

Development of a Chromatographic Fingerprint for the Quality Control of *Mallotus apelta* by Using HPLC-DAD-FLD-ELSD with *Malloapelta B* as Marker Compound

Tu VA¹, Cuong NX¹, Thanh NV¹, Dimitrov V² and Nguyen NH^{1*}

¹Institute of Marine Biochemistry, VAST, 18 Hoang Quoc Viet, Hanoi, Vietnam

²Institute of Organic Chemistry, BG-1113 Sofia, Bulgaria

Abstract

High performance liquid chromatography diode array detector and Ion trap MS combined with some statistical methods was developed for the quality control of *Mallotus apelta* with *malloapelta B* as marker compound. In the ESI-MS experiment, both negative and positive ESI modes were used for the detection of *malloapelta B*. All the results showed that the developed fingerprint assay was specific and could further serve for quality identification and quantitative the content of *malloapelta B* in the *Mallotus* sample.

Keywords: LC-MS; Fingerprint analysis; *Mallotus apelta*; *Malloapelta B*

Introduction

The *Mallotus* genus, belonging to the Euphorbiaceae family, is widely distributed in Vietnam and the south of China. In Vietnam, roots, stem barks, leaves and fruits of *Mallotus* species have been used for hundreds of years in traditional medicine for the treatment of chronic hepatitis and enteritis [1]. In previous studies concerned the chemical components and pharmaceutical activities of *Mallotus apelta*, several benzopyran compounds were isolated and pharmacologically active constituents were determined [2,3]. The major components of *Mallotus apelta* as a series of benzopyrans such as a new chromene derivative with benzopyran skeleton was isolated and identified as 1-(5,7-dimethoxy-2,2-dimethyl-2H-chromen-8-yl)-but-2-en-1-one or *malloapelta B* (1) with high yield. Different chromatographic techniques were applied to purify several new compounds 8-(1'-oxo-3'(R)-hydroxy-butyl)-5,7-dimethoxy-2,2-dimethyl-2H-1-benzopyran; 8-(acetic acid 1'-oxo-3'(R)-hydroxy-butyl ester)-5,7-dimethoxy-2,2-dimethyl-2H-1-benzopyran; 6-(1'-oxo-2'-en-butyl)-5,7-dimethoxy-2,2-dimethyl-2H-1-benzopyran; 6-[1'-oxo-3'(R)-hydroxy-butyl]-5,7-dimethoxy-2,2-dimethyl-2H-1-benzopyran; 6-[1'-oxo-3'(R)-methoxy-butyl]-5,7-dimethoxy-2,2-dimethyl-2H-1-benzopyran and 6-(1'-oxo-2',3'-epoxy-butyl)-5,7-dimethoxy-2,2-dimethyl-2H-1-benzopyran from the leaves of *M. apelta* which were named as *malloapelta C*, *D*, *E*, *F*, *G*, and *H*, respectively [4-6], among which *malloapelta B* is present in the greatest amounts. *malloapelta B* with α,β -unsaturated carbonyl moiety in side chain presented a potent NF- κ B (Nuclear Factor-kappa B), which is a major transcription factor that regulates genes responsible for both the innate and adaptive immune response, inhibitory activity ($IC_{50}=5.0 \mu\text{M}$), and NFAT (Nuclear factor of activated T-cells) which is a general name applied to a family of transcription factors shown to be important in immune response, transcription factor inhibitory ($IC_{50}=2.48 \mu\text{M}$) and cytotoxic activity against two human cancer cell lines (Hep-G2 and KB with $IC_{50}=0.49 \mu\text{g/mL}$ and $0.54 \mu\text{g/mL}$, respectively) [7]. *Mallotus apelta* is widely distributed and used in Vietnam. The content of each component in the herb varies significantly due to difference in geographic origin, climate condition, environment and other factors. Therefore, controlling the quality of herbal medicines and their derivatives is difficult [8]. Therefore, a holistic approach for quality control is necessary. Recently, the chromatographic fingerprint technique was regarded as a useful method to control the quality of the herbal medicines and their derivatives because this technique emphasizes the systemic characterizations of compositions of samples and focuses on the identification and assessment of the stability of the

components [9,10]. In the present study, we used high performance liquid chromatography diode array detector and Ion trap MS combined with some statistical methods to quality control of Vietnamese medicinal preparations of *malloapelta B* as marker of *Mallotus apelta* sample.

Experimental

Standards and materials

All *Mallotus apelta* Muell.-Arg samples were authenticated by Professor Nguyen Nghia Thin (Hanoi National University, Vietnam). A voucher specimen (INPC 2847) was deposited in the herbarium of institute of Natural Products Chemistry (Hanoi, Vietnam). *Malloapelta B* (Figure 1) was isolated from the leaf of *Mallotus apelta*, this work carried out in Institute of Natural Products Chemistry, Vietnam Academy of Science and Technology. HPLC grade methanol, acetonitrile (both from Fisher Scientific, Leicestershire, UK), formic acid (Sigma-Aldrich, Steinheim, Germany), and MilliQ water (obtained

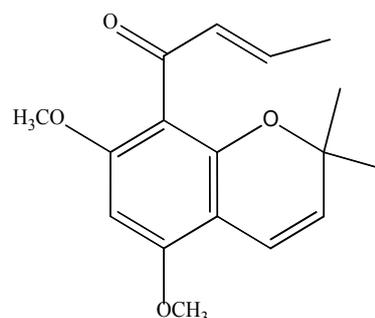


Figure 1: Structure of *malloapelta B* isolated from *M. apelta*.

*Corresponding author: Hoai Nam Nguyen, Institute of Marine Biochemistry, VAST, 18 Hoang Quoc Viet, Hanoi, Vietnam, Tel: +847917053; E-mail: namnguyenhoai@imbc.vast.vn

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from a MilliQ water purification system, Millipore, Bedford, MA) were used to prepare the mobile phases.

Instrumentation

HPLC/MS was performed using ion trap MS instrument Agilent 1200 series (Agilent, USA) equipped with electrospray ionization (ESI) source that was connected to Agilent LC pump. This Agilent 1200 HPLC system (Agilent, USA), consisting of a vacuum degasser, quaternary pump, auto sampler and a diode array detection detector, was used. Chemstation for LC (Agilent) acquired and processed the analytical data. Helium was used as the damping and collision gas. Parent ions in ESI were examined under negative ESI (-) and positive ESI (+) ion modes after the direct injection of a standard solution (1 µg/mL) in 80% methanol through a syringe at a flow rate of 3 µL/min. MS fragment analysis of *malloapelta B* and MSⁿ experiments were also performed. The following ESI inlet conditions were used: nitrogen was used both as the nebulising gas and as a drying gas at a temperature of 350°C; capillary temperature was set at 285°C; In the positive mode, the capillary voltage was set to 10 V, the source voltage to 5 kV and the source current to 5 µA; In the negative mode, the capillary voltage was set to -4 V, the source voltage to 4.5 kV and the source current to 80 µA, a collision energy of 25 V was applied in both modes.

For LC/MS chromatographic separation, a Zorbax Eclipse XDB C₁₈ (4.6 × 150.0 mm, 5 µm) with a Zorbax Eclipse XDB C₁₈ guard column (4.6 × 12.5 mm, 5 µm) was used at ambient temperature with a flow rate of 0.4 mL/min. The mobile phase consisted of (A) 0.1% formic acid in ACN, and (B) 0.1% formic acid in water; 5 µL of sample was injected. The gradient was programmed as follows: starting at 20% (A), increasing to 40% (A) over 15 min, increasing to 80% (A) over 30 min, holding at 80% A for 10 min, and then returning to 20% A over 5 min. DAD detector was set at 254 nm for acquiring chromatograms, UV spectra and 3D-plots were recorded between 200 and 450 nm.

Preparation of samples

To prepare the herbal extract, 2.5 g plant sample (leaves) was weighed and extracted three times with 25 mL methanol in an ultrasonic bath (Branson Ultrasonic Corporation, Connecticut, US), each time at a temperature between 40-50°C during 60 minutes. Then the combined extracts were filtered through a 240 nm pore size filter paper (Whatman, Hanoi, Vietnam) and evaporated at decreased pressure (60 Pa) at a temperature of 50°C. The sample solutions were diluted 50 times. Then the solution was followed by filtration through a 25 mm syringe filter (VWR International, Leuven, Belgium) having a polypropylene membrane with 0.2 µm pore size.

Calibration curves

To prepare *malloapelta B* sample, a 1000 µg/mL standard stock solution of *malloapelta B* was prepared and diluted to 10 µg/mL to provide the working solution. Then this solution was used to prepare a series of standards having concentrations of 1 µg/mL, 0.8 µg/mL, 0.5 µg/mL, 0.25 µg/mL, 0.1 µg/mL, 0.05 µg/mL, 0.025 µg/mL in pure 80% methanol solution and also in the extract from *Mallotus apelta* to allow calibration of the sample. A calibration curve was obtained by plotting the ratio of the peak areas of the analyte (Figure 2).

Results and Discussion

Selection of suitable chromatographic conditions

In the course of optimizing the conditions of separation, the influence of the stationary and mobile phases were firstly investigated.

Considering the presence of benzopyrans in the herbal extraction, a little amount of formic acid was added to the mobile phase to reduce the ionization of these compounds. Different elution conditions with methanol-water, acetonitrile-water and different concentrations of formic acid in water and in organic mobile phase were compared to get the most suitable mobile phase. The results showed that best resolution and shortest analysis time were achieved when the 0.1% formic acid in ACN, and 0.1% formic acid in water system was used as mobile phase. As seen in Figure 3a, not all of the peaks but most of the main peaks include peak's *malloapelta B* could be well separated by the optimum gradient elution. It is not necessary to strive for baseline separation of all components in one run, to fulfill the identification of objectives of fingerprint of herbal medicines. So the resolution of the chromatograms of *Mallotus apelta* sample under the optimized conditions was acceptable.

Two columns (Zorbax Sil C₁₈ column with 2.5 × 250 mm i.d. 5 µm; Zorbax Eclipse XDB C₁₈ column with 4.6 × 150.0 mm i.d., 5 µm) were screened. The best selectivity and resolution can be observed on the Zorbax Eclipse XDB C₁₈ (5 µm; 4.6 × 150.0 mm I.D.) column as shown in Figure 3a. Over 18 sharp and symmetrical peaks were obtained. Column temperature was an important parameter influencing retention, selectivity, system pressure and column stability. The column temperature at 15, 25 and 35°C also were investigated and the results indicated that 35°C was the optimum temperature. Selection of detection wavelength was one of the key factors contributing to a reliable and reproducible HPLC fingerprint of *Mallotus apelta*. Diode array detector (DAD) was applied to select the optimum wavelength. From the 3D-plots, it was observed that the UV absorption maximum for the *malloapelta B* was at 254 nm (Figure 3b). Hence, 254 nm was selected as the detection wavelength.

Validation of the quantitative analysis

As shown in Figure 2, the calibration curve showed good linear regression ($R^2 > 0.9991$) and the limit of detection (LOD) was less than 0.025 µg/mL per injection, indicating that this method is precise and sensitive for the quantitative evaluation of *malloapelta B* in *Mallotus apelta* sample. The current method was also utilized to analyze the quantitative of *malloapelta B* in *Mallotus apelta* sample collected in Tamdao province. In this sample, content of *malloapelta B* was found to be 0.2%, while other samples collected in other provinces were found to be lower than that in Tamdao province (Table 1).

General characters and analysis MS data of the main peaks

MS spectra was recorded after the direct injection of *malloapelta B*, *malloapelta B* exhibited its most intense adduct ions under ESI positive mode; it appeared at m/z 289 [M+H]⁺ (Figure 4). *malloapelta B* do not exhibit its most intense adduct ions under ESI negative mode.

LC-MS was adopted to identify the present of *malloapelta B* in the chemical constituents of *M. apelta*. ESI in both negative and positive

Sample number	Species	Collection time	Origin	(%) <i>malloapelta B</i>
1	<i>Mallotus apelta</i>	August, 2009	VanBan-Laocai	0.18
2	<i>Mallotus apelta</i>	November, 2009	TamDao-Vinhphuc	0.20
3	<i>Mallotus apelta</i>	November, 2009	Dongdang-Langson	0.172
4	<i>Mallotus apelta</i>	December, 2009	Hamyen-Tuyenquang	0.156
5	<i>Mallotus apelta</i>	June, 2010	Paco-Hoabinh	0.19

Table 1: *Mallotus apelta* samples with indication of collection time, origin and percent of *malloapelta B*.

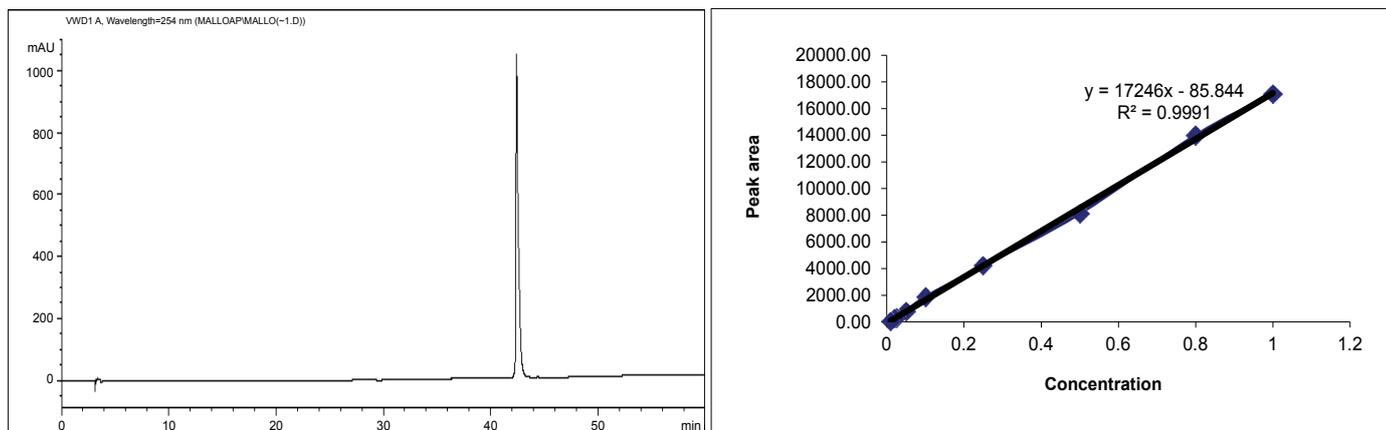


Figure 2: Calibration curves and chromatogram of malloapelta B.

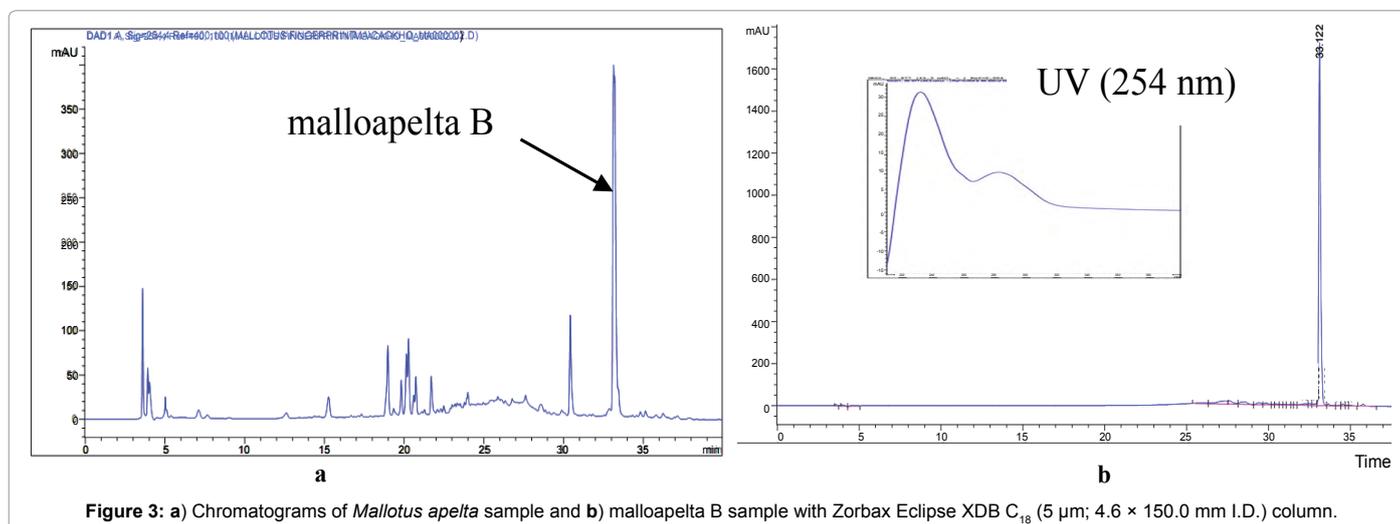


Figure 3: a) Chromatograms of *Mallotus apelta* sample and b) malloapelta B sample with Zorbax Eclipse XDB C₁₈ (5 μm; 4.6 × 150.0 mm I.D.) column.

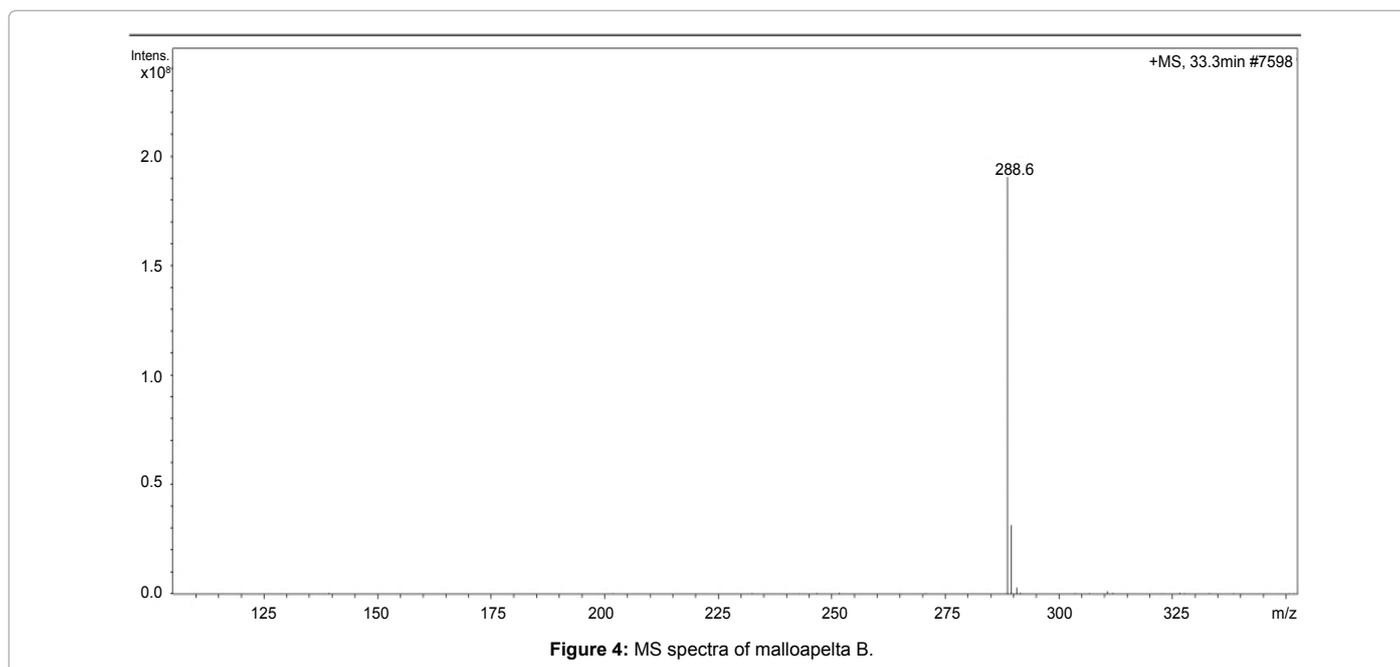


Figure 4: MS spectra of malloapelta B.

modes was used for the detection of *malloapelta B*. Positive mode ESI was found to be sensitive for *malloapelta B*. In the ESI-MS experiment, the molecular weight of *malloapelta B* was obtained (Figure 4). Then on the basis of the comparison of UV spectrum, HPLC retention time and mass spectra with the reference above. We tentatively identified and deduced the *malloapelta B* in the *Mallotus apelta* samples.

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