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Optimizing ultrasound-assisted extraction for enhanced quantification of 7-hydroxymitragynine and mitragynine in kratom

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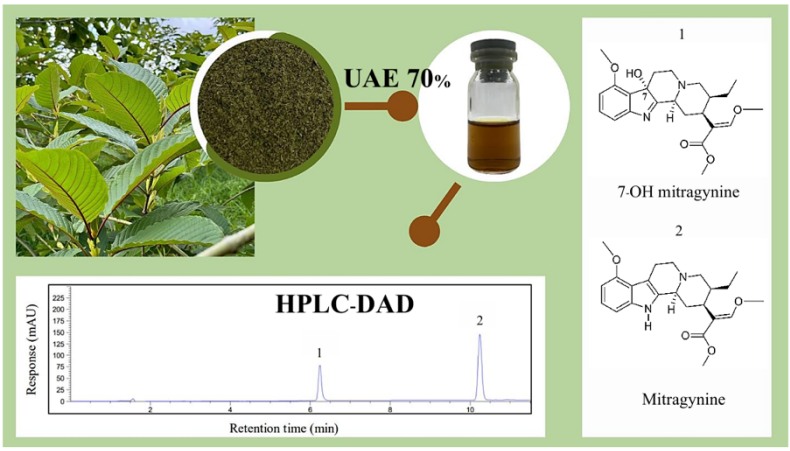
Abstract

This study focuses on optimizing the extraction of two key alkaloids, mitragynine and 7-hydroxy mitragynine, from kratom (*Mitragyna speciosa*) leaves using ultrasound-assisted extraction (UAE). The aim was to identify optimal extraction conditions by varying sample-to-liquid ratio, solvent types, methanol concentrations, ultrasonic power, and extraction time. High-performance liquid chromatography with diode array detection (HPLC-DAD) was used to quantify the alkaloids accurately. The results indicate that using a sample-to-liquid ratio of 0.5:10 g mL^{-1}, 70% v/v methanol as the solvent, with an ultrasonic power of 175 watts, and a 10 min for extraction time, yielded the highest amounts of mitragynine and 7-hydroxymitragynine. This method offers a reliable, efficient approach for extracting and quantifying these bioactive compounds, supporting the development of standardized kratom products for quality control. The study has practical applications for local farmers in Phetchabun province, improving the extraction process for kratom leaves.

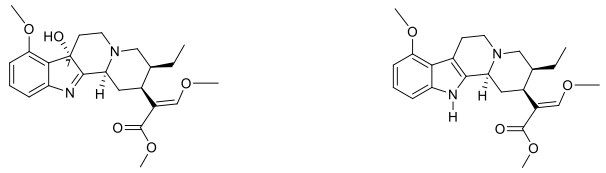


Keyword: Kratom; Mitragynine; 7-Hydroxymitragynine; Ultrasound-Assisted Extraction; HPLC

1. Introduction

Mitragyna speciosa, known as kratom, is a traditional herbal plant native to Southeast Asia. It is mainly found in Malaysia, Thailand, Myanmar, Papua New Guinea, and Indonesia. Depending on the region, the plant goes by different local names, such as ketum or biak in Malaysia, keton or Kadamba in Indonesia, and ithang, thom, and kratom in Thailand [1,2]. In Thailand, there are at least three identified varieties of kratom: tengkwa, yaksayai, and kratom khao daeng (red stem) [3]. Kratom has

been removed from the list of controlled substances and can now be legally cultivated, consumed, and sold for economic and community benefits. However, legal restrictions still exist on its processing into food, herbal products, or medicines. Producers must obtain proper authorization and comply with the Ministry of Public Health's guidelines to ensure product quality and prevent misuse [4]. In traditional medicine, kratom has been used for many years to treat a variety of ailments, including diarrhoea, pain relief, muscle aches, and sedation. It has also been used as a remedy for more complex conditions, such as opioid withdrawal, headaches, toothaches, coughs, fever, and insomnia. In addition, it has been applied in treating stroke symptoms, typhoid fever, cholesterol issues, hypertension, diabetes, and anxiety. Kratom is also known for stimulating appetite, healing wounds, and expelling parasites [1-3,5].



7-OH mitragynine Mitragynine

Fig. 1 Structures of 7-OH mitragynine and mitragynine.

The alkaloids in kratom, especially mitragynine and 7-hydroxy (OH) mitragynine (Fig. 1 shows their structures), have gotten much attention because they have effects similar to opioids and could be used in medicine. Mitragynine, the dominant alkaloid, acts as a partial agonist at the μ-opioid receptor, contributing to its pain-relieving properties. It also serves as a competitive antagonist at κ- and δ-opioid receptors, which gives it a unique pharmacological profile. 7-OH mitragynine, on the other hand, is a metabolite of mitragynine that binds much more strongly to the β-opioid receptor, strengthening its pain-relieving effects [6]. Mitragynine, being the dominant alkaloid in kratom, is frequently used as a marker compound for quality control and characterization of kratom leaves in analytical testing, making it crucial for the standardization and quality assurance of kratom products [7]. Most analyses are carried out using chromatographic techniques like HPLC-UV [8-9], LC-MS/MS [10], and UPLC-MS/MS [11] for the quantification of alkaloids in kratom.

Ultrasound-assisted extraction (UAE) uses ultrasonic waves to induce cavitation in liquid media, producing shock waves that disrupt plant cell walls and release target compounds into the solvent. There are two types of UAE: probe-type, which delivers intense, direct energy for efficient, small-scale extractions, and bath-type, which provides more uniform energy distribution suitable for large-scale operations but with reduced intensity [12]. Previous research has highlighted the effectiveness of UAE in isolating mitragynine from kratom [9,13]. This method is recognized for achieving high yields of this specific alkaloid. Past studies have focused on refining the conditions of the UAE to optimize the extraction process, emphasizing its critical role in extracting targeted compounds from plants.

This research aimed to refine the extraction of 7-OH mitragynine and mitragynine from kratom using probe-type UAE. We systematically adjusted the sample-to-liquid ratio, solvent type, ultrasonic power, and extraction time to optimize efficiency. The optimized conditions were then used to analyze kratom samples from local entrepreneurs, facilitating fair trade and enhancing commercial success for both sellers and growers.

2. Materials and Methods

Reagents and standard preparation

The certified reference materials of 7-OH mitragynine (100 μg mL^{-1} in with 0.1N NH_3) and mitragynine (100 μg mL^{-1} in methanol) were obtained from Merck and stored at a temperature of -20 ˚C until use. HPLC-grade acetonitrile was purchased from RCI Labscan. Analytical grade methanol, ammonium bicarbonate, and ethanol were acquired from Carlo Erba and Merck. Purified water was produced from the arium® pro ultrapure water

system (18.2 MΩcm, Sartorius, Germany). Standard solutions of 7-OH mitragynine and mitragynine were prepared using certified reference materials, with concentrations for the calibration curve ranging from 0.5 to 50 μg mL^{-1}. These solutions were prepared at ten different concentration levels (0.5, 1, 2, 3, 5, 10, 20, 30, 40, and 50 μg mL^{-1}) using methanol as the solvent. The prepared solutions were filtered through a 0.22 μm nylon membrane filter before analysis by HPLC-DAD technique, with each concentration analyzed in triplicate. The results of the experiment were used to construct a calibration curve between the average peak areas and the concentrations of each standard solution. The correlation coefficient for the calibration curve was no less than 0.995.

To evaluate the sensitivity of the method, the limit of detection (LOD) and limit of quantification (LOQ) were calculated using the following formulas (1) and (2) based on the calibration curve [14]:

$$LOD = 3.3 \times (\sigma/S) \quad (1)$$

$$LOQ = 10 \times (\sigma/S) \quad (2)$$

where σ represents the standard deviation of the response, and S is the slope of the calibration curve. This approach allows for precise determination of the method's sensitivity in detecting and quantifying target compounds.

Instrumentation

The Agilent 1260 Infinity (USA) was used for HPLC-DAD analysis. It had a thermostatted column compartment (G1316A), a diode array detector (G1315D), an autosampler (G1329B), a degasser (G1322A), and a quaternary pump VL (G1311C). The data processing and evaluation were performed using the OpenLab CDS ChemStation-Edition software (Rev.C.01.07). The samples were prepared using an ultrasonic homogenizer, model JY88-IIN (Drawell, China), for ultrasound-assisted extraction, and a bench-top centrifuge, model V18R (Dynamica Scientific, UK) for centrifugation.

Chromatographic condition

The analysis of 7-OH mitragynine and mitragynine utilized chromatographic condition adapted from the research of Mudge and Brown [8]. Chromatographic separation was achieved using a ZORBAX Eclipse Plus-C18 column (4.6×150 mm, 3.5 μm, Agilent Technologies), maintained at 30°C. The mobile phases consisted of 5mM ammonium bicarbonate (A) and acetonitrile (B). Each component of the mobile phase was filtered through a 0.22 μm nylon membrane filter and degassed by sonication for 20 min before use. The gradient elution program was set as follows: 45% B (0-2 min), 45-70% B (2-5 min), 70-80% B (5-7 min), and 80% B (7-12 min). The column was equilibrated for 15 min before each analysis. The total run time was 27 min. The flow rate was set at 1.0 mL min^{-1} and the injection volume was 5 μL. The wavelengths were set to 222 nm for 7-OH mitragynine and 226 nm for mitragynine, respectively.

Optimal conditions using ultrasound- assisted extraction

This study focuses on establishing the optimal conditions for extracting 7-OH mitragynine and mitragynine from kratom leaf samples using UAE techniques. The experimental design is based on a completely randomized design. This research was adapted from the study of Rusydan et al. [13], and the following parameters of the extraction process, including sample-to-liquid (0.1:10 to 2:10 g mL^{-1}), the type of solvent (water, ethanol and methanol), methanol concentration (50-100%v/v), ultrasonic power (25-200W), and extraction time (5-60 min). All experiments will be replicated three times to ensure robust results.

Quantitative analysis of 7-OH mitragynine and mitragynine in kratom

This study analyzed 15 samples, consisting of 14 kratom leaf samples (S1 to S14) and one kratom flower sample (S15), for mitragynine and 7-OH mitragynine content. All these

samples were obtained from entrepreneurs who sell kratom leaves, which were collected from various farmers' gardens in the Lom Sak district of Phetchabun province. Each sample was dried, ground into a powder, and sifted through an 80-mesh sieve before analysis. In this experiment, each sample weighing 500 mg was precisely weighed using a four-decimal balance and placed into a 50-mL centrifuge tube. Then, 10 mL of 70%v/v methanol solution was added, and the weight was measured again before undergoing UAE at 175 W for 10 min. After extraction, once the solution had cooled, it was weighed again to adjust the weight to its original amount using 70%v/v methanol. The solution was then centrifuged at 20 °C for 15 min at 10,000 rpm to separate the supernatant and filtered through a 0.22 µm nylon membrane filter before being injected into an HPLC. Each sample was analyzed in triplicate. The concentrations of mitragynine and 7-OH mitragynine were calculated based on the standard curve of the standards. Mitragynine from samples was quantified in %w/w using the following equation (3), and 7-OH mitragynine from samples was quantified in µg g^{-1} using the following equation (4).

$$\text{Mitragynine, (\%w/w)} = \frac{C \times V \times D}{W \times 10{,}000} \quad (3)$$

$$\text{7-OH mitragynine, (µg g}^{-1}\text{)} = \frac{C \times V}{W} \quad (4)$$

Where;

C = Concentration of mitragynine or 7-OH mitragynine, determined from the calibration curve, µg mL^{-1}

V = Volume of the test solution, mL

W = Dry weight of sample, g

D = Dilution factor

Statistical analysis

All assays were performed in triplicate with the results expressed as mean ± standard deviation (SD). The data were analyzed using SPSS software for windows. An analysis of variance (ANOVA) was conducted, and Duncan's multiple range test (DMRT) at a significance level of 0.05 was used to determine if there were significant differences between the means.

3. Results and Discussion

Before determining the optimal way to employ ultrasound-assisted extraction to extract 7-OH mitragynine and mitragynine from kratom leaves, the chromatographic conditions for separating these two compounds were assessed. The study adopted the method of Mudge and Brown [8], with slight modifications to the chromatographic settings. The mixed standard solution of 7-OH mitragynine and mitragynine provided retention times of 6.25 min for 7-OH mitragynine and 10.23 min for mitragynine (Fig. 2a). Chromatograms of the kratom leaf (S14) and flower (S15) samples are shown in Figs. 2 b and 2c. The method demonstrated specificity, as it successfully separated both compounds without interference. Calibration curves are also provided, demonstrating linearity from 0.5 to 50 µg mL^{-1} (coefficient of determination, R^2 > 0.999). Table 1 details the regression analysis and the LOD and LOQ values.

Table 1 The regression analyses of the linearity of 7-OH mitragynine and mitragynine.

Parameter	7-OH MG	MG
Concentration range (µg mL^{-1})	0.5-50.0	0.5-50.0
Slope	16.7258	32.3720
Intercept	-4.4719	-4.8017
R^2	0.9995	0.9996
LOD (µg mL^{-1})	1.27	1.19
LOQ (µg mL^{-1})	4.25	3.97

MG: mitragynine

Effects of ultrasound- assisted extraction on 7-OH mitragynine and mitragynine in kratom

UAE was used in single-factor studies to investigate the factors influencing the extraction of mitragynine and 7-OH mitragynine from Kratom. The experiments were designed using a completely randomized design with three replicates for each experiment. The initial extraction process followed the methodology of

Rusydan *et al.* [13]. Four key factors were analyzed: sample-to-liquid ratio, solvent type and methanol concentration, ultrasonic power, and extraction time. Each factor was adjusted while keeping the others constant to determine the optimal conditions.

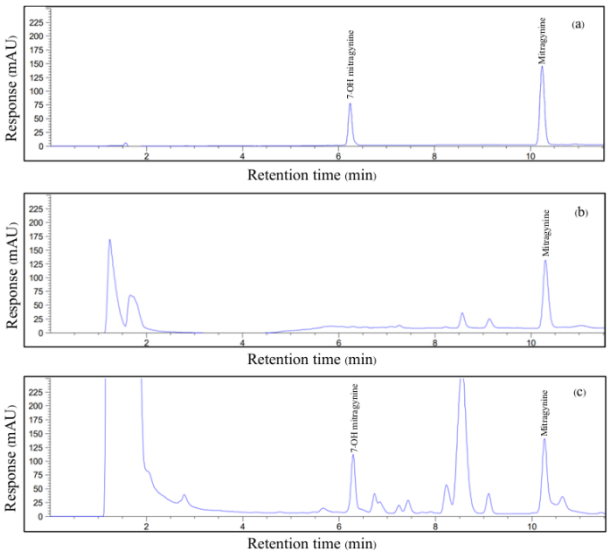


Fig. 2 The chromatograms of (a) mixed standard solution of 7-OH mitragynine and mitragynine at 30 µg mL^{-1}, (b) the kratom leaf sample solution diluted 10 times (S14) and (c) the kratom flower sample solution (S15).

Effects of sample-to-liquid ratio

The sample-to-liquid ratio significantly affected the extraction yields of 7-OH mitragynine and mitragynine. Under 200 W ultrasonic power, 100%v/v methanol, and a 10 min for extraction time, various ratios (0.1:10 to 2:10 g mL^{-1}) were tested. The highest yields were achieved at a 0.5:10 g mL^{-1} ratio, with 59.28 µg g^{-1} for 7-OH mitragynine and 1.24%w/w for mitragynine, as shown in Table 2. The ratios of 0.2:10, 0.5:10, and 1:10 g mL^{-1} did not significantly differ in mitragynine yields ($p>0.05$), indicating that 0.5:10 g mL^{-1} was the optimal condition.

Effects of solvent type and methanol concentration

In the preliminary experiments of this study, water, methanol, and ethanol were selected as solvents for extraction. The results showed that methanol had the highest extraction efficiency, making it the optimal solvent for further experiments. The 7-OH mitragynine and mitragynine yields were tested with methanol concentrations of 50%, 60%, 70%, and 100% v/v. The experiment used 200 W of ultrasonic power, a 10 min for extraction time, and a sample-to-liquid ratio of 0.5:10 g mL^{-1}. The results, presented in Table 3, showed that 70%v/v methanol was the most effective, yielding 146.51 µg g^{-1} for 7-OH mitragynine and 1.35%w/w for mitragynine.

Table 2 Effect of sample- to- liquid ratio on 7- OH mitragynine and mitragynine yields.

Sample/Liquid ratio (g mL^{-1})	7-OH MG (µg g^{-1})	MG (%w/w)
0.1:10	nd	1.19± 0.00^{ab}
0.2:10	nd	1.24± 0.03^{a}
0.5:10	59.28± 0.71^{a}	1.24±0.01^{a}
1.0:10	44.85± 2.53^{b}	1.24± 0.015^{a}
1.5:10	42.47± 1.48^{b}	1.17± 0.02^{b}
2.0:10	37.80± 2.16^{c}	1.07± 0.05^{c}

Values are presented as means ± standard deviation (n=3). Values in the same column with the same superscript letter are not significantly different (Duncan, $p>0.05$). nd: not detected. MG: mitragynine.

Table 3 Effect of solvent type and methanol concentration on 7- OH mitragynine and mitragynine yields.

Solvent (% v/v)	7-OH MG (µg g^{-1})	MG (%w/w)
water	45.63±2.01^{e}	0.32±0.01^{b}
100% EtOH	52.25±0.09^{d}	1.08±0.01^{c}
100% MeOH	59.28±0.71^{c}	1.24±0.01^{b}
80% MeOH	84.88±3.26^{b}	1.36±0.03^{a}
70% MeOH	146.51±5.65^{a}	1.35±0.05^{a}
60% MeOH	81.55±3.52^{b}	1.33±0.04^{a}
50% MeOH	62.58±2.75^{c}	1.28±0.02^{b}

Values are presented as means ± standard deviation (n=3). Values in the same column with the same superscript letter are not significantly different (Duncan, $p>0.05$). MG: mitragynine.

Effects of ultrasonic power

Ultrasonic power was identified as a critical factor in enhancing the extraction efficiency of kratom with a methanol concentration of 70% (v/v), a sample-to-liquid ratio of 0.5:10 g mL^{-1}, and an extraction duration of 10 min, the impact of ultrasonic powers at 25, 50, 125, 175, and 200 W on the yields of 7-OH mitragynine and mitragynine are detailed in Table 4. The yields of 7-OH mitragynine and mitragynine increased

significantly with the increase in ultrasonic power from 25W to 200W. However, the yield of 7-OH mitragynine slightly decreased with further increases in ultrasonic power. This phenomenon is attributed to the enhancements in ultrasonic power, which intensify the cavitation, mechanical, and thermal effects, thus increasing cell disruption and mass transfer rates. The optimal yields of 7-OH mitragynine and mitragynine were achieved at an ultrasonic power of 175W. Therefore, an ultrasonic power of 175W was chosen for further optimization.

Table 4 Effect of ultrasonic power on 7-OH mitragynine and mitragynine yields.

Power (W)	7-OH MG (μg g^{-1})	MG (%w/w)
25	58.17±1.37^{e}	1.12±0.02^{b}
50	105.21±9.10^{d}	1.11±0.03^{b}
125	126.66±4.00^{c}	1.29±0.07^{a}
150	139.22±2.39^{b}	1.30±0.07^{a}
175	148.94±4.30^{a}	1.35±0.05^{a}
200	146.51±5.65^{ab}	1.35±0.05^{a}

Values are presented as means ± standard deviation (n=3). Values in the same column with the same superscript letter are not significantly different (Duncan, p>0.05). MG: mitragynine.

Table 5 Effect of extraction time on 7-OH mitragynine and mitragynine yields.

UAE Time (min)	7-OH MG (μg g^{-1})	MG (%w/w)
5	145.17±3.95^{a}	1.28±0.02^{b}
10	146.96±1.72^{a}	1.35±0.04^{a}
15	147.07±2.57^{a}	1.34±0.03^{a}
20	146.15±2.38^{a}	1.32±0.03^{ab}
30	147.07±1.73^{a}	1.33±0.03^{ab}

Values are presented as means ± standard deviation (n=3). Values in the same column with the same superscript letter are not significantly different (Duncan,p>0.05). MG: mitragynine.

Effects of extraction time

Under the optimized extraction conditions (175W ultrasonic power, 70% (v/v) methanol concentration, and 0.5:10 g mL^{-1} sample-to-liquid ratio) , the extraction efficiency for different durations (5, 10, 15, 20, and 30 min) was examined. According to Table 5, the yields of 7-OH mitragynine and mitragynine were observed. It was noted that the extraction time had no significant impact on the yield of mitragynine for durations ranging from 10 to 30 min. However, the yields of both 7-OH mitragynine and mitragynine showed a slight decrease beyond these times. Consequently, 10 min was determined to be the optimal extraction time.

The experimental results from this study revealed differences in solvent usage compared to the research by Rusydan *et al*. [13]. This study used 70%v/v methanol, yielding better extraction results for mitragynine and 7-OH mitragynine than 100% methanol, as shown in Table 3. In addition, Table 2 shows that a sample-to-liquid ratio of 0.5:10 g mL^{-1} was better for extracting mitragynine and 7-OH mitragynine than a ratio of 1.5:10 g mL^{-1}. For extraction time, both studies employed 10 min.

Quantification of 7-OH mitragynine and mitragynine in kratom

The experimental results revealed that the quantification of 7- OH mitragynine and mitragynine in kratom extracts varies significantly between the leaf samples (S1-S14) and the flower sample (S15), as shown in Table 6. The kratom flower sample (S15) stands out due to its exceptionally high concentration of 7-OH mitragynine at 914.92 μg g^{-1}, much higher than all the leaf samples. However, S15 had the lowest mitragynine content at only 0.05%, indicating a very different alkaloid profile than the leaf samples. This suggests that the chemical composition of kratom flowers could differ substantially from that of the leaves, possibly due to differences in plant part function, growth stage, or environmental factors. These findings underscore the need for further research to fully understand the factors contributing to these differences.

For the kratom leaf samples from Lom Sak District, Phetchabun Province, it was found that sample S4 had the highest mitragynine content, at 1.87% w/w. The 7-OH mitragynine content across all 14 samples was shallow, ranging from 76.44 to 354.82 μg g^{-1} (0.03% w/w). This finding aligns with several other studies, which had consistently reported that mitragynine is the predominant alkaloid in kratom leaves, generally not exceeding 2% w/w. In contrast, 7-OH mitragynine was found in trace amounts [15-16]. Similarly, Shuayprom *et al.* looked at the amount of mitragynine in kratom leaf samples from Trang, Chumphon, Krabi,

Nonthaburi, Ayutthaya, Nakhon Pathom, and Bangkok provinces, finding concentrations ranging from 0.99 to 1.89% w/w [3]. Kratom leaves contain different levels of alkaloids, influenced by genetic factors, cultivation practices, harvesting methods, and environmental conditions such as the season, water availability, calcium and magnesium content in the soil, sunlight exposure, and the location of growth. These elements collectively determine the alkaloid concentrations in kratom leaves [17-19].

Table 6 7-OH Mitragynine and mitragynine content in UAE kratom leaf extracts obtained of kratom leaf samples from various sources.

Samples	7-OH MG ($\mu g\ g^{-1}$)	MG (%w/w)
S1	76.44 ± 0.93^{l}	1.52 ± 0.04^{c}
S2	90.34 ± 2.25^{k}	1.44 ± 0.08^{d}
S3	354.82 ± 3.66^{b}	1.05 ± 0.03^{g}
S4	114.53 ± 3.80^{g}	1.87 ± 0.04^{a}
S5	161.09 ± 7.76^{f}	1.36 ± 0.04^{de}
S6	255.00 ± 4.29^{c}	1.04 ± 0.03^{g}
S7	182.61 ± 4.62^{e}	1.04 ± 0.05^{g}
S8	114.53 ± 3.80^{g}	1.36 ± 0.04^{de}
S9	164.46 ± 8.66^{f}	0.96 ± 0.04^{h}
S10	152.46 ± 0.86^{g}	1.55 ± 0.02^{c}
S11	224.72 ± 4.56^{d}	1.35 ± 0.06^{e}
S12	134.75 ± 1.09^{h}	1.21 ± 0.01^{f}
S13	125.79 ± 1.01^{i}	1.29 ± 0.06^{e}
S14	149.15 ± 5.90^{g}	1.68 ± 0.02^{b}
S15	914.92 ± 8.49^{a}	0.05 ± 0.00^{i}

Values are presented as means ± standard deviation (n=3). Values in the same column with the same superscript letter are not significantly different (Duncan, $p>0.05$). nd: not detected. MG: mitragynine.

4. Conclusion

This study successfully optimized the UAE method for quantifying 7-OH mitragynine and mitragynine in kratom (*Mitragyna speciosa*) leaf and flower samples. The optimal conditions identified for the extraction process include using 70%v/v methanol as the solvent, with an ultrasonic power of 175 W and an extraction time of 10 min. Under these conditions, the kratom flower sample (S15) exhibited the highest concentration of 7-OH mitragynine, while the kratom leaf sample (S4) demonstrated the highest mitragynine content. These findings suggest that kratom leaves and flowers possess distinct alkaloid profiles, with possible variations attributable to differences in plant parts, genetics, and environmental factors. The method developed in this study provides a reliable and efficient approach for extracting and quantifying these bioactive compounds, supporting the standardization and quality control of kratom products.

5. Acknowledgement

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