ORIGINAL ARTICLE

Evaluation of Phytochemical Composition, Antioxidant and anti-Diabetic Activities of *Mitragyna speciosa* Methanolic Extract (MSME)

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ABSTRACT

Introduction: The therapeutic potential of plant-based or herbal medicine has been widely embraced by the public as a prevention and remedy for many illnesses. Mitragyna speciosa is one of the medicinal plant that exhibit the opioid-like effects of analgesia. This study aimed to evaluate the phytochemical content, antioxidant activity, and alpha-amylase enzyme inhibition of Mitragyna speciosa methanolic extract (MSME). **Methods:** The phytochemical composition of MSME was analyzed for secondary metabolites using UHPLC-TWIMS-QTOF-MS/MS. The total phenolic content (TPC), total flavonoid content (TFC), antioxidant scavenging activities (2,2-diphenyl-1-picrylhydrazyl [DPPH] and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) [ABTS] assays) and α-amylase enzyme inhibition activities of MSME were analyzed in comparison to positive control Pterostilbene and acarbose, respectively. **Results:** Analysis of UHPLC-TWIMS-QTOF-MS/MS has characterized the presence of at least five different bioactive compounds, mostly derivatives of flavonoids and polyphenols. A significantly higher level of TFC (347.72±15.97 mg QE/g extract; p = 0.0005), but a significantly lower level of TPC (167.43±13.50 mg GAE/g extract; p = 0.002) was detected in MSME (1 mg/mL) compared to Pterostilbene. MSME presented antioxidant activity which has no significant difference compared to Pterostilbene as determined by DPPH (MSME IC_{50} = 4.34±1.79 µg/mL; p = 0.727) and ABTS (MSME IC_{50} = 4.25±1.59 µg/mL; p = 0.311) assays. Moreover, MSME also exhibited anti-diabetic effects through inhibition of α-amylase activity (IC_{50} = 0.01±7.18 mg/mL) which shows a significant difference (p = 0.009) compared to acarbose. **Conclusion:** This finding suggests that MSME has bioactive phytochemicals and exhibits potential antioxidant and anti-diabetic properties.

Malaysian Journal of Medicine and Health Sciences (2022) 18(SUPP21): 92-99. doi:10.47836/mjmhs18.s21.15

Keywords: *Mitragyna speciosa*, Phytochemical, Antioxidant, alpha-amylase, Anti-diabetic

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INTRODUCTION

The application of medicinal plants as a therapy or health supplement is growing rapidly across the world. The indigenous plant of Thailand and Malaysia, *Mitragyna speciosa* Korth. (Rubiaceae) which is also known as Ketum has long been used to treat various symptoms and conditions including pain, fever, diarrhea, fatigue, and diabetes (1). *Mitragyna speciosa* exhibits an opioid-like effect that can cause addiction due to the presence of mitragynine in the plant (2). Besides its antipyretic, analgesic, and anesthetic effects, many local inhabitants also consume *Mitragyna speciosa* juice as an energy booster (2). However, there is a lack of scientific evidence to relate *Mitragyna speciosa* with antioxidant and anti-diabetic properties.

Antioxidants are natural substances that inhibit free radicals from causing cell or tissue damage. In normal homeostasis, moderate production of reactive oxygen species (ROS) including hydroxyl radical, superoxide, and hydrogen peroxide are crucial in several cellular activities and functions including gene transcription, signaling transduction, and immune response (3). Conversely, excessive ROS levels can trigger oxidative stress and eventually injure the cell (4). The oxidative damage initiated by free radicals can lead to the pathogenesis of many chronic diseases such as cancer, respiratory, cardiovascular, neurodegenerative, and digestive diseases (3). Therefore, stabilizing the ROS activity by inhibiting the lethal effect of ROS may reduce

the susceptibility to the disease (5).

Endogenously produced antioxidants in the human body include glutathione peroxidase, catalase, and superoxide dismutase (6). Nevertheless, these antioxidants may not be adequate to sustain optimal cellular functions in many disease conditions as a result of increased oxidative stress. Adding antioxidant supplements or high antioxidant food to the diet may be essential to support the antioxidative defense (7). However, consumption of synthetic antioxidants such as butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), tertiary butylated hydroquinone, and gallic acid esters may exhibit several unfavorable side effects (8). Hence, the intake of natural antioxidants from leafy plant vegetables has recently gained much interest due to its high content of phytochemicals which are known as plant secondary metabolites (6).

Phenolic compounds are the largest group of phytochemicals known as good hydrogen donors and thus have been acknowledged for having the most antioxidant activity in plants or plant products (9). Flavanoids are the predominant group of phenolics in plants that act as antioxidants and defense against diseases such as cardiovascular disease, cancer, and degeneration of cell components (9). In addition, increased activities or levels of antioxidant has been found to reduce diabetic complications (10). This has gained much interest to further investigate the antioxidant-containing natural product for its potential effect in the diabetic study.

The pancreatic alpha (α)-amylase enzyme is responsible for breaking down carbohydrates, mostly starch, into a variety of smaller oligosaccharides, including maltose, maltotriose, and several α-(l-6) and α-(1-4) oligoglucans (11). However, excessive activity of α-amylase can lead to rapid carbohydrate digestion in diabetic patients, resulting in hyperglycemia, which raises blood glucose levels (11). Thus, inhibiting α-amylase activity is an essential therapeutic target to delay carbohydrate digestion and therefore limit the rate of glucose absorption, decreasing postprandial blood glucose levels in diabetic individuals (12).

Therefore, this present study analyzed the phytochemical content in *Mitragyna speciosa* methanolic extract (MSME) and subsequently evaluated the potential antioxidant and anti-diabetic effects through assessment of free radical scavenging activity and inhibition of the alpha (α)-amylase enzyme, respectively. Methanol was used as a solvent for the extraction of bioactive compounds from *Mitragyna speciosa* due to its high polarity and the highest extraction efficiency and yield compared to distilled water, ethanol, chloroform, dichloromethane, and acetone (13).

MATERIALS AND METHODS

Plant collection

The collection of fresh plant leaves of *Mitragyna speciosa* Korth. was done in Kampung Ayer Hitam, Kubang Pasu, Kedah, Malaysia. The plant species were identified and the voucher number (KM0024/22) was issued by the Institute of Biosciences, Universiti Putra Malaysia. Any adhering material and dirt on the leaves were removed by washing thoroughly under running tap water. The leaves were dried in an oven at 50°C for 48 hours (h) and ground into a fine powder.

Methanolic extraction of *M. speciosa*

The methanolic extraction of *Mitragyna speciosa* was performed by the soxhlet extraction method by Senik et al. with a slight modification (2). 100 g of dry powdered leaves were extracted in 200 mL of absolute methanol at 60°C by a soxhlet extractor for 4 h followed by solvent evaporation at 40°C under reduced pressure by using a rotary evaporator. The extract was left overnight in an oven at 30°C to remove excess moisture and any residue of the solvent. The dried extract was then sealed and stored at -20°C until further use.

Screening of phytochemical compounds in MSME

The screening of phytochemical compounds in MSME was done qualitatively by ultra-high-performance liquid chromatography (UHPLC) method by using the ACQUITY UPLC I-Class system (Waters, UK) in Makmal Khidmat Analisis, Lembaga Koko Malaysia, Nilai, Negeri Sembilan, Malaysia. The isolation of the phenolic compounds was performed chromatographically at 40°C by the column ACQUITY UPLC HSS T3 (100 mm x 2.1 mm x 1.8 µm) (Waters, UK). A linear binary gradient of water (0.1% formic acid) and acetonitrile were used in mobile phases A and B, respectively. During the process, the mobile phase setting was changed as follows: 1% B at 0 min, 1% B at 0.5 min, 35% B at 16 min, 100% B at 18 min, and 1% B at 20 min. The flow rate and injection volume were maintained at 0.6 mL/min and 1 µL, respectively. The UHPLC method was combined with traveling-wave ion mobility spectrometry-quadrupole time of flight mass spectrometry (TWIMS-QTOF-MS/MS) method by using a Vion IMS QTOF hybrid mass spectrometer (Waters, UK). Data were acquired in a high-definition MSE (HDMSE) mode with the m/z range from 50 to 1500 at 0.1 s/scan. During the run, a low-energy (LE) scan at fixed collision energies (CE) of 4 eV, and a high-energy (HE) scan at CE of 10-40 eV were obtained.

Total antioxidant contents

Total Phenolic Content (TPC) assay

Pterostilbene (Cat. No. P1499, Sigma-Aldrich), a naturally occurring polyphenol with strong antioxidant

properties, was used as a positive control (14).

The TPC of MSME was evaluated by using a well-established Folin-Ciocalteau reagent process. 100 µL of (0.1, 1, 10 mg/mL) MSME or Pterostilbene, was mixed with 1 mL of Folin-Ciocalteau Reagent (1:10). Following the addition of 3 mL of 7.5% sodium carbonate (Na_2CO_3), the mixture was incubated for 30 min at room temperature. The absorbance was then measured by a spectrophotometer at 765 nm. The value is presented in milligrams of gallic acid equivalent (GAE) per gram of extract (mg GAE/g sample).

Total Flavonoid Content (TFC) assay

The TFC was measured using the colorimetric method of aluminum chloride. 500 µL of (0.1, 1, 10 mg/mL) MSME or Pterostilbene was mixed with 100 µL of 10% w/v aluminum chloride ($AlCl_3$), 100 µL of 1 M potassium acetate ($C_2H_2O_2K$) and added with distilled water to 5 mL. After 30 min incubation, the absorbance was read at 415 nm by using a spectrophotometer. The result was expressed as milligrams of quercetin equivalent (QE) per gram of extract (mg QE/g sample).

Antioxidant assay

Radical scavenging activity of MSME was performed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays. For the DPPH assay, an equal volume (1 mL) of 0.2 mM DPPH solution was combined with MSME, Trolox (standard) or Pterostilbene followed by incubation for 30 min. The absorbance was measured at 517 nm by a spectrophotometer. In the ABTS assay, ABTS solution [7 mM of ABTS and 2.45 mM of potassium persulfate ($K_2S_2O_8$)] was diluted (1:50) with 100% methanol until the absorbance value reached approximately 0.7 at 734 nm. Then, an equal volume (1 mL) of MSME, Trolox, or Pterostilbene was combined with diluted ABTS and incubated for 10 min. The absorbance was read at 734 nm by a spectrophotometer. A mixture of methanol and DPPH or ABTS solution was used as blank. The percentage of ABTS or DPPH scavenging activity was performed by using the formula below. The result was expressed as mmol of Trolox equivalent (TE) per gram of extract (mmol TE/g sample). The IC_{50} value was determined by plotting a graph of log concentration vs percentage of scavenging activity.

$$\% \text{ scavenging activity} = [Ac - As] / Ac \times 100$$

where Ac is the absorbance of the negative control and As is the absorbance of the sample.

Alpha-amylase enzyme inhibition

Dinitrosalicylic acid (DNSA) protocol by Alsawalha et al. (15) with slight modification was used to measure the α-amylase inhibition activity of MSME and acarbose (Cat. No. AB141891, Abcam). An equal volume (200 µL) of α-amylase solution (2 units/mL) and MSME or acarbose were mixed and incubated at 37°C. After 30 min, 200 µL of 1% starch solution was added and further incubated for 15 min at 37°C, followed by the addition of 500 µL of DNSA (Cat. No. 156441000, Acros) reagent. The reaction was stopped by heating the tubes in a boiling water bath. The absorbance of the reaction mixture in serial dilution was measured at 540 nm using a microplate reader.

Statistical Analysis

The expressed values are the means of two independent experiments ± standard error mean (SEM). The statistical analysis was performed by multiple T-test to identify a significant difference between the means of the control group and experimental group by Graphpad Prism 7 software. A p-value <0.05 is considered statistically significant.

RESULTS

The phytochemical composition in MSME

Table I shows the summary of the identified potential phytochemical compounds in MSME. The identification of the phytochemical compounds was done by comparing the retention times, neutral and observed mass, and theoretical fragmentation. The isolated compounds showed mass accuracy of less than 5 ppm and a minimum of one fragment ion. In addition, the compounds were evaluated and compared with the available standard of similar compounds in the Waters library based on the expected retention time.

Table I: Qualitative screening of phytochemical compounds in MSME by UHPLC-TWIMS-QTOF-MS/MS analysis

Compound class	Compound	Neutral mass (Da)	Observed mass (m/z)	Retention time (min)
Flavonoid	Rutin	610.15	609.15	8.51
Flavonoid	Epicatechin	290.08	289.07	6.12
Flavonoid	Quercetin	302.04	301.04	12.04
Flavonoid	Procyanidin B2	578.14	577.14	5.74
Polyphenol	Chlorogenic acid	354.09	353.09	5.15

Five phytochemical bioactive compounds were identified in MSME. Four out of five are classified as flavonoid groups which include rutin, epicatechin, quercetin, and procyanidin B2 while chlorogenic acid is classified under the polyphenol group. Fig. 1 shows the chemical structure of each identified compound in MSME. Fig. 2 represents the MSME bioactive compound identified by UHPLC-TWIMS-QTOF-MS/MS chromatograms with mass spectra at low and high collision energy.

Total phenolic and flavonoid content of MSME

Table II shows the bioactive content of MSME that was measured by evaluating TPC based on gallic acid equivalent (GAE) and TFC based on the quercetin equivalent in comparison with Pterostilbene. The data

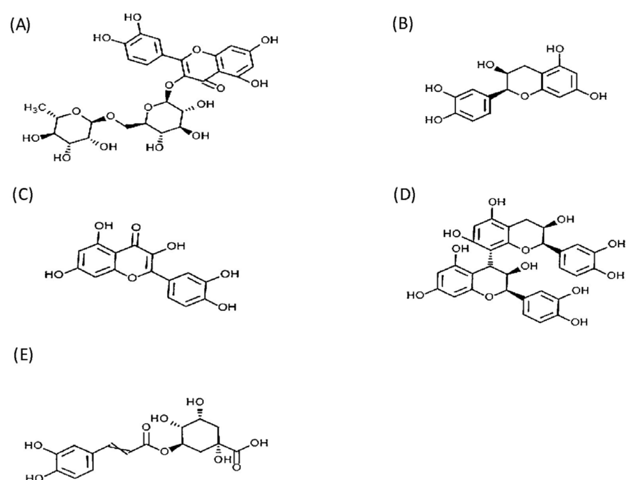

Figure 1: Chemical structure of flavonoids and polyphenols found in MSME. Chemical structure of (A) rutin $C_{27}H_{30}O_{16}$; molecular weight (MW): 610.517 g/mol, (B) epicathecin $C_{22}H_{18}O_{10}$; MW: 442.37 g/mol, (C) quercetin $C_{15}H_{10}O_{7}$; MW: 302.236 g/mol, (D) procyanidin B2 $C_{30}H_{26}O_{12}$; MW: 578.52 g/mol, and (E) chlorogenic acid $C_{16}H_{18}O_{9}$ 5; MW: 354.31 g/mol.

demonstrate that MSME has a significantly higher TFC (p = 0.0005) (in 1 mg/mL), but lower TPC (p = 0.002 and p = 0.000) (in 1 and 10 mg/mL) in comparison with Pterostilbene.

Free radical scavenging activity of MSME

The antioxidant properties of MSME were determined by DPPH and ABTS assays through evaluation of its free radical scavenging activity. The comparison of DPPH and ABTS radical scavenging activity between Pterostilbene and MSME was presented in Table III. Data shows that, in the DPPH assay, MSME indicated a considerable Trolox equivalent antioxidant capacity (TEAC) (8.65±0.25 mmol TE/g sample) with IC_{50} value determined at 4.34±1.79 µg/mL which was not significantly different (p = 0.727) to Pterostilbene (4.39±0.82 µg/mL). Similarly, in the ABTS assay, MSME also revealed a non-significant difference (p = 0.311) in TEAC (MSME = 6.76±0.18 mmol TE/g sample; IC_{50} = 4.25±1.59 µg/mL) when compared to Pterostilbene (7.66±0.64 mmol TE/g sample; IC_{50} = 1.55±0.04 µg/mL).

MSME inhibits α-amylase enzyme activity

The inhibition of α-amylase activity by MSME in comparison with acarbose was shown in Table IV. The results demonstrated that the inhibition of α-amylase digestive enzyme activity by MSME (IC_{50} of 0.01±7.18 mg/mL) was significantly different (p = 0.009) compared to acarbose (0.05±7.63 mg/mL).

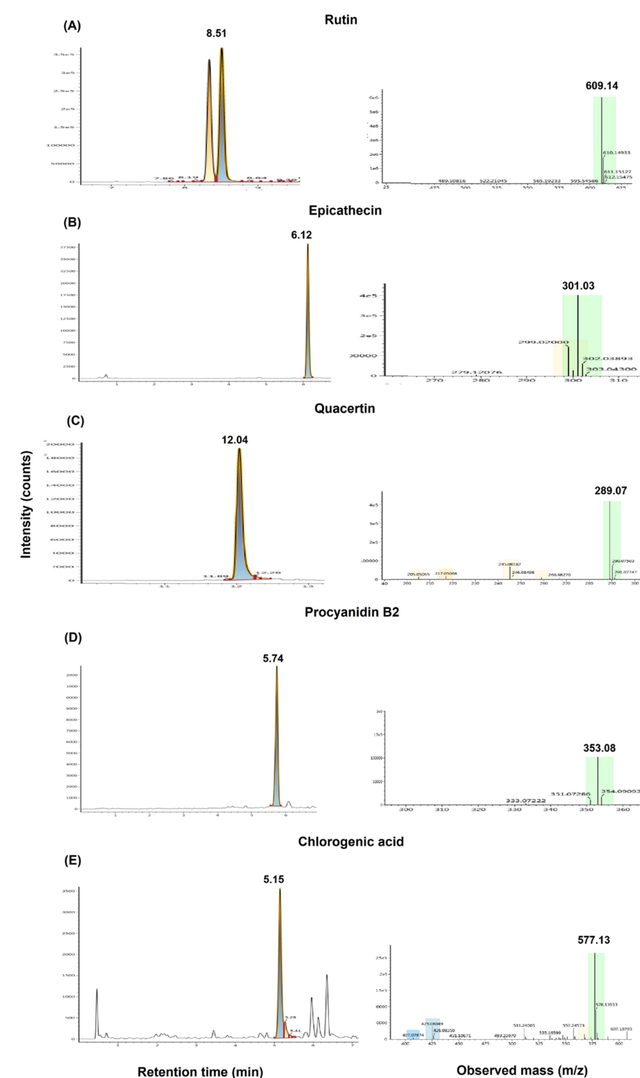

Figure 2: The UHPLC-TWIMS-QTOF-MS/MS analysis of phytochemicals in MSME. Depicted chromatograms are intensity of mass spectrometry (MS/MS) over low collision energy at specific retention time (min) (left panel) and high collision energy observed in molecular ion mass (m/z) (right panel) of (A) rutin (8.51 min; 609.14 m/z), (B) epicathecin (6.12 min; 301.03 m/z), (C) quercetin (12.04 min; 289.07 m/z), (D) procyanidin B2 (5.74 min; 353.08 m/z), and (E) chlorogenic acid (5.15 min; 577.13 m/z).

DISCUSSION

Phytochemicals are natural elements in plants that possess biological activity (16). The presence of

Table III: DPPH and ABTS radical scavenging activity of MSME

	DPPH				ABTS			
	mmol TE / g sample	Scavenging activity (%)	IC_{50} (µg/mL)	*p*-value^	mmol TE/ g sample	Scavenging activity (%)	IC_{50} (µg/mL)	*p*-value^
MSME	8.65±0.25	91.65±2.08	4.34±1.79	0.727	6.76±0.18	82.32±0.84	4.25±1.59	0.311
Pterostilbene	8.53±0.17	90.64±1.41	4.39±0.82		7.66±0.64	86.58±3.06	1.55±0.04	

TE: Trolox equivalent
Data are expressed as mean ± SEM of two independent measurements
^ indicates multiple T-test statistical analysis

Table IV: α-amylase digestive enzyme inhibition activity of MSME expressed in IC_{50}.

	MSME	Acarbose
IC_{50} (mg/mL)	0.01±7.18	0.05±7.63
p-value^	0.009*	

Data are expressed as mean ± SEM of two independent measurements
* indicates significant differences at p<0.05 in comparison to Acarbose
^ indicates multiple T-test statistical analysis

phytochemicals in plants including alkaloids, tannins, saponins, flavonoids, phenols, steroids, and carotenoids is essential for the prevention of many diseases due to their defensive properties such as anti-inflammatory, anti-diabetic, anti-aging, anti-microbial, anti-parasitic, anti-depressant, anti-cancer, antioxidant, and wound healing (17). A previous study on the ethanol extract of *Mitragyna speciosa* has found the presence of various organic compounds including alkaloids, phenols, saponins, tannins, flavonoids, and steroids/ triterpenoids (18). In this present study, preliminary screening of phytochemical compounds in MSME by UHPLC-TWIMS-QTOF-MS/MS analysis has identified four flavonoids (rutin, epicatechin, quercetin, and procyanidin B2) and one polyphenol (chlorogenic acid) compounds. Other previous studies also confirmed the presence of similar bioactive phytochemicals (including rutin, quercetin, and chlorogenic acid) in ethanol and methanol extracts of *Mitragyna speciosa* by UHPLC-ESI-QTOF-MS/MS analysis (19).

Each of the phytochemical compounds that were detected in MSME has its known benefits for health. Rutin is a bioflavonoid with anti-inflammatory and antioxidant which was also found to have protective effects on the brain, kidney, and liver against diseases (20). The dietary supplement of rutin has been reported to show a significant reduction in glucose levels (21). Besides, epicatechin which can mostly be found in green tea, grapes, cocoa, and chocolate is reported to exhibit antibacterial, anti-inflammatory, and anti-cancer properties (22). It has also been reported to help in the prevention of Type 2 Diabetes Mellitus onset with high insulin sensitivity and lower insulin resistance (23). In addition, quercetin is one of the most abundant dietary flavonoids, prominent for its antioxidant activity, anti-allergic, and anti-inflammatory properties (24). Procyanidin B2 is a natural flavonoid primarily found in cocoa, red grapes, apples, and cinnamon by which a previous study revealed its cellular defense against oxidative stress on PC12 cells (25). In addition, procyanidin B2 also helps in accelerating wound healing and increases angiogenesis in diabetic mice (26). Chlorogenic acid was commonly known to exhibit hypoglycemic, hypolipidemic, anti-bacterial, antioxidant, and anti-inflammatory properties (27). Thus, this phytochemical characterization and qualitative analysis of MSME suggests that the extract may contain potential agents of antioxidant, anti-diabetic, anti-inflammatory, and wound healing.

Antioxidants are well-known therapeutic agents for the prevention of damage by free radicals (28). Several studies investigated that both flavonoid and phenolic compounds exhibit the ability to act as antioxidants. Therefore, this present study investigated the antioxidant content and activity through the determination of the TPC and TFC of MSME based on GAE and quercetin equivalent respectively in comparison with Pterostilbene as the positive control.

Pterostilbene (trans-3,5-dimethoxy-4-hydroxystilbene) is a polyphenol that has a natural antioxidant compound that can be found in blueberries, grapes, almonds, leaves of Vitis vinifera, and heartwood of Pterocarpus marsupium and Pterocarpus santalinus (16). In comparison with Pterostilbene, MSME has statistically significant lower TPC levels (p = 0.002) but significantly higher TFC levels (p = 0.0005). In addition, a previous study revealed a significantly higher TPC and TFC in a methanol extract of *Mitragyna speciosa* in comparison to alkaloid and aqueous extracts (29). This data suggests that MSME could be a prospective of natural antioxidants due to its bioactive phytochemical compounds.

Radical scavenging activity plays a major role in preventing the damaging effects of free radicals in many diseases (30). The scavenging activities of an extract are determined by the ability of the antioxidant component to reduce hydrogen and the structural conformation of the free radicals (31). The DPPH and ABTS assays were used in our study to evaluate the radical scavenging activity of MSME and compared to positive control drugs Pterostilbene. The interpolation of linear regression analysis has given the IC_{50} value of MSME that defines it as the number of antioxidants needed to reduce 50% of DPPH or ABTS, in which a lower IC_{50} shows a higher antioxidant activity of a compound (32). ABTS assay is a well-known method used to determine the antioxidant ability to scavenge the ABTS which is formed in the aqueous phase. On the other hand, the DPPH assay is commonly used to determine antioxidant activity in phenolic compounds of plant extracts (33). DPPH is relatively known as a free radical with high stability and the antioxidant effect on DPPH radical scavenging is caused by the ability to donate hydrogen (34). In the DPPH assay, changing the DPPH solution to a yellow-colored product, diphenylpicryl hydrazine, indicated the presence of the antioxidant compound in plant extract (30). Contrarily, the evaluation of scavenging activity in ABTS assay is due to the reduction of a blue/green $ABTS^+$ color by antioxidants (35). This present study showed that MSME exhibits a comparatively similar antioxidant scavenging activity with Pterostilbene in both DPPH and ABTS assays, as the data indicated a non-significant difference (p = 0.727 and p = 0.311) in TEAC of MSME when compared to Pterostilbene. MSME showed a slightly higher DPPH but slightly lower ABTS scavenging activity than Pterostilbene. These results are possibly due to the high content of phenolic compounds in the

non-aqueous phase of MSME, thus, a better scavenging activity in the DPPH assay in comparison to ABTS. A previous study demonstrated a strong antioxidant activity of *Mitragyna speciosa* methanol extract in the DPPH method in comparison to alkaloid and water extracts (29).

The inhibition of α-amylase enzyme activity by MSME was also investigated in this present study by using DNSA reagent, an aromatic compound that reacts to the reducing sugars, and then reduced to form 3-amino-5-nitrosalicylic acid (36). The results showed a significant difference (p = 0.009) in the inhibitory activity of α-amylase by MSME with a lower IC_{50} value compared to acarbose. Thus, MSME could be a good candidate for an effective α-amylase inhibitor which is favorable for diabetes mellitus treatment in reducing postprandial hyperglycemia. The α-glucosidase inhibition study could provide a strong justification for the present study as it is allied to the final step of carbohydrate digestion.

CONCLUSION

In comparison to Pterostilbene, MSME exhibits a comparable antioxidant scavenging activity associated with statistically significant higher in flavonoid content. In addition, MSME was also found to exhibit anti-diabetic activity through inhibition of α-amylase enzyme activity which is associated with carbohydrate digestion. Therefore, our present findings propose a potential therapeutic application of *Mitragyna speciosa* related to antioxidant and anti-diabetic effects. Further study is suggested, particularly focusing on the quantitative analysis of the main bioactive compounds of MSME and the mechanisms implemented in its antioxidant and anti-diabetic properties.

ACKNOWLEDGEMENT

This study was funded by Geran Putra Siswazah (GP-IPS/2019/9679200) of the Universiti Putra Malaysia. We thank Associate Professor Dr. Uswatun Hasanah Zaidan and her student, Nur Izzati Mohamad Zen from the Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia for their kind assistance in the extraction of MSME.

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