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Phytochemical Screening and FTIR Analysis for Fractions of Ethanol Extract from *Mitragyna speciosa* leaves

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Abstract

Introduction: *Mitragyna speciosa*, also referred to as kratom, has long been utilised for its stimulant and analgesic effects, which are mostly due to its high alkaloid content. Most of the study of *M. speciosa* utilised methanol as the solvent of extraction. However, ethanol is a more environmentally friendly and nontoxic solvent that can also be used for the extraction process. The purpose of this study was to use ethanol to extract phytochemicals from *M. speciosa* leaves and separate the bioactive compounds by using Vacuum Liquid Chromatography (VLC). **Methods:** *M. speciosa* leaves underwent ethanol maceration followed by phytochemical screening with Dragendorffs, Shinoda, Salkowski, $FeCl_3$, foam tests and VLC fractionation. The resulting five fractions were analysed by TLC and screened for alkaloids, phenolic, unsaturated compounds and flavonoids along with analysation of the functional groups by using the Fourier Transform Infrared Spectroscopy (FTIR). **Results:** Screening of the crude ethanol extract exhibits positive reactions for alkaloids, flavonoids, terpenoids, phenolics, tannins and saponin. Meanwhile, the TLC analysis of the fractions using solvent system n-hexane: ethyl acetate (30:20) shows presence of alkaloid compound with Rf value of 0.80 for fraction 3 and 0.83 for fraction 2,4 and 5 which aligns with the literature for mitragynine. FTIR results exhibit presence of important peaks of mitragynine such as C=O, N-H and C-H for the fractions while C=C, can only be found in fraction 3. **Conclusion:** This study supports the use of ethanol as solvent of extraction and proved that VLC is highly efficient in its ability to fractionate the ethanol extract of *M. speciosa.*

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Introduction

Mitragyna speciosa commonly known as Kratom or Ketum is a tropical tree that can be found in Southeast Asia and is a member of the Rubiaceae family (**Fig.1**). It is widely used in countries such as Thailand, Indonesia, Papua New Guinea and Malaysia by the communities. Acording to Ramanathan and McCurdy (2020), *M. speciosa* is currently used as recreational drugs commonly in the Western countries to reduce the usage of opioid drugs and as a method for mood enhancement. This is due to the increasing research attention on kratom's alkaloid along with phenolic acids and flavonoids, particularly mitragynine and 7-hydroxymitragynine due to their potential use in treating mental health conditions like anxiety and depression, managing pain and treating opiate addiction.

The species used in this study is *M. speciosa* leaves with red colour veins which originate from Kedah, Malaysia. The traditional ways of using the *M. speciosa* is by chewing, smoking or drunken as a tea. According to Sokup and Pippin (2025), it is traditionally used as a stimulant and sedative due to its physiologic effect being like opioids. Besides, it is also commonly used in Southeast Asia to increase the stamina of outdoor labourer for their daily works (Ramanathan & McCurdy, 2020).

The psychoactive effects exhibited by *M. speciosa* is due to the active compounds mitragynine(**1**) and 7-hydroxymitragynine **(2)** found in the leaf of the plant. Despite the fact that over forty (40) of compounds have been discovered in *M. speciosa* so far, only four (4) of them are known to exhibit pharmacological activity which are corynantheidine, mitragynine, 7-hydroxymitragynine (7-OH-mitragynine) and speciociliatine (**3**) (Eastlack et al., 2020). Mitragynine which is an indole alkaloid (Karunakaran et al., 2022) acts on the brain's μ-, δ- and κ-opioid receptors to produce effects similar to those of opium (Annuar et al., 2024). Mitragynine may also be hepatically metabolised to 7-hydroxymitragynine which has forty-six (46) times the affinity for opioid receptors compared to mitragynine. This is proven by Masriani et al. (2023) where it is mentioned that secondary metabolites found in kratom leaves have been reported to be responsible for its pharmacological actions.

Despite its traditional and modern uses, comprehensive phytochemical profiling of *M. speciosa* is still limited. *M. speciosa* extracts with varying chemical compositions and potencies are usually due to variations in the plant's origin, extraction processes and preparation techniques. The extraction process, especially the solvent of choice for extraction, plays a very important role in derivation of the phytochemical compounds from the leaves. Most of the current research focuses on methanolic or aqueous extractions, however ethanol extraction is still not well studied for its effectiveness and selectivity in extracting important phytochemicals. Thus, this study seeks to fill these gaps by performing ethanol extraction, phytochemical screening,VLC fractionation and Fourier Transform Infrared Spectroscopy (FTIR) analysis of *M. speciosa* leaf fractions.

Fig. 1: *Mitragyna speciosa* Plant (a) Leaves, (b) Fruit, (c) Tree, (d) Plantation (Hassan et al., 2013)

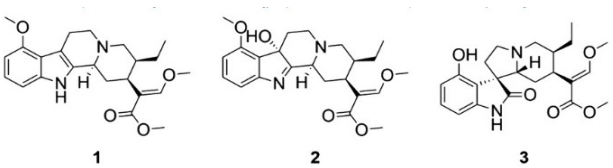

Fig. 2: Indole Alkaloid Found in *M.speciosa* mitragynine (1), 7-hydroxymitragynine (2) and speciofoline (3) (Todd et al., 2020)

Materials and methods

Materials

Fresh leaves of *M. speciosa*, HmbG Chemicals Ethanol Absolute, TLC plate, Dragendorff's reagent, Magnesium powder, Hydrochloric acid, Fischer Scientific UK Dichloromethane, concentrated sulphuric acid, $FeCl_3$, KIESELGEL silica gel 60 PF254, Whatman No. 1 filter paper, HmbG Chemicals n-Hexane, HmbG Chemicals Ethyl Acetate, R&M Chemicals Methanol and distilled water.

Method

Plant leaves preparation

1.0 kg of dried leaves of *M. speciosa* (voucher no. PIIUM 0358) obtained from Dr Siti Hadijah Binti Shamsudin was thoroughly cleaned to remove any debris and dirt. Then, the clean leaves were oven-dried at a 40 °C for 48 hours to eliminate moisture. Once dried, the leaves were grounded using the Cutting Mill Machine (FRITSCH PULVERISETTE 19).

Ethanol extraction of M. speciosa

Finely ground and dried *M. speciosa* leaf powder was put inside a 5 L conical flask for the maceration process. 832 g of the powdered leaves were macerated with a total of 3 L HmbG Chemicals Ethanol Absolute for 5 days at room temperature, followed by filtration using Whatman No. 1 filter paper to obtain the liquid ethanol extract. The *M. speciosa* powders were then reintroduced to another 1.5 L of the HmbG Chemicals Ethanol Absolute and macerated for another 2 days and filtered. The filtered ethanol extract was subjected to Rotavapor system at 40℃ to produce a concentrated thick extract. The final weight of the ethanol extract was recorded, and the percentage yield of the ethanol extract was calculated using the formula 1 (Masriani et al., 2023).

$$\% \text{ yield} = \frac{(weight\ of\ extract)}{(\text{weight of powder})} \times 100 \quad (\mathbf{1})$$

Phytochemical screening of the ethanol extract of M. Speciosa

Preparation of solution

3g of ethanol extract of *M. speciosa* was diluted in 150 mL of 75% ethanol in a beaker by using a water bath at 40℃ to produce ethanol extract solution with concentration of 40 mg/mL. 1 mL of the solution was then subjected to each qualitative phytochemical test. Each qualitative phytochemical screening was done in triplicates (n=3) to ensure reliability of the colour changes.

Alkaloid Screening by Dragendorff's reagent

1 mL of the previous solution was put into a 5mL test tube. 2 drops of reagent were added to the test tube and changes were observed and recorded. Presence of alkaloids can be confirmed with the presence of brick-red precipitate (Devmuravi, 2010).

Flavonoid Screening by Shinoda reagent

1 mL of the prepared solution was put into a 5 mL test tube along with 2 drops of concentrated 12M HCl. Then, a pinch of magnesium chips was added to the mixture and colour change was observed and recorded (Masriani et al., 2023).

Terpenoid Screening by Salkowski reagent

2 mL of diethyl chloromethane and 3 drops of concentrated H_2SO_4 were added into a test tube containing 1 mL of prepared solution. Positive reactions are confirmed upon formation of brownish-red colour interface indicating the presence of triterpenoids (Masriani et al., 2023).

Phenolic Screening

1% of $FeCl_3$ were added into a test tube containing 1 mL of prepared solution. Formation of blackish green to solid black precipitate confirms the presence of phenol in the extract (Masriani et al., 2023).

Saponin Screening

2 mL of aquadest was added into a test tube containing the prepared solution. The test tube was shaken vigorously for 10 minutes until there was formation of foams. The presence of saponins is indicated when there is formation of stable foam with a height of 1-10 cm (Masriani et al., 2023).

Tannin Screening

The prepared solution of ethanol extract of *M. speciosa* was dissolved in aquadest and heated using water bath at 40℃. Then, 1 mL of 1% $FeCl_3$ solution was added into the test tube. The presence of tannins can be confirmed with the presence of a brownish-green or bluish-green color precipitate (Masriani et al., 2023).

Fractionation of ethanol extract of M.speciosa

12.1864 g of activated silica gel 60 (0.2-0.5 mm) for column chromatography (Merck KGaA, Germany) were added to 9.2415 g crude ethanol extract of *M. speciosa* producing 19.4683 g dried sample. The KIESELGEL silica gel 60 PF_{254} was packed into a sintered glass funnel until it reached an even distribution of 7 cm height. The vacuum was applied using a vacuum pump until it was packed to 5 cm height. The prepared sample of ethanol extract of *M. speciosa* were loaded into the vacuum liquid chromatography with filter paper Whatman as separator to the silica column. Gradient elution was carried out using increasing polarity of binary mixtures of n-Hexane and Ethyl Acetate (EtOAc). The fractionation ratio includes n-Hex: EtOAc (70:30) (Fraction 1), n-Hex: EtOAc (50:50) (Fraction 2), n-Hex: EtOAc (30:70) (Fraction 3), n-Hex: EtOAc (20:80) (Fraction 4) and 100% EtOAc (Fraction 5). The samples were eluted according to its polarity. A total of 5 fractions of 800 mL each were collected and evaporated by using rotary evaporator BUCHI with the temperature according to the solvent temperature limit.

Thin Layer Chromatography (TLC) analysis and visualisation

A small number of fractions was applied to a silica gel plate by using a capillary tube, then inserted into a developing chamber with a suitable solvent system. The TLC was left to develop until it reached the 1 cm line from the top of the TLC plate. The developed plates were observed under short wave UV (254nm), long wave UV (365nm) and visualised by spraying Ferric Chloride ($FeCl_3$), Dragendorff, Vanillin and developed in iodine gas chamber. All observed spots were measured and Rf values for each spot were recorded.

Fourier Transform Infrared Spectroscopy (FTIR) analysis on the selected fractions

Fractions 2, 3, 4 and 5 were selected for FTIR analysis due to the strong visualisation of alkaloid upon visualisation with Dragendorffs reagent. The FTIR (PerkinElmer) was cleaned with ethanol before the sample was put on it. FTIR spectra of each sample were obtained within the range of 4000 - 400 cm^{-1}. The wave numbers obtained for each fraction were recorded and analysed. The FTIR spectroscopic analyses for each fraction were performed in triplicate (n=3) to ensure the reliability and reproducibility of the result.

Results and discussion

Percentage yield of ethanol extract of M. speciosa

The weight of *M. speciosa* powder used is 832 g which produced extract of 129.13g. The percentage yield of *M. speciosa* extraction by using ethanol is 15.52%. The percentage yield obtained is within a high range. The percentage yield obtained by using maceration technique is acceptable as it has higher yield when compared with literature, which yields 9.3% of the extract (Tuntiyasawasdikul et al., 2024). The crude ethanolic extract yield of 15.5% achieved in this study can be validated as a highly efficient return as it substantially exceeds the yield of 4.44% reported in studies by (Phoonket et al., 2025) and 12.6% yield reported by (Prasetya et al., 2026). The high percentage yield obtained shows that ethanol which were used during the extraction process is effective in extracting the compounds in the leaves of *M. speciosa.* Besides, there may be some loss during filtration and transfer process that should

Table 1: Phytochemical Screening Results of Crude Ethanol Extract of *M. speciosa.*

Phytochemical Test	Reagents	Result	Observation
Alkaloids	Dragendorff	+++	Colour change from green to orange-brown
Flavonoids	Shinoda	++	Colour change from green to orange
Terpenoid	Salkowski	+++	Formation of reddish-brown colour at interface
Phenolic	1% $FeCl_3$	+++	Immediate change of colour to blackish green colour
Saponin	Aquadest and shaking	++	Formation of a stable 2 cm foam that stays for 5 minutes
Tannin	Heating and $FeCl_3$	+++	Immediate colour change to blue-black

Description:
(+) positive: Contains the compound being examined in low intensity of colour/precipitate/foam formation
(++) positive: Contains the compound being examined in moderate intensity of colour/precipitate/foam formation
(+++) positive: Contains the compound being examined in high intensity of colour/precipitate/foam formation

be taken into account.

$$\% \text{ yield} = \frac{(weight\ of\ extract)}{(\text{weight of powder})} \times 100$$

$$\% \text{ yield} = \frac{(129.13\text{g})}{(832\ \text{g})} \times 100$$

% yield =15.5168%

Phytochemical screening of the crude ethanol extract of M. speciosa.

After evaporation by using BUCHI Rotary Evaporator, the crude ethanol extract of *M. speciosa* was subjected to phytochemical screening. **Table 1** shows the results obtained during the phytochemical screening of the crude ethanol extract of *M. speciosa.*

Alkaloid Screening

Upon testing the crude ethanol extract of *M. speciosa* with Dragendorffs reagent, the green colour of the extract quickly turns into orange-brown colour (**Fig.3**(a)). This indicates the presence of alkaloid in the crude ethanol extract of *M. speciosa*. Dragendorff reagent is used for detecting alkaloids and other nitrogen-containing compounds (Dragendorff Reagent | Alkaloids Detection Agent | MedChemExpress, n.d.). The basic nitrogen group of alkaloids reacted with the tetraiodide ion complex present in the Dragendorff reagent to produce an ion pair effectively pairing the positively charged protonated alkaloid with the negatively charged bismuth complex. The insoluble orange-red precipitate produced indicates the presence of alkaloid in the ethanol extract of *M. speciosa*.

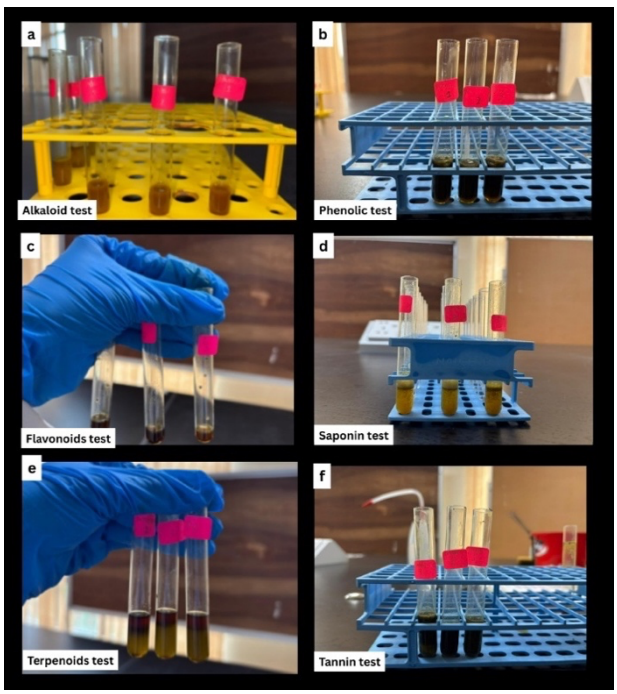

Fig. 3: Phytochemical Screening Results of Crude Ethanol of *M. speciosa* (a) Alkaloid test b) Phenolic test c) Flavonoid test d) Saponin test e) Terpenoid test f) Tannin test.

Flavonoid Screening

The crude ethanol extract of *M. speciosa* was screened for the presence of flavonoid with the Shinoda test by using concentrated HCl and Magnesium chips. The result showed that the crude ethanol extract of *M. speciosa* contained flavonoids indicated by the colour change from green to orange (**Fig.3**(b)). In the flavonoid test, the orange colour formed when magnesium metal in hydrochloric acid reduces polyhydroxy molecules from flavanones present in the sample, forming benzopyrylium salts (Masriani et al., 2023)(**Fig.4**). The orange colour changes that happened during the Shinoda test indicates that the flavonoids present in the crude ethanol extract of *M. speciosa* are flavones.

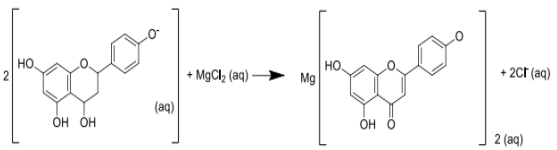

Fig. 4: Shinoda Reaction (Masriani et al., 2023)

Terpenoids Screening

The ethanol extract of *M. speciosa* was tested by using Salkowski test to detect the presence of terpenoids in the extract. The formation of reddish-brown colour at the interface confirmed the presence of terpenoids in the ethanol extract of *M. speciosa* (**Fig.3**(c)). The red colour formed in Salkowski test was due to the formation of bi-sulphonic acid of bi-cholestadiene. The concentrated H_2SO_4 used in this reaction will remove two molecules of water from two molecules of cholesterols, forming bi-cholestadiene. Simultaneously, the sulphuric acid sulfonated the bi-cholestediene at position 7,7 of the aromatic ring. Formation of bi-cholestadiene indicates the presence of cholesterols which was shown through the formation of red colour (Gupta A., 2019). The presence of terpenoids in the ethanol extract of *M. speciosa* aligns with the finding from Masriani et al. (2023).

Phenolic Screening

The result for the phenolic testing showed that the ethanol extract of *M. speciosa* contains phenolic compounds (**Fig.3**(d)). The ethanol extract of *M. speciosa* quickly turns blackish green right after 1% $FeCl_3$ was added which indicates the presence of phenolic compounds (Masriani et al., 2023)

Saponin Screening

The foam test was used to confirm the presence of saponin in the ethanol extract of *M. speciosa*. By the addition of aquadest, saponins will act as a surfactant forming a stable foam when shaken. The formation of 2 cm of stable foam during the screening of ethanol extract of *M. speciosa* confirms the presence of saponin (**Fig.3**(e)).

Tannins Screening

Tannin compounds in the ethanol extract of *M. speciosa* were identified by reacting the extract with $FeCl_3$ (Setyawati, 2020). The development of a blackish-green colour during the screening indicates the presence of tannins in the ethanol extract of *M. speciosa* (**Fig.3**(f)). This change shows that the hydroxyl group of the tannin molecule underwent an inter condensation process. It is believed that the tannins in kratom leaves have antioxidant properties (Begum et al., 2025).

All the phytochemical screening shows positive reactions which shows presence of alkaloids, flavonoids, phenolics, terpenoids, tannins and saponins in the crude ethanol extract of *M. speciosa*. This shows that ethanol effectively and successfully extracts the semi polar and polar compounds.

Qualitative phytochemical screening provides an initial indication of the presence or absence of various phytochemical groups in plant extracts. However, the results are based on color changes or precipitate formation, which may be subjective and prone to interpretation errors. The method does not quantify the concentration of phytochemicals present and may produce false-positive or false-negative results due to interference from other compounds. Therefore, the findings should be considered preliminary and confirmed using more specific and sensitive analytical such as chromatography or spectroscopy.

Fractionation of Ethanol Extract of M. speciosa

Table 2: Thin Layer Chromatography (TLC) Analysis of the *M.speciosa* fractions by using n-hexane:EtOAC (80:20)

Fraction No	Solvent System	TLC Analysis					
		Short Wavelength UV (254 nm) (Conjugated system compound	Long Wavelength UV (365 nm) (Aromatics)	Dragendorff (Alkaloids)	$FeCl_3$ (Phenolics)	Vanillin (Flavonoids)	Iodine (Unsaturated compounds)
1	n-Hexane:EtOAc (70:30)	Rf1: 0.97 Rf2: 0.94 Rf2: 0.85 Rf3: 0.77 Rf4: 0.61 Rf5: 0.56 Rf6: 0.35 Rf7: 0.3	Rf1: 0.95 Rf2: 0.89 Rf3: 0.82 Rf4: 0.71 Rf5: 0.58 Rf6: 0.54 Rf7: 0.43	-	-	Rf1: 0.99 Rf2: 0.95 Rf3: 0.81 Rf4: 0.73 Rf5: 0.69 Rf6: 0.60 Rf7: 0.55 Rf8: 0.43	Rf1: 0.99 Rf2: 0.88
2	n-Hexane:EtOAc (50:50)	Rf1: 0.85 Rf2: 0.59 Rf3: 0.55 Rf4: 0.46 Rf5: 0.40 Rf6: 0.38 Rf7: 0.23 Rf8: 0.15	Rf1: 0.83 Rf2: 0.73 Rf3: 0.44 Rf4: 0.35 Rf5: 0.28 Rf6: 0.23 Rf7: 0.18	Rf1: 0.43	-	Rf1: 0.83 Rf2: 0.73 Rf3: 0.44 Rf4: 0.35 Rf5: 0.28 Rf6: 0.23 Rf7: 0.18	Rf1: 0.73 Rf2: 0.59 Rf3: 0.50 Rf4: 0.28
3	n-Hexane:EtOAc (30:70)	Rf1: 0.96 Rf2: 0.91 Rf3: 0.82 Rf4: 0.79 Rf5: 0.62 Rf6: 0.56 Rf7: 0.49 Rf8: 0.46 Rf9: 0.30 Rf10: 0.15	Rf1: 0.96 Rf2: 0.91 Rf3: 0.76 Rf4: 0.55 Rf5: 0.40 Rf6: 0.31 Rf7: 0.14	Rf1: 0.4	-	Rf1: 0.49	Rf1: 0.35 Rf2: 0.41

Table 2: Thin Layer Chromatography (TLC) Analysis of the *M.speciosa* fractions by using n-hexane:EtOAC (80:20) (cont.)

4	**n-Hexane:EtOAc (20:80)**	Rf1: 0.88 Rf2: 0.76 Rf3: 0.73 Rf4: 0.59 Rf5: 0.46 Rf6: 0.41	Rf1: 0.41 Rf2: 0.36 Rf3: 0.18	Rf1: 0.43	-	Rf1: 0.36	Rf1: 0.31
5	EtOAc (100%)	Rf1: 0.41 Rf2: 0.36	Rf1: 0.40 Rf2: 0.36	Rf1: 0.43	-	Rf2: 0.36	Rf1: 0.36 Rf2: 0.41

All the fractions had undergone TLC analysis with n-hexane:EtOAc (80:20) being the solvent system of choice (Beng et al., 2011)(**Table 2**). The TLC were then tested with Vanillin, Dragendorff, iodine and $FeCl_3$ for the phytochemical screening. Black and fluorescence colours were detected on the plates under short UV light (254 nm) and long UV light (365nm) respectively. This indicates that all fractions contain aromatics. Fraction 2 shows the highest separation due to the highest amount of Rf observed. All fractions exhibit presence of alkaloids except for fraction 1. This was confirmed due to the presence of orange spots when the TLC plate was sprayed with Dragendorff (Masriani et al., 2023). The absence of alkaloids in fraction 1 may be due to medium polarity characteristics of alkaloids present in *M. speciosa*, which makes the alkaloid to not be eluted in the low polarity system of fraction 1 as mitragynine, which is the most dominant alkaloid in *M. speciosa*, is an intermediate polar compound (Mohd Zubri et al., 2025). According to Beng et al. (2011), the Rf value for standard mitragynine in the solvent system n-hexane:EtOAc (80:20) is 0.82. However, based on the result obtained, the Rf value for alkaloids obtained are 0.40 for fraction 3 and 0.43 for fraction 2,4 and 5. The difference in Rf value for the alkaloid in the fractions may be due to different alkaloids present in fraction 3 compared to the other fractions. This is due to the leaves of M. speciosa containing a multicomponent mixture of alkaloids (Mustafa et al., 2020). The fractions were tested using a different solvent system which is n-hexane:EtOAc (30:20), the alkaloid testing of fractions 2,4 and 5 has the Rf value of 0.83 which aligns with the mitragynine standard by Mustafa et al. (2020) which is 0.82, while fraction 3 possess the Rf value of 0.80. The Rf values for each fraction are comparable to the standard (Mustafa et al., 2020). This indicates that the alkaloid is a major compound in the fraction. Fraction 2,3,4 and 5 has similar Rf value for the alkaloid, this may suggest that the alkaloids presence in the fractions are alkaloids of similar structures. The $FeCl_3$ visualisation showed no formation of blue spots on the TLC plates. This indicates that there is no presence of phenolic groups for all the fractions as phenolic compounds are not detected. However, undetected does not always imply absence as it might signify that the amount was too little for the TLC method to detect (Azman & Mat So'ad, 2024). This may be due to the high polarity of the phenolic group which were not eluted during fractionation with n-hexane and ethyl acetate solvent. Phenolic groups in *M. speciosa*, for example, epicatechin are eluted by using $CHCl_3$/MeOH (1:1) which are polar solvent (León et al., 2009). In addition, the phenolic may not be separated during the TLC analysis due to the low polarity of the solvent system used which cause the phenolic compounds to be more attracted to the silica plate compared to the solvent. With the increasing polarity of the fractions, the flavonoids that can be separated and visualised on the TLC plate decreases. The flavonoids detected in fraction 4 and 5 have the exact same Rf value and colour which is purple. Terpenoids are detected with purple colours when sprayed with Vanillin/H_2SO_4 (Garg et al., 2024). This confirms the presence of terpenoids in the fractions.

Table 3: Functional groups in fraction 2 of *M. speciosa*

Wavenumber ($\mathbf{cm^{-1}}$)	Functional Group	Class
3383.82	N-H stretching	amines
2924.33	C-H stretching	alkane/aromatics
2854.38	C-H stretching	alkane/aromatics
1690.09	C=O stretching	Conjugated ketone/ester
1514.30	C-H bending	aromatics

Table 4: Functional groups in fraction 3 of *M. speciosa*

Wavenumber ($\mathbf{cm^{-1}}$)	Functional Group	Class
3355.76	N-H stretching	amines
2923.39	C-H stretching	alkane/aromatics
2852.17	C-H stretching	alkane/aromatics
1692.05	C=O stretching	Conjugated ketone/ester
1645.77	C=C stretching	alkene
1514.27	C-H bending	aromatics

Table 5: Functional groups in fraction 4 and 5 of *M. speciosa*

Wavenumber ($\mathbf{cm^{-1}}$)		Functional Group	Class
Fraction 4	Fraction 5		
3361.97	3358.18	N-H stretching	amines
2925.15	2925.72	C-H stretching	alkane/aromatics
1687.56	1686.85	C=O stretching	Conjugated ketone/amide
1513.14	1513.44	C-H bending	aromatics

FTIR Analysis of The Fractions

Based on their physical and chemical structures, FTIR spectroscopy was used to identify the functional groups in the active compounds of the fractions and to identify the types of chemical bonding.

Fraction 2 (**Table 3**, Appendix A1) displayed several distinct absorption peaks. A peak at 3383.82 cm^{-1}corresponds to N-H stretching available in amines class. Due to the presence of only a single peak, the amines can be defined as secondary amines. This aligns with the phytochemical profile of *M. speciosa* (Haspi et al., 2025). Besides, the two peaks at 2924.33 cm^{-1} and 2854.38 cm^{-1} are attributed to C-H stretching in either alkane or aromatic rings. Additionally, the strong sharp absorption peak at 1690.09 cm^{-1} corresponds to C=O stretching of conjugated ketone or ester. Lastly, another absorption peak can be seen at 1514.30 cm^{-1} which is consistent with C-H bending in aromatics. This further confirms the presence of aromatics in this fraction.

The FTIR result of fraction 3 (**Table 4**, Appendix A2) shows an absorption peak at 3355.76 cm^{-1} which corresponded to N-H stretching in amine functional group. Besides, the absorption peaks at both 2923.39 cm^{-1} and 2852.17 cm^{-1} are attributed to C-H stretching in either alkane or aromatics. The absorption peak at 1692.05 cm^{-1} and 1514.27 cm^{-1} indicates the C=O stretching in conjugated ketone or ester and C=H bending in aromatics respectively. The FTIR spectrum of fraction 1 and

fraction 2 are similar to one another with an additional sharp absorption peak in fraction 3 at 1645.77 cm^{-1} which may be attributed to C=C stretching in alkene.

Fraction 4 and fraction 5 (**Table 5**, Appendix A3) have the exact same functional groups in the FTIR result. The N-H stretching which belongs to amines can be seen in the absorption peak of 3361.97 cm^{-1} and 3358.18 cm^{-1} in fraction 4 and fraction 5 respectively. Besides, both the absorption peak of 2925.15 cm^{-1} in fraction 4 and 2925.71 cm^{-1} in fraction 5 corresponded to the C-H stretching in alkane or aromatic. Additionally, the peak at 1687.56 cm^{-1} of fraction 4 and 1686.85 cm^{-1} of fraction 5 are indicative of C=O stretching in conjugated ketone or amide. Lastly, both fraction 4 and fraction 5 has shown absorption peak at 1513.14 cm^{-1} and 1513.44 cm^{-1} respectively which are indicative of C-H bending. This indicates the presence of aromatic rings in the fractions.

All the functional groups that present in the fractions align with the functional groups in the main alkaloid of *M. speciosa* which is mitragynine. Aromatic C-H stretching vibrations at 2825-2925 cm^{-1} across all VLC fractions confirm the indole ring scaffold characteristic of mitragynine, supported by C-H bending of aromatic in all the fractions (Haspi et al., 2025). Secondary amine (N-H), carbonyl functional group (C=O), C=C alkene and aromatic moieties are the important peaks which are used to identify mitragynine compounds (Ernawati et al., 2024). The important peaks of mitragynine such as N-H, C=O and C=C can be found in fraction 3, tentatively representing the presence of mitragynine in the fraction. This finding aligns with previous study by Haspi et al. (2025) which also found the presence of N-H, C-H and C=O functional group in the FTIR findings of polar extract of *M. speciosa*. Meanwhile, fraction 2, 4 and fraction 5 are missing the C=C peaks. These findings can be compared to the TLC result of fraction 3 as when compared, the Rf value for fraction 3 is different compared to the other fractions. Therefore, the alkaloid present in fraction 3 may be mitragynine,. Meanwhile, the alkaloid present in the other fractions may be the derivatives of mitragynine or other structurally similar alkaloids. However, the presence of mitragynine in the fraction cannot be confirmed yet as confirmatory analysis such as Gas Chromatography Mass Spectrometry (GC-MS) or Nuclear Magnetic Resonance (NMR) analysis are required for final alkaloid identification in the fractions of *M. speciosa* due to FTIR restrictions.

Conclusion

In conclusion, this study successfully screened the phytochemical compounds presence in the ethanol extracts of *M. speciosa* and its fractions. This includes alkaloids, flavonoids, phenolics, tannins, saponins and triterpenoids in the crude ethanol extract and the fractions, while identifying the absence of phenolic compounds in the fractions of binary mixture of n-hexane: EtOAc under solvent system n-Hexane: EtOAc (80:20). Besides, the putative presence of mitragynine is supported by the presence of secondary amine (N-H), carbonyl functional group (C=O) and C=C alkene in the fractions during FTIR analysis. Therefore, this study is important as it supports the use of ethanol as solvent of extraction for *M. speciosa* as most of the study utilise methanol as their solvent of extraction. Besides, this study demonstrated that VLC is highly efficient in its ability to fractionate the compounds according to its polarity. While FTIR confirms the presence of functional groups, future work should utilise GC-MS to confirm the presence of mitragynine in the fractions and High-Performance Liquid Chromatography (HPLC) to quantify the exact concentration of mitragynine and its derivatives within the fractions. Besides, it is recommended to analyse the isolated fractions for particular biological activities, such as antioxidant, antibacterial or analgesic capabilities to associate the presence of particular phytochemicals such as the detected triterpenoids and alkaloids with therapeutic outcomes.

Authors contributions

Study design, S.Z.M.S. and N.A.M..; direction and coordination, S.Z.M.S. investigation, N.A.M; resources, S.Z.M.S; writing—original draft preparation, N.A.M.; writing—review and editing, S.Z.M.S and N.A.M; supervision, S.Z.M.S.; project administration, S.Z.M.S and N.A.M.

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Conflict of interest

There is no conflict of interest

Declaration of generative AI and AI-assisted technologies in the writing process

The author declared that the Perplexity AI assistant was utilised during preparation of the study. No generative AI was used for data fabrication or result interpretation. All experimental data represent original laboratory work and analysis. The final content was reviewed and edited as needed.

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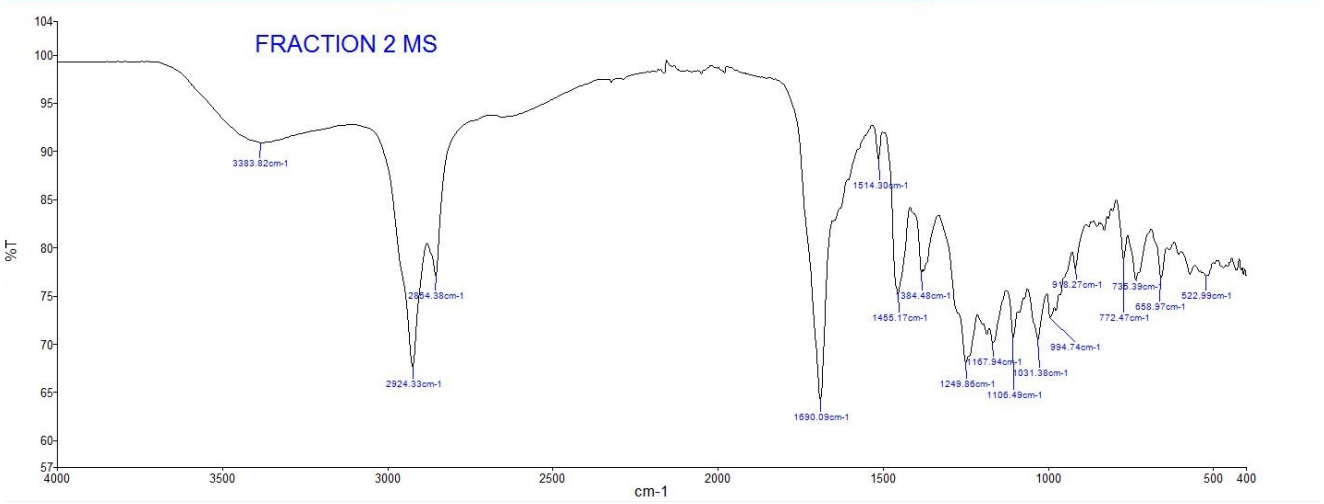
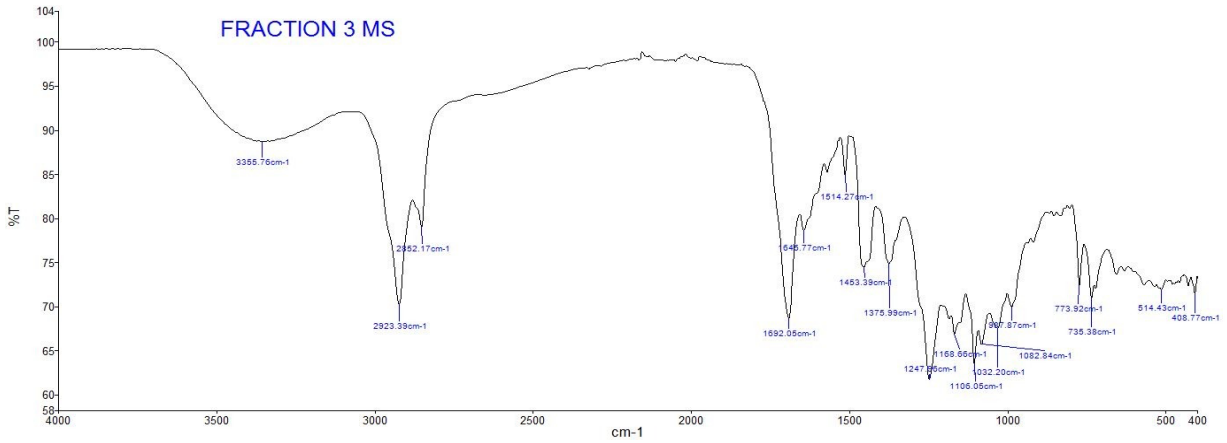
Appendix A (if applicable)

Appendix A1



Appendix A2



Appendix A3