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Research paper

Optimization of *Mitragyna speciosa* (Korth.) Havil. extraction techniques

Mohd Kamal N.H., Ihsan Safwan K., Fauziah A., Zaridah M.Z., Azman M., Nazrin C.S., Alif Haikal M.

Natural Product Division, Forest Research Institute Malaysia (FRIM), 52109 Kepong, Selangor Darul Ehsan, Malaysia

*Corresponding author: mohdkamal@frim.gov.my
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ABSTRACT

Context: This study was conducted to investigate the relationship between the use of appropriate temperature and time with the antioxidant activity of *Mitragyna speciosa* (Kratom) extract. ***Methods:*** aqueous extract prepared at various time and temperature were tested using DPPH, TPC, FRAP, and MDA tests. ***Results:*** the most active extracts were obtained 60°C for more than one hour extraction. ***Conclusion:*** the extraction temperature and time for the preparation of *M. speciosa* extract are important parameters to ensure its high antioxidant potential activity and best extraction of phenolics.

Keywords: antioxidant, extraction, malondialdehyde

INTRODUCTION

Oxidative stress is a condition in the body where there is a mismatch between the levels of reactive oxygen species (ROS) and the antioxidant defences of the human body (Pizzino *et al.*, 2017). It is one of the main causes in the pathogenesis of several chronic diseases (García-Sánchez *et al.*, 2020). These include neurodegenerative diseases, cardiovascular diseases and age-related disorders (Korovesis *et al.*, 2023). Meanwhile, antioxidants are molecules that can neutralise ROS and thus protect cells from oxidative damage (Muchtaridi *et al.*, 2024). Antioxidants have become an intriguing subject of study due to their potential therapeutic applications (Losada-Barreiro *et al.*, 2022). Natural products, especially plant extracts, are suitable sources of antioxidant bioactive

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molecules and are therefore promising targets for the development of pharmaceutical and nutraceutical products (Lourenço *et al*., 2019).

Mitragyna speciosa (kratom in Malay) is a tropical evergreen tree native to Southeast Asia (Botejue *et al*., 2021). It has traditionally used for its analgesic, mood-enhancing and stimulant efficacy (Eastlack *et al*., 2020). In addition, *M. speciosa* is known for its bioactive components, mitragynine and 7-hydroxymitragynine, and much attention has been paid to its alkaloid constituents (Kruegel *et al*., 2019). Parthasarathy *et al*. (2009) reported that extracts from *M. speciosa* leaves have potential as natural antioxidants and antimicrobial agents, with the methanol extract being particularly rich in antioxidants and the alkaloid extract showing stronger antibacterial properties. The focus of previous studies on *M. speciosa* was only on alkaloid compounds (Karunakaran *et al*., 2022). Although alkaloids from *M. speciosa* contribute to various pharmacological activities, their use and possession are subject to national laws (Bergen-Cico *et al*., 2016). As a result, recent advances in phytochemistry have focused on the importance of non-alkaloid compounds from *M. speciosa*. However, there has been insufficient research on M. speciosa's antioxidant activity during the extraction process, particularly in terms of optimising the extraction process and its bioactivity. Therefore, this study focused on measuring the level of phenolic groups in *M. speciosa* extracts. This is because phenolic and flavonoid compounds contribute to the bioactivity of the plant (Parthasarathy *et al*., 2009).

In vitro assays, such as the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging assay, Ferric Reducing Antioxidant Power (FRAP) assay, Total Phenolic Content (TPC), and Malondialdehyde (MDA) assay, are largely used to assess the antioxidant capacity of plant extracts (Clarke *et al*., 2013). The DPPH assay measures the ability of compounds to scavenge free radicals (Baliyan *et al*., 2022), while the FRAP assay analyses their reducing power (Benzie and Strain, 1996). The TPC assay quantifies phenolic compounds, key contributors to antioxidant activity (Muflihah *et al*., 2021), and the MDA assay quantifies the extent of lipid peroxidation, a marker of oxidative stress (Cordiano *et al*., 2023). These assays provide an overall idea of the antioxidant potential of *M. speciosa* extracts and can provide hints about their therapeutic value.

Recent studies have identified the importance of optimising extraction conditions to obtain optimum bioactivity of antioxidants from *M. speciosa*. Temperature, extraction time, and solvent employed are all essential factors in the yield and stability of bioactive molecules (Yim *et al*., 2013). For instance, low extraction temperatures have been observed to facilitate the release of phenolic and flavonoids, while high temperatures could lead to degradation (Dai and Mumper, 2010). Understanding these parameters is crucial to the development of standardised extraction protocols, which are required for the

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optimisation of herbal extract to prepare an effective extract (Zhang *et al*., 2018).

METHODS

Water extract: water extract was done according to Nik Hasan *et al*. (2020). The leaves of *M. speciosa* from Malaysia were collected (identified by one of us), washed and then cut into small pieces. The pieces were dried in the oven at 55°C for 48 hours and ground into fine powder. Approximately 10 grams of the powdered sample were accurately weighed using an electronic balance and filled into glass tubes. Each tube was filled with 100 mL of distilled water. The tubes were then placed under various temperatures (40°C, 60°C, 80°C, and 100°C) and various extraction times (15 minutes, 30 minutes, 1 hour, 2 hours, and 3 hours). The solutions were filtered out after extraction to remove any solid impurities, and the resulting extracts were poured into appropriately labelled tubes. Finally, the extracts were freeze-dried and stored at 4°C until they were analyzed.

DPPH test: The DPPH test was performed according to Hussen and Endalew (2023). A 0.1 mM solution of DPPH in ethanol was prepared, and 200 µL of the extract at different concentrations was incubated with 2 mL of the DPPH solution. The mixture was shaken in the dark at room temperature for 30 minutes, and the absorbance was then measured at 517 nm using a spectrophotometer. The percentage inhibition of DPPH radicals was measured.

FRAP test: The FRAP assay was conducted using the Benzie and Strain (1996) method. A FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), TPTZ (10 mM in 40 mM HCl), and ferric chloride solution (20 mM) in a 10:1:1 ratio. 200 µL of the extract sample was mixed with 3 mL of FRAP reagent and incubated at 37°C for 30 minutes. Absorbance was taken at 593 nm, and results were expressed as µmol Fe^{2+} equivalents per gram of extract.

TPC test: The TPC assay was conducted using the Folin-Ciocalteu method (Pérez *et al*., 2023) with slight modification. 200 µL of the extract was added to 1.5 mL of Folin-Ciocalteu reagent (10% v/v) and 1.5 mL sodium carbonate (7.5%). The sample was left at room temperature for 2 hours, and a reading was taken at 765 nm. Gallic acid was used as the standard, and results were expressed as mg gallic acid equivalents (GAE) per gram of extract.

MDA test: Lipid peroxidation was measured by using the Ohkawa *et al*. (1979) method. Tissue homogenates were centrifuged at 4000 rpm for 10 minutes, and

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the supernatants were mixed with thiobarbituric acid (TBA) reagent. The reaction mixture was heated at 95°C for 1 hour and allowed to cool. Absorbance was measured at 532 nm. The concentration of MDA was determined and expressed as nmol of MDA per mg of protein.

RESULTS

The results of the DPPH assay showed that the extraction time and temperature influence the antioxidant activity. The DPPH assay is one of the most common tests used to evaluate plant extracts' free radical scavenging activity, and higher percentages indicate greater antioxidant activity (Kedare and Singh, 2011). Figure 1 shows that the percentage of DPPH inhibition was highest at temperatures between 60°C but decreased at higher temperatures. The highest antioxidant activity was achieved at 60°C for 2 hours, suggesting that temperature and time are related to the release of bioactive compounds during the extraction process of herbs with antioxidant potential, such as phenols and flavonoids. Previous studies have reported that heat treatment improves the extraction of antioxidant compounds and their bioavailability (Benzie and Strain, 1996; Maghsoudlou *et al.*, 2019). However, prolonged use of high temperatures during the extraction process was found to reduce the quality of the antioxidant compounds present in the extract (Che Sulaiman *et al.*, 2017). The results of the studies on the lower percentage of DPPH inhibition proved this. This trend is consistent with the finding that excessive heat can degrade polyphenols and flavonoids and reduce antioxidant activity (Singleton and Rossi, 1965; Dai and Mumper, 2010). The increase in antioxidant activity at a moderate temperature could be due to the improved solubility and extraction effect of phenolic compounds (Antony and Farid, 2022). If suitable temperatures are used, the active compounds in the plant cells can be released and dissolved in water (Nortjie *et al.*, 2022). On the other hand, thermal degradation at high temperatures may involve the oxidation of polyphenols, the degradation of flavonoid structures or the volatilization of certain antioxidant compounds (Ohkawa *et al.*, 1979; ElGamal *et al.*, 2023). The trend observed in this study is consistent with previous research on the preparation of medicinal herbal extracts, where antioxidant activity was maximal at intermediate extraction temperatures (60–80°C) but decreased with excessive heat treatment (Benzie and Strain, 1996; Kedare and Singh, 2011; Abd Ghani *et al.*, 2023). Other herbal extracts have also shown similar trends where optimal extraction temperature and time increased phenolic yield and radical scavenging activity (Singleton and Rossi, 1965; Abd Ghani *et al.*, 2023). These results highlight the importance of selecting appropriate extraction conditions to optimize the DPPH inhibition activity of *M. speciosa*. The optimal conditions for the extraction process with higher antioxidant activity were 60°C for 2

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hours. The findings indicated that the extraction process should avoid extended heating times at high temperatures to retain its bioactive compounds.

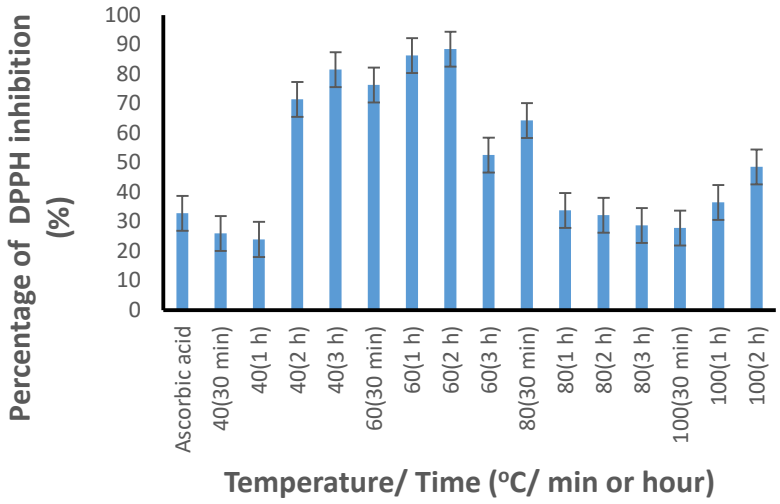

Figure 1. DPPH test

FRAP test

The FRAP assay is commonly used to assess the reducing power of antioxidants in plant extracts by measuring the conversion of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) (Benzie and Strain, 1996). Figure 3 illustrates that the highest FRAP value was achieved at 60°C for 2 hours, suggesting that prolonged moderate heating facilitates the release of antioxidant compounds with reducing capacity (Ross *et al.*, 2011). This finding is consistent with previous studies that suggest phenolic compounds, flavonoids, and other antioxidants exhibit enhanced bioavailability under optimal extraction conditions (Prior *et al.*, 2005). At lower temperatures (40°C), FRAP values were comparatively low, possibly due to partial extraction of antioxidant components. However, at higher temperatures (80°C and 100°C), FRAP values varied, suggesting the likely degradation of bioactive components upon exposure to severe heat for long periods. Similar trends have been observed in other medicinal plants, where antioxidant activity decreases beyond the optimal temperature ranges due to thermal degradation of flavonoids and polyphenols (Singleton and Rossi, 1965; Baharuddin *et al.*, 2018). Unexpectedly, extended extraction times at high temperatures (80–100°C) did not significantly enhance the FRAP values, indicating that extensive heating does not necessarily result in increased antioxidant potential. This can be explained through oxidative degradation or conformational modifications of phenolic compounds, leading to the loss of their reducing power (Ohkawa *et al.*, 1979). The trend that was found is 60°C for 12 hours as the optimum

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extraction condition for maximum antioxidant capacity, as in previous research on plant antioxidants (Kedare and Singh, 2011).

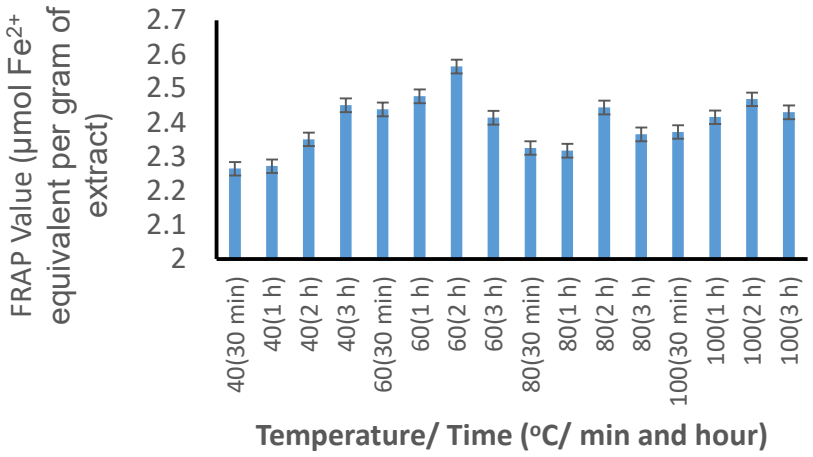

Figure 2. FRAP test

TPC test

Total phenolic content is used extensively for the quantification of total phenolic compounds in plant extracts, which are significant contributors to antioxidant activity (Singleton and Rossi, 1965; Pérez *et al.*, 2023). A peak TPC of 60°C for 3 hours was seen, reflecting improved release of the phenolic compounds after moderate heating. This aligns with previous research that thermally processing will facilitate the extraction of bound phenolic compounds by making them soluble and bioavailable (Dai and Mumper, 2010). Temperature as low as 40°C gave moderate TPC values, suggesting that the extraction of some phenolic compounds improves with increasing temperature. However, over-extraction at higher temperatures (100°C/3 hours for longer times) gave lower TPC values. The trend in the pattern is that high heat can lead to phenolic compound degradation or polymerization, which lowers their overall concentration (Miliauskas *et al.*, 2004). Other medicinal plants have also shown that extended heating adversely affects the stability of polyphenols (Zhou and Yu, 2006; Chew *et al.*, 2011). Results show that 80°C for 3 hours is the optimum extraction time to achieve maximum phenolic content without degradation. Determination of the specific phenolic compounds using chromatographic procedures such as HPLC and LC-MS to correlate their occurrence with biological activity should be done in future research. In addition, combining TPC data with other antioxidant assays such as FRAP and DPPH will provide a better indication of *M. speciosa*'s antioxidant potential.

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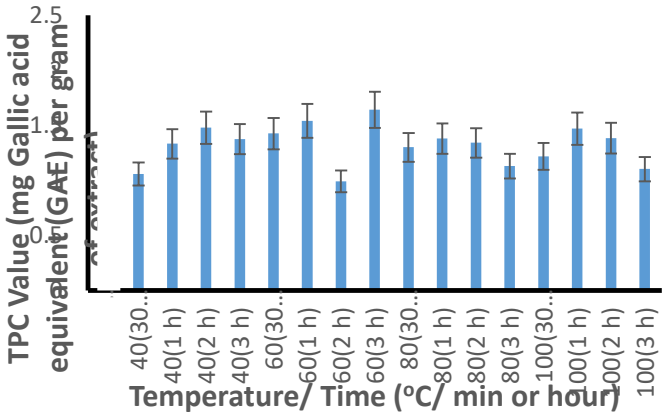

Figure 3: TPC test

MDA test

Figure 4 illustrates the changes in MDA contents at different extraction temperatures and times. Malondialdehyde has long been established as the major indicator for lipid peroxidation (Ayala *et al.*, 2014) and the oxidative breakdown of lipids, which indicates an increased oxidative rate in samples and show compromised antioxidant capability when subjected to those treatments. The MDA contents ranged from around 0.099 to 0.106 µM per mg protein, which suggests that mild thermal processing does not result in a significant change in the antioxidant activity of the extract. The greatest contents of MDAs were found when extracts were prepared at 40°C/30 min, 40°C/3 h, 60°C/3 h, and 80°C/1 h, i.e., these extracts had the lowest antioxidant activity and were unable to inhibit lipid peroxidation. At temperatures greater than 60°C and 80°C, MDA content declined minimally, suggesting that higher temperatures could facilitate the extraction of bioactive compounds with the capacity to stabilize lipid oxidation. The trend is inconsistent with the outcomes of DPPH, FRAP, and TPC assays, in which 60°C yielded the most active extract. In the MDA analysis, however, extracts heated at 80°C and 100°C were more potent in inhibiting lipid peroxidation, showing potential temperature-dependent differences in antioxidant activity. These findings highlight the critical role of temperature optimization in extraction processes to ensure maximum preservation of bioactive components. To further enhanced the therapeutic effects of *M. speciosa,* future studies should be aim to elucidate the biochemical processes controlling MDA content under different extraction conditions to further enhance the therapeutic effects of *M. speciosa*. Further studies are required to explore the stability and bioavailability of some antioxidant compounds of *M. speciosa* and their mechanisms of action in vivo. In

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conclusion, this study provides a fundamental understanding of the antioxidant activity of *M. speciosa* and proposes subsequent research, i.e., in vivo studies and clinical trials, for the confirmation of said findings and establishment of its therapeutic applications.

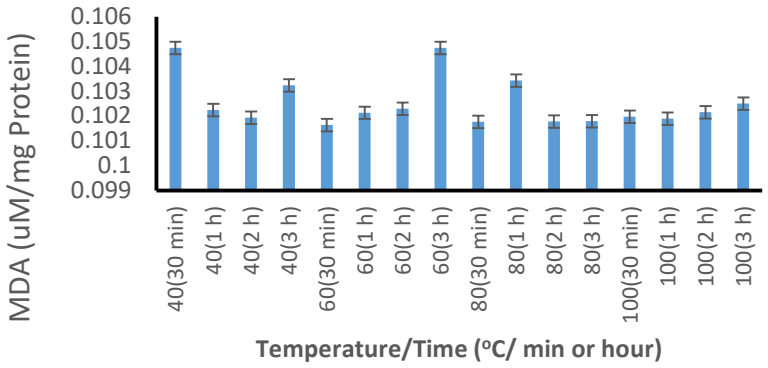

Figure 4. MDA test

CONCLUSION

Antioxidant properties of *M. speciosa* highlight its potential for application in the development of nutraceutical and pharmaceutical products aimed at preventing and treating pathologies associated with oxidative stress, such as neurodegenerative disorders, cardiovascular diseases, and ageing. The treatment duration of 2 hours at 60°C would be employed for achieving *M. speciosa* extract with elevated levels of total phenolic content (TPC) and acceptable antioxidant activity with minimal values of DPPH percentages, as well as with a high FRAP value. However, preparing extracts at 80°C or 100°C appears to be the most effective method for reducing the levels of MDA.

DECLARATIONS OF INTEREST

None.

DECLARATION OF HONOUR

We declare in our honor that our results are not fake and made up.

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