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Characteristic Fingerprint Analysis of *Mallotus philippinensis* by Ultra Performance Liquid Chromatography Electrospray Ionization Mass Spectrometry

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Abstract

The developing countries mostly trust on traditional remedies, which include the use of different plant extracts or the bioactive phytoconstituents. For this purpose, analysis such as chemical fingerprinting strongly represents one of the best possibilities in searching of new economic and therapeutically effective plants for medicine. *Mallotus philippinensis* Muell. Arg (Euphorbiaceae) is a large genus of the trees and shrubs mainly distributed in the tropical and subtropical regions and are reported to have widespread range of pharmacological activities. A new, simple and rapid ultraperformance liquid chromatography (UPLC) method with photodiode array (PDA) detector has been developed. Further confirmation was performed by electrospray ionization mass spectrometry (ESI-MS) for the chemical fingerprint analysis in extracts of *Mallotus philippinensis*. The chromatographic separations were obtained on a Waters ACQUITY UPLC BEH C18 (2.1 mm × 50 mm, 1.7 µm) column using a gradient elution with 0.1% (v/v) formic acid/water and acetonitrile as mobile phase at a flow rate of 0.4 mL/min. The UPLC-PDA-ESI-MS characteristic fingerprints were established and 7 characteristic peaks were identified along with 5 unknown peaks within 4.5 min by comparing the retention times, λ max (nm), and MS spectra with the literature data. Therefore, this fingerprint analysis method can be applied for the identification and quality control of *Mallotus philippinensis*.

Keywords: *Mallotus philippinensis*; Euphorbiaceae; UPLC; PDA; Chemical Fingerprinting; ESI-MS

Introduction

Mallotus philippinensis Muell. (commonly called Kamala, Kampillaka, and Kapila) is belonging to the family Euphorbiaceae and is a very common perennial shrub or small tree found in the outer Himalayas ascending to 1500 meters [1,2]. Mature fruits have glandular hairs/trichomes collected as a reddish brown powder by shaking and rubbing the fruits by hand. The collected material is fine, granular powder, dull red, or madder red-colored and easily floats on water. *Mallotus philippinensis (M. philippinensis)* has a widespread natural distribution, from the western Himalayas, Western Ghats through India, Sri Lanka, to southern China, and throughout Malaysia to Australia.

M. philippinensis possess various pharmacological activities such as, antifilarial [3], anticancer, antimicrobial, antiparasitic, anti-inflammatory, immune-regulatory and antioxidant [4-7]. This plant is traditionally used as an anthelmintic, purgative, anti-allergic, carminative and also useful in the treatment of bronchitis, abdominal diseases, spleen enlargement [1,8]. Major phytochemicals present in this genus comprise of different natural compounds, generally phenols, diterpenoids, steroids, flavonoids, cardenolides, triterpenoids, coumarin and isocoumarins [1,9-11]. Current knowledge about this endangered medicinal plant is still inadequate concerning its phytochemistry and biological activity. Although, some researchers have contributed towards the isolation of certain novel constituents and their activity. One of the major anticancer potential phytoconstituent of *M. philippinensis* is Rottlerin (1-[6-[(3-acetyl-2,4,6-trihydroxy-5-methylphenyl)methyl]-5,7-dihydroxy-2,2-dimethyl-2H-1-benzopyran-8-yl]-3-phenyl-2 propen-1-one) [12]. In recent years, chromatographic fingerprinting has been found to be a suitable approach for the quality assessment and control of many natural product/herbal medicines. Few analytical studies were performed on *M. philippinensis* using high-performance liquid chromatography technique [5,7,13-16]. These methods reported chemical fingerprinting limited only for those peaks which are responsible for particular pharmacological activity and were not extensive. Though, the above methods are very time-consuming with longer run times, low sensitivity and specificity, so they are inappropriate for the analysis of multiple compounds for the authentication of *M. philippinensis*. As a modern-day separation and detection method, ultra-performance liquid chromatography (UPLC) with photodiode array (PDA) coupled with electrospray ionization (ESI) mass spectrometry has attracted increasing attention because of its short analysis time, high throughput, greater resolution, higher peak capacity, lower solvent consumption and extremely high sensitivity. In the present study, UPLC-PDA method have been developed for the chemical fingerprint analysis of chemical constituents in *M. philippinensis*. Also, UPLC coupled with mass spectrometry with electrospray ionization (ESI) interface is defined for the confirmation of phytoconstituents.

Materials and Methods

Reagents and chemicals

Methanol, acetonitrile (LC-MS grade) and formic acid (analytical grade) were of from Fluka (Sigma Aldrich, St. Louis, MO, USA). Ultra high purity water was prepared using a Milli-Q water purification system (Millipore Corporation, Bedford, MA, USA).

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M. philippinensis fruits were collected from the plants grown in western ghats of Maharashtra region, India in the month of February. A voucher specimen of *M. philippinensis* (RAAMAP3) was deposited in the Botanical Survey of India, Western Region Centre, Pune, India. The glandular trichomes from fruits were dried at room temperature in shade and ground to powder using a stainless-steel grinder.

Extraction and sample preparation

In the extraction process, 2.5 g powdered glandular trichomes was weighed and extracted with 50 mL methanol in an ultrasonic bath (Equitron, Medica Instrument Mfg. Co., Mumbai, India) at a frequency of 53 KHz and temperature between 40 and 50°C for 60 min. The extract was filtered through a 240 nm pore size filter paper (Whatman, USA). The residues were then re-extracted twice with 25 mL methanol using the same conditions. The extraction solution was collected, pooled and evaporated to dryness under reduced pressure (60 Pa) using rotatory evaporator (Buchi Rotavapor-R2, Flawil, Switzerland) at elevated temperature (50°C) [17]. Dried extract (1 mg) was weighed accurately and dissolved in 1 mL of 100% methanol using ultrasonicator and filtered through a 0.2 µm PTFE syringe filter (Whatman, USA). The filtrate was diluted with methanol to final working solutions and analyzed directly by UPLC-PDA-ESI-MS. Samples were stored in a refrigerator at 4°C until analysis.

Instrumentation

The chromatographic analysis was performed on a Waters ACQUITY UPLC system (Waters, Milford, MA, USA) equipped with autosampler, a binary pump and PDA detector. The chromatographic separation was performed on a Waters ACQUITY UPLC BEH C18 column (2.1 mm × 50 mm, 1.7 µm). The column oven temperature was maintained at 25°C. The mobile phase consisted of 0.1% v/v formic acid in water (A) and acetonitrile (B) with a gradient elution program, i.e., 0–1.5 min, 50-90% B; 1.5–3.5 min, 90% B; 3.5–4.5 min, 90–50% B; and 1 min post-run, 50% B. The flow rate was set at 0.4 mL/min and the injection volume was 5 µL.

For phytoconstituents confirmation, samples were analysed using the same UPLC conditions mentioned above and mass spectrometry detection was conducted on an Applied Biosystem 4000 QTRAP (Applied Biosystems/ MDS Sciex, Concord, ON, Canada) connected to the UPLC system, operating in positive (ESI+) electrospray ionization mode with Q1MS Scan.

The source parameters were: ion spray voltage set at 5500 V; turbo spray source temperature, 450°C; nebulizer gas (gas 1), 50 psi; heater gas (gas 2), 50 psi; collision gas, medium; and the curtain gas (CUR) was set at 20 psi. Q1MS spectra were recorded at unit resolution by scanning in the range of m/z 100–1000 at a cycle time of 9 s with a step size of 0.1 Da. Nitrogen was used as the nebulizer, heater, and curtain gas as well as the collision activation dissociation (CAD) gas. Declustering potential (DP) and entrance potential (EP) were set at 78 and 10 V, respectively as compound dependent parameters. Analyst 1.5.1 software package (AB Sciex) used for instrument control and data processing.

Results and Discussion

Optimization of UPLC conditions

To attain optimum separation in a shorter analysis time, the chromatographic conditions such as the mobile phases, column temperatures, and elution programs were optimized in the initial test. Two different brands of analytical columns, the ACQUITY CSH C18 column (2.1 mm × 100 mm, 1.7 µm) and the ACQUITY BEH C18 column (2.1 mm × 50 mm, 1.7 µm) were compared. The results showed that the ACQUITY BEH C18 column (2.1 mm × 50 mm, 1.7 µm) produced chromatograms with improve resolution within a shorter run time. In this analysis, different combinations of mobile phases (water-methanol, 0.1% formic acid in water-methanol, water-acetonitrile and 0.1% formic acid in water-acetonitrile) flow rates (0.2, 0.3, and 0.4), column temperatures (25,30 and 40°C) and time were optimized for better chromatographic behavior and appropriate ionization. A suitable chromatographic separation was achieved within 4.5 minutes using gradient elution with 0.1% formic acid in water and acetonitrile at 25°C column temperature with a flow rate of 0.4 mL/min. The PDA detection wavelength was optimized within the range of 190-400 nm, and finally the λ max (nm) with good response for most of the phytoconstituents was selected (Table 1).

Fingerprint analysis of *M. philippinensis*

In order to identify the compounds in the glandular trichomes of *M. philippinensis*, UPLC-PDA and ESI-MS technique displaying the protonated molecular ion ($[M+H]^+$, positive ion mode) was used. There are 7 characteristic peaks (peak 2, 3, 4, 7, 8, 10, 11) (Figure 1) were identified along with 5 unknown peaks (peak 1, 5, 6, 9, 12) in the UPLC fingerprint, and their respective retention time, λ max (nm) and protonated molecular ion is shown in Table 1. By comparing the retention times (t_R), λ max (nm) of the UV and ESI-MS spectra with previously reported literature, the compounds were unambiguously recognized. UPLC-UV chromatogram at 290 nm of *M. philippinensis* extract and MS spectra of identified phytoconstituents were shown in Figures 2 and 3 respectively. Unknown peaks were identified due to the lack of appropriate data and literature support.

The major phytoconstituent of *M. philippinensis* is rottlerin (peak 10), which elute at t_R 2.89, exhibit a $[M+H]^+$ peak at m/z 517.1 [1,18-20] as shown in Table 1. Two compound (peak 3, 4) were identified at t_R 1.85 i.e., mallotophilippen D (m/z 491.2, $[M+H]^+$) [1,21] and 4-hydroxyrottlerin (m/z 532.9, $[M+H]^+$) [18,19]. Red compound (peak 2) was identified at t_R 1.67 with $[M+H]^+$ m/z 337.2 [20,22]. Similarly at t_R 2.09 (peak 7,8), byakangelicin and mallotus A were exhibit a $[M+H]^+$ peak with m/z 335.3 [23] and m/z 339.1 [24,25], respectively. Kamalachalcone C (peak 7) exhibit a $[M+H]^+$ peak with m/z 531.4 [18] and elute at t_R 3.36. In the similar way, peak 1 was identified as unknown 1 that exhibit a $[M+H]^+$ peak with m/z 322.9 and 581.0 at t_R 1.58. Peak 5,6 (unknown 2 and 3) elute at t_R 1.92 and 2.02 represents $[M+H]^+$ peak with m/z 429.1 and 391.1, 407.2, respectively. The other high intensity peaks (peak 9 and 12) identified as unknown 4 and 5 exhibit intense $[M+H]^+$ peaks with m/z 461.2 and 338.1, elute at t_R 2.40 and 3.98, respectively.

Peak No.	t_R (min)	m/z [Ion species]	Molecular Formula	Identification	λ max (nm)
1	1.58	322.9, 581.0 $[M+H]^+$	-	Unknown 1	276, 384
2	1.67	337.2 [M+H]+	$C_{21}H_{20}O_4$	Red compound	273
3	1.85	491.2 [M+H]+	$C_{30}H_{34}O_6$	Mallotophilippen D	288, 377
4	1.85	532.9 [M+H]+	$C_{30}H_{28}O_9$	4-Hydroxyrottlerin	288, 377
5	1.92	429.1 [M+H]+	-	Unknown 2	273, 290
6	2.02	391.1, 407.2 [M+H]+	-	Unknown 3	292, 351
7	2.09	335.3 [M+H]+	$C_{17}H_{18}O_7$	Byakangelicin	297, 351
8	2.09	339.1 [M+H]+	$C_{20}H_{18}O_5$	Mallotus A	297, 351
9	2.40	461.2 [M+H]+	-	Unknown 4	351
10	2.89	517.1 [M+H]+	$C_{30}H_{29}O_8$	Rottlerin	290, 351
11	3.36	531.4 [M+H]+	$C_{31}H_{30}O_8$	Kamalachalcone C	351
12	3.98	338.1 [M+H]+	-	Unknown 5	339

Table 1: The $[M+H]^+$ ions and UV absorption maxima for compounds identified from fruit glandular trichomes extract of *Mallotus philppinensis* by using UPLC/ESI-MS experiment.

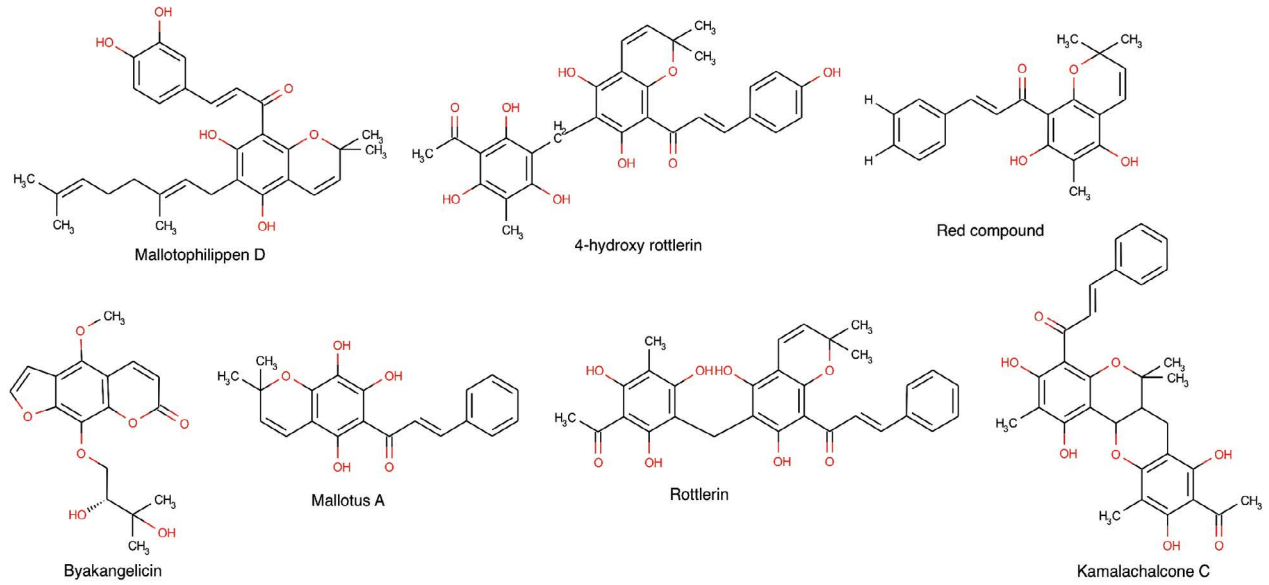

Figure 1: Chemical structure of 7 investigated characteristic compounds.

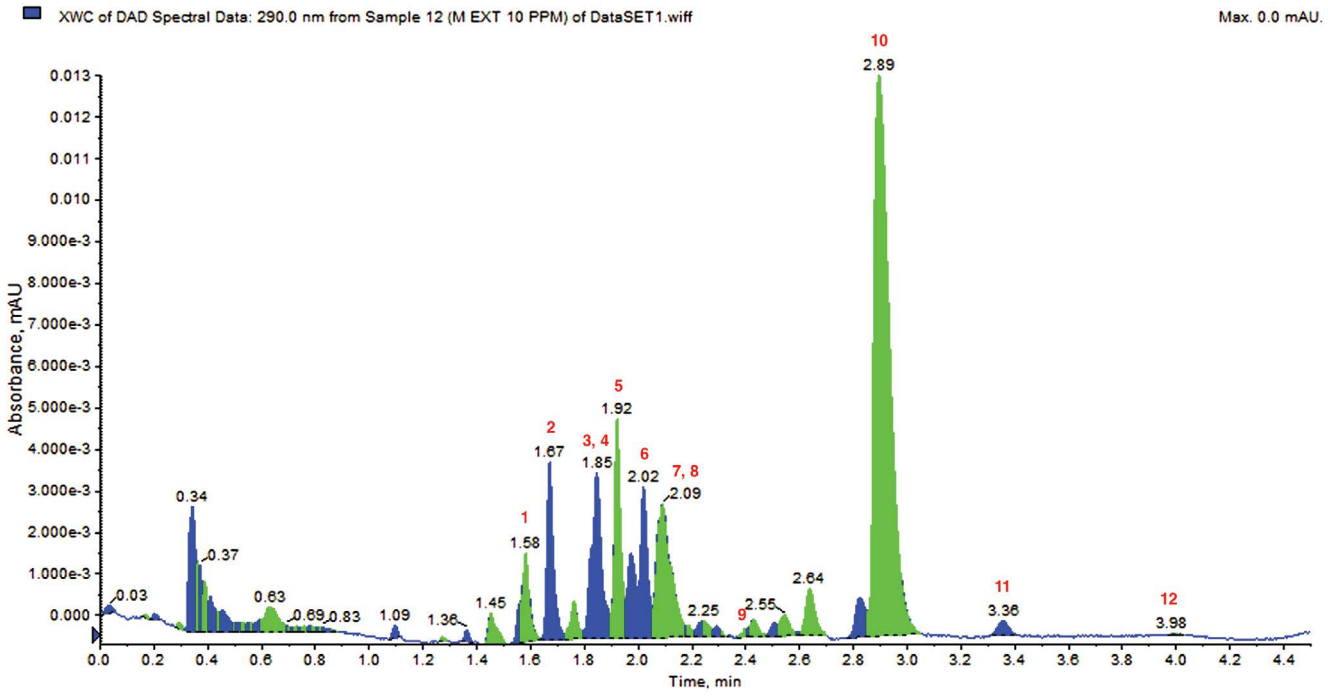

Figure 2: UPLC-PDA chromatogram of *Mallotus philippinensis* extract at 290 nm.

These results suggest that fingerprint analysis method of *M. philippinensis* may be used for authentication and to control adulteration from other species. In addition, this method can also help to standardize the presence of major phytoconstituents in other *M. philippinensis* products.

To summarize, for the first time we have established here a simple and rapid UPLC- PDA and ESI-MS method for the quality control of *M. philippinensis* which plays a significant role in the effectiveness of its clinical purposes. Chromatographic fingerprinting, which has been popular and accepted by experts and scientists universally, is supposed to be a good methodology for quality assessment and control of *M. philippinensis*. The UPLC-PDA combined with ESI-MS offers a powerful instrument for separating and qualifying individual phytoconstituents and creates a characteristic fingerprint profile. The individual peaks are a primary source for quality monitoring of *M. philippinensis* products.

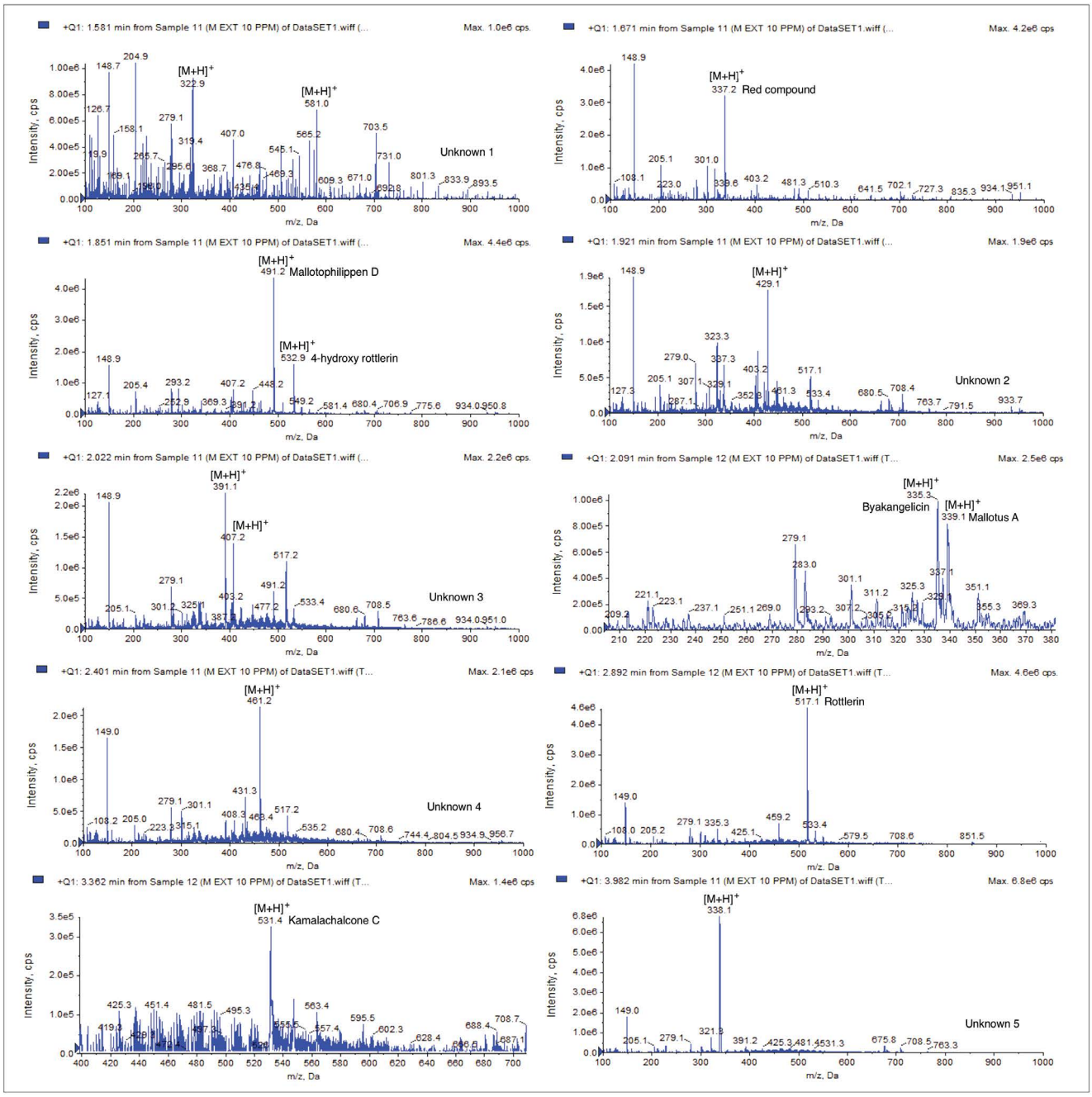

Figure 3: MS spectra of identified compounds.

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