Optimisation of ultrasonic-assisted extraction and biological activity of total flavonoids from leaves of *Murrayae exotica* using response surface methodology

**Yao Wen**, **Manchun Liu**, **Xueying Mai**

School of Pharmacy, Guangdong Pharmaceutical University, Guangzhou 510006, China

**ABSTRACT**

*Murrayae exotica* is a traditional Chinese medicine widely grown in southeast China. A Box–Behnken design of response surface methodology was employed to further optimise ultrasonic-assisted extraction conditions for *M. exotica* leaves total flavonoids (MELTF). The results showed that the optimised extraction conditions were an ultrasonic power of 240 W, an ultrasonic temperature of 60 °C, a solvent concentration of 76%, an ultrasonic time of 55 min and a liquid–solid ratio of 22 mL · g⁻¹. Under these conditions, 8.59 ± 0.34 mg · g⁻¹ was achieved as the mean experimental value of extraction yield, which amounts to 2.56 times that of ethanol leaching extraction. As compared to MELTF, MELTF after purification (MELPTF) had a higher purity of 9.96%, which was nearly nine times higher than that of MELTF (1.26%). As compared to MELTF, MELPTF had higher α-glucosidase and α-amylase inhibitory activities as well as DPPH· and ABTS⁺ scavenging activities with IC₅₀ values of 0.021, 0.094, 0.245 and 0.113 mg · mL⁻¹, which are 1.33, 2.12, 3.17 and 1.78 times higher than those of MELTF (0.028, 0.199, 0.777 and 0.201 mg · mL⁻¹). The study thus demonstrates the eligibility of MELPTF to be considered as a multifunctional bioactive ingredient having potential applications in anti-hyperglycaemic pharmaceutical formulation and as an antioxidant in functional foods.

**Keywords**: anti-hyperglycaemic activity, *M. exotica* leaves, response surface optimisation, total flavonoids, ultrasonic-assisted extraction

**INTRODUCTION**

Diabetes mellitus (DM), one of the most difficult health issues of the 21st century, is a sign of hyperglycaemia brought on by insufficient insulin production or decreased insulin sensitivity (Schmidt and Hickey, 2009). DM can result in numerous consequences, including diabetic ketoacidosis and cardiovascular disease and so on, if adequate therapy is not provided (Cambell, 2011). As is common knowledge, managing blood glucose levels is essential for DM therapy. Additionally, the postprandial digestive enzymes α-amylase and α-glucosidase play crucial roles in the breakdown of carbohydrates, which raises blood sugar levels (Joshi et al., 2015).

The α-glucosidase inhibitor acarbose is useful in reducing postprandial hyperglycaemia. However, the use of acarbose inevitably causes some side effects (Deng et al., 2015). Therefore, finding natural sources of α-glucosidase and α-amylase inhibitors with no or relatively fewer adverse effects has become a research priority in treating DM (Kwon et al., 2006).

*Murrayae exotica* L., a member of the Rutaceae family, is a dwarf tree or an evergreen shrub. Owing to its glossy green leaves and clusters of fragrant white flowers, it is commonly cultivated in gardens as ornamental plant in southern China and many tropical and subtropical
regions (Sharma and Arora, 2015). Meanwhile, as one of the source plants of traditional Chinese medicine (TCM), Murrayae Folium et Cacumen ("Jiulixiang" in Chinese) is documented in the Chinese Pharmacopoeia (2020 edition), and its official medicinal parts are the fresh leaves and twigs of plants (Commission, 2020). Besides flavouring food, M. exotica has been widely used for treatment of various diseases such as toothache, rheumatic arthralgia, stomach-ache, swelling and pain (College, 1986). Modern research has shown that M. exotica contains coumarin, alkaloids, flavonoids and volatile oils, and has a variety of pharmacological activities, including anti-diabetic, antioxidant, anti-inflammatory, insecticidal, antinociceptive and antimicrobial activities (Wu et al., 2010; Huang et al., 2013; Kaur et al., 2016; Xu et al., 2016; Liu et al., 2018). This herb is also a basic ingredient in Sanjü Weitai granule, a well-known Chinese complex prescription for gastric diseases (Liu et al., 2018). M. exotica has been subjected to phytochemical studies, which have led to the discovery that flavonoids are the main active compounds in M. exotica leaves (Kaur et al., 2016). At present, most researches are concentrated on the volatile oils and coumarin of M. exotica, as well as their anti-inflammatory and antioxidant activities (Huang et al., 2013; Liang et al., 2020). However, there are no available reports about the extraction of M. exotica leaves total flavonoids (MELTF) and their anti-diabetic activity. Consequently, it is of great importance to develop a high efficiency method for extracting flavonoid constituents from MELs (MELTF).

In the present study, a three-level, three-variable (solvent concentration, ultrasonic time and liquid–solid ratio) Box–Behnken design (BBD) of response surface methodology (RSM) was employed to further optimise UAE conditions for MELTF. The total flavonoids that were extracted were then refined using AB-8 macroporous resin. The extracted anti-diabetic total flavonoids’ inhibitory effects on α-glucosidase and α-amylase were also identified.

MATERIALS AND METHODS

Materials and reagents
Fresh leaves of M. exotica were collected from Guangdong Province, China. The material was authenticated by Professor Y. H. Wang, Guangdong Pharmaceutical University, Guangzhou, China. Voucher specimens were deposited in the School of Pharmacy at Guangdong Pharmaceutical University. AB-8 macroporous resin was acquired from a tetramethyl biochemical plastics factory (Luqiao district, Taizhou, Zhejiang, China). Rutin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), α-glucosidase, p-nitrophenyl-α-D-glucopyranoside (PNPG), α-amylase, starch, 3,5-dinitrosalicylic acid and acarbose were purchased from Guangzhou Chemical Reagent Company, China. All other chemicals and solvents were of analytical grade.

Ultrasonic-assisted extraction of MELTF

For the experiment’s UAE protocol, we followed the one created by Mai et al. (2020) with minor modifications. The required quantity of powdered dried M. exotica leaves (2 g) was weighed and placed in a conical flask. The flask was then filled with 40 mL of ethanol (60% concentration). The mixtures were then extracted using a Kunshan Ultrasonic Instrument Co., Ltd., Kunshan, China, KQ-400KDB ultrasonic extraction reaction workstation at the designated extraction power (240 W), extraction temperature (50 °C), and extraction time (30 min). The solution in the flask was then centrifuged at 4,390 g for 5 min in a low-speed centrifuge, and the supernatant was used to calculate the content of total flavonoids from M. exotica leaves (MELTFC).

Determination of the content of flavonoids in samples

With certain modifications, the technique outlined by Li et al. (2019) was used to examine the MELTFC. In brief, 200 mL of 5% NaNO₂ solution and 100 mL of the supernatant were combined, and left for 6 min. A total of 200 μL of a 10% Al(NO₃)₃ solution was then added, and the mixture was maintained for 6 min. Subsequently, 200 μL of a 4% NaOH solution was added and then mixed to obtain the test solution. Rutin was used as a standard to evaluate the test solution’s absorbance at 510 nm. The total flavonoids content of the leaves of M. exotica was calculated using the following formula:

\[
\text{Extraction yield of total flavonoids (mg \cdot g^{-1})} = \frac{C \times V \times N}{M} \times 100\%
\]

where C is the concentration of the MELTFC according to the standard curve (mg \cdot mL^{-1}); V is the volume of the extract solution (mL); N is the dilution multiple; and M is the sample weight (g).

Experimental design

Single-factor experiment

A one-way test was used to obtain a preliminary range of extraction variables using the total flavonoid yield of M. exotica leaves as an indicator. The mixture was extracted by weighing 2 g of M. exotica leaf powder precisely in a conical flask, concomitant with the use of an ultrasonic extraction device. The experiments were carried out with the five variables of ethanol concentration (50%, 60%, 70%, 80% and 90%), sonication power (200 W, 240 W, 280 W, 320 W and 360 W), sonication temperature (30 °C, 40 °C, 50 °C, 60 °C and 70 °C), sonication time (15 min, 30 min, 45 min, 60 min and 75 min) and material–liquid ratio (1:5, 1:10, 1:15, 1:20 and 1:25). The five variables were tested in turn. The extracts were then transferred...
to centrifuge tubes and centrifuged at 4,390 g for 5 min and the supernatant was used to determine MELTFC.

RSM experiments

According to the results of the single-factor test, the main influencing factors for the extraction rate of MELTF were ethanol concentration (X₁, %), sonication time (X₂, min) and liquid-to-material ratio (X₃, mL·g⁻¹). A three-factor (Table 1), three-level BBD of RSM response surface method was used for the optimisation of the extraction process of MELTF, with MELTF extraction rate as the index. Each variable was assigned three levels, coded as +1, 0 and –1 to indicate high, medium and low values, respectively.

Comparison of optimised UAE with ethanol leaching extraction

Two grams of the required material were weighed and placed into a conical flask. The flask was then filled with 40 mL of ethanol and left to soak for 1 hr. The solution in the flask was then centrifuged at 4,390 g for 5 min to determine how many total flavonoids were present in the supernatant (MELTCF). Following response surface optimisation, the yields of the MELTF extracted by ultrasonic extraction and ethanol leaching extraction were compared.

Purification of M. exotica flavonoids

Pretreatment of AB-8 macroporous resin

After being soaked in 95% ethanol for 24 hr, AB-8 macroporous resin was packed onto a glass column (20 mm × 40 cm). Since the resin’s height was determined to be 18 cm, its bed volume (BV) was around 60 mL. The macroporous resin was washed with 95% ethanol until the effluent mixed with water (1:5) did not become white and turbid. After that, deionised water was used to wash the column until the smell of ethanol was completely gone.

Adsorption and elution experiments

The purification of total flavonoids was ascertained according to the method reported by Yao et al. (2013), with certain modifications. Following the packing of the column with 1 BV of the sample solution, the column was washed with 3 BV of deionised water at a flow rate of 1.5 BV · hr⁻¹. After that, a flow rate of 1.5 BV · hr⁻¹ of 60% ethanol was used to elute the column. The effluents were collected, and the quantity of flavonoids present, as well as the combined, concentrated and lyophilised effluents inclusive of the total flavonoid content, was ascertained based on studying the colour reaction between NaNO₂-Al(NO₃)₃ and NaOH. The combined, concentrated and lyophilised effluents that included total flavonoids. For the next assays, the purified total flavonoids [MELTF after purification (MELPTF)] were kept in a freezer at 4 °C. The total flavonoids purity of the leaves of M. exotica was calculated using the following formula:

\[
Purity(\%) = \frac{C \times V}{M} \times 100\%
\]

where C is the concentration of the total flavonoids of the leaves of M. exotica (mg · mL⁻¹); V is the volume of the effluents (mL); and M is the sample weight (g).

Anti-hyperglycaemic activity assay

\(\alpha\)-Glucosidase inhibitory activity assay

The literature was used to evaluate the \( \alpha \)-glucosidase inhibitory activity with a little modification (Striegel et al., 2015). A 96-well plate was incubated for 5 min at 37 °C with a combination of 40 μL of sample at various concentrations (0, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 mg · mL⁻¹, dissolved in 0.1 M phosphate buffer) and 40 μL of \(\alpha\)-glucosidase solution (1 U · mL⁻¹, dissolved in 0.1 M phosphate buffer). A total of 50 μL of PNPG (10 mM, dissolved in 0.1 M phosphate buffer) was then added, and the mixture was left at 37 °C for 30 min. A total of 90 μL of Na₂CO₃ solution (0.1 M) was added to end the catalytic process. The positive control was acarbose. The absorbance was measured at 405 nm with a microplate reader, and the inhibition percentage was calculated as follows:

\[
\text{Inhibition percentage (\%)} = \left[ 1 - \frac{A_S - A_D}{A_0} \right] \times 100\%
\]

where \(A_S\) represents the absorbance of the sample reaction solution; \(A_D\) is the absorbance of the reaction system without \(\alpha\)-glucosidase; and \(A_0\) is the absorbance of the reaction system without sample.

\(\alpha\)-Amylase inhibitory activity assay

The \(\alpha\)-amylase inhibitory activity was determined according to a previously published method (Eleazu et al., 2016). A 96-well plate was incubated at 37 °C for 15 min with a combination of 40 μL of sample at various concentrations (0, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 mg · mL⁻¹, dissolved in 0.1 M phosphate buffer) and 40 μL of \(\alpha\)-amylase (0.5 mg · mL⁻¹, dissolved in 0.1 M phosphate buffer). Thereafter, 20 μL of 1% starch solution (dissolved in 0.1 M phosphate buffer) was added, and it was left at 37 °C for 15 min. A total of 100 μL of 3,5-dinitrophenylsalicylic acid was then added and heated for 15 min. Subsequently, the mixture was cooled to room temperature. The positive control was acarbose. The absorbance was measured at 540 nm with a microplate reader, and the inhibition percentage was calculated as follows:

\[
\text{Inhibition percentage (\%)} = \left[ 1 - \frac{A_S - A_D}{A_0} \right] \times 100\%
\]
where $A_o$ represents the absorbance of the sample reaction solution; $A_i$ is the absorbance of the reaction system without α-amylase; and $A_s$ is the absorbance of the reaction system without sample.

**Antioxidant activity assay**

**Determination of DPPH scavenging activity**

In assessing the DPPH-scavenging activity of MELTF and MELPTF, an approach presented in the literature (Chen et al., 2019) was adopted after carrying out a slight modification. We combined all the test samples and DPPH solution (180 μL, 0.2 mM, absolute ethanol) with vitamin C (VC, 20 μL, 0–3.2 mg · mL⁻¹, distilled water). After the reaction was allowed to take place at room temperature for 20 min in the dark, the absorbance was measured at 517 nm on a microplate reader. The formula for calculating the DPPH scavenging rate is as follows:

$$\text{Scavenging rate} (%) = \left[1 - \frac{A_s - A_1}{A_o}\right] \times 100\% \quad (5)$$

where $A_o$ is the absorbance of DPPH mixed with distilled water; $A_1$ is the absorbance of DPPH mixed with the sample; and $A_s$ is the absorbance of anhydrous ethanol mixed with the sample.

**Determination of ABTS⁺ scavenging activity**

The literature-based methodology used to determine ABTS⁺ scavenging ability has been somewhat changed (Rozi et al., 2019). To create an ABTS⁺ stock solution, 7 mM ABTS and 2.45 mM potassium persulphate were combined in an equal volume and heated to 25 °C for 12 hr in the dark. The stock solution was diluted to have an absorbance between 0.2 and 0.8 at 734 nm. Thereafter, we combined all of the samples or the VC stock solution, reaction solution; $A$ is the absorbance of the sample; and $A_i$ is the absorbance of anhydrous ethanol mixed with the sample.

**Statistical analysis**

SPSS 25.0 software (SPSS Inc., Chicago, IL, USA) was used to analyse the bioactivity experiments and single-factor data. For the determination of the experimental design and regression analysis of the experimental data, Design-Expert 10.0.7 (trial version, Stat-Ease Inc., Minneapolis, MN, USA) was used.

**RESULTS AND DISCUSSION**

**Effect of ultrasonic temperature on yield of MELTF**

The cavitation action of ultrasound can encourage the collision of the extracts, resulting in speedy and complete dissolution of the active components in the solvent (Senrayan and Venkatachalam, 2020). While maintaining the solvent concentration, liquid–solid ratio, ultrasonic power and ultrasonic duration at 60%, 40 mg · mL⁻¹, 240 W, and 30 min, respectively, the current study examined the effects of various ultrasonic temperature points within 40–80 °C employed on the extraction yield of MELTF. The MELTF concentration in the extract test rose as the ultrasonic temperature climbed from 40 °C to 60 °C, as shown in Figure 1A, and peaked at around 60 °C. However, increasing the temperature further led to a reduction in the amount of MELTF in the extract. The extraction yield of the MELTF was observed to undergo a gradual rise with the increase of temperature below 60 °C, as a result of the thermal movement of the molecules intensifying with temperature, which is favourable for the exudation and diffusion of flavonoids (Pompeu et al., 2009). Contrastingly, when the temperature exceeded 60 °C, the extraction yield of total flavonoids dropped, and this is likely attributable to the role played by high temperatures in degrading the flavonoid glycosides in *M. exotica* leaves (Jing et al., 2015). Since there were no significant differences for the ultrasonic temperature evaluated, according to the findings of the statistical analysis ($p > 0.05$), 60 °C was selected as the temperature for MELTF extraction. This finding is an exact match with what Pompeu et al. (2009) stated.

**Effect of ultrasonic power on yield of MELTF**

It is crucial to maintain the ultrasonic-extraction appliance at its ideal working power for the extraction of total flavonoids (Ma et al., 2009). While maintaining the solvent concentration, liquid–solid ratio, ultrasonic temperature and ultrasonic time at 60%, 40 mg · g⁻¹, 60 °C and 30 min, respectively, in the present investigation, the influence of various ultrasonic power points within 200–360 W, employed on the extraction yield of MELTF, was examined. As can be seen in Figure 1B, the amount of MELTF in the extract assay rose as the ultrasonic power increased from 200 W to 240 W, peaking at about 240 W. The faster the flavonoids in *M. exotica* leaves release and disperse from the cells, the higher the cavitation effect produced by the ultrasonic application, and this could be the cause of the increase in extraction yield (Zheng et al., 2022). However, further power enhancement led to a reduction in MELTF concentration in the extract. The power being used may be the cause of the decreased extraction yield since it damages part of the flavonoids’ cell structures as well as the *M. exotica* leaves’ cell walls. This decreases
Effect of solvent concentration on yield of MELTF

A high extraction efficiency for total flavonoids was achieved owing largely to solvent concentration (Lin et al., 2005). The impact of solvent concentration on extraction yield was examined in this work, as seen in Figure 1D. The yield of total flavonoids extracted from the *M. exotica* leaves increased steadily in tandem with the rise in the ethanol concentration. When the ethanol concentration was less than 70%, the extraction yield increased quickly, and when the ethanol concentration was higher than 70%, the extraction yield increased gradually. The extraction yield of total flavonoids even dropped at 90% ethanol concentration. Polyhydroxy flavonoids, which have low polarity, make up the majority of the total flavonoids in *M. exotica* leaves. Consequently, the extraction yield of total flavonoids rises as ethanol concentration grows. But when the ethanol concentration is too high, less polar molecules such as chlorophyll could be removed, which lowers the extraction yield of total flavonoids (Wu et al., 2020). As a result of statistical analysis, it was determined that the solvent concentration examined had significant differences ($p < 0.01$). Consequently, 240 W was used as the power for MELTF extraction. This finding exactly matched what Zheng et al. (2022) stated.

**Effect of ultrasonic time on yield of MELTF**

In the case of all flavonoids, a high extraction efficiency was achieved owing largely to ultrasonic time (Silva et al., 2007). It will have an impact on the ultimate MELTF output as well as the cost of energy and extraction efficiency. In this study, MELTF extraction increased as ultrasonic duration increased from 15 min to 75 min, peaking at 45 min. However, as ultrasonic time increased further, MELTF content decreased (Figure 1C). It might be because certain flavonoids’ structures change as ultrasounds get longer (Liyanapathirana and Shahidi, 2005). Based on statistical analysis, it was determined that there were significant differences for the ultrasonic time examined ($p > 0.05$). Therefore, 240 W was used as the power for MELTF extraction. This finding exactly matched what Zheng et al. (2022) stated.

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that an ideal ratio of 70% would be desirable for the manufacture of flavonoids when the solvent cost issue is taken into account. Yu et al. (2019) reported comparable outcomes.

**Effect of liquid–solid ratio on yield of MELTF**

In the UAE, the liquid–solid ratio played a crucial role in achieving a high extraction efficiency for total flavonoids (Lai et al., 2014). A liquid–solid ratio range of 5–25 mL · g⁻¹ was examined, with the ultrasonic power, solvent concentration, ultrasonic temperature and ultrasonic duration all maintained at 240 W, 70%, 60 °C and 45 min, respectively, to examine the impact of liquid–solid ratio on the extraction yield of MELTF. MELTF extraction might be improved by increasing the measured ratio of ethanol to raw material (from 5 mL · g⁻¹ to 25 mL · g⁻¹); however, the improvement levelled out at 20 mL · g⁻¹ (Figure 1E). A quicker exudation of the flavonoids is observed at this juncture, which is possibly attributable to the attendant higher liquid–solid ratio, as well as to the increased surface area of the M. exotica leaves that have been used in the preparation of the extraction solvent (Volpi et al., 2004). However, the greater the increase of extraction solvent, the longer it takes for the total flavonoids of M. exotica to diffuse. When, as a result, the liquid–solid ratio rises over a finite period of time, the extraction yield of M. exotica’s total flavonoids increases more slowly. The extraction yield of total flavonoids fell when the liquid–solid ratio was 25:1. The findings of the statistical analysis revealed that the liquid–solid ratio examined had significant differences (p < 0.01). In the current experiment, a liquid–solid ratio of 20 mL · g⁻¹ was determined to be ideal. Silva et al.’s investigation (2007) yielded a comparable outcome.

**Optimisation of extraction conditions of MELTF**

**Statistical analysis and the model fitting**

As shown in Table 2, the extraction yield of MELTF values varied from 4.39 mg · g⁻¹ to 8.38 mg · g⁻¹. The results of extraction yield affected by solvent concentration, ultrasonic time and liquid–solid ratio were fitted to a second-order polynomial equation, and the values of regression coefficients were calculated. The effects of the three variables on the extraction yield of MELTF were highly significant (Table 3). The predicted model of the extraction yield value was obtained using the following second-order polynomial equation:

\[
Y_1 = 8.00 + 0.93X_1 + 0.61X_2 + 0.63X_3 + 0.26X_1X_2 \\
- 0.43X_1X_3 + 0.19X_2X_3 - 0.78X_3^2 - 0.65X_2^2 - 0.52X_1^2
\]

\(7\)

According to the data obtained (Table 3), the p-value is 0.0024, indicating that the model is highly significant. The p-value of lack of fit is 0.3255, which is greater than 0.05 and not significant. In addition, the \(R^2 = 0.9329\) and \(R_{adj}^2 = 0.8467\), which are not very different and both close to 1.0, indicating that the model is reliable and statistically significant. Secondly, when we checked the p-values, \(p = 0.0007 < 0.01\) for variable \(X_1\) (ethanol concentration), \(p = 0.0068 < 0.01\) for variable \(X_2\) (ultrasonic time) and \(p = 0.0060 < 0.01\) for variable \(X_3\) (liquid–solid ratio), which proved that the effects of ethanol concentration, ultrasonic time and liquid–solid ratio on the extraction yield of MELTF were highly significant; additionally, while the effects of interaction terms of variables \(X_iX_j\) and \(X_iX_k\) on the extraction of MELTF were significant, the effect of the interaction term \(X_iX_j\) on the extraction of MELTF was not significant. This indicates that the degree of influence of factors on the yield was the following: ethanol concentration > liquid–solid ratio > ultrasonic time.

**Analysis of response surface**

The graphical representations of the regression equation were the 3D response surface and the 2D contour plots. They offered a means to show the interactions between two test factors, as well as the relationship between responses and experimental levels of each variable. The circular or elliptical contour plots show whether or not there are substantial mutual interactions between the variables. The elliptical contour map shows strong interactions between the relevant variables, whereas the circular contour plot shows minor interactions between the corresponding variables. In this study, the results of extraction yield of MELTF affected by solvent concentration, liquid–solid ratio and ultrasonic time were presented in Figure 2 and Figure 3.

As shown in Figure 2A, the 3D response surface plot of ethanol concentration was steeper than

<table>
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RSM, response surface methodology.
that of ultrasonic time, indicating that the ethanol concentration has a greater influence on the extraction yield of the MELTF than that of ultrasonic time, which is consistent with the conclusion drawn by the statistical and model-fitting analyses. As shown in Figure 3A, the contour plot was similar to an ellipse, indicating that the ethanol concentration and ultrasonic time have a certain interactive effect on the extraction yield of the MELTF.

As shown in Figure 2B, the 3D response surface plot of ethanol concentration was steeper than that of the liquid–solid ratio, which shows that the influence of ethanol concentration on the extraction yield of total flavonoids is greater than that of the liquid–solid ratio, which is the same as the conclusion drawn from the statistical and model-fitting analyses. The contour plot in Figure 3B presented an ellipse, which shows that the interaction between the liquid–solid ratio and the ethanol concentration has a greater impact on the extraction yield of the total flavonoids from the leaves of *M. exotica*.

As shown in Figure 3C, the steepness of the 3D response surface plot of the liquid–solid ratio in Figure 2C was similar to that of ultrasonic time, which indicates that the two factors have similar effects on the MELTF, which demonstrates consistency with the conclusion drawn from the statistical analysis presented in Table 3 (liquid–solid ratio, \( p = 0.0068 \); and ultrasonic time, \( p = 0.0060 \)). As shown in Figure 3C, the contour plot was slightly elliptical, indicating that the interaction between ultrasonic time and liquid–solid ratio has a certain effect on the extraction yield of the MELTF.

### Verification of predictive model

In comparison to the conventional single parameter optimisation, response surface optimisation is more favourable since it conserves time, space and raw materials. Response surface analysis was conducted through Design-Expert, and the optimised extraction conditions were a solvent concentration of 75.72%, an ultrasonic time of 54.84 min and a liquid–solid ratio of 22.44 mL · g\(^{-1}\). In order to validate the adequacy of the model equations, a verification experiment was carried out under the actual optimal conditions, namely the following: a solvent concentration of 76%, an ultrasonic time of 55 min and a liquid–solid ratio of 22 mL · g\(^{-1}\), considering the feasibility of actual operation. As shown in Table 4, the RSM model was validated by the mean extraction yield (8.59 ± 0.34 mg · g\(^{-1}\)), which was derived from actual trials. The MELTF had an estimated extraction yield of 8.62 mg · g\(^{-1}\). The validation result showed no discernible discrepancy between experimental and projected values, indicating that the response model was sufficient for capturing the anticipated optimisation and that the model presented in Eq. (7) is reliable and accurate. So, the response surface method is a reliable means for optimisation of the extraction process of *M. exotica* leaves.

### Comparison analysis of optimised UAE with ethanol leaching extraction

The goal of this experiment is to present a reasonably effective and straightforward extraction method for...
Optimisation of ultrasonic-assisted extraction

the production and application of *M. exotica* leaves’ total flavonoids. In order to evaluate the impact of the improved extraction process, the optimal process parameters established in this experiment were contrasted with the ethanol extraction technique based on the extraction yield. As shown in Table 4, comparing ethanol leaching extraction to the UAE technique, the ethanol leaching extraction had an average extraction yield of $3.36 \pm 0.08 \text{ mg} \cdot \text{g}^{-1}$. UAE had a higher extraction yield of $8.59 \pm 0.34 \text{ mg} \cdot \text{g}^{-1}$ following the response surface optimisation, which was 2.56 times
more than that of ethanol leaching extraction (3.36 ± 0.08 mg · g⁻¹). This conclusion supports earlier observations that, when it comes to total flavonoid content, ultrasonic extraction is preferable in terms of increasing efficiency, cutting down on extraction time and using less solvent. The overall amount of flavonoids in this study is greater than what has previously been published. This may have been influenced in part by improved extraction effectiveness.

**Purification analysis of MELPTF**

The collected 60% ethanol eluate was developed using the colour reaction with NaNO₂-Al(NO₃)₃-NaOH to monitor the total flavonoid content. The elution profile was obtained based on the volume of elution and the concentration of solute therein and is given in Figure 4. It can be seen from Figure 4 that the total flavonoids were completely eluted by approximately 90 mL eluent at a flow rate of 1.5 BV · hr⁻¹. So the elution volume of 60% ethanol is determined to be 1.5 BV. As compared to the purity of the unpurified total flavonoids (MELTF), the purity of the total flavonoids of *M. exotica* leaves after purification (MELPTF) by the macroporous resin was higher at 9.96%, which is nearly nine times higher than that of MELTF (1.26%).

**Anti-hyperglycaemic activities of MELTF and MELPTF in vitro**

*Inhibitory effects of MELTF and MELPTF on α-glucosidase activity*

Since α-glucosidase inhibitors may considerably lower postprandial blood glucose levels, which is a critical component in the treatment of DM, the inhibition percentage of α-glucosidase can be used to quantify the anti-diabetic impact of medications (Ademiluyi et al., 2014). Acarbose, MELTF and MELPTF were tested for their ability to inhibit glucosidase, and the findings are displayed in Figure 5. As shown in Figure 5, the total

**Table 4. Result of model validation experiments.**

<table>
<thead>
<tr>
<th>No.</th>
<th>Optimum conditions</th>
<th>Extraction yield (mg · g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Solvent concentration (%)</td>
<td>Ultrasonic time (min)</td>
</tr>
<tr>
<td>1</td>
<td>76</td>
<td>55</td>
</tr>
<tr>
<td>2</td>
<td>76</td>
<td>55</td>
</tr>
<tr>
<td>3</td>
<td>76</td>
<td>55</td>
</tr>
<tr>
<td>4</td>
<td>76</td>
<td>55</td>
</tr>
<tr>
<td>5</td>
<td>76</td>
<td>55</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ethanol leaching extraction

| 6   | 0                  | 20                        | 20                          | 3.38        |
| 7   | 0                  | 20                        | 20                          | 3.28        |
| 8   | 0                  | 20                        | 20                          | 3.42        |
| Average |                         |                           |                             | 3.36        |

*Figure 4. Elution profile of MELPTF on AB-8 macroporous resin column. MELPTF, MELTF after purification.*
flavonoids of the leaves of *M. exotica* before and after purification showed dose-dependent inhibitory effects on α-glucosidase activity when the concentration of total flavonoids was in the range of 0.1–3.2 mg · mL\(^{-1}\). The IC\(_{50}\) values of MELTF and MELPTF were determined to be 0.028 mg · mL\(^{-1}\) and 0.021 mg · mL\(^{-1}\). As compared to MELTF, MELPTF had higher α-glucosidase inhibitory activity, with an IC\(_{50}\) value of 0.021 mg · mL\(^{-1}\), which was 1.33 times higher than that of MELTF (0.028 mg · mL\(^{-1}\)). In the range of 0.1–3.2 mg · mL\(^{-1}\), the inhibition percentage of acarbose reached more than 90%, and the inhibition percentage of MELPTF was 88.29% at a concentration of 3.2 mg · mL\(^{-1}\), which was close to that of acarbose. After purification, the inhibition percentage of the total flavonoids from the leaves of *M. exotica* has shown improvement, and this percentage was relatively high, indicating that the total flavonoids from the leaves of *M. exotica* have development value in the direction of being an α-glucosidase inhibitor. At the same concentration, MELPTF’s α-glucosidase inhibitory activity was superior to that of the flavonoid-rich extracts made from the peel of *Ficus carica* (Meziant et al., 2021). The outcome indicated that MELPTF could be the active ingredient in charge of *M. exotica’s* anti-diabetic action.

**Inhibitory effects of MELTF and MELPTF on α-amylase activity**

Dietary starch and glycogen can be broken down by the α-amylase to provide glucose and maltose. As a result, delaying a rise in the blood glucose level through inhibition of α-amylase activity is crucial for the treatment of DM (Lordan et al., 2013). Acarbose, MELTF and MELPTF were tested for their ability to inhibit amylase, and the findings are displayed in Figure 6. As shown in Figure 6, the α-amylase inhibitory activities of all samples correlated positively with increasing concentrations in the range of 0.1–3.2 mg · mL\(^{-1}\). The IC\(_{50}\) values of acarbose, MELTF and MELPTF were determined to be 0.043, 0.199 and 0.094 mg · mL\(^{-1}\), respectively. As compared to MELTF, MELPTF had higher α-amylase inhibitory activity, with an IC\(_{50}\) value of 0.094 mg · mL\(^{-1}\), which was 2.12 times higher than that of MELTF (0.199 mg · mL\(^{-1}\)). MELPTF strongly inhibited α-amylase, with an IC\(_{50}\) value of 0.094 mg · mL\(^{-1}\), which was close to that of acarbose (0.043 mg · mL\(^{-1}\)). In the range of 0.1–3.2 mg · mL\(^{-1}\), the inhibition percentage of acarbose reached 92.45%, and the inhibition percentage of MELPTF was 71.13% at a concentration of 3.2 mg · mL\(^{-1}\), which was close to that of acarbose. As compared to MELTF, MELPTF had a higher α-amylase inhibitory activity, with an inhibition percentage of 71.13%, which was higher than that of MELTF (64.28%) at 3.2 mg · mL\(^{-1}\). Following purification, the MELTF showed better inhibition percentages, and these percentages were reasonably high, indicating that the MELTF have potential for development as α-amylase inhibitors. At the same dose, MELPTF showed exceptional amylase inhibitory action, outperforming certain flavonoids that had been previously reported (Liu et al., 2013; Wang et al., 2018).

**Antioxidant activities of MELTF and MELPTF in vitro**

**DPPH scavenging activities of MELTF and MELPTF**

A traditional technique used in the food business and agriculture to assess the antioxidant potential of foods is the DPPH-scavenging experiment. Figure 7 displays the findings from the analysis of the DPPH-scavenging abilities of VC, MELTF and MELPTF. When the concentration of total flavonoids was between 0.1 mg · mL\(^{-1}\) and 3.2 mg · mL\(^{-1}\), as seen in Figure 7, the total flavonoids of *M. exotica* leaves both before and after purification demonstrated dose-dependent scavenging effects on DPPH-scavenging activity. The IC\(_{50}\) values of
VC, MELTF and MELPTF were determined to be 0.027, 0.777 and 0.245 mg · mL⁻¹, respectively. As compared to MELTF, MELPTF had a higher DPPH scavenging activity, with an IC₅₀ value of 0.245 mg · mL⁻¹, which was 3.17 times higher than that of MELTF (0.777 mg · mL⁻¹). In the range of 0.1–3.2 mg · mL⁻¹, the inhibition percentage of VC reached more than 90%, and the inhibition percentage of MELPTF was 85.34% at a concentration of 3.2 mg · mL⁻¹, which was close to that of VC. The total flavonoids from *M. exotica*’s leaves offer potential for development as antioxidants, as shown by the enhanced scavenging rate of these compounds after purification. This scavenging rate was also rather high. MELPTF demonstrated exceptional DPPH-scavenging activity, which was even higher than that of the extracts from *Sophora flavescens* that were rich in flavonoids (0.984 mg · g⁻¹) (Zhou et al., 2018). The outcome indicated that MELPTF could be the active ingredient in charge of *M. exotica*’s antioxidant action.

**ABTS⁺ scavenging activities of MELTF and MELPTF**

It is possible for flavonoids to scavenge ABTS⁺, a specific kind of free radical. VC, MELTF and MELPTF were tested for their ability to scavenge ABTS⁺, and the findings are displayed in Figure 8. All samples had ABTS⁺ scavenging activities that increased in a positive correlation with concentrations between 0.1 mg · mL⁻¹ and 3.2 mg · mL⁻¹, as shown in Figure 8. The IC₅₀ values of VC, MELTF and MELPTF were determined to be 0.047, 0.201 and 0.113 mg · mL⁻¹, respectively. As compared to MELTF, MELPTF had a higher ABTS⁺ scavenging activity, with an IC₅₀ value of 0.113 mg · mL⁻¹, which was 1.78 times higher than
that of MELTF (0.201 mg · mL\(^{-1}\)). MELPTF strongly scavenged ABTS\(^{+}\), with an IC\(_{50}\) value of 0.094 mg · mL\(^{-1}\), which was close to that of VC (0.043 mg · mL\(^{-1}\)). In the range of 0.1–3.2 mg · mL\(^{-1}\), the scavenging rate of VC reached 100.00%, and the scavenging rate of MELPTF was 98.95% at a concentration of 3.2 mg · mL\(^{-1}\), which was close to that of VC. As compared to MELTF, MELPTF had higher ABTS\(^{+}\) scavenging activity, with an scavenging rate of 98.95%, which was higher than that of MELTF (87.59%) at 3.2 mg · mL\(^{-1}\). The total flavonoids from *M. exotica*’s leaves offer potential for development as antioxidants, as shown by the enhanced scavenging rate of these compounds after purification. This scavenging rate was also rather high, and thus demonstrated a greater effectiveness compared with the flavonoid-rich extracts from *Crataegus* (0.39 mg · mL\(^{-1}\)); accordingly, we are led to infer that MELPTF is capable of providing significant ABTS\(^{+}\) scavenging activity (Čopra-Janičijević et al., 2018).

CONCLUSIONS

The leaves of *M. exotica* were extracted using the ultrasonic aided extraction method and the ethanol leaching extraction method in the present investigation, and the extracts showed variable yields. The best strategy for increasing yield was determined to be ultrasonic aided extraction. In cases involving the usage of ethanol as a solvent, the optimal extraction conditions for ultrasonic-assisted extraction of MELTF are obtained as the following: an ultrasonic power of 240 W, an ultrasonic temperature of 60 °C, a solvent concentration of 76%, an ultrasonic time of 55 min and a liquid–solid ratio of 22 mg · g\(^{-1}\). Under this condition, the mean experimental value of extraction yield (8.59 ± 0.34 mg · g\(^{-1}\)) was achieved, which corresponds well with the predicted value and was 2.56 times higher than that of ethanol leaching extraction (3.36 ± 0.08 mg · g\(^{-1}\)). As compared to MELTF, MELPTF had a higher purity of 9.96%, which was nearly nine times higher than that of MELTF (1.26%). As compared to MELTF, MELPTF had higher α-glucosidase and α-amylase inhibitory activities, with IC\(_{50}\) values of 0.021 mg · mL\(^{-1}\) and 0.094 mg · mL\(^{-1}\), which were 1.33 and 2.12 times higher than those of MELTF (0.028 mg · mL\(^{-1}\) and 0.199 mg · mL\(^{-1}\)), respectively. Moreover, as compared to MELTF, MELPTF had higher DPPH and ABTS\(^{+}\) scavenging activities, with IC\(_{50}\) values of 0.245 mg · mL\(^{-1}\) and 0.113 mg · mL\(^{-1}\), which were 3.17 and 1.78 times higher than those of MELTF (0.777 mg · mL\(^{-1}\) and 0.201 mg · mL\(^{-1}\)), respectively. The aforementioned findings point to the potential that this method of extraction possesses for improving the *in vitro* hypoglycaemic and antioxidant activities of MELPTF, and thereby offer a rationale for conducting further research into and utilisation of the total flavonoids of *M. exotica* leaves, as well as a fresh approach towards the development of DM therapies by making use of these desirable properties of *M. exotica*.

To further understand the pharmacological mechanism and related structure, more research is warranted. The aforementioned findings imply that MELPTF are the active ingredients in charge of exerting the discussed anti-diabetic actions via inhibition of α-glucosidase and α-amylase. As a result, MELPTF may be studied and developed as one of the possible functional foods for anti-diabetic drugs.

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AUTHOR CONTRIBUTIONS

Y.W. – funding acquisition, methodology, project administration, resources, supervision and writing: original draft preparation, review and editing. Y.W., M.L. and X.M. – investigation and formal analysis.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest germane to this work.

DATA AVAILABILITY

The data used to support the findings of this study are available from the corresponding author upon request.

ETHICS APPROVAL

This study involves no experiments with human participants or animals performed by any of the researchers.

CONSENT TO PARTICIPATE

Not applicable. This study did not use any human or animal subjects.

REFERENCES


Comparison of different extraction methods for polysaccharides from bamboo shoots (Chimonobambusa quadrangularis) processing by-products. International Journal of Biological Macromolecules, 130, 903–914.


Chemical composition and antioxidant activity of the endemic Crataegus microphylla Koch subsp. malyana K. I. Chr. & Janjić from Bosnia. Industrial Crops and Products, 113, 75–79.


Evaluation of inhibitory properties of flavonoid-rich fig (Ficus carica L.) peel extracts against Rhus typhina L. and their using starch digestibility, alpha-amylase and alpha-glucosidase inhibitory capacities of raw and processed forms of three varieties of Livingstone potato (Plectranthus esculentus). Innovative Food Science & Emerging Technologies, 37, 37–43.

Optimization of ultrasonic-assisted water extraction of flavonoids from Psidium guajava leaves by response surface analysis. Preparative Biochemistry & Biotechnology, 49, 21–29.

Coumarin derivatives from the leaves and twigs of Murraya exotica L. and their anti-inflammatory activities. Phytochemistry, 177, 112416.

Effects of solvent polarity and acidity on the extraction efficiency of isoflavones from soybeans (Glycine max). Journal of Agricultural and Food Chemistry, 53, 3795–3800.


Optimization of extraction of phenolic compounds from wheat using response surface methodology. Food Chemistry, 93, 47–56.


Simultaneous extraction of phenolic compounds of citrus peel extracts: Effect of ultrasound. Ultrasonics Sonochemistry, 16, 57–62.

Optimization of ultrasound-assisted polyethylene glycol-based extraction coupled to temperature-induced cloud point extraction. Ultrasonics Sonochemistry, 66, 105073.

Assessment of inhibitory properties of flavonoid-rich fig (Ficus carica L.) peel extracts against tyrosinase, alpha-glucosidase, urease and cholinesterases enzymes, and relationship with antioxidant activity. European Journal of Integrative Medicine, 43, 101272.

Optimisation of the solvent extraction of phenolic antioxidants from fruits of Euterpe oleracea using Response Surface Methodology. Bioresource Technology, 100, 6076–6082.


Yu, M., Wang, B., Qi, Z., Xin, G., and Li, W. (2019). Response surface method was used to optimize the ultrasonic assisted extraction of flavonoids from *Crinum asiaticum*. *Saudi Journal of Biological Sciences*, 26, 2079–2084.


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