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Herbal monograph methodology for identification of *Mitragyna speciosa* (Korth.) Havil. leaves

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Abstract
Mitragyna speciosa (Korth.) Havil is a plant native to Malaysia and Thailand. Although it has medicinal properties, it also has psychotropic effects and is widely abused. Previous studies have focused on evaluating its beneficial effects or the identification and detection of its primary active component, mitragynine. However, there is a lack of data available for leaf identification. The present study investigated pharmacognostical analysis and to evaluate physicochemical properties in order to assist in the identification of this plant and to characterize *M. speciosa* leaves. The present data is used to create monograph and document for pharmacopoeia, which will aid with plant identification and will assist in the identification of misuse of the leaves in herbal products. At the same time, this identification method may aid regulatory authorities in identifying the plant. The present results may also facilitate the standardization of *M. speciosa* in nutraceutical products and botanical drugs.

Keywords: *Mitragyna speciosa* (Korth.) Havil. ketum, kratom, physicochemical, pharmacognostical, chromatographic

1. Introduction
Mitragyna speciosa (Korth.) Havil. belongs to the family Rubiaceae. It is native to Southeast Asia and grows in the wild of Thailand, Malaysia, the Philippines, and Papua New Guinea. Demand for the plant has led to its cultivation in many other parts of East Asia [1].
The plant is known locally as 'ketum' and 'kutuk' in Malaysia, whereas in Thailand it is called 'kratom' or 'kadam' [1]. Synonym include *Nauclea luzoniensis* Blanco, *Nauclea speciosa* (Korth.) Miq., and *Stephegyne speciosa* Korth. [2] The major chemical constituents of the leaves are mitragynine indole alkaloids, which have been classified as poisonous psychotropic substances; these substances are illegal but widely abused in Malaysia, Thailand, Australia, and Myanmar [3]; however, they also possess medicinal effects.
M. speciosa. leaves are valued as a medicinal herb. In Malaysia, the leaves are traditionally used as an external remedy for wounds and to treat enlarged spleens. They are chewed, or infused and ingested similarly to tea [1]. Mitragynine and 7-hydroxymitragynine compound of *M. speciosa* leaves has been found to stimulate opioid receptors, leading to analgesic and antinociceptive activities [4-6]. In addition, aqueous extract of *M. speciosa* leaves administered to male albino Swiss mice decreases the duration of immobility in force swimming test and tail suspension test and mitragynine isolated from the methanol extract of M. speciosa leaves administered intraperitoneally as a single dose to male ICR mice decrease in the duration of immobility time in force swimming test and tail suspension test (indicating antidepressant effects) [7, 8]. Furthermore, methanol extract of *M. speciosa* leaves has been reported to contain anti-inflammatory properties [9].
Analytical methods have been used to quantify the amount of mitragynine from *M. speciosa* leaves. Studies using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) [10] and high-performance liquid chromatography with diode array detection (HPLC-DAD) [11, 12] have been conducted to identify mitragynine in the raw material or in the final product. Nuclear magnetic resonance using ^{1}H and ^{13}C has been used to detect and confirm the alkaloid compounds in the leaves [13]. Furthermore, new indole alkaloids have been isolated and characterized using nuclear magnetic resonance (NMR), circular dichroism (CD), and mass spectrometry (MS). Various methods of sample extraction, preparation, and instrument detection have focused on detecting psychotropic substances in this plant [14, 15].
Currently in the literature there are pharmacognostic studies but they lack information on physicochemical data which includes ash content, extractive value, and moisture content of the

raw material. The objectives of this study are to conduct pharmacognostical analysis and to evaluate physicochemical properties in order to assist in the identification of this plant and to characterize *M. speciosa* leaves.

2. Material and methods

2.1 Materials

Fresh leaves of *M. speciosa* were collected from Sanglang, Kedah. The authenticity of the leaves was confirmed at the University Putra Malaysia, Institute of Bioscience. A dried sample of the leaf voucher specimen was deposited at the Institute for Bioscience Herbarium with voucher number SK 2118/13.

The collected plant material was visually inspected for contamination. The leaves were washed with reverse osmosis water. The leaves were dried in a drying chamber at 40 °C and then ground using a grinding machine. Subsequently, the powdered samples were examined for contamination.

3. Methods

3.1 Macroscopic examination

The morphological features of the plant were inspected and compared with information from other literature reviews. The leaves and other parts of the plant were visually inspected to identify the form and structure of the specimen. Observed features were recorded in a qualitative and quantitative format, and photographs of the leaves' main features were captured and stored as a record of the specimen.

During the storage period, the powdered leaves were examined for any change in colour, fungus growth, or insect contamination.

3.2 Microscopic examination

Powdered leaves of *M. speciosa* were mounted with 60% chloral hydrate aqueous solution and observed under a light microscope. Photomicrographs of the sample were captured and processed using CellSens Standard software.

3.3 Colour test

Ten mg of powdered leaves was weighed in test tubes. Then, 5 to 10 drops of a reagent were added to test tubes containing the powdered herb using a narrow-tipped Pasteur pipette, and the tubes were shaken gently to mix the contents. Colour and clarity were recorded immediately after addition of the reagent and after 15 minutes by using the Federal Standard 595 Color Server as a guide. This test was repeated using seven different reagents: deionized ultrapure water, concentrated 95-97% sulphuric acid (H_2SO_4), concentrated 37% hydrochloric acid (HCl), 5% sodium hydroxide (NaOH), 5% potassium hydroxide (KOH), 25% ammonium solution (NH_4OH), and 5% ferric chloride ($FeCl_3$).

3.4 HPTLC method

Three samples were prepared for HPTLC analysis. One sample of powdered dried plant material (2 g) was sonicated in 5 mL of deionized ultrapure water for 15 minutes. A second powdered plant sample (2 g) was sonicated with ethanol for 15 minutes. The third sample (2 g) was sonicated with methanol for 15 minutes. The samples were then filtered through with 0.45 µm nylon membrane filter. The standard for HPTLC band verification was prepared by dissolving mitragynine (100 µg/mL) in ethanol.

For the mobile phase, a toluene: ethyl acetate: methanol: ammonia 25% solution was used (volume ratio of 30:30:15:1). Mitragynine standard solution and 3 µL of each sample were spotted on a plate silica gel 60 F_{254} 20 x 10 cm plate supplied by Merck as 6 mm bands. The developed plate was observed with a CAMAG TLC visualizer and winCATS software under ultraviolet light (254 nm and 366 nm), and then sprayed with Dragendorff's reagent spray. The derivatized plate was examined under visible light.

3.5 HPLC-DAD-UV method

Powdered dried plant material (0.5 g) was macerated with 10.0 mL of ultrapure ionized water for 30 minutes and filtered through a 0.45-µm nylon membrane filter. The standard solution was prepared by dissolving 94.3% pure mitragynine (primary standard (P) grade Chemical Abstracts Service (CAS) number 4098-40-2, lot number 00013890-1741; ChromaDex Inc., USA) in ethanol to a concentration of 180 µg/mL.

High-performance liquid chromatography was performed using a Waters HPLC Alliance 2695 separation module system with a 996 photodiode array (PDA) detector (Waters XBridge Ethylene Bridged Hybrid (BEH) C18; 2.5 µm 4.6 x 150 mm column; part number: 186006711; Milford, USA). The mobile phase consisted of 0.05% trifluoroacetic acid (TFA) in deionized ultrapure water and 0.05% TFA in acetonitrile (ACN) using gradient mode, with a 0.6 mL/min flow rate. A gradient mobile phase was used for the separation mode (Table 1), and 10 µL of the sample was injected. The column was kept at 35 ± 5 °C. The detector wavelength was set at 210.0 - 400 nm. Empower System Suitability software version 2.0 was used to analyze the system suitability in accordance with the United States Pharmacopeia.

Table 1: HPLC gradient elution table.

Time (min)	% A	% B
0.01	80	20
10.0	70	30
15.0	40	60
18.0	20	80
20.0	60	40
25.0	80	20
30.0	80	20

(A) 0.05% TFA in deionized ultrapure water; (B) 0.05% TFA in acetonitrile.

3.6 Foreign matter

A magnifying glass were used to examine 100.0-500.0 g of powdered plant material for foreign matter.

3.7 Ash analysis

3.7.1 Total ash

A sample of powdered leaves (2-4 g) was weighed using a preheated (500-600 ºC) crucible disc. The sample was burned in a furnace at 500-600 ºC until no trace of carbon remained and a constant ash weight was achieved. The percentage of total ash in the powdered samples was then calculated:

$$total\ ash\ value\ (\%\ w/w) = \frac{weight\ of\ the\ residue\ (g)\ \times 100\%}{weight\ of\ the\ sample\ (g)}$$

3.7.2 Acid-insoluble ash

The total ash residue (3.7.1) was digested in 25 ml of 2 M hydrochloric acid and filtered with ashless filter paper. The insoluble residue was ignited in a furnace at 500-600 °C until it was of a constant weight. The percentage of acid-insoluble ash in the powdered samples was calculated:

3.8 Loss on drying

A preheated weighing bottle (100-105 °C for 10 minutes) was used to weigh 2 g of powdered sample. The sample was dried at 100-105 °C for five hours. At that point, the sample weight was checked every 15 minutes, and the drying time was extended until a constant weight was achieved. The percentage of loss on drying was then calculated:

$$acid\text{-}insouble\ ash\ value\ (\%\ w/w)\ = \frac{weight\ of\ residue\ (g)\ \times 100\%}{weight\ of\ sample\ (g)}$$

3.9 Extractive value

The extractive values were determined by employing ultrapure water and ethanol (95%) as solvents in the methods below. Ultrapure water was used first, and then the method was repeated with ethanol.

3.9.1 Hot solvent method

The solvent (100 mL) was added to 4 g of powdered leaves and mixed using a shaker. This mixture was left standing for 1 hour and then refluxed for 1 hour at low heat. From this solution 25 mL was pipetted and the solvent was evaporated using a water bath. The remaining residue was heated for six hours at 105 °C.

3.9.2 Cold solvent method

Powdered leaves (4 g) were macerated for six hours in 100 mL of a solvent, with frequent shaking using a shaker. After the shaking process, the sample was kept standing for 18 hours. From this solution 25 mL was pipetted and the solvent was evaporated using a water bath. The remaining residue was heated for 6 hours at 105 °C.

4.0 Heavy metal detection

4.0.1 Sample preparation: microwave digestion

Powdered samples (5 g each) were digested in microwave vessels with 6 mL of HNO_3, 1 mL of H_2O_2, and 1 mL of HCl. After digestion, each vessel of solution was filtered with a 0.45-µm syringe filter and topped off with 50 mL of deionized water.

Graphite furnace atomic absorption spectroscopy (GFAAS) was employed for heavy metal detection using the following lamps: arsenic electrode discharge lamp (193.7 nm), mercury hollow cathode lamp (253.7 nm), lead electrode discharge lamp (283.7 nm), and cadmium electrode discharge lamp (228.8 nm).

4.1 Microbial contamination tests

The total viable aerobic count or total aerobic microbial count (TAMC) and fungi-total yeast and mold count (TYMC) of the herbal material were determined as specified in the British Pharmacopeia [16].

Tests for bile-tolerant gram-negative bacteria and specific microorganisms (*Escherichia coli*, *Salmonella*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*) in the herbal material were also determined as specified in the British Pharmacopoeia [16].

4. Results and Discussion

4.1 Macroscopic characteristics of the leaves and other parts of the plant

Morphological examination revealed dark green leaves that were simple, opposite, entire, oblong-ovate, with abruptly acuminate apexes. The leaves measured 8-15 cm long and 4-10 cm wide. The base of each leaf was broadly rounded. The leaves were glabrous, i.e., the veins beneath the leaf surface were puberulous. Each leaf had 12-15 pairs of veins. The petioles were 2-5 cm long. The stipules were lanceolate, 2 cm long, and pubescent, with nine veins inside and colleters at the base.

Most of the plants were 3.7-9.1 m tall. The flowers were crowded in a round terminal. The inflorescences at the terminal of the lateral branches were composed of 3-7 globose heads, with one head subsessile between two others on 5-cm-long peduncles. The heads were 2.5 cm in diameter when flowering, and 1.5 cm in diameter when fruiting. The receptacles were hairy and the leafy bracts were up to 4 cm long, and there were petiolate leaves. The interflora bracteoles measured up to 3.5 mm long.

4.2 Microscopic examination

The powdered leaves consisted of fragments of spirally thickened vessels with parenchyma cells and bordered pitted vessels, a few fragments of fibres, and simple unicellular trichomes with non-glandular one-celled hairs. Microscopic analysis of powdered leaves revealed an abundance of epidermis cells with straight or slightly sinuous walls. Fragments of palisade parenchyma cells, thin-walled and long in shape, were also present. Calcium oxalate crystals were numerous and formed druses, often appearing as crystal chains inside the veins of the leaves, which is characteristic of the plant. Fragments of abaxial epidermis cells and stomata were present. An abundance of vessels, i.e., spiral vessels, longitudinally pitted vessels, and scalariform vessels, was also observed. Also noted was a large quantity of thin-walled fibres appearing in groups and simple unicellular trichomes with rough surfaces (Figure 1) [17].

Microscopic examination and literature review revealed micromorphological and anatomical similarities between species within the same genus. Based on these factors, it was determined that the anatomical characteristics of plants should not be used as the sole method for identifying *M. speciosa* (Korth.) Havil. However, microscopic examination of powdered leaf samples can be used to complement information and confirm the identity *M. speciosa*.

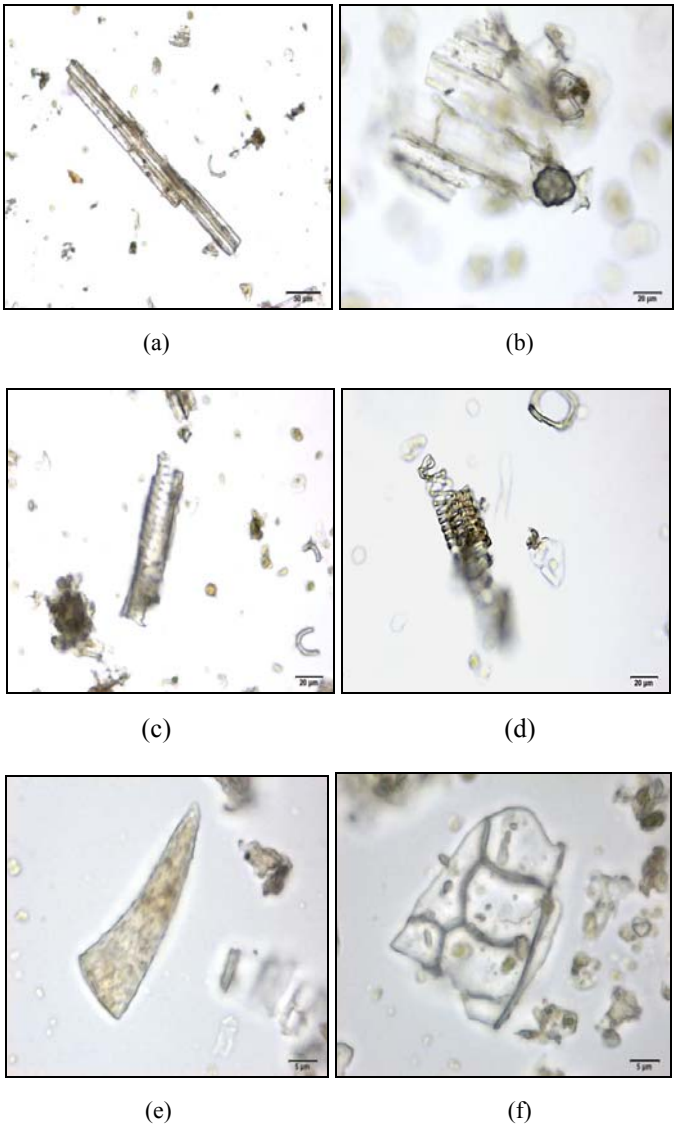


Fig 1: Microscopic characteristics of *M. speciosa* leave powder. (a) Group of fibres (10 x); (b) parenchyma cells (100 x); (c) fragment of pitted thickened vessel (20 x); (d) fragment of spirally thickened vessel (20 x); (e) one-celled non-glandular trichome (100 x); (f) epidermal cells (100 x).

4.3 Colour test analysis

The powdered leaves samples were light brown. Changes in colour upon addition of the reagent solution to powdered samples were recorded (Table 3). Colour changes indicate a chemical reaction between the reagent solution and the presence of chemical constituents in the leaves.

Table 2: Colour test observation result

Reagent	Observation	
	Immediately	At 15 minutes
Deionized ultrapure water	Light green	Light green
Conc. H_2SO_4	Brown	Dark brown
Conc. HCI	Green	Green
5% NaOH	Brown	Dark brown
5% KOH	Yellow	Dark brown
25% NH_4OH	Brown	Dark brown
5% $FeCl_3$	Yellow	Dark green

4.4 Chromatographic separation analysis

4.4.1 HPTLC fingerprinting

The developed HPTLC chromatogram displayed many bands for the methanol extract compared to the aqueous and ethanol extracts. Specific yellowish bands appeared under white light with a retention factor (R_f) of 0.74 after derivatization, indicating that mitragynine was present in the leaves. The yellowish band in the methanol extract was very intense and broader than the yellowish bands in the ethanol and aqueous extracts. Under the ultraviolet–visible (UV-Vis) lamp (366 nm), mitragynine appeared as a greenish band before derivatization. In addition, light green bands appeared with R_f 0.20, and red bands were observed with R_f 0.20. The compounds in the identified mitragynine bands were strongly non-polar. Minimal changes in the R_f values were observed when the mobile phase polarity was optimized.

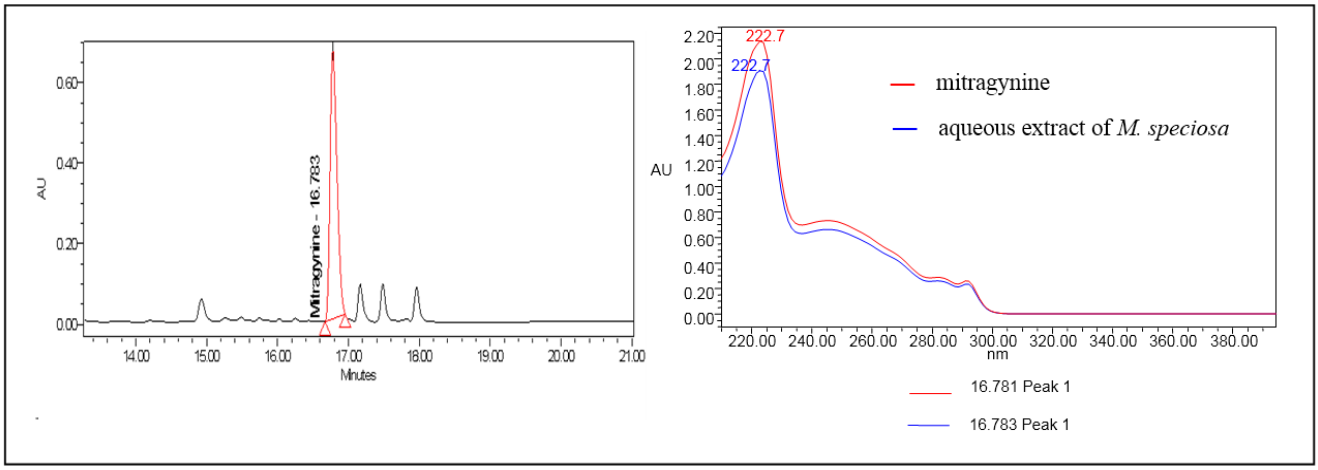
4.4.2 HPLC - DAD - UV fingerprinting

System suitability testing was conducted using the mitragynine standard as a reference marker. The performance and stability of the instrument were verified with five injections of the reference marker. The mean retention time (RT) for the five injections was 16.8 minutes, with a 0.1% relative standard deviation (RSD) and 0 standard deviation (Table 2). The wavelength at which maximum absorption was observed (λ_{max}) was 246 nm.

Table 3: System suitability test using mitragynine solution (180 µg/mL)

No.	Sample Name	RT	USP Plate Count	USP Tailing	Symmetry Factor
1	Mitragynine	16.8	125353.5	1.5	1.5
2	Mitragynine	16.8	125519.5	1.5	1.5
3	Mitragynine	16.8	126734.6	1.5	1.5
4	Mitragynine	16.8	127353.1	1.5	1.5
5	Mitragynine	16.8	129926.2	1.4	1.4
Mean		16.8	126977.4	1.5	1.5
% RSD		0.1	1.5	2.3	2.3
Std Dev		0	1847.8	0	0

The sample profile peak had the same retention time as the reference marker's peak at 16.8 minutes, with 0.8 absorption units (AU) at 222.7 nm for the aqueous extract. The UV absorption spectrum for the reference and aqueous extract showed the same lambda max (λ_{max}) at 222.7 nm (Figure 2).



(a) (b)

Fig 2: HPLC chromatogram profile of *M. speciosa* leaves: (a) aqueous extract chromatogram profile, (b) comparison of UV absorbent spectrum of mitragynine standard and aqueous extract sample.

The linearity of the calibration curve (R^2) equalled 1.0. The amount of mitragynine detected was higher than 20.0 µg/mL (aqueous maceration), with a mean purity angle of 0.26, standard deviation 0.0, and RSD of 6.5%. The mean United States Pharmacopeia (USP) tailing factor was 1.5, with a 2.3% RSD. The USP plate count reached 126977.4 mean plates, with a 1.5% RSD. The chromatography separation profile indicated that the target peak of the reference marker mitragynine was spectrally pure, with a purity threshold (0.4) higher than the purity angle (0.3).

The acceptance criteria for system suitability were constructed based on the HPLC results and were as follows: resolution (R)> 1.5 for five injections, repeatability, relative standard deviations of peak response (< 2.0%) and retention time (< 2.0%), column efficiency (N) > 2000, and tailing factor (T) 0.8- 1.5. The criteria were used to monitor system specificity, precision, and column stability.

4.5 Physicochemical parameters

Before the leaves were processed, it was verified that no other parts of the plant was present. This was further confirmed by the foreign matter test where the powdered leaves was visually observed under a magnifying glass. Additionally the microscopic analysis will support whether there is any foreign matter present in the sample.

For the ash analysis, the temperature was set at 500.0-600.0 °C to remove organic matter. The inorganic compound content of the sample was below 7.0%, with only 0.2 ± 0.1% (w/w) soluble in 2 M hydrochloric acid. High ash content would have indicated the presence of sand, calcium oxalate crystals, and many other inorganic materials in the sample.

The loss of mass after drying (100.0 - 105.0 °C for five hours) was measured to calculate the level of moisture and other volatile matter present in the powdered sample. This information is important for preserving the raw material, as powdered samples are prone to microorganism contamination. Lower levels of moisture prevent the growth of microorganisms. In this study, heating resulted in an 8.5 ± 1.3% (w/w) loss of mass from evaporation of water and volatile compounds.

Analysis of the extractive values indicated a higher presence of chemical constituents in hot extracts compared to cold extracts. The hot ethanol extractive value was 22.5 ± 0.7% compared to the cold ethanol extractive value of 13 ± 3.5% (Table 4).

Table 4: Physicochemical analysis of leaves.

Physicochemical parameters		Value*
Foreign matter value		< 2.5
Total ash value		6.0 ± 0.4
Acid-insoluble ash value		0.2 ± 0.1
Loss on drying value		8.5 ± 1.3
Water soluble extractive value	Hot	25.9 ± 0.9
	Cold	20.1 ± 2.3
Ethanol soluble extractive value	Hot	22.5 ± 0.7
	Cold	13.0 ± 3.5

* % w/w: weight/weight

4.6 Elemental analysis

Powdered leaves were analyzed for arsenic, cadmium, lead, and mercury. Traceable heavy metal elements were recorded (Table 5). Arsenic and mercury levels found to be above the detection limit of the instrument; however, these elements were found to be at the lowest level. Lead and cadmium were found to be below the detection limit of the instrument. Arsenic and toxic metal contamination in the leaf samples was found to be at the lowest level. This contamination of trace elements was most likely from environmental factors or from contamination during sample processing.

Table 5: Traceable heavy metal detection in the leaves

Element	Results, ppm*
Arsenic	0.022
Mercury	0.007
Lead	Not detected (<0.004)
Cadmium	Not detected (<0.003)

* Limit of detection mg/kg

4.7 Microbial contamination test

Microbiological analysis revealed a total bacteria count of approximately 10^5 cfu/g compared to a total yeast and mold count of approximately 10^3 cfu/g (Table 6). No toxic pathogens were detected. The microbial limit test specifies the absence of *Salmonella* spp in 25 g rather than 1 g in comparison to bile-tolerant gram-negative *Escherichia coli, Staphylococcus aureus,* and *Pseudomonas aeruginosa* because a higher sample size is associated with higher power or a higher probability for detecting the microbial load in a sample. *Salmonella* is specific pathogen and can cause serious infection in humans; thus, it is important to confirm absence of this microbe even from raw materials because it is capable of withstanding processing or treatment.

Four major factors may have affected the variety of microbes contained in the samples: environment, sample collection and handling, sample storage, and the moisture level of the sample. In this study, the growth of microbes in the raw material was reduced by maintaining the moisture level below 10%.

Table 6: Detection of microbial limits in the powdered leaves

Microbial limits	cfu/g
Total bacteria count	8.2 x 10 ^{5}
Total yeast and mold count	1.4 x 10 ^{3}
Bile-tolerant Gram-negative	Absent in 1 g
Salmonella spp.	Absent in 25 g
Escherichia coli	Absent in 1 g
Staphylococcus aureus	Absent in 1 g
Pseudomonas aeruginosa	Absent in 1 g

5. Conclusion

This study evaluated the pharmacognostical, physicochemical, and phytochemical properties of *M. speciosa* leaves. Although the leaves have highly medicinal qualities, illegal drug abuse and addiction have caused many countries to ban the leaves and classify them as poisonous or psychotropic substances.

Acceptable physicochemical parameters and the safety parameters of heavy metal elements and microbial levels are currently determined using pharmacopoeias, but available pharmacopoeias lack data on *M. speciosa*. There are currently no proper pharmacognostical, physicochemical, or photochemical data available as references for drug manufacturers to meet pharmacy laws and regulations. The need is now even greater because the active alkaloid mitragynine is being isolated and marketed as a raw drug. To resolve this problem, *M. speciosa* leaves were analysed in the present study, and these parameters were recorded.

The methodology developed in the present study will help detect mitragynine and identify *M. speciosa*. In addition, law enforcement agencies may employ it to quickly identify suspected material. The present findings will aid in developing pharmacopoeias that might be used for identification of misuse of the leaves and that may assist regulatory authorities in identify the plant. The present findings may also help to standardize drug development and facilitate the quality control of *M. speciosa* in nutraceutical products or botanical drugs.

The present findings may be further improved with more studies. In collaboration with other researchers, in-house studies are presently under way using transmission electron microscopy and scanning electron microscopy, which may reveal more details of the anatomy and structure of *M. speciosa* leaves. Further research may include profiling with infrared spectroscopy, differential scanning calorimetry, and thermogravimetric analysis.

By defining the characteristics of *M. speciosa*, this study will assist other researchers, the pharmaceutical and nutraceutical industries, law enforcement, and government agencies in identifying kratom, formulating industry standards, and preventing adulteration of products.

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