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Sequential Extraction and Phytochemical Study of the Extracts of *Mitragyna speciosa*

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Mitragyna speciosa, commonly known as kratom, is traditionally used for its analgesic and mood-enhancing effects. However, concerns about its toxicity, particularly from the presence of alkaloids like mitragynine, highlight the need for safe extraction methods. This study aimed to develop a sequential extraction approach to isolate bioactive compounds while mitigating the material's toxicity. *M. speciosa* leaves were sequentially extracted using hexane, ethyl acetate, and methanol, and the resulting extracts were analyzed using Thin Layer Chromatography (TLC), Fourier Transform Infrared Spectroscopy (FTIR), and Gas Chromatography-Mass Spectrometry (GC-MS). TLC analysis indicated the presence of distinct compounds with Rf values of 0.76 and 0.50 for ethyl acetate and methanol extracts, aligning with the literature for squalene and mitragynine, respectively. FTIR confirmed the presence of functional groups such as N-H and C=O, while GC-MS identified mitragynine in the methanol extract at a 93% match, consistent with reported values. This study demonstrates the effectiveness of solvent polarity in selectively extracting bioactive compounds and provides a foundation for further quantitative and bioactivity assessments to validate the therapeutic potential of *M. speciosa*.

Keywords: *Mitragyna speciosa*; chemical compounds; spectroscopy analysis; TLC; FTIR; GC-MS

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Mitragyna speciosa, commonly known as kratom or ketum, is a tropical medicinal plant belonging to the Rubiaceae family, prevalent in Southeast Asia. Known by various names in different regions, *M. speciosa* has historical usage for both medicinal and recreational purposes. More than 25 alkaloids have been identified in the plant, with mitragynine and 7-hydroxymitragynine being the primary active compounds responsible for their morphine-like effects [1]. Additionally, other compounds have also been identified in the leaves part of *M. speciosa*, such as mitragynine, speciogynine, speciociliatine, and mitracilianite [2]. In general, the alkaloid content in *M. speciosa* leaves varies between 0.5% and 1.5%, depending on geographical location, environmental factors, and plant maturity [3].

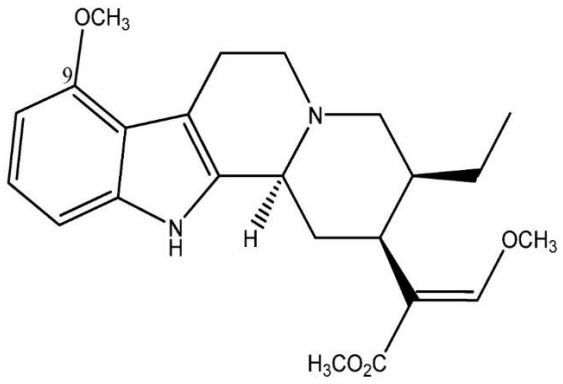

Figure 1. Chemical structure of mitragynine [4].

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Mitragynine as shown in Figure 1 is the most prevalent alkaloid in *M. speciosa*, acts on human μ-opioid and ƙ-opioid receptors by producing effects such as pleasure, pain relief, and energy boosting. In comparison, 7-hydroxymitragynine is significantly more potent than both mitragynine and morphine [5]. Lower doses of *M. speciosa* have been determined to induce stimulant effects, while higher doses can produce opiate-like effects. Long-term consumption of the plant can lead to constipation, dehydration, and weight loss. Meanwhile, acute toxicity symptoms as a result of ingesting *M. speciosa* include abdominal pain, jaundice, and, in severe cases, fatal outcomes [6].

Sequential extraction is a critical analytical method for isolating plant alkaloids with high purity. It involves using solvents with high polarity to extract different classes of compounds. Non-polar solvents extract fats and waxes, intermediate polar solvents extract flavonoids, whereas highly polar solvents like methanol extract alkaloids [7]. According to a previous study, sequential extraction using methanol yields a higher concentration of mitragynine, making it suitable for detoxification processes [8].

Thin Layer Chromatography (TLC) is a technique used to identify and quantify compounds in plant extracts. To perform TLC, a small amount of the extract is applied to a silica gel-coated plate, which is then placed in a solvent. The solvent moves up the plate by capillary action, separating the compounds into distinct spots. This process allows TLC to identify and separate various phytochemicals within the extracts. Next, the extract is placed on the TLC plate and developed in a suitable solvent, and the resulting spots are visualized using UV light or a reagent. The distance the compounds move (Rf values) is compared with known standards to confirm their presence and concentration.

Two (2) spectroscopy analyses can be employed to detect chemical fingerprints in the extracts: Fourier Transform Infrared (FT-IR) and Gas Chromatography-Mass Spectrometry (GC-MS). FT-IR spectroscopy identifies the extract's functional groups and chemical bonds. It offers advantages such as simultaneous wavelength observation, higher signal-to-noise ratio, and better throughput compared to dispersive spectrometers [9]. The characterization is performed by exposing the organic substance to infrared light, where the absorbed frequencies indicate the molecule's structure. The resulting spectrum is then used to identify functional groups and bond types [10].

GC–MS combines gas chromatography's separation capabilities with mass spectrometry's identification power, which distinguishes compounds based on their mass-to-charge ratios. The method is highly effective for analyzing volatile and semi-volatile compounds in plant extracts [11]. GC-MS involves using a carrier gas to move the sample through a column, separating components based on their solubility in gas and liquid phases. The mass spectrometer then identifies and quantifies the compounds to provide detailed chemical findings [12].

EXPERIMENTAL

Plant Sample Preparation

5.0 kg of fresh leaves of *M. speciosa* (voucher no. PIIUM 0358) were thoroughly washed to remove any debris and dirt. Then, the clean leaves were oven-dried at a temperature between 35 - 40°C for a week to eliminate moisture. Once dried, the leaves were ground using a Pulverisette 25 Cutting Mill and stored in airtight containers.

Sequential Extraction Method

1.0 kg of finely ground and dried *M. speciosa* leaf powder was sequentially macerated three times at room temperature using 1.8 L of solvents such as hexane, ethyl acetate, and methanol for 48 hours. The extracts were individually concentrated using the Buchi R-215 Rotavapor System to obtain crude extracts.

Thin Layer Chromatography (TLC)

TLC analysis was used to identify and separate various phytochemicals within the extracts. The silica gel plates were activated in an oven to remove moisture, cut to dimensions of 3 cm x 6.5 cm, and marked with lines. The mobile phase consisting of various ratios of ethyl acetate and methanol (50:50, 70:30, 80:20, 90:10 and 100:0) were prepared. 10 mg of each extract were diluted with 2 mL of their respective solvents. The dissolved extract samples were spotted onto the TLC plates using capillary tubes and labeled accordingly. The plates were positioned vertically in a sealed chamber containing the mobile phase. After the solvent reached the top of the TLC plates, the plates were removed, air-dried, and visualized under UV light at 254 nm. The observed spots were circled, and their Rf values were calculated based on formula (1).

$$Rf = \frac{Distance\ traveled\ by\ solute}{Distance\ traveled\ by\ solvent} \quad (1)$$

Fourier Transform Infrared (FT-IR) Spectroscopy

Before the analysis was performed, the plates were thoroughly cleaned with distilled water and ethanol to remove any impurities. Background data was collected to ensure the software registered a zero reading. Each extract (10 mg) was deposited onto the sample detector of the Attenuated Total Reflectance FTIR (ATR-FTIR) Perkin-Elmer SPECTRUM 100 FTIR. The sample was scanned from 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1}. The plate was cleaned before starting the analysis of a new sample to ensure

no cross-contamination occurred. The obtained IR spectra were analyzed to identify functional groups, chemical bonds, and molecular structures based on the peak positions.

Gas Chromatography-Mass Spectrometry (GC–MS)

GC analysis was conducted using Clarus™ 680/GC SQ8T/MS equipped with Elite-5MS capillary column (30 m x 0.25 mm x 1.4 µm). The oven temperature was programmed to start at 50°C, then ramped at 20°C/ min up to 110°C. This was followed by a ramp at 10°C/ min to 280°C, where it was held isothermally for 10 minutes, with a total run time of 30 minutes. The temperature of the injector was set to 220°C in the splitless mode. Extracts were diluted with a volatile solvent at a ratio of 1:100 and filtered. 1 µL extract was injected, and the column flow was set at 1.0 mL/ min. The carrier gas used was helium. The MS was operated in electron impact mode (70eV) and scanned from 50 to 500 m/z at one scan per second with a source temperature of 250°C and a quadrupole at 150°C. The data was analyzed using software, and compound mass spectra with >90% match were identified by comparing their spectra to those of the libraries of the NIST installed in the GCMS and mass spectra from literature.

RESULTS AND DISCUSSION

Sequential Extraction Method

The extraction process involved the sequential use of solvents with varying polarities: hexane, ethyl acetate, and methanol. Hexane, a non-polar solvent, was used to extract non-polar compounds such as chlorophyll. This initial hexane extract displayed an intense dark green color that indicate a high chlorophyll content. Subsequent hexane extracts, along with extracts from ethyl acetate and methanol, exhibited an intense dark yellow color, which suggests a significant reduction in chlorophyll content.

The average mass percentages and percentage yields of the crude extracts were as follows: hexane yielded 2.436 g (0.27%), ethyl acetate yielded 16.052 g (1.78%), and methanol yielded 40.692 g (4.52%). The yield differences indicate the efficiency of each solvent in isolating compounds based on their polarity. From the analysis, methanol extract provided the highest yield due to its ability to extract a broader range of polar compounds. Despite the systematic extraction approach, the overall percentage yield from the maceration process was relatively low. This can be attributed to several factors, including frequent transfers of solvents between beakers, which may have led to loss of material, improper handling during the filtration process, and insufficient maceration time [13].

Even though maceration is often favored for its simplicity and cost-effectiveness [14], these results highlight its limitations in terms of efficiency. The low yield observed in this study aligns with other reports that have noted the inefficiency of maceration as an extraction method. To improve the extraction yield, alternative methods such as Soxhlet extraction, ultrasound-assisted extraction, or supercritical fluid extraction could be considered. These methods may provide better solvent penetration and compound recovery, thereby increasing the overall efficiency of the extraction process.

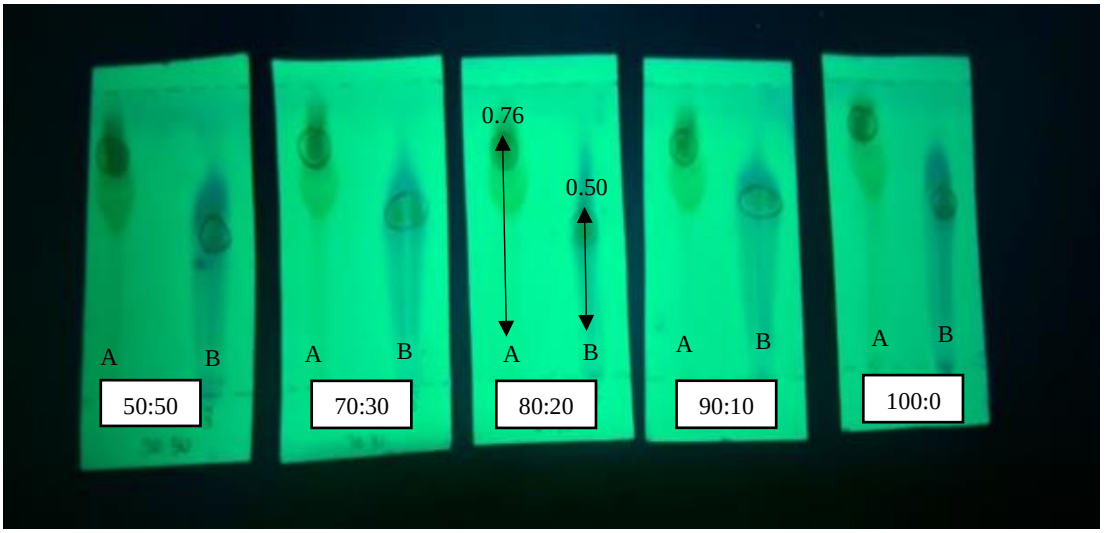

Figure 2. TLC chromatogram under UV short wave (A = ethyl acetate extract and B = methanol).

Thin Layer Chromatography (TLC)

TLC is a widely used preliminary technique for separating compounds in complex mixtures. Although the approach provides an initial insight into the separation of major compounds, it is not considered a confirmatory test due to its inability to specifically identify individual compounds. The current study utilized different ratios of ethyl acetate to methanol (50:50, 70:30, 80:20, 90:10, and 100:0) to determine the most effective solvent system for the separation of major compounds in both ethyl acetate and methanol extracts of *M. speciosa*.

As depicted in Figure 2, the solvent ratio of 80:20 exhibited the most distinct separation of compounds in both extracts. The successful separation observed suggests that the maceration process effectively isolated the major compounds present in the extracts. This observation aligns with the findings by [13], whom also reported that an 80:20 ratio is ideal for analyzing complex mixtures. This particular ratio was selected for its superior separation capabilities, especially for compounds with varying polarities.

The TLC analysis was performed for both ethyl acetate and methanol extracts, but the hexane extract was excluded due to its non-polar nature, which limits the effective separation of compounds on a polar TLC plate. The observed Rf values of 0.76 cm for ethyl acetate and 0.50 cm for methanol indicate distinct separation patterns, suggesting the presence of different compounds in each extract. Mitragynine, a known alkaloid in *M. speciosa*, has been documented to exhibit an Rf range of 0.45 – 0.55 cm in methanol-based TLC systems [7]. This means that the result obtained from this study aligned with the Rf value of 0.50 observed in the methanol extract. The similarity suggests that the compound observed in the methanol extract may be mitragynine or a structurally similar alkaloid.

Squalene is a non-polar compound that typically exhibits Rf values between 0.70 and 0.80 in ethyl acetate systems [7]. The Rf value of 0.76 recorded for the ethyl acetate extract aligns with this range, indicating the possible presence of squalene or other non-polar phytochemicals in the ethyl acetate fraction. Moreover, the Rf values obtained in this study are consistent with those reported in previous research, which helps validate the presence of specific compounds in the *M. speciosa* extracts. The Rf value of the methanol extract at 0.50 closely matches literature values for mitragynine, suggesting that the sequential extraction with methanol effectively isolated this polar alkaloid. Similarly, the Rf value observed in the ethyl acetate extract supports the potential presence of non-polar compounds like squalene, consistent with the extraction profile expected from an intermediate-polarity solvent like ethyl acetate.

The lower Rf value of the methanol extract indicates the presence of more polar compounds. Polar compounds such as mitragynine interact more strongly with the silica gel and are able to bind more effectively to it, which slows their migration and results in shorter distances traveled on the TLC plate. In short, the TLC result indicated that methanol extract has higher polar compounds than ethyl acetate extract. Although TLC is primarily a qualitative analysis, these Rf values provide a semi-quantitative insight into the polarity differences in the major compounds extracted by each solvent.

Fourier Transform Infrared (FT-IR) Spectroscopy

FTIR spectroscopy was employed to identify the functional groups in the active compounds of *M. speciosa* leaf extracts and determine the types of chemical bonds based on their physical and chemical structures. The FTIR spectrum of the hexane extract exhibited several characteristic absorption peaks (Table 1). Specifically, a broad absorption peak at 3405.64 cm^{-1} can be attributed to N-H stretching. Upon closer examination, this peak appears consistent with secondary amines since it does not possess additional sharp signals typically associated with primary amines. This peak, therefore, likely corresponds to a secondary amine structure commonly found in alkaloids, which aligns with the phytochemical profile of *M. speciosa*.

Table 1. Functional groups in the hexane extract of *M. speciose.*

Wavenumber (cm^{-1})	Functional Group
3405.64	N-H
2953.80	sp^3 C-H (stretching)
2917.20	sp^3 C-H (stretching)
2849.21	sp^2 C-H (stretching)
1734.30	C=O
1711.76	C=O
1461.84	C-H (bending)
1376.03	C-N

In addition, peaks at 2953.80 cm^{-1} and 2917.20 cm^{-1} correspond to sp^3 C-H stretching vibrations, while the peak at 2849.21 cm^{-1} is attributed to sp^2 C-H stretching. Notably, the strong absorption bands at 1734.30 cm^{-1} and 1711.76 cm^{-1} are indicative of C=O stretching, suggesting the presence of carbonyl groups that are likely from aromatic esters. The absorption at 1461.84 cm^{-1} is consistent with C-H bending. Meanwhile, the peak at 1376.03 cm^{-1} corresponds to C-N stretching and indicates the presence of amines or alkyl groups in the extract.

The ethyl acetate extract displayed distinct absorption peaks (see Table 2). The peak at 3395.80 cm^{-1} is attributed to N-H stretching of the secondary amines functional group, while peaks at 2925.67 cm^{-1} and 2856.00 cm^{-1} correspond to sp^3 and sp^2 C-H stretching, respectively. The strong absorption at 1707.47 cm^{-1} suggests C=O stretching in carbonyl-containing functional groups. Given the additional presence of C-O stretching around 1243.56 cm^{-1}, these peaks can be associated with aromatic esters rather than carboxylic acids. The absence of an O-H stretching peak between 3200 and 3600 cm^{-1} further supports the statement, as it rules out carboxylic acid functionality in these extracts. Meanwhile, the absorption peak at 1441.07 cm^{-1} is attributed to C-H bending. The peak at 1374.42 cm^{-1} indicates C-N stretching, suggesting the presence of amines or alkyl groups in the extract.

The FTIR results of the methanol extract in Table 3 revealed an absorption peak at 3363.67 cm^{-1} that corresponded to N-H stretching, suggesting the presence of secondary amine groups. Peaks at 2922.54 cm^{-1} and 2852.34 cm^{-1} correspond to sp^3 and sp^2 C-H stretching, respectively. Other than that, the absorption peaks at 1691.31 cm^{-1} and 1625.42 cm^{-1} are indicative of C=O stretching, while the peak at 1452.67 cm^{-1} corresponds to C-H bending. Additionally, C-N and C-O stretching were observed at 1375.74 cm^{-1} and 1251.21 $cm^{-1,}$ respectively, further confirming the presence of aromatic esters in the extract.

While certain functional groups were consistently identified across all extracts, variations in peak intensity and presence of specific groups were observed, as shown in the spectra of Appendix 1. These results align with previous studies by [15], which also identified similar functional groups in their research. The observed variations demonstrate the influence of solvent polarity on the extraction of specific phytochemicals, an aspect that further highlights the selective affinity of different solvents for various compounds within *M. speciosa* leaves.

An important consideration in this study is the presence of mitragynine, a major active alkaloid in *M. speciosa*, which contains a pyrrole ring in its chemical structure. The pyrrole ring is an aromatic amine heterocyclic compound recognized for its role in stimulating opioid receptors one of the pharmacological effects associated with *M. speciosa* [2]. The N-H stretching peaks observed in the FTIR spectra for each extract suggest the presence of amine groups. However, only the methanol extract showed mitragynine, as confirmed by GC-MS analysis with a retention time of 20.71 minutes. This supports the selective extraction of mitragynine using polar solvents like methanol. A semi-quantitative comparison of peak heights further underscores methanol's efficacy in extracting amine-containing compounds from *M. speciosa* leaves as compared to ethyl acetate.

Table 2. Functional groups in ethyl acetate extract of *M. speciose.*

Wavenumber (cm^{-1})	Functional Group
3395.80	N-H
2925.67	sp^3 C-H (stretching)
2856.00	sp^2 C-H (stretching)
1707.47	C=O
1441.07	C-H (bending)
1374.42	C-N
1243.56	C-O (aromatic ester)

Table 3. Functional groups in methanol extract of *M. speciose.*

Wavenumber (cm^{-1})	Functional Group
3363.67	N-H
2922.54	sp^3 C-H (stretching)
2852.34	sp^2 C-H (stretching)
1691.31	C=O
1625.42	C=O
1452.67	C-H (bending)
1375.74	C-N
1251.21	C-O (aromatic ester)

Table 4. Isolated compounds in hexane, ethyl acetate, and methanol extracts of *M. speciose.*

Type of Extract	Compounds	Retention Time (RT)	Quality (%)
Hexane	26-Methyl-octacosanoic acid, pyrrolidide	20.93	95
	Squalene	21.46	94
	Diethylmalonic acid, 4-chlorobenzyl pentadecyl ester	13.47	89
	3, 7, 11, 15-Tetramethyl-2-hexadecen-1-ol	13.04	85
	n-Hexadecanoic acid	14.27	82
Ethyl Acetate	Squalene	24.72	94
	n-Hexadecanoic acid	17.44	93
	Bicyclo[10.1.0]tridec-1-ene	19.17	91
	Vitamin E	27.30	76
	Bicyclo[2.2.1]heptane, 2-methyl-, exo-	19.11	64
Methanol	Mitragynine	20.71	93
	Ethanol, 2,2'-oxybis-	4.85	83
	1,2,3-Propanetriol, monoacetate	6.77	56
	Myo-Inositol, 2-C-methyl-	14.8	53
	3,4,6-Tri-O-methyl-d-glucose	15.54	53

Gas Chromatography-Mass Spectrometry (GC–MS)

GC analysis proved to be effective in detecting polar compounds within the plant extracts. The primary function of GC is to separate compounds based on their retention time within the chromatographic column and allow for a clear differentiation of components. The Mass Spectrometry (MS) component complements this approach by identifying the various compounds present and providing the corresponding data on their relative molecular mass and structure.

As presented in Table 4, five major compounds isolated from *Mitragyna speciosa* leaves were identified in each of the hexane, ethyl acetate, and methanol extracts. Notably, according to Appendix 2, the highest intensity peak observed in the GC-MS spectrum for the hexane extract was squalene, with a retention time of 21.46 minutes and a match quality of 94%. For the ethyl acetate extract, the two most prominent peaks corresponded to squalene (retention time 24.72 minutes, 93% match) and *n*-hexadecanoic acid (retention time 17.44 minutes, 94% match). In contrast, the methanol extract's GC-MS spectrum was dominated by 3,4,6-Tri-O-methyl-d-glucose, detected at a retention time of 15.54 minutes with a match quality of 53%.

The overall quality of the GC-MS spectra was high, with well-resolved peaks that indicate a successful separation process due to the suitability of the maceration method employed. A notable finding was the detection of mitragynine at a retention time of 20.71 minutes in the methanol extract, with a 93% match, according to the library database. Interestingly, mitragynine was not detected in the hexane and ethyl acetate extracts, possibly because of the solvents' polarity.

These results showed the successful isolation of major compounds from *M. speciosa* leaves, with the solvent polarity playing a crucial role in the efficiency of the extraction process. The findings also highlight the potential for selective extraction of bioactive compounds based on solvent choice, which can be critical for optimizing the yield of desired phytochemicals in future studies.

A comparative analysis between FTIR and GC-MS data revealed consistent identification of several compounds. For instance, the presence of mitragynine in the methanol extract was corroborated by both the N-H peak in FTIR (3363.67 cm^{-1}) and the GC-MS peak at a retention time of 20.71 minutes. This dual confirmation strengthens the identification of mitragynine as a major compound, particularly in the methanol extract, which is in line with its known solubility in polar solvents.

Mitragynine was detected at a retention time of 20.71 minutes in the methanol extract, consistent with findings in previous studies. For instance, [16] reported mitragynine as a major alkaloid in methanol extracts of *M. speciosa*, with considerable variability in concentration based on extraction methods and plant sources. Although specific quantification was not performed in this study, the presence of mitragynine in the methanol extract corroborates literature findings that suggest methanol is an effective solvent for isolating alkaloids from *M. speciosa*.

Other identified compounds, such as squalene and *n*-hexadecanoic acid, are similarly consistent with prior research. [17] reported the presence of squalene in non-polar solvent extracts like hexane, highlighting

its affinity for less polar solvents. This supports the selectivity of the sequential extraction process in isolating compounds based on solvent polarity, with hexane favoring non-polar compounds and methanol targeting polar alkaloids like mitragynine.

The presence of mitragynine and other bioactive compounds in the methanol extract suggests potential therapeutic applications of *M. speciosa*, particularly for analgesic and anti-inflammatory uses. Further in vitro and in vivo studies are recommended to validate the bioactivity of these extracts and confirm their efficacy and safety. These studies will be crucial for establishing the medicinal potential of *M. speciosa* and its applicability in therapeutic settings.

CONCLUSION

This study successfully utilized sequential extraction using hexane, ethyl acetate, and methanol to isolate and analyze phytochemicals from *Mitragyna speciosa* leaves. In addition, the results identified key compounds like mitragynine, squalene, and various esters through the analysis of TLC, FTIR, and GC-MS. The findings align with the values of the literature, especially for the methanol extract, where mitragynine was confirmed as the primary alkaloid. This discovery is critical as it supports the selective affinity of polar solvents for bioactive compounds. While the study provides foundational insights into the chemical profile of *M. speciosa* and highlights its therapeutic potential, future research is recommended to include quantitative analyses and in-depth bioactivity studies to understand its medicinal applications and optimize extraction efficiency.

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