehydrogenase (SDH) in both cephalopodium and liver, were also detected through experiments, which also explored esterase isozyme (EST) exposed to AN2 in liver tissue. The results showed that AN2 significantly inhibited the activities of SDH, MDH and esterase isozyme, as AN2 significantly stimulated the activities of ALP, ALT and AST to increase at a low concentration (e.g. 25 mg/L), while significantly inhibited the activities of these enzymes at a high concentration (100 mg/L). These results indicated that AN2 not only inhibited protein synthesis, and respiratory chain oxidative phosphorylation, but also caused hepatocellular injury and reduced the detoxification ability of liver.

1. Introduction

Schistosomiasis is the second most prevalent endemic disease after malaria in tropical and subtropical regions [1] and remains a serious disease in China (*Schistosoma japonicum*) by devastating human health [2]. Recent monitoring data revealed that schistosomiasis was re-emerging in China, especially along the Yangtze River and in the lakes region of Jiangxi and Hunan provinces [3]. As the snail *Oncomelania hupensis* is the only intermediate host of *Schistosoma japonicum* that causes schistosomiasis in China, the extermination of snails is efficient for control of schistosomiasis. Niclosamide, the only commercially available molluscicide recommended by WHO for large scale use in schistosomiasis control [4], however, is limited by its high cost of synthetic compounds, along with increasing concern of possible snail resistance to these compounds and their toxicity in non-target organisms, which have raised the study of plant molluscicides [5]. The use of plants with molluscicidal properties is a technology that is simple, inexpensive, biodegradable and appropriate for local control of the snail vector, especially in rural areas of developing countries where schistosomiasis is endemic [6].

As a perennial plant, *Macleaya cordata* (Willd) R. Br. is a member of the Macleaya genus in the family of Papaveraceae, which is mainly distributed in North America, Europe, South and Northwest China. *M. cordata* has been widely used as a folk herbal medicine in China to cure cervical cancer and thyroid cancer, and to relieve insect bites and ringworm infection [7,8]. Currently, *M. cordata* is utilized as a traditional Chinese medicine for the treatment of inflamed wounds, arthritis, rheumatism arthralgia, and trichomonas vagi-nalis [9]. In our initial study, it was found that plant *M. cordata* had strong molluscicidal activity against snails *O. hupensis*, with the molluscicidal effect stronger than that of *Nerium indicum* Mill and *Arisaema erubescens* Schott [10,11]. The objectives of this study were: (1) to furtherly examine the molluscicidal activities of *M. cordata* extracts and try to find out the strongest molluscicidal component in the plant; and (2) to reveal the possible physiological toxicity mechanism of the molluscicidal component from activity changes in Alkaline phosphatase (ALP), Alanine aminotransferase (ALT), Aspartate transaminase (AST), Malic dehydrogenase (MDH), Succinate dehydrogenase (SDH) and esterase isoenzyme (EST).

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2. Materials and methods

2.1. Oncomelania hupensis

Adult *O. hupensis* snails (9–11 mm in length) were collected from farmfield of Taihu countryside of Jingzhou, Hubei Province, China, amongst which the healthy ones were selected by cercariae escaping method to get rid of the infected [12]. Then the snails were kept in a laboratory at 20 °C for a week before experiment.

2.2. Plants

Leaves of the plant *M. cordata* at vegetative growth phase before flowering were collected from Lushan Mountain in central China, with their fresh and dry weights recorded.

2.3. Extracts of alkaloids

The total alkaloids of *M. cordata* were extracted according to the modified ethanol extract method [13,14]. Weigh dryly pulverized *M. cordata* powder for 500 g with 95% ethanol at M:V = 1:3 added, and immerse it for 3 days. Filter the ethanol for the distillation concentration in a cyclotron evaporimeter. With the immersion repeated for 3 times, mix and dry the concentrate to obtain the extractum. Then, solve the total alkaloid extractum of *M. cordata* by 1% hydrochloric acid solution, extract it via chloroform at a volume ratio of 1:1, remove the lipids and pigments in acid water, and keep the acid aqueous phase. Adjust the pH of acid water to around 6.8 through ammonia, and remove the tannin fraction by filtration. Finally, continue to adjust the pH of acid water to 8.0–9.0, and again, extract the solution via chloroform at a volume ratio of 1:1, retain the chloroform phase, and remove the chloroform layer via the cyclotron distillation, so as to obtain the total alkaloids of *M. cordata*.

Components of the total alkaloids were separated with column chromatography. D-101 Macroporous resin, selected as the stationary phase, with ethanol as the moving phase, was columned by the wet packing column method. Firstly fill the chromatographic column with distilled water, and weigh the macroporous resin for 10 g to gradually pour the resion into the chromatographic column with a glass rod for drainage. Then fill the samples as the columning was finished. For the last step, elution was formed by water, gradient aqueous ethanol (10%, 20%, …, 90%), and absolute ethanol in turn with each moving phase of 100 ml. The moving speed was controlled, as every 5 ml was collected in a tube until the eluent was detected as no precipitation via Dragendorff's reagent.

The collected liquors were further analyzed by TLC(thin-layer chromatography) method. Development solvent (chloroform-methanol (19:1)) was used to separate the alkaloids and iodine vapor was used to test the separation point in TLC. Rf values were measured after finishing of the separation, whereas the liquids from different tubes with same Rf values were merged into one tube as one component. Liquids of seven alkaloid components (NO. 1–7) (AN1-AN7) were gained in the end and then dried (freeze drying) for next experiment.

2.4. Testing for molluscicidal activity

Seven different alkaloids components separated from leaves of *M. cordata* were respectively dissolved with distilled water and three treatments (0, 25 mg/L, 50 mg/L and 100 mg/L) were made to test the molluscicidal activity. The snails were collected in a nylon mesh bag (30 snails per bag) and immersed into 500 mL solution of a known concentration in the extracts. For each treatment, five bags (30 snails per bag) of snails were exposed for 24, 48, 72, 96 and 120 h, respectively, with dechlorinated tap water exposure as the control. For checking the mortality, no response to a needle probe under dissecting microscope was the evidence of death. The experiments were conducted at 21 °C, each of which was replicated three times.

2.5. Enzyme assay

According to the testing result of molluscicidal activity above, the strongest molluscicidal active component of alkaloid (AN2) was used to examine the snail enzyme assay. Two hundred snails were randomly divided into four groups and exposed to each concentration level of AN2 (0, 25 mg/L, 50 mg/L, and 100 mg/L) for 48 h, with dechlorinated water as the control. Afterwards, the snails were washed with dechlorinated water, and their cephalopodium and liver tissues were excised for biochemical analysis - a process recommended by Li [15]. Phosphatic buffer solutions (pH 6.8) were added (1 drop/snail) to the cephalopodium and liver which were then homogenated in ice incubation condition, where the homogenates were centrifuged at 4000 rpm for 10 min at 0 °C. Then, one part of supernatant was stored at − 20 °C for the measurement of enzyme activities and electrophoresis. The processes of preparation of snail cephalopodium and liver were replicated for 3 times in the same conditions.

2.5.1. Assay of enzyme activities

The enzyme activities were measured according to Guilbault et al.'s [16] methods of enzyme kinetic assay. Enzyme activities were expressed as the amount of substrate hydrolyzed or production liberated in 1 mol/min/g protein in the supernatant.

Total protein level of supernatants was estimated according to Bradford's method [17]. Enzyme activities were expressed as the amount of substrate hydrolyzed or production liberated in 1 mol/min/g protein in the supernatant.

ALP kit was purchased from Nanjing Jiancheng Bioengineering Institute, and the ALP activities were determined at 405 nm with 4-nitrophenyl phosphate as the substrate. ALT, AST, LDH, SDH and MDH kits were purchased from Shanghai AILEX Technology Co., Ltd., and the enzyme activities were determined at 340 nm.

2.5.2. Electrophoresis assay of EST

Similar to the snail treatments as above, two treatment levels (0 and LC_{50}) were designed. Having exposure for 24 h and 48 h, the snails were carefully crushed so that the liver tissues could be extracted. Phosphatic buffer solutions (pH 6.8) were added (1 drop/snail) to the liver tissues, which were then homogenated on ice. Then the homogenates were centrifuged at 8000 *g* for 10 min at 0 °C. The supernatant was stored at − 20 °C for enzyme electrophoresis.

The supernatant (10 μL each sample, 2 replicates) of snail liver tissues was electrophoresed with a vertical electrophoresis system ECP3000. Polyacrylamide gel electrophoresis (PAGE) was carried out with 20 mA constant current until the tracing dye reached the bottom of the gel. The chemicals were purchased from Sigma, with the separating gel consisted of 7.5% (acrylamide + bisacrylamide) and 0.04 mol/L Tris-HCl, with pH of 8.8; the stacking gel consisted of 3.75% (acrylamide + bisacrylamide) and 0.01 mol/L Tris-HCl, with pH of 6.8; and the buffer was Tris-glycine buffer, with pH of 8.3. After electrophoresis, gels were stained, washed, fixed and then photographed. Acetic acid (7.5%) was the stationary phase.

2.6. Data analysis

The mortalities of snails were expressed as the mean of three replicates. The effect of alkaloid components on *O. hupensis* was expressed by LC_{50} and LC_{90} and their 95% confidence limit (95% cl). The results of enzyme activities were expressed as means ± SE of the three replicates. One way ANOVA and SSR (Duncan's repeat comparison) were used to detect significant differences ($P < 0.05$ or 0.01).

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