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AN OPTIMISED RECOVERY OF MITRAGYNINE FROM MITRAGYNA SPECIOSA USING FREEZE DRYING AND ULTRASONIC-ASSISTED EXTRACTION METHOD

ABSTRACT

Mitragynine isolated from Mitragynaspeciosa, has been a subject of interest of late due to its analgesic properties and potential use in opioid drug replacement therapy. To further explore its therapeutic potential, large quantity of mitragynine is required. However, there is limited data on the influence of extraction strategies on the extraction efficiency of mitragynine, which is important to increase mitragynine production. The present study aims to develop an ultrasonic-assisted extraction method to increase the extraction efficiency and recovery of mitragynine from the host plant. By carefully optimising the pH and extraction temperature of the choice solvent,combined with the appropriate sample preparation technique, a substantial increase in the recovery ofmitragynine was observed.The present study concluded that the optimum condition for the extraction of mitragynine should couple the freeze-drying technique with the ultrasonic-assisted extraction procedure that uses the solvent combination of $CHCl_3$: MeOH 1:4 (v/v) at pH 9.5, at 60 °C. The identity and amount of mitragynine recovered from the leaves were determined by means of a hyphenated gas chromatography and mass spectrometry (GC-MS) system.

keywords Mitragynine,Mitragynaspeciosa,freeze-drying, ultrasonic-assisted extraction, GC-MS

INTRODUCTION

Mitragynine is a prominent lead compound isolated from MitragynaspeciosaKorth. (Rubiaceae) – a plant used traditionally in Thailand and Malaysia for the enhancement of work tolerance and as a substitute for opium. This compound was found to have analgesic effect mediated through the μ- and δ-opioid receptors, [1] and interacts with the central nervous system via descending noradrenergic and serotonergic systems.[2]Although studies have demonstrated that the analgesic properties of mitragynine was only a quarter that of morphineand has poor bioavailability, [3]one of its naturally occurring derivatives, 7α-hydroxy-7H-mitragynine was found to be 13 times more potent than morphine.[4]In addition, this compound has good oral absorption compared to morphine, thus making it a more attractive drug candidate.[4]In order to further evaluate the potential of mitragynineand its derivatives for the development into new analgesia and also possibly be used for the treatment of opioid addiction in replacement therapy, extensive studies on the pharmacological effect, pharmacodynamics, pharmacokinetics, toxicology and various other aspects need to be conducted. However, a reasonable amount of mitragynine is required for these studies. At present, although it is possible to synthesizemitragynine,the synthetic procedure is lengthy and laborious. [5, 6]On the other hand the commercially available mitragynine is extremely costly. As such, isolation of the compound from its mother plant is still the most feasible option.

For most researchers who worked on Mitragynaspeciosa, methanol or methanol in combination with small amount of water were used as the solvent of extraction. [7-13]The method of extraction was either maceration at room temperature, [7-9,12]or soxhlet, [11, 13]except for the work of Kikura-Hanajiri et al. [10]and Chan et al.[14]who utilised ultrasonication. There are a few cases where other solvents were used for extraction, such as ethyl acetate [15]and chloroform in combination with methanol. [14]Recently, Orio et al. [16]evaluated the use of other extraction technologies and solvent combinations on the extraction of Mitragynaspeciosa. Results showed that microwave at 110 °C in a closed vessel with methanol-water, 1:1 (v/v) and ultrasound-assisted extraction with an immersion horn at 25 °C with methanol were able to increase the yield of mitragynine significantly. However, the rationale for choosing to work at these particular temperatures was not discussed. [16]In the present work, we intend to further explore the use of ultrasound to extract mitragynine by optimising the extraction conditions such as the pH and temperature of the extraction solvent.To further enhance sample stability and extraction efficiency ofmitragynine, several sample dryingtechniques were also explored, including freeze drying. Studies have shown that freeze-dried samples are stable up to at least half-a-year, while extraction carried out on these freeze-dried

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samplesresulted ina significant increase of extraction yield. [17, 18]

EXPERIMENTAL

Plant material

The aerial parts of MitragynaspeciosaKorth.(Rubiaceae) was obtained from Sanglang, Kedah, Malaysia. The plant was identified by a botanist from the School of Biological Sciences, UniversitiSains Malaysia, where a voucher specimen (no. 11290) was deposited.

Sample preparation

Method A: Freshwater-cleaned leaves were frozen at -30 °C immediately upon received. The sample was then dried using a freeze dryer and crushed into powder using a Shimono SKP-3150 high-speed blender equipped with a dome-shaped collection vessel (Shimono Holdings, Malaysia).

Method B: Fresh cleaned leaves were immersed in hot methanol (approximately 60 °C)for 5 min immediately upon received. The leaves were then air-dried in a hood at room temperature (25 – 30 °C). The dried material was then ground into powder using the high-speed blender equipped with a dome-shaped collection vessel.

Method C: Fresh cleanedleaves were immersed in hot methanol similar to method B and subsequently dried in the oven at 120 °C for 24 hr. The dried material was then ground into powder using the high-speed blender equipped with a dome-shaped collection vessel.

A sample of the pulverised material prepared using methods A, B and C was observed under the Leica EZ4D stereomicroscope (Leica Microsystem GmbH, Germany) and an image of each sample was recorded.

Extraction procedure

1.00 g of each of the pulverised samples prepared using methods A, B and C was extracted for 10 min in 50 ml of $CHCl_3$:CH_3OH, 1:4 (v/v) in an ultrasonic cleaner bath(Branson Ultrasonics Corporation, USA). The operating frequency of the ultrasonic bath is 40 kHz, while the power generated is 200 W. Following that, the leaf extracts were filtered and the filtrate was allowed to dryon an evaporating dish placed on a water bath. The dried residue was then reconstituted in 2 ml of CH_3OH and analysed by GC-MS.

Evaluation of the effect of pH on the extraction of mitragynineforMitragynaspeciosa

A series of solvent mixture consisted of $CHCl_3$:CH_3OH, 1:4 (v/v) with different pH was prepared. 5 M HNO_3 was added to the mixture to produce extraction solvents of pH 3 and 5.5, respectively, while ammonia solution (25%) was added to produce solvents of pH 9.5 and 11.5, respectively. The set of solvent mixture which was not added with acid or base was found to have a pH of 7.5. Measurement of pH was carried out using a pH510 pH meter (Eutech Instruments, Singapore). Extraction ofM.speciosa prepared via method Awas then carried outusing the series of solventwith varied pH. The amount of mitragynine extracted was analysed using GC-MS.

Evaluation of the effect of temperature on the extraction of mitragynine fromMitragynaspeciosa

Ultrasonic-assisted extraction was carried out on M.speciosa prepared using method A. The extraction was carried out inan ultrasonic bath at different temperatures, i.e. 25 °C(room temperature), 40°C and 60°C. The content of mitragynine extracted was then analysed using GC-MS.

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Instrumentation

GC-MS analysis of the content of M.speciosa was carried out on a 7890A Gas Chromatographic system coupled with a 5975C Mass Spectrometry Detector (Agilent, Germany). Chromatographic separation was achieved on a HP-5MS column, 30 m × 0.25 mm × 0.25 µm film thickness. The oven temperature was initially held at 60°C for 5 min, following which the temperature was raised at 15°C/min until280°C and was held at 280 °C for 25 min. The sample injection volume of 1 µl was performed with a split ratio of 14:1.The identity of mitragynine was determined by comparing the mass spectrum with that from the NIST library. Further confirmation of the identity was done by spiking the sample with mitragynine standard purchased from Chromadex, USA.

RESULTS AND DISCUSSION

Sample preparation methods

The fresh leaves samples were prepared using 3 different methods. In method A, water-cleaned leaves were frozen at -30 °C before subjecting them to freeze-drying under vacuum (0.01 mbar).In methods B and C, cleaned leaves were dipped in hot methanol prior to the drying process in order to denature the plant's enzyme which may otherwise lead to degradation of natural products as a result of enzymatic activities. [19]After treating the leaves with methanol, the plant was air-dried slowly at ambient temperature (25 – 30 °C) in method B, while in method C the leaves were further dried at elevated temperature (120 °C) for 24 hr. The dried leaves were then pulverised using a high-speed blender equipped with a dome-shaped collection vessel. A blender of such design has less dead volume, thus enables the leaves to be crushed more rapidly and evenly compared to a normal blender.

The marked distinction in the texture of the product prepared by methods A, B and C can be realised simply via direct visual observation. The product prepared using method A appeared as fine greenish powder. The sample emits aroma akin to the freshly picked leaves. Product obtained using method B appeared coarser compared to that using method A, and the material appeared light brown. Method C produced fine powder similar to that of method A, but the colour of the product was brown. The images of these 3 productsas observed under thestereomicroscope aredepicted in **Figure 1**.As expected, the products obtained using methods A and C consisted of smaller particles compared to that of B. Residual amount of water in B caused the material in the leaves to agglomerate, thus it remained soft and difficult to crush. On the other hand, both the products of A and C were thoroughly dried, therefore the material were highly brittle and easy to crush. However the product of A appeared to be more favourable compared to Cowing to its greenish texture and fresh smell althoughthey both had fairly similarparticle sizes. Due to the long heating process in method C, some of the thermally labile compounds, including the chlorophyll, were lost.

An extract of the product of each preparation method was obtained using a solvent combination of $CHCl_3$: CH_3OH, 1:4 (v/v) in an ultrasonic bath. This is an extraction method developed by the Forensic Division, Department of Chemistry, Malaysia for the analysis of mitragynine in M. speciosa. [14]The addition of $CHCl_3$ to CH_3OH is crucial in order to get a more defined chromatogram for accurate quantification ofmitragynine. [14]GC-MS analysis of the extracts of products A, B and C was carried out to compare the mitragynine content. The elution time for mitragynine was 30.26 min, while the peaks eluted at 30.81 and 31.41 min corresponded to compounds with the molecular masses (M^+) of m/z 396.20 and 398.50, respectively (**Figure 2**). By observing the fragmentation pattern, these two compounds that elutes after mitragynine were tentatively assigned as paynantheine (MW: 396.5) and speciogynine (MW: 398.5), which are the naturally occurring analogues of mitragynine.

As shown in **Figure 2**, mitragyninewere found in higher concentrations in the extract of product A, followed by that inproduct B. The extract of product C contained the least amount of mitragynine.Since product A has a smaller particle size compared to that of product B,it assumed a larger surface area/mass. This enhances the mass transfer of mitragynine and the other components into the solvent. Forproduct C however, although the particle size is quite similar to that of product A, the drying process at 120 °C for 24 hr had possibly

resulted in the degradation of mitragynine, thus the amount of mitragynine recovered is less compared to that ofproducts A and B. On this basis, product prepared using method A was chosen for further evaluation. This product retained its texture for at least 6 months when wrapped in an aluminium foil and kept at 4 °C in an air tight container.

The effect of pH on the extraction efficiency ofmitragynine

The quantity of mitragynine extracted was found to be largely dependent on the pH of the extraction solvent. As illustrated in **Figure 3**, at pH 3, hardly any or no mitragyninewas detected. As the pH increased from 5.5 to 9.5, a drastic change in the amount of mitragynine recovered was observed. By comparison, the amount of mitragyninewhen extracted at pH 9.5 was more than twice the amount of that carried out under neutral condition. Increment of pH above9.5 showed no improvement in the extracted amount of mitragynine. The results suggest that at low pH, mitragynineform organic salt with the acid which does notdissolve in the extraction solvent. When the pH increases, mitragynine is converted into free organic bases which dissolve well in the extraction solvent. At pH 9.5, all the alkaloids have been fully converted into its basic form and thus further increment of the pH does not increase the amount of alkaloids extracted into the solvent.

The effect of temperature on the extraction efficiency of mitragynine

Figure 4shows the amount of the extracted mitragynineat different extraction temperature. The amount of mitragynine extracted was found to increase proportionally as the temperature increases. At 60 °C, which is nearing the boiling point of the solvent, the amount of mitragynine extracted into the solvent was approximately 9 times the amount of mitragynine extracted at room temperature. This result suggests that mitragynine can be best extracted at high temperature as the extraction efficiency outweighs possible decomposition of the compound.

The improved sample preparation and extraction procedure

The study above clearly demonstrates the improvement made to the existing extraction methods. By using the freeze-drying technique in the sample preparation step and coupling it with an optimised extraction strategy where the optimal pH and temperature were used, the concentration of mitragynine extracted from M. speciosaincreased significantly. The present extraction strategy has proven to be fast, simple, straightforward and does not require the use of sophisticated instruments. It can be usedto step up the production of mitragynine for drug development purposes.

CONCLUSION

Major improvements were attainedon the extraction efficiencyof mitragynine from M. speciosaby adopting the appropriate sample preparation methods and optimised ultrasonic-assisted extraction method. The freeze-drying methodthoroughly removes water content in the leaves without disrupting the thermally labile compounds. This effective drying processenables the leaves to be crushed into very fine particles which is an important factor for the enhancement ofextraction efficiency of mitragynine into the solvent system.Other parameters such as pH and extraction temperature were also found to influence the extraction efficiency greatly. From this study, mitragynine was found to be best extracted with a combination of $CHCl_3$: MeOH (pH 9.5), 1:4 (v/v) using the ultrasonic-assisted method at 60 °C.Although further studies need to be carried out before the extraction procedure could be scaled-up to production level, this finding has pointed out several important factors when considering the sample preparation and extraction of M. speciosa alkaloids. The information presented in this work shall undoubtedly contribute to the area of pharmacognosyand drug development of mitragynineand its related compounds.

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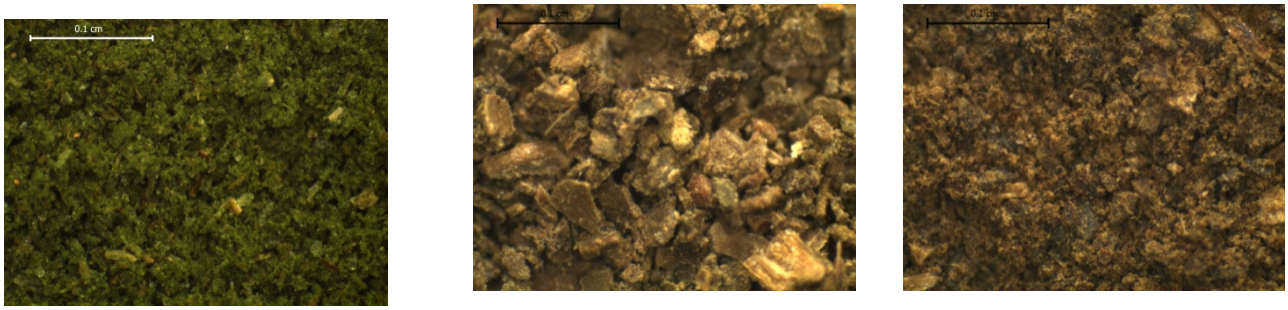

Figure 1: Stereomicroscopic images of the products of (a) method A; (b) method B; (c) method C. Original magnification is 35×.

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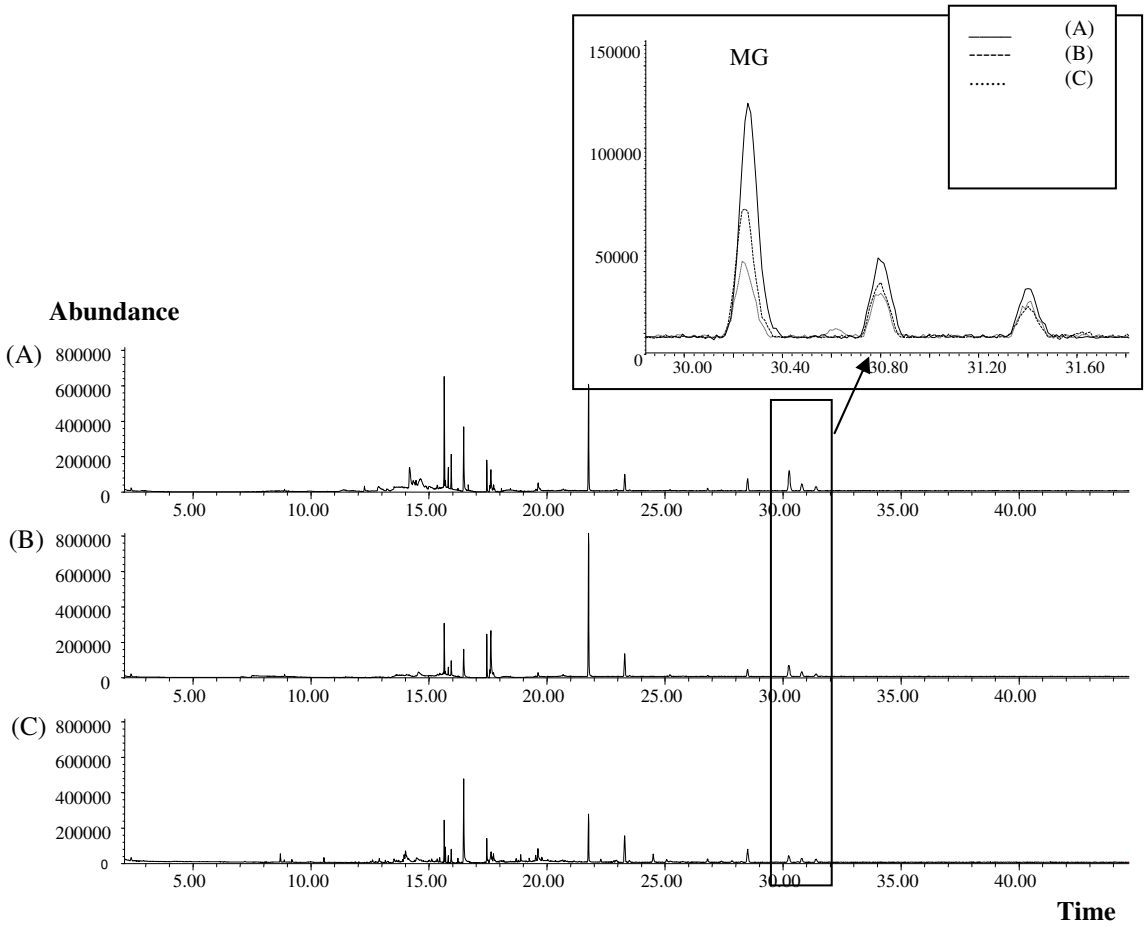

Figure 2: GC-MS (TIC) chromatogram of the extracts of products A, B and C.

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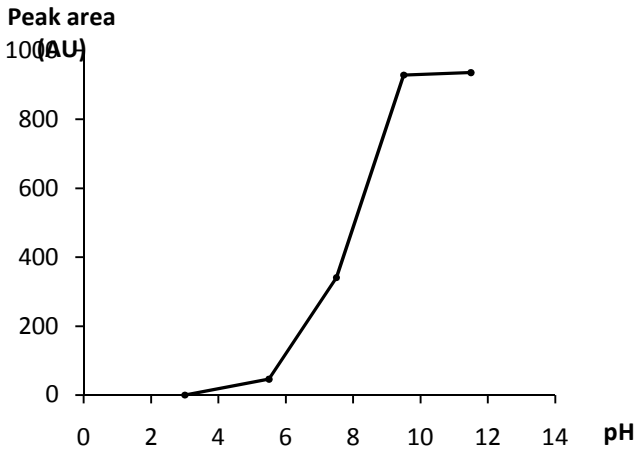

Figure 3: Relative mitragynine amount extracted from product A at various pH.

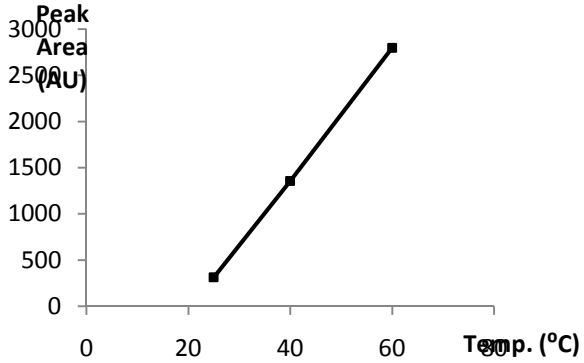

Figure 4: Relative mitragynine amount extracted from product A at various temperature.

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