

Article

Optimization of the Antioxidant Activities of Mixtures of Melastomataceae Leaves Species (*M. malabathricum Linn Smith, M. decemfidum,* and *M. hirta*) Using a Simplex Centroid Design and Their Anti-Collagenase and Elastase Properties

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Received: 19 August 2020; Accepted: 15 September 2020; Published: 8 October 2020



Abstract: In this study, a simplex-centroid mixture design (SCMD) approach was used to select the optimal proportions of three different Melastomataceae leaves species (M. malabathricum, *M. decemfidum*, and *M. hirta*) extracts to determine the optimum antioxidant activities of total phenolic compound (TPC) and ABTS (2, 2'-azino-bis (ethylbenzthiazoline-6-sulfonic acid)) radical-scavenging activities. Twelve experimental designs were set up, consisting of points that were equally weighted mixtures of 0 to 1 components, which were pure blends $(1, 0, \ldots, 0)$, binary blends $(1/2, 1/2, 0, \ldots, 0)$, tertiary blends (1/3, 1/3, 1/3, 0, ..., 0), and a control point. The in vitro anti-elastase and collagenase activities were evaluated in order to determine the anti-aging efficacy of the optimized mixture extracts. A high-performance liquid chromatography (HPLC) analysis was employed to identify the flavonoid content (rutin and quercetin) present in the optimized mixture extracts. The results showed that the best proportions of the optimum phenolic compounds and ABTS activity corresponded to 30%, 40%, and 30% of M. malabathricum, M. hirta, and M. decemfidum leaf extracts, respectively. The invitro anti-collagenase and elastase activity evaluation of the optimized mixture extracts showed 70% inhibition against both elastase and collagenase enzymes. The HPLC analysis revealed two flavonoids (rutin and quercetin) at retention time 7.770 and 8.769 min, respectively, in the mixture extracts. This study suggests the potential exploitation of mixtures of Melastomataceae leaves (M. malabathricum, M. decemfidum, and M. hirta) as cosmetic ingredients for antioxidant and anti-wrinkles applications.

Keywords: *Melastoma malabathricum; M. hirta; M. decemfidum;* simplex centroid; rutin; quercetin; antioxidant; collagenase; elastase



1. Introduction

Since ancient times, plants have been used to treat various ailments due to their antioxidant activities. According to Saeed et al. [1], numerous medicinal plants have been investigated for their antioxidant properties. The active compounds present in natural antioxidants; such as phenolic compounds, flavonoids, folic acid, carotenoids, benzoic acid, and tocopherol are secondary metabolites of the plants that play an important role in the body's defense system [2]. It has been reported that the active compounds found in phenolic or polyphenol compounds (caffeic, benzoic acid, and chlorogenic acid) and flavonoids (catechin, rutin, and quercetin) are potent antioxidants, and they have been identified as metal chelators and free-radical terminators used to prevent harmful reactive oxygen species (ROS) from damaging tissues and cells, such as DNA, proteins, and lipids [3].

To date, Melastomataceae is one of the natural plant sources that has gained attention within the scientific world due to its significant therapeutic value. It is one of the most abundant and diversified groups of pantropical families, having 4200 to 4500 species in 166 genera and approximately 1000 species predominantly cultivated in tropical Asia [4]. In Malaysia, particularly, the genus Melastoma consists of 22 species, 2 subspecies, and 3 varieties, which can be distinguished by the color of their flower petals, such as Melastoma malabathricum (dark-purple magenta), Melastoma decemfidum (white), and Melastoma hirta [4-7]. Studies have reported that members of this family have been used by practitioners of traditional medicines to treat diarrhea, pox scars, inflamed wounds, gastric ulcers, and diabetes [8–10]. The phytochemistry of these plants has revealed numerous secondary metabolites or bioactive compounds, such as flavonoids and phenolic compounds [5]. Moreover, previous reports have shown the isolation of several flavonoids from *M. decemfidum* yields naringenin, kaempferol, kaempferol-3-O-D-glucoside, and kaempferol-3-O-[2'6'-di-O-p-trans-coumaroyl], which are responsible for their antioxidant activities [11]. In addition, due to the fact that plant extracts usually occur as a combination of diverse type of bioactive compounds, the identification and isolation of active constituents in plant extracts are commonly achieved using the application of spectroscopic or chromatographic techniques such as high-performance liquid chromatography (HPLC) and gas chromatography mass spectrometry (GCMS).

Optimization of the antioxidant compound is a crucial step for a variety of applications, especially in industrial fields such as pharmaceuticals. To date, the mixture design experiment (MDE) has been one of the most powerful tools used as a systemic statistical approach and an effective method of determining the proportions of variables (ingredients) of a blend. In addition, it is used to minimize the number of experiments and, at the same time, identify the effect of the simultaneous interactions between the variables and the target response [12]. The MDE has provided satisfactory results for the optimization of the combination procedures, and it has been successfully applied in scientific research and development and in real-world problems. For instance, Fadil et al. [13] successfully optimized the amount of fermented soy flour extract solvents to obtain maximum phenolic and antioxidant activities using an MDE. Despite numerous studies of MDEs, the simplex-centroid mixture design (SCMD) experiment was used in this study to determine the optimum proportions or combinations for a target response of three different Melastomataceae leaf species (M. malabathricum, M. decemfidum, and M. hirta) extracts for the optimum antioxidant activities of phenolic compounds and optimum ABTS (2, 2'-azino-bis (ethylbenzthiazoline-6-sulfonic acid)) radical-scavenging activities. The development of better formulations of Melastomataceae leaf extracts will be reviewed in order to optimize the in vitro collagenase and elastase properties of the species for anti-aging applications.

2. Materials and Methods

2.1. Chemicals

ABTS (2, 2'-azino-bis (ethylbenzthiazoline-6-sulfonic acid)), Folin-Ciocalteu reagent, sodium carbonate, and gallic acid standard were purchased from Sigma-Aldrich Co., Ltd. (Sigma Chemical

Co., St. Louis, MO, USA). All solvents (methanol) and reagents (potassium persulphate) used in this study were of high purity and were analytical grade.

2.2. Plant Materials

The leaves of *M. malabathricum* and *M. hirta* were collected from their natural habitat at Ayer Hitam, Hutan Puchong, Selangor, Malaysia from August 2017 to October 2017. Meanwhile, *M. decemfidum* leaves were obtained in Johor in the south of Malaysia. All leaves were further identified by Dr. Paiman bin Bawon from the Faculty of Forestry, Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia. A voucher herbarium specimen for all samples was deposited at the Institute of Bioscience (IBS), UPM, Serdang, Selangor, Malaysia, with voucher numbers SK3377/18, SK3378/18, and SK3379/18 for *M. malabathricum*, *M. hirta*, and *M. decemfidum*, respectively.

2.3. Extraction of Plants

The whole leaf samples were washed with tap water to remove all dust and debris and dried under shade at room temperature $(27 \pm 2 \,^{\circ}C)$ for two weeks. According to the literature, this process does not cause any destruction to the bioactive compounds of the leaves, as proven by the presence of antioxidant, anti-inflammatory, anti-nociceptive, and anti-bacterial activities [10]. Next, the air-dried leaves were ground into a powder using a mechanical grinder machine (120 V to 60 Hz, Waring Laboratory Blenders). Twenty grams of *Melastoma* leaf powder was subjected to extraction using a maceration method with 200.0 mL of methanolic solvent carried out under shaking at 200 rpm for 72 h (3 days) at room temperature. The mixtures were filtered using filter paper (Whatman No. 1). After filtration, the solvent was completely removed in a rotary evaporator (Yamato, Rotary Evaporator, model-RE 801, Japan) until crude extracts were obtained. Ten milligrams of dry extract was then weighed and re-dissolved in 10.0 mL of methanol to obtain a solution of 1000 mg/mL for each sample extract.

2.4. Preparation of Extract Mixtures

Ten milligrams of each crude extract was diluted with 10.0 mL of methanol until the final volume of the mixtures was 1000.0 μ L. The combination of three *Melastoma* leaf extracts and the observed response of each experiment are listed in Table 2. The experiments were performed after randomization, and every response of interest is the average of three replicates.

2.5. Antioxidant Activity Assay

2.5.1. Abts Radical-Scavenging Activity

This assay was adopted by Shalaby et al. [14] and it is based on the ability of extracts to scavenge the 2,2'-azino-bis (ethylbenzthiazoline-6-sulfonic acid (ABTS⁺) radical cation. First, the ABTS radical cation was prepared by mixing 10.0 mL of 7.0 mM ABTS stock solution with 10.0 mL of 2.45 mM potassium persulphate ($K_2S_2O_8$), and the mixture was left in the dark at ambient temperature for 16 h until the reaction was complete. Next, the generated ABTS⁺ solution was diluted with methanol to an absorbance of 0.70 ± 0.05 at 734 nm for measurements. Fifty microliters of *Melastoma* leaf extract was reacted with the generated ABTS solution, and the mixture was vortexed for 10 s. Finally, the absorbance of the reaction mixture was recorded spectrophotometrically (Tecan, Microplate reader) at 734 nm. The antioxidant activity was evaluated as the percent inhibition according to the decolorization of the ABTS radical cation using the following Equation (1):

% ABTS scavenging activity = (Abts control – Abts extract)/Abts control
$$\times$$
 100 (1)

where Abts control and Abts extract are the absorbances of the tested samples.

2.5.2. Total Phenolic Content (TPC)

The total phenolic content (TPC) was determined using the Folin-Ciocalteu method by Singleton and Rossi (1965), with minor modifications [15]. In brief, 100.0 μ L (1.0 mg/mL) of the sample extract was mixed with 50.0 μ L of Folin solution that was previously diluted with 7.0 mL of distilled water. Next, 1.5 mL of a 7.5 w/v° sodium carbonate solution was added to the mixture. The samples were incubated in the dark at room temperature for about 2 h for the reaction to take place. The absorbance of the samples was measured at 765 nm using a UV-VIS (Ultraviolet-Visible Spectroscopy) microplate reader (Infinite M200, Tecan Austria, Grödig/Salzburg, Austria). All samples were prepared in triplicate for each analysis, and the mean absorbance values were determined. A gallic acid standard with a concentration of 25–1000 μ g/L was prepared in this study to generate a standard calibration curve. The phenolic contents of a mixture of *Melastoma* leaf extracts were calculated based on the standard calibration curve and are expressed as mg gallic acid equivalent (mg/g GAE) of extract sample.

2.6. Experimental Design

2.6.1. Mixture Design

The mixture design method is applied to obtain an optimal formulation with the least number of experiments [13,16]. This strategy determines the relationship between the variables and the measured experimental responses. Moreover, according to Fadil et al. [13] mixture design is the easiest possible design to set up where variables are ingredients or proportions. In this study, mixture design, namely simplex-centroid design, allowed for the investigation of the synergistic or antagonistic effects of the mixture components on response variables because the proportion of the components is interdependent on the mixture [17]. This statistical tool helps to clarify the relationship between the mixture compositions of *Melastoma* leaf extracts and higher antioxidant activity.

2.6.2. Experimental Matrix and Mathematical Model

A simplex-centroid design using Design-Expert software[®] version 7.0 (Stat-Ease Inc., Minneapolis, MN, USA) was selected for this optimization. In Figure 1, this design consists of 12 experimental runs set up as follows: (1) the 3 *Melastoma* leaf extracts in the vertices of the triangle (points 1, 2, and 3); (2) the 0.5/0.5 binary mixtures (points 4, 5, and 6); (3) the equal proportionate mixture of the 3 constituents (points 7); and (4) the control points (experiments 8, 9, and 10). Experiment 7 was replicated 3 times in order to determine the pure error and compare it with the lack of fit from the analysis of variance (ANOVA) in MDE. This method provides the optimal proportion of the variables (in this case, the proportion of each *Melastoma* extract, x_1 , x_2 , and x_3). To express the responses as a function of the independent variables based on the mixture method, the special cubic regression model was used to describe the response of a ternary mixture (Equation (2)), as follows [12]:

$$y = b_{1}^{*}x_{1} + b_{2}^{*}x_{2} + b_{3}^{*}x_{3} + b_{12}^{*}x_{1}x_{2} + b_{13}^{*}x_{2}x_{3} + b_{23}^{*}x_{2}x_{3} + b_{123}^{*}x_{1}x_{2}x_{3}$$
(2)

The response of interest predicted by the model and x_1 , x_2 , x_3 , x_{12} , x_{23} , and x_{123} are the estimated variables corresponding to the pure components, $mm(x_1)$, $mh(x_2)$, and $md(x_3)$, and their binary and ternary interactions are predicted by the model.

2.6.3. Statistical Analysis

All analyses were performed in triplicate. The mathematical models were subjected to analysis of variance (ANOVA), and the regression analysis was conducted using Design-Expert version 7.0 software. In this analysis, the coefficients of determination (R^2) were also determined to measure how well the regression equation (model) fit the experimental data and, at the same time, provided the correlation between the observed and the predicted responses. The significance of the model was statistically validated by the ratio between the mean square regression (MSR), the mean square residual

(MSr), *F*-value, and *p*-value ratio at a 95% significance level. The variability of the data around its mean is adequately explained by the greater *F*-value. The ratio between the mean square lack of fit (MSLOF) and the mean square pure error (MSPE) was determined to evaluate if the model was well adjusted to the observations [13].



Figure 1. Positions of experimental points for the simplex-centroid design.

2.6.4. Optimization

The simultaneous optimization mixture formulations were determined from the overall desirability function and graphical analysis of the response surface (contour plot) from adjusted models [17]. The models were finally validated by performing the real experiments on each optimal point mixture.

2.7. Identification of Isolated Rutin and Quercetin Using High-Performance Liquid Chromatography (HPLC)

A simple and accurate high-performance liquid chromatography (HPLC) method was developed for the identification of isolated quercetin and rutin in the optimized *Melastoma* leaf extract. The chromatographic condition was developed as follows. The optimized separation was achieved on a reversed-phase C18 column using a Zorbax Eclipse Plus C18 column (6.6×150 mm, 3.5μ m particle size; Agilent, Palo Alto, CA, USA). The mobile phase consisted of a mixture of water acidified with 0.1% formic acid (mobile phase A) and acetonitrile (mobile phase B) in the gradient flow method (Table 1). The HPLC system was operated at a flow rate of 0.750 mL/min, and the temperature of the column was set at 40 °C. The sample injection volume was 20.0 μ L, and the identified compounds were detected using UV detection at 254 nm. The isolated quercetin and rutin were dissolved in methanol, and the ultraviolet radiation absorption peaks were determined and compared with standard quercetin and rutin.

Table 1. The gradient flow method in mobile phase.

Time (min)	%A	%B
0.00	90	10
0.50	90	10
10.50	55	45
12.00	30	70
14.00	30	70
16.00	90	10

2.8. Neutrophil Elastase Inhibition Assay

The determination of elastase inhibition activity using a mixture of *Melastoma* leaf extracts was carried out using the Neutrophil Elastase Colorimetric Drug Discovery Kit, which was

purchased from Enzo Life Sciences (BML-AK497). This kit provides a complete assay system that is designed to screen inhibitors of neutrophil elastase (purified human neutrophil elastase; $2.2 \mu U/\mu L$), a potential therapeutic agent, using the chromogenic substrate MeOSuc-Ala-Ala-Pro-Val-pNA (100.0 μ M), which was spectrophotometrically measured at 405 nm using a microplate reader. In this study, a pancreatic elastase inhibitor scientific name is Elastatinal (N-((S)-1-carboxyisopentyl)) carbamoyl- α -(2-iminohexa-hydro-4(S)-glycyl-(S)-glutaminyl-(S)-alaninal) was used as a positive control. During the experimentation, the kit and reagent were thawed to room temperature from -80 °C and were kept in a cool environment. This is to ensure maximal enzymatic activity. Initially, $2.0 \mu L$ of the inhibitor, elastastinal, was diluted with 78.0 μ L of buffer solutions (100.0 mM HEPES (N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)), pH 7.25, 500 mM NaCl, 0.05% Tween 20 in dimethyl sulfoxide (DMSO) to make up a 100.0 μ M inhibitor solution. Next, the substrate solution was prepared by diluting 7.5 μ L of substrate in 67.5 μ L of assay buffer in a separate tube. Subsequently, in another vial, 1.0 µL of the neutrophil elastase enzyme was diluted with 89.0 µL of assay buffer. To ensure elastase inhibition activity, 20.0 µL of the Melastoma extract mixture was prepared and diluted with 65.0 µL of buffer solution in a 96-well plate. Next, 10.0 µL of the neutrophil elastase enzyme solution was added to the appropriate well containing test sample or control (elastastinal inhibitor), followed by incubation for 30 min at a reaction temperature of 37 °C to allow interaction of the inhibitor and enzyme. Finally, the absorbance at 405 nm was monitored for 10 min after the addition of substrate to the respective well. The blank and control were prepared in the present work consisting of 5.0 µL of substrate diluted with 95.0 µL of buffer solution and for the negative control consisting of 5.0 μ L of the substrate and 10.0 μ L enzyme in an 85.0 μ L assay buffer solution.

2.9. Matrix Metalloproteinase (MMP-1) Collagenase Inhibition Assay

Matrix metalloproteinase-1 (MMP-1) is an interstitial collagenase or fibroblast collagenase. These enzymes play a significant role in targeting collagen, gelatine, entactin, pro-TNF- α , and the chemokine SDF-11-4. The MMP-1 Colorimetric Drug Discovery kit was purchased from Enzo Life Sciences (BML-AK404). This kit is a complete assay system designed to screen MMP-1 inhibitors using a thiopeptide as a chromogenic substrate (Ac-PLG-(2-mercapto-4-methyl-pentanoyl)-LG-OC₂H₅). During this assay, the MMP cleavage site peptide bond is replaced by a thioester bond in the thiopeptide (substrate), which produces a sulfhydryl group that reacts with DTNB (5,5'-dithiobis (2-nitrobenzoic acid)). The final product was detected at a wavelength of 412 nm. During experimentation, the kit and reagent were thawed to room temperature from -80 °C and were kept in a cool environment. This is to ensure that maximal enzymatic activity is retained. Initially, in a separate vial, 1.0 µL of inhibitor (MMP, N-isobutyl-N-(4-Methoxyphenylsulfonyl) glycyl hydroxamic acid) was diluted with 199.0 µL of assay buffer (500.0 mM HEPES, 10.0 mM CaCl₂, 0.05% Brij-35, and 1.0 mM DTNB; pH 7.5). Subsequently, a substrate solution was prepared by diluting 6.4 µL of the substrate (thiopeptide, Ac-PLG-(2-mercapto-4-methyl-pentanoyl)-LG-OC₂H₅; 100.0 mM) with 153.6 µL of assay buffer in a separate tube. Next, an enzyme solution was prepared by diluting 1.0 μ L of the MMP-1 enzyme (human recombinant) with 40.0 µL of assay buffer. To ensure collagenase inhibition activity, 20.0 µL of the *Melastoma* extract mixture was prepared and diluted with 65.0 µL of buffer solution in a 96-well plate. Next, 20.0 µL of the enzyme was added to each respective well containing an active extract, followed by incubation for 30 min at a reaction temperature of 37 °C to allow interaction of the inhibitor and enzyme. Then, 10.0 μ L of the substrate solution was added to each well, and the absorbance 412 nm was monitored for 10 min. The blank, 90.0 μ L of the assay buffer, and 10.0 μ L of the substrate were added to the well. For the positive and negative control, 20.0 µL of the test inhibitor was added to 10.0 μ L of the enzyme and 10.0 μ L of the substrate, except for the negative control; the test inhibitor was excluded from the well.

3. Results

3.1. Antioxidant Activity

The optimization of antioxidant activity on ABTS-radical scavenging activity and phenolic content were studied using the simplex-centroid mixture design with three different *Melastoma* extract components (x_1 : *M. malabathricum*, x_2 : *M. hirta*, x_3 : *M. decemfidum*). The proportions of the mixtures used are presented in Table 2 The experiments (1, 6, and 7) represent the pure components, the binary mixtures (2, 9, and 10), the tertiary mixtures (3, 4, and 11), and the center point (5, 8, and 12). The oxidant-ABTS radical cation is generated by persulphate oxidation of 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid). In this work, the radical-scavenging activity of each designed mixture was tested by measuring the decrease in its absorbance value at 734 nm, while the phenolic content of the mixtures of *Melastoma* leaf extracts was measured based on the amount of phenolic compounds in dry weight gallic acid. The results show that the antioxidant activity at 93.11% and phenolic content at 29.25 mg/GAE. From this study, there is no significant difference between *M. hirta* (92.96%) and *M. malabathricum* (92.94%) in terms of ABTS radical activity. However, there were differences in the phenolic content of *M. hirta* and *M. malabathricum* which were 25.25 and 22.25 mg/GAE, respectively.

	Coded Level			ABTS (2, 2'-azino-bis (ethylbenzthiazoline-6-sulfonic acid)) Radical Assay			Total Phenolic Content		
Experiment. No ^a	M. malabathricum (x ₁)	M. hirta (x ₂)	M. decemfidum (x ₃)	Measured Inhibition ^b (%)	Approx. Inhibition (%)	Residues	Measured Phenolic Content ^b (mg/g GAE)	Approx. Phenolic Content (mg/g GAE)	Residues
1	0.00	1.00	0.00	92.96	92.99	0.03	25.25	25.39	0.14
2	0.50	0.50	0.00	93.71	93.72	0.01	24.75	24.73	-0.02
3	0.17	0.67	0.17	93.83	93.84	0.01	30.50	29.87	-0.63
4	0.17	0.17	0.67	94.33	94.14	-0.19	29.50	28.53	-0.97
5	0.33	0.33	0.33	93.70	93.76	0.06	30.25	30.78	0.53
6	0.00	0.00	1.00	93.11	93.15	0.04	29.25	29.50	0.25
7	1.00	0.00	0.00	92.94	92.91	-0.03	22.25	22.09	-0.16
8	0.33	0.33	0.33	93.71	93.76	0.05	30.90	30.78	-0.12
9	0.00	0.50	0.50	94.57	94.64	0.07	29.50	29.88	0.38
10	0.50	0.00	0.50	94.89	94.91	0.02	16.50	16.59	0.09
11	0.67	0.17	0.17	93.74	93.77	0.03	24.10	24.34	0.24
12	0.33	0.33	0.33	93.77	93.76	-0.01	30.50	30.78	0.28

Table 2. Mixture experimental design and obtained responses for the dependent variables.

^a Experiments were carried out after randomization; ^b Each response is the average of three replicates with standard error.

Furthermore, it can also be seen that the percentage inhibition in ABTS radical assays of all *Melastoma* samples increased in each experimental mixture compared to its actual pure component. For instance, the binary mixture of *M. malabathricum* and *M. decemfidum* increased up to approximately 2%, yielding the highest antioxidant activity of ABTS (94.89%). It also showed that the combination of *M. hirta* and *M. decemfidum* increased the inhibition activities of the ABTS radical (94.57%) as compared to the pure components. Tertiary proportions (experiments 3, 4, and 11) represented the percentage inhibition of ABTS radical scavenging activities significantly increased compared to its pure components and binary combination. Moreover, the center point (experiments 5, 8, and 12), which corresponds to the equal proportions, also showed that percentage inhibition of ABTS and phenolic content increased. Recently, a similar study from Baj et al. [18] revealed that the antioxidant potential measured in the mixtures of essential oils (EOs), basil, marjoram, and rosemary gave higher results than the pure samples.

The antioxidant activity of the *Melastoma* leaf extract used in this study correlates with previous literature. For example, the decoction from the leaves of *M. decemfidum* has been known to exhibit the phenol 2,4-bis (1,1-dimethylethyl) compound, which belongs to one of the important polyphenol

antioxidant constituents, exhibiting antifungal effects [19]. Phenolic compounds or polyphenols can be characterized as antioxidants, attractants (flavonoids and carotenoids), structural polymers (lignin), and defense response chemicals (tannins and phytoalexins) [20]. Meanwhile, major antioxidant compounds were also found in both *M. malabathricum* and *M. hirta*. For example, Joffry et al. [21] reported that the leaves of *M. malabathricum* yielded a new compound of flavonol glycoside derivatives. Meanwhile, the phytochemical analysis of *M. hirta* found positive phenolic compounds, such as flavonoids, tannins, and terpenoids [22]. Hence, the presence of secondary metabolites in each *Melastoma* leaf plays a vital role in human healthcare due to their free-radical scavenging effect, which is likely responsible for preventing various chronic diseases.

3.2. Statistical Analysis of the Model

Twelve mixture formulations were run according to the experimental design, and the respective coefficients of the regression model and ANOVA of the mathematical models were adjusted to the response function (Tables 3 and 4). Based on Tables 3 and 4, the model *F*-value of ABTS radical activity was 45.41, while the phenolic content was 87.90, which implies that the models are significant. There is only a 0.03% and <0.0001% chance that a model *F*-value this large could occur due to noise. Moreover, according to Fadil et al. [13], it can be concluded that since the *p*-values were 0.0003 and <0.0001, which are less than <0.05, the main effect of regression was statistically significant. Therefore, in this case, it shows that the model is significant.

Source	Sum of Square	Degrees of Freedom (df)	Mean Square	F-Value	<i>p</i> -Value	
Model	3.93	6	0.66	45.41	0.0003	significant
Linear mixture	0.40	2	0.20	13.78	0.0092	
$x_1 x_2$	0.39	1	0.39	27.28	0.0034	
$x_1 x_3$	2.38	1	2.38	164.86	< 0.0001	
$x_2 x_3$	1.67	1	1.67	115.74	0.0001	
$x_1 x_2 x_3$	1.29	1	1.29	89.61	0.0002	
Residual	0.07	5	0.014			
Lack of fit	0.069	3	0.023	16.12	0.059	not significant
Pure error	0.00028	2	0.00014	45.41	0.0003	0
Cor total	4.01	11	0.66	13.78	0.0092	
R ²	0.9820					
Adj. R ²	0.9604					
ĆV	0.13					

Table 3. Analysis of variance (ANOVA) for the ABTS radical antioxidant activity.

Source	Sum of Square	Degrees of Freedom (df)	Mean Square	F-Value	<i>p</i> -Value	
Model	215.10	6	35.85	87.90	< 0.0001	significant
Linear mixture	68.33	2	34.17	83.77	0.0001	
$x_1 x_2$	0.66	1	0.66	1.62	0.2585	
$x_1 x_3$	57.30	1	57.30	140.51	< 0.0001	
$x_2 x_3$	4.03	1	4.03	9.89	0.0255	
$x_1 x_2 x_3$	61.50	1	61.50	150.81	< 0.0001	
Residual	2.04	5	0.41			
Lack of fit	1.82	3	0.61	5.66	0.1539	not significant
Pure error	0.21	2	0.11			0
Cor total	217.14	11				
R ²	0.9906					
Adj. R ²	0.9793					
ĆV	2.37					

Table 4. Analysis of variance (ANOVA) for the total phenolic content activity.

Meanwhile, the entire model terms in the ABTS radical activity response (x_1 , x_2 , and x_3), where the values of probability > F are less than 0.05, indicate that the model terms are also significant. In this study, the linear mixture components x_1x_2 , x_1x_3 , x_2x_3 , and $x_1x_2x_3$ are significant model terms. According to the model terms in Table 3, the ABTS antioxidant activity was highly significant (*p*-value < 0.0001) for binary (x_1x_3) *M. malabathricum* and *M. decemfidum*, followed by (x_2x_3) *M. hirta* and *M. decemfidum* and tertiary ($x_1x_2x_3$) *M. malabathricum*, *M. decemfidum*, and *M. hirta*. The mathematical model of ABTS antioxidant activity is presented in Equation (3), as follows:

$$ABTS = +92.917_{x1} + 92.988_{x2} + 93.154_{x3} + 3.052_{x1x2} + 7.502_{x1x3} + 6.286_{x2x3} - 30.088_{x1x2x3}$$
(3)

Meanwhile, the model terms for the phenolic content response (x_1 , x_2 , and x_3), where the values of probability > F are less than 0.05, indicate that the model terms are significant. In this study, the linear mixture components x_1x_3 , x_2x_3 , and $x_1x_2x_3$ are significant model terms, except for the interaction between x_1x_2 with a larger *p*-value (0.2585), indicating that the combination of the mixtures produced the least response of interest. According to the model terms in Table 4, the phenolic content activity was highly significant (*p*-value < 0.0001) for tertiary ($x_1x_2x_3$) *M. malabathricum*, *M. decemfidum*, and *M. hirta*, followed by binary (x_1x_3) *M. malabathricum* and *M. decemfidum* and binary (x_2x_3) *M. hirta* and *M. decemfidum*. The mathematical model of phenolic content activity is presented as follows in Equation (4):

Phenolic content =
$$+22.094_{x1} + 25.385_{x2} + 29.498_{x3} + 3.958_{x1x2} - 36.814_{x1x3} + 9.767_{x2x3} + 207.468_{x1x2x3}$$
 (4)

This study shows that the models of the coefficients of determination were 98.20% for ABTS radical activity and 99.06% for phenolic content, which present the maximum degree of correlation between the observed and predicted values. Moreover, the high value of the adjusted R^2 (96.04% and 97.93%) also indicates a significant correlation between the experimental and predicted runs by the software. These results can be explained by a similar study by Yolmeh et al. [23] where the model was considered accurate when R^2 was close to one. Figures 2 and 3 show a linear curve for the experimental values in terms of the predicted ones. Next, the fitness of the models was also studied through a lack of fit test, where the values for all responses were not significant (*p*-value > 0.05), which showed the suitability of models selected to predict the responses [23].



Figure 2. Actual ABTS (2, 2'-azino-bis (ethylbenzthiazoline-6-sulfonic acid)) radical activity versus predicted ABTS radical activity.



Figure 3. Actual phenolic content versus predicted phenolic content.

3.3. Response Surface Analysis and Optimization

According to Ladeira et al. [17], in the statistical methods, the response surface design is used to discover the effects of process variables on the specific responses of a system. In our study, the response surface for ABTS antioxidant activity with respect to the different proportions of the three *Melastoma* leaf extracts, *M. malabathricum* (x_1), *M. hirta* (x_2), and *M. decemfidum* (x_3), are shown in Figure 4. The mixture contour plots in Figure 4A,B show that the percentage inhibition of ABTS radical scavenging activity significantly increased with the addition of the x_3 component to both interactions of x_1 and x_2 components. This interaction can also be supported through the greater *F*-values of these coefficients, x_1x_3 , x_2x_3 , and $x_1x_2x_3$, which yielded 164.86, 115.74, and 89.61, respectively in Table 3.



Figure 4. (A) Contour charts. (B) 3-Dimensional response surface of ABTS radical activity.

Simultaneously, the response surface for the phenolic content activity of the *Melastoma* leaf mixture extract is shown in Figure 5. The mixture contour plots in Figure 5a,b show that the phenolic content activity also increased with the addition of the x_3 component to both interactions of mixtures x_1 and x_2 components. From this result, the highest interaction between the mixtures can be seen from the coefficients of x_1x_3 and $x_1x_2x_3$ where the greater *F*-values of both coefficients yielded 140.51 and 150.81, respectively, in Table 4. This result can be justified using the study of antimicrobial efficacy of essential oils by Quedrhiri et al. [24] where different compounds can interact to either reduce or increase the response outcomes. On the other hand, in terms of ABTS radical activity, the component x_1 had a slight effect on the interaction of both x_2 and x_3 components, where the synergistic effect was found between the mixtures of x_1 and x_3 , while antagonistic effects were found in the mixture of x_1 and x_2 . Meanwhile, for the phenolic content activity of the mixture of *Melastoma* extracts, the component x_3 showed a significant synergistic effect on either x_1 or x_2 coefficients. Therefore, it can be seen that the interaction between the three *Melastoma* leaf extracts resulted in a significant effect on the scavenging activity of radical species and on the phenolic content activity.



Figure 5. (a) Contour plot. (b) 3-Dimensional optimal points of phenolic content.

The optimization technique was further evaluated after considering the simultaneous response surface and contour plot from the interaction between the independent variables and the response. This technique has been applied by Moreira et al. [25] where a visual inspection of the contour charts was evaluated and studied in order to determine good operational conditions. In this study, a desirability function was generated to predict (1.00) the optimal points for each *Melastoma* leaf extract. By performing real experiments on each optimal point mixture based on the prediction, the best optimum antioxidant activity for the simultaneous three *Melastoma* leaf extracts was 30% *M. malabathricum*, 40% *M. hirta*, and 30% *M. decemfidum*, yielding 93.0% antioxidant activity against radical species for the ABTS assay and 30.96 mg/g GAE for phenolic content. Table 5 shows the optimal points for optimum antioxidant activity in the mixture of *Melastoma* leaf extracts for both the ABTS radical assay and phenolic content.

M. malabathricum	M. hirta	M. decemfidum	Response	Predicted Value	Experimental Value	Residue
0.30	0.40	0.30	ABTS radical assay (%)	93.73	93.0 ± 0.23	0.73
0.30	0.40	0.30	Phenolic content (mg/g) GAE	31.43	30.96 ± 0.15	0.47

Table 5. The optimum formulation for each dependent variable.

3.4. Isolated Bioactive Compounds in Melastoma Leaf Extract Using HPLC

The isolation and identification of major and unique compounds in medicinal plants as markers are crucial steps in analytical methodologies for marker-based standardization. HPLC has recently emerged as a preferred analytical tool since it is simple, rapid, and precise for fingerprinting isolated compounds in plants. For instance, HPLC is commonly used by many researchers due to the small particle size and length in the HPLC column, which allows for an efficient separation and good resolution for biomarker identification. The HPLC method was developed in this study for the simultaneous identification of flavonoid constituents, such as quercetin and rutin, from the mixture of Melastoma leaf extracts. The HPLC system used in the present work was a reverse-phase C18 column, which can produce promising results with most common eluents. In the present work, the binary solvent system in gradient mode with acetonitrile and water/formic acid was able to achieve good peak resolution and symmetry due to buffering in the solution. Meanwhile, the separations of quercetin and rutin in a mixture of *Melastoma* plants were accomplished by gradient elution with a mobile phase consisting of water, formic acid, and acetonitrile. Furthermore, applying gradient solvents of acetonitrile with water gives sharp and symmetrical peaks with minimal noise, which supports the precise measurement of the peak area ratio. In this study, 0.1% formic acid was used due to its buffering capacity in the pH range of 2 to 4, and it provided a good volatile mobile phase that was efficient for separating flavonoid constituents.

HPLC chromatograms of flavonoid constituents (quercetin and rutin) of all the samples and standards were separated within 18 min and showed good resolution between the matrix and analyte peaks recorded at 254 nm, as shown in Figure 6. From the observations, there were four peaks in the chromatogram of the optimized mixtures of *Melastoma* extract. The first and second peaks were excluded in this study, which belongs to solvent and unknown peaks. Meanwhile, the third and fourth peaks were identified and confirmed from significant antioxidant constituents. In this analysis, both rutin and quercetin were identified in the mixture of *Melastoma* leaf extract. The retention times of the mixture of *Melastoma* leaf extracts were 7.770 min for rutin and 8.769 min for quercetin (Table 6). They were confirmed and identified by comparing the retention time with two reference standard compounds from the standard response, such as in rutin (RT = 7.024 min) and quercetin (RT = 8.741 min) in Table 7. On the other hand, it can be seen that the retention of rutin was shorter than quercetin. Wu et al. [26] also found the same elution order in the chromatogram of rutin and quercetin obtained with an high performance liquid chromatography- ultraviolet (HPLC-UV) detector from a sample extract of Flos Sophorae Immaturus. The differences in retention time are mainly due to the

role of flavonoid constituents of their differences in chemical structure and chemical variation, such as hydroxylation, methoxylation, the type of conjugation (glycosylation, sulfonation, malonylation), and the degree of polymerization [27]. Meanwhile, the HPLC-UV peak area of rutin and quercetin shows that quercetin exhibits the largest quantity mass fraction (1498.90 mAU) as compared to rutin, which was 391.7 mAU. Saraf and Sankhla [28] also found that the amount of quercetin is large compared to rutin in the methanolic extract of *Tecomella undulata*.



Figure 6. The overall chromatogram for the mixture of Melastoma leaf extracts.

Sample	Peak No.	Retention Time (min)	Peak Area (mau)	Compound Identification
	i	1.978	10,273.4	-
Optimized Melastoma	ii	2.535	3207.2	-
Extract	iii	7.770	391.7	Rutin hydrate
	iv	8.769	1489.9	Quercetin hydrate

Table 6. The retention time and peak area of the optimized mixture extract.

1 mg/1 mL of extract in HPLC-grade methanol.

Table 7. The retention time and pe	eak area of the standard
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Standard	Retention Time (min)	Peak Area (mAU)
Rutin hydrate	7.024	21,171.9
Quercetin hydrate	8.741	19,730.0
Quercetin	11.508	8955.2
Apigenin	13.074	9479.5
Myricetin	9.758	10,513.1
Kaempferol	13.101	7045.6

1 mg/2 mL of standard in HPLC-grade methanol.

Plant polyphenols, such as flavonoids, are a class of secondary metabolites that are also collectively known as "Vitamin-p" [28]. The chemical structure of flavonoids consists of a C15 (C6–C3–C6) skeleton, and they are grouped into four major classes according to the position of the aromatic ring in the phenyl-benzopyran rings [29]. They are widely distributed throughout the plant kingdom, and, to date, there are about 300 known flavonoids [30]. Quercetin and rutin are considered the two main and most significant flavonoid compounds. Quercetin is known as 5,7,31,41-tetra hydroxy flavonol. Meanwhile, according to Ashok and Saini [31], rutin is the rhamnoglucoside of the flavonoid quercetin (quercetin-3- rutinoside or 3,3',4',5,7-pentahydroxy flavones-3-rutinoside), and it is present in several plant parts. They both possess important phytochemicals and medicinal properties [28].

For example, they provide several defense protections against ROS in humans. Moreover, flavonoids display a remarkable role in various pharmacological activities, such as antioxidant, anti-inflammatory, anti-diabetic, and anti-cancer effects [31]. Therefore, these significant bioactive compounds of rutin and quercetin in the mixture of *Melastoma* leaf extracts provide therapeutic value as the natural antioxidants against attacks caused by reactive oxygen species.

3.5. In Vitro Anti-Elastase and Collagenase Activity of the Mixture of Melastoma Leaf Extracts

Human skin is a complex structure that provides various important functions, including thermoregulation and protection against harmful substances. For instance, under normal conditions, the skin produces enzymes, such as elastase and collagenase, at a normal rate through the aging process. However, overexposure to ultraviolet radiation and harmful pollution results in enzymes that are produced at a faster rate following faster degradation of elastin and collagen, which are the main basis of the extracellular matrix (ECM) of the dermis [32]. Additionally, the imbalance of ROS on the skin can interact with protein, DNA, and lipids and alter their cellular functions, thus causing premature aging and other skin-related disorders [33]. Therefore, more research is needed to determine the inhibitors of these enzymes that can be used in cosmetics and medications to protect the skin from aging.

In this study, the in vitro inhibitory potential of bioactive compounds from the optimum mixture of *Melastoma* extract was carried out against elastase and collagenase enzymes. Prior to the analysis, the elastase inhibitory activity was determined by inhibition of the studied sample with the neutrophil elastase enzyme, which is a purified human neutrophil elastase combined with synthetic peptides as substrate molecules. Meanwhile, the collagenase inhibitory activity was determined by inhibition of the studied sample with the MMP-1 enzyme (interstitial collagenase) combined with synthetic peptides as substrates molecules. In this study, a slope of remaining activity for the tested sample against the control (without sample) was determined and the percentage and inhibition percentage were obtained by subtracting the obtained value from 100.

From the results in Table 8, the elastase reduction activity of the optimum mixture of Melastoma extract gave a good R^2 (0.9508), showing that it is not significantly different between the studied sample and positive inhibitor. Based on the results, the studied sample exhibited 27.78% in remaining elastase activity when compared to the positive control (elastastinal), which was recorded to have 27.77% (Figure 7). Moreover, Table 8 also revealed that there was about 72.22% inhibition of the elastase enzyme by an optimum mixture of the Melastoma extract versus the positive inhibitor, which exhibited 72.23% inhibition. Meanwhile, for collagenase inhibition activity, it can be seen that the optimum mixture of the Melastoma extract exhibited 29.13% remaining collagenase activity compared to the positive inhibitor (20.43%) (Table 9, Figure 7). It also shows that there was about 70.87% inhibition of collagenase activity by an optimum mixture of the *Melastoma* extract versus the positive inhibitor, which exhibited 79.57% inhibition. From the results, the higher percentage inhibition activity for both elastase and collagenase enzymes by optimum *Melastoma* extract can be supported by the bioactive compounds that are present in the Melastoma leaf. For instance, rutin and quercetin compounds that are found in the leaf can significantly inhibit or slow down the generation of enzymes and radical activities. Moreover, an optimum mixture of Melastoma extract exhibited numerous terpenoid compounds that can increase the rate of inhibition enzyme activity, as it was previously reported that terpenoid compounds in plant extracts can act as natural inhibitor agents [32]. Therefore, it is interesting to note that the optimum mixture of the Melastoma extract demonstrated good anti-elastase and anti-collagenase activity against premature skin aging.



Table 8. Inhibition elastase activity of a mixture of *Melastoma* leaf extracts.

Figure 7. The percentage of remaining elastase and collagenase activity for a mixture of *Melastoma* extracts.

Table 9. Inhibition collagenase activity of a mixture of *Melastoma* leaf extracts.

Sample	R ²	Remaining Elastase Activity (%)	Inhibition of Elastase Activity (%)
Mixture of <i>Melastoma</i> extract	0.9891	29.13	70.78
Inhibitor (collagenase)	0.9829	20.43	79.57
Negative (control)	0.9649	100.00	0.00

4. Conclusions

The simplex-centroid mixture design was found to be the best design for this study, and it was successfully developed for the evaluation of mixtures from different leaf extracts of the Melastomataceae family species in the formulation of the highest antioxidant activity. By using the desirability function, the optimal formulation obtained for each *Melastoma* extract composed of *M. malabathricum*, *M. hirta*, and *M. decemfidum* was found to be in the ratio of 3:4:3 mixtures, respectively. In addition, the present study also showed that each *Melastoma* leaf extract contained a significant bioactive antioxidant compound of rutin and quercetin through the HPLC method. The in vitro anti-collagenase and anti-elastase activity of optimum *Melastoma* extract exhibited good inhibition activity of more than 70% against both elastase and collagenase enzymes. There could be clear alternatives for the utilization of *Melastoma* plant mixtures for formulations in the cosmetics industry to overcome and prevent various degenerative diseases, especially aging.

Author Contributions: N.F.A. participated in the design of the study and performed the experiments and statistical analysis. S.S.A.G., U.H.Z., and P.B. were involved in the design of the study, supervised the experimental workflow, and helped draft the manuscript. M.I.E.H. participated in the sequence alignment and drafted the manuscript. All authors read and approved the final manuscript.

Funding: This work was financially supported by the Fundamental Research Grant Scheme (FRGS) under the Ministry of Higher Education, Malaysia (grant number: 5540164), within the project title "The Antioxidants and Functional Cosmeceuticals Properties of Leaves Melastomataceae Family Species and Its Potential for Stratum Corneum Applications."

Acknowledgments: The authors would like to acknowledge the Laboratory of Halal Science Research, Halal Products Research Institute, Universiti Putra Malaysia (UPM), for consultation and expert advice to complete the research work.

Conflicts of Interest: The authors declare that there are no conflict of interest regarding the publication of this paper.

References

- 1. Saeed, N.; Khan, M.R.; Shabbir, M. Antioxidant activity, total phenolic and total flavonoid contents of whole plant extracts *Torilis leptophylla* L. *BMC Complement. Altern. Med.* **2012**, *12*, 221. [CrossRef]
- Azahar, N.F.; Abd Gani, S.S.; Mohd Mokhtar, N.F. Optimization of phenolics and flavonoids extraction conditions of Curcuma Zedoaria leaves using response surface methodology. *Chem. Cent. J.* 2017, 11, 96. [CrossRef] [PubMed]
- Ghazal, G.M.; Shahhat, I.; Shahhat, A. Polyphenols, flavonoids, caretenoids and antioxidant activity of lupine (*Lupinus termis* L.) seeds affected by vitamin C, vitamin B3 and turmeric rhizomes extract. *Adv. Environ. Biol.* 2013, 7, 4914–4924.
- 4. Haron, N.W.; Anuar, N.; Veeramohan, R. The taxonomic significance of leaf micromorphology in the Genus *Melastoma* L. (Melastomataceae). *Sains Malays.* **2015**, *44*, 643–650. [CrossRef]
- Danladi, S.; Wan-Azemin, A.; Sani, Y.N.; Mohd, K.S.; Us, M.R.; Mansor, S.M.; Dharmaraj, S. Phytochemical screening, total phenolic and total flavonoid content, and antioxidant activity of different parts of Melastoma Malabathricum. J. Teknol. 2015, 77, 63–68. [CrossRef]
- 6. Ismail, I.N.; Razak, W.R.W.A.; Sidik, N.B.J.; Said, S.S.M. Antibacterial activity of flower, leave and stem extract of melastoma decemfidum. *Int. J. Life Sci. Biotechnol. Pharma Res.* **2017**, *6*, 13–17.

- Lopez, T.; Corbin, C.; Falguieres, A.; Doussot, J.; Hano, C.; Montguillon, J.; Hagege, D.; Laine, E. Secondary metabolite accumulation, antibacterial and antioxidant properties of in vitro propagated *Clidemia hirta* L. extracts are in fl uenced by the basal culture medium In fl uence de la composition du milieu de culture sur la pr. *C. R. Chim.* 2016, *19*, 1071–1076. [CrossRef]
- 8. Alnajar, Z.A.A.; Abdulla, M.A.; Ali, H.M.; Alshawsh, M.A.; Hadi, A.H.A. Acute toxicity evaluation, antibacterial, antioxidant and immunomodulatory effects of Melastoma malabathricum. *Molecules* **2012**, *17*, 3547–3559. [CrossRef]
- 9. Zabidi, Z.; Wan Zainulddin, W.N.; Mamat, S.S.; Shamsahal Din, S.; Kamisan, F.H.; Yahya, F.; Rodzi, R.; Hasan, H.; Mohtarrudin, N.; Somchit, M.N.; et al. Antiulcer activity of methanol extract of Melastoma malabathricum leaves in rats. *Med. Princ. Pract.* **2012**, *21*, 501–503. [CrossRef]
- Zakaria, Z.A.; Rofiee, M.S.; Mohamed, A.M.; Teh, L.K.; Salleh, M.Z. In vitro antiproliferative and antioxidant activities and total phenolic contents of the extracts of Melastoma malabathricum leaves. *J. Acupunct. Meridian Stud.* 2011, 4, 248–256. [CrossRef]
- 11. Jamalnasir, H.; Wagiran, A.; Shaharuddin, N.A.; Samad, A.A. Isolation of high quality RNA from plant rich in flavonoids, Melastoma decemfidum Roxb ex. Jack. *Aust. J. Crop Sci.* **2013**, *7*, 911–916.
- Munhoz, V.M.; Longhini, R.; Souza, J.R.P.; Zequi, J.A.C.; Leite, E.V.S.; Lopes, G.C.; Mello, J.C.P. Extraction of flavonoids from tagetes patula: Process optimization and screening for biological activity. *Rev. Bras. Farmacogn.* 2014, 24, 576–583. [CrossRef]
- Fadil, M.; Fikri-benbrahim, K.; Rachiq, S.; Ihssane, B.; Lebrazi, S.; Chraibi, M.; Haloui, T.; Farah, A. Combined treatment of *Thymus vulgaris* L., *Rosmarinus officinalis* L. and *Myrtus communis* L. essential oils against Salmonella typhimurium: Optimization of antibacterial activity by mixture design methodology. *Eur. J. Pharm. Biopharm.* 2018, 126, 211–220. [CrossRef] [PubMed]
- 14. Shalaby, E.A.; Shanab, S.M.M. Comparison of DPPH and ABTS assays for determining antioxidant potential of water and methanol extracts of Spirulina platensis. *Indian J. Geo-Mar. Sci.* **2013**, *42*, 556–564.
- 15. Singleton, V.; Rossi, J.A.J. Colorimetry of total phenolics with phosphomolybdic-phosphotungtic acid reagents. *Am. J. Enol. Vitic.* **1965**, 144–158.
- 16. Orives, J.R.; Galvan, D.; Coppo, R.L.; Henrique, C.; Rodrigues, F.; Angilelli, K.G.; Borsato, D. Multiresponse optimisation on biodiesel obtained through a ternary mixture of vegetable oil and animal fat: Simplex-centroid mixture design application. *Energy Convers. Manag.* **2014**, *79*, 398–404. [CrossRef]
- 17. Handa, C.L.; De Lima, F.S.; Guelfi, M.F.G.; Georgetti, S.R.; Ida, E.I. Multi-response optimisation of the extraction solvent system for phenolics and antioxidant activities from fermented soy flour using a simplex-centroid design. *Food Chem.* **2016**, *197*, 175–184. [CrossRef]
- Baj, T.; Baryluk, A.; Sieniawska, E. Application of mixture design for optimum antioxidant activity of mixtures of essential oils from *Ocimum basilicum* L., *Origanum majorana* L. and *Rosmarinus officinalis* L. *Ind. Crop. Prod.* 2018, 115, 52–61. [CrossRef]
- 19. Adam, N.A.M.; Sidik, N.J.; Osman, N.I.; Razak, W.A. In vitro regeneration and phytochemical profile of medicinal plant Melastoma decemfidum. *Int. J. Pharm. Sci. Rev. Res.* **2016**, *37*, 36–41.
- 20. Lin, D.; Xiao, M.; Zhao, J.; Li, Z.; Xing, B. An overview of plant phenolic compounds and their importance in human nutrition and management of type 2 diabetes. *Molecules* **2016**, *21*, 1374. [CrossRef]
- Joffry, S.M.; Yob, N.J.; Rofiee, M.S.; Affandi, M.M.R.M.M.; Suhaili, Z.; Othman, F.; Akim, A.; Desa, M.N.M.; Zakaria, Z.A. *Melastoma malabathricum* (L.) Smith ethnomedicinal uses, chemical constituents, and pharmacological properties: A review. *Evid. Based Complement. Altern. Med.* 2011, 2011, 258434. [CrossRef]
- 22. Musa, N.; Musa, N.; Ibrahim, W.N.W.; Zahrol, M.; Zamani, A.I.; Ranzi, M.; Tee, L.; Marip, M.; Razak, L.A.; Sokhawi, A. Methanolic activities of selected weeds on bacteria isolated from macrobrachium rosenbergii larvae. *Thai J. Vet. Med.* **2011**, *41*, 535–539.
- 23. Yolmeh, M.; Khomeiri, M.; Ahmadi, Z. Application of mixture design to introduce an optimum cell-free supernatant of multiple-strain mixture (MSM) for Lactobacillus against food-borne pathogens. *LWT Food Sci. Technol.* **2017**, *83*, 298–304. [CrossRef]
- 24. Ouedrhiri, W.; Bouhdid, S.; Balouiri, M.; El Ouali Lalami, A.; Moja, S.; Chahdi, F.O.; Greche, H. Chemical composition of Citrus aurantium L. leaves and zest essential oils, their antioxidant, antibacterial single and combined effects. *J. Chem. Pharm. Res.* **2015**, *7*, 78–84.

- 25. Moreira, G.C.; Dias, F.D.S. Mixture design and Doehlert matrix for optimization of the ultrasonic assisted extraction of caffeic acid, rutin, catechin and trans -cinnamic acid in Physalis angulata L. and determination by HPLC DAD. *Microchem. J.* **2018**, 141, 247–252. [CrossRef]
- 26. Wu, H.; Chen, M.; Fan, Y.; Elsebaei, F.; Zhu, Y. Determination of rutin and quercetin in Chinese herbal medicine by ionic liquid-based pressurized liquid extraction-liquid chromatography—chemiluminescence detection. *Talanta* **2012**, *88*, 222–229. [CrossRef]
- 27. Wang, S.P.; Huang, K.J. Determination of flavonoids by high-performance liquid chromatography and capillary electrophoresis. *J. Chromatogr. A* **2004**, *1032*, 273–279. [CrossRef]
- 28. Saraf, A.; Sankhala, S. Simultaneous determination of rutin and quercetin in different parts of of tecomella undulata (seem): An endangered medicinal plant. *Int. J. Pharmacogn. Phytochem. Res.* **2014**, *6*, 434–439.
- 29. Kuppusamy, P.; Lee, K.D.; Song, C.E.; Ilavenil, S.; Srigopalram, S.; Arasu, M.V.; Choi, K.C. Quantification of major phenolic and flavonoid markers in forage crop Lolium multiflorum using HPLC-DAD. *Braz. J. Pharmacogn.* **2018**, *28*, 282–288. [CrossRef]
- 30. Subramanian, G.; Meyyanathan, S.N.; Karthik, Y.; Karunakaranair, A.; Palanisamy, D. Development and validation of hplc method for the simultaneous estimation of quercetin and rutin in Aganosma dichotoma (Roth) K. Schum. *Int. J. Pharm. Pharm. Sci.* **2014**, *6*, 606–608.
- 31. Ashok, P.K.; Saini, B. HPLC analysis and isolation of rutin from stem bark of *Ginkgo biloba* L. *J. Pharmacogn. Phytochem.* **2013**, *2*, 68–71.
- 32. Karim, A.A.; Azlan, A.; Ismail, A.; Hashim, P.; Gani, A.S.S.; Zainudin, B.H.; Abdullah, N.A. Phenolic composition, antioxidant, anti-wrinkles, and tyrosinase inhibitory activities of cocoa pod extract. *BMC Complement. Altern. Med.* **2014**, *14*, 381.
- 33. Tu, P.T.B.; Tawata, S. Anti-oxidant, anti-aging, and anti-melanogenic properties of the essential oils from two varieties of *Alpinia zerumbet*. *Molecules* **2015**, *20*, 16723–16740. [CrossRef] [PubMed]



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