*Malaysian Journal of Chemistry, 2025, Vol. 27(5), 80-94*
(Special Issue: *5th IKMPB Online Symposium, IKMPB 2025*)

# Phytochemical Screening and Evaluation of *in vitro* Antioxidant, Antibacterial and Antibiofilm Activities of *Mitragyna speciosa* Leaf Extract

**Taslima Begum[1], Qamar Uddin Ahmed[1]*, Mohd Hafiz Arzmi[2], A. B. M. Helal Uddin[1], Alfi Khatib[1], Sharifah Nurul Akilah Syed Mohamad[1], Syarifah Nurhikmah Izzati Syed Nasarudin[3], Syed Atif Abbas[4] and Zul Hadif Abd Aziz[1]**

[1]Drug Discovery and Synthetic Chemistry Research Group, Department of Pharmaceutical Chemistry, Kulliyyah of Pharmacy, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia
[2]Department of Fundamental Dental and Medical Sciences, Kulliyyah of Dentistry, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia
[3]Biomedical Science, Kulliyyah of Allied Health Sciences, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia
[4]Department of Pharmacology, Faculty of Pharmacy, Quest International University, Ipoh 30250 Perak, Malaysia
*Corresponding author (e-mail: quahmed@iium.edu.my)

*Mitragyna speciosa* leaves have long been utilised in traditional Southeast Asian medicine, including in Malaysia, for treating ailments such as diarrhoea, intestinal infection and wound healing. In light of the global rise in antibiotic resistance, this study aimed to explore the phytoconstituent composition, antioxidant capacity, antibacterial and antibiofilm properties of *M. speciosa* leaf extract. A 100% methanol extract was prepared using maceration (referred to as 100% M) and subjected to comprehensive phytochemical screening, antioxidant assays, antibacterial and antibiofilm evaluations, and Q-ToF-LCMS analysis. The extract demonstrated high levels of bioactive constituents, with a total phenolic content (TPC) of 257.46 ± 1.32 mg gallic acid equivalent/g of extract and a total flavonoid content (TFC) of 50.75 ± 0.24 mg quercetin equivalent/g of extract. Antioxidant activity was notable, with DPPH $IC_{50}$ of 7.94 ± 0.12 µg/mL and FRAP values of 2103.46 ± 5.67 mg ascorbic acid equivalent/g of extract. Antibacterial testing revealed strong activity against *Staphylococcus aureus* (zone of inhibition [ZOI] at 100 mg/mL: 9.89 ± 0.10 mm; MIC: 5 mg/mL), and *Streptococcus mutans* (ZOI at 100 mg/mL: 7.22 ± 0.19 mm; MIC: 10 mg/mL). The extract also showed significant antibiofilm activity with % inhibition ranging from 88.68 ± 0.40 to 93.96 ± 0.33 against *S. aureus*, and 22.97 ± 1.28 to 40.62 ± 2.11 against *S. mutans*. Q-ToF-LCMS analysis identified a diverse array of phenolic, flavonoid, terpenoids, alkaloid and other types of compounds, which likely contribute to the observed biological activities. These findings suggest that *M. speciosa* leaves methanol extract holds promise as a source of novel antibacterial agents, supporting its traditional use and potential for pharmaceutical development.

**Keywords**: *Mitragyna speciosa*; phytochemical analysis; DPPH; FRAP; disc diffusion; antibiofilm; Q-ToF-LCMS.

*Received: April 2025; Accepted: August 2025*

Kratom (*Mitragyna speciosa* Korth.), a native plant of Southeast Asia (Thailand, Malaysia, Borneo, Philippines), is widely used by the inhabitants as a traditional medicine. It is locally named as "biak-biak or ketum" in Malaysia, "mambog" in the Philippines and "ithang, kakuam, thom or kratom" in Thailand. It is an evergreen tree with relatively large oval-shaped and glossy dark green leaves. Kratom based preparation is a mixture of alkaloids, where mitragynine is the principal alkaloid [1]. Both mitragynine and 7-hydroxymitragynine (a highly oxidized metabolite of mitragynine) are competitive antagonists for kappa (κ) and delta (δ) opioid receptors and partial agonists of mu (μ) opioid receptor [2]. Apart from the alkaloids, some other secondary metabolites such as epicatechin (flavonoid), daucosterol (saponin), quinovic acid 3-O-β-D-quinovopyranoside, quinovic acid 3-O-β-D-glucopyranoside (triterpenoid saponins), and 1-O-feruloyl-β-D-glucopyranoside, benzyl-β-D-glucopyranoside, 3-oxo-α-ionyl-O-β-D-glucopyranoside, roseoside, vogeloside, and epivogeloside (glycoside derivatives) have been isolated from kratom leaves [1].

†Paper presented at the 5th IKMPB Online Symposium: Sustainable Chemistry Innovations Shaping the Future Life

**Figure 1.** Kratom (*Mitragyna speciosa*) leaves [3].

Kratom has been known to have therapeutic properties. In this light, it has been used in traditional medicine as relief for pain, fever, cough, diarrhoea, intestinal infections, wound healing, anxiety, opioid withdrawal, prevention of cancer, increasing sexual stimulation, hypertension and diabetes [3]. Several scientific studies have explored the traditional claims and medicinal potential of kratom and have demonstrated its analgesic and antinociceptive properties, antipyretic effects, anxiolytic benefits antioxidant activity, antimicrobial and antibiofilm capabilities, anticancer potential, antidiabetic and antihyperlipidemic effects, antiparasitic properties, antitussive effects, and antispasmodic activities [1].

Antioxidants are a class of compounds that neutralize free radicals and reactive oxygen species (ROS) in cells, thereby protecting cells from oxidative damage [4]. In biological systems, free radicals, including RNS (reactive nitrogen species) and ROS, such as nitric oxide, hydroxyl and superoxide radicals can impair DNA which trigger the oxidation of proteins and lipids in cells. Under normal physiological conditions, the endogenous antioxidant system can neutralize these free radicals to maintain balance. However, due to aging or other exogenous stimuli, the production of free radicals increases, which in turn disturbs the antioxidant- free radical balance in the body [5].

Additionally, oxidative stress plays a dual role in the context of infections. While it contributes to immune defense mechanisms, excessive oxidative activity can lead to cellular damage. The pathological outcomes associated with infections are often linked to oxidative trauma and the overproduction of reactive species, which may result in severe or even lethal consequences. In this regard, phytochemicals such as polyphenols (including phenolic acids, flavonoids, anthocyanins, lignans, stilbenes, and tannins), terpenoids, carotenoids (xanthophylls and carotenes), and vitamins (notably vitamins E and C) function as natural antioxidants capable of effectively neutralizing free radicals and mitigating oxidative damage [6].

Oral infections represent a significant global health challenge. According to the WHO Global Oral Health Status Report, around 3.5 billion people get infected by oral diseases globally, with nearly 75% of these individuals residing in middle-income countries [7]. The report underscores the urgent need for action, highlighting stark inequalities and the widespread burden of oral conditions across all age groups. These oral infections are caused by microorganisms such as bacteria, fungi, and viruses, which exist in the oral cavities in biofilm forms. In cases of dysbiosis, these biofilms can promote inflammation. Alarmingly, these oral biofilms have recently developed resistance to conventional antibacterial therapies [8], complicating the treatment of oral infections. Moreover, synthetic drugs can lead to undesirable outcomes, including toxicity, adverse side effects and the development of drug resistance [9]. Apart from this, prolonged oral infections can also cause oral cancer, with the role of oral bacteria such as *Staphylococcus aureus* and *Streptococcus mutans* in cancer development being well-documented [10, 11].

Therefore, there is a pressing need for alternative treatment approaches that offer effective antimicrobial activity with lower toxicity and better selectivity. In this context, *M. speciosa* (Kratom) shows potential as an antimicrobial agent, supported

by traditional use and several scientific studies. Hence, the chief aim of this research was to evaluate the phytochemical composition, along with the antioxidant and antibacterial properties of kratom (*M. speciosa*) leaf extract, in order to assess its medicinal potential as a natural antioxidative and antibacterial agent.

## METHODOLOGY

### Collection and Preparation of Plant Materials

Fresh leaves of *M. speciosa* (3.8 kg) were collected from Kedah, Malaysia. Species identification and authentication were conducted by a botanist at the Kulliyyah of Pharmacy, IIUM, and documented under voucher number PIIUM 0358. The collected leaves were thoroughly cleaned and subsequently dried in a controlled-temperature dryer at 40 °C for 72 hours. Once dried, the leaves were finely ground using a Universal Cutting Mill (FRITSCH Pulverisette 19, Germany). to obtain a uniform powder suitable for extraction.

### Preparation of Extract by Maceration Method

To prepare the 100% methanol maceration extract (100% M), 100 g of dried leaf powder was placed in a round-bottom flask and immersed in methanol at a 1:3 (w/v) ratio. This mixture was macerated for 24 h at room temperature, followed by filtration using Whatman No. 3 filter paper. The resultant filtrate was then concentrated by using a rotary evaporator at 40 °C to obtain the methanol extract. The recovered methanol was subsequently reintroduced to the previously filtered plant residue, and the filtration and concentration steps were repeated. This entire process was performed three times to ensure maximum extraction of methanol-soluble compounds. After drying, the final weight of the methanol extract (100% M) was recorded, and the percentage yield was calculated [12].

### Qualitative Phytochemical Screening

*Test for Phenolic Compounds (Ferric Chloride Test)*

Initially, a 5% $FeCl_3$ aqueous solution was prepared and transferred to a test tube containing 2 mL of the extract. The presence of phenolic compounds was indicated by the change in the color of the extract solution to brownish green or orange [13].

*Test for Flavonoids*

The presence of flavonoids in the extract was assessed using the Shinoda test and the Zinc-HCl reduction test. For the Shinoda test, 3 mL of the leaf extract (dissolved in ethanol) was placed into separate test tubes. To each tube, five drops of concentrated hydrochloric acid (HCl) were added, followed by approximately 1 mg of magnesium turnings. When magnesium and hydrochloric acid are added to the extract, flavonoids undergo a reduction reaction that produces a characteristic red or pink coloration, indicating their presence. In the Zinc-HCl reduction test, a small piece of zinc and a few drops of concentrated HCl were added to 3 mL of the ethanolic leaf extract in a test tube. The mixture was then heated in a water bath for one minute. A colour change to crimson red or orange was considered a positive indication of flavonoid presence [13].

*Test for Phytosterols (Salkowski Test)*

5 mL of chloroform was added to 1 mL of the extract. Subsequently, a few drops of concentrated sulphuric acid were added. This resulted in the formation of two layers; the lower layer exhibited a yellow colour with green fluorescence, while the upper layer turned red. The formation of these layers indicated the presence of phytosterols [14].

*Test for Saponins*

To test for the presence of saponins, 10 mL of distilled water was mixed with 10 mL of the leaf extract in a separating funnel. The mixture was then vigorously shaken for at least 10 minutes. The formation of a persistent foam layer at the top of the solution was taken as a positive indication of saponins in the extract [13].

*Test for Terpenoids*

To identify the presence of terpenoids, the leaf extract (1 mL) was combined with 1.5 mL of concentrated sulphuric acid and 1 mL of chloroform. The formation of a reddish-brown coloration at the interface was considered a positive result, indicating the presence of terpenoids in the extract [14].

*Test for Glycosides (Borntrager's Test)*

To test for the presence of glycosides, 50 mg of the leaf extract was first dissolved in 30 mL of distilled water and hydrolysed with 1 mL of hydrochloric acid in a water bath for two hours. The mixture was then filtered to obtain the hydrolysed extract. Next, 2 mL of acetic acid and 2 mL of chloroform were added to 2 mL of the hydrolysed extract. The solution was allowed to stand for 5 minutes. Afterwards, a few drops of concentrated sulphuric acid were carefully added. The appearance of a green or reddish-brown coloration indicated a positive result for glycosides [14].

*Test of Alkaloids (Mayer's Test)*

To detect the presence of alkaloids, 1 mL of the leaf extract was placed in a test tube, followed by the addition of 2 drops of Mayer's reagent. The formation

of a white, creamy precipitate was taken as a positive indication of alkaloids in the extract [14].

**Quantitative Phytochemical Screening**

*Total Phenolic Content Test*

In this test, gallic acid monohydrate served as the standard for the calibration curve. DMSO was used to prepare extract solution (1 mg/mL), which was also used as a blank. In each microplate well, 20 µL of extract, gallic acid or DMSO was added to100 µL of 10% Folin Ciocalteu reagent, shaken for 1 minute, and incubated for 4 minutes. Then, 80 µL of 7.5% sodium carbonate was added, shaken, and incubated for 2 hours. Absorbance was measured at 765 nm using a microplate reader (Tecan Nano Quant Infinite M200PRO, Tecan, Saphire, Switzerland). The results were reported as mg of gallic acid equivalent per gram of extract (mg GAE/g) [15].

*Total Flavonoid Content Test*

In this test, quercetin dihydrate (98% purity) was used as a standard to generate the calibration curve. The extract solution (1 mg/mL) was prepared in methanol. In each microplate well, 50 µL of extract or standard solution was mixed with 100 µL of methanol, with one well containing 150 µL of methanol as a blank for the microplate absorbance reading. Then, 20 µL of 10% $AlCl_3$ was added, gently shaken, and incubated at room temperature for 3 minutes. Next, 20 µL of 1M CH3COONa and 60 µL of methanol were added. The plate was then incubated in the dark at room temperature for 40 minutes. Absorbance was measured at 415 nm using a microplate reader. The outcome was expressed in terms of mg of quercetin equivalent per gram of extract (mg QE/g) [16].

*DPPH Scavenging Assay*

In this assay, several concentrations of extract or standard were prepared by serial dilution with methanol from a stock solution of 1 mg/mL. DPPH solution (0.2 mM) was also prepared with methanol. In a 96 well-plate, 100 µL of each extract or standard solution was added to 100 µL of DPPH solution. After adding, the reaction mixture was shaken well and subjected to incubation at ambient temperature for around 30 minutes by ensuring darkness. The absorbance was recorded at 517 nm using a microplate reader. The blanks were absent from the DPPH solution and contained 100 µL extract or ascorbic acid and 100 µL methanol. Moreover, 100 µL of methanol and 100 µL of DPPH solution were used as a control, while 200 µL of methanol acted as a blank control. The DPPH scavenging activity (% inhibition) was calculated by using the following equation [17].

$$\% \text{ Inhibition} = \left(\frac{\text{Acontrol} - \text{Asample}}{\text{Acontrol}}\right) \times 100$$

Here, Acontrol = Absorbance of control; Asample = Absorbance of sample (extract/standard).

*FRAP Assay*

In this assay, ascorbic acid was used as a standard to prepare a standard curve. The extract solution was prepared with DMSO having a concentration of 250 µg/mL, therefore, DMSO was used as the control. The FRAP reagent was instantly prepared by combining ferric chloride hexahydrate, 2,4,6-tris(2- pyridyl)-S-pyrazine, and acetate buffer with a pH of 3.6. After preparation, the resultant reagent was incubated for 10 minutes at 37 °C in an incubator shaker. Post incubation, in the 96-well plate, 30 µL of extract or ascorbic acid or DMSO was added with 270 µL of freshly prepared FRAP reagent. Next, it was shaken for 1 minute and incubated for 30 minutes at 37 °C in dark and the absorbance was recorded at 593 nm using a microplate reader. The FRAP activity was expressed as milligrams of ascorbic acid equivalent per gram of extract (mg AAE/g) [18].

**Evaluation of Antibacterial Activity of *M. speciosa* Leaves Extract**

Antibacterial tests of the *M. speciosa* leaves extract were performed via disc diffusion, broth microdilution, and antibiofilm assays against *Streptococcus mutans* (ATCC 25175) and *Staphylococcus aureus* (ATCC 25923). The Brain Heart Infusion (BHI) agar was used to subculture these pathogens and from the pure culture of *S. aureus* and *S. mutans*, four to five colonies were taken with a wire loop and transferred to BHI broth. For *S. aureus*, OD 595 nm of 0.5, and *S. mutans*, OD 620 nm of 0.5 were maintained to have a bacterial load of $10^8$ CFU/mL [19,20].

*Disc Diffusion Assay*

To perform the disc diffusion test, the extract was used in three concentrations (100, 50, and 25 mg/mL) using methanol. Sterile, blank discs (6 mm) were impregnated with 20 µL of each dilution from the extract. The discs, made from Whatman No. 1 filter paper, were autoclaved at 121 ºC for 20 minutes. Each disc was alternately spotted with 5 µL of the extract on both sides, allowing each layer to dry before applying the next. Methanol-loaded discs served as the negative control. All discs were fully dried before being applied to the bacterial lawn. Chlorhexidine 0.12% was used as the standard. The standardized bacterial inoculums were streaked over Mueller-Hinton agar (MHA) plates with a sterile cotton swab three times, rotating the plate 60° each time to ensure full coverage. The inoculated plates were left ajar to dry. Using sterile forceps, discs were applied and pressed gently for uniform contact, placing 6 discs per plate to avoid overlapping zones of inhibition. The plates were incubated and inverted at 37 ºC overnight. Zones of inhibition were observed the next day and were measured to the nearest millimetre. The assay

was carried out in triplicate and the mean diameters were calculated [21].

**Broth Microdilution Test to Determine MIC and MBC**

The extract was evaluated for its antimicrobial activity using the broth microdilution method to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). A stock solution (100 mg/mL) was prepared by dissolving 100 mg of the extract in 1 mL of a solvent system consisting of methanol and phosphate-buffered saline (PBS) in a 10:90 ratio. Serial dilutions were made to obtain eight different concentrations. In a sterile 96-well plate, 10 µL (for *S. aureus*) or 20 µL (for *S. mutans*) of extract were added to 90 µL (for *S. aureus*) or 80 µL (for *S. mutans*) PBS, followed by 50 µL of bacterial inoculum ($10^8$ CFU/mL) and 50 µL of BHI broth, and incubated for 24 hours. Methanol concentration did not exceed 1%. Controls included Extract + PBS + BHI, PBS + culture + BHI, PBS + BHI, 0.12% Chlorhexidine + PBS + BHI and 0.12% chlorhexidine + PBS + culture + BHI. MIC was detected by adding 20 µL of 5 mg/mL MTT and incubating at 30 °C for 30 minutes. The lowest concentration showing a colour change was considered the MIC. MBC was determined by streaking broth from MIC wells onto sterile MHA and incubating at 37 °C for 24 hours. The lowest concentration with no growth was the MBC [22].

**Antibiofilm Assay**

To evaluate the antibiofilm effects of the extract on *S. aureus* and *S. mutans* biofilms, extract solutions were prepared using the same solvent system as the broth microdilution method. Two concentrations (1XMIC and 2XMIC) were used. In each well of a sterile microplate, the extract solution, PBS, 50 µL of bacterial culture ($10^8$ CFU/mL), and 50 µL BHI broth were added to make a final volume of 200 µL. Controls included Extract + PBS + BHI, PBS + culture + BHI, PBS + BHI, 0.12% of chlorhexidine + PBS + culture + BHI and 0.12% of chlorhexidine + PBS + BHI. Plates were incubated at 37 °C for 72 hours, with the medium replenished aseptically every 24 hours

Following incubation, biofilm formation was assessed using a crystal violet staining method. The wells were emptied and washed three times with sterile distilled water to remove planktonic (non-adherent) cells. Plates were then air-dried, followed by oven-drying at 60 °C for 45 minutes. To fix adherent cells, 200 µL of 100% methanol was added to each well and incubated for 20 minutes. After fixation, the wells were emptied and stained with 200 µL of 0.1% crystal violet solution for 20 minutes at room temperature. Excess stain was removed by washing the plates five times with distilled water. To evaluate biofilm biomass semi-quantitatively, the bound crystal violet was resolubilized with 200 µL of absolute ethanol. After 20 minutes, 100 µL from each well was transferred to a new microplate, and absorbance was measured at 590 nm using a microplate reader [48]. The mean absorbance (OD590nm) of the sample was determined, and results were recorded as percentage inhibition using the equation below [23].

$$(\%)\ \text{Inhibition} = \left(\frac{\text{OD( Negative control)} - \text{OD (Sample)}}{\text{OD (Negative control)}}\right) \times 100$$

**Identification of Bioactive Compounds of *M. speciosa* Leaves Extract by Quadrupole Time-of-Flight Liquid Chromatography Mass Spectrometry (Q-ToF-LCMS) Analysis**

The plant sample was analysed using an LC-MS-QTOF instrument (Agilent 1290 Infinity and 6550 iFunnel, Santa Clara, CA, USA) with an electrospray interface (ESI). To prepare the sample, 1 mg of plant extract was dissolved in 250 µL of methanol, vortexed, sonicated for 15 minutes, and then mixed with 250 µL of water. After centrifugation for 15 minutes, the clear supernatant was filtered into a glass vial. The plant sample was injected into a Phenomenex Kinetex C18 column (250 mm × 4.6 mm, 5 µm) at 27 °C, using a gradient elution from 5% methanol in water with 0.1% formic acid to absolute MeOH over 20 minutes, followed by 10 minutes with absolute MeOH at 0.7 mL/min. MS/MS data were acquired in the m/z range of 50 to 1500 with a collision energy ramp of 35 eV. Source parameters were gas temperature 200 °C, flow rate 14 L/min, nebulizer 35 psig, sheath gas temperature 350 °C, and flow rate 11 L/min. Data produced were analysed using ACD/Spec Manager and pre-processed with MZmine software, including baseline correction, peak detection, filtering, alignment, smoothing, and gap filling, and saved in CSV format [24].

**Statistical Analysis**

The DPPH, antibacterial and antibiofilm data were analysed using Minitab software version 21.4. A one-way analysis of variance (ANOVA) was done, along with a multiple comparison test using the Tukey post hoc test. Results were considered statistically significant when $p < 0.05$.

## RESULTS AND DISCUSSION

**Percentage Yield, Phytochemical Screening and Antioxidant Test of *M. speciosa* Leaves Methanol Extract (100% M)**

The percentage yield, phytochemical screening and antioxidant tests of the *M. speciosa* leaves methanol extract (100% M) are shown in Table 1. The qualitative phytochemical screening revealed that the extract contained flavonoids, phytosterols, alkaloids and terpenoids, but was devoid of saponins. Additionally, the quantitative phytochemical screening and

antioxidant tests demonstrated significant antioxidant properties (TPC: 257.46 ± 1.32 mg gallic acid monohydrate equivalent/g of extract; TFC: 50.75 ± 0.24 mg quercetin dihydrate equivalent/g of extract; DPPH: $IC_{50}$ 7.94 ± 0.12 µg/mL; FRAP: 2103.46 ± 5.67 mg ascorbic acid equivalent/ g of extract). In this regard, the DPPH results were statistically significant compared to the standard ($p < 0.05$). The standard curves for determining TPC, TFC, and FRAP are shown in Figure 2.

**Table 1.** Results of % yield, phytochemical screening, and antioxidant tests of 100% M.

| Extract/ Standard | % Yield | Qualitative test | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Phenolic | Flavonoid | Phytosterol | Saponin | Terpenoid | Glycoside | Alkaloid |
| 100% M | 30.169 | ++ | +++++ | ++++ | - | +++++ | + | ++ |
| | Quantitative test | | | Antioxidant Test | | | | |
| | TPC (mg gallic acid monohydrate equivalent/g of extract) | | TFC (mg quercetin dihydrate equivalent/g of extract) | DPPH ($IC_{50}$ µg/mL) | | FRAP (mg ascorbic acid equivalent/ g of extract) | | |
| 100% M | 257.46 ± 1.32 | | 50.75 ± 0.24 | 7.94 ± 0.12[a] | | 2103.46 ± 5.67 | | |
| Ascorbic acid | NA | | NA | 5.26 ± 0.03[b] | | NA | | |

**Note:** Here, - means absence, + means very low presence, ++ means moderate presence, +++ means high presence, ++++ means very high presence and +++++ means extremely high presence. Results are expressed as Mean ± SD. For the DPPH test, means that do not share a letter are significantly different ($p<0.05$).

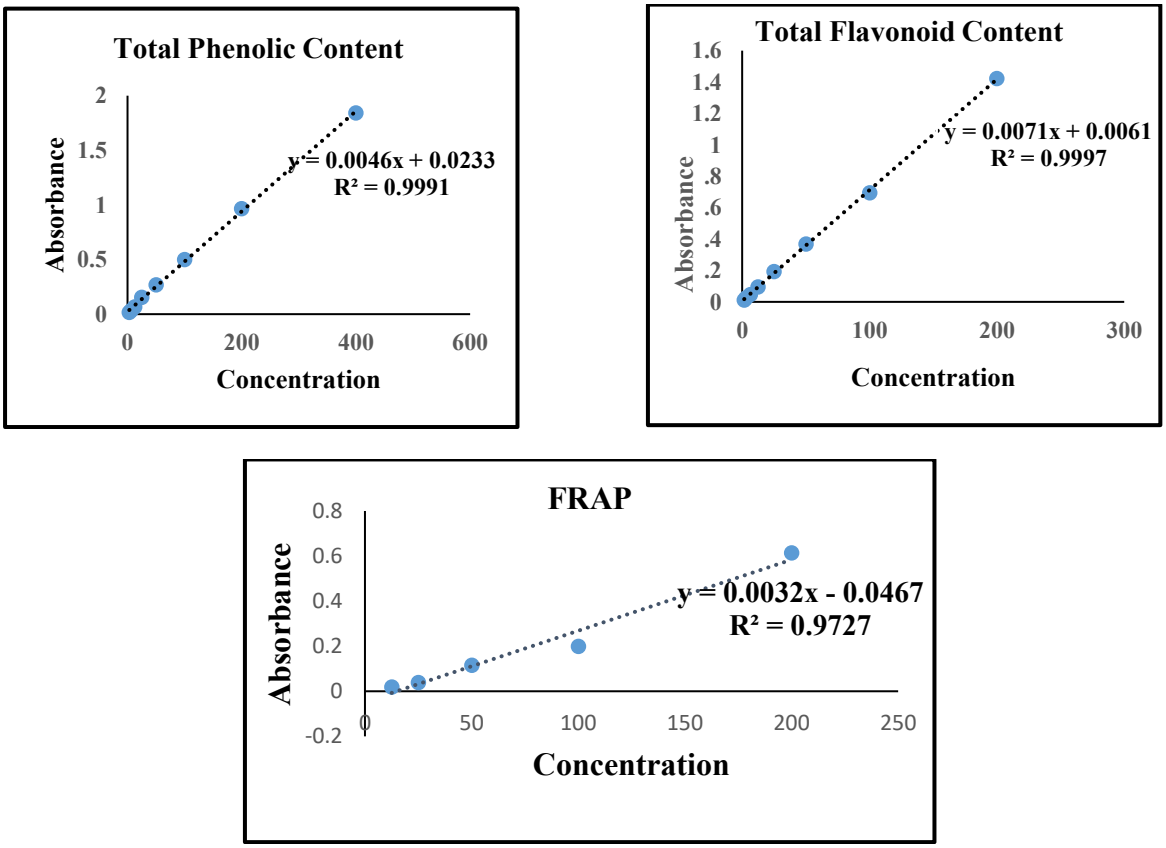



**Figure 2.** Standard curves for TPC, TFC, and FRAP tests.

For TPC, the regression equation was derived from the standard curve of gallic acid and determined to be y = 0.0046x + 0.0233, with a coefficient of determination ($R^2$) of 0.9991. indicating excellent linearity. Similarly, for TFC, the quercetin standard curve yielded a regression equation of y = 0.0071x + 0.0061, with an $R^2$ value of 0.9997, reflecting a highly reliable calibration. In both cases, the $R^2$ values of the standard curves were very close to 1, indicating high accuracy. Using these standard curves, the TPC and TFC of the extract were determined, showing high TPC and TFC values. Furthermore, the extract's antioxidant property was investigated through DPPH and FRAP tests (*in vitro*). In the DPPH assay, linear regression interpolation was employed to determine the $IC_{50}$ value of the extract, representing the concentration of extract required to scavenge 50% of DPPH radicals. In the DPPH assay, standard ascorbic acid showed an $IC_{50}$ value of 5.26 ± 0.03 µg/mL, whereas the $IC_{50}$ value for the 100% M extract was 7.94 ± 0.12 µg/mL. In the FRAP assay, the ascorbic acid standard curve yielded a regression equation of y = 0.0032x - 0.0467, with an $R^2$ value of 0.9727. The high $R^2$ value, being close to 1, reflects strong linearity and accuracy of the calibration. FRAP values for the 100% M extract were calculated based on this equation.

Phytochemicals are bioactive compounds naturally found in plants, known for their potential health-promoting properties and roles in plant defense mechanisms. The prevalence of these phytochemicals, including saponin, terpenoids, steroids, alkaloids, phenols and flavonoids, is responsible for various biological activities in the human body, such as wound healing, antiaging, antimicrobial, anti-inflammatory, antidiabetic, antiparasitic, antidepressant, anticancer, and antioxidant [25]. Qualitative phytochemical screening revealed the presence of various bioactive constituents, with particularly high levels of flavonoids and terpenoids. These findings underscore the therapeutic potential of the extract, suggesting its possible efficacy in the treatment of a range of disease conditions due to the well-documented antioxidant, anti-inflammatory, and antimicrobial properties of these compounds.

In the case of quantitative phytochemical screening, the extract showed high TPC and TFC values. Based on numerous studies, phytochemicals such as flavonoids and phenols can act as antioxidants [26]. This statement was justified by the antioxidant tests, where the extract exhibited a high FRAP value and low $IC_{50}$ value in the DPPH test. The FRAP test is a colorimetric assay used to evaluate the antioxidant capacity of a sample. It measures the ability of antioxidants to reduce ferric ions ($Fe^{3+}$) complexed with a ligand to the ferrous form ($Fe^{2+}$) under acidic conditions. The reduction results in the formation of an intensely blue ferrous complex, which can be quantified spectrophotometrically. The intensity of the blue coloration is directly proportional to the antioxidant power of the sample. Absorbance is typically measured at 593 nm, and results are often expressed in terms of Trolox or ascorbic acid equivalents or compared against a standard curve. The DPPH assay utilizes the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•), which is neutralized by antioxidants through electron donation, resulting in a measurable decrease in absorbance. This reduction reflects the radical scavenging ability of the sample [27]. The strength of antioxidants iscommonly classified into four categories based on $IC_{50}$ values as follows: very strong ($IC_{50}$ < 50 µg/mL), strong ($IC_{50}$: 50 – 100 µg/mL), moderate ($IC_{50}$: 101 – 150 µg/mL), and weak ($IC_{50}$: 250 – 500 µg/mL [28]. Here, the extract demonstrated a very strong antioxidant property, with an $IC_{50}$ value below 50 µg/mL, which was comparable to that of ascorbic acid, a known potent antioxidant. These findings, along with the FRAP assay results, clearly indicate that the extract possesses robust antioxidant activity.

**Table 2.** Results of disc diffusion and broth microdilution tests of 100% M against *S. aureus,* and *S. mutans*.

| **Extract/0.12% Chlorhexidine** | | **Disc Diffusion Test** | | **Broth Microdilution Test** | | | |
|---|---|---|---|---|---|---|---|
| | | ***S. aureus*** | ***S. mutans*** | ***S. aureus*** | | ***S. mutans*** | |
| | | **Zone of Inhibition (mm)** | | **MIC (mg/mL)** | **MBC (mg/mL)** | **MIC (mg/mL)** | **MBC (mg/mL)** |
| 100% M | 25 mg/mL | 7.94 ± 0.10$^{d}$ | - | 5 | - | 10 | - |
| | 50 mg/mL | 8.78 ± 0.26$^{c}$ | 6.56 ± 0.10$^{b}$ | | | | |
| | 100 mg/mL | 9.89 ± 0.10$^{b}$ | 7.22 ± 0.19$^{b}$ | | | | |
| 0.12% Chlorhexidine | | 21.89 ± 0.51$^{a}$ | 20.11 ± 0.69$^{a}$ | - | - | - | - |

**Note** Here, - means absence. Results are expressed as Mean ± SD. Means that do not share a letter are significantly different ($p<0.05$).

**Antibacterial Tests**

The disc diffusion test was performed with three different concentrations of 100% M, against *S. aureus,* and *S. mutans.* The extract showed zones of inhibition against *S. aureus* (25 mg/mL, 50 mg/mL, and 100 mg/mL) and *S. mutans* (50 mg/mL and 100 mg/mL). The zones of inhibition for the extract were statistically significant ($p < 0.05$) compared to the positive control, 0.12% chlorhexidine (Table 2). In the broth microdilution test, the MIC values obtained against *S. aureus* and *S. mutans* were 5 mg/mL and 10 mg/mL, respectively. Further subculturing from the wells of MIC to the highest concentration did not show MBC.

The disc diffusion method is a widely used, simple, and cost-effective technique for assessing antimicrobial susceptibility. It is known for its reproducibility and reliability, making it a preferred initial screening tool in clinical laboratories [29]. When new antimicrobial agents are approved for clinical use, disc diffusion is often the first method cleared for routine testing. This assay provides a preliminary indication of the efficacy of test substances such as antibiotics or plant extracts against specific microbial strains. In contrast, the broth microdilution method is employed to determine the Minimum Inhibitory Concentration (MIC), defined as the lowest concentration of an antimicrobial agent that visibly inhibits microbial growth. Due to its sensitivity, reproducibility, and convenience, broth microdilution is considered a standard method for evaluating compounds with potential antimicrobial activity. Furthermore, colorimetric assays using tetrazolium salts such as 2,3,5-triphenyltetrazolium chloride (TTC), tetrazolium red, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and *p*-iodonitrotetrazolium violet (INT) can be incorporated into microdilution protocols to enhance visual detection of microbial viability. These reagents undergo colour changes in response to metabolic activity, providing a rapid and quantifiable measure of microbial inhibition [30].

The results of the disc diffusion assay are aligned with those obtained from the antioxidant tests. Among the two bacterial strains tested, the 100% M extract exhibited stronger activity against *S. aureus*, producing zones of inhibition at all three concentrations. However, when compared to the standard antibiotic, the extract showed relatively small zones of inhibition against both *S. aureus* and *S. mutans*. Despite this limitation, a broth microdilution assay was subsequently performed to evaluate its antibacterial properties further. According to several studies, while disc diffusion assays offer a preliminary indication of bacterial susceptibility, they are inherently qualitative and rely solely on the diffusion capacity of the compound from the disc [31]. As a result, compounds with poor diffusive properties may not spread adequately through the medium, leading to smaller or absent of zones of inhibition. Disc diffusion has also been shown to yield false resistant (negative) results in certain cases, where broth microdilution assays later confirmed microbial susceptibility to the tested agents [32]. Given that the extract used in the present study is a complex mixture of bioactive compounds, solely relying on disc diffusion results is insufficient and could potentially be misleading. Therefore, additional quantitative methods such as broth microdilution, are essential to assess its antibacterial potential accurately.

Both disc diffusion and broth microdilution assays were conducted on planktonic bacterial cells. However, in physiological conditions, microorganisms predominantly exist as biofilms structurally complex communities that exhibit enhanced resistance to conventional antimicrobial treatments [33]. Hence, to simulate *in vivo* conditions and assess the therapeutic relevance of the extract, an antibiofilm assay was performed to evaluate its ability to inhibit biofilm formation. Two concentrations of the extract viz. 1× MIC and 2× MIC for each bacterial strain were tested. The results demonstrated a reduction in biofilm biomass, with greater inhibition observed at higher extract concentrations. Statistical analysis confirmed significant differences ($p < 0.05$) in biofilm biomass between treated and untreated groups, and also in comparison to 0.12% chlorhexidine, a standard antimicrobial agent (Table 3).

Consistent with the findings from planktonic assays, the extract exhibited strong antibiofilm activity against *S. aureus*, while its effect against *S. mutans* was comparatively weaker. Notably, this study represents the first investigation into the antibiofilm potential of *M. speciosa* extract against *S. mutans*. Although the 100% methanolic extract (100% M) showed less than 50% inhibition of biofilm formation at its highest concentration, this outcome is not uncommon for natural compounds, which often act through multifactorial mechanisms distinct from those of conventional antibiotics. While standard drugs typically exert strong bactericidal effects, natural extracts may interfere with biofilm formation through mechanisms such as glucosyltransferase (GTF) inhibition, quorum sensing disruption, or extracellular polymeric substance (EPS) matrix destabilization, which may not immediately result in high inhibition percentages. Recent studies support this distinction. For example, Atta et al. [34] demonstrated that targeting GTFs, key enzymes in *S. mutans* biofilm formation, can significantly compromise biofilm integrity even when overall bacterial inhibition remains modest. Their molecular docking and dynamics simulations revealed that certain natural compounds bind effectively to GTFs, disrupting biofilm architecture and pathogenicity despite lower MIC values compared to standard drugs. Therefore, the extract's antibiofilm potential remains scientifically relevant and promising, particularly in the context of biofilm-associated oral infections, where conventional antibiotics often fail due to resistance and limited biofilm penetration.

**Table 3**. The effect of 100% M and 0.12% chlorhexidine on the biofilm biomass of *S. aureus* and *S. mutans*.

| **Extract** | **Biofilm biomass** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ***S. aureus*** | | | | | | ***S. mutans*** | | | | | |
| | **Concentrations (mg/mL)** | **Biomass (Treated)** | **Biomass (Untreated/ Neg. Control)** | **Biomass (0.12% chlorhexidine)** | **% inhibition of extract (Treated)** | **% inhibition of 0.12% chlorhexidine** | **Concentrations (mg/mL)** | **Biomass (Treated)** | **Biomass (Untreated/ Neg. Control)** | **Biomass (0.12% chlorhexidine)** | **% inhibition of extract (Treated)** | **% inhibition of 0.12% chlorhexidine** |
| 100% M | 5 | 0.059 ± 0.002[b] | 0.523 ± 0.002[a] | NG | 88.68 ± 0.40[c] | 100 ± 0.0[a] | 10 | 0.092 ± 0.002[b] | 0.186 ± 0.006[a] | NG | 22.97 ± 1.28[c] | 100 ± 0.0[a] |
| | 10 | 0.032 ± 0.002[c] | | | 93.96 ± 0.33[b] | | 20 | 0.071 ± 0.003[c] | | | 40.62 ± 2.11[b] | |

**Note:** NG means No Growth. Results are expressed as Mean ± SD. Means that do not share a letter are significantly different ($p<0.05$).

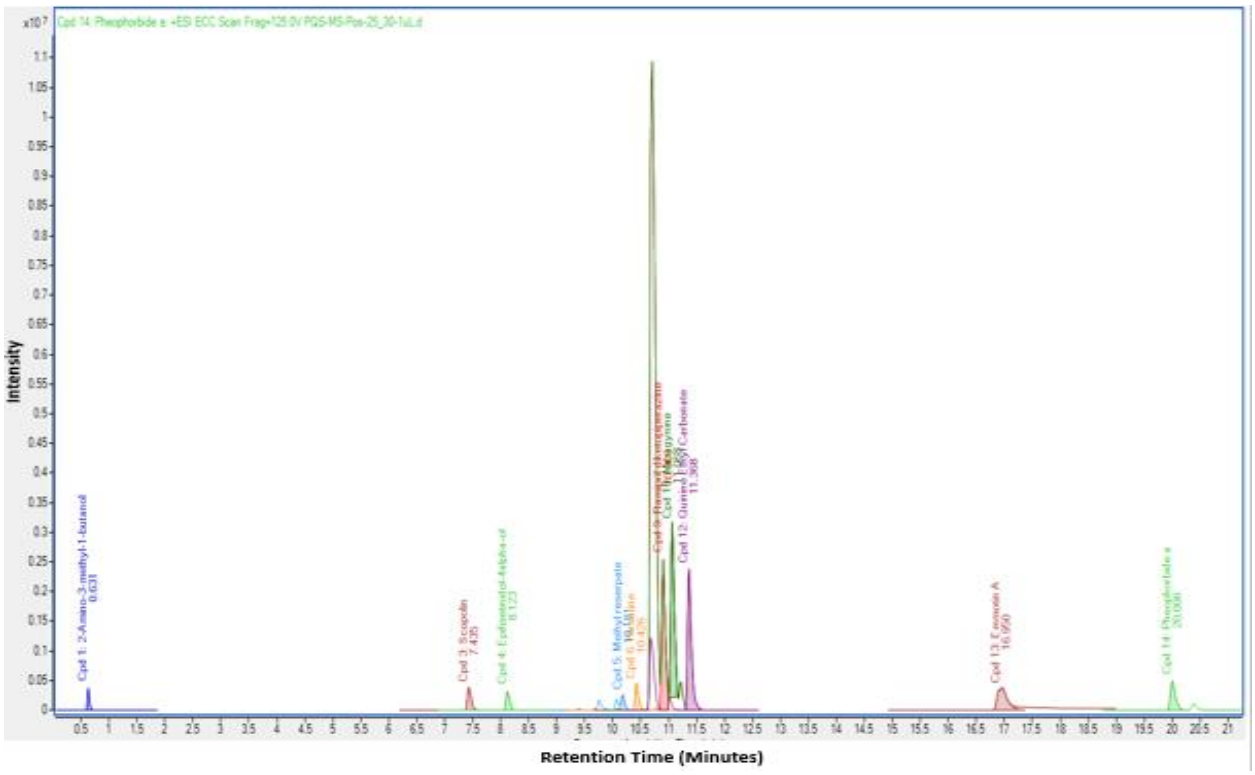



**Figure 3.** LC-MS QTOF chromatogram of *M. speciosa* leaves (100% M) in positive ionization mode.

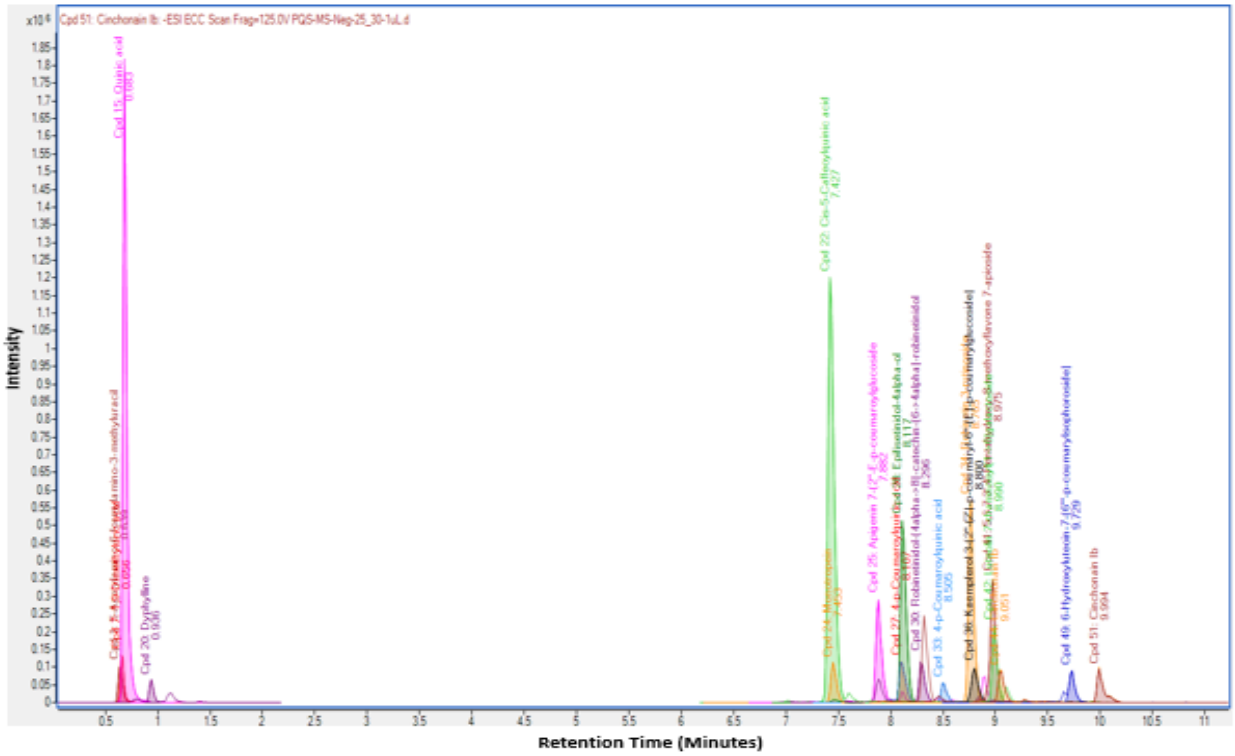



**Figure 4.** LC-MS QTOF chromatogram of *M. speciosa* leaves (100% M) in negative ionization mode.

**Q-ToF-LCMS Analysis**

Q-TOF-MS (Quadrupole Time-of-Flight Mass Spectrometry) is a powerful analytical technique that integrates the strengths of two distinct mass analysers quadrupole and time-of-flight (TOF), to form a unique hybrid system. This configuration combines the high fragmentation efficiency of quadrupole technology with the rapid analysis speed and high mass resolution of TOF, enabling precise identification

and characterization of complex compounds. Mass spectrometers function by converting analyte molecules into charged ions, which are then analysed based on their mass-to-charge ratio (m/z). Ionization typically involves either the loss of a proton ($M-H^-$) in negative ion mode or addition of a proton ($M+H^+$) in positive ion mode. The choice of mode for ionization depends on the chemical nature of the analyte; the positive ion mode is optimal for detecting basic compounds. Negative ion mode is more suitable for acidic compounds. This dual-mode capability allows Q-TOF-MS to comprehensively profile a wide range of chemical constituents, making it especially valuable in phytochemical analysis, drug discovery, and metabolomics. [35,36].

The Q-ToF-LCMS analysis of 100% M provided chromatograms in both positive and negative ionization modes, as shown in Figures 3 and 4, respectively.

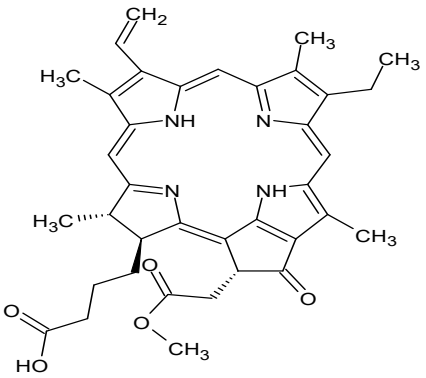
Several compounds were identified after the Q-ToF-LCMS analysis of 100% M using both positive and negative ionization modes. The bioactive compounds identified in the negative ionization mode were primarily phenolic and flavonoid compounds, such as, quinic acid, *cis*-5-caffeoylquinic acid, apigenin 7-(2''-E-p-coumaroylglucoside), 4-p-coumaroylquinic acid, robinetinidol-(4alpha->8)-catechin-(6->4alpha)-robinetinidol, robinetin 3-rutinoside, kaempferol 3-(2”-(Z)-p-coumaryl-6”-(E)-p-coumarylglucoside), 5,6,7,3’,4'-pentahydroxy-8-methoxyflavone 7-apioside, luteolin 7-rhamnosyl(1->6)galactoside, 6-hydroxyluteoin-7-(6'''-p-coumarylsophoroside) and cinchonain Ib. Conversely, through positive ionization mode, several alkaloids were identified (methyl reserpate, hirsutine, mitragynine, quinine ethyl carbonate) along with a flavonoid (epifisetinidol-4alpha-ol), a terpenoid (emmotin A), a glycoside (scopolin), a chlorophyll derivative (pheophorbide A), and ramipril derivative (ramipril diketopiperazine). The predicted compounds for each ionization mode are summarized in Tables 4 and 5. Moreover, the identified compounds chemical structures are depicted in Figure 5.

**Table 4.** Compounds identified in *M. speciosa* leaves (100% M) through LC-MS QTOF negative ionization mode.

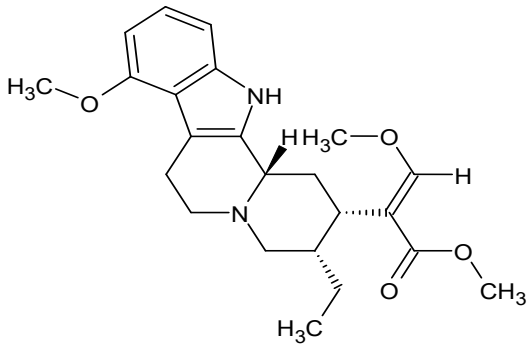
| Identified compounds | Formula | m/z | Score | RT |
|---|---|---|---|---|
| 5-Acetylamino-6-formylamino-3-methyluracil | $C_8H_{10}N_4O_4$ | 225.0631 | 95.24 | 0.639 |
| 1,9-Dimethyluric acid | $C_7H_8N_4O_3$ | 195.0522 | 93.93 | 0.656 |
| Quinic acid | $C_7H_{12}O_6$ | 191.0559 | 99.68 | 0.683 |
| Dyphylline | $C_{10}H_{14}N_4O_4$ | 253.0938 | 98.18 | 0.936 |
| Cis-5-Caffeoylquinic acid | $C_{16}H_{18}O_9$ | 353.0895 | 95.67 | 7.427 |
| Monotropein | $C_{16}H_{22}O_{11}$ | 389.1093 | 91.1 | 7.453 |
| Apigenin 7-(2"-E-p-coumaroylglucoside) | $C_{30}H_{26}O_{12}$ | 577.1363 | 97.7 | 7.882 |
| 4-p-Coumaroylquinic acid | $C_{16}H_{18}O_8$ | 337.0928 | 99.75 | 8.107 |
| Epifisetinidol-4alpha-ol | $C_{15}H_{14}O_6$ | 289.0723 | 98.73 | 8.117 |
| Robinetinidol-(4alpha->8)-catechin-(6->4alpha)-robinetinidol | $C_{45}H_{38}O_{18}$ | 865.1982 | 99.37 | 8.296 |
| Robinetin 3-rutinoside | $C_{27}H_{30}O_{16}$ | 609.1479 | 95.09 | 8.765 |
| Kaempferol 3-(2"-(Z)-p-coumaryl-6"-(E)-p-coumarylglucoside) | $C_{39}H_{32}O_{15}$ | 739.1672 | 98.64 | 8.8 |
| 5,6,7,3',4'-Pentahydroxy-8-methoxyflavone 7-apioside | $C_{21}H_{20}O_{12}$ | 463.0891 | 96.62 | 8.975 |
| Luteolin 7-rhamnosyl(1->6)galactoside | $C_{27}H_{30}O_{15}$ | 593.152 | 99.07 | 8.99 |
| Cinchonain Ib | $C_{24}H_{20}O_9$ | 451.1039 | 99.03 | 9.051 |
| 6-Hydroxyluteoin-7-(6'''-p-coumarylsophoroside) | $C_{36}H_{36}O_{19}$ | 771.1779 | 99.52 | 9.729 |

**Table 5.** Compounds identified in *M. speciosa* leaves (100% M) through LC-MS QTOF positive ionization mode.

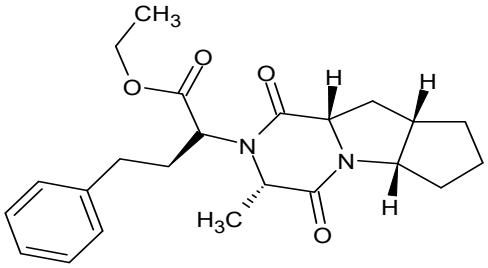
| Identified compounds | Formula | m/z | Score | RT |
|---|---|---|---|---|
| 2-Amino-3-methyl-1-butanol | $C_5H_{13}N_O$ | 104.1068 | 99.63 | 0.631 |
| Scopolin | $C_{16}H_{18}O_9$ | 355.1024 | 99.58 | 7.435 |
| Epifisetinidol-4alpha-ol | $C_{15}H_{14}O_6$ | 291.086 | 99.58 | 8.123 |
| Methyl reserpate | $C_{23}H_{30}N_2O_5$ | 415.2229 | 99.25 | 10.181 |
| Hirsutine | $C_{22}H_{28}N_2O_3$ | 369.217 | 99.51 | 10.426 |
| sRamipril diketopiperazine | $C_{23}H_{30}N_2O_4$ | 399.2286 | 98.55 | 10.906 |
| Mitragynine | $C_{23}H_{30}N_2O_4$ | 399.2281 | 99.6 | 11.068 |
| Quinine Ethyl Carbonate | $C_{23}H_{28}N_2O_4$ | 397.2124 | 98.63 | 11.368 |
| Emmotin A | $C_{16}H_{22}O_4$ | 279.1576 | 94.76 | 16.95 |
| Pheophorbide A | $C_{35}H_{36}N_4O_5$ | 593.2777 | 95.15 | 20.006 |



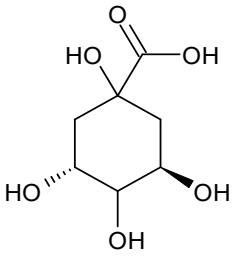
Pheophorbide A



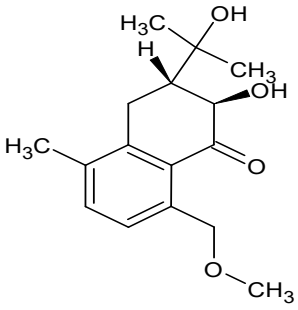
Mitragynine



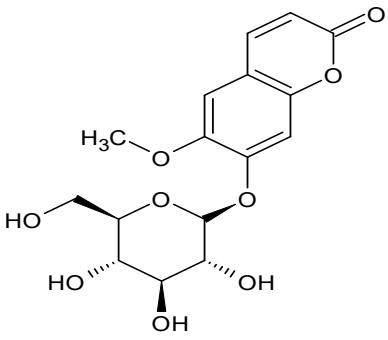
Ramipril diketopiperazine



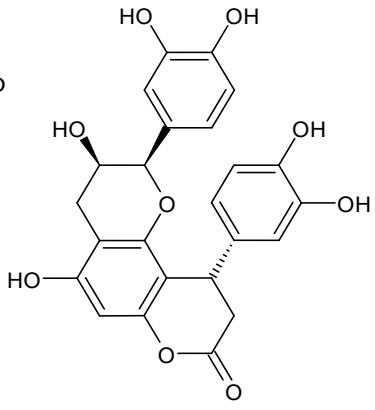
Quinic acid



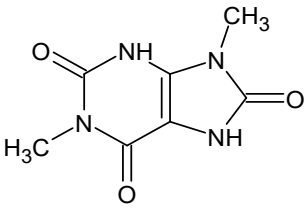
Emmotin A



Scopolin



Cinchonain Ib



1,9-Dimethyluric acid

**Figure 5.** Chemical structures of identified compounds in *M. speciosa* leaves (100% M).

Phenolic and flavonoid compounds have been proven to exhibit antioxidant, anticancer, antidiabetic, antihypertensive, antibacterial, antispasmodic, and antidepressant activities [37]. For instance, *cis*-5-caffeoylquinic acid exhibits significant antimicrobial properties, including activity against *E. coli*, *S. aureus,* and other pathogens [38]. Apigenin and its derivatives such as apigenin 7-(2"-E-p-coumaroylglucoside), are known for their antimicrobial activities, including antibacterial, antifungal, and antiviral properties [39]. Kaempferol and its derivatives such as kaempferol 3-(2”-(Z)-p-coumaryl-6”-(E)-p-coumarylglucoside), exhibit antibacterial, antifungal, and antiprotozoal activities [40]. Cinchonains such as cinchonain Ib are a group of naturally occurring flavonolignans that exhibit antioxidant, anti-inflammatory, and antimicrobial activities [41]. Moreover, Uracils have been reported to possess antiviral, anticancer and bactericidal activities [42]. Thus, the identified uracil compounds namely 5-acetylamino-6-formylamino-3-methyluracil and 1,9-dimethyluric acid may possess potent antimicrobial properties. Additionally, Pheophorbide A, a chlorophyll-breakdown product, has anticancer, anti-inflammatory, antiviral, antiparasitic, antioxidant, and immunostimulatory activities [43].

Thus, the identification of compounds in the extract through Q-ToF-LCMS analysis, demonstrated the occurrence of primarily phenolic and flavonoid compounds, along with alkaloids and other classes of phytoconstituents. These findings are consistent with the obtained results of phytochemical screening, antioxidant and antibacterial tests. Additionally, the outcome of the Q-ToF-LCMS analysis also corroborates the ethnobotanical use of *M. speciosa* for treating various diseases such as diarrhoea, intestinal infections, wound poultices, and inflammation as the identified phytochemical compounds in the extract have been scientifically proven to exert biological activity against these conditions.

## CONCLUSION

In conclusion, the study highlights the significant therapeutic potential of the phytoconstituents present in the methanol extract of *M. speciosa* leaves. The qualitative phytochemical screening confirmed the occurrence of a diverse array of biologically active compounds, known for their broad spectrum of biological activities. The extract demonstrated strong antioxidant properties, as evidenced by high TPC and TFC values, a high FRAP value, and a low $IC_{50}$ in the DPPH radical scavenging assay. Furthermore, the antimicrobial evaluations using disc diffusion and broth microdilution methods revealed notable efficacy against specific pathogens. The results indicated that the extract is particularly active against *S. aureus*, and *S. mutans* at the tested concentrations. The Q-ToF-LCMS analysis showed the presence of various phenolics, flavonoids, terpenoids, alkaloids and other phytoconstituents, which likely contribute to the observed results in the phytochemical screening, as well as the antioxidant, and antibacterial demonstrated in the assays. Overall, the study helps build a comprehensive insight into the phytochemical profile and biological activities of the methanol extract of *M. speciosa*, highlighting its potential as a promising natural source for the development of therapeutic agents.

## ACKNOWLEDGEMENTS

All authors are thankful to the Regal Malay Capital Berhad, Malaysia (SPP22-137-0137) and CPS Sejahtera Award and Research Management Centre, IIUM, Malaysia for the research support.

## REFERENCES

1. Begum, T., Arzmi, M. H., Khatib, A., Uddin, A. H., Aisyah Abdullah, M., Rullah, K., So’ad, S. Z. M., Haspi, N. F. Z., Sarian, M. N., Parveen, H., Mukhtar, S. and Ahmed, Q. U. (2025) A review on *Mitragyna speciosa* (Rubiaceae) as a prominent medicinal plant based on ethnobotany, phytochemistry and pharmacological activities. *Natural Product Research*, **39(6)**, 1636–1652.

2. Kruegel, A. C., Gassaway, M. M., Kapoor, A., Váradi, A., Majumdar, S., Filizola, M., Javitch, J. A. and Sames, D. (2016) Synthetic and receptor signaling explorations of the *Mitragyna* alkaloids: mitragynine as an atypical molecular framework for opioid receptor modulators. *Journal of the American Chemical Society*, **138(21)**, 6754–6764.

3. Begum, T., Arzmi, M. H., Uddin, A. H., Khatib, A., Abbas, S. A. and Ahmed, Q. U. (2024) *Mitragyna speciosa* Korth toxicity: Experimental findings and future prospects. *Journal of Taibah University Medical Sciences*, **19(6)**, 1143–1156.

4. Zehiroglu, C. and Ozturk Sarikaya, S. B. (2019) The importance of antioxidants and place in today’s scientific and technological studies. *Journal of Food Science and Technology*, **56**, 4757–4774.

5. Ahmed, M., Sajid, A. R., Javeed, A., Aslam, M., Ahsan, T., Hussain, D., Mateen, A., Li, X., Qin, P. and Ji, M. (2022) Antioxidant, antifungal, and aphicidal activity of the triterpenoids spinasterol and 22, 23-dihydrospinasterol from leaves of *Citrullus colocynthis* L. *Scientific Reports*, **12(1)**, 4910.

6. Abeyrathne, E. D. N. S., Nam, K., Huang, X. and Ahn, D. U. (2022) Plant-and animal-based anti-oxidants’ structure, efficacy, mechanisms, and applications: A review. *Antioxidants*, **11(5)**, 1025.

7. World Health Organization Expert Committee on Drug Dependence (2021) Pre-review report: Kratom (*Mitragyna speciosa*), mitragynine, and 7-

hydroxymitraginine. *Expert Committee on Drug Dependence Forty-fourth Meeting, Geneva*.

8. Szafranski, S. P., Winkel, A. and Stiesch, M. (2017) The use of bacteriophages to biocontrol oral biofilms. *Journal of Biotechnology*, **250**, 29–44.

9. Niculescu, A. G. and Grumezescu, A. M. (2021) Natural compounds for preventing ear, nose, and throat-related oral infections. *Plants*, **10(9)**, 1847.

10. Wang, Y., Liu, S., Jiang, Y., Zhou, X., Jing Chen, M., Li, B. R., Peng, X., Zhou, X. and Cheng, L. (2019) *Staphylococcus aureus* induces COX-2-dependent proliferation and malignant transformation in oral keratinocytes. *Journal of Oral Microbiology*, **11(1),** 1643205.

11. Tsai, M. S., Chen, Y. Y., Chen, W. C. and Chen, M. F. (2022) *Streptococcus mutans* promotes tumor progression in oral squamous cell carcinoma. *Journal of Cancer*, **13(12)**, 3358–3367.

12. Limcharoen, T., Pouyfung, P., Ngamdokmai, N., Prasopthum, A., Ahmad, A. R., Wisdawati, W., Prugsakij, W. and Warinhomhoun, S. (2022) Inhibition of α-glucosidase and pancreatic lipase properties of *Mitragyna speciosa* (Korth.) Havil. (kratom) leaves. *Nutrients*, **14(19),** 3909.

13. Ananda, Z. K., Begum, T., Ahda, M., Rofiee, M. S., Shah, S. A. A., Salleh, M. Z., Almutairi, B. O., Azmi, S. N. H., Sah, P, Wardani, A. K., Khatib, A., Abbas, S. A., Mia, M. A. R. and Ahmed, Q. U. (2025) Comparative evaluation of phytochemical screening, *in vitro* antioxidant & α-Glucosidase inhibitory properties of *Ceiba pentandra* & *Basella rubra* leaf extracts: Identification of active principles by Q-TOFLCMS, ADMET prediction & molecular docking approach. *Journal of King Saud University–Science*, **37**, 162024.

14. Settaluri, V. S., Al Anbari, T. M., Al Shukaili, S. K., Al Rawahi, R. M., Azmi, S. N. H., Sah, P., Reddy, S, H., Ahmed, Q. U., Hussain, S, A., Daddam, J. R., Sah, S. and Mahmood, S. (2024) Phytochemical screening and *in vitro* evaluation of antioxidant and antimicrobial efficacies of *Pteropyum scoparium* (Jaub. & Spach) Sidaf crude extracts. *Journal of King Saud University-Science*, **36(1)**, 102995.

15. Ladeska, V., Elya, B. and Hanafi, M. (2022) Anti-oxidants, Total Phenolic and Flavonoid Content and Toxicity Assay of Ampelas (*Tetracera macrophylla* Wall. Ex Hook. F. & Thoms) From Kalimantan-Indonesia. *Pharmacognosy Journal*, **14(5)**, 642–648.

16. Sari, K. R. P., Ikawati, Z., Danarti, R. and Hertiani, T. (2023) Micro-titer plate assay for measurement of total phenolic and total flavonoid contents in medicinal plant extracts. *Arabian Journal of Chemistry*, **16(9)**, 105003.

17. Shretha, D., Dhakal, K., Pokhrel, T., Sharma, P. and Adhikari, A. (2022) *In vitro* antioxidant and alpha-glucosidase inhibition activity of *Polygonatum verticillatum* of Karnali, Nepal. *Current Drug Therapy*, **17(3)**, 217–220.

18. Nipun, T. S., Khatib, A., Ahmed, Q. U., Nasir, M. H. M., Supandi, F., Taher, M. and Saiman, M. Z. (2021) Preliminary phytochemical screening, *in vitro* antidiabetic, antioxidant activities, and toxicity of leaf extracts of *Psychotria malayana* Jack. *Plants*, **10(12)**, 2688.

19. Arzmi, M. H., Dashper, S., Catmull, D., Cirillo, N., Reynolds, E. C. and McCullough, M. (2015) Coaggregation of *Candida albicans*, *Actinomyces naeslundii* and *Streptococcus mutans* is *Candida albicans* strain dependent. *FEMS Yeast Research*, **15(5)**, fov038.

20. Qiao, J., Zheng, L., Lu, Z., Meng, F. and Bie, X. (2021) Research on the biofilm formation of Staphylococcus aureus after cold stress. *Microorganisms*, **9(7)**, 1534.

21. Ab Rahim, N., Zakaria, N., Dzulkarnain, S. M., Othman, N. and Abdulla, M. A. (2019) Antibacterial activity of *Alstonia angustifolia* leaf extract against *Staphyloccoccal* and *Bacilli*. *Biomedical Research*, **30(1)**, 11–15.

22. Njeru, S. N., Obonyo, M. A., Ngari, S. M., Onsarigo, S. N., Njeru, J. M. and Kamweru, P. K. (2015) Antituberculous, antimicrobial, cytotoxicity and phytochemical activity study of Piliostigma thonningii extract fractions. *Journal of Medicinal Plants Research*, **9(22)**, 655–663.

23. Olawuwo, O. S., Famuyide, I. M. and McGaw, L. J. (2022) Antibacterial and antibiofilm activity of selected medicinal plant leaf extracts against pathogens implicated in poultry diseases. *Frontiers in Veterinary Science*, **9**, 820304.

24. Nipun, T. S., Khatib, A., Ibrahim, Z., Ahmed, Q. U., Redzwan, I. E., Saiman, M. Z., Supandi, F., Primaharinastiti, R., El-Seedi, H. R. (2020) Characterization of α-glucosidase inhibitors from *Psychotria malayana* Jack leaves extract using LC-MS-based multivariate data analysis and *in silico* molecular docking. *Molecules*, **25**, 5885.

25. Asao, T. and Asaduzzaman, M. (Eds.) (2018) Phytochemicals: Source of Antioxidants and Role in Disease Prevention. *InTech, London*.

26. Zailan, N. F. Z., Zaidan, U. H., Mahayidin, H. and Hassan, M. (2019) *Mitragyna speciosa* methanol extract (MSME) exhibits potential anti-oxidative

property and inhibitory activity on tumour growth stimulating cytokines in SW480 colorectal cancer cell lines. *Malaysian Journal of Medical Sciences*, **15(108)**, 35–35.

27. Munteanu, I. G. and Apetrei, C. (2021) Analytical methods used in determining antioxidant activity: A review. *International Journal of Molecular Sciences*, **22(7)**, 3380.

28. Itam, A., Wati, M. S., Agustin, V., Sabri, N., Jumanah, R. A. and Efdi, M. (2021) Comparative study of phytochemical, antioxidant, and cytotoxic activities and phenolic content of *Syzygium aqueum* (Burm. f. Alston f.) extracts growing in West Sumatera Indonesia. *The Scientific World Journal*, 5537597.

29. Webber, D. M., Wallace, M. A. and Burnham, C. A. D. (2022) Stop waiting for tomorrow: disk diffusion performed on early growth is an accurate method for antimicrobial susceptibility testing with reduced turnaround time. *Journal of Clinical Microbiology*, **60(5)**, e03007-20.

30. Veiga, A., Maria da Graça, T. T., Rossa, L. S., Mengarda, M., Stofella, N. C., Oliveira, L. J., Gonçalves, A. G. and Murakami, F. S. (2019) Colorimetric microdilution assay: Validation of a standard method for determination of MIC, IC50%, and IC90% of antimicrobial compounds. *Journal of Microbiological Methods*, **162**, 50–61.

31. Eloff, J. N. (2019) Avoiding pitfalls in determining antimicrobial activity of plant extracts and publishing the results. *BMC Complementary and Alternative Medicine*, **19**, 1–8.

32. Hudzicki, J. (2009) Kirby-Bauer disk diffusion susceptibility test protocol. *American Society for Microbiology*, **15(1),** 1–23.

33. Bovo, F., Lazzarotto, T., Ambretti, S. and Gaibani, P. (2023) Comparison of broth microdilution, disk diffusion and strip test methods for cefiderocol antimicrobial susceptibility testing on KPC-producing *Klebsiella pneumoniae*. *Antibiotics*, **12(3)**, 614.

34. do Socorro Costa, M., da Silva, A. R. P., Araújo, N. J. S., Paulo, C. L. R., Alencar, G. G., de Alencar, M. A. S., Moura, T. F., Lima, C. M. G., de Freitas, T. S., Filho, J. M. B., Andrade-Pinheiro, J. C. and Coutinho, H. D. M. (2024) Assessment of the potential susceptibility of planktonic cells and bacterial biofilms by diosgenin. *Food Bioscience*, **62**, 105106.

35. Atta, L., Siddiqui, A. R., Mushtaq, M., Munsif, S., Nur-e-Alam, M., Ahmed, A. and Ul-Haq, Z. (2025) Molecular insights into antibiofilm inhibitors of *Streptococcus mutans* glucosyltransferases through *in silico* approaches. *Scientific Reports*, **15(1),** 14160.

36. Allen, D. R. & McWhinney, B. C. (2019) Quadrupole time-of-flight mass spectrometry: a paradigm shift in toxicology screening applications. *The Clinical Biochemist Reviews*, **40(3)**, 135.

37. Pitt, J. J. (2009) Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry. *The Clinical Biochemist Reviews*, **30(1),** 19.

38. Chen, Q., Wang, X., Yuan, X., Shi, J., Zhang, C., Yan, N. and Jing, C. (2021) Comparison of Phenolic and Flavonoid Compound Profiles and Antioxidant and α-Glucosidase Inhibition Properties of Cultivated Soybean (*Glycine max)* and Wild Soybean *(Glycine soja). Plants,* **10(83)**, 1–14.

39. Fahsi, N., Mahdi, I., Annaz, H., Bitchagno, G. T. M., Mahmoud, M. F. & Sobeh, M. (2024) Unlocking the therapeutic potential of cinchonains: a comprehensive review. *Phytochemistry Reviews*, **24(1)**, 197–233.

40. Wang, M., Firrman, J., Liu, L. and Yam, K. (2019) A review on flavonoid apigenin: Dietary intake, ADME, antimicrobial effects, and interactions with human gut microbiota. *BioMed Research Internation*al, **2019(1)**, 7010467.

41. Periferakis, A., Periferakis, K., Badarau, I. A., Petran, E. M., Popa, D. C., Caruntu, A., Costache, R. A., Scheau, C., Caruntu, C. and Costache, D. O. (2022) Kaempferol: antimicrobial properties, sources, clinical, and traditional applications. *International Journal of Molecular Sciences*, **23(23)**, 15054.

42. Pałasz, A. and Cież, D. (2015) In search of uracil derivatives as bioactive agents. Uracils and fused uracils: Synthesis, biological activity and applications. *European Journal of Medicinal Chemistry*, **97**, 582–611.

43. Saide, A., Lauritano, C. and Ianora, A. (2020) Pheophorbide a: State of the Art. *Marine Drugs*, **18(5)**, 257.