JCNaR
Journal of Chemical Natural Resources
Journal homepage: https://jcnar.usu.ac.id

# Long-Term Ambient Storage and Retained Hypnotic–Sedative Activity of a Confiscated *Mitragyna speciosa* Specimen: A Forensic Chemical and Pharmacological Evaluation

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**ARTICLE INFO**
**Article history:**
Received 12 June 2026
Revised 10 July 2026
Accepted 15 July 2026
Available online 26 July 2026

E-ISSN: 2656-1492
P-ISSN:

**How to cite:**
Supiyani and Sarah Salsabil. Long-Term Ambient Storage and Retained Hypnotic–Sedative Activity of a Confiscated *Mitragyna speciosa* Specimen: A Forensic Chemical and Pharmacological Evaluation. Journal of Chemical Natural Resources. 2025, 7(2):133-148.


https://doi.org/10.32734/jcnar.v7i2.25976

**ABSTRACT**

*Mitragyna speciosa* (kratom) is a Southeast Asian medicinal plant whose principal alkaloid, mitragynine, exhibits stimulant and sedative properties. Long-term preservation of bioactive constituents in stored plant material is a quality control concern. This study investigated alkaloid stability and pharmacological recoverability of *M. speciosa* leaf powder stored under ambient, uncontrolled conditions for over ten years following confiscation in 2016. Three extraction solvents, ethanol, acetone, and water–methanol, were employed and their phytochemical profiles compared by Thin-Layer Chromatography with iodoplatinate visualization and Gas Chromatography–Mass Spectrometry (GC–MS). Phytochemical screening revealed consistent presence of alkaloids, sterols, triterpenoids, fatty acids, terpenoids, phenolics, and nitrogen-containing heterocycles across all solvent systems. Acetone yielded the highest relative mitragynine content (8.52%) compared with ethanol (7.01%) and water–methanol (3.64%). Preserved pharmacological activity of the acetone extract was confirmed in vivo using the glass cylinder escape latency test in male mice (Mus musculus), demonstrating a statistically significant, concentration-dependent hypnotic–sedative effect across doses of 4.5–114 mg/kg. One-way ANOVA with Tukey's HSD and Duncan's post hoc tests revealed statistically significant differences among treatment groups ($p < 0.001$). Within the limitations of this single-specimen evaluation, findings indicate that a detectable alkaloid profile and measurable hypnotic–sedative activity of *M. speciosa* are retained over extended ambient storage, with solvent selection influencing relative mitragynine recovery; absolute quantification and comparison with a fresh reference specimen were not possible and are recommended. This study provides reference data for forensic evaluation of aged kratom specimens and underscores the need for standardized storage and quality control protocols for kratom preparations.

**Keyword:** Forensic Pharmacognosy, Hypnotic–Sedative, Mitragynine, *Mitragyna speciosa*, Long-Term Stability.

**ABSTRAK**

*Mitragyna speciosa* (kratom) adalah tanaman obat Asia Tenggara yang alkaloid utamanya, mitraginin, menunjukkan sifat stimulan dan sedatif. Pelestarian jangka panjang konstituen bioaktif dalam bahan tanaman yang disimpan merupakan masalah pengendalian mutu. Studi ini menyelidiki stabilitas alkaloid dan pemulihan farmakologis bubuk daun *M. speciosa* yang disimpan dalam kondisi lingkungan yang tidak terkontrol selama lebih dari sepuluh tahun setelah disita pada tahun 2016. Tiga pelarut ekstraksi, etanol, aseton, dan air-metanol, digunakan dan profil fitokimianya dibandingkan dengan Kromatografi Lapis Tipis dengan visualisasi iodoplatinat dan Kromatografi Gas-Spektrometri Massa (GC-MS). Skrining fitokimia mengungkapkan keberadaan alkaloid, sterol, triterpenoid, asam lemak, terpenoid, fenolik, dan heterosiklik yang mengandung nitrogen secara konsisten di semua sistem pelarut. Aseton menghasilkan kandungan mitraginin

relatif tertinggi (8,52%) dibandingkan dengan etanol (7,01%) dan air-metanol (3,64%). Aktivitas farmakologis ekstrak aseton yang terjaga dikonfirmasi secara in vivo menggunakan uji latensi pelarian silinder kaca pada tikus jantan (Mus musculus), yang menunjukkan efek hipnotik-sedatif yang signifikan secara statistik dan bergantung pada konsentrasi pada dosis 4,5–114 mg/kg. ANOVA satu arah dengan uji HSD Tukey dan uji post hoc Duncan mengungkapkan perbedaan yang signifikan secara statistik antar kelompok perlakuan ($p < 0,001$). Dalam keterbatasan evaluasi spesimen tunggal ini, temuan menunjukkan bahwa profil alkaloid yang terdeteksi dan aktivitas hipnotik-sedatif yang terukur dari *M. speciosa* dipertahankan selama penyimpanan suhu ruangan yang lama, dengan pemilihan pelarut memengaruhi pemulihan mitraginin relatif; kuantifikasi absolut dan perbandingan dengan spesimen referensi segar tidak mungkin dilakukan dan direkomendasikan. Studi ini memberikan data referensi untuk evaluasi forensik spesimen kratom yang sudah tua dan menggarisbawahi perlunya protokol penyimpanan dan kontrol kualitas yang terstandarisasi untuk sediaan kratom.

**Kata kunci**: Farmakognosi Forensik, Hipnotik-Sedatif, Mitraginin, *Mitragyna speciosa*, Stabilitas Jangka Panjang.

## 1. Introduction

*Mitragyna speciosa* Korth. (Havil.) is a tropical tree of the family Rubiaceae, indigenous to Southeast Asia, commonly known as kratom. It has been traditionally used for its stimulant, analgesic, and sedative properties [1]. The pharmacological activity of *M. speciosa* is attributed primarily to its complex alkaloid mixture, with *mitragynine* as the predominant bioactive constituent. *Mitragynine* exerts significant modulatory effects on the Central Nervous System (CNS) via partial agonism at μ-opioid receptors and interactions with adrenergic, serotonergic, and dopaminergic systems [2]. Analytical characterization of *M. speciosa* has been undertaken using multiple platforms, including TLC, LC/GC–MS, UPLC–HRMS, and quantitative NMR (qNMR), each offering distinct advantages in sensitivity and throughput [3–5].

The selection of extraction solvent critically determines the yield and composition of bioactive compounds recovered from plant material. Chatepa et al. [6] demonstrated that solvent polarity substantially influences phytoconstituent solubility and antioxidant yield in medicinal plant extracts. Conventional solvents such as water, ethanol, and acetone exhibit distinct polarity profiles, enabling differential extraction of hydrophilic and lipophilic phytochemicals [7]. Aqueous extraction is consistent with traditional consumption practices (kratom tea), but typically yields lower alkaloid concentrations due to the limited aqueous solubility of non-polar alkaloids. In contrast, organic solvents such as ethanol and acetone demonstrate higher extraction efficiency for alkaloids and other secondary metabolites, potentially enhancing bioactive yield and pharmacological relevance [8]. Despite these known solvent effects, systematic comparative analyses evaluating the effect of solvent polarity on mitragynine content, particularly in aged specimens, remain limited.

*Mitragyna speciosa* is consumed and commercially distributed in various forms, including dried leaves, teas, powders, capsules, and liquid extracts [9]. Product stability is a major quality control concern, as prolonged storage, drying processes, and environmental exposure may alter the alkaloid profile and reduce biological potency [10]. Such physicochemical transformations challenge the standardization of kratom preparations, particularly when aged or confiscated materials are subjected to analytical or pharmacological evaluation.

The existing kratom literature, however, has characterized alkaloid content and pharmacological activity almost exclusively in freshly collected leaf material or in specimens maintained under defined laboratory storage conditions [3,4]. Neither scenario reflects the reality of forensic casework, in which confiscated botanical evidence is typically retained for years in evidence facilities where temperature, humidity, and light exposure are neither regulated nor recorded. Whether the chemical and pharmacological properties established for fresh or laboratory-archived kratom generalize to material recovered from long-term, uncontrolled evidence storage has, to our knowledge, not been directly examined. The present study addresses this gap by characterizing the alkaloid profile of *M. speciosa* leaf material subjected to prolonged ambient storage without controlled temperature or humidity, a scenario frequently encountered in forensic repositories. This same specimen is used to characterize the alkaloid profile of *M. speciosa* leaf powder stored for over ten years following law enforcement confiscation, using TLC and GC–MS analysis across three extraction solvent systems. Furthermore, the neuropharmacological activity of the acetone extract, which yielded the highest *mitragynine* content, was assessed *in vivo* using the glass cylinder escape latency (fireplace) test in male mice.

This study thus provides a case-level evaluation of whether kratom alkaloids and their associated hypnotic–sedative activity remain detectable after long-term ambient storage, and offers comparative solvent-selection data of practical interest to forensic laboratories handling similarly aged exhibits.

The novelty of this research, compared to previous studies on kratom alkaloid characterization and pharmacology, lies not in the chemical or pharmacological methodology itself, but in the systematic evaluation of forensic specimens stored under uncontrolled, natural environmental conditions for over ten years. To the best of the authors' knowledge, this scenario has not previously been documented with combined confirmation from both chemical analysis and in vivo pharmacological studies.

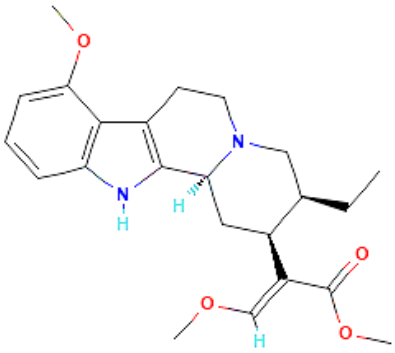

Figure 1. Chemical structure of mitragynine (the principal alkaloid of *Mitragyna speciosa*).

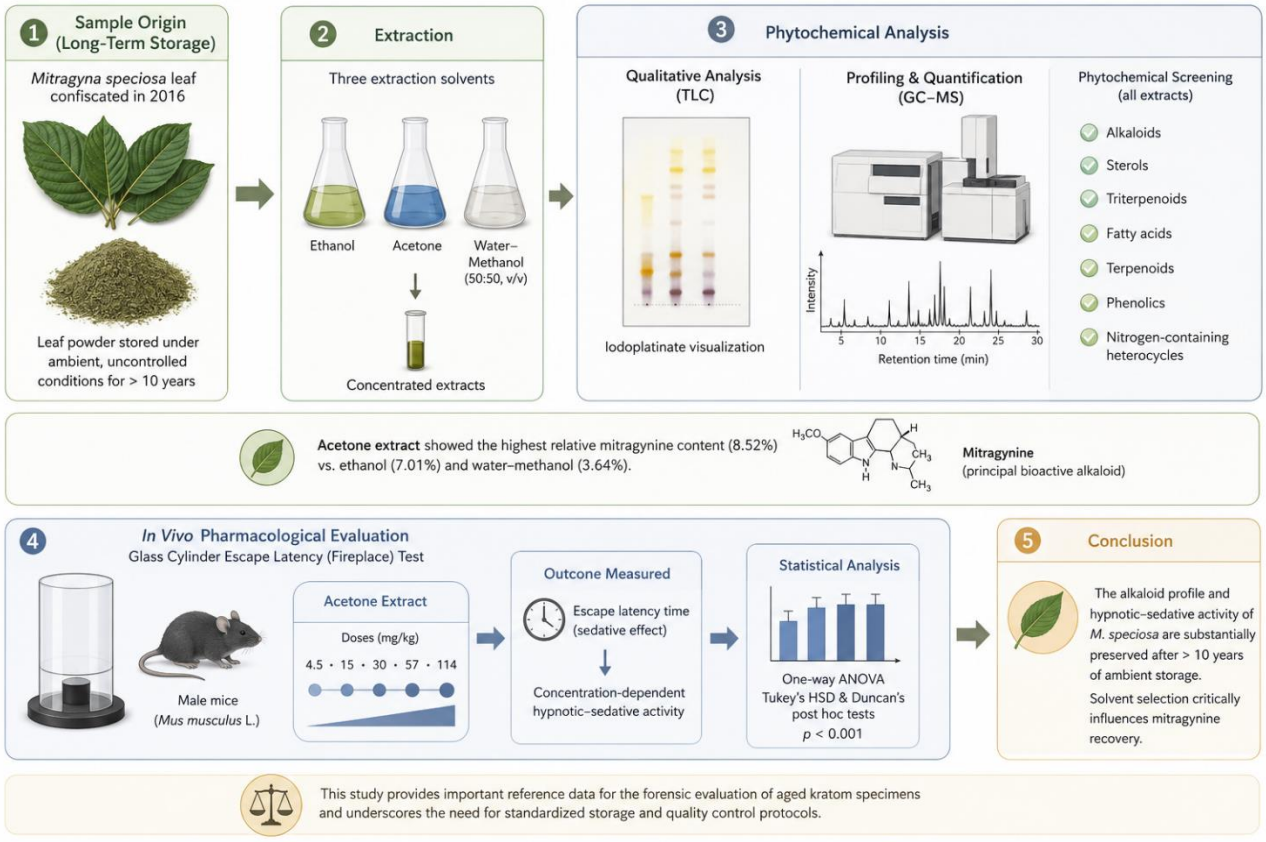


Figure 2. Overall experimental workflow, from long-term ambient storage of the confiscated leaf material through the three parallel solvent extractions, TLC and GC–MS characterization, selection of the acetone extract, in vivo hypnotic–sedative evaluation, and forensic interpretation.

## 2. Experimental

### *2.1 Materials*

Analytical-grade reagents, methanol, ethanol, and acetone (Merck, Darmstadt, Germany) and distilled water were used throughout. Diazepam tablets (PT. Meprofarm, Bandung, Indonesia) served as the positive pharmacological reference control. Sodium carboxymethylcellulose (Na-CMC) was used as vehicle (1% w/v aqueous solution) for both the negative control and extract suspension. The dried *M. speciosa* leaf sample consisted of leaf powder confiscated by the Aceh Regional Police in 2016 (registration number TA/13/IV/2016/NNF) from Drin Rampak Village, Johan Pahlawan District, West Aceh Regency, and maintained in the forensic archive of the North Sumatra Regional Police Laboratory since the date of confiscation. The GC–MS system comprised an Agilent Technologies GC 7890B / MSD 5977A instrument (Agilent Technologies, Santa Clara, CA, USA). As is typical of evidence retained in forensic repositories rather than climate-controlled archival facilities, the custody record documents the chain of possession and storage duration but does not include temperature, relative humidity, light exposure, or packaging logs for the storage period. These parameters could not be reconstructed retrospectively and are not reported here. The implications of this gap for interpreting the present findings are discussed in Section 3.8.

### *2.2 Sample Preparation*

Dried *M. speciosa* leaves were ground in a blender and sieved to a particle size of 50 mesh. Three separate batches of the fine leaf powder were subjected to independent, parallel extraction using ethanol, acetone, and water–methanol (1:1, v/v), respectively, each at a solid-to-solvent ratio of 1:10 (w/v), based on the procedure of Mustafa et al. [11] with modifications. Each batch was agitated using an orbital shaker (Oregon KJ-201B D, Oregon Scientific, Portland, OR, USA) for 1 h at room temperature, followed by ultrasonication for 30 min [12]. Mixtures were subsequently centrifuged at 2500 rpm for 10 min, and the supernatants were filtered through Whatman No. 42 filter paper (particle retention: 2.5 μm). Filtrates were dried at ambient temperature to constant weight, and the dried extracts were stored at 4°C until analysis. Each extract was characterized by TLC and GC–MS. Because the aim of the in vivo phase was to establish whether the aged specimen retained measurable hypnotic–sedative bioactivity at all, a single extract was selected for behavioral testing following chemical screening, rather than testing all three solvent systems in parallel. Acetone was selected because it yielded the highest relative mitragynine content among the three solvents tested (8.52%, versus 7.01% in ethanol and 3.64% in water–methanol) and, unlike the water–methanol system, can be fully removed by evaporation at ambient temperature without residual solvent toxicity, making it compatible with safe oral administration. This selection criterion reflects analytical performance and practical safety rather than a direct measure of comparative biological activity; comparative in vivo evaluation of the ethanol and water–methanol extracts was not performed, owing to the limited quantity of specimen available from a single confiscated batch (Section 3.8). Extractions and GC–MS analyses were performed once per solvent, without technical replicates, reflecting the limited quantity of confiscated specimen available for this single forensic exhibit. Gravimetric extraction yield (% w/w) was not recorded for the three solvent systems. Solvent performance is instead compared through the relative GC–MS peak area of mitragynine, as reported in Tables 1–3. Both constraints, absence of replicate determinations and of gravimetric yield data, limit the precision and reproducibility that can be claimed for the reported chemical profile and are addressed further in Section 3.8.

### *2.3 Characterization by TLC*

Kratom extracts were applied onto silica gel 60 $F_{254}$ TLC plates (Merck, Darmstadt, Germany) using a capillary tube (internal diameter 1.55 ± 0.05 mm). Sample tracks were applied at 1.5 cm inter-track spacing, 1.0 cm from the bottom edge of the plate, within a Camag TLC development chamber. The mobile phase consisted of ethyl acetate : methanol : ammonia (8.5 : 1.0 : 0.5, v/v/v). Elution was performed for approximately 8 min. The plate was heated at 100°C for 5 min to remove residual ammonia, and developed plates were examined under UV light at 254 nm and 366 nm, then sprayed with iodoplatinate reagent for detection of secondary amine-containing alkaloids. A certified methamphetamine hydrochloride reference standard (forensic laboratory archive) was applied in a dedicated reference track (track 9) to serve as a secondary amine positive control for iodoplatinate reactivity. $R_f$ values were calculated and compared with literature values for *mitragynine*.

*2.4 Characterization by GC–MS*

GC–MS analysis was performed on an Agilent Technologies system (GC 7890B / MSD 5977A) using an HP-5MS capillary column (30 m × 0.250 mm × 0.25 μm film thickness). Helium carrier gas (purity ≥99.999%) was delivered at a constant flow rate of 1.0 mL/min. Sample extracts (1 μL) were injected in splitless mode. The oven temperature program was: initial hold at 100°C for 2 min, ramped at 15°C/min to 290°C, with a final isothermal hold of 12 min. The MS scan was operated in full-scan mode (*m/z* 35–550) using electron ionization at 70 eV. Compound identification was performed by library matching against the NIST14 mass spectral database using MassHunter software. A minimum match quality threshold of 80% was applied; compounds below this threshold are included in the tables but are flagged for further verification. Compounds identified with library match scores at or below this threshold are reported here as tentative rather than confirmed. Before being treated as confirmed plant metabolites, they should ideally be verified using complementary analytical techniques such as LC–MS/MS or authentic reference standards. Mitragynine and rhynchophylline, the two alkaloids on which the chemical and pharmacological conclusions of this study depend, matched at 99% in every extract in which they were detected and are treated throughout as confidently identified. The compounds affected by the 80% threshold are minor or trace constituents (Tables 1–3), several of which are noted below as likely solvent-derived artifacts.

*2.5 Testing Hypnotic–Sedative Activity in Mice (Mus musculus L.)*

*2.5.1 Adaptation of Test Animals*

Twenty-one male ICR mice (*Mus musculus* L.), aged approximately 2 months and weighing 25–30 g, were acclimatized for one week prior to treatment under standard laboratory conditions (12:12 h light:dark cycle, 22 ± 2°C, *ad libitum* water access). Food was withheld for 18 h immediately prior to each pharmacological experiment, with water access maintained throughout [13]. Mice are commonly used as test animals in pharmacological studies due to their anatomical and physiological parallels to mammalian CNS pharmacology.

*2.5.2 Treatment of Test Animals*

This study received ethical approval from the Animal Research Ethics Committee (AREC) of the Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara (Approval No. 0007/KEPH-FMIPA/2025), and was conducted in accordance with established guidelines for the use of laboratory animals. Mice were randomly allocated into seven groups ($n$ = 3/group): a negative control group receiving 1% Na-CMC vehicle (0.1 mL/10 g body weight by oral gavage); a positive control group receiving diazepam (human therapeutic dose: 2 mg/70 kg, converted to a mouse-equivalent dose of approximately 0.35 mg/kg using the established FDA $K_m$-based allometric scaling factor [human $K_m$ = 37; mouse $K_m$ = 3]); and five treatment groups receiving the acetone extract of *M. speciosa* at ascending dose levels. Both diazepam solution and extract preparations were administered as a single oral dose by gavage. Each group consisted of 3 mice.

Group P1: *M. speciosa* acetone extract 0.5% (w/v), volume 0.025 mL/mouse (≈4.5 mg/kg)
Group P2: *M. speciosa* acetone extract 1.0% (w/v), volume 0.05 mL/mouse (≈18.2 mg/kg)
Group P3: *M. speciosa* acetone extract 1.5% (w/v), volume 0.075 mL/mouse (≈40.9 mg/kg)
Group P4: *M. speciosa* acetone extract 2.0% (w/v), volume 0.10 mL/mouse (≈72.7 mg/kg)
Group P5: *M. speciosa* acetone extract 2.5% (w/v), volume 0.125 mL/mouse (≈113.6 mg/kg)

All gavage volumes (0.025–0.125 mL/mouse) were within the accepted capacity of the murine stomach (maximum approximately 0.4 mL) as reported by McConnell et al. [18]. The incremental dosing scheme was designed to characterize the dose–response relationship and to reflect the range of exposures associated with escalating human consumption of *M. speciosa* preparations.

*2.5.3 Hypnotic–Sedative Test (Glass Cylinder Escape Latency Test)*

Sedative activity was assessed using the glass cylinder escape latency test (locally referred to as the "fireplace test") [13,15], a validated rodent behavioral assay for evaluating CNS depressant activity. Each mouse was placed individually inside a vertically oriented borosilicate glass cylinder (250 mL capacity, open top) placed on a flat surface. The latency (in seconds) for the animal to exit the cylinder was recorded over a maximum observation period of 5 min. In non-sedated animals, escape latency is typically less than 30 s; sedated animals display a prolonged escape latency reflecting reduced voluntary motor activity and CNS depression. Measurements were recorded for each mouse at defined time intervals following oral administration of the respective treatment.

*2.5.4 Statistical Analysis*

All data were expressed as mean ± standard deviation (SD). Prior to ANOVA, the normality of data distribution within each group was confirmed using the Shapiro–Wilk test ($p > 0.05$ in all groups). Dose effects on escape latency were analyzed by one-way ANOVA using IBM SPSS Statistics v26. Where significant *F*-ratios were obtained, pairwise comparisons were performed using Tukey's Honest Significant Difference (HSD) and Duncan's Multiple Range post hoc tests. The significance threshold was set at $\alpha = 0.05$.

## 3. Results and Discussion

*3.1 Phytochemical Analysis of M. speciosa Leaf Extracts by Thin-Layer Chromatography*

Figure 3 presents TLC plates from the elution of acetone (A), ethanol (B), and water–methanol (C) extracts of kratom leaves, developed in ethyl acetate : methanol : ammonia (8.5 : 1.0 : 0.5, v/v/v) and visualized following iodoplatinate staining. Each plate was spotted with eight sample tracks (tracks 1–8) and one reference track (track 9). Multiple separated bands were observed across all plates, reflecting the complex multi-component profiles of the three extracts.

All three extract plates displayed a characteristic reddish-violet to gray coloration on tracks 1–8 at $R_f \approx 0.91$, corresponding in color and $R_f$ value to the methamphetamine reference standard (track 9) on the same plate. The iodoplatinate reagent selectively stains secondary and tertiary amine-containing compounds, producing purple-violet colorations characteristic of alkaloids [16]. The methamphetamine reference standard serves as a secondary amine positive control for iodoplatinate reactivity; the comparable staining pattern observed in the kratom extract tracks qualitatively confirms the presence of secondary amine-bearing alkaloids in all three extraction systems. The reddish-purple coloration at $R_f \approx 0.91$ is consistent with the behavior of *mitragynine*, an indole alkaloid bearing a secondary amine moiety [3], supporting its qualitative identification in all three solvents.

The detection of alkaloids by TLC in leaf powder stored for over ten years without controlled temperature or humidity provides preliminary evidence of chemical stability over the storage period. This observation is corroborated quantitatively by the GC–MS data described in subsequent sections. TLC was used here as a rapid, preliminary screening step to compare the general alkaloid profiles of the three extracts ahead of GC–MS characterization. The resulting spot patterns and Rf values are qualitative and are not treated as confirmatory of compound identity. Quantitative techniques such as HPLC or LC–MS/MS would provide stronger, concentration-based evidence of mitragynine preservation than either TLC or relative-area GC–MS can offer, and are recommended for future confirmatory work on aged forensic kratom specimens.

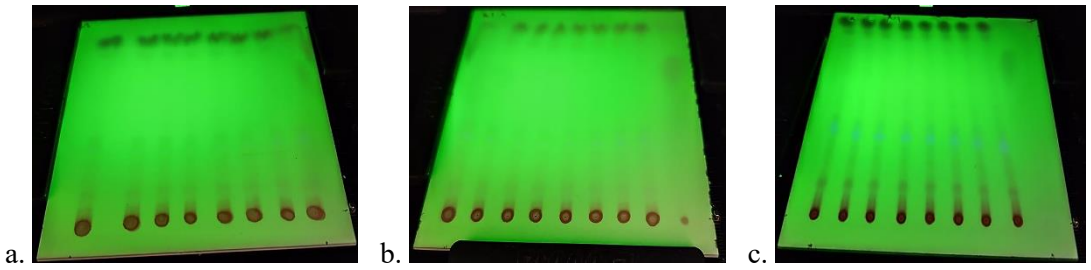


Figure. 3. TLC plates from elution of kratom leaf extracts: a. Plate A (acetone extract), b. Plate B (ethanol extract), c. Plate C (water–methanol extract). Mobile phase: ethyl acetate : methanol : ammonia (8.5 : 1.0 : 0.5, v/v/v). Visualization: iodoplatinate spray.

*3.2 Identification of Phytochemicals in Ethanol Extract of M. speciosa Leaves*

The GC–MS profile of the ethanol extract identified 17 major compounds (Figure 4; Table 1). Ethanol is an amphiprotic solvent capable of extracting semi-polar to moderately non-polar compounds through both hydrogen bonding and van der Waals interactions [17]. The dominant alkaloids identified were *rhynchophylline* (28.43%) and *mitragynine* (7.01%), consistent with their amphiphilic character and established ethanol extractability. The phytosterols γ-sitosterol (15.69%) and campesterol (5.18%) were also prominent, attributable to their polycyclic steroidal skeletons. Retention times for these key components were 19.381, 25.063, 23.797, and 22.37 min, respectively. Fatty acid derivatives (*n*-hexadecanoic acid and its esters)

and terpenoids (phytol, neophytadiene) were also recovered, reflecting the broad polarity range accessible to ethanol extraction.

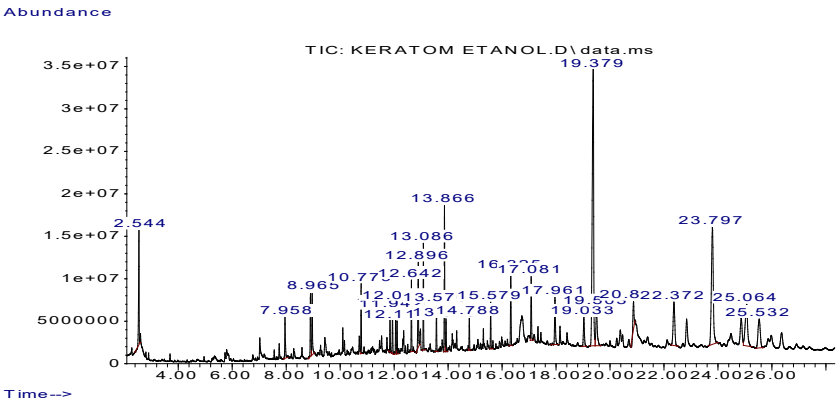


Figure. 4. Total ion chromatogram (TIC) of the ethanol extract of *M. speciosa* leaves (GC–MS analysis).

Table 1. Phytochemicals identified in the ethanol extract of *M. speciosa* leaves by GC–MS.

| Group | Compound Name | RT (min) | Area (%) | Quality |
|---|---|---|---|---|
| Alkaloids | *Rhynchophylline* | 19.381 | 28.43 | 99 |
| | *Speciofoline* | 20.875 | 1.49 | 93 |
| | *Mitragynine* | 25.063 | 7.01 | 99 |
| Sterol/Triterpenoids | *Campesterol* | 22.37 | 5.18 | 94 |
| | *γ-Sitosterol* | 23.797 | 15.69 | 99 |
| | *Stigmasta-3,5-dien-7-one* | 25.53 | 4.55 | 86 |
| Fatty acids & derivatives | *Butanoic acid, 2-[(phenylmethoxy)imino]-, trimethylsilyl ester* | 2.544 | 4.43 | 76 |
| | *Hexadecanoic acid, methyl ester* | 12.641 | 2.36 | 98 |
| | *n-Hexadecanoic acid* | 12.895 | 3.79 | 99 |
| | *Hexadecanoic acid, ethyl ester* | 13.087 | 3.47 | 99 |
| Terpenoids/Isoprenoids | *Neophytadiene* | 12.06 | 1.81 | 99 |
| | *2-Pentadecanone, 6,10,14-trimethyl-* | 12.112 | 1.15 | 99 |
| | *Phytol* | 13.865 | 5.01 | 99 |
| Phenolics & Aromatic | *2-Methoxy-4-vinylphenol* | 7.955 | 1.87 | 95 |
| | *Benzaldehyde, 4-hydroxy-* | 8.967 | 2.73 | 86 |
| Nitrogen-containing/Other | *Guaifenesin* | 11.852 | 1.46 | 90 |
| | *2-Hydroxy-1,8-naphthyridine* | 13.57 | 1.64 | 90 |

*3.3 Identification of Phytochemicals in Acetone Extract of M. speciosa Leaves*

GC–MS analysis of the acetone extract identified 25 major compounds (Figure 5; Table 2). Acetone, a medium-polarity ketonic solvent, selectively solubilizes compounds with intermediate polarity and specific heteroatom functionalities. The alkaloids *rhynchophylline* (15.09%) and *mitragynine* (8.52%) were identified as major constituents. Notably, the relative *mitragynine* content in the acetone extract (8.52%) exceeded that in both the ethanol extract (7.01%) and the water–methanol extract (3.64%), indicating that acetone preferentially partitions *mitragynine* relative to co-extracted matrix components. This is consistent with the intermediate log *P* of *mitragynine* (≈1.28) and the capacity of acetone to dissolve compounds across a broad mid-polarity range. γ-Sitosterol (13.63%) was again a prominent sterol. Several early-eluting compounds in the acetone extract, including 1,2,4-triazolidin-3-one (RT 2.601 min; 13.14%), silanediol dimethyl (RT 2.673 min; 2.9%), and 2-pentanone-4-hydroxy-4-methyl (RT 3.529 min; 7.13%) displayed retention times consistent with low molecular weight volatiles. These compounds are likely solvent-related artifacts or chromatographic contaminants and are marked accordingly in Table 2. These entries should be interpreted with caution and verified by independent methods prior to attribution as genuine plant metabolites.

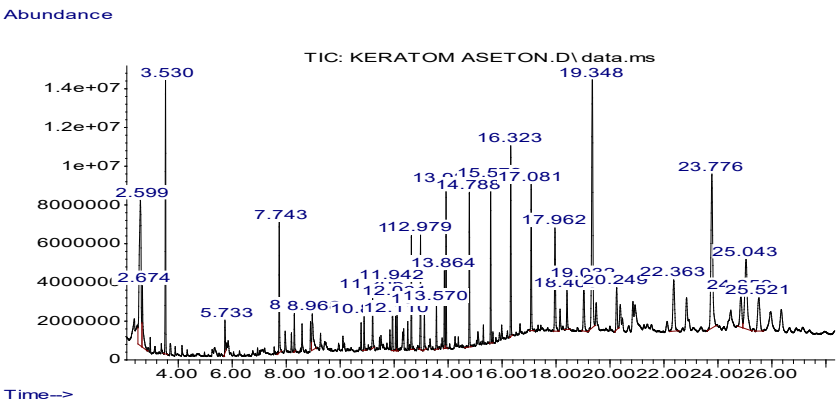


Figure 5. Total ion chromatogram (TIC) of the acetone extract of *M. speciosa* leaves (GC–MS analysis).

Table 2. Phytochemicals identified in the acetone extract of *M. speciosa* leaves by GC–MS.

| Group | Compound Name | RT (min) | Area (%) | Quality |
|---|---|---|---|---|
| Alkaloids | *Pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)-* | 8.303 | 0.95 | 97 |
| | *Rhynchophylline* | 19.35 | 15.09 | 99 |
| | *Mitragynine* | 25.042 | 8.52 | 99 |
| Sterol/Triterpenoids | *Campesterol* | 22.364 | 4.47 | 90 |
| | *γ-Sitosterol* | 23.776 | 13.63 | 99 |
| Fatty acids & derivatives | *2-Tetradecene, (E)-* | 10.887 | 0.89 | 98 |
| | *Dodecyl isobutyl ether* | 11.203 | 1.56 | 87 |
| | *Hexadecanoic acid, methyl ester (methyl palmitate)* | 12.641 | 2.71 | 96 |
| | *Undecane* | 13.123 | 1.12 | 90 |
| | *n-Nonadecanoic acid, pentamethyldisilyl ester* | 15.577 | 4.04 | 94 |
| | *Eicosane* | 18.411 | 1.43 | 97 |
| | *Octadecane* | 20.247 | 2.18 | 98 |
| Terpenoids/Isoprenoids | *Neophytadiene* | 12.06 | 1.17 | 95 |
| | *2-Pentadecanone, 6,10,14-trimethyl-* | 12.111 | 0.85 | 98 |
| | *Phytol* | 13.865 | 1.83 | 99 |
| Phenolics & Aromatic | *Benzaldehyde, 2-methyl-* | 5.735 | 0.90 | 97 |
| | *4'-Methylpropiophenone* | 7.743 | 3.57 | 83 |
| | *Phenol, 2,6-dimethyl-* | 8.967 | 2.40 | 90 |
| | *Coumarin, 3,4-dihydro-4,4,6,8-tetramethyl-* | 24.85 | 3.03 | 86 |
| Nitrogen-containing/Other[1] | *1,2,4-Triazolidin-3-one, 4-methyl-2-phenyl-5-thioxo-* [1] | 2.601 | 13.14 | 80 |
| | *Silanediol, dimethyl-* [1] | 2.673 | 2.90 | 90 |
| | *2-Pentanone, 4-hydroxy-4-methyl-* [1] | 3.529 | 7.13 | 76 |
| | *Cyclononasiloxane, octadecamethyl-* | 11.94 | 1.69 | 91 |
| | *2-Hydroxy-1,8-naphthyridine* | 13.569 | 1.19 | 86 |
| | *3H-pyrazol-3-one, 5-amino-2,4-dihydro-4-(4-methylphenoxy)-2-phenyl-* | 25.519 | 3.62 | 76 |

*[1] Compounds marked with superscript 1 elute at very short retention times and may represent solvent artifacts or chromatographic contaminants rather than genuine plant metabolites; further verification is recommended.*

### *3.4 Identification of Phytochemicals in Water–Methanol Extract of M. speciosa Leaves*

GC–MS analysis of the water–methanol extract identified 30 major compounds (Figure 6; Table 3). The water–methanol solvent system preferentially extracts highly polar and semi-polar compounds through extensive hydrogen bonding and ionization interactions under aqueous conditions. Accordingly, polar alkaloids, including *rhynchophylline* (23.90%), *3-epi-isorotundifoline* (6.43%), *speciofoline* (3.83%), and *mitragynine* (3.64%) were detected. *Rhynchophylline* reached its highest relative abundance across all three solvents in this system (23.90%), while *mitragynine* showed its lowest relative recovery (3.64%), reflecting its intermediate polarity and comparatively lower affinity for highly polar aqueous-organic systems. Polar phenolic derivatives (2-methoxy-4-vinylphenol, 4-hydroxy-benzaldehyde) and terpenoids (phytol, neophytadiene) were also present.

It should be noted that methanol is toxic and the water–methanol solvent system, while analytically powerful for resolving polar metabolites, produces extracts unsuitable for direct *in vivo* administration without complete solvent removal and redissolution in a biocompatible vehicle [18]. For pharmacological testing, acetone which yields the highest *mitragynine* recovery and can be fully removed by evaporation at ambient temperature (boiling point: 56°C) is the preferred extraction solvent.

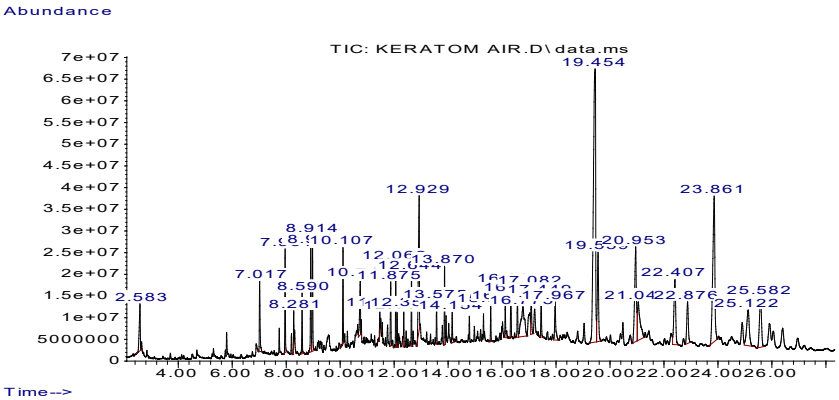


Figure 6. Total ion chromatogram (TIC) of the water–methanol extract of *M. speciosa* leaves (GC–MS analysis).

Table 3. Phytochemicals identified in the water–methanol extract of *M. speciosa* leaves by GC–MS.

| Group | Compound Name | RT (min) | Area (%) | Quality |
|---|---|---|---|---|
| Alkaloids | *Rhynchophylline* | 19.453 | 23.90 | 99 |
| | *3-Epi-isorotundifoline* | 20.953 | 6.43 | 95 |
| | *Speciofoline* | 21.052 | 3.83 | 94 |
| | *Mitragynine* | 25.12 | 3.64 | 99 |
| Sterol/Triterpenoids | *Methyl 3β-hydroxyolean-18-en-28-oate* | 16.776 | 3.94 | 90 |
| | *Campesterol* | 22.406 | 4.50 | 99 |
| | *Stigmasterol* | 22.878 | 2.44 | 99 |
| | *γ-Sitosterol* | 23.864 | 11.63 | 99 |
| | *Cholesta-3,5-dien-7-one* | 25.581 | 3.91 | 80 |
| Fatty acids & derivatives | *Hexadecanoic acid, methyl ester* | 12.646 | 1.30 | 99 |
| | *n-Hexadecanoic acid* | 12.931 | 4.91 | 99 |
| | *Octadecanoic acid* | 14.156 | 0.70 | 99 |
| | *Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester* | 16.122 | 0.73 | 87 |
| Terpenoids/Isoprenoids | *Neophytadiene* | 12.065 | 1.62 | 99 |
| | *2-Pentadecanone, 6,10,14-trimethyl-* | 12.111 | 0.64 | 91 |
| | *3,7,11,15-Tetramethyl-2-hexadecen-1-ol* | 12.361 | 0.69 | 78 |
| | *Phytol* | 13.87 | 1.59 | 99 |
| Phenolics & Aromatic | *2-Methoxy-4-vinylphenol* | 7.96 | 2.09 | 95 |
| | *Phenol, 2,6-dimethoxy-* | 8.282 | 0.56 | 97 |
| | *Benzaldehyde, 4-hydroxy-* | 8.915 | 2.69 | 80 |
| | *(E)-Stilbene* | 10.109 | 1.97 | 82 |
| | *4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol* | 11.473 | 0.48 | 96 |
| Nitrogen-containing/Other | *Butanoic acid, 2-[(phenylmethoxy)imino]-, trimethylsilyl ester* | 2.585 | 1.88 | 76 |
| | *Benzofuran, 2,3-dihydro-* | 7.016 | 1.92 | 86 |
| | *2-Pyrazolin-5-ol, 5-tert-butyl-3-trifluoromethyl-* | 8.588 | 1.28 | 86 |
| | *1-(3-methylbenzoyl)-* | | | |
| | *1-propyl-1H-indole* | 10.747 | 1.88 | n.d. |
| | *Guaifenesin* | 11.873 | 1.47 | 84 |
| | *2-Hydroxy-1,8-naphthyridine* | 13.575 | 0.86 | 86 |
| | *3,5,6-Trimethyl-p-quinone, 2-(2,5-* | 16.574 | 1.07 | 95 |
| | *dioxotetrahydrofuran-3-yl)thio-* | | | |
| | *Ethanone, 1-[4-[4-(dimethylamino)benzylideneamino]phenyl]-* | 17.451 | 1.06 | 83 |

*3.5 Comparative GC–MS Phytochemical Profiles and Implications for Long-Term Stability*

Across all three extraction systems, the GC–MS data demonstrated a broadly conserved phytochemical profile in *M. speciosa* leaf powder stored for over ten years, comprising alkaloids, phytosterols, fatty acids, terpenoids, phenolics, and nitrogen-containing heterocycles. The consistent detection of *mitragynine* and *rhynchophylline* pharmacologically active alkaloids known for CNS modulation, opioid receptor partial agonism, calcium channel blocking, and neuroprotective activity, indicates remarkable chemical stability under ambient storage conditions without controlled temperature, humidity, or light exposure. This is significant because alkaloid degradation in plant material is typically accelerated by moisture, heat, oxidative stress, and microbial activity; the present findings suggest that these degradative forces were insufficient over the approximately ten-year storage period to substantially deplete the major alkaloid fraction.

The selective enrichment of *mitragynine* in the acetone extract relative to ethanol and water–methanol is consistent with the physicochemical properties of *mitragynine* (log $P \approx 1.28$, molecular weight 398.49 g/mol) and acetone's ability to efficiently solubilize medium-polarity indole alkaloids while excluding more polar co-extractables. The co-occurrence of phytosterols (γ-sitosterol, campesterol, stigmasterol) and fatty acid derivatives across all solvents confirms their robust extractability and suggests these lipophilic classes are equally stable over long-term storage.

The broader phytochemical complexity of the water–methanol extract (30 compounds) compared with ethanol (17) and acetone (25) suggests that the aqueous-organic system achieves greater total metabolite coverage, which may be relevant to comprehensive metabolic profiling. However, for targeted mitragynine recovery, which is relevant to potency standardization and forensic quantification, acetone outperformed the other systems tested.

The stability data presented here have direct implications for the forensic evaluation of confiscated kratom samples, where storage conditions prior to analysis are rarely controlled. The results suggest that dried leaf material may retain analytically detectable and pharmacologically relevant alkaloid profiles for periods exceeding ten years under typical ambient storage, challenging assumptions about rapid alkaloid degradation in aged specimens. Future investigations should examine the quantitative impact of specific storage variables (temperature, humidity, UV exposure, oxygen) on alkaloid stability using validated absolute quantification methods. These findings have practical relevance for forensic laboratory practice. Analysts and courts frequently must interpret confiscated botanical evidence retained for years pending legal proceedings, typically without environmental monitoring or a matched fresh reference. That this single confiscated kratom specimen retained a chemically detectable, tentatively identifiable alkaloid profile and measurable in vivo hypnotic–sedative activity after more than ten years of uncontrolled ambient storage supports the broader premise that long-retained botanical narcotic evidence can still yield analytically and pharmacologically meaningful results, relevant to retrospective investigations, appeals, or re-examination of evidence using updated methods. We are careful, however, not to extend this beyond what a single specimen can support. Translating it into specific forensic protocols, for example minimum retesting intervals or confidence thresholds for aged samples, would require validation across multiple confiscated specimens spanning a range of documented storage durations and conditions, which this study was not designed to provide. We present these findings as motivation for such validation work rather than as a basis for immediate protocol change.

Detection of mitragynine and rhynchophylline by GC–MS after more than ten years of ambient storage indicates that these compounds retain sufficient structural and spectral integrity to be recovered and identified. Chemical stability is the compounds which remain detectable and chromatographically resolvable, and biological or pharmacological stability is the specimen retaining its original capacity to produce an equivalent magnitude of physiological effect. Both are related but conceptually distinct properties, and are linked here only through the separate in vivo confirmation described in Section 3.6. The behavioral findings reported there indicate that the acetone extract retained measurable hypnotic–sedative activity. Whether this activity is diminished, unchanged, or altered in character relative to a hypothetical fresh extract from the same source cannot be determined from the present data. This is compounded by the fact that this study evaluated a single confiscated

batch of plant material without a freshly collected reference sample, so the absolute magnitude of any change relative to a time-zero baseline cannot be determined

Comparison with freshly harvested *M. speciosa*, and inclusion of additional samples with different storage histories, would substantially strengthen the generalizability of these conclusions. A related question is why a chemical and behavioral signal persisted at all after such prolonged ambient storage. One observation from the present data may bear on rhynchophylline, a corynanthe-type oxindole alkaloid, was the most abundant alkaloid by relative peak area in all three extracts (28.43% ethanol, 15.09% acetone, 23.90% water–methanol), exceeding mitragynine, the corresponding indole alkaloid (7.01%, 8.52%, and 3.64%, respectively), in every case. The indole system of mitragynine-type alkaloids is known to be oxidatively labile at the position corresponding to mitragynine's C-7, and oxidative rearrangement of this indole scaffold to a spirocyclic oxindole structure is a documented transformation for this alkaloid class, most thoroughly characterized as an enzymatic, CYP3A-mediated metabolic pathway in vivo [22]. Whether an analogous, non-enzymatic transformation contributed to the comparatively higher oxindole-to-indole alkaloid ratio observed in this decade-old specimen is, at present, a hypothesis rather than a finding: rhynchophylline is also a naturally occurring constituent of *M. speciosa*, and its relative abundance is known to vary between individual plants independently of any storage history [3,4]. We cannot distinguish between these two explanations, natural chemotype variation versus storage-related oxidative conversion, with the data available, since no unstored reference from the same source plant exists and the GC–MS method used here was not designed to search for specific transformation products [23]. We present the oxidative-conversion hypothesis as a concrete, testable direction for future work rather than as an explanation we can confirm here.

*3.6 Hypnotic–Sedative Activity of Acetone Extract of M. speciosa*

Sedative activity was assessed using the glass cylinder escape latency test in male mice. Escape latency serves as an indirect index of CNS depression: sedated animals exhibit reduced voluntary motor activity and a delayed escape response. The acetone extract of *M. speciosa* produced a progressive, concentration-dependent increase in mean escape latency across the five dose groups (Table 4; Figure 7), confirming preserved hypnotic–sedative bioactivity in the long-term stored material.

Table 4. Hypnotic–sedative activity of acetone extract of *M. speciosa* (glass cylinder escape latency test). Values expressed as mean ± SD; *n* = 3/group.

| Treatment | Dose (mL \| mg/kg) | Escape Latency (s) |
|---|---|---|
| Positive Control (+): Diazepam | 0.05 \| ≈0.35 | 39.67 ± 3.512 |
| Negative Control (−): Na-CMC 1% | 0.10 \| vehicle | 11.67 ± 3.055 |
| *M. speciosa* 0.5% | 0.025 \| ≈4.5 | 36.67 ± 1.528 |
| *M. speciosa* 1.0% | 0.05 \| ≈18.2 | 41.00 ± 1.000 |
| *M. speciosa* 1.5% | 0.075 \| ≈40.9 | 50.33 ± 4.509 |
| *M. speciosa* 2.0% | 0.10 \| ≈72.7 | 55.33 ± 1.528 |
| *M. speciosa* 2.5% | 0.125 \| ≈113.6 | 66.33 ± 3.215 |

*Dose (mg/kg) calculated for mean mouse body weight of 27.5 g. Diazepam dose converted from human dose (2 mg/70 kg) using FDA $K_m$-based allometric scaling.*

The dose-dependent increase in escape latency across groups P1–P5 is consistent with a concentration-dependent pharmacological effect. At the lowest dose (P1: ≈4.5 mg/kg), the mean escape latency (36.67 ± 1.53 s) did not differ significantly from the positive control (diazepam, ≈0.35 mg/kg; 39.67 ± 3.51 s), placing both groups in the same post hoc subset. At the highest dose tested (P5: ≈113.6 mg/kg), escape latency (66.33 ± 3.22 s) significantly exceeded the diazepam reference. These results indicate that the acetone extract produces a dose-dependent sedative effect at this behavioral endpoint that, at the lowest dose tested, is statistically indistinguishable from that of diazepam; they are not evidence of equivalent pharmacodynamic potency, for reasons discussed below. Diazepam and the kratom alkaloids act through distinct primary pharmacodynamic mechanisms, direct $GABA_A$ positive allosteric modulation versus opioid-receptor partial agonism, so a statistically indistinguishable escape-latency value at one dose does not imply equivalent receptor-level potency, therapeutic index, or safety profile.

Even at the minimal gavage volume of 0.025 mL (P1: ≈4.5 mg/kg), a measurable and statistically significant sedative response was observed. This finding provides direct evidence that the alkaloids in *M. speciosa* stored for over ten years under ambient, uncontrolled conditions retain sufficient biological activity to elicit CNS depressant effects *in vivo*. The incremental dose groups were designed to reflect the range of exposures associated with escalating human *M. speciosa* consumption, which commonly involves progressive increases in leaf quantity and intake frequency during habituation.

The mechanistic basis of the observed sedative effects likely involves multiple receptor systems. *Mitragynine* is a partial agonist at μ-opioid receptors and has been reported to modulate adrenergic and serotonergic pathways, contributing to both CNS stimulation at low doses and depression at higher doses [21]. Diazepam, used here as the positive control, produces sedation through positive allosteric modulation of $GABA_A$ receptors, opening chloride ion channels and hyperpolarizing the neuronal membrane [22]. Mitragynine reaches its effect through a different primary route, opioid-receptor partial agonism, described above; this is a mechanistically distinct path to a superficially similar behavioral endpoint, and is the reason the diazepam comparison above is presented as a behavioral benchmark rather than evidence of shared mechanism. The extract tested here, moreover, was not isolated mitragynine but the whole acetone extract, in which GC–MS also identified rhynchophylline and several minor corynanthe-type alkaloids at appreciable relative abundance. Whole kratom extracts have been reported to engage opioid and monoaminergic signaling in ways not fully predicted by mitragynine content alone, with contributions from co-occurring indole and oxindole alkaloids [2,23]. We therefore regard the sedative activity observed in this study as most plausibly reflecting the combined action of mitragynine together with these co-occurring alkaloids, whose relative contributions, and whether they act additively, synergistically, or in some respects in opposition to one another, cannot be resolved from a whole-extract dosing design. This mechanistic account remains an inference rather than a demonstrated one for the present material, for the reasons just described, and should be read accordingly.

The glass cylinder escape latency test is a validated and widely used sedative screen, but it assesses a single behavioral endpoint. Incorporating complementary assays, such as the open-field test for locomotor and anxiety-related activity or the rotarod test for motor coordination, would allow a more comprehensive characterization of the hypnotic–sedative profile of this extract and would help distinguish genuine sedation from generalized motor impairment

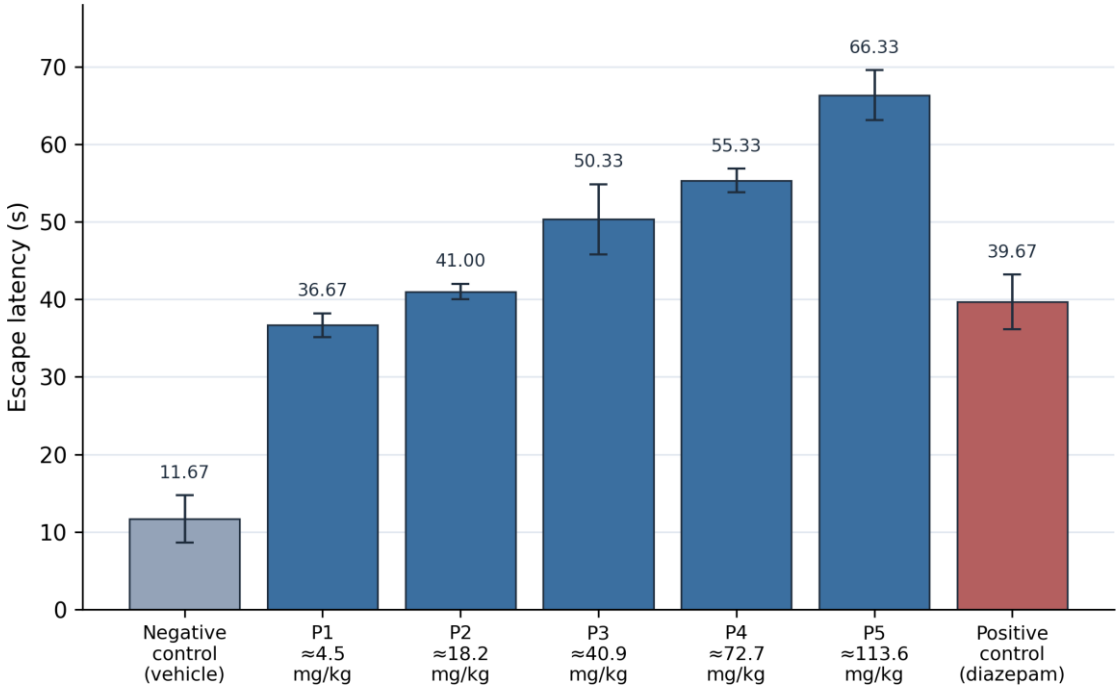


Figure 7. Concentration–response relationship for the glass cylinder escape latency test following administration of *M. speciosa* acetone extract. Points represent group means (n = 3).

*3.7 Statistical Analysis*

Parametric statistical analysis was performed using one-way ANOVA. The Shapiro–Wilk test confirmed normal distribution within all groups prior to ANOVA. ANOVA demonstrated statistically significant effects of treatment on escape latency when compared with both the negative control ($F(5, 12) = 139.877$, $p < 0.001$) and the positive control ($F(5, 12) = 47.485$, $p < 0.001$), as shown in Table 5.

Table 5. One-way ANOVA results for the effect of *M. speciosa* acetone extract on escape latency.

| Comparison | df | Sum of Squares | Mean Square | F | p-value |
|---|---|---|---|---|---|
| Treatment vs. Positive Control | 5 | 1925.778 | 385.156 | 47.485 | <0.001 |
| Treatment vs. Negative Control | 5 | 5323.111 | 1064.622 | 139.877 | <0.001 |

*df: degrees of freedom.*

The higher *F*-value in the comparison with the negative control (139.877) relative to the positive control comparison (47.485) reflects the larger contrast between vehicle-treated mice and all pharmacologically treated groups, consistent with the near-zero sedative baseline of the negative control. Post hoc analysis using Tukey's HSD and Duncan's Multiple Range tests confirmed the pattern of group differences (Tables 6 and 7). All kratom-treated groups differed significantly from the negative control ($p < 0.05$). The 0.5% and 1.0% doses were not significantly different from each other (Tukey's HSD subset b) but both differed from the negative control. The 1.5% and 2.0% groups shared a subset under Tukey's HSD (less conservative) but were separated by the more sensitive Duncan test, consistent with the higher discriminatory power of the latter [24].

Table 6. Tukey's HSD and Duncan's post hoc subsets (comparison with negative control). Groups sharing the same letter do not differ significantly ($p > 0.05$).

| Treatment | Tukey HSD Subset | Duncan Subset |
|---|---|---|
| Negative Control (−) | 11.67[a] | 11.67[a] |
| *M. speciosa* 0.5% | 36.67[b] | 36.67[b] |
| *M. speciosa* 1.0% | 41.00[b] | 41.00[b] |
| *M. speciosa* 1.5% | 50.33[c] | 50.33[c] |
| *M. speciosa* 2.0% | 55.33[c] | 55.33[d] |
| *M. speciosa* 2.5% | 66.33[d] | 66.33[e] |

*Significance within Tukey's HSD subsets: a (p = 1.000), b (p = 0.434), c (p = 0.297), d (p = 1.000). Within-subset p-values > 0.05 confirm homogeneity within each subset.*

Comparison against the positive control (Table 7) placed the diazepam group (39.67 ± 3.51 s), the 0.5% dose (36.67 ± 1.53 s), and the 1.0% dose (41.00 ± 1.00 s) in the same statistically homogeneous subset (Tukey's and Duncan's HSD, subset a; $p > 0.05$). This indicates that low-dose *M. speciosa* acetone extract produces sedative effects statistically equivalent to the diazepam reference at the dose tested. Higher extract concentrations (1.5–2.5%) formed progressively distinct subsets with significantly greater escape latencies than the diazepam group.

Table 7. Tukey's HSD and Duncan's post hoc subsets (comparison with positive control). Groups sharing the same letter do not differ significantly ($p > 0.05$).

| Treatment | Mean ± SD (s) [Subset] |
|---|---|
| Positive Control (+): Diazepam | 39.67[a] |
| *M. speciosa* 0.5% | 36.67[a] |
| *M. speciosa* 1.0% | 41.00[a] |
| *M. speciosa* 1.5% | 50.33[b] |
| *M. speciosa* 2.0% | 55.33[b] |
| *M. speciosa* 2.5% | 66.33[c] |

*Tukey's HSD within-subset significance: a (p = 0.101), b (p = 0.053), c (p = 1.000). All within-subset p-values > 0.05, confirming homogeneity within each subset.*

Together, the ANOVA and post hoc results confirm a statistically significant, linear, concentration-dependent sedative response in the acetone extract of long-term stored *M. speciosa*, with effects ranging from statistically equivalent to (low doses) and significantly greater than (high doses) the diazepam reference. The preserved bioactivity after more than ten years of ambient storage, evidenced both analytically (GC–MS) and pharmacologically (escape latency), constitutes the central finding of this study.

*3.8 Limitations*

Several limitations should be considered when interpreting these findings. Chemical and pharmacological characterization was performed on a single confiscated batch of *M. speciosa*. Alkaloid ratios can vary naturally between individual plants and populations [3,4], so this specimen's profile cannot be assumed to represent confiscated kratom generally. The storage history is documented with respect to duration and custody but not environmental conditions (Section 2.1); no freshly collected reference sample from the same source exists, so the extent of any change from an unstored baseline cannot be determined. Compound identification relied on GC–MS library matching without authenticated mitragynine or rhynchophylline standards; several minor constituents returned match scores ≤80% and are reported as tentative (Section 2.4). Alkaloid content is expressed as relative peak area rather than absolute concentration, and extractions and GC–MS analyses were performed once per solvent rather than in replicate, reflecting the limited, non-renewable quantity of forensic specimen available; gravimetric extraction yield (% w/w) was not recorded and cannot be reconstructed retrospectively. Our GC–MS method was not designed to specifically search for degradation products or contamination introduced during storage, so neither can be confirmed nor excluded from the present data. The in vivo evaluation used a single behavioral endpoint (glass cylinder escape latency) in a small number of animals per group (n = 3), which limits statistical power and the breadth of behavioral domains assessed; complementary assays such as open-field or rotarod testing were not performed.

Despite these constraints, this study offers a rare case-level demonstration that a botanical narcotic exhibit can retain a chemically detectable and behaviorally active alkaloid profile after more than ten years of uncontrolled ambient storage, a scenario common in forensic practice but largely absent from the published literature. We present it as a methodological template, solvent screening, TLC/GC–MS characterization, and in vivo confirmation of retained bioactivity, that could be applied prospectively to additional confiscated specimens with fuller custody documentation, rather than as a definitive statement about kratom stability in general.

## 4. Conclusion

This study demonstrated that ambient, long-term storage of *M. speciosa* leaf material for over ten years does not result in complete degradation of its alkaloid profile or pharmacological activity. GC–MS analysis confirmed the preserved presence of mitragynine, rhynchophylline, phytosterols, fatty acid derivatives, and other bioactive metabolites across all three extraction systems (ethanol, acetone, and water–methanol), with acetone yielding the highest relative mitragynine recovery (8.52% area) owing to its compatibility with mitragynine's intermediate polarity. In vivo hypnotic–sedative testing confirmed dose-dependent CNS depressant activity (≈4.5–114 mg/kg equivalent) in male mice, with effects statistically indistinguishable from the diazepam reference at low doses and significantly exceeding it at higher doses (one-way ANOVA with post hoc analysis, $p < 0.001$), establishing that mitragynine and associated alkaloids in prolonged-storage kratom leaves retain biological activity sufficient to produce measurable CNS depression. Given the small per-group sample size (n = 3) and reliance on relative quantification from a single confiscated batch, these findings offer a case-level reference point for the pharmacognostic evaluation of aged, forensically relevant kratom specimens and comparative solvent data of practical interest to forensic laboratories, though broader validation across multiple specimens is needed before they inform forensic casework protocols or are extended to commercial kratom product standardization, which raises distinct quality-control questions this single specimen was not designed to address.

## 5. Ethical Approval

This study complies with animal testing guidelines, as it has obtained an ethical review letter from the Animal Research Ethics Committee (AREC) of the Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, No. 0007/KEPH-FMIPA/2025.

## 6. Author Contributions

S.Y. contributed substantially to the conceptualization, experimental design, data collection, data analysis, and manuscript preparation. S.S. contributed to data collection, data analysis, and manuscript writing. Both authors have read and approved the final manuscript.

## 7. Acknowledgements

The authors acknowledge Universitas Sumatera Utara for providing ethical approval and laboratory support for this research.

## 8. Conflicts of Interest

The authors declare no conflict of interest.

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