Antioxidant, Antimicrobial, and Cytotoxic Activities of the Hexane and Dichloromethane extracts of Malaysian *Mitragyna spesioca* Korth. Leaves

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The leaves of *Mitragyna speciosa* were traditionally used by natives as a substitute for opium. Nowadays, recreational use of *M. speciosa* leaves is increasing and has become a concern because of the lack of information and studies about its potential health risks. This study evaluated the antioxidant, antimicrobial, and cytotoxic activities of the crude hexane and DCM extracts of *M. speciosa*. Antioxidant activity was tested against DPPH, ABTS, beta carotene bleaching, FRAP, and total phenolic content (TPC) assays. Antimicrobial activity was investigated by disc diffusion and microdilution methods to determine the zone of inhibition, MIC and MBC values. The cytotoxic activity was evaluated using MTT assays. Two breast cancer cell lines (MCF7 and MDA-MD-231), two colon cancer cell lines (SW948 and HT29) and a lung cancer cell line were tested with the DCM and hexane extracts (A549). The DCM leaf extract showed the highest total phenolic content (TPC) (84.63±9.01 µg GE/mg) while the hexane extract showed the lowest total phenolic content (74.04±7.07 µg GE/mg). However, the total flavonoid (TFC) content was higher in the hexane extract (97.14±1.74 µg QE/mg) than the DCM extract (59.88±4.25 µg QE/mg). Both extracts showed DPPH free radical scavenging (EC_{50} >1mg/mL), ABTS (hexane extract: 20.84±0.92 mg/mL; DCM extract: 39.49±2.28 mg/mL) and beta carotene bleaching (hexane extract: 78.29 µg/mL; DCM extract: 75.17±3.26 µg/mL), FRAP (hexane extract: 11.45±0.99 µg/mL; DCM extract: 7.94±0.96 µg/mL). The cytotoxic assay for the DCM extract was moderately toxic (IC_{50} value ranging 2.86 to 14.81 µg /mL) on tested cancer cells, while the hexane extract showed IC_{50} values of more than 100 µg/mL for most of the tested cells. All extracts exhibited moderate to weak action, with MIC/MBC values ranging from 0.625 to 10 mg/mL. These findings demonstrated that the *M. speciosa* extracts possessed a variety of important biological properties.

Key words: *M. speciosa;* Rubiaceae; antioxidant; antimicrobial; cytotoxic assay

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M. speciosa, which belongs to the Rubiaceae family and found in both Asia and Africa, is a good example of a new psychoactive substance (NPS) of natural origin. It is known as kratom, kakuam, Kraton, ketum, thing, or thom in Thailand and biak-biak in Malaysia or krypton when combined with O-demethyltramadol [1-5]. Two types of kratom can be identified based on the colour of the leaf vein, which can be either green or red. Locals usually prefer the red vein type, characterized by its bitterness and prolonged effects which can invigorate workers under harsh conditions [1-5]. At a dosage of 10 to 30 fresh leaves per day, these leaves are normally chewed and swallowed as a powder, but they can also be dried for smoking or used to make tea [6]. Unfortunately, the use of novel psychotropic "herbal highs" as legal alternatives to conventional controlled drugs may cause adverse effects in humans if consumed in large amounts (>15g) [7]. Anxiety, irritability, and enhanced aggression have been described as side effects, while long-term high dose consumption has been related to several atypical effects. In individuals with long-term addiction, hyperpigmentation of the cheeks, tremors, anorexia, weight loss, and psychosis have been noted. Herbal preparations of kratom are increasingly found and used in Europe and the US [8]. Labourers in Malaysia and Thailand have chewed, smoked, and brewed kratom leaves as a herbal remedy to cure mild illnesses. Kratom is commonly used as a narcotic panacea to combat fatigue, as it helps to improve the

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productivity of labourers under the sweltering sun.

Globally, the statistics on the consumption of NPS of natural origin are not clear. According to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), the proportion of seizures recorded under the category of other NPS (including those of natural origin) was 12% in 2013 [9]. Vicknasingam et al. studied the major reasons for kratom consumption by its users' socio-demographic characteristics. The study was conducted on 136 active users, of which 76.5% had a previous history of drug use [10]. The presented reasons for kratom consumption included reduced addiction to other drugs, improved opiate addiction withdrawal symptoms, and its affordability relative to heroin. Many short and long-term users claimed to have felt an increase in their capability for hard work, activeness, and heightened sexual desire [10,11].

Analysis of kratom extracts has showed that its antioxidant activities are correlated with the total phenolic content. This suggests that the relatively high antioxidant activity of the methanolic extract compared to the aqueous and alkaloid extracts could be due to its high phenolic content [12]. Phytochemical screening of the kratom leaf ethanol extract showed that it had an IC_{50} value of 38.56 µg/mL. The results indicated that the kratom ethanol extract had strong antioxidant activity [13].

Kratom was previously reported to exhibit inhibitory effects against *Salmonella typhi* and *Bacillus subtilis*. The minimum inhibitory concentrations (MICs) of the extracts as determined by the broth dilution method ranged from 3.12 to 6.25 mg/mL. The alkaloid extract was found to be most effective against all the tested organisms [12]. It had an inhibitory effect on the growth of *Escherichia coli* bacteria and was also able to inhibit the growth *of Propionibacterium acnes* at a concentration of 5% with a diameter of inhibition of 8.6 mm [14].

Extracts of *M. speciosa* and mitragynine have been found to induce concentration-dependent cytotoxicity in different human cell lines such as cHol, MCL-5, HEK 293, and SH-SY5Y, with the neuronal SH-SY5Y cells being the most affected [15]. Moreover, higher cytotoxicity was observed in the metabolically competent MCL-5 cells compared to the non-competent cHol cells [16]. As recommended by the FDA, cell-based models that can predict toxicity in rodents should be incorporated into the early stage of lead optimization to rule out high-risk molecules, and to minimize development time, cost, and animal usage [17]. Ethical concerns have always limited the use of animals in toxicological studies, thus cellular toxicity is often used as a rough approximation of in vivo toxicity. Cancer cell lines have many advantages over primary cell lines for primary cytotoxicity screening in vitro [18, 19].

As part of our ongoing investigation of bioactive compounds from plant species [20-25], we are now also investigating the biological activities of *M. speciosa* leaves. Hence, the screening of its hexane and dichloromethane (DCM) extracts to determine antioxidant and antimicrobial activities, as well as its cytotoxic value. It is hoped that the findings of this study will provide an improved understanding of the benefits of these leaves, and an assessment of the potential and overall value of this herb.

MATERIALS AND METHODS

Plant Material

M. speciosa (Voucher number. TM 1047) was reported in September 2018 at Hulu Bernam, Selangor. The species was identified by a botanist from Universiti Pendidikan Sultan Idris' Faculty of Science and Mathematics.

Extraction Method

Plant samples (leaves) were air-dried for seven days before being ground and extracted sequentially by maceration with hexane, dichloromethane (DCM), and methanol, each for 72 hours. To acquire crude extracts for additional biological screening, the extracts were filtered and condensed under decreased pressure using a rotatory evaporator. For determining its antioxidant, antibacterial, and cytotoxicity properties, only the hexane and DCM dry extracts were used.

Solvents and Chemicals

Analytical grade *n*-hexane, dichloromethane (DCM) and methanol used for extraction were purchased from Merck (Germany). Antioxidants: 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzo thiazoline-6 sulphonic acid) (ABTS), butylated hydroxytoluene (BHT), Ferric reducing antioxidant power (FRAP), and β-carotene bleaching were obtained from the Department of Pharmacy, Faculty of Medicine, Centre for Natural Product Research & Drug Discovery, University of Malaya.

Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

The Folin-Ciocalteu assay [26] was used to assess the total phenolic content (TPC) of each extract. The TPC test was performed in a 96-well flat-bottom plate. Gallic acid was utilised as a reference. Each crude extract's TPC was expressed as gallic acid equivalents, GAE (g of gallic acid/mg extract). Adedapo et al. [27] evaluated the total flavonoid content (TFC) of the extracts using a technique which was adopted in this study. In this experiment, quercetin was utilised as a control. TFC was calculated as quercetin equivalents, QE (g of QU/mg of crude extract).

Anti-oxidant Assay

The DPPH radical-scavenging activities of all extracts were assessed using a previously described technique [31]. In this experiment, gallic acid was employed as a reference, and absorbance was measured at 517 nm using a microplate absorbance reader (Infinite M200PRO) after 30 minutes at room temperature. The b-carotene bleaching experiment was carried out in accordance with the methodology [32], with BHT serving as a positive control. At 0 and 2 hours, the absorbance was measured at 470 nm. The proportion of BCB was compared between all extracts (1 mg/mL) and the positive control (100 g/mL). The reducing power of the extracts and gallic acid was determined [31]. To evaluate the reducing power (FRAP) of these extracts, a standard curve of gallic acid was created and a standard equation was derived. The reducing power was reported in terms of gallic acid equivalents (GAE, g of gallic acid/mg of extract). Each extract was tested at a concentration of 1 mg/mL. The Total Anti-oxidant Capacity (TAOC) assay is a non-enzymatic technique for determining antioxidant capacity. This experiment was carried out in accordance with a procedure previously described [33]. At 695 nm, all extracts were screened at 1 mg/mL and evaluated. The overall antioxidant activity was reported in terms of ascorbic acid equivalents. The ABTS technique [34] was used with minor modifications to perform the free radical scavenging test. The procedure comprised pipetting 10 L of 1 mg/mL sample (diluted in DMSO) onto a 96-well microplate, followed by 300 L of ABTS+● solution. As a control, DMSO was utilized. The dish was maintained at 30 °C for 10 minutes. The absorbance was measured at 743 nm. The ABTS was decolorized due to the anti-scavenging oxidant.

Antimicrobial Assay

The minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) values were determined using the broth microdilution technique in 96-well microplates. Microbial strains were generated from 24-hour broth cultures, and the suspensions were corrected to 0.5 McFarland standard turbidity. To make a 1000 g/mL stock solution, extracts (1 mg) were diluted in DMSO (1 mL). In each plate, several wells (A–H) were set aside for positive and negative controls. Sterile broth (100 L) was poured into the wells in rows B through H. The sample stock solutions (100 L) were introduced to the wells in rows A and B. The combination of samples and sterile broth (100 L) was then added to each well for serial dilution to generate sample concentrations ranging from 7.8 to 1000 g/mL. Each well received 100 L of inoculum. Each well had an ultimate volume of 200 L. The plates were incubated for 24 hours at 37 °C. Turbidity and the presence of a pellet at the bottom of the well showed microbial development. Samples from the MIC investigation that did not demonstrate any bacterial growth were withdrawn from each well (10 mL) and sub-cultured in disposable Petri plates on the surface of the newly produced nutrient agar. The Petri plates were then inverted and incubated at 37 °C for 16-20 hours. The number of surviving organisms was then assessed.

Bacterial and fungal stock cultures were preserved at 4 °C on Muller-Hinton agar and potato dextrose agar, respectively. Their antimicrobial activities were investigated against six bacteria: three Gram-positive strains (*Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (B145), and *Staphylococcus epidermidis* (a clinical isolate)] and three Gram-negative strains (*Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (a clinical isolate), and *Klebsiella pneumoniae* (ATCC). All the bacterial strains were acquired from the Microbiology Laboratory, Medical Faculty, Universiti Putra Malaysia.

Disk Diffusion Assay

The diffusion method was used for the antibacterial assays [32], with each extract having a concentration of 10 mg/mL. After incubating the plates at 37 °C for 24 hours, antibacterial activity were assessed by measuring the width of the inhibitory zone. Streptomycin sulphate (100 g/mL) was utilised as a positive control, and dimethyl sulfoxide (DMSO) as a negative control. The broth microdilution test was used to determine the minimum inhibitory concentration (MIC) for each plant extract. The plant extracts were serially diluted (10 mg/mL - 19.53 ug/mL) for the test. The samples were then mixed with 20 L of an aqueous solution of 2,3,5-triphenyl-tetrazolium chloride (TTC, 5 mg/mL) and incubated at 37 °C for 24 hours. The existence of microbial growth was indicated by the emergence of a pink tint. The MIC was calculated using the lowest concentration that remained colourless.

Cytotoxicity Assay

MTT tests were used for the cytotoxicity assays [33]. The extracts were examined with five cancer cell lines: two breast cancer cell lines (MCF7 and MDA-MD-231), two colon cancer cell lines (SW948 and HT29), and one lung cancer cell line (A549). All cancer cells were obtained from the American Type Culture Collection, USA (ATCC). All cells were grown and allowed to reach log phase in RPMI1640 media containing 5% foetal bovine serum. Cancer cells were sown in 96-well plates at different doses. SW948 and MCF7 cells were concentrated at 1 x 105 cells per well in 100 L aliquots of media, whereas the others were at 2 x 105 cells per well. Each compound was put into the 96-well plates at varying quantities. In a 5 percent CO_2 humidifier incubator, the plates were incubated for 72 hours at 37 °C. After 72 hours of exposure to the extract, 20 L of MTT solution (5 mg/mL) was added to each well. The plates were then incubated for another 3 hours at 37 °C in the 5 percent CO_2 humidifier incubator. After discarding 80% of the

medium from each well, the same quantity of DMSO was added to dissolve any purple formazan crystals that had developed. Finally, using a microplate reader, the absorbance of the content of each well was measured at 550 nm. Following the formula below, the findings were computed as a percentage of cell inhibition:

$$\text{Percentage of cell inhibition} = [(A-B)/A] \times 100\%$$

where A represents the average absorbance of the cell without treatment and B represents the average absorbance of the cell with treatment.

The cytotoxicity of the substances was displayed as IC_{50} values on a graph showing percentage cell inhibition vs extract concentration. Tamoxifen and paclitaxel were utilized as positive controls in each experiment, which was carried out in triplicate.

RESULTS AND DISCUSSION

Decolorization assays were used to identify the existence of antioxidants and the development of the ABTS and DPPH radicals. The antioxidant activities of the *M. speciosa* extracts are summarised in Table 1. The results show that the DCM extract (116.85 ± 4.02 µg/mg) had a higher total antioxidant capacity (µg Ascorbic acid/ mg extract) than the hexane extract (108.21 ± 13.57 µg/mg). For the DPPH assays, both hexane and DCM extracts showed a DPPH EC_{50} value of more than 1mg/mL. The DCM extract (39.49 ± 2.28 1mg/mL) had a higher ABTS percentage of scavenging than the hexane extract (20.84 ± 0.92 1mg/mL). These results were supported by Ghatak et al., whereby the DPPH radical scavenging activity of the more polar solvent ethyl acetate was higher (47.33 ± 0.65%) than hexane (46.48 ± 0.54%) [34]. The ABTS activity was strongly correlated with DPPH because both methods are responsible for the same chemical property of H- or electron-donation to the antioxidant [35,36]. The FRAP assay showed that the hexane extract (11.45 ± 0.99 µg gallic acid/mg) had a higher value than the DCM extract (7.94 ± 0.96 µg gallic acid/mg extract), while the b-carotene bleaching (% of β-carotene bleaching of extract) for the hexane extract was higher (78.29 ± 2.04 1mg/mL) compared to the DCM extract (78.29 ± 2.04 1mg/mL).

Table 1. The total antioxidant activity, DPPH EC_{50}, ABTS, FRAP, and Beta carotene bleaching of various *M. speciosa* leaf extracts

Sample	Total antioxidant capacity (µg Ascorbic acid/ mg extract)	DPPH (EC_{50}), mg/mL	ABTS % of scavenging of extract at 1mg/mL	FRAP (µg gallic acid/ mg extract)	Beta carotene bleaching (% of β-carotene bleaching of extract at 1mg/mL)
TM1047 Hexane	108.21 ± 13.57	>1mg/mL	20.84 ± 0.92	**11.45 ± 0.99**	**78.29 ± 2.04**
TM1047 DCM	**116.85 ± 4.02**	>1mg/mL	**39.49 ± 2.28**	7.94 ± 0.96	75.17 ± 3.26
Standard compound	nil	Gallic acid EC_{50} =5.56 ± 0.70 µg/mL	Gallic acid (100µg/mL) 94.05 ± 0.25	nil	Gallic acid (100µg/mL) 88.58 ± 1.42

Table 2. Total phenolic content (µg Gallic extract/ mg extract) and total flavonoid content (µg quercetin/ mg extract) of the hexane and DCM leaf extracts.

Sample	Total phenolic content (µg Gallic extract/ mg extract)	Total flavonoid content (µg quercetin/ mg extract)
TM1047 Hexane	74.04 ± 7.07	97.14 ± 1.74
TM1047 DCM	84.63 ± 9.01	59.88 ± 4.25

The total phenolic content (TPC) and total flavonoid content (TFC) values are shown in Table 2. The DCM extract had a higher TPC (84.63 ± 9.01) than the hexane extract (74.04 ±7.07), which in turn had a significantly higher TFC (97.14 ± 1.74) than the DCM extract (59.88 ± 4.25). Because of their abilities as reducing agents, hydrogen donors, and singlet oxygen scavengers, phenolic chemicals such as flavonoids are well-known as plant antioxidant agents [37].

Table 3 shows the zone of inhibition, MIC, and MBC results obtained from the leaf extracts of *M. speciosa* against 6 microorganisms. Overall, the DCM extract of *M. speciosa* had better results for the three tests conducted with 5 out of 6 microorganisms, while the hexane extract showed better results with *Escherichia coli*. These results also indicated that both the hexane and DCM extracts had moderate to weak action against three gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, and *Enterococcus faecalis*) and three gram-negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumonia*). It is commonly claimed that gram-negative bacteria are more resistant to plant extracts because their hydrophilic cell wall structures are mostly composed of lipopolysaccharides, which limit the passage of hydrophobic oils and prevent extract build-up in the target cell membrane.

Table 3. Zone of inhibition, MIC, and MBC results for the leaf extracts of *M. speciosa* and streptomycin sulfate salt against six microorganisms.

Organism	**TM1047 Hexane**			**TM1047 DCM**			**Streptomycin sulfate salt**		
	ZOI (mm) (10mg/mL)	MIC (mg/mL)	MBC (mg/mL)	ZOI (mm) (10 mg/mL)	MIC (mg/mL)	MBC (mg/mL)	ZOI (mm) (10 mg/mL)	MIC (mg/mL)	MBC (mg/mL)
S. aureus	Nil	Nil	Nil	Nil	Nil	Nil	23.5 ± 2.1	0.125	0.5
B. subtilis	15.5 ± 2.1	2.5	5.0	16.5 ± 0.7	0.625	5.0	30.5 ± 0.7	0.031	>1.0
S.epidermidis	16.5 ± 0.7	2.5	5.0	16.0 ± 1.4	1.25	10.0	21.5 ± 2.1	0.25	0.5
K.pneumoniae	Nil	Nil	Nil	12.0 ± 1.4	Nil	Nil	29 ± 1.4	0.031	0.5
E. coli	16.0 ± 0.0	2.5	5.0	Nil	Nil	Nil	29 ± 0.0	0.125	0.5
P. aeruginosa	15.0 ± 0.0	1.25	>10.0	14.5 ± 0.7	2.5	>10.0	28.5 ± 0.7	0.25	0.5

ZOI = Zone of inhibition

Table 4. IC_{50} Cytotoxicity results for the hexane and DCM crude extracts on selected cancer cell lines.

Sample	**IC_{50} (µg/mL)**				
	A549	**MCF 7**	**MDA-MB-231**	**SW948**	**HT29**
TM1047 Hexane	>100	>100	>100	92.62 ± 4.53	>100
TM1047 DCM	27.76 ± 5.25	14.18 ± 0.97	31.52 ± 1.34	20.11 ± 1.37	11.76 ± 0.19
Paclitaxel	2.23 ± 0.11	Nil	Nil	0.26 ± 0.02	0.16 ± 0.01
Tamoxifen	Nil	2.28 ± 0.10	3.15 ± 0.04	Nil	Nil

Table 4 shows the cytotoxicity results for the hexane and DCM extracts of *M speciosa,* Paclitaxel and Tamoxifen on A549 = lung cancer, MCF7 and MDA-MB-231 = breast cancer; SW948 and HT29 = colon cancer. Based on the general screening protocol, the cytotoxicity of the plant extracts was scored into four categories: very active (IC_{50} ≤ 20 µg/mL), moderately active (IC_{50} >2 0–100 µg/mL), weakly active (IC_{50} > 100–1000 µg/mL) and inactive (IC_{50} >1000 µg/mL) [38,39]. The hexane extract had an IC_{50} value >100 µg/mL on A549, MCF7, MDA-MB-231, HT29 and 92.62 ± 4.53 µg/mL on SW948 = colon cancer. The DCM extract had an IC_{50} value of 27.76 ± 5.25 µg/mL on A549, 14.18 ± 0.97 µg/mL on MCF 7, 31.52 ± 1.34 µg/mL on MDA-MB-231, 20.11 ± 1.37 µg/mL on SW948 and 11.76 ± 0.19 µg/mL on HT29. The DCM extract had a cytoxicity approximately 5 times higher than the hexane extract. DCM was thus considered as an extract with active compounds that had potential cytotoxic effects.

CONCLUSION

The *M. speciosa* plant is gaining popularity as it has been used by Southeast Asian locals for a long time. Kratom is becoming popular among young people for recreational purposes. It is still highly appealing for people to experiment with drugs that can improve their capabilities. Several new psychoactive substances (NPS) or so-called "designer drugs" are often utilized in non-medical settings as synthetic alternatives for illegal drugs of abuse. The use of NPS for boosting or treating the body has not been approved by health experts or societies throughout the world. Unfortunately, kratom appears to be addictive, resulting in withdrawal symptoms when people stop using it. It has also been linked to an increase in blood pressure, hepatotoxicity, nephrotoxicity, emphysema, alveolar over-inflation, and cytotoxicity in human brain cells. Furthermore, there have been multiple accounts of fatalities following kratom ingestion, thus there is good reason to pay closer attention to this plant. More studies are needed to identify the chemical compounds present in the leaves of this plant, and to understand the mechanisms behind kratom's pharmacological effects.

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