



Extraction of Cantharidin from *Mylabris phalerata* Pallas with Compound Enzyme and HPLC Analysis

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Cantharidin is a monoterpene anhydride released from beetle *Mylabris phalerata* Pallas or *Mylabris cichorii* Linnaeus as a defensive substance showing versatile pharmacological activities especially anticancer. Now efficient extraction methods for cantharidin have been established so far. Here, four extraction methods, including hydrochloric acid, NaOH solution, mixed extraction and compound enzyme (cellulase and pectinase) methods were compared with traditional Chinese pharmacopoeia extraction method. A quantitative HPLC method was developed for the estimation of cantharidin in *Mylabris phalerata* extract. The compound enzyme method was revealed to present the highest yield of cantharidin among these methods, followed in order by hydrochloric acid method, mixed extraction method, Chinese pharmacopoeia method and NaOH solution method. The HPLC conditions comprise: diamonsil C₁₈ (250 mm × 4.6 mm, 5 μm), flow rate of 1.0 mL/min, detection wavelength of 273 nm, column temperature at 25 °C, mobile phase with acetonitrile: water = 40:60. Under these conditions, cantharidin could be well extracted from *Mylabris phalerata*.

Key Words: Cantharidin, *Mylabris phalerata* Pallas, Extraction, Cellulase, Pectinase.

INTRODUCTION

Cantharidin or 2,3-dimethyl-7-oxabicyclo[1,2,2]heptane-2,3-dicarboxylic anhydride is a monoterpene anhydride released from beetle *Mylabris phalerata* Pallas or *Mylabris cichorii* Linnaeus as a defensive substance. Of diverse insects, only *M. phalerata* and *M. cichorii* have been so far recognized as cantharidin producer. Cantharidin has been employed to cure abdominal lumps, amenorrhoea, stubborn dermatitis, scrofula, malignant sore, as well as carbuncle. This record goes back to the ancient medical book of China "Shen Nong's Materia Medica" in Ming dynasty¹ and *M. phalerata* or *M. cichorii* were indexed by State Pharmacopoeia of the People's Republic of China². Nowadays, cantharidin has been centered as an antitumor agent to treat hepatocirrhosis, gastric carcinoma, breast cancer, hepatogenic cancer, hepatitis carrier, esophageal carcinoma and lung cancer in clinic³⁻⁶. Unfortunately, the current chloroform and acid extraction methods usually lead to considerable yield loss⁷, therefore, there is an urgent demand for efficient extraction methods. Inspired by the extraction of flavonoids from Ginkgo biloba leaves using compound enzyme⁸, whereby high yield of active ingredients were achieved because cellulase and pectinase degraded cell wall, in this present study, we employed cellulase and pectinase

to extract cantharidin from *M. phalerata*. Apart from comparison with other extraction methods, a reliable analysis method was also required.

EXPERIMENTAL

Mylabris phalerata was purchased from Anguo Medical Market in Hebei Province of China, voucher specimens of the beetles were deposited in Biochemical Engineering College of Beijing Union University. Cantharidin was purchased from Nanjing Ze Long Medical Technology Company Limited (98 % purity). Cellulose (activity of 6 × 10⁵ U/g) and pectinase (activity of 8 × 10⁵ U/g) were purchased from Sunson Group, Beijing, China. All chemicals were of analytical or chromatography grade.

Extraction methods: Beetle materials (100 g for each method) were pulverized to 30 mesh and the following procedures were operated.

Chinese pharmacopoeia method: *Mylabris phalerata* was shaken with chloroform of 2000 mL for 15 min, soaked with chloroform for 6 h at room temperature, filtrated, the fluid was collected and evaporated in a rotary evaporator, followed by a constant chloroform volume to fill the concentrated extracts.

Mixed extraction method (chloroform, HCl, ethanol):

The materials were extracted with 750 mL of mixed solution (chloroform:HCl:ethanol = 1:1:1) for 23 h in a 63 °C water bath pot, the extraction was filtrated at once, the residue was extracted twice with above mentioned mixed solution. The filtered fluid was combined, evaporated in a rotary evaporator, the extract was dissolved with mixed solution (water:chloroform = 1:1), the organic phase was evaporated and dissolved with the mixed solution (petroleum ether:ethanol = 1:1) for detection.

Enzyme extraction method (cellulase, pectinase):

Mylabris phalerata were mixed with compound enzyme (cellulose 8 g, pectinase 16 g) based on pre-experiment, the pH of water solution was adjusted to 5.6 with citric acid, extracted at 40 ± 2 °C for 2 h in a water bath pot, the residue was extracted as described before.

Extraction with hydrochloric acid: 600 mL chloroform, 400 mL acetone and 20 mL hydrochloric acid were added to *Mylabris phalerata* for reflux extraction of 12 h, filtrated, the fluid was collected and evaporated in a rotary evaporator, followed by a constant chloroform volume to fill the concentrated extract for detection.

Extraction with NaOH solution: The materials were soaked with 600 mL of NaOH solution (0.2 mol/L) for 0.5 h, shook at 80 °C for 2 h. After centrifugation at 3000 g/min for 20 min, the supernatants were collected and the pH was adjusted to 1, followed by a chloroform extraction, the organic phase was evaporated in a rotary evaporator, a constant chloroform volume was filled for detection.

TLC analysis: Extracts from *Mylabris phalerata* were respectively loaded to a silica gel plate (10 cm × 20 cm, GF₂₅₄, Taizhou Si-Jia Biochemical Plastic Company, China) and developed with mobile phase of chloroform:acetone = 49:1 (v/v), 0.1 % bromocresol green alcohol solution was sprayed, heated, yellow spots appeared.

HPLC detection and analysis: To quantify the cantharidin in extracts, HPLC analytical parameters including mobile phase, separation column, temperature, flow rate were explored. Consequently, the optimized chromatographic condition was established as follows: Preparative HPLC: Shimadzu, analytical HPLC, LC-10AT vp HPLC pump, CTO-10AS vp thermostated column compartment, SPD-10A vp detector and controller. Injection volume of all sample and standard solutions was 20 μL. The cantharidin was calculated according to the standard curve. The increase rate (content_x compared with the pharmacopoeia method, content_{ph}) was calculated according to the following formula:

$$\text{Increase rate (\%)} = (\text{content}_x - \text{content}_{\text{ph}}) / \text{content}_{\text{ph}} \times 100 (\%)$$

RESULTS AND DISCUSSION

TLC results: TLC results for the extracts by five methods were shown in Fig. 1. The cantharidin was confirmed at R_f 0.628 for each method. Much more spots were shown in enzyme extraction method, implying the generation of new substance when cellulase and pectinase were employed to degrade *M. phalerata*. This finding is coincided with the following HPLC result (Fig. 3).

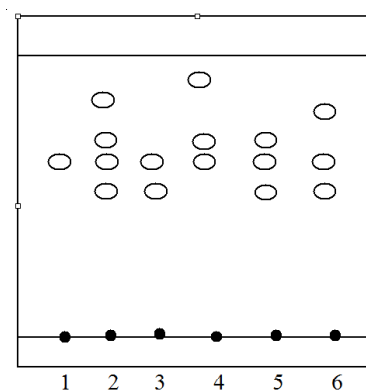


Fig. 1. TLC analysis for enzymatic and other methods. (1. Cantharidin; 2. Enzyme extraction solution; 3. Hydrochloric acid extraction solution; 4. NaOH extraction solution; 5. Mixed extraction solution; 6. Pharmacopoeia extraction solution)

HPLC analysis of cantharidin in extract: Considering gas chromatography had ever been used to determine cantharidine^{9,10}, in the present work, gas chromatography was used to determine the cantharidin in extract. However, we failed to get an appropriate condition. For this reason, HPLC was alternatively used to identify cantharidin in the extract. Two kinds of chromatogram column PLatisil ODS and diamonsil C₁₈ (4.6 mm × 250 mm, 5 μm, DIKMA, American), different wavelength (230, 260, 273 nm), as well as three mobile phase systems (water:methanol; water:acetonitrile: formic acid; acetonitrile:water) were tried. To achieve better chromatogram, different flow rate (0.8, 0.6 and 1.0 mL/min) and temperature (25, 30 and 35°C) were also tried. At last, the optimum HPLC conditions were listed as follows: Diamonsil C₁₈ (250 mm × 4.6 mm, 5 μm); the flow rate was 1.0 mL/min; detection wavelength is 273 nm; column temperature was maintained at 25 °C; mobile phase was acetonitrile: water = 40:60. Under these conditions, cantharidine could be well separated (Figs. 2 and 3).

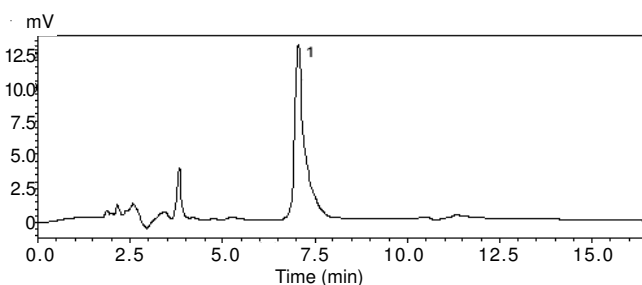


Fig. 2. Chromatogram analysis of cantharidin standard (1= cantharidin)

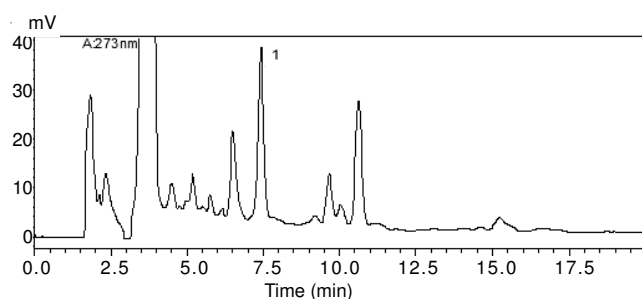


Fig. 3. Chromatogram analysis of *Mylabris phalerata* Pallas extraction (1 = cantharidin)

Standard curve of cantharidin: Cantharidin of 10 mg was accurately weighed and added to a 10 mL volumetric flask, dissolved in 5 mL chloroform and the solution was diluted to 10 mL with the same solvent. The retention time of standard cantharidin was 7.032 min. The working calibration curve based on standard solutions showed good linearity. The standard curve of cantharidin was

$$Y = 196707X + 23419 \quad (R^2 = 0.9998, n = 8)$$

Comparison of extraction methods: Total cantharidin for each method was shown in Table-1, cantharidin was increased when sample was treated with combined enzymes. The total extraction yield of cantharidin was 1.327 mg/mL, which ranked the highest among these methods. Compared with Chinese pharmacopoeia extraction method (0.461 mg/mL), the increase rate was 187.85 % (ca. 3 folds). For mixed extraction method, the total cantharidin was 0.593 mg/mL, which was also higher than Chinese pharmacopoeia extraction method (increase rate was 16.92 %). Similarly, hydrochloric acid extraction method presented total cantharidin of 0.704 mg/mL, with increase rate of 52.71 % compared with Chinese pharmacopoeia method. Conversely, NaOH solution method led to lower cantharidin content (0.230 mg/mL). For acid and alkali methods, the former presented higher cantharidin than the latter, because cantharidin was an anhydride which might bind Na, Mg or other metal ions to form sodium salt or magnesium salt. In acidic environment, cantharidin would dissociate from the main conjugated body, thereby resulted in higher yield of cantharidin. Clearly, enzyme extraction method was suitable for extraction of cantharidin from *M. phalerata*. It might be attributed to cellulose and pectinase which degraded cell wall. In fact, similar conclusion has been reported by Chen *et al.*⁸ and in-depth study was needed to investigate the adaptability in large-scale industry.

TABLE-1
EFFECT OF DIFFERENT EXTRACTION
METHODS ON CANTHARIDIN CONTENT

Extraction methods	Contents (mg/mL)	Increase rate (%)
Mixed extraction method	0.539	16.92
Enzyme extraction method	1.327	187.85
Extracting with hydrochloric acid	0.704	52.71
Extracting with NaOH solution	0.230	-50.11
Chinese pharmacopoeia extraction method	0.461	–

Conclusion

Two major conclusions could be drawn from this study: (i) Of the extraction methods, enzyme extraction method was found to be simple, easy to operate, benign to the environment and advantageous for the highest yield of cantharidin, followed in turn by hydrochloric acid extraction, mixed extraction method, Chinese pharmacopoeia method and NaOH solution method; (ii) HPLC analysis method was suitable for determining cantharidin, the optimum conditions comprise: diamonsil C₁₈ (250 mm × 4.6 mm, 5 μm), flow rate of 1.0 mL/min, detection wavelength of 273 nm, column temperature at 25 °C, mobile phase of acetonitrile:water = 40:60.

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