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A SIMPLE AND COST EFFECTIVE ISOLATION AND PURIFICATION PROTOCOL OF MITRAGYNINE FROM *MITRAGYNA SPECIOSA* KORTH (KETUM) LEAVES

(Satu Protokol Pengasingan Mitragina daripada Daun *Mitragyna speciosa* Korth (Ketum) yang Mudah dan Kos Efektif)

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Abstract

The objective of the present study was to develop a simple and cost effective method for the isolation of mitragynine from *Mitragyna speciosa* Korth leaves. The results of our study showed that around 0.088 % (w/w) pure mitragynine was obtained by this newly developed method with a purity of 99.0 % (w/w) based on GC-MS analysis. Moreover, the recovery of the pure mitragynine from the chloroform extract was also more than 95.0 %. Advantages of this new method were simpler, faster and more economical. In conclusion this simple and cost effective method helps to isolate mitragynine with higher purity in comparison with the published methods.

Keywords: *Mitragynine speciosa* Korth, mitragynine, mitragynine extraction

Abstrak

Objektif kajian ini adalah untuk membangunkan satu kaedah yang mudah dan kos efektif untuk pengasingan mitraginina daripada daun *Mitragyna speciosa* Korth. Keputusan kajian menunjukkan bahawa lebih kurang 0.088 % (w/w) mitraginina tulen telah diperolehi dengan menggunakan kaedah baru yang dibangunkan ini dengan ketulenan 99.0 % (w/w) berdasarkan analysis GC-MS. Malahan, hasil pemerolehan semula mitraginina tulen daripada ekstrak kloroform juga melebihi 95.0 %. Faedah kaedah baru ini adalah lebih mudah, cepat dan ekonomikal. Kesimpulannya, kaedah yang lebih mudah dan kos efektif ini membantu mengasingkan mitraginina dengan ketulenan yang lebih tinggi berbanding dengan kaedah yang telah pun diterbitkan.

Kata kunci: *Mitragynine speciosa* Korth, mitragina, pengekstrakan mitragina

Introduction

Mitragynine is the main alkaloid present in *Mitragyna speciosa* leaf with opioid agonistic activity [1,2,3,4]. The mitragynine content in *M. speciosa* leaves varied from place to place depending on the geographical location and season [4]. The mitragynine content of Thai *M. speciosa* was 66% of the total alkaloids while the Malaysian species contained only 12% of the total alkaloids [5]. This necessitates finding a simple, fast and reproducible method to obtain mitragynine from Malaysian species of *M. speciosa*.

Currently, several methods are available for the extraction and isolation of mitragynine from *Mitragyna speciosa* leaf. These extraction and isolation methods published have their own advantages and disadvantages. Most of these methods commonly employs soxhlet extraction method using methanol as solvent to obtain the crude extract of *M. speciosa* leaves [6,7,8]. However, these methods were very slow and require days to obtain crude extract of *M. Speciosa*. The extract obtained by these methods were sticky, agglutinated and might need sonication to facilitate the extraction of mitragynine from the crude extracts. Moreover, the process required to obtain pure mitragynine

from the crude extract was too lengthy and laborious which involves acidification, neutralisation followed by liquid-liquid extraction and finally column chromatography with a series of mobile phase with increasing polarity [6,7,8]. Hence, our main objective of this study is to find a simple and rapid method with maximum output of pure mitragynine from dried leaves of *M. speciosa* as it is not commercially available at cheaper price.

Experimental

Chemicals and solvents

Picric acid, potassium bromide (KBr) and acetanilide were obtained from Sigma-Aldrich, St. Louis, USA. Pure mitragynine standard was obtained from IMR (Institute of Medical Research, Kuala Lumpur, Malaysia). Commercial pure mitragynine standard (ASB-00013890-025) was obtained from Chromadex, Irvin, USA. Solvents hexane, petroleum ether, methanol, acetone, chloroform, ethyl acetate and HCL used were of analytical grade purchased from Fischer Scientific, Loughborough, U.K.

Plant material identification

Leaves of *M. speciosa* were collected from Perlis, Malaysia and were identified by a botanist in the School of Biological Science, Universiti Sains Malaysia (USM). A voucher specimen (No 11074) was deposited at the Herbarium of the School of Biological Science, USM for further references.

Extraction and isolation of mitragynine

Fresh *Mitragyna speciosa* Korth leaves (1.0 kg) were washed with water to remove dirt and adhering material, oven dried at 45-50 °C for three days and milled into fine powder using a blender to get the dry powder (297.0 g). Soxhlet extraction was carried out using 297.0 g powdered leaves with 4 L petroleum ether (40-60 ^{o}C) for 8 hour, then petroleum ether solution was discarded and the defatted powdered leaves were dried and the extraction was repeated with 4 L chloroform for 8 hour. The chloroform solution obtained was filtered, concentrated, evaporated to dryness under the reduced pressure using rotary evaporator and was kept in a refrigerator (-20^{0}C). The dried crude chloroform extract was subjected for flash chromatography according to the method of Still *et al.*[10]. Crude fraction containing mitragynine was obtained by eluting with hexane and ethyl acetate (80:20 v/v) and this fraction (100 mL) was subjected to liquid-liquid fractionation using petroleum ether (100 mL X 3 times) for further purification. The petroleum ether layer was discarded and the remaining solution was concentrated under the reduced pressure using a Buchi R215 Rotavapor (Flawil, Switzerland) to obtain crude mitragynine.

Purification of mitragynine by crystallization

Approximately 0.80 g of the isolated crude mitragynine was dissolved in minimum quantity of methanol and this solution was added to a 5 mL saturated methanolic picric acid solution. Side of test tube was scratched and this solution was kept in refrigerator for 20 minutes to obtain orange colored mitragynine picrate crystals. These crystals were filtered, washed with methanol followed by acetone and the melting point of these crystals was determined. The mitragynine picrate crystals were converted to mitragynine free base by dissolving them in hot saturated acetone solution and by adding this solution to an excess of dilute ammonia solution lead to the liberation of free base which was extracted with diethyl ether. This solution was dried to obtain a pale brown, amorphous compound. This compound along with IMR pure standard was subjected for TLC for the comparison of hR_f value.

Qualitative characterisation of mitragynine free base

Various analytical methods such as UV-Visible, IR, NMR, mass spectroscopy, CHN analysis, HPTLC, melting point and determination of dissociation constant were carried out to characterize the isolated pure mitragynine free base. UV-Visible spectroscopy was carried out using Shimadzu UV 160-A double beam spectrophotometer (Kyoto, Japan), the UV spectra of 0.015 ppm pure mitragynine in methanol was obtained against a blank containing methanol. FTIR spectra were recorded using KBr pellets by a Thermal Scientific Nicolet 6700 spectrophotometer with Omnic software (Franklin, USA). ^{1}H-NMR and ^{13}C –NMR were performed using 400 MHz and 100 MHz Bruker spectrometer (Karlsruhe, Germany), respectively. GC-MS spectra were taken using HP 6890A GC system (Santa Clara, USA) with HP-5MS Polyimide coated capillary column (30 m x 0.25 mm i.d. x 0.1 μm), heated from 100^{o}C to 280^{o}C at 10^{o}C / min, 10 Kpa helium at 1.00 mL/min flow rate and an injection volume of I μL with split ratio of 5 : 1. CHN elemental analysis was conducted using Perkin Elmer CHN Analyzer Model 2400-2 (Massachusetts, USA). HPTLC study was performed on a 0.50 mm thick Silica gel 60 G_{254} (10 cm x 20 cm) using a

mobile phase hexane:ethyl acetate (80:20 v/v). TLC plates were then visualized under UV light (254 and 366 nm). Melting point was measured by Koffler Block Electrothermal Melting Point (London, UK) with Acetanilide (113-114^{o}C) as calibration standard. pKa of isolated and purified mitragynine free base was determined by titration method using 0.0001 N HCL with Cyberlab 14 L pH meter (Millbury, USA) in pH modes. In this procedure, 50 mg pure mitragynine in 30 mL methanol was used. The long term stability of the stock solution of mitragynine (1000 ng mL^{-1}) refrigerated at -20 ^{o}C was tested after storage period of 60 days. The stock was diluted to 1 ng mL^{-1} with pure methanol in three replicates prior to the analysis. The result was expressed as the difference from time t = 0 day. The percentage difference from time t=0 day not exceeded ±15% indicating that the stock solution of mitragynine was stable [11].

Statistical analysis

Results were expressed as the mean ± S.E.M of three independent experiments. Student's *t*-test was used for statistical analysis; P values < 0.05 were considered to be significant [12]. Statistical calculations were carried out with the Minitab 15.0 English for windows software package.

Results and Discussion

Extraction is an important step involved in the isolation of bioactive compounds from the plants with medicinal value. In our study we followed simple soxhlet extraction method to defat *Mitragyna speciosa* Korth leaves using petroleum ether followed by extraction with chloroform which yielded 7.22 g of crude chloroform extract. It was evident from Table 1 that chloroform extract obtained was pure and rich in mitragynine content (70.0 %) than the methanol extract of the first stage from the published method [6,7,8] with a mitragynine content of only 19.0%. Approximately 0.80 g of crude mitragynine was obtained by flash chromatography of the crude extract followed by liquid-liquid fractionation and the mitragynine in this extract appeared as one single peak in GC-MS with relative purity of 98.0 %. This newly developed method even employed only one step flash chromatography to obtain 98.0 % pure mitragynine (Table. 1) whereas the published methods followed a series of mobile phase to obtain the same result [7]. Thus, this newly developed method required less solvent, incurred less steps and more economical.

Table 1: Comparison of GC-MS purity of mitragynine extracts at various stage between our method and published methods

Stage of Extraction	GC-MS Purity of Mitragynine Using Our Method	Using Published Method [6, 7, 8]
Methanol	-	*19.0
Acid solubilisation	-	
Filtration	-	
Base neutralization	-	
Chloroform	70.0	*43.0
First column	92.0	*76.0
Second column	-	96.0
Petroleum ether washing	98.0	-
Crystallization	99.0	-

The data is the percentage area of GC-MS based on *ca.*300.0 g *M. speciosa* dried leaves at each stage
* Results obtained in our lab using published method [6,7,8]

Results of crystallisation showed that orange colored crystals of mitragynine picrate salt (*ca.* 0.60 g.) obtained from crude mitragynine have a melting point of 220-225 °C. Precipitation of these orange colored crystals leads to the formation of a pale brown, amorphous mass of mitragynine free base (*ca*. 0.26 g). It was very soluble in methanol and gives a very strongly fluorescent solution. The result of TLC study shown that the hR_f (81.9 cm) value of the isolated and purified free base of mitragynine was in accordance with that of IMR pure mitragynine standard

(Table 5). Moreover, the purity of the mitragynine obtained by this newly developed method was approximately 99.0 %. In addition, the purified mitragynine showed a single chromatographic peak upon spectroscopic analysis by GC-MS. The spectrum of purified mitragynine ($C_{23}H_{30}N_2O_4$, exact molecular mass = 398.2207) was confirmed with the IMR pure standard and to the NIST 98 mass spectral library. Mitragynine obtained from this newly developed method was 99% pure, when peak area of mitragynine was compared to other minor impurities in the GC-MS analysis (Table 2), and was used as the analytical standard to build the calibration curve (Figure 1) for quantitative analysis.

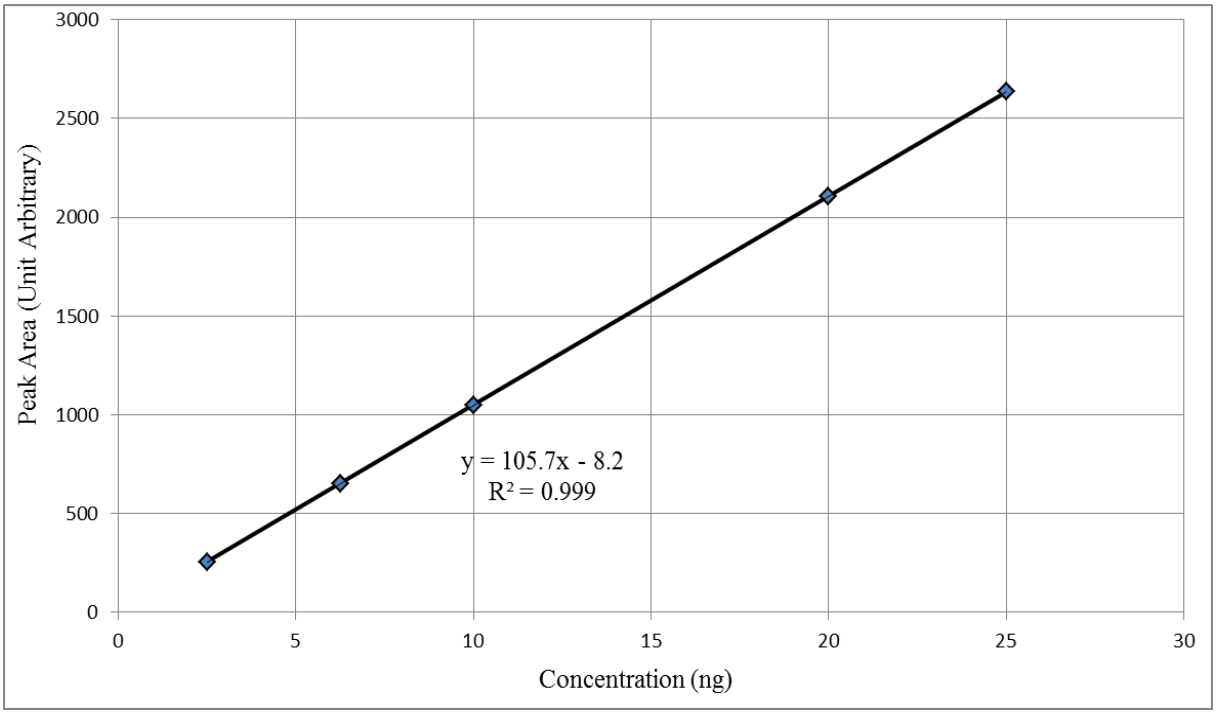


Figure 1: Pure mitragynine calibration curve

The pure mitragynine standard calibration curve was linear with the equation of y= 105.7x – 8.2 over the range of 0.5-50 ng (n=5, r^2 = 0.999±0.002) (Figure 1).The mean coefficients of variation for within day assay precision was 2.33±0.25 % at a concentration range of 0.5-50 ng. For the day to day variation, the mean coefficient of variation was 4.90±0.60 % for the same concentration range. The detection limit (LOD) and quantification limit (LOQ) of assay for mitragynine was 0.50 ng and 2.00 ng respectively. The concentrations giving signal-to-noise ratios of 3:1 and 10:1 were considered to be the LOD and the LOQ respectively.

It is clear from Table 2 that the pure mitragynine obtained (99.0 %) by this newly developed method was stable for six months and its strength was 100 % comparable with that of IMR pure mitragynine standard (Table. 2). The recovery of pure mitragynine from chloroform extract of *M. speciosa* leaves from Perlis, Malaysia by this method was more than 95.0 % at each stage (Table. 3).

Table 2: Comparison of purity and stability of mitragynine obtained by our method and standard mitragynine (Chromadex, Irvin, USA)

Characteristic of Pure Mitragynine	Isolated mitragynine	Standard mitragynine
Purity (%)	99.0	96.0
*Purity strength (%)	100.0	98.0
Stability, 1000 $ngmL^{-1}$ Difference from t=0 ($ngmL^{-1}$), after 6 months at -20°C	0.40	0.30

*Purity strength (%) was in comparison with IMR standard

Table 3: Recovery (%) of mitragynine from chloroform extract of *M. speciosa* leaves from Perlis, Malaysia by our method.

Type of extract	Actual yield (g)	Theoretical yield (g)	Recovery from chloroform extract (%)
Chloroform	7.22 ± 0.33	-	-
Column chromatography	1.54 ± 0.09	1.55	99.35 ± 0.10
Petroleum ether Washing	0.78 ± 0.06	0.80	97.50 ± 0.12
Crystalization	0.26 ± 0.01	0.27	96.29 ± 0.15

Values are the means of three replicates ± standard error of the mean (S.E.M.).

From Table 3, it was confirmed that our method is feasible to isolate pure mitragynine. The pure mitragynine (99.0 %) yield obtained by this method was 0.26±0.01 g from *ca.*300.0 g of *M. speciosa* dry leaves (Table. 4) and the content of mitragynine we obtained from the *M. speciosa* leaves of Perlis, Malaysia was 0.088 % w/w (0.88 mg/g of dried leaves). This was within the range of 0.08-0.10% as reported in the literature for leaves extracts of *M. speciosa* [6, 9].

Table 4: Yields (%) and GC-MS purity (%) of mitragynine obtained from *M. speciosa* leaves of Perlis by our method at each stage.

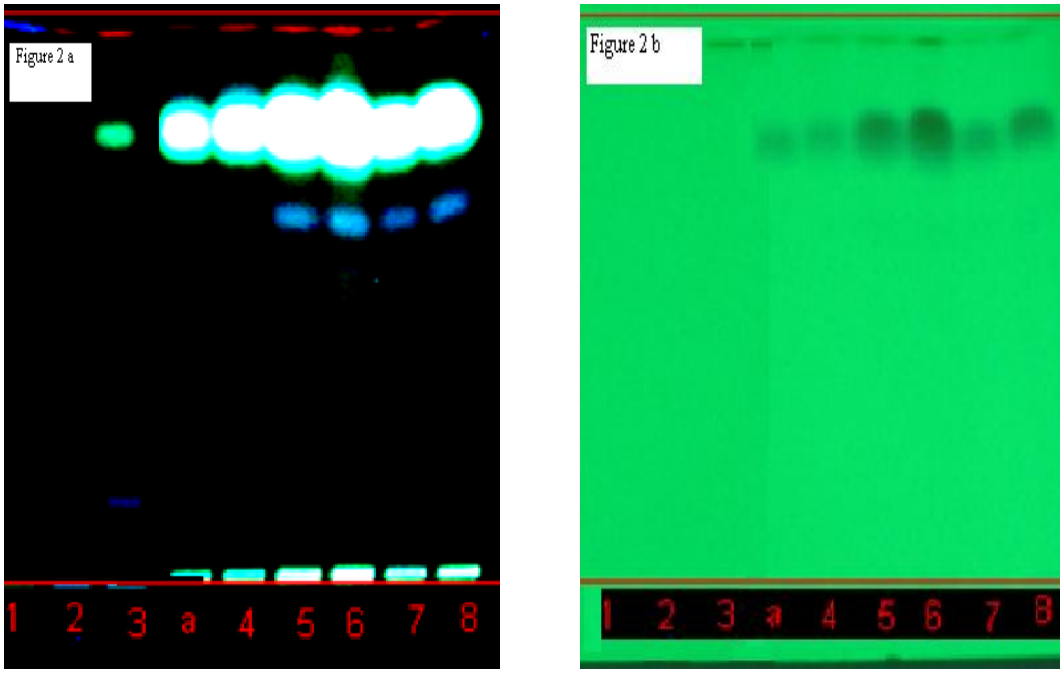
Stage of extraction	Actual yield (g)	Actual yield (%)	GC-MS Purity (%)
Chloroform	7.22 ± 0.33^{a}	2.60	70.0 ± 0.20^{e}
Column Chromatography	1.54 ± 0.09^{b}	0.56	92.0 ± 0.54^{f}
Hexane washing	0.78 ± 0.06^{c}	0.26	98.0 ± 0.32^{g}
Crystallization	0.26 ± 0.01^{d}	0.088	99.0 ± 0.25^{h}

Values are the means of three replicates ± standard error of the mean (S.E.M). The different alphabet of a,b,c and d represents the values different significantly for the yield obtained whereas the different alphabet e,f,g and h represents the values different significantly for the GC-MS purity at $P<0.05$ based on Student t-Test Minitab (Illinois USA) ($^{a,b}P< 0.05$ and $^{e,f}P<0.05$ in comparison between chloroform extraction and column chromatography, $^{b,c}P<0.05$ and $^{f,g}P<0.05$ in comparison between column chromatography and hexane washing, $^{c,d}P< 0.05$ and $^{g,h}P< 0.05$ in comparison between hexane washing and crystallization for both yields and GC-MS purity respectively.

The details about the HPTLC results of isolated and pure mitragynine are given in Table 5. The hR_f of isolated mitragynine (81.9) was comparable with the hR_f of IMR standard (82.0) which shows that the isolated compound is mitragynine. Figure 2a, 2b shows the HPTLC profile of fractions collected (1-8) from flash chromatography as well as IMR mitragynine standard under UV 365 and 254 nm. It is noted from the Figure 2a and 2b that fractions 1-3 does not contain mitragynine whereas fractions 4-8 containing mitragynine. The hR_f of mitragynine (81.6) present in the fractions 4-8 were in comparison with the hR_f of IMR standard (82.0).

Table 5: HPTLC fingerprint of isolated mitragynine and the standard mitragynine (IMR)

Parameters	TLC conditions / results	
	Isolated mitragynine	Standard mitragynine (IMR)
Quantity sample applied	2 ul	2 ul
Detector	12 W VL. 6.1C UV detector	12 W VL. 6.1C UV detector
	6 W : 254 nm	6 W : 254 nm
	6 W : 365 nm	6 W : 365 nm
Stationary Phase	Silica Gel $60G_{254}$	Silica Gel $60G_{254}$
Mobile Phase	Hexane : Ethyl acetate (80 : 20)	Hexane : Ethyl acetate (80 : 20)
Applied Concentration, mg/ml	0.03	0.03
Elution Length, cm	8.0-10.0	8.0-10.0
hR_f, cm	81.9	82.0
Visual appearance of Band :		
White light	Reddish brown	Reddish brown
UV 365	Greenish brown	Greenish brown
UV 254	Brownish	Brownish
Iodine	Yellowish brown	Yellowish brown
Dragendorff reagent	Orange	Orange



Figures 2a and 2b**:** HPTLC profile of different fractions (1-8) of flash chromatography and standard mitragynine (a) under UV 365 and UV 254 nm.

Mitragynine free base: Yellow amorphous solid. m.p.: 103-105^{0}C. pKa = 5.5. UV/Vis λ_{max} (MeOH) nm (log ε): 225 (4.76),290 (3.94). IR (KBr): 1567.2, 1704.5, 3439.3 cm^{-1}. hR_f : 0.82 (C_6H_{14}: EtOOCMe, 80:20). GCMS (EI, 70 eV): *m/z* (%) = 398 [M $^+$] (92), 214. (100),383 (44), 200 (26). CHN : Calcd for $C_{23}H_{30}N_2O_4$: C, 69.35; H, 7.54; N,7.04. Found C, 69.35; H , 7.50; N, 7.00. ^{1}H NMR (400 MHz, MeOD): δ 7.68 (1H, br-s, N-H), 7.53 (1H, s, H-17), 6.91 (1H, dd, J=7.9, 7.9 Hz, H-11), 6.89 (1H, d, J=7.9 Hz, H-12), 6.41 (1H, d,J=7.9, H-10), 3.84 (3H, s, 9-OCH_3), 3.78 (3H, s, 17-OCH_3), 3.68 (3H, s, 22-OCH_3), 3.15 (1H, br-d, J=9.8 Hz, H3), 3.07 (1H, m, H-6), 3.04-3.07 (2H, overlapped, H-15, H-21), 2.90-3.00 (2H, overlapped, H-5 and H-6), 2.40-2.55 (3H, overlapped, H-5, H-14, H-21), 1.75-1.80 (2H, overlapped, H-14, H-19), 1.64(1H, br-d,J=11.3, H-20), 1.30(1H, m, H-19), 0.87 (3H, dd, H-18).^{13}C NMR (100 MHz, MeOD): 169.36 (C-22), 160.65 (C-17), 154.49 (C-9), 137.33 (C-13), 133.69 (C-2), 121.91 (C-11), 117.72 (C-8), 111.55 (C-16), 107.91 (C-7), 104.26 (C-10), 99.80 (C-12), 61.65 (17-OCH_3), 61.20 (C-3), 57.77 (C-21), 53.85 (C-5), 51.45 (22-$COOCH_3$),55.40 (9-OCH_3), 39.95 (C-20), 31.99 (C-15), 29.78 (C-14), 22.78 (C-6), 14.22 (C-19), 12.95 (C-18).

Conclusion

In conclusion, we have developed an alternative method to isolate and purify the mitragynine from *M. speciosa* leaves. The main advantage of this method was simple, fast and cost effective with high reproducibility for the extraction of mitragynine. Morover this method give improved yield of mitragynine (0.088 % w/w) with purity of 99.0 % based on GCMS analysis which was better than the purity of the mitragynine obtained from the other published methods.

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References

1. Takayama, H., Ishikawa, H., Kurihara, M., Kitajima, M., Aimi, N., Ponglux, D., Koyama, F., Matsumoto, K., Moriyama,T., Yamamoto, L.T., Watanabe, K., Horie, K. 2002. Studies on the synthesis and opioid agonistic activities ofmitragynine-related índole alkaloids: Discovery of opioid agonists structurally different from other opioid ligands.*Journal of Medicinal Chemistry*, **45**: 1949-1956.
2. Grewal, K.S. 1932. Observations on the pharmacology of mitragynine. *Journal of Pharmacology and Experimental Therapeutics*, **46**: 251-271.
3. Suwanlert, L.S. 1975. A study of Kratom eaters in Thailand. *Bulletine on Narcotics*, **27**: 21-27.
4. Shellard, E.J. 1989. Enthnopharmacology of Kratom and the Mitragyna alkaloids. *Journal of Ethnopharmacology*, **25**(1): 123-124.
5. Takayama, H. 2004. Chemistry and pharmacology of analgesic indole alkaloids from the Rubiaceous plant, *Mitragyna speciosa*. *Chemical and Pharmaceutical Bulletin*, **52**(8): 916-928.
6. Houghton, P.J., Latiff, A., and Said, I.M. 1991. Alkaloids from *Mitragyna Speciosa*. *Phytochemistry*, **30**: 347-350.
7. Ponglux, D., Wongseripipatana, S., Takayama, H., Kikuchi, M., Aimi, M. and Sakai,S. 1994. A new indole alkaloid, 7 hydroxy-7H mitragynine, from *Mitragyna Speciosa* in Thailand. *Planta Medica*, **60**: 580-581.
8. Jansen, K.L.R. and Prast, C.J. 1988b. Enthnopharmacology of kratom and the Mitragyna alkaloids. *Journal of Enthnopharmacology*, **23**: 115-119.
9. Takayama,H. and Maruyama, T. (2009). Simultaneous analysis of mitragynine, 7-hydroxymitragynine, and other alkaloids in the psychotropic plant "kratom" (Mitragyna speciosa) by LC-ESI-MS. *Forensic Toxicology*, **27**: 67-74.
10. Still, W. C.; Kahn, M. and Mitra, A. (1978). Rapid chromatographic techniques for preparative separation with moderate resolution. *Journal of Organic Chemistry*, **43**(14): 2923-2925.
11. Janchawee, B., Keawpradub, N., Chittrakarn, S., Prasettho, S., Wararatananurak, P., Sawangjareon, K. 2007. A high performance liquid chromatographic method for determination of mitragynine in serum and its application to a pharmacokinetic study in rats. *Biomedical Chromatography*, **21** (2): 176-183.
12. James A. D., John M. F., Graeme A.P., Catherine E. S., Nigel B. P. and Ray A. L. 2010. Seasonal variation of biomass and bioactive alkaloid content of goldenseal, *Hydrastis Canadensis*. *Fitoterapia*, **81**: 925-928.