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A simple HPLC–DAD method for the detection and quantification of psychotropic mitragynine in *Mitragyna speciosa* (ketum) and its products for the application in forensic investigation

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ABSTRACT

Mitragyna speciosa, a native plant of Thailand and Malaysia known as 'ketum', is a plant of considerable interest. It exhibits strong antinociceptive effect and yet, acts like a psychostimulant. Due to the affordability and its ease of availability, the abuse of this plant as a substitute for other banned narcotics has become a major concern in many societies. In countries such as Thailand, Myanmar, Australia and Malaysia, the use of ketum is illegal. However, for a person to be charged for possessing or selling ketum, a reliable analytical method is needed in order to detect and identify the plant and its products. Mitragynine is the major alkaloid of ketum. This compound manifests its antinociceptive effects by acting on the opioid receptors. Since *M. speciosa* contain large quantity of mitragynine and it is exclusive to the species, the present analytical method is developed and validated for the purpose of screening ketum products based on this unique compound as the analytical marker. The method uses a HPLC–DAD system with Inertsil C8 (4.6 mm × 150 mm, 5 μm) as the column and a mixture of acetonitrile and formic acid, 50:50 (v/v), as the mobile phase. This method not only detects mitragynine, it can also be used to quantify the amount of mitragynine in the sample. The limit of detection is 0.25 μg/ml, while the limit of quantification is 0.50 μg/ml. The method is quick, simple and reliable with an accuracy of 97.27–101.74% and coefficient of variations of between 0.91 and 3.96%. The method has been tested and found suitable for the identification and quantification of mitragynine in dried plants, a variety of ketum extracts, as well as ketum drink obtained from the market.

1. Introduction

Mitragyna speciosa Korth (Rubiaceae) is a tropical plant indigenous to the Northern region of Peninsular Malaysia as well as the Central and South Thailand. The plant is known as "biak-biak" or "ketum" in Malaysia, and as "kratom" in Thailand. The physiological effect of ketum is dose dependent. At lower concentrations, ketum appears to be stimulating, producing a coca-like and euphoric effect but at higher concentrations, the plant acts like opium in that it is able to suppress pain and is used to mitigate opioid withdrawal syndromes [1]. Ketum is often a more economical choice compared to other opioids as it is available at a very low price [2]. In Malaysia, 1 kg of fresh ketum leaves can be obtained at a price of RM 24 (USD 8), while a glass of ketum drink is sold only for RM 2–4 (USD 0.70–1.30). Ketum products can also be purchased over the internet in various forms such as dried leaves, powdered leaves, extracts and tinctures. The ease of availability and its extremely low price have attracted many young people to seek ketum as an alternative to other banned narcotics such as heroin or cannabis. This trend has been growing and is now a serious concern in the country. Although ketum has been used traditionally to help reduce the dependency on other drugs, it itself causes addiction and abstinence from it results in deleterious withdrawal effects [3,4]. Moreover, ketum is often sold adulterated with other substances such as cough mixtures, traditional herbs and even synthetic pyrethroid from mosquito coil, which may lead to more serious complications [5].

As a mean to curb ketum abuse, the Malaysian government has called on a ban to selling and possessing ketum. Effective from August 2003, a person if convicted, can be fined up to RM 10,000 or jailed up to 4 years, or both, under section 30 (5) of the Poisons Act 1952 [6]. Ketum is also illegal in other countries like Thailand, Myanmar and Australia, while in Japan, it is listed as 'designated

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substances' [7]. Since ketum can be sold in several forms, it is difficult to determine the presence of such material merely by visual observation, especially when it is in the form of packet drink, dried leaf or plant extract. Hence, a rapid and reliable analytical method is required for the detection and quantification of ketum and its preparations. Mitragynine (Fig. 1) is the major active alkaloid of ketum. It accounts for 12% (w/w) of the total alkaloid content in Malaysian ketum and 66% (w/w) in Thai ketum on dry weight basis [8]. Mitragynine is found exclusively in *M. speciosa* but not in any other species of *Mitragyna* [9]. As such it can be used as the marker compound for identification of ketum.

To date, few methods on the analysis of mitragynine (MG) in biological fluids and its application in pharmacokinetic studies have been reported [10–13]. However, there is still a lack of suitable analytical methods for the detection and quantification of MG in raw materials, extracts and ketum preparations for routine analysis. A GC method has been described by Chan et al. However, this method is only applicable for qualitative detection of MG and it has not been evaluated and validated for quantitative studies. Moreover, the detection sensitivity of this method is also not reported [14]. Another method which has been reported is based on LC–PDA–ESI-MS. This method simultaneously detects MG, 7-hydroxymitragynine and other alkaloids in ketum and its product. The total run time of each analysis is 55 min, with MG appearing at approximately 20 min. After the analysis of each sample, another 11 min is needed to prepare the system for the next injection [7]. Although the method has the advantage of detecting several ketum alkaloids simultaneously, the lengthy analysis time and high capital expenditure on the instrument limit its application and availability for routine use. Hence, there is a need for the development of a faster and more economical method for routine detection of ketum and its products based on its unique marker, mitragynine. This report describes the validation of a HPLC–DAD method which is simple, rapid and reliable for the detection and routine quantification of MG in ketum raw materials, alkaloid extracts and ketum drinks.

2. Methodology

2.1. Plant material

Fresh leaves of *M. speciosa* Korth. (Rubiaceae) were collected from the state of Perak, Malaysia. A voucher specimen was deposited at the herbarium of Universiti Kebangsaan Malaysia (Specimen No. UKMB06509).

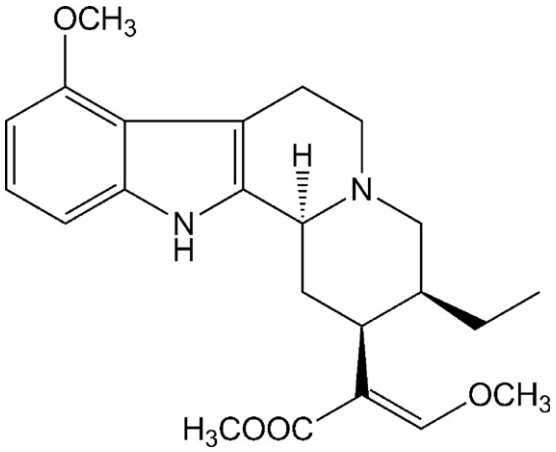

Fig. 1. Chemical structure of mitragynine (MG).

2.2. Chemicals

Solvents used for chromatographic separation were of HPLC grade. Formic acid, methanol (MeOH) and acetonitrile (ACN) were purchased from Fisher Scientific (Loughborough, UK), while NaOH was purchased from R&M Chemicals (Essex, UK). Petroleum ether (PE), chloroform ($CHCl_3$) and MeOH used for extracting the plant material were of analytical grade purchased from Merck (Darmstadt, Germany). Mitragynine (94.3% purity) was purchased from Chromadex Inc. (CA, USA). Deionized water for HPLC was prepared using an Elga Classic UVF ultrapure water purifier system (Elgastat, Bucks, UK).

2.3. Chromatographic conditions

The analytical method was developed on an Agilent 1200 series HPLC system which was coupled to a photodiode array detector (Agilent, CA, USA). Chromatographic separation was achieved at room temperature (25 °C) on a C8 reversed-phase Inertsil column (4.6 mm × 150 mm, 5 μm) (GL Sciences Inc., Tokyo, Japan) which was protected with a guard column (4.0 mm × 20 mm, 5 μm). Mobile phase was a mixture of ACN and 0.05% formic acid (adjusted to pH 5 with NaOH), 50:50 (v/v) running at an isocratic mode at flow rate 1.0 ml/min. The sample injection volume was 20 μl and the total analytical run time was 6 min with mitragynine (MG) eluting at 5.5 min. Detection was carried out at 200–400 nm, while the UV signal at 223 nm was extracted for quantification purposes. Identification of MG was done by comparing the HPLC retention time and UV spectrum of the analyte with that of MG standard. Peak purity test was also performed using the ChemStation LC3D software to ensure method selectivity for reliable quantification of MG.

2.4. Analytical method

2.4.1. Validation of the HPLC method

A 50 μg/ml stock solution of MG reference standard was prepared in MeOH. From the stock solution, working standard solutions of 0.5, 1.0, 2.5, 5.0, 7.5, and 10.0 μg/ml were prepared. Calibration curve was constructed on each day of analysis. To evaluate the within-day and between-day precision, as well as the accuracy of the method, five replicates of MG at six different concentrations were analysed on five consecutive days. Extraction recovery was carried out by spiking MG standards to blank samples. Extraction was then carried out as described in Section 2.4.2 and the recovery value was expressed in terms of percentage of spiked MG obtained from the extraction over that of an equivalent amount of MG in the pure standards.

2.4.2. Preparation and analysis of M. speciosa extracts

The analytical method was developed and tested with authentic samples prepared from *M. speciosa*. Fresh leaves were washed with clean water and dried in the oven at 45 °C until a constant weight was obtained. Following that, the dried leaves were pulverised with a mill grinder and extracted using various means of extraction methods. To produce water extract, 5 kg of the dried leaves were boiled in 8 L of water for 2 h. The extract was then removed and the plant residue was extracted again with the same amount of water for another 2 h. Following that, both extracts were combined and dried using a freeze-dryer. Methanol extract of the plant was prepared by macerating dried leaves (10 g) in MeOH (50 ml) at room temperature for 5 days. The maceration procedure was repeated twice with fresh MeOH to ensure that the materials were fully extracted. Solvent was then evaporated under vacuum to yield the MeOH extract. The alkaloid-rich extract was obtained by extracting the alkaloids from the MeOH extract of *M. speciosa*. The