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Antibacterial activity of *Mitragyna speciosa* Korth. leaves

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ABSTRACT

The crude extracts of red vein kratom leaves (hexane, dichloromethane, ethyl acetate, ethanol, and 50% acetic acid) and mitragynine were tested for antibacterial activity against gram-positive *Staphylococcus aureus* and gram-negative *Escherichia coli* using the disk diffusion method. The minimum inhibitory concentration (MIC) values against *S. aureus* and *E. coli* were determined. The acetic acid crude extract was effective against both *S. aureus* and *E. coli* with an inhibition zone of 5.52±0.44 and 4.65±1.02 mm, at a minimum concentration of 6 and 9 mg/mL, respectively. Mitragynine was active against *S. aureus* with an inhibition zone of 4.35±0.68 mm and a MIC of 6 mg/mL.

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Keywords: crude extract; antibacterial; kratom leaves; *Mitragyna speciosa*; mitragynine

1. INTRODUCTION

According to a study, antibiotic resistance was identified as a last resort drug used to treat infection caused by drug-resistant bacteria (McGann et al., 2016). This indicates that the problem of antibiotic resistance of bacteria has reached a critical stage (Ventola, 2015). Traditional medicine books around the world show that plants are an important source of antimicrobial compounds (Cowan, 1999). Scientists have researched and developed many active compounds from plants and herbs such as berberine from huanglian, epicatechin from peppermint, ellagitannin from pomegranate, and eugenol from clove (Kosalec et al., 2022; Arruda et al., 2017; Machado et al., 2002; Bai et al., 2023).

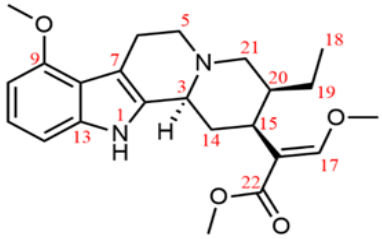
In addition to medicinal plants, there are also studies of local plants in countries, especially in Thailand, which is considered a country with vast plant diversity. Unfortunately, the majority of these plants have not been studied, and most of the ones that have been investigated lack thorough analysis.

A very interesting plant ubiquitous in Thailand is kratom (*Mitragyna speciosa* Korth), a perennial plant of the Rubiaceae family, which is mainly found in Southeast Asia, especially Thailand, Malaysia. The main active compound of kratom is mitragynine (Figure 1), an alkaloid that is illegal in Thailand under the Narcotics Act, 1979 (Narcotics Act B.E. 2522, 1979).

Some studies have reported the effects of kratom leaf consumption. It has been shown to possess the same neurostimulation effects as cocaine, including nausea and vomiting (Grewal, 1932a, 1932b). Kratom extract has also been reported to be toxic to the liver and kidneys (Harizal et al., 2010). In addition, Thai medicine textbooks indicate that kratom is used as an ingredient in folk formulations for diarrhea, such as "ya prasa kratom" (Nakaphan et al., 2016). In some regions, it is said that

kratom can treat and alleviate the symptoms of diabetes and relieve body aches. Traditional healers have used the bark and leaves of kratom to treat diarrhea, abdominal pain, bloody diarrhea, stomach ailments, diabetes, high blood pressure, skin diseases, and purulent wounds (Gordon and Lowy, 2008; Suwanlert, 1975; Weese, 2010), but research on kratom in Thailand is scarce, compared to research in Malaysia and Indonesia (Parthasarathy et al., 2009; Salim et al., 2021).

(a) Red vein kratom leaves



(b) Mitragynine

Figure 1. (a) A red vein kratom leaf and (b) the structure of mitragynine

The Narcotics Affairs Section (NAS) at the U.S. Embassy in Thailand, Office of the Narcotics Control Board, Ministry of Justice, encouraged the publication of a book "kratom leaves in Thai society". Part of the text shows the percentage of diseases for which southern healers used kratom for treatment of issues such as diarrhea and purulent wounds (Suwanlert, 1975). The present study evaluates the antibacterial activity of red vein kratom leaves crude extracts and mitragynine. The activity was assessed against gram-positive *Staphylococcus aureus*, a human and animal skin parasite that causes boils and ulcers, and gram-negative *Escherichia coli*, the most common cause of diarrhea in both children and adults (Gordon and Lowy, 2008). In this regard, the trend in action of kratom leaves is considered as feasible as claimed by traditional healers in the sense that kratom leaves can be used to treat diarrhea and purulent wounds according to the aforementioned folk wisdom by means of disc diffusion and minimum inhibitory concentration. This research aimed to study the antibacterial activity of crude extracts and mitragynine from red vein kratom leaves.

2. MATERIALS AND METHODS

2.1 Materials

Dichloromethane, ethanol, and glacial acetic acid were purchased from Lab-Scan (Bangkok, Thailand). Hexane, ethyl acetate, and sodium sulfate anhydrous were purchased from Sigma-Aldrich (Saint Louis, USA). Acetone and ammonium hydroxide were purchased from Merck (Darmstadt, Germany). All solvents were purified by distillation before use. Analytical thin-layer chromatography (TLC) was conducted using TLC plates from Merck (silica gel 60 F_{254} on an aluminum sheet). Solvents were evaporated using a rotary evaporator (Buchi Rotavapor R-114).

2.2 Preparation of crude extracts of red vein kratom leaves

Red vein kratom leaves were collected from Ratchaburi province in April 2022. The leaves were washed with water and dried at 30 ˚C until the weight was unchanged. The dried red vein kratom leaves were ground into powder. Crude extracts were prepared using 20 g of the powder and 200 mL of one of the following solvents: hexane, dichloromethane, ethyl acetate, ethanol, or 50% acetic acid; they were then allowed to stand at room temperature overnight. The suspension was filtered and dried using a rotary evaporator. Finally, each crude extract was weighed and the yield and R_f were determined.

2.3 Purification and characterization of mitragynine

Mitragynine was extracted following the process reported by Muandao et al. (2018). Briefly, red vein kratom leaves were dried at 30 ˚C and then ground into powder. Afterward, 2.5 g of this powder was extracted with 40 mL of 50% v/v acetic acid at 80 ˚C for 30 min. The mixture was cooled to room temperature, then the pH was adjusted to the range of 9–10 with 25% v/v ammonium hydroxide, and 40 mL of ethyl acetate added to the mixture. The sludge was filtered and the liquid phase was placed into a separatory funnel. It was left until the substance phase separated. The organic phase was collected and dried using a rotary evaporator. Compound 1, mitragynine, was separated by preparative thin layer chromatography (PTLC), then the R_f was determined. Fourier transform infrared (FTIR) spectra were recorded on a Perkin Elmer Spectrum GX FTIR spectrometer. The proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectra were recorded using a Bruker Avance-300 spectrometer.

2.4 Antibacterial activity test

2.4.1 Preliminary evaluation of crude extracts

The antimicrobial activity of the crude extracts was initially evaluated *via* the disc diffusion method, adapted from the Clinical and Laboratory Standards Institute (CLSI, 2009). Initially, one colony of *S. aureus* ATCC25913 and *E. coli* W3110S were selected from a culture dish. Each bacterium was inoculated into Mueller-Hinton broth (MHB) medium and incubated at 37 ˚C for 3–5 h. The turbidity was then adjusted using McFarland 0.5 standard, resulting in a cell concentration of approximately 1.5×10^8 CFU/mL. The paper disc was

prepared by piercing a 6-mm diameter hole on a Whatman No.1 filter paper, which was then sterilized at 121 ˚C for 20 min. The crude extract was dissolved in dimethyl sulfoxide (DMSO) to achieve a concentration of 10 mg/mL. Subsequently, 10 µL of the solution was placed into the middle of the paper disc and left to dry for approximately 24 h before use. A sterile cotton swab was dipped in the test pathogen culture and spread onto the surface of a plate with Mueller-Hinton agar (MHA) medium. The paper disc was then placed on the infected MHA plate using sterile forceps. A total of six paper disks were placed 15–20 mm apart on each plate and 15 mm away from the edge of the petri dish. Plates were incubated at 37 ˚C for 24 h. The diameter of the inhibition zone surrounding the paper disk was measured thrice using Vernier calipers. The negative and positive controls, DMSO and tetracycline respectively, were prepared at the same concentration as the samples. The experiment was repeated three times and the standard deviation was calculated.

2.4.2 Minimum inhibitory concentration (MIC)

The MIC was determined using a microdilution method adapted from CLSI. *S. aureus* and *E. coli* cultures were prepared as indicated in section 2.4.1. The crude extracts were prepared at a concentration of 0–10 mg/mL. The MHB medium was added to a 96-well plate, then, 100 µL of 1×10^6 CFU/mL concentrated inoculation was added to each well and incubated at 37 ˚C for 18–24 h. The MIC values were obtained by observing the turbidity or transparency of the unleavened medium. For comparisons, wells with only MHB medium and with crude extracts at the lowest concentrations without inoculation were also evaluated. Tests were carried out in triplicate.

3. RESULTS AND DISCUSSION

3.1 Crude extract yield

Table 1 shows the extraction yields of red vein kratom leaf crude extracts obtained using different solvents. In addition, the extracts were analyzed using TLC. As expected, different solvents yielded different mixtures because of variation in polarity of the solvent.

Table 1. Weight and yield of crude extracts from various solvents

Solvents	Crude extracts	
	Weight (g)	Yield (%)
Hexane	0.027	0.135
Dichloromethane	0.303	1.517
Ethyl acetate	0.105	0.523
Ethanol	4.191	20.953
50% Acetic acid	4.572	22.862

It was found that the crude extracts of red vein kratom leaves with different solvents were able to separate many other mixtures. This difference could be observed primarily by physical characteristics such as the color of the crude extract and the solution when applied to the TLC with different results. This may be due to the polarity of the solvent, which affects the extraction capacity of heterogeneous substances. The 50% acetic acid solvent extract was expected to contain mitragynine because it was too acidic and then adjusted to a base with an aqueous solution of NH_3 for the extraction of the alkaloids. Chromatograms were viewed as the most mitragynine in the fraction of crude extract of 50% acetic acid when separated by TLC, as shown in Figure 2.

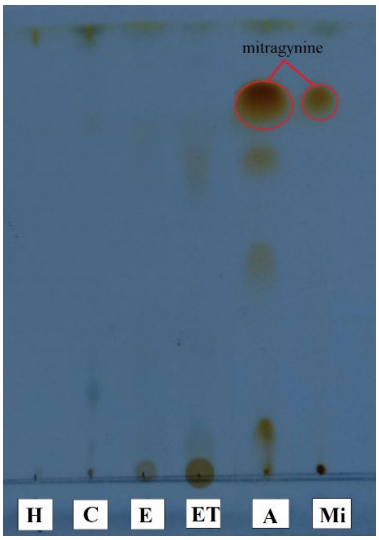


Figure 2. TLC of the crude extracts (H=hexane, C=dichloromethane, E=ethyl acetate, ET=ethanol, A=50% acetic acid and Mi=mitragynine)

3.2 Purification and characterization of compound 1

Compound 1 was obtained by PTLC purification of the 50% acetic acid crude extract of dried red vein kratom powder and adjusting the pH value to be in the range of 9–10 using ammonia solution. The mixture solution was extracted with ethyl acetate to provide the crude extracts (28.3 mg, 1.132 % yield) using ethyl acetate: hexane (7:3) as mobile phase (Muandao et al., 2018). The R_f was 0.77. The ^{1}H-NMR and ^{13}C-NMR spectra of compound 1 were compared to those reported by Flores-Bocanegra et al. (2020) Table 2.

Table 2. Comparison of ^{1}H-NMR and ^{13}C-NMR in $CDCl_3$ of compound 1 and reported mitragynine (δ in ppm)

Position	Type	^{1}H-NMR		^{13}C-NMR	
		Compound 1	Mitragynine[a]	Compound 1	Mitragynine[a]
1-NH	NH	7.86 (1H, s)	7.74 (1H, bs)	-	–
2	C	-	-	133.3	133.5
3	-CH-	3.19 (1H, d)	3.20 (1H, d)	61.2	61.3
5	-CH_2-	2.95 (1H, m) 2.56 (1H, m)	2.97 (1H, m) 2.55 (1H, m)	53.6	53.8
6	-CH_2-	3.10 (1H, m) 2.98 (1H, m)	3.11 (1H, m) 2.97 (1H, m)	23.7	23.9
7	C	–	–	107.7	108.0
8	C	–	–	117.6	117.7
9	C	–	–	154.5	154.6
10	-CH-	6.45 (1H, d)	6.45 (1H, d)	99.7	99.9
11	-CH-	6.99 (1H, t)	7.00 (1H, t)	121.7	122.0
12	-CH-	6.89 (1H, d)	6.90 (1H, d)	104.2	104.3
13	C	–	–	137.3	137.4
14	-CH_2-	2.53 (1H, m) 1.83 (1H, m)	2.55 (1H, m) 1.81 (1H, m)	29.7	30.0
15	-CH-	3.07 (1H, m)	3.06 (1H, m)	39.7	39.9
16	C	–	–	111.4	111.5
17	-CH-	7.43 (1H, s)	7.43 (1H, s)	160.6	160.7
18	-CH_3	0.88 (3H, t)	0.87 (3H, t)	12.8	13.0
19	-CH_2-	1.76 (1H, m) 1.25 (1H, s)	1.75 (1H, m) 1.19 (1H, qd)	19.1	19.3
20	-CH-	1.67 (1H, d)	1.64 (1H, dt)	40.5	40.7
21	-CH_2-	3.01 (1H, m) 2.45 (1H, m)	3.00 (1H, m) 2.44 (1H, m)	57.5	57.7
22	C	–	–	169.2	169.4
9-OCH_3	-CH_3	3.86 (3H, s)	3.87 (3H, s)	55.3	55.5
17-OCH_3	-CH_3	3.72 (3H, s)	3.73 (3H, s)	61.6	61.7
22-OCH_3	-CH_3	3.70 (3H, s)	3.71 (3H, s)	51.3	51.5

[a] Data reported by Flores-Bocanegra et al. 2020

The FTIR spectrum of compound 1 showed a signal corresponding to C-H bond at 2950 cm^{-1}; C-C and C=C of aromatics at 1462–1500 cm^{-1}, and 1580–1698 cm^{-1}; C=O at 1702 cm^{-1}; C-O from the ester bond at 1284 cm^{-1}, and N-H at >3000 cm^{-1}. These results were consistent with the functional groups of mitragynine.

Spectroscopic analysis confirmed that compound 1 obtained from red vein kratom leaves, was mitragynine. The number of alkaloids in kratom leaves, especially mitragynine, depended on many factors such as the age of the plant, soil nutrients, environment, and age of harvest (León et al., 2009).

3.3 Antibacterial activity test

The antibacterial activity of five crude extracts (hexane, dichloromethane, ethyl acetate, ethanol, 50% acetic acid) and compound 1 were evaluated against *S. aureus* and *E. coli* by the disc diffusion method. The results are summarized in Table 3.

Only the acetic acid crude extract and compound 1 showed antibacterial activity against *S. aureus*. The inhibition zone diameter values were 5.52±0.44, and 4.35±0.68 mm, respectively. Against *E. coli*, only the acetic acid crude extract was active, with an inhibition zone of 4.65±1.02 mm.

Table 4 shows the MIC values (OD_{600} = 0.05) against gram-positive *S. aureus* and gram-negative *E. coli* of the acetic acid crude extract and the pure compound had the same MIC against *S. aureus*, 6 mg/mL. The MIC of the acetic acid crude extract against *E. coli* was 9 mg/mL (Table 4, Figure 3 and Figure 4).

Table 3. Inhibition zone on the agar disc diffusion method of crude extracts and mitragynine at a concentration of 10 mg/mL

Concentration (mg/mL)	Bacteria for testing	Inhibition zone (mm)							
		H	C	E	ET	A	Mi	P	N
10	*S. aureus*	NA	NA	NA	NA	5.52±0.44	4.35±0.68	7.52±0.35	NA
	E.coli	NA	NA	NA	NA	4.65±1.02	NA	6.82±0.45	NA

Remark: NA=No Activity. H=hexane, C=dichloromethane, E=ethyl acetate, ET=ethanol, A=extract 50% acetic acid, Mi=mitragynine, P=positive control (tetracycline) and N=negative control (DMSO)

Table 4. Minimal inhibitory concentration (MIC), OD_{600} = 0.05

Extract concentration (mg/mL)	*S. aureus*		*E. coli*
	Crude extract of 50% acetic acid	Mitragynine	Crude extract of 50% acetic acid
0	++	++	++
0.5	++	++	++
1	++	++	++
2	++	++	++
3	++	++	++
4	++	++	++
5	++	++	++
6	--	--	++
7	--	--	++
8	--	--	++
9	--	--	--
10	--	--	--

Remarks: ++ means growth bacteria and -- means non-growth bacteria

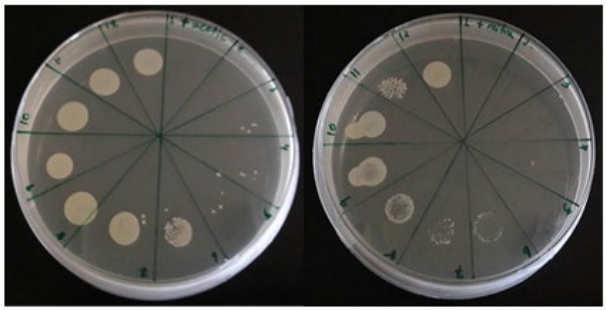


Figure 3. Antibacterial activity against gram-positive *S. aureus* of the acetic acid crude extract and compound 1 at the following concentrations: 12=0, 11=0.5, 10=1, 9=2, 8=3, 7=4, 6=5, 5=6, 4=7, 3=8, 2=9, and 1=10 mg/mL

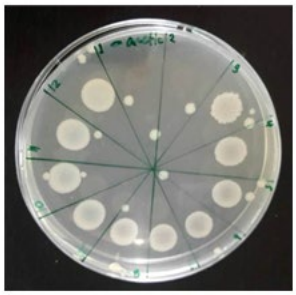


Figure 4. Antibacterial activity against gram-negative *E. coli* of the acetic acid crude extract at the following concentrations: 12=0, 11=0.5, 10=1, 9=2, 8=3, 7=4, 6=5, 5=6, 4=7, 3=8, 2=9, and 1=10 mg/mL

Overall, the results indicate that the acetic acid crude extract and mitragynine from red vein kratom leaves were effective against *S. aureus* and *E. coli* bacteria. This is consistent with previous reports that found methanolic kratom extract was active against *Bacillus subtilis*, which is a gram-positive bacteria, similar to *S. aureus* (MIC = 6.25 mg/mL) (Parthasarathy et al., 2009). Similarly, the authors reported that the ethanolic extract had no antibacterial activity. Mitragynine was active against *S. aureus* at a concentration as low as 6 mg/mL. Crude extracts of hexane, dichloromethane, ethyl acetate, and ethanol were inactive against the evaluated bacteria.

The phytochemicals of the active crude extracts should be thoroughly studied and tested before further clinical use. This research represents a starting point for the development of kratom as a safe natural drug to replace antibiotics in the future.

4. CONCLUSION

The 50% acetic acid extract of red vein kratom leaves showed antibacterial activity against *S. aureus* and *E. coli*, with MIC values of 6 and 9 mg/mL, respectively.

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