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Optimization of the Extraction Process for Bioactive Compounds from the Root Barks of *Moringa oleifera*

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Abstract – The optimal condition for *Moringa oleifera* root barks extraction was determined using response surface methodology and Box-Behnken Design. The actual optimal condition of the factors was 65°C, ethanol 60%, 40 (mL/g) liquid-to-solid ratio with 240 minutes extraction time. The enrichment of phenolic compounds sharply affected the antioxidant, and inhibitions of α-amylase enzyme, as well as, the anti-inflammatory effect of the extract from *M. oleifera* root barks. The extract in the optimal condition exhibited better 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging and α-amylase inhibitory activities than those of positive controls. Also, the extract showed weak hydroxyl free radical scavenging and nitric oxide (NO) production inhibitory effects. These revealed a simple and promising method for the preparation of bioactive products from the root bark of *M. oleifera.*
Keywords – *Moringa oleifera*, Phenolic, Antioxidant, Anti-inflammation, α-Amylase

Introduction

Moringa oleifera Lam. (Moringaceae) is a small deciduous tree which is widely cultivated in Asia. Parts of the plants, such as leaves, flowers, seeds, and roots, have been used as foods and herbal medicine.[1,2] Phytochemical studies indicated that *M. oleifera* leaves and seeds have a number of secondary metabolites, including phenolics, flavonoids, glycosides, and alkaloids which exhibited considerable antioxidant, anti-inflammatory, anti-tumor, and antimicrobial effects.[3-10] Though many studies focused on the phytochemicals of leaves and seeds of *M. oleifera*, [3,4,6,7,9,10] few investigations on the root barks of the plant have been conducted. Phenolics were detected as major bioactive constituents in the roots of *M. oleifera* which possessed antimicrobial and anticancer activities.[11-14]

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Response surface methodology (RSM) is an effective statistical model to design, develop, and optimize a process when multiple independent factors simultaneously affect one or several responses.[15] The Box-Behnken design (BBD) is a method for RSM, which is frequently used to optimise the extraction process in biochemistry, chemical engineering, or the food industry.[16-18] This study presents the optimization of the condition for the extraction of phenolics from *M. oleifera* root barks using Box-Behnken experimental design and evaluates *in vitro* free-radical scavenging and nitric oxide (NO) production inhibitory effects of the extract.

Experimental

Instruments and chemicals – UV-Vis absorbance was measured on a BioTek Synergy HTX multimode reader (Agilent, US). The incubator and the sonication bath were supplied by Daihan Scientific, Korea. Ethanol (EtOH), water, DMSO, $Fe(NH_4)_2(SO_4)_2$, $AlCl_3$, $NaNO_2$, Na_2CO_3, K_3PO_4, K_2HPO_4, KH_2PO_4, NaCl, NaOH, and HCl (36%) were purchased from Daihan Scientific, Korea. Folin-Ciocalteu reagent, gallic acid, lipopolysaccharides from *Escherichia coli* O26:B6 (LPS), 2,2-diphenyl-1-picrylhy-

drazyl (DPPH), α-amylase, 3,5-dinitro salicylic acid (DNSA), sodium potassium tartrate tetrahydrate, trichloroacetic acid (TCA), deoxyribose, thiobarbituric acid, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N^G-Methyl-L-arginine acetate salt (L-NMMA), Griess reagent, catechin, acarbose and quercetin were supplied by Merck, Germany.

Plant material – *M. oleifera* root barks were collected in July 2022 in Quang Ninh province, Vietnam. The samples were identified by Dr. Nguyen The Cuong, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology. A voucher specimen of the plant was deposited in the Center for Research and Technology Transfer with code number CR2207009. The sample was cleaned under water taps, dried at 50–55°C, powdered, and preserved for further experiments.

Total polyphenol assay – The total phenolic contents (TPC) of the samples were evaluated by the Folin–Ciocalteu method.[19] The standard solutions were prepared with several concentrations of gallic acid (10–400 μg/mL). The analytes were prepared by dissolving the extracts or subfractions in methanol at certain concentrations. Each 100 μL of the sample or standard solution was mixed with 900 μL of Folin-Ciocalteu 10% and 1000 μL of Na_2CO_3 6% and incubated for 15 minutes at 40°C. Its absorbance was measured with a UV–Vis. spectrophotometer at 750 nm. The total phenolic content was calculated as mg of gallic acid equivalent (mg GAE/g) by using the gallic acid calibration curve.

Antioxidant assays – The antioxidant activities of extracts and subfractions were evaluated by DPPH and hydroxyl radicals scavenging assays. DPPH radical-scavenging activity was conducted by modifying a previous method.[20] Briefly, each sample (20 μL) was mixed with 380 μL of DPPH in methanol and then dark incubated at 37°C for 20 minutes. The absorbance was measured at 517 nm. Ascorbic acid was used as a positive control.

The hydroxyl radical scavenging assay was measured based on quantification of the degradation product of 2-deoxyribose by condensation with thiobarbituric acid.[21] 50 μL of the test sample was incubated with 100 μL of the phosphate buffer 50 mM pH 7.8, 100 μL of deoxyribose 2.8 mM, and 100 μL of $Fe(NH_4)_2(SO_4)_2$ 500 μM for 1 h at 37°C. Next, 250 μL of trichloroacetic acid (10%, w/v) and 250 μL of thiobarbituric acid (1% w/v) were added, and the reaction mixture was boiled for 15 minutes in a water bath. The colour development was measured at 532 nm.

The IC_{50} values were measured based on experiments in several concentrations. The statistically significant difference in IC_{50} values was evaluated by one-way ANOVA and Tukey's HSD posthoc analysis.

NO production inhibition – The effects of extracts on the NO production in LPS-stimulated RAW264.7 macrophage cells were examined as a reported method.[22] The cells were cultivated in 96-well plates at 2×10^5 cells/well and incubated for 18 h. The plates were treated with the extracts (from 0.1 μg/mL to 100 μg/mL) for 30 minutes and then incubated for another 24 h with or without 1 μg/mL LPS. 100 μL of the culture supernatant was transferred to another 96-well plate, and 100 μL of Griess reagent was added. The absorbance of the reaction solution was read at the wavelength 570 nm. The remaining cell solutions in a cultured 96-well plate were used to evaluate cell viability by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[23] L-NMMA, a well-known NO production inhibitor,[24] was used as a positive control.

Preliminary single-factor experiments – Ranges of single factors for the extraction of *M. oleifera* root bark were selected from the following experiments. Firstly, the effect of extraction temperature on TPC was investigated by extracting the material in ethanol 70% at 30–80°C for 120 minutes with the liquid-to-solid ratio at 30 mg/L. Next, the influence of extraction time on TPC was evaluated by extracting *M. oleifera* root bark in ethanol 70% at 30–80°C with the liquid-to-solid ratio at 30 mg/L for 60–300 minutes. Lastly, the impact of liquid-to-solid ratio was studied by extracting the material in ethanol 70% at the ratio of solvent to material between 10 to 60 while the temperatures and time were set at 70°C and 120 minutes.

Experimental design – The response surface method (RSM) was used to determine the optimal extraction conditions. Box-Behnken Design (BBD) was employed to design the experimental data on the software Design-Expert 12.0 (Stat-Ease, Inc., Minneapolis, US). Extraction temperature (°C, X_1), ethanol concentration (%, X_2), extraction time (minute, X_3), and solvent-to-material (mL/g, X_4) were selected as independent factors, while total phenolic content (TPC). Ethanol 0% (distilled water), 48%, and 96% were used as the solvents, meanwhile, the range and centre points of the other factors were selected based on the results of preliminary single-factor experiments. Each experiment was conducted in triplicate and the mean values were stated as an observed response.

Results and Discussion

Extraction temperature, time, and liquid-to-solid ratio

would deeply affect the total phenolic content (TPC) of the extract from *M. oleifera* root barks. The effects were seen in Fig. 1.

As can be seen, the TPC of the extracts increased significantly from 39.31 mg GAE/g to 72.37 mg GAE/g when raising the extraction temperature from 30 to 80ºC (Fig. 1a). A higher temperature could be ineffective because it was over the boiling point of the solvent and might decompose the bioactive metabolites in the extract. In contrast, the low temperature might be not efficient due to the higher viscosity of the liquid solvent and lower penetration of the liquid solvent into the matrix of solid material.[25] Thus, in this study, we would select the range between 50 and 80ºC to optimize the extraction process. Whereas, when extraction time increased from 60 to 240 minutes, the TPC of the extracts showed an upward trend and peaked at 82.17 mg GAE/g as the extraction time was 240 minutes, then remained static during the longer extraction period (Fig. 1b). This result indicated that 4 hours was enough for the extraction of phenolic from *M. oleifera* root barks. Besides the extraction temperature and time, the ratio between the volume of the solvent and the weight of the material (liquid-to-solid ratio) might sharply affect the TPC of the extract. A too-small ratio may restrict the diffusion of the material's compositions into the solvent. In contrast, a too-big ratio will cause higher processing costs. Thus, a suitable liquid-to-solid ratio should be selected for the extraction of *M. oleifera* root bark. As shown in Fig. 1c, when other factors were fixed, the TPC of the extracts increased from 58.96 to 74.70 mg GAE/g as the ratio increased from 10 to 50. When the ratio continued increasing, the TPC value no longer rose. While the ratio of 10 mL/g was the minimum for the adsorption of the solvent to the raw material, the ratio of 50 mL/g should be the maximum value for the optimization of the extracting procedure.

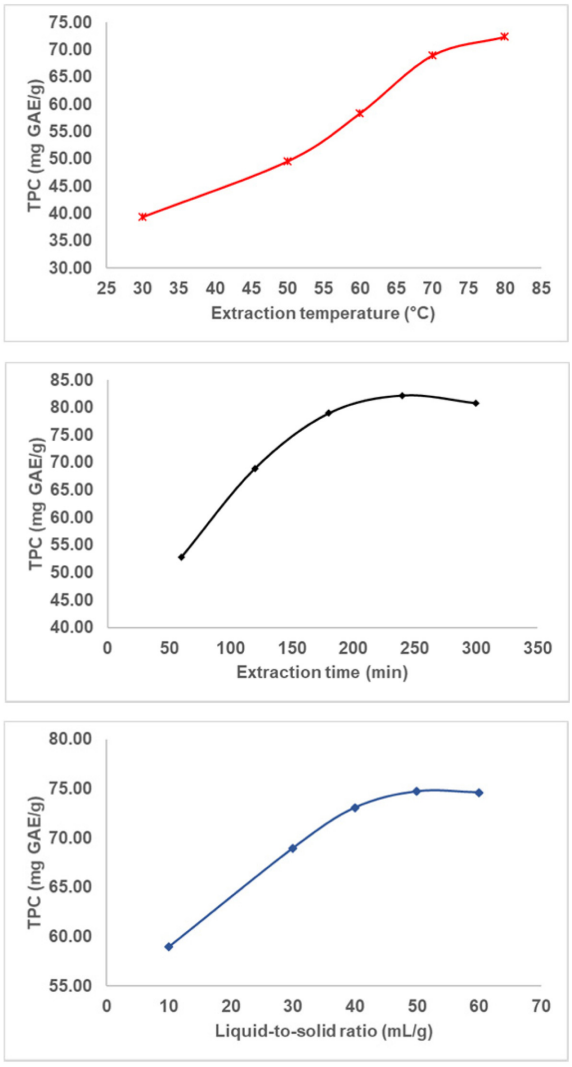

Fig. 1. Effect of extraction temperature (a), time (b), and liquid-to-solid ratio (c) to total phenolic content of *M. oleifera* root bark extracts.

According to those singer-factor studies, ranges of extraction temperature, time, and liquid-to-solid ratio for RSM experiments were 50–80ºC, 60–240 minutes, and 10–50, respectively.

The TPC extraction from *M. oleifera* root barks was further optimized through the RSM approach, using Box-Behnken design (BBD). Codes and actual levels of four variables were selected as in Table 1. Consequently, 30 experiments were designated.

The results of 30 runs were presented in Table 2. The highest TPC was recorded at 97.17 mg GAE/g under the condition of X_1 = 80ºC, X_2 = 48%, X_3 = 240 minutes, X_4 = 30 mL/g. In this case, the total extraction yield was

Table 1. Codes and levels of the variables

Variables	Unit	Code levels		
		–1	0	1
Temperature (X_1)	ºC	50	65	80
Ethanol concentration (X_2)	%	0	48	96
Time (X_3)	minutes	60	150	240
Liquid-to-solid ratio (X_4)	mL/g	10	30	50

Table 2. Responses of TPC of the extracts to independent variables using Box-Behnken design

No.	Variables				TPC (mg GAE/g)
	X_1	X_2	X_3	X_4	
1	50	96	150	30	64.05
2	65	96	150	50	75.36
3	65	0	150	50	45.86
4	65	48	240	50	92.75
5	50	48	60	30	37.64
6	80	48	150	50	67.91
7	50	48	150	50	75.17
8	80	48	150	10	70.84
9	80	0	150	30	49.44
10	65	48	150	30	72.90
11	65	48	150	30	82.41
12	65	96	60	30	53.43
13	50	0	150	30	32.43
14	65	48	150	30	76.73
15	65	0	150	10	42.68
16	65	48	150	30	94.56
17	65	48	240	10	84.78
18	65	0	240	30	80.06
19	50	48	150	10	46.83
20	65	96	150	10	62.14
21	65	96	240	30	88.14
22	65	48	60	50	51.67
23	65	48	60	10	40.28
24	50	48	240	30	92.41
25	80	48	60	30	44.28
26	65	48	150	30	79.13
27	65	0	60	30	22.13
28	65	48	150	30	72.74
29	80	96	150	30	75.47
30	80	48	240	30	97.17

12.37%. RSM from the experimental design was evaluated using the analysis of variance (ANOVA). The quadratic model was significant ($p < 0.05$) with the model F-value at 25.08 while the lack of fit F-value of 0.25 ($p = 0.9706$) implied the lack of fit was not significant relative to the pure error and there was a 97.06% chance that a lack of fit F-value could occur due to the noise. The predicted R^2 value of 0.8821 was in reasonable agreement with the adjusted R^2 of 0.9208. Meanwhile, the signal-to-noise ratio of 19.15 indicated the model could be used to navigate the design space. The responses TPC showed relations with four independent factors by the following second-order polynomial equations:

$$TPC = -260.06552 + 5.79003X_1 + 1.15602X_2 + 0.51368X_3 + 3.02598X_4 - 0.00194X_1X_2 - 0.00035X_1X_3 - 0.02606X_1X_4 - 0.00134X_2X_3 + 0.00262X_2X_4 - 0.00054X_3X_4 - 0.03499X_1^2 - 0.00681X_2^2 - 0.00049X_3^2 - 0.01886X_4^2$$

The result of ANOVA revealed that X_1 (temperature), X_2 (ethanol concentration), X_3 (time), X_4 (solid-to-liquid ratio), X_1X_4 (temperature × solid-to-liquid ratio), X_1^2 (temperature × temperature), X_2^2 (ethanol concentration × ethanol concentration), X_4^2 (solid-to-liquid ratio × solid-to-liquid ratio) were significant model terms. In detail, these factors could deeply affect the TPC of the extraction product.

Three-dimension response surface plots are presented in Fig. 2. As shown, a longer extraction time could increase the TPC of the products. Meanwhile, the extraction tem-

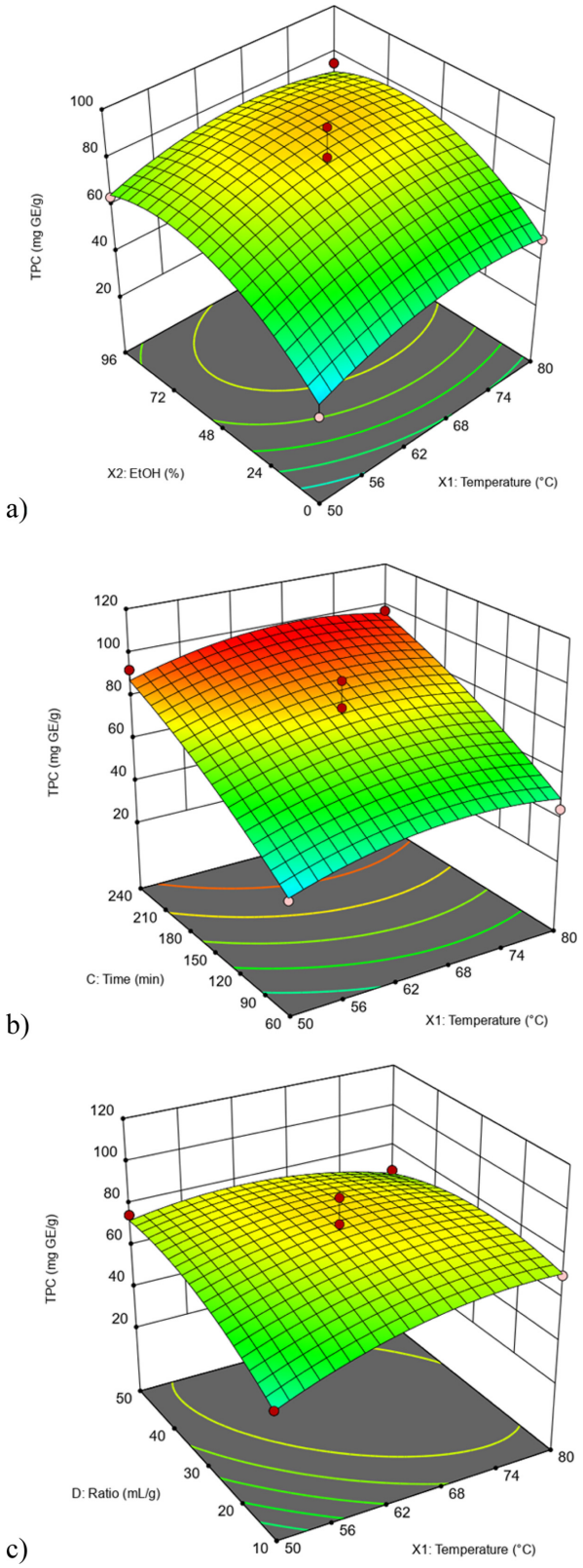

Fig. 2. Response surfaces of (a) temperature vs ethanol concentration, (b) temperature vs time, and (c) temperature vs liquid-to-solid ratio to total phenolic contents of *M. oleifera* root bark extracts.

perature, ethanol concentration, and liquid-to-solid ratio should be raised to certain levels to maximize the contents of phenolic compounds. The optimal values of those factors were selected from RSM and calculated by Design-Expert software. The optimal condition to extract phenolics from *M. oleifera* root bark was the extraction temperature of 64.5°C, ethanol 58.9%, and liquid-to-solid ratio of 37.9 mL/g with the extraction time of 240 minutes with the optimal TPC response should be 101.00 mg GAE/g. Therefore, the actual condition to confirm the result was selected as 65°C, ethanol 60%, and the liquid-to-solid ratio of 40 mL/g with an extraction time of 240 minutes. The actual TPC value was 102.93 ± 6.87 mg GAE/g (triplicate test), which was fitted to the calculated result.

Free radicals are molecules or atoms that carry one or some unpaired electrons. They are highly reactive and very unstable and can easily attack closet stable molecules. In a healthy body condition, there is a balance between reactive free radicals and endogenous antioxidant defence mechanisms. However, if this equilibrium is disturbed, it can lead to oxidative stress and associated damage which can affect DNA and protein, or lead to cell death. As a result, this can cause numerous diseases which include diabetes, cardiovascular diseases, inflammation, cancer, etc.[26] In this study, the antioxidant activity of *M. oleifera* root bark extract in the optimal condition was evaluated by DPPH and hydroxyl radicals scavenging effects. Data were shown in 0. The extract exhibited a strong DPPH scavenging effect with IC_{50} at 18.8 µg/mL, which was lower than the value of ascorbic acid, the positive control (IC_{50} 32.49 µg/mL). Besides, the extract demonstrated weak scavenging effects of hydroxyl radical with the IC_{50} value of 83.30, which was higher than those of catechin, the positive control. Moreover, the anti-inflammatory effect of the *M. oleifera* root bark extract was evaluated via the inhibition of NO production in LPS-stimulated RAW264.7 macrophage cells. As shown in Table 3, the extracts exhibited weaker NO production inhibition than L-NMMA, the positive control.

In addition, *α*-amylase inhibitory activities of *M. oleifera* root bark extract in the optimal condition were evaluated. The extract exhibited considerable inhibition of the enzyme α-amylase with IC_{50} at 104.72 µg/mL, which was lower than acarbose, a common oral antidiabetic drug (IC_{50} at 108.31 µg/mL). *α*-amylase is the enzyme that catalyses the hydrolysis of starch to smaller chains. Activations of the enzyme may cause an increase in post-prandial blood glucose, which could harm diabetic patients. *α*-amylase inhibitors could reduce the activation of the enzyme which could delay the increase of blood glucose

Table 3. Free radicals scavenging, NO production, and α-amylase inhibitory activities of *M. oleifera* root bark extract in the optimal extracting condition

Sample	DPPH (IC_{50}, μg/mL)	Hydroxyl (IC_{50}, μg/mL)	NO inhibition (IC_{50}, μg/mL)	α-amylase inhibition (IC_{50}, μg/mL)
M. oleifera root-bark extract	24.42 ± 1.58	83.30 ± 6.72	44.25±1.41	104.72 ± 8.34
Ascorbic acid *	32.49 ± 2.63	-		
Catechin **	-	34.44 ± 3.06		
L-NMMA#			8.48±0.78	
Acarbose				108.31 ± 9.53

*, **, #, & positive controls

levels. Thus, the extract in the optimal condition of *M. oleifera* root bark in this study could be a considerable anti-diabetic product. The levels of phenolics may strongly correlate with the bioactivities of the extracts. These compounds were reducing agents which could easily react to active free radicals, inhibit the activities of the enzyme, or exhibit anti-inflammatory effects. In this study, the extract under the optimal condition had high contents of phenolic compounds that showed significant free-radicals scavenging and α-amylase inhibition. It also exhibited a weak inhibitory effect on NO production in LPS-stimulated RAW264.7 macrophage cells. Therefore, the phytochemical composition of the polyphenol enrichments of *M. oleifera* root barks should be further investigated. Moreover, these results revealed that the optimal method could be applied on a large scale for the preparation of the bioactive phenolic enrichment from *M. oleifera* root barks.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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