PHARMACIA

Pharmacia 71: 1–9
DOI 10.3897/pharmacia.71.e115528

Research Article

Optimization of ultrasound-assisted extraction using response surface methodology and quantification of polyphenol compounds in *Avicennia officinalis* L. from Vietnam

Ngoc-Van Thi Nguyen[1], Cuong Van Nguyen[2], Ngan Tuyet Duong[1], Xuan-Trang Thi Dai[3], Kien Trung Nguyen[1], Cam-Thuy Thi Le[4]

1 *Can Tho University of Medicine and Pharmacy, CanTho, Vietnam*
2 *College of Pharmacy, Can Tho Medical College, Ninh Kieu District, Can Tho, Vietnam*
3 *College of Natural Sciences, Can Tho University, Ninh Kieu District, Can Tho, Vietnam*
4 *Drug, Cosmetic, Food Quality Control Center of Can Tho City, Ninh Kieu District, Can Tho, Vietnam*

Corresponding author: Ngoc-Van Thi Nguyen (ntnvan@ctump.edu.vn)

Received 9 November 2023 ♦ **Accepted** 10 December 2023 ♦ **Published** 11 January 2024

Citation: Nguyen N-VT, Nguyen CV, Duong NT, Dai X-TT, Nguyen KT, Le C-TT (2024) Optimization of ultrasound-assisted extraction using response surface methodology and quantification of polyphenol compounds in *Avicennia officinalis* L. from Vietnam. Pharmacia 71: 1–9. https://doi.org/10.3897/pharmacia.71.e115528

Abstract

Response surface methodology was used to estimate the optimum extraction parameters, for which the total phenolic content and total flavonoid content of *Avicennia officinalis* L. extract were the highest. Based on the 3D surface plots and regression analysis of the independent variables, the optimal conditions for the ultrasound-assisted extraction were as follows: methanol content, 55.27%; liquid-to-solid ratio, 14:1 (mL/g, v/w); temperature, 48.8 °C, and time, 9.66 min. Optimal extraction conditions were applied to validate the quantification. The bioactive compounds from the *Avicennia officinalis* L. extract were identified through ultra-fast liquid chromatography coupled to diode array detector for the first time. These methods were applied to quantify phenolic acids and flavonoids from *A. officinalis* extracts collected at six different locations in Vietnam. The results showed that the concentrations of these compounds varied from 0.319 ± 0.022 to 3.524 ± 0.125 mg/g in the methanol extract. The result showed that cinnamic acid is the major compound in *A. officinalis* collected in Vietnam. In the present study, an optimized ultrasound-assisted extraction was first designed to improve the content of phenolic acids and flavonoid compounds from *A. officinalis*. Additionally, the bioactive compounds from the *A. officinalis* extract were identified through UFLC-DAD for the first time.

Keywords

Avicennia officinalis L., flavonoid, phenolic acid, response surface methodology

Introduction

There are eight species of mangrove plants in the genus *Avicennia*, which is found on every continent. The bark of mangrove plants contains a significant amount of tannin, which is used in the leather and dye industries (Kathiresan et al. 2001; Bandaranayake et al. 2002). One of the most significant mangrove species is *Avicennia officinalis* L., an evergreen plant found throughout Europe, Western Asia, and North Africa (Das et al. 2019). In Vietnam, mangrove

habitats in coastal provinces, such as Ca Mau, Kien Giang, and Tra Vinh, are home to a large population of this species (Duong et al. 2022).

Several *Avicennia officinalis* L. components have been used in traditional medical procedures. The seeds are used topically for ulcers, boils, and abscesses to speed up the suppuration process. Roots are used in the treatment of skin conditions, including scabies, rheumatism, paralysis, asthma, and snakebites, and as aphrodisiac; bark is used as a diuretic. Fruits are used as tumor plasters (Das et al. 2018; Thatoi et al. 2018). The pharmacological significance of this plant has been reported in several studies. *A. officinalis* is used because of its therapeutic benefits as antidiabetic (Das et al. 2018), anti-inflammatory (Sumithra et al. 2011a), anticancer (Sumithra et al. 2011b), and antibacterial (Shanmugapriya et al. 2012; Valentin et al. 2016). Furthermore, *A officinalis* has significant antioxidant effects (Thirunavukkarasu et al. 2011; Bakshi et al. 2018; Bui et al. 2022), and natural antioxidant particles may inhibit viral replication by denaturing enzymes. The phytochemicals in this plant might be a source of substances that can inactivate SARS-CoV-2 Mpro (Mahmud et al. 2021).

According to a phytochemical investigation of *A. officinalis* leaf extracts, phenolic compounds, such as trans-cinnamic acid (Muhammad et al. 2013), n-hexadecanoic acid, 9-octadecenoic acid methyl ester, and phenethyl alcohol, are present in the extract (Mahmud et al. 2021). Solvent extraction is frequently used for the preparation of bioactive chemicals. The extraction yield is considerably affected by several variables, including solvent composition, solvent-to-solid ratio, extraction temperature, and extraction duration (Chirinos et al. 2007; Ghafoor et al. 2009; Omwamba et al. 2009; Nguyen et al. 2015). The one-factor-at-a-time approach, in which only one component is changed at a time while others are fixed at constant values, is a traditional assay to explore the optimization conditions. However, this method takes considerable time and cannot assess the interaction of different elements. Response surface methodology (RSM) is a useful method for solving these problems. When various factors may have an impact on the results, RSM can investigate the links between the response values and the independent variables and improve procedures or goods (Bas et al. 2007). System-related biochemical and biotechnological processes have been effectively modeled and optimized using RSM (Pompeu et al. 2009; Karacabey et al. 2010; Gong et al. 2012; Nguyen et al. 2022).

The objective of this study was to determine the effect of methanol concentration, extraction temperature, and time on the total phenolic content (TPC) and total flavonoid content (TFC) yields and antioxidant activity in *A. officinalis* extracts. The optimal extraction parameters were determined using RSM with the *A. officinalis* extract having the highest concentration of bioactive compounds. Using ultra-fast liquid chromatography coupled to diode array detector (UFLC-DAD), we developed and validated a method for quantifying phenolic acid and flavonoid components from a methanol extract of *A. officinalis* from Vietnam using the optimal extraction conditions. Additionally, this study is the first to simultaneously quantify six polyphenols in *A. officinalis*.

Materials and methods

Chemicals and instruments

Methanol, ethanol, acetone, ethyl acetate, dichloromethane, chloroform and n-hexane (analytical grade) used for sample extraction were purchased from Sigma-Aldich, Germany. 2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were all purchased from Sigma-Aldrich Chemical Co., Ltd. (St. Louis, Missouri, USA). Other analytical-grade reagents used in the study were purchased from Tianjin Kemiou Chemical Reagent Co., Ltd. (Tianjin, China). The chemicals used to prepare DESs were purchased from Aladdin Biochemical Technology Co., Ltd. (Guangzhou, China).

Methanol, acetonitrile, acetic acid, and formic acid (Sigma-Aldich, Germany) were HPLC grade and water was purified by a MilliQ system (Milllipore Corporation, Bedford, MA, USA). The standards of taxifolin (98%), luteolin-7-O-glucosid (98%), sinapic acid (98%) and cinnamic acid (98%) used for HPLC analysis were purchased from ChemFaces Biochemical Company, Wuhan. Chlorogenic acid (98%) and *p*-coumaric acid (98%) standards were purchased from Sigma-Aldich, Germany.

UFLC-DAD was performed on an UFLC Shimadzu system (LC-20AD), detector DAD SPD-M20A, all piloted by Labsolutions software.

Plant material

Avicennia officinalis L. leaves were obtained from the Mekong Delta region of Ca Mau, Vietnam, and identified at the Can Tho University Department of Biology. To remove dirt and grime, the leaves were washed with water. The moisture content of the dried samples was less than 13%. The raw ingredients were crushed into a fine powder with a size of 2 mm. *A. officinalis* powder was maintained at room temperature in black glass containers until use.

Preliminary extraction experiments

The aim of the preliminary studies was to choose the ideal conditions (solvent concentration, solid-to-liquid ratio, temperature, and time) for the ultrasonic-assisted extraction of the total phenolic acid content (TPC) and total flavonoid content (TFC) from the extract of *A. officinalis* leaves. The first step of the experiment was to determine the effect of the solvent on the production of phenolic acids and flavonoids. Leaf powder (1 g) and solvent (15 mL) with varying methanol concentrations (50–100%, v/v)

were used for extraction. The extraction flask was submerged in an ultrasonic bath at 40 °C for 15 min. The flask was covered to prevent solvent loss during the extraction. Each filtered extract from the solid-liquid mixture was diluted to a volume of 50 mL using the same extraction solvent after the solid-liquid mixture was filtered. The second step of the experiment determined the solid-to-liquid ratio (1:5 to 1:25). Setting the temperature and proper duration for the ultrasound-assisted extraction was the final step of the experiment. The extraction was conducted between 30 °C and 50 °C, and lasted between 10 and 25 min. The extraction effectiveness was assessed through the sum of the peak regions for TPC and TFC.

Optimization of the extraction procedure

RSM was used to optimize the phenolic acid and flavonoid extraction parameters from the *A. officinalis* leaf extract for the highest antioxidant potential. The methanol concentration (X_1), solid-to-liquid ratio (X_2), temperature (X_3), and duration (X_4) are the independent variables. The levels of independent parameters were set based on early experimental findings. For 20 runs of the five stages of the variable optimization process, a rotatable Box-Behnken design was used. Table 1 shows the ranges and numbers of the independent variables in their coded forms. To represent the TPC (Y_1) and TFC (Y_2) yields as a function of the independent factors, linear models were fitted to the experimental data as follows:

$$Y_n = a_0 + \sum_{i=1}^{4} a_i X_i + \sum_{i=1}^{4} a_{ii} X_{ii} + \sum_{i=1}^{4} a_{iii} X_{iii} + \sum_{i=j=1}^{4} a_{ij} x_i x_j$$

where Yn represents the response variables, a0 is a fixed amount, and ai, aii, and aij represent the linear, quadratic, and interaction coefficients, respectively. The independent variables are Xi and Xj. Three-dimensional surface response plots were produced by altering the two variables within the experimental range and maintaining the third constant at the center point. The coefficients of the response surface equation were calculated using Design Expert 11. A 95% confidence level ($p < 0.05$) based on the total error criterion was used to test for statistical significance.

Table 1. Experimental design factors and levels of design of experiments.

Independent variable	Units	Experimental value	
		Low (-1)	High (+1)
A: Methanol concentration	v/v,%	50	100
B: Liquid: Solid (L/S)	mL/g	5	25
C: Ultrasonic temperature	°C	30	50
D: Ultrasonic time	Min	5	25

Analysis of samples through UFLC-DAD

A UFLC Shimadzu (LC-20AD) system with a DAD detector, autosampler, injection system, and quaternary pump was used to identify and quantify phenolic acids and flavonoids in the crude methanol extracts. The system was controlled using LabSolution software. A 4.6 × 250 mm Agilent C18 column with 5 µm particle size was used to measure phenolic acid and flavonoid components. The complete loop injection option was used to inject 20-µL samples. The mobile phase was used to elute the column at a constant flow rate of 1 mL/min and consisted of HPLC-grade acetonitrile (solvent A), HPLC-grade methanol (solvent B), and water containing 0.2% ammonium acetate and 0.1% formic acid. The gradient elution program included: 0–17 min, 3% B and 90% C; 32 min, 9% B and 90% C; 41 min, 10% B and 83% C; 48 min, 11% B and 82% C; 55 min, 15% B and 74% C; 72 min, 18% B and 73% C; 86 min, 20% B and 80% C; and 90–95 min, 7% B and 90% C. The DAD detector was set at 280 nm.

Standard solutions

Stock solutions of chlorogenic, *p*-coumaric, sinapic, and cinnamic acids, taxifolin, and luteolin-7-O-glucosid were prepared in methanol at 1000 µg/mL and stored at 4 °C. Then, solutions were diluted in methanol to obtain seven concentrations (from 1.25 to 150 µg/mL). The peak areas and concentrations of each standard were fitted to linear regression and linear regression after square root transformation to select the most suitable regression model.

Validation quantitative method

The validation of the method was performed with three independent series of experiments to analyze the following criteria: selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, and accuracy according to the Association of Official Analytical Chemists (AOAC) guidelines (Association of Analytical Communities International 2013).

Applications

Six crude methanol extracts of *A. officinalis* leaves collected from different coastal provinces of Vietnam were used. Leaves were harvested from many different *A. officinalis* trees to obtain 1 kg of dry leaf samples. The methanol leaf extracts were analyzed using validated methods for their concentrations of chlorogenic, *p*-coumaric, sinapic, and cinnamic acids, taxifolin, and luteolin-7-O-glucosid to analyze variations according to the collection location and determine the best one.

Statistical analyses

Statistical analyses were performed using Microsoft Excel and GraphPad Prism 8. All measurements were performed in triplicates. Data and figures from the preliminary experiments were generated using Microsoft Excel. Analysis of variance (ANOVA) of the results of

the RSM test and the correlations of extraction parameters with TPC and TFC were analyzed using Design Expert 11.

Results and disscussion

Preliminary extraction experiments

The extraction is very important for the isolation and identification of phenolic acid and flavonoid compounds in plants. In this study, a one-factor-at-a-time approach was used to select the appropriate extraction conditions (solvent concentration, solid-to-liquid ratio, temperature, and time). TPC and TFC were chosen as the indices for the extraction process. The effect of different methanol concentrations (50%, 60%, 70%, 80%, and 100%; v/v) on the TPC and TFC yields was tested at 40 °C for 15 min. The results showed that TPF and TFC depended on methanol concentration (Fig. 1a). The total phenolic acid and flavonoid yields increased with an increase in methanol concentration from 50% to 80% and then decreased when 100% methanol was used. The maximum TPC and TFC yields were 126.47 µg/mL and 75.36 µg/mL, respectively, when methanol concentration was 80%. These results were similar to those previously reported (Liyana-Pathirana et al. 2005; Silva et al. 2007), and might be attributed to the change in solvent polarity with changes in methanol proportion. Furthermore, a high methanol concentration may cause protein denaturation, which prevents the dissolution of polyphenols and affects the extraction rate (Yang et al. 2009). Considering the limitation of the highest methanol concentration in the design, 80% was chosen as the center value for the RSM.

The effect of the solid-to-liquid ratio was investigated using 80% methanol as the solvent at 40 °C for 15 min. To evaluate the effect of this factor on the extraction yields, we examined different ratios ranging from 1:5 to 1:25 g/mL. Fig. 1b shows that TPC and TFC were significantly affected by the solid-to-liquid ratio. Therefore, a 1:15 ratio was selected as the center point for the RSM.

The effect of the temperature (30 °C, 35 °C, 40 °C, 45 °C, and 50 °C) was investigated using 80% methanol with a solid-to-liquid ratio of 1:15 for 15 min. Fig. 1c demonstrates that TPC and TFC were significantly affected by the temperature. When the temperature increased from 30 to 45 °C, a substantial increase in the TPC and TFC was observed. However, a further increase in temperature induced a decrease in TPC and TFC. The rise in temperature may benefit the extraction of TPC and TFC by decreasing the viscosity coefficient, increasing the diffusion coefficient, and enhancing the solubility of phenolics (Cacace et al. 2003). Simultaneously, heat-sensitive compounds may be degraded at higher temperatures. Therefore, a temperature of 45 °C was fixed as the center point for the RSM.

The effect of the ultrasonic time (5 min, 10 min, 15 min, 20 min and 25 min) was investigated using 80% methanol with a solid-to-liquid ratio of 1:15 at 45 °C. Fig. 1d

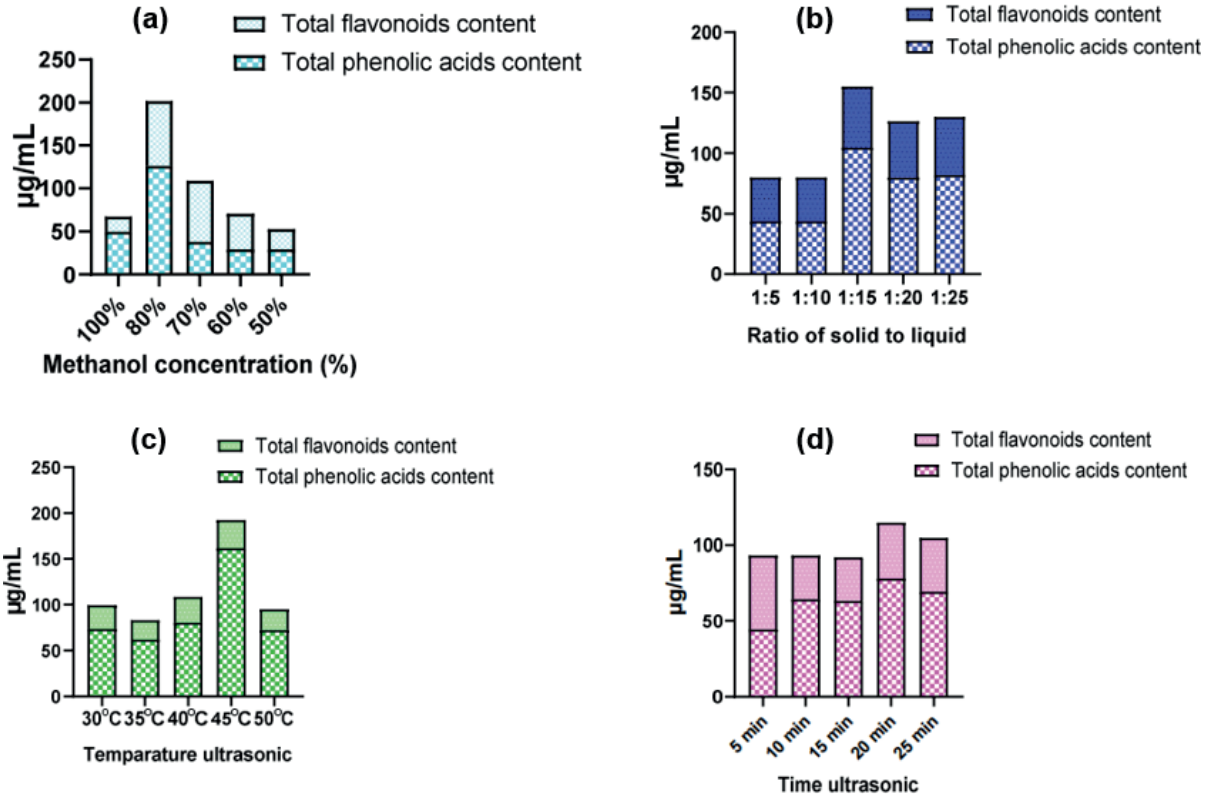

Figure 1. Influence of methanol concentration, ratio of solid to liquid, temperature and time ultrasonic on the extration of TPC and TFC from *A.officinalis* leave extracts.

demonstrates that TPC and TFC were significantly affected by ultrasonic time. When the ultrasonic time increased from 5 to 20 minutes, a substantial increase in the TPC and TFC was observed. However, a further increase in ultrasonic time at 25 minutes induced a decrease in TPC and TFC. Therefore, 20 minutes was fixed as the center point for the RSM.

Optimization of the extraction procedure by RSM

Model fitting

The experimental modeling results showed that TPC varied from 29.39 to 162 μg/mL and TFC from 17.03 to 75.38 μg/mL (Table 2). The software generated two regression equations that demonstrated the empirical relationship between the response values and extraction parameters of methanol concentration (X_1), solid-to-liquid ratio (X_2), temperature (X_3), and time (X_4) of the ultrasound-assisted extraction.

Table 2. Rotatable central composite design setting in the original and coded forms of the independent variables (X_1, X_2, X_3, X_4) and experimental results of TPC (Y_1) and TFC (Y_2).

Run	Independent variables				Responses	
	A	B	C	D	TPC (μg/mL)	TFC (μg/mL)
1	50	15	40	5	162.00	75.38
2	100	15	40	5	126.49	71.02
3	100	15	50	15	104.58	23.34
4	75	15	40	15	78.07	36.04
5	75	15	30	5	81.90	36.03
6	50	15	40	25	80.64	30.47
7	75	15	40	15	80.01	28.08
8	75	25	40	25	43.71	21.06
9	100	15	30	15	64.46	36.78
10	75	5	30	15	63.24	25.97
11	75	25	30	15	73.71	22.58
12	75	15	40	15	72.61	28.84
13	75	5	40	5	38.04	46.35
14	50	25	40	15	29.43	50.38
15	50	5	40	15	29.39	48.58
16	100	5	40	15	44.49	48.06
17	75	25	50	15	43.75	41.33
18	50	15	50	15	49.95	28.76
19	75	5	50	15	79.51	17.03
20	75	15	40	15	62.09	45.18

Regression analysis and ANOVA were used to fit the model and examine the statistical significance of the terms. The results of the ANOVA are presented in Table 3. The corresponding coefficients of determination (R^2) for the models were 0.9674 and 0.9571 for TPC and TFC, respectively. No significant difference in the lack of fit ($p > 0.05$) was observed between the two models, indicating that the models could be used to predict responses. The generated 3D response surface graphs corresponding to all responses showed the interactive effects of the variables (Figs 2, 3).

Table 3. ANOVA for the effect of methanol concentration (X_1), solid-to-liquid ratio (X_2), temperature (X_3), and time (X_4) of the ultrasound-assisted extraction on the total phenolic content (TPC) and total flavonoid content (TFC) using a quadratic response surface model.

Term	Df	TPC			TFC		
Mode		SS	F-ratio	P-value	SS	F-ratio	P-value
Model	14	19605.75	10.59	0.0083	4444.55	7.96	0.0158
X_1	1	458.92	3.47	0.1215	80.20	2.01	0.2154
X_2	1	2.34	0.0177	0.8993	171.48	4.30	0.0928
X_3	1	7.60	0.0575	0.8201	15.95	0.3997	0.5550
X_4	1	61.91	0.4684	0.5242	855.12	21.44	0.0057
X_1X_2	1	2.73	0.0207	0.8913	39.35	0.9864	0.3662
X_1X_3	1	694.88	5.26	0.0704	115.79	2.90	0.1419
X_1X_4	1	1824.08	13.80	0.0138	65.72	1.65	0.2556
X_2X_3	1	534.35	4.04	0.1006	191.59	4.80	0.0799
X_2X_4	1	3384.79	25.61	0.0039	299.59	7.51	0.0408
X_3X_4	1	3213.73	24.31	0.0044	34.63	0.8681	0.3942
X_1^2	1	428.37	3.24	0.1317	225.90	5.66	0.0632
X_2^2	1	1265.60	9.57	0.0270	255.85	6.41	0.0524
X_3^2	1	531.84	4.02	0.1012	783.76	19.65	0.0068
X_4^2	1	5937.68	44.92	0.0011	199.52	5.00	0.0755
Lack of fit	2	467.24	3.92	0.1586	9.61	0.0760	0.9286
Pure error	3	193.67			189.83		
R^2		0.9674			0.9571		
Adj R^2		0.8761			0.8368		

Effect of the extraction variables on TPC

The linear effects of X_1, as well as the quadratic effects of X_1^2, X_2^2, X_3^2, and X_4^2 showed highly significant effects on TPC. In addition, the p-values verified that TPC depended mainly on X_1X_3, followed by X_1X_4, X_2X_4, X_3X_4, X_2^2, and X_4^2 (Table 3). Furthermore, out of the four interactive terms, the interactive effect of X_1 and X_4 was significant. The relationship between TPC and variables is described by the following second-order polynomial equation (1):

$$Y_1 = 73.19 + 10.46X_1 - 0.5884X_2 - 1.21X_3 - 4.52X_4 + 1.16X_1X_2 + 19.06X_1X_3 + 32.19X_1X_4 - 11.59X_2X_3 - 83.67X_2X_4 - 86.52\,X_3X_4 - 11.93X_1^2 - 23.15X_2^2 - 15X_3^2 + 74.5X_4^2 \quad (1)$$

A 3D response surface (Fig. 2A–F) was applied to clarify the interactive effects of the four variables on the TPC of *A. officinalis* leaf extract. X_1 and X_4 showed an interactive effect on TPC, which increased readily up to 162 μg/mL with increasing TPC (Fig. 2C). Fig. 2E, F show that X_4 interacted significantly with X_2 and X_3 ($p < 0.05$). Fig. 2A, B showed X_1 had no significant effect on TPC with X_2 and X_3 ($p > 0.05$).

Effect of the extraction variables on TFC

The linear effects of X_1 and the quadratic effects of X_1^2, X_2^2, X_3^2, and X_4^2 showed highly significant effects on TFC. TFC was mainly affected by X_4, X_2X_4, and X_3^2 ($p < 0.05$). The TFC model is represented through the following equation (2):

$$Y_2 = 34.54 + 4.37X_1 + 5.03X_2 + 1.76X_3 - 16.79X_4 + 4.38X_1X_2 - 7.78X_1X_3 + 6.11X_1X_4 + 6.92X_2X_3 - 24.89X_2X_4 - 8.98X_3X_4 + 8.67X_1^2 + 10.41X_2^2 - 18.21X_3^2 + 13.66X_4^2 \quad (2)$$

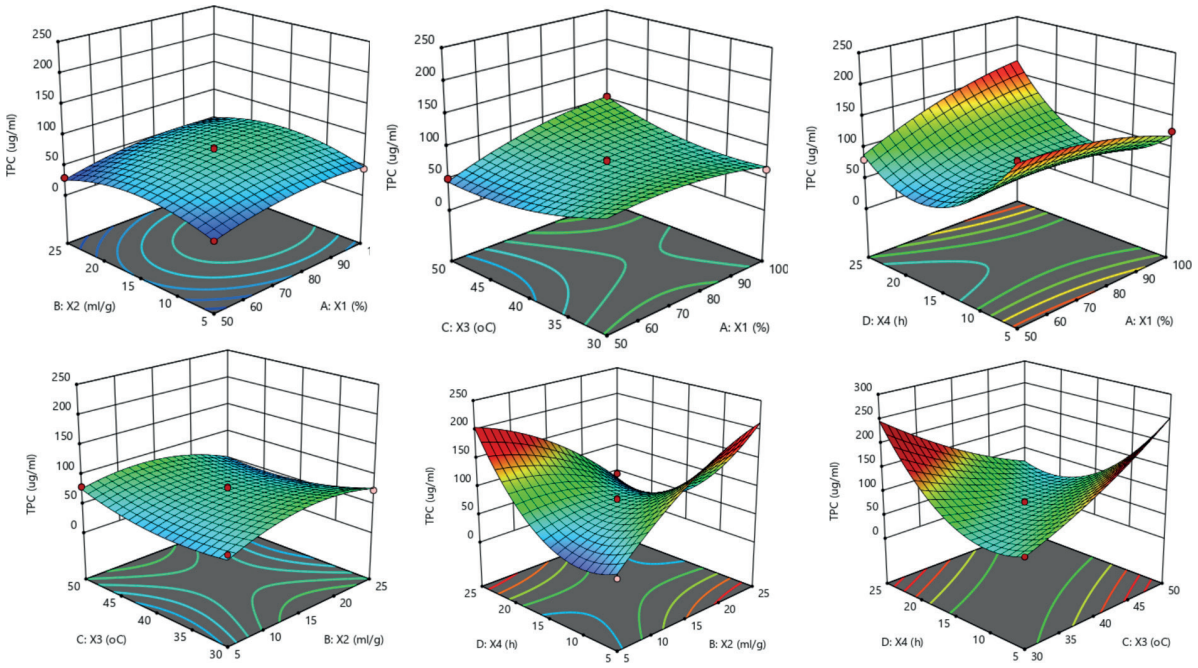

Figure 2. 3D response surface curve showing the influences of independent variables on the TPC (**A–F**).

To visualize the effects of the four independent variables on TFC from *A. officinalis* leaf extracts, 3D response surface plots (Fig. 3A–F) were generated according to equation (2). X_1 and X3 showed a positive effect on all the responses. Negative quadratic effects were observed at the time of extraction, which confirmed a decrease in the extraction yield. Thus, an excessive time is not effective in the extraction process.

Optimization of the extraction conditions

An experiment was conducted to confirm the reliability of the RSM design under the ideal ultrasound-assisted extraction conditions established through the model. The ideal conditions for TPC extraction were determined using the following parameters: 55.27% methanol concentration, 14:1 (mL/g, v/w) for the liquid-to-solid ratio, 48.8 °C as the temperature, and 9.66 min as the duration of the ultrasound-assisted extraction at optimal conditions. TPC and TFC were 127.841 µg/mL and 46.18 µg/mL, respectively. In contrast, the greatest 162 µg/mL TPC and 75.38 µg/mL TFC were projected with 80% of methanol concentration and a 15:1 (mL/g, v/w) liquid-to-solid ratio at 45 °C for 20 min. When phenolics from *Inga edulis* were extracted, a similar result was observed (Silva et al. 2007). This could be caused by process flaws, including

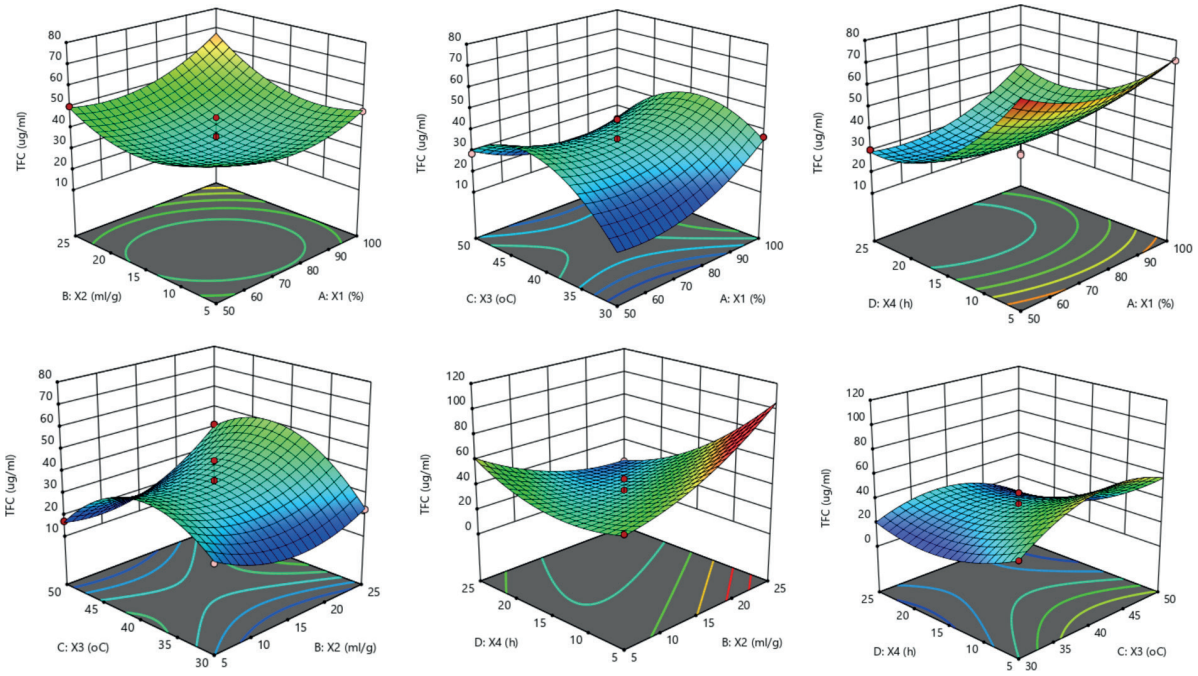

Figure 3. 3D response surface curve showing the influences of independent variables on the TFC (**A–F**).

phenolic breakdown at high temperatures, prolonged extraction times, or use of high solvent concentrations.

Quantification method and validation

Four standards of phenolic acids, including chlorogenic, *p*-coumaric, sinapic, and cinnamic acids, and two standards, including taxifoli and luteolin-7-O-glucosid were selected to quantify the major phenolic compounds from the methanol extracts of *A. officinalis* leaves from Vietnam by the validated method we developed.

Selectivity

Selectivity was validated by comparing the retention times and UV spectra of peaks of the crude extract and standards of six analytes at retention times corresponding to the beginning, middle, and end of these peaks. Similar results indicated the selectivity of the method (Fig. 4).

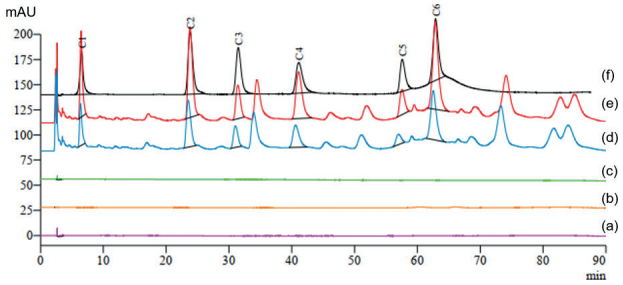

Figure 4. HPLC chromatogram of *A.officinalis* leaves extract. **a** Mobile phase solvent; **b** Solute sovent; **c** Extraction solvent; **d** Spiked sample; **e** Mixed standards solution; **f** Standard addition. (C1: Chlorogenic acid, C2: *p*-coumaric acid, C3: Sinapic acid, C4: Taxifolin, C5: Luteolin -7-O-glucoside, C6: Cinnamic acid).

Linearity and limits of detection and quantification

The stock solutions were diluted and mixed to seven concentrations ranging from 0.05 to 100 µg/mL for the six analytes. To evaluate linearity, each mixed standard sample was injected in triplicate into the HPLC system, and calibration curves were obtained by plotting the average of the peak area responses versus the concentration of each sample. Square correlation coefficients (R^2) were higher than 0.999 (Table 4). The LOD was estimated as 0.33 µg/mL for chlorogenic, *p*-coumaric, and cinnamic acids, 0.7 µg/mL for taxifolin, 1.0 µg/mL for luteolin-7-O-glucoside, and 1.32 for sinapic acid by the signal-to-noise ratio of three as mentioned in 3.0. The LOQ varied from 0.1 to 0.4 µg/mL for the six analytes. This was determined as the lowest concentration level of the test, in agreement with the accuracy profiles.

Precision

Precision was expressed as relative standard deviation (RSD %) values to evaluate repeatability (intra-day) and intermediate precision (inter-day). The results showed that the repeatability and intermediate precision were less than 4% for the six analytes (Table 2). The precision values (≤ 6%) agreed with the AOAC guidelines criteria (Association of Analytical Communities International 2013).

Accuracy

The accuracy of this method was investigated through recovery studies. Three different concentrations of the reference compounds, including low (50%), medium (100%), and high (150%), were added to the blank samples. Spiked samples were then added and quantified according to the methods described above. The results indicated that the method developed exhibited good accuracy, with an overall recovery ranging from 88.44 to 103.86%. Based on the results of the recovery test, this method was deemed accurate.

Application to samples

The leaves of *A. officinalis* used in this study were collected from six provinces in Vietnam. Crude methanol extracts were analyzed using the methods developed. Quantification results (Table 5) showed that the concentration of phenolic acids and flavonoids in the methanol extract of the six samples varied from 0.319 to 3.524 mg/g. The highest total content of bioactive components was found in samples collected in the Kien Giang province.

Cinnamic acid is the major phenolic acid component in the leaves of *A. officinalis* in Vietnam. The highest content of cinnamic acid reached 2.531 mg/g in the crude extract in samples from the Kien Giang province, while no cinnamic acid was detected in the leaf samples collected from the Ben Tre province, and the lowest phenolic acid content was chlorogenic acid, which ranged from 0.005 to 0.102 mg/g.

The leaf samples from the Soc Trang Province had the highest TFC (0.856 mg/g) and contained the highest concentration of luteolin-7-O-glucoside (0.841 mg/g). The sample collected in the Bac Lieu province had no detected luteolin-7-O-glucoside and the content of taxifolin was low (0.018 mg/g).

Table 4. Precision, recovery, calibration parameters, LOD and LOQ.

Substance	Calibration curve		Precision (n=6)		Recovery (%)			LOD	LOQ
	Regression equation	R^2	Intra-day RSD (%)	Inter-day RSD (%)	Low-level	Mid-level	High-level	(µg/ mL)	(µg/ mL)
Chlorogenic acid	y = 41266x – 32257	0.9996	3.22	0.77	91.59	100.09	101.26	0.33	0.10
***p*-coumaric acid**	y = 74152x – 61700	0.9996	3.19	1.03	88.44	91.13	89.03	0.33	0.10
Sinapic acid	y = 64417x – 68771	0.9992	3.54	2.61	96.16	94.21	101.42	1.32	0.40
Cinnamic acid	y = 76966x + 65516	0.9994	3.35	2.78	103.86	102.58	100.33	0.33	0.10
Taxifolin	y = 52877x – 71296	0.9995	3.64	1.28	99.51	99.11	100.86	0.70	0.20
Luteolin -7-O-glucoside	y = 25716x – 23233	0.999	3.31	2.45	98.71	99.48	98.61	1.00	0.30

Table 5. Phenolic acid and flavonoid content in samples of *A.officinalis* leaves collected in different provinces in Vietnam by the validated methods.

Samples (mg/g dry weight)	Bac Lieu	Ben Tre	Soc Trang	Tra Vinh	Kien Giang	Ca Mau
Chlorogenic acid	0.102 ± 0.007	0.032 ± 0.001	0.014 ± 0.002	0.017 ± 0.001	0.005 ± 0.001	0.006 ± 0.001
p-coumaric acid	0.236 ± 0.007	0.007 ± 0.001	0.107 ± 0.006	0.081 ± 0.005	0.198 ± 0.003	0.033 ± 0.008
Sinapic acid	0.042 ± 0.009	0.008 ± 0.002	0.035 ± 0.005	0.016 ± 0.001	0.037 ± 0.001	0.008 ± 0.001
Cinnamic acid	1.698 ± 0.005	ND	1.114 ± 0.017	0.496 ± 0.045	2.531 ± 0.059	0.141 ± 0.007
Total phenolic acid content (a)	2.078 ± 0.029	0.046 ± 0.004	1.332 ± 0.029	0.611 ± 0.053	2.771 ± 0.063	0.189 ± 0.016
Taxifolin	0.018 ± 0.001	0.032 ± 0.002	0.015 ± 0.001	0.013 ± 0.001	0.043 ± 0.002	0.012 ± 0.002
Luteolin -7-O-glucoside	ND*	0.336 ± 0.012	0.841 ± 0.041	0.522 ± 0.042	0.710 ± 0.060	0.118 ± 0.004
Total flavonoid content (b)	0.018 ± 0.001	0.369 ± 0.014	0.856 ± 0.042	0.535 ± 0.043	0.753 ± 0.062	0.130 ± 0.006
Total (a+b)	2.078 ± 0.03	0.412 ± 0.018	2.188 ± 0.071	1.146 ± 0.096	3.524 ± 0.125	0.319 ± 0.022

(n = 3, mean = ± SD) in mg/g methanol extract.
*ND: not detected

Huang studied polyphenol-rich *Avicennia marina* leaf extracts (Huang et al. 2016). The content of bioactive compounds in the water extracts included chlorogenic acid (0.0089 mg/g), p-coumaric acid (0.0058 mg/g), and luteolin (0.0022 mg/g). The *A. officinalis* polyphenol content in Vietnam was 10 times higher than that of *A. marina* in Taiwan. To the best of our knowledge, this is the first report on the sinapic acid and taxifolin content in *A. officinalis*.

Conclusions

In the present study, an optimized ultrasound-assisted extraction was first designed to improve the content of phenolic acids and flavonoid compounds from *A. officinalis*. From the results of the antioxidant assay, the methanol extract was selected as the solvent for the extraction of compounds from *A. officinalis* leaves. Based on the 3D surface plots and regression analysis of the independent variables, the optimal conditions for the extraction were: 55.27% methanol content, 14:1 (mL/g, v/w) liquid-to-solid ratio, and 48.8 °C and 9.66 min as the temperature and time, respectively, of the ultrasound-assisted extraction. Optimal extraction conditions were applied to validate the quantification. The bioactive compounds from the *A. officinalis* extract were identified through UFLC-DAD for the first time. These methods were used to quantify phenolic acids and flavonoids from six extracts of *A. officinalis* collected at six locations in Vietnam. The results showed that the concentrations of these compounds varied from 0.319 to 3.524 mg/g in the methanol extract. This study showed that cinnamic acid is the major compound in *A. officinalis* collected in Vietnam.

Conceived and designed research: Van Cuong Nguyen, Cam-Thuy Le Thi, Xuan-Trang Dai Thi, Ngoc-Van Nguyen Thi. Conducted experiments: Van Cuong Nguyen, Tuyet Ngan Duong. Data analysis: Tuyet Ngan Duong, Van Cuong Nguyen. Manuscript writing: Tuyet Ngan Duong, Ngoc-Van Nguyen Thi. All authors read and approved the manuscript.

Acknowledgements

The authors would like to express their hearty gratitude to Can Tho University of Medicine and Pharmacy. We also thank all of our colleagues for their excellent assistance.

References

Association of Analytical Communities International (2013) Association of Analytical Communities International. Guidelines for single laboratory validation of chemical methods for dietary supplements and botanicals. AOAC International, 1–38.

Bakshi M, Ghosh S, Chakraborty D, Hazra S, Chaudhuri P (2018) Assessment of potentially toxic metal (PTM) pollution in mangrove habitats using biochemical markers: A case study on *Avicennia officinalis* L. in and around Sundarban, India. Marine Pollution Bulletin 133: 157–172. https://doi.org/10.1016/j.marpolbul.2018.05.030

Bandaranayake W (2002) Bioactivities, bioactive compounds and chemical constituents of mangrove plants. Wetlands Ecology and Management 10: 421–452. https://doi.org/10.1023/A:1021397624349

Bas D, Boyaci IH (2007) Modeling and optimization I: Usability of response surface methodology. Journal of Food Engineering 78: 836–845. https://doi.org/10.1016/j.jfoodeng.2005.11.024

Cacace JE, Mazza G (2003) Mass transfer process during extraction of phenolic compounds from milled berries. Journal of Food Engineering 59: 379–389. https://doi.org/10.1016/S0260-8774(02)00497-1

Chirinos R, Rogez H, Campos D, Pedreschi R, Larondelle Y (2007) Optimization of extraction conditions of antioxidant phenolic compounds from mashua (*Tropaeolum tuberosum* Ruíz & Pavón) tubers. Separation and Purification Technology 55: 217–225. https://doi.org/10.1016/j.seppur.2006.12.005

Das SK, Samantaray D, Thatoi H (2017) Evaluation of in vitro antidiabetic and antioxidant activities and preliminary phytochemical screening of leaf extracts of *Avicennia officinalis*. Journal of Bioanalysis and Biomedicine 9: 173–176. https://doi.org/10.4172/1948-593X.1000174

Das SK, Samantaray D, Mahapatra A, Pal N, Munda R, Thatoi H (2018) Pharmacological activities of leaf and bark extracts of a medicinal

mangrove plant *Avicennia officinalis* L. Clinical Phytoscience 4: 13–23. https://doi.org/10.1186/s40816-018-0072-0

Das SK, Samantaray D, Sahoo SK, Patra JK,Samanta L, Thatoi H (2019) Bioactivity guided isolation and structural characterization of the antidiabetic and antioxidant compound from bark extract of *Avicennia officinalis* L. South African Journal of Botany 125: 109–115. https://doi.org/10.1016/j.sajb.2019.07.011

Duong TN, Nguyen NVT, Pham TLT (2022) Simultaneous determination of common phenolic acid in leaves extract of *Avicennia officinalis*. Journal of Multidisciplinary Sciences 4(2): 10–16. https://doi.org/10.33888/jms.2022.422

Ghafoor K, Choi YH, Jeon JY, Jo IH (2009) Optimization of ultrasound-assisted extraction of phenolic compounds, antioxidants, and anthocyanins from grape (*Vitis vinifera*) seeds. Journal of Agricultural and Food Chemistry 57: 4988–4994. https://doi.org/10.1021/jf9001439

Gong Y, Hou Z, Gao Y (2012) Optimization of extraction parameters of bioactive components from defatted marigold (*Tagetes erecta* L.) residue using response surface methodology. Food and Bioproducts Processing 90(1): 9–16. https://doi.org/10.1016/j.fbp.2010.12.004

Huang C, Lu C-K, Tu M-C, Chang J-H, Chen Y-J, Tu Y-H, Huang H-C (2016) Polyphenol-rich *Avicennia marina* leaf extracts induce apoptosis in human breast and liver cancer cells and in a nude mouse xenograft model. Oncotarget 7(24): 35874–35893. https://doi.org/10.18632/oncotarget.8624

Karacabey E, Mazza G (2010) Optimisation of antioxidant activity of grape cane extracts using response surface methodology. Food Chemistry 119: 343–348. https://doi.org/10.1016/j.foodchem.2009.06.029

Kathiresan K, Bingham BL (2001) Advances in marine biology. In: Southward A, Young C, Fuiman L, Tyler P (Eds) Biology of Mangrove and Mangrove Ecosystem. Academy Press, New York, 84–88. https://doi.org/10.1016/S0065-2881(01)40003-4

Liyana-Pathirana C, Shahidi F (2005) Optimization of extraction of phenolic compounds from wheat using response surface methodology. Food Chemistry 93: 47–56. https://doi.org/10.1016/j.foodchem.2004.08.050

Mahmud S, Paul GK, Afroze M (2021) Efficacy of phytochemicals derived from *Avicennia officinalis* for the management of COVID-19: A combined in silico and biochemical study. Molecules 26(8): e2210. https://doi.org/10.3390/molecules26082210

Muhammad N, Saeed M, Adhikari A, Khan KM, Khan H (2013) Isolation of a new bioactive cinnamic acid derivative from the whole plant of *Viola betonicifolia*. Journal of Enzyme Inhibition and Medicinal Chemistry 28(5): 997–1001. https://doi.org/10.3109/14756366.2012.702344

Ngoc-Van TN, Ngan TD, Kim-Ngan HN (2022) Effect of extraction solvent on total phenol, flavonoid content, and antioxidant activity of *Avicennia officinalis*. Biointerface Research in Applied Chemistry 12(2): 2678–2690. https://doi.org/10.33263/BRIAC122.26782690

Nguyen TTT, Chollet-Krugler M, Lohézic-Le Dévéhat F, Rouaud I, Boustie J (2015) Mycosporine-like compounds in chlorolichens: Isolation from *Dermatocarpon luridum* and *Dermatocarpon miniatum*, and their photoprotective properties. Planta Medica Letters 2(1): e1-e5. https://doi.org/10.1055/s-0034-1396321

Nguyen TTL, Le Minh T, Do DQ (2022) Optimization of alcohol extraction and spray-drying conditions for efficient processing and quality evaluation of instant tea powder from lotus and green tea leaves. Pharmacia 69(3): 621–630. https://doi.org/10.3897/pharmacia.69.e84650

Omwamba M, Hu Q (2009) Antioxidant capacity and antioxidative compounds in barley (*Hordeum vulgare* L.) grain optimized using response surface methodology in hot air roasting. European Food Research and Technology 229: 907–914. https://doi.org/10.1007/s00217-009-1128-7

Pellegrini N, Visioli F, Buratti S, Brighenti F (2001) Direct analysis of total antioxidant activity of olive oil and studies on the influence of heating. Journal of Agricultural and Food Chemistry 49(5): 2532–2538. https://doi.org/10.1021/jf001418j

Pompeu DR, Silva EM, Rogez H (2009) Optimisation of the solvent extraction of phenolic antioxidants from fruits of *Euterpe oleracea* using response surface methodology. Bioresource Technology 100: 6076–6082. https://doi.org/10.1016/j.biortech.2009.03.083

Ramadan MF, Kroh LW, Mörsel JT (2003) Radical scavenging activity of black cumin (*Nigella sativa* L.), coriander (*Coriandrum sativum* L.), and niger (*Guizotia abyssinica* Cass.) crude seed oils and oil fractions. Journal of Agricultural and Food Chemistry 51(24): 6961–6969. https://doi.org/10.1021/jf0346713

Samarakoon SR, Shanmuganathan C, Ediriweera MK, Tennekoon KH, Piyathilaka P, Thabrew I, de Silva ED (2016) *In vitro* cytotoxic and antioxidant activity of leaf extracts of mangrove plant, *Phoenix paludosa* Roxb. Tropical Journal of Pharmaceutical Research 15(1): 127–132. https://doi.org/10.4314/tjpr.v15i1.18

Shanmugapriya R, Ramanathan T, Renugadevi G (2012) Phytochemical characterization and antimicrobial efficiency of mangrove plants *Avicennia marina* and *Avicennia officinalis*. International Journal of Pharmaceutical & Biological Archive 3: 348–351.

Silva EM, Rogez H, Larondelle Y (2007) Optimization of extraction of phenolics from *Inga edulis* leaves using response surface methodology. Separation and Purification Technology 55: 381–387. https://doi.org/10.1016/j.seppur.2007.01.008

Sumithra M, Janjanam VK, Kancharana VS (2011a) Influence of methanolic extract of *Avicennia officinalis* leaves on acute, subacute and chronic inflammatory models. International Journal of PharmTech Research 3: 763–768.

Sumithra M, Anbu J, Nithya S, Ravichandiran V (2011b) Anticancer activity of methanolic leaves extract of *Avicennia officinalis* on ehrlich ascitis carcinoma cell lines in rodents. International Journal of PharmTech Research 3: 1290–1292.

Thatoi H, Das SK, Samantaray D (2016) The genus *Avicennia*, a pioneer group of dominant mangrove plant species with potential medicinal values: A review. Frontiers in Life Sciences and Related Technologies 9: 267–291. https://doi.org/10.1080/21553769.2016.1235619

Thirunavukkarasu P, Ramanathan T, Ramkumar L, Shanmugapriya R, Renugadevi G (2011) The antioxidant and free radical scavenging effect of *Avicennia officinalis*. Journal of Medicinal Plants Research 5: 4754–4758.

Valentin BB, Meenupriya J, Elsa JL, Naveena DE, Kumar S, Thangaraj M (2010) Antibacterial activity and characterization of secondary metabolites isolated from mangrove plant *Avicennia officinalis*. Asian Pacific Journal of Tropical Medicine 1: 412–420. https://doi.org/10.1016/S1995-7645(10)60131-9

Yang L, Jiang JG, Li WF, Chen J, Wang DY, Zhu L (2009) Optimum extraction process of polyphenols from the bark of *Phyllanthus emblica* L. based on the response surface methodology. Journal of Separation Science 32(9): 1437–1444. https://doi.org/10.1002/jssc.200800744