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Research Article

Optimum Conditions and LC-ESI-MS Analysis of Phenolic Rich Extract from *Eucalyptus marginata* L. under Maceration and Ultrasound-Assisted Extraction Methods Using Response Surface Methodology

Soumaya Hasni,[1] **Ghayth Rigane**,[1,2] **Hanene Ghazghazi**,[3] **Hajer Riguene**,[1] **Amir Bouallegue**,[4] **Olfa Khedher**,[1] **Moufida A. Oueslati**,[5] **and Ridha Ben Salem**[1]

[1]*Laboratory of Organic Chemistry LR17ES08, Sciences Faculty of Sfax, B.P 1171, University of Sfax, Sfax 3038, Tunisia*
[2]*Physic and Chemistry Department, Faculty of Sciences and Technology of Sidi Bouzid, B.P «380» 9100, Sidi Bouzid, University of Kairouan, Kairouan, Tunisia*
[3]*Laboratory of Management and Valorization of Forest Resources, National Research Institute of Rural Engineering, Water and Forestry (INRGREF), Kairouan, Tunisia*
[4]*Laboratory for the Improvement of Plants and Valorization of Agro-Ressources, National School of Engineering of Sfax (ENIS), University of Sfax, Sfax 3038, Tunisia*
[5]*College of Applied Medical Sciences Al Jubail, Deanship of Preparatory Year and Supporting Studies and the Department of Respiratory Care, Imam Abdulrahman Bin Faisal University, PO. Box 1982, Dammam 31441, Saudi Arabia*

Correspondence should be addressed to Ghayth Rigane; gaith.rigane@yahoo.fr

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Eucalyptus marginata L. has a significant value in traditional medicine and recently has been shown to possess many pharmacological properties in vitro. The main goal of the present study was to optimize the extraction parameters of phenolic compounds from *Eucalyptus marginata* L. leaves using the extraction technique assisted by ultrasound in comparison with maceration using response surface methodology as a predicted tool. Therefore, total phenolic and flavonoid contents have been optimized, taking into account four variables: extraction time, temperature, liquid-to-solid ratio, and ethanol concentration. The optimum ultrasound-assisted extraction method for total phenolic and total flavonoid contents was obtained by ensuring the following parameters: $t = 49.9$ min, $T = 74.9^{\circ}$C, liquid-to-solid ratio = 39.5 ml/g, and ethanol = 58.48%. The optimum extract has been subjected to LC-ESI-MS analysis. This technique allowed us to identify ten phenolic compounds: four phenolic acids mainly gallic acid ($27.77 \pm 0.06\,\mu$g/g DW) and protocatechuic acid ($37.66 \pm 0.04\,\mu$g/g DW) and six flavonoid compounds such as quercetrin ($150.78 \pm 0.02\,\mu$g/g DW) and hyperoside ($39.19 \pm 0.03\,\mu$g/g DW). These green and efficient procedures should be a promising option to guide industrial design for the production of phenolic-rich plant extracts.

1. Introduction

The Myrtaceae is a large evergreen tree that is known in the literature scientific with many synonyms including *Calyptranthes oneilli* Lundell, *Calyptranthes jombolona* Wild, and *Eugenia cumini* Druce. It contains around 3000 species such as *Eucalyptus*. It is a big and strong tree belonging to the family; however, some species are now distributed all over the world. It represents about 27% of the total timber volume and is one of the most important. *Eucalyptus marginata* is easily recognized by its flowers and fruits. Many research studies showed that the antioxidant activity, which is

generally attributed with the interesting phenolic composition, allows this plant to be widely used in food and pharmaceutical industries [1–7]. Previous work showed that *Eucalyptus* is like any medicinal plant present an original chemical composition. Each organ apart has properties important because it contains essential oil and phenolic compounds as phenolic acids, flavonoids, and tannins. The composition makes the plant more magnificent and presents itself as a natural treasure since it is used in various fields, mainly the field of medicine [1, 3]. Nowadays, nothing describes the particularity of *Eucalyptus* more clearly than the nature of its essential oil and phenolic composition, which is now very popular with the general public who aspires to treat themselves effectively with simple and natural means and which are designated for interesting biological and physiological purposes and activities. Indeed, many research studies' team showed that *Eucalyptus marginata* L. presents several properties of antioxidant, antimicrobial, anticancer, anti-inflammatory, and antifungal. In addition, it is designed for relaxation, mood disorders, and to relieve fever, coughs, and respiratory problems, even for the treatment of acne all by reducing the production of sebum [3]. Ultrasound-assisted extraction is a new technology used in several fields such as cosmetics, pharmaceutical, chemical, and food industries. Furthermore, Saifullah et al. [8] and Ezzoubi et al. [9] mentioned that ultrasound extraction allowed them to get extracts rich in biomolecules in a shorter extraction time with comparison to conventional extraction techniques. In addition, Chemat et al. [10] showed that the carvone yield extracted from *Carum carvi* L. seeds was higher using ultrasound treatment than those obtained using Soxhlet. A comparative study has been done by [11] who studied the extraction of phenolic compounds yields from *Acacia confusa* using ultrasound, maceration, and extraction assisted by heat. They confirmed that ultrasound treatment is the most fast and efficient technique, which allows to significantly increase the rate of phenolic content compared to other methods studied.

The aim of this work was to maximize the obtained total phenolic and flavonoid contents extracted from *Eucalyptus marginata* L. using ultrasound and maceration methods. In order to achieve this objective, we used the Box–Behnken design in conjunction with a response surface methodology (RSM) to optimizing four parameters: the extraction time (min), temperature (°C), liquid-to-solid ratio (ml/g), and ethanol concentration (%). In addition, the individual phenolic compounds present in the optimum extraction condition have been identified and quantified using LC-ESI-MS.

2. Materials and Methods

2.1. Plant Material and Sample Preparation. *Eucalyptus marginata* L. leaves were collected in January 2020, from Souiniet arboreta from northeastern provinces of Tunisia (35°54 N and 8°48 *E*, 492 m) under semiarid bioclimate. A voucher specimen of *Eucalyptus marginata* L. (LGVR 2020) was deposited at the Laboratory of Organic Chemistry LR17ES08, Faculty of Sciences of Sfax, University of Sfax, Tunisia. Only healthy leaves have been harvested at different heights and immediately transported to our laboratory. In the same day, leaves were all ground using an electric mill (Retsch Muhle, Grindomix, GM200, Kurt Retsch GmbH and Co., KG, Haan, Germany), at a speed of 10000 rpm/min, using a 0.5 mm mesh screen to improve contact with the solvent.

2.2. Maceration and Ultrasound-Assisted Extraction

2.2.1. Maceration Extraction. Briefly, twenty grams of *Eucalyptus marginata* leaves powders were extracted by maceration according to experimental design and under continuous agitation using a magnetic laboratory shaker TT-SSMS-200, TT-DMS series with 1800 rpm (11 310 rad min^{-1}) (Table 1).

2.2.2. Ultrasound-Assisted Extraction. The extraction method was performed using an Elmasonic S60H ultrasonic bath (Elma Hans Schmidbauer GmbH and Co., Singen. Germany) [12]. The *Eucalyptus marginata* L. leaves powders' (2.5 g) was placed in a beaker (100 ml) and mixed with an appropriate amount of the extraction solution (according to experimental design) (Table 1). The beaker with the suspension was immersed in water in the ultrasonic device and irradiated for the preset extraction time.

For both extraction methods, the resulting extract was then filtered through Whatman no. 4 paper and evaporated under vacuum at 40°C on a rotary evaporator until dryness. Each sample was kept in the refrigerator at +4°C until use. Experiments were performed in triplicate (Table 1). Each extractions method was replicated three times.

2.3. Experimental Design. Response surface methodology (RSM) was used for investigating the influence of four independent variables on total phenolic and flavonoid content in *Eucalyptus marginata* L. leaves extracts [13]. The extraction time (min, X_1), temperature (°C, X_2), liquid-to-solid ratio (ml/g, X_3), and ethanol concentration (%, X_4) were selected as independent variables that should be optimized for the extraction. The samples were kept at room temperature to avoid the degradation of temperature-sensitive compounds. In the study, the experiments were performed on the central composite design (CCD). The level values of the experimental factors are given in Tables 1 and 2.

2.4. Total Phenols and Flavonoids Contents. Total phenol content was evaluated according to the Folin–Ciocalteu method according to Khedher et al. [5] with slight modification using a UV-visible spectrophotometer (Beckman DU 800). Total phenol content was calculated based on a gallic acid calibration curve ($R^2 = 0.9978$) and expressed as mg gallic acid equivalent (GAE)/g dry weight (DW). In addition, total flavonoids content was estimated as reported previously by Ben Hmed et al. [6]. The total flavonoids content was quantified using quercetin standard curve

Table 1: The operating conditions according to an experiment plan.

N	X_1 ME t (min)	X_1UAE t (min)	X_2 T (°C)	X_3 R (ml/g)	X_4 ethanol (%)
1	30	10	25	20	20
2	30	10	25	20	60
3	30	10	25	40	20
4	30	10	25	40	60
5	30	10	75	20	20
6	30	10	75	20	60
7	30	10	75	40	20
8	30	10	75	40	60
9	90	50	25	20	20
10	90	50	25	20	60
11	90	50	25	40	20
12	90	50	25	40	60
13	90	50	75	20	20
14	90	50	75	20	60
15	90	50	75	40	20
16	90	50	75	40	60
17	60	30	50	30	40
18	60	30	50	30	40
19	60	30	50	30	40
20	17.58	17.2	50	30	40
21	102.42	58.2	50	30	40
22	60	30	14.65	30	40
23	60	30	85.35	30	40
24	60	30	50	15.9	40
25	60	30	50	44.1	40
26	60	30	50	30	16.56
27	60	30	50	30	96.56

ME, maceration extraction; UAE, ultrasound-assisted extraction; t, extraction time expressed in min; T, temperature expressed in °C; R, solvent-solid ratio expressed in mg/g.

Table 2: The selected factors and their levels in the CCD design.

	Factors	Unit	Symbol	Factor levels		
				−1	0	1
ME	Extraction time	min	X_1	30	60	90
	Temperature	°C	X_2	25	50	75
	Solvent-to-material ratio	ml/g	X_3	20	30	40
	Ethanol	%	X_4	20	40	60
UAM	Extraction time	min	X_1	10	30	60
	Temperature	°C	X_2	20	50	75
	Solvent-to-material ratio	ml/g	X_3	20	30	40
	Ethanol	%	X_4	20	40	60

ME, maceration extraction; UAM, ultrasound-assisted extraction.

($R^2 = 0.9954$) and expressed as milligram of quercetin equivalent (QE)/g DW.

2.5. LC-ESI-MS Analysis. The LC-ESI-MS analysis was carried out using a LC-electrospray ionization-tandem mass spectrometry (Shimadzu, Kyoto, Japan) equipped with electrospray ionization (ESI). An Aquasil C18 column (Thermo Electron, Dreieich, Germany) (150 mm × 3 mm × 3 μm) proceeded by an Aquasil C18 guard column (10 mm × 3 mm, 3 μm, Thermo Electron) were used for the analysis. The mobile phase was A (0.1% formic acid in H_2O, v/v) and B (0.1% formic acid in methanol, v/v) with a linear gradient elution: 0–45 min, 10–100% B; 45–55 min, 100% B. Reequilibration duration was 5 min between individual runs. The column temperature was maintained at 40°C, the mobile phase flow rate was 0.4 ml/min, and the injection volume was 5 μl. The mass spectrometer was operated in the negative ion mode with a capillary voltage of −3.5 V, nebulizing gas flow of 1.5 L/min, a dry gas flow rate of 12 L/min, a block source temperature of 400°C, a DL (dissolving line) temperature of 250°C, the full scan spectra from 50 to 2000 Da, and the negative ionization mode source voltage of −4500 V. The quantification of phenolic compounds was performed at 280 and 335 nm using four-point regression curve ($R^2 = 0.989$) standards [7].

2.6. Software. NemrodW 2007 software has been used in order to build the experimental designs and regression analysis of the experimental data.

3. Results and Discussion

To find the conditions, which increase the extraction of phenolic compounds from *Eucalyptus marginata* leaves using maceration and ultrasound-assisted methods. It was very important to take into account the variables that affect the system behavior. Therefore, preliminary tests were reviewed individually to determine their experimental domain in order to obtain an appropriate RSM design by analyzing their general model responses.

3.1. Preliminary Study of Single Factor Experiments for Maceration and Ultrasound Extraction Methods

3.1.1. Influence of Extraction Time. The extraction time is an important parameter to minimize the energy and the cost of the extraction process [14, 15]. Therefore, extraction time has been ranged from 30 to 105 min for maceration and from 10 to 60 for ultrasound-assisted extraction methods. As shown in Figure 1(a) and Figure 2(a), we could note that the maximum TPC has been showed at 60 min and 30 min of extraction for maceration and ultrasound extraction, respectively (147.18 and 209.15 mg GAE/g of DW, respectively). In addition, extraction time > 60 and > 30 min for the both extraction methods decreased not only TPC but also TFC (Figure 1(a) and Figure 2(a)). The obtained results were in agreement with those reported by Khedher et al. [5] who showed that the highest TPC (332.55 mg GAE/g DW) has been obtained for a period of extraction $t = 60$ min. The last results were also in accordance to those reported by Ghafoor et al. [16], Alu'datt et al. [17], Rubio-Senent et al. [18], and Fan et al. [19] who claimed that longer extraction time of alperujo under hydrothermal conditions could provoke degradation or polymerization reactions of the phenolic extract and longer extraction times increased total phenolic content but reduced antioxidant activity and also caused the oxidation of the targeted compounds, mainly flavonoids.

3.1.2. Influence of Temperature. The solubility of phenolic compounds increased with increasing temperature used for extraction because the rise in temperature allows mass transfer easily between solvent and solid matter [17]. As indicated in Figure 1(b) and Figure 2(b), temperature extraction influenced significantly ($p < 0.05$) the TPC and TFC contents; for maceration and ultrasound extraction methods, our research team showed the highest TPC at 50°C (147.18 and 209.15 mg GAE/g DW, respectively). On the other hand, using temperature >50°C decreased significantly ($p < 0.05$) the TPC and TFC (~100–160 mg GAE/g DW and ~30–50 mg QE/g DW, respectively) for the both studied extraction methods. By comparing the obtained results to those reported in the literature, it can be concluded that the temperature is an important factor to have a large part of the phenolic content; however, these compounds were sensitive to heat; it is estimated that the increase in the extraction temperature causes the decrease essentially when the temperature exceeds the boiling point of the solvent. This will be evaporated in the air which causes the reduction in volume which destroys the extraction efficiency [20, 21], Samaram et al., 2015 [23–25].

3.1.3. Influence of Liquid-to-Solid Ratio. The liquid-to-solid ratio plays an essential role in the extraction influencing the recovery of phenolic compounds. Indeed, its role behaves in improving the extraction yield because it influences the concentration gradient between the plant and the solvent which ensures the transfer of material as it is cited in the literature [26]. Therefore, to study the effect of different liquid-solid ratios on the extraction of phenolic compound from *Eucalyptus marginata* leaves, a different liquid-to-solid ratio R varied from 15 to 40 ml/g. Figure 1(c) and Figure 2(c) show that the highest values of TPC and TFC were obtained for 30 ml/g: 147.18 mg GAE/g DW and 49.75 mg QE/g DW for the maceration extraction method and 209.15 mg GAE/g DW and 75.07 mg QE/g DW for the ultrasound extraction method. These data allowed us to check the importance of solvents amount in extraction, but that does not prevent us to say that more than 30 ml/g of the obtained phenolic content do not be more desirable as discussed by Zhu et al. [26] and Mohammadpour et al. [27]. However, we can conclude that the existence of an additional solvent in the system is equivalent to a low concentration of the solid, which causes a decrease in the phenomenon of cavitation. As indicated in the literature, the liquid-to-solid ratio has an effect considerable in obtaining phenolic compounds [20, 28].

3.1.4. Influence of Ethanol Concentration. The nature of the solvent is important for extracting molecules selectively, and it must have a strong affinity with a great capacity of dissolution. Water is used as a solvent for the extraction of biomolecules present in plant sources; its polarity dissolved several polar phenolic compounds. In addition, other solvents such as ethanol, methanol, acetone, ethyl acetate, and their mixtures with water were widely used for the extraction of phenolic compounds for the reason of absence of toxicity and abundance even if other more effective solvents than water and ethanol [29]. The different concentrations of ethanol have been varied from 0% to 100%; these significant effects are shown in Figures 1(d) and 2(d). The highest phenolic content has been showed using 40% of hydroethanolic solvent, while extraction with mixture 100% ethanol has a low value. Several similar results showed that the percentage of ethanol presents an important role in improving performance for phenolic extraction using ultrasound treatment [30–33].

3.2. Response Surface Methodology

3.2.1. Model Fitting and Response Surface Analysis. The response surface methodology (RSM) is performed for the 27 experimental tests. The regression equations were obtained by fitting the experimental data of each response in a polynomial model like the following equation (Table 3). The good adaptation of the regression model is estimated by the coefficient of determination (R^2), which measured the adaptability in the response values, due to variation of the experiment's factors and their interactions. The model can fit well with the actual data when R^2 is close to one. The coefficients of determination of the model (R^2) were 0.881 and 0.923, which indicate that the obtained results would have good accuracy, which proved the capacity of the established model within the limits of the range of use.

The three-dimensional (3D) response surface presented 2 factors (axe X and axe Y) and the response (axe Z) (Figures 3 and 4). Figures 3(a) and 4(a) show the interaction between the time and the temperature using ultrasound

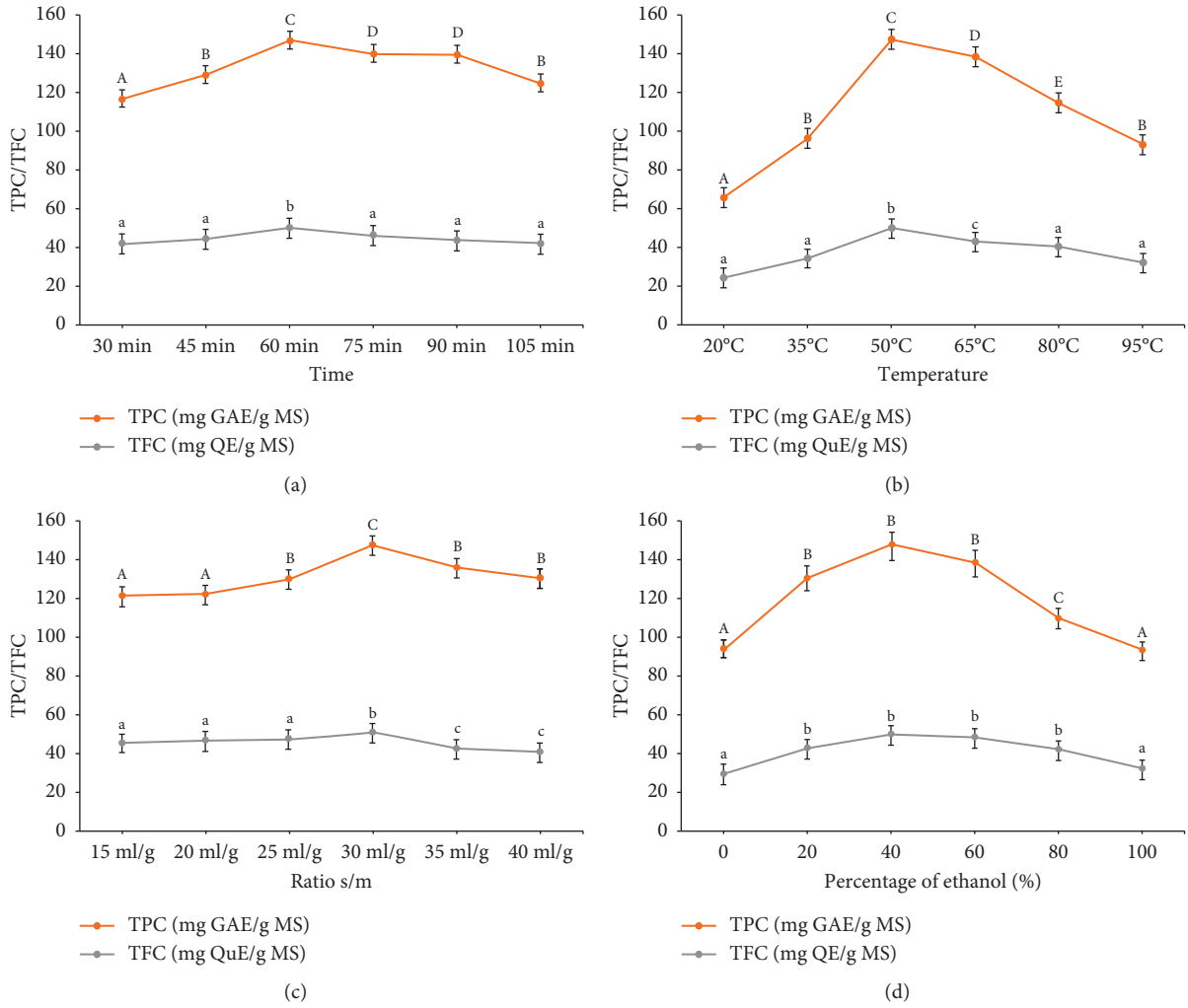


Figure 1: Effect of extraction time (a), temperature (b), ratio s/m (c), and ethanol (%) (d) on total phenol and flavonoid contents from *Eucalyptus marginata* L. leaves using the maceration extraction method.

extraction and maceration methods, while s/m ration and ethanol concentration have been fixed. Increasing t and T affects significantly TPC and TFC. However, after a long extraction time and high temperature, TPC and TFC decreased. These results could be explained by a degradation process of some phenolic compounds as mentioned previously by Zhang et al. (2019) and [34]. The effect of the t and ratio is shown in Figures 3(b) and 4(b). The TPC and TFC increased with increasing these two factors where our research team concluded that increasing the ratio s/m enhanced the solubility of the phenolics compounds. TPC and TFC reached its maximum after increasing t and the percentage of ethanol (Figures 3(c) and 4(c)). As described by Do et al. [35], several polyphenols are soluble in organic solvent (ethanol). TPC and TFC increased with increasing the T and the ratio s/m (Figures 3(d) and 4(d)). According to Kamarudin et al. [36], increasing T enhanced the penetration of the solvents into the cells.

3.2.2. Optimization of the Extraction. Using the ultrasound method, the maximum of TPC (~210 mg GAE/g DW) was obtained when the ultrasonic time was 49.9 min, the temperature was 74.9°C, the liquid-to-solid ratio was 39.5 ml/g, and the percentage of ethanol was 58.48%. However, the optimum conditions using maceration methods were as follows: $t = 88$ min, $T = 74.42$°C, liquid-to-solid = 40 ml/g, and the percentage of ethanol equal to 59.65% to maximize the total phenolic compounds (~150 mg GAE g DW). For that reason, UAE could be considered as an economic and green extraction method for extraction of the bioactive compounds. The obtained results indicated that the UAE-RSM approach was very useful in order to improve the phenols and flavonoids contents in the plant material extracts.

3.3. Identification and Quantification of Phenolic Compounds in Eucalyptus marginata L. Extract. The chemical composition of the two *Eucalyptus marginata* L. extracts obtained after maceration and ultrasound-assisted extraction parameters was analyzed with LC-ESI-MS in the negative mode (Figure 5). Based on the mass spectra and comparison with reference compounds and with literature data [4, 6, 7, 37–39], the detected compounds were classified as

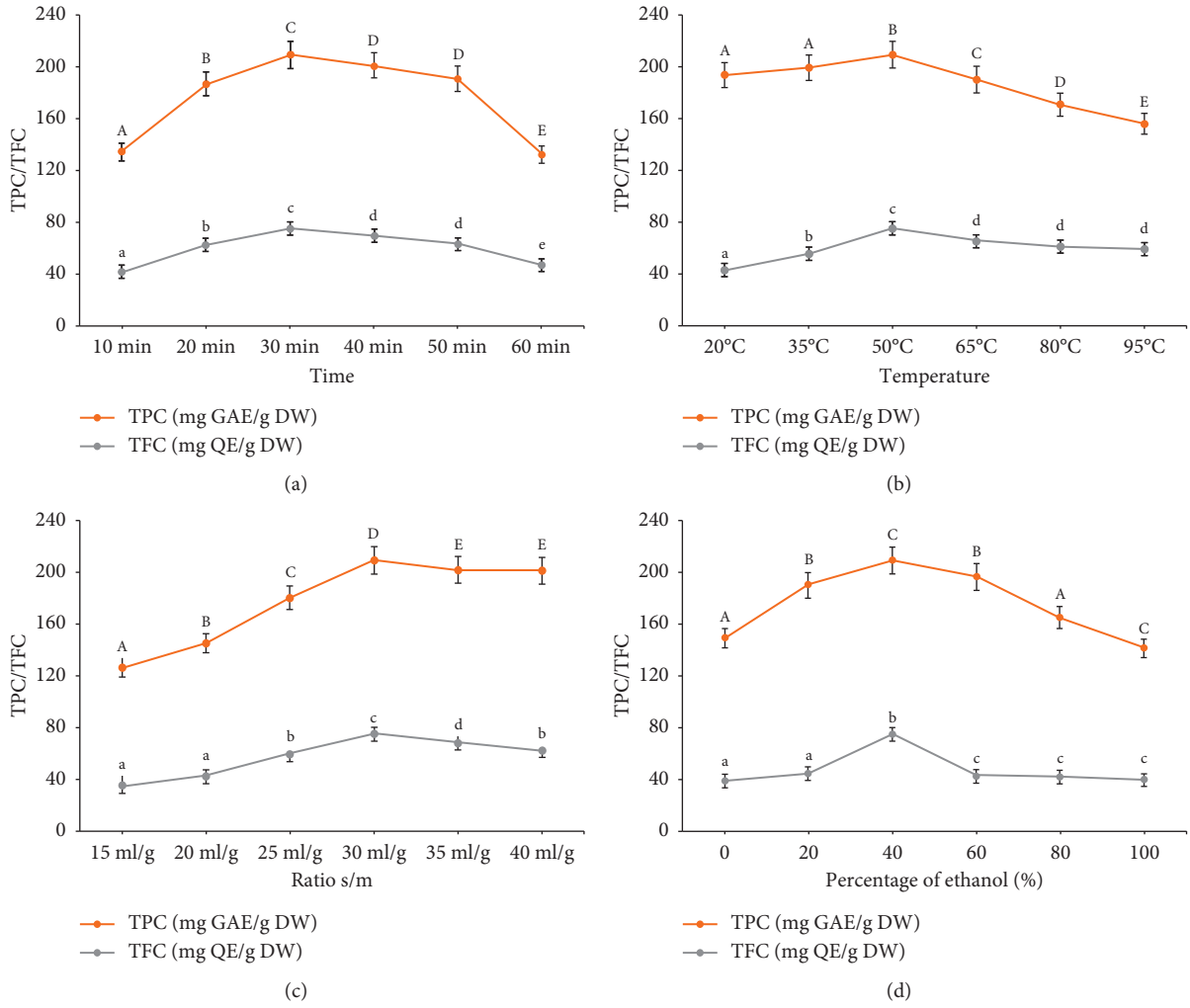


Figure 2: Effect of extraction time (a), temperature (b), ratio s/m (c), and ethanol (%) (d) on total phenol and flavonoid contents from *Eucalyptus marginata* L. leaves using the ultrasound-assisted extraction method.

Table 3: The responses in TPC and TFC for the two extraction methods.

$$Y(\mathrm{TPC})_{ME} = 150.789 + 7.180X_1 + 1.056X_2 + 2.948X_3 + 2.769X_4 - 11.638X_2^1 - 19.335X_2^2 - 12.9X_3^2 - 20.815X_4^2 - 0.335X_1X_2 - 0.083X_1X_3 - 2.124X_2X_3 + 0.581X_1X_4 - 0.991X_2X_4 + 0.061X_3X_4.$$

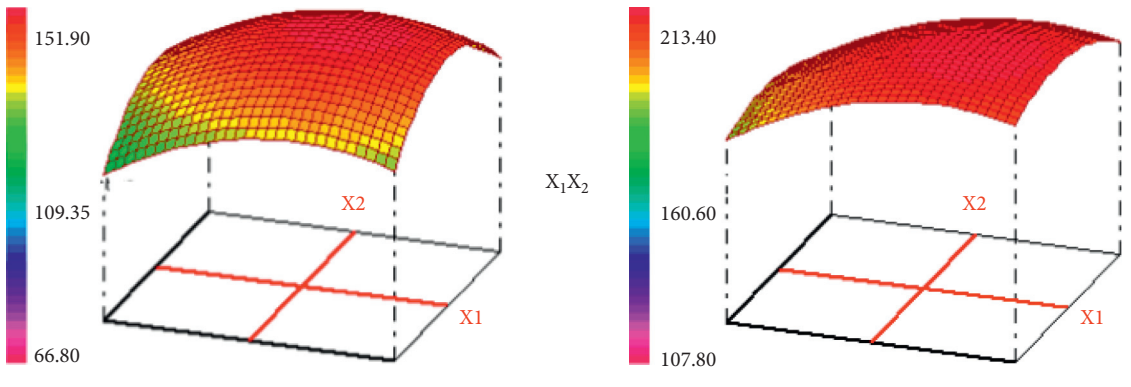
$$Y(\mathrm{TFC})_{ME} = 50.457 + 0.690X_1 - 0.506X_2 - 1.349X_3 + 3.938X_4 - 1.502X_1^2 - 4.098X_2^2 - 4.176X_3^2 - 7.014X_4^2 - 0.190X_1X_2 + 0.335X_1X_3 - 0.001X_2X_3 + 0.371X_1X_4 - 0.335X_2X_4 - 0.350X_3X_4.$$

$$Y(\mathrm{TPC})_{\mathrm{UAE}} = 209.729 + 12.39X_1 - 6.036X_2 + 20.175X_3 + 10.762X_4 - 13.156X_1^2 - 11.804X_2^2 - 18.185X_3^2 - 8.463X_4^2 + 0.663X_1X_2 - 0.368X_1X_3 + 0.372X_2X_3 + 0.354X_1X_4 + 0.402X_2X_4 - 0.917X_3X_4.$$

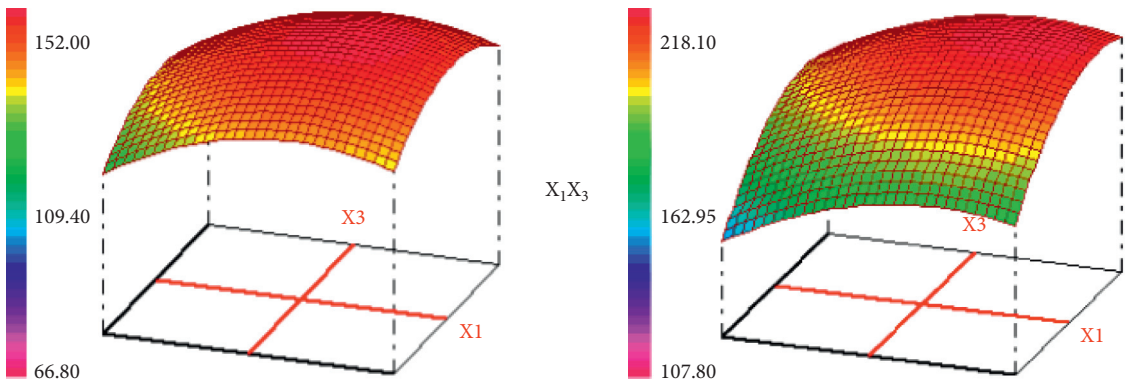
$$Y(\mathrm{TFC})_{\mathrm{UAE}} = 70.521 + 0.973X_1 - 2.142X_2 + 6.232X_3 + 2.786X_4 - 10.584X_1^2 - 4.328X_2^2 - 2.392X_3^2 + 0.244X_4^2 - 0.172X_1X_2 + 1.237X_2X_3 + 0.469X_1X_4 - 2.591X_2X_4 - 0.196X_3X_4.$$

TPC, total phenol content expressed in mg GAE/g DW; TFC, total flavonoid content expressed in mg QE/g DW; ME, maceration extraction; UAM, ultrasound-assisted extraction; X_1, extraction time; X_2, temperature; X_3 solvent-solid ratio; X_4, ethanol concentration.

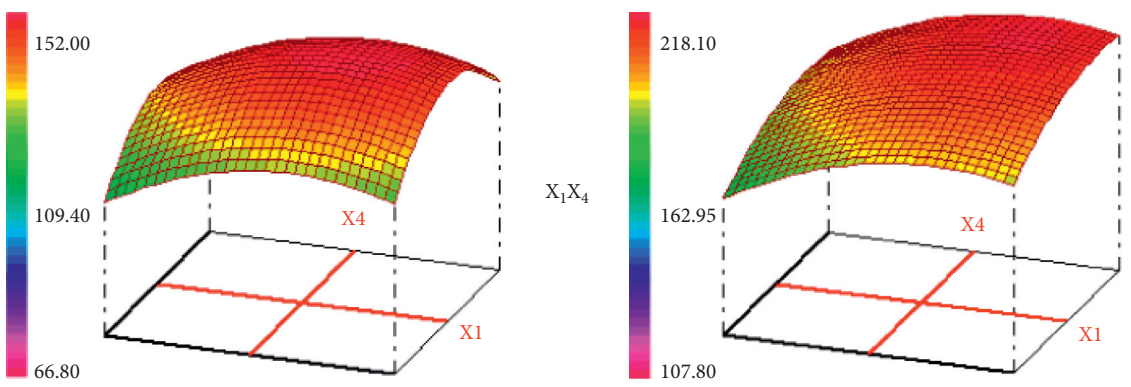
phenolic acids such as quinic, gallic, protocatechuic, *p*-coumaric, salviolinic, *trans*-ferulic, and *trans*-cinnamic acids and flavonoids such as hyperoside, rutin, quercetrin, naringin, quercetin, and naringenin. Retention times, pseudomolecular ions, and the concentration of each identified phenolic compound are given in Table 4. For example, compound 7 (t_R 21.742 min) was identified as quercetin aglycon that was assigned according to the presence of a main peak at m/*z* 609 as well as a strong peak at m/*z* 301 in its ESI-mass spectrum at the negative mode. The compound 7 MS^2 mass spectrum's showed fragments at m/*z* 463 and 301, which could be attribute to loss of rhamnosyl and glucosyl moieties, respectively. These results confirmed the presence of a rutin [7]. Additionally, the mass spectrum of compound 12 showed a peak at m/*z* 301 whose spectrum of MS^2 fragmentation indicated various ionic species: 273, 257, 193,



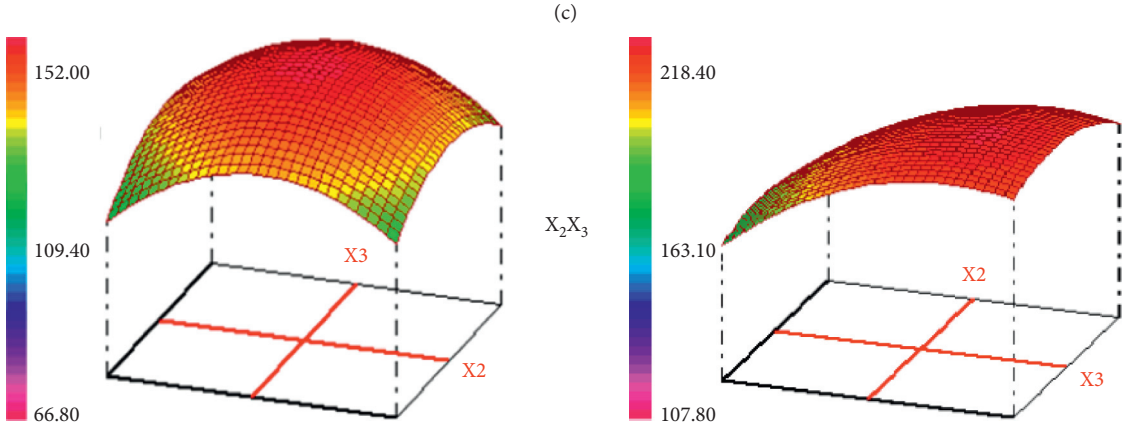
(a)



(b)



(c)



(d)

Figure 3: Continued.

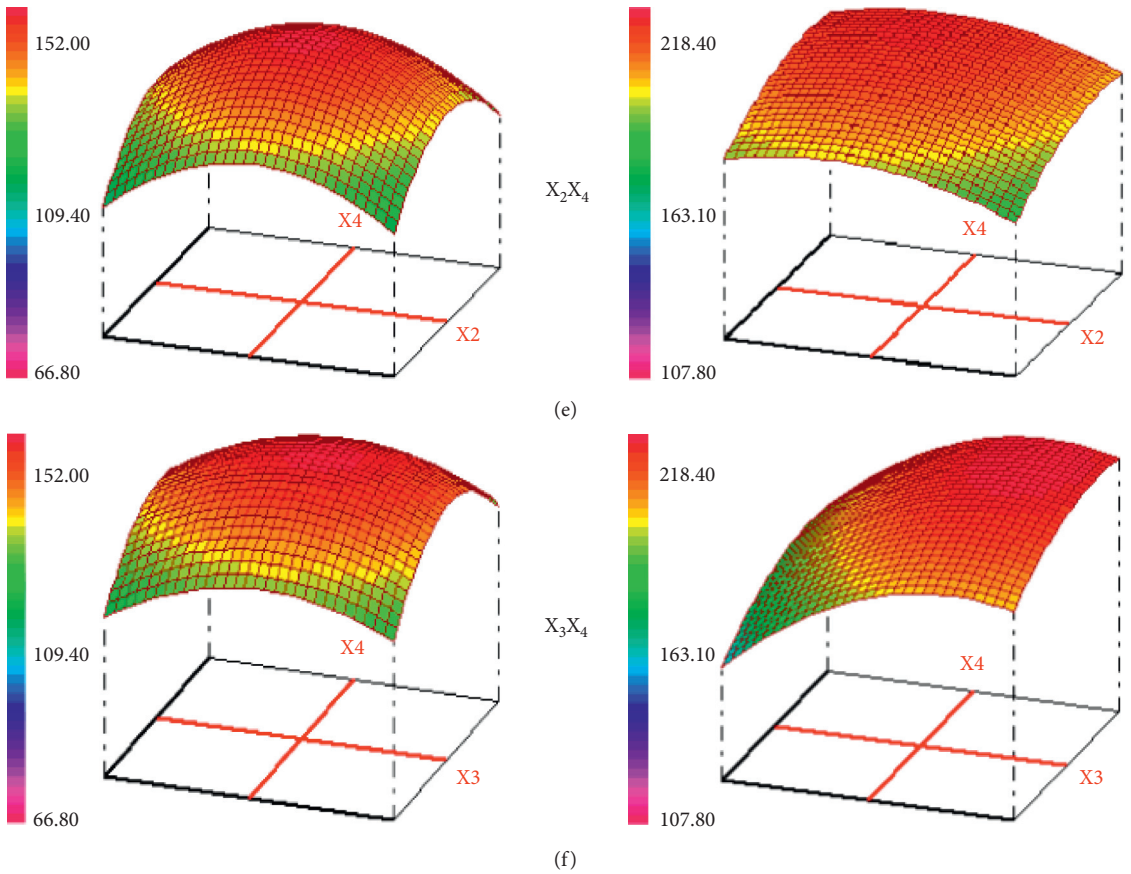


Figure 3: Response surface plot of TPC (mg GAE g-1 DW) of *Eucalyptus marginata* leaves extracts as a function of time, temperature, ratio s/m, and ethanol concentration obtained by maceration and ultrasound-assisted extraction methods. The extraction time (min, X_1), temperature (°C, X_2), liquid/solid ratio (ml/g, X_3), and ethanol concentration (%, X_4).

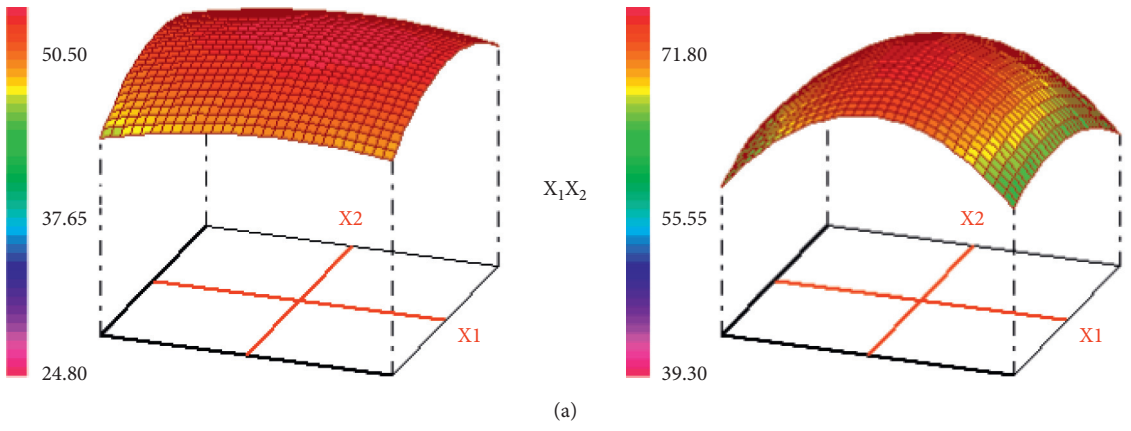
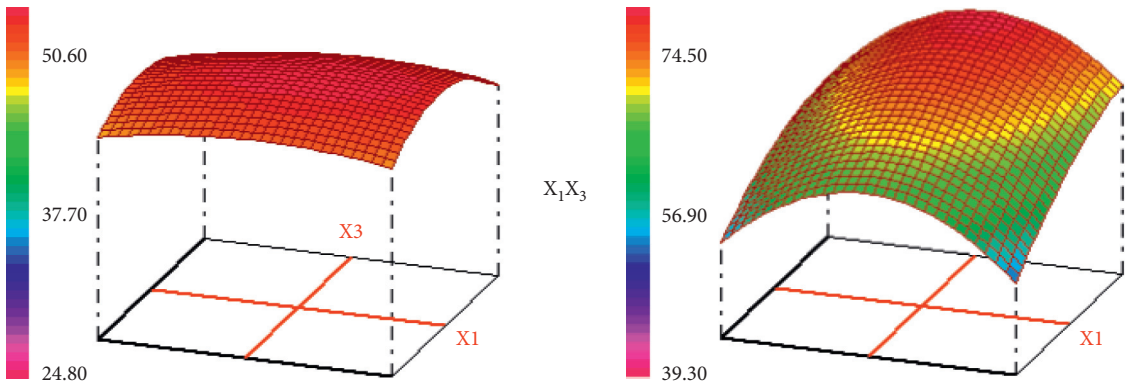
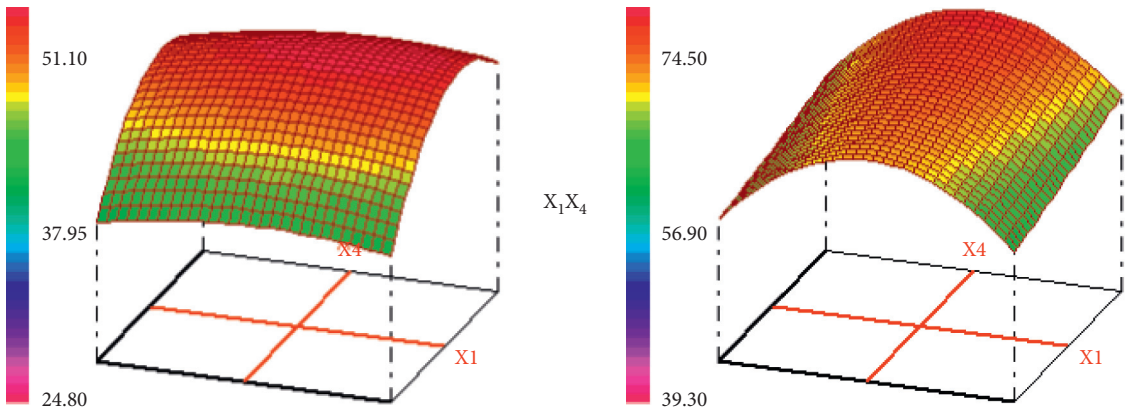


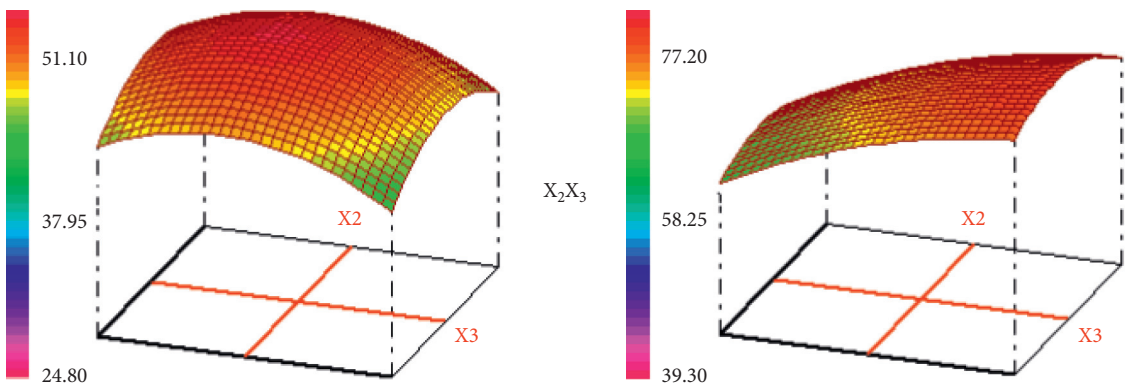
Figure 4: Continued.



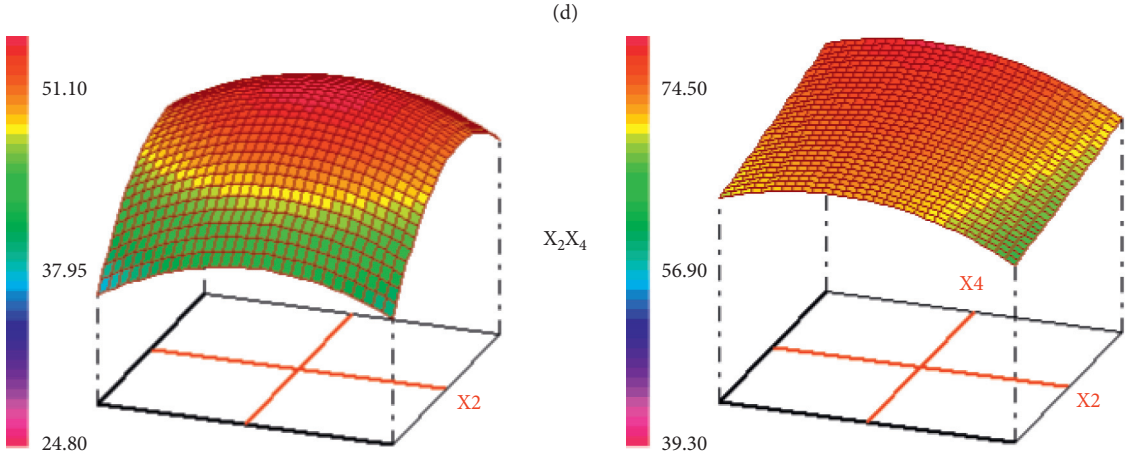
(b)



(c)

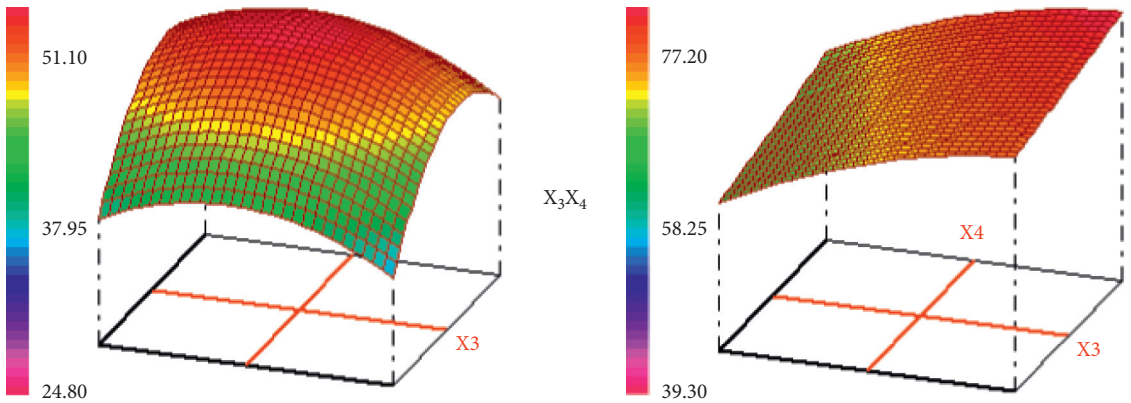


(d)



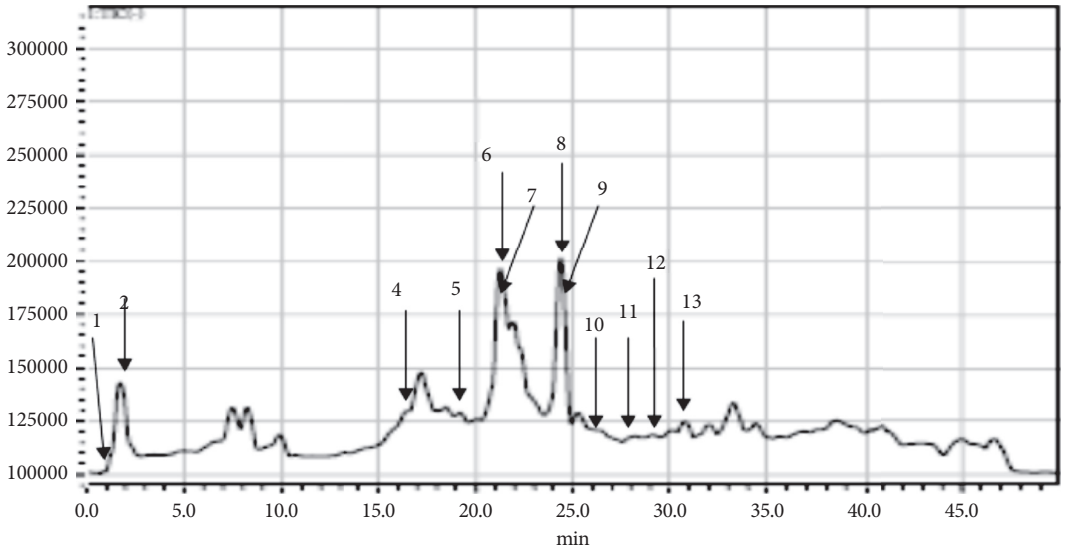
(e)

Figure 4: Continued.

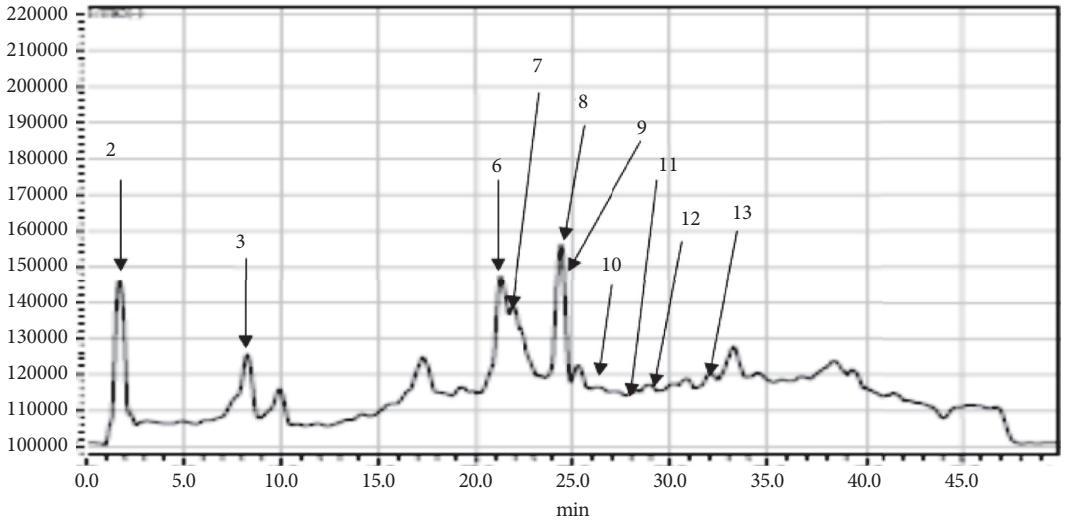


(f)

Figure 4: Response surface plot of TFC (mg QE g-1 DW) of *Eucalyptus marginata* leaves extracts as a function of time, temperature, ratio s/m, and ethanol concentration obtained by maceration and ultrasound-assisted extraction methods. The extraction time (min, X_1), temperature (°C, X_2), liquid/solid ratio (ml/g, X_3), and ethanol concentration (%, X_4).



(a)



(b)

Figure 5: HPLC chromatograms of the phenolic compounds of the extracts obtained by maceration (a) and ultrasound (b) extraction methods. 1, quinic acid; 2, gallic acid; 3, protocatechuic acid; 4, *p*-coumaric acid; 5, *trans*-ferulic acid; 6, hyperoside; 7, rutin; 8, quercetrin; 9, naringin; 10, salviolinic acid; 11, *trans*-cinnamic acid; 12, quercetin; 13, naringenin.

Table 4: Phenolic compounds detected in *Eucalyptus marginata* L. leaves optimum extraction condition extracts.

N	Compounds	Formula	Molecular mass	$[M-H]^-$ m/*z*	Retention time (min)	Maceration (μg/g DW) *	Ultrasound (μg/g DW) *
1	Quinic acid	$C_7H_{12}O_6$	192	191	1.750	1.65 ± 0.02^a	ND
2	Gallic acid	$C_7H_6O_5$	170	169	2.627	12.17 ± 0.06^a	27.77 ± 0.06^b
3	Protocatechuic acid	$C_7H_6O_4$	154	153	8.617	ND	37.66 ± 0.04^b
4	*p*-Coumaric acid	$C_9H_8O_3$	164	163	16.217	4.67 ± 0.06^a	ND
5	*trans*-Ferulic acid	$C_{10}H_{10}O_4$	194	193	19.150	0.12 ± 0.03^a	ND
6	Hyperoside (quercetin-3-*O*-galactoside)	$C_{21}H_{20}O_{12}$	464	463	20.744	96.47 ± 0.08^a	39.19 ± 0.03^b
7	Rutin (quercetin-3-*O*-rutinoside)	$C_{27}H_{30}O_{16}$	610	609	21.742	0.23 ± 0.06^a	0.08 ± 0.09^b
8	Quercetrin (quercetin-3-*O*-rhamnoside)	$C_{21}H_{20}O_{11}$	448	447	24.147	181.10 ± 0.05^a	150.78 ± 0.02^b
9	Naringin (naringenin-7-*O*-neohesperidoside)	$C_{27}H_{32}O_{14}$	580	579	24.246	19.00 ± 0.05^a	8.80 ± 0.07^b
10	Salviolinic acid	$C_7H_6O_3$	138	137	26.643	0.74 ± 0.04^a	0.75 ± 0.05^a
11	*trans*-Cinnamic acid	$C_9H_8O_2$	148	147	28.794	7.11 ± 0.07^a	21.99 ± 0.01^b
12	Quercetin	$C_{15}H_{10}O_7$	302	301	29.118	3.21 ± 0.01^a	2.06 ± 0.02^b
13	Naringenin	$C_{15}H_{12}O_5$	272	271	31.478	1.23 ± 0.09^a	1.03 ± 0.06^b

*Concentration expressed as μg/g of DW. ND, not detected; DW, dry weight. Results are expressed as mean ± standard deviation of three determinations. Means with different letters in the same line were significantly different at $p < 0.05$.

and 121. The main one fragment caused a loss of 28 Da, giving rise to a fragment ion at m/*z* 273. The fragment ion at m/*z* 257 was also obtained by the loss a CO_2 molecule. While, the ions at m/*z* 193 and 121 were obtained, respectively, by the elimination of $-C_6H_4O_2$ and $C_7H_4O_2$ from the ion to m/*z* 301. These results are consistent with the presence of quercetin, as described above, previously by Rigane et al. [37]. On the other hand, compound 13 was identified as naringenin, according to the $[M-H]^-$ ion at m/*z* 271 as well as the ions produced in the MS^2 spectrum at m/*z* 227, 177, 151, 119, and 107, which are in agreement with the fragments obtained for its standard [40].

The quantification of the identified phenolic compounds given in Table 4 showed a significant difference between the two extraction methods ($p < 0.05$). Therefore, our research team showed that the main phenolic acid found in the *Eucalyptus marginata* L. obtained by ultrasound-assisted extraction was protocatechuic acid which was present with 37.664 μg/g DW followed by gallic acid (27.77 μg/g DW). On the other hand, gallic acid was the major phenolic acid present in the *Eucalyptus marginata* leaves obtained by maceration (12.17 μg/g DW). On the other hand, salviolinic acid was present in the two studied samples (~0.75 μg/g DW). In addition, from Table 4, we can conclude that quercetrin was the main flavonoid compound quantified in the two studied extracts (181.10 and 150.78 μg/g DW obtained, respectively, by maceration and ultrasound-assisted extraction), followed by hyperoside (96.47 and 39.19 μg/g DW obtained, respectively, by maceration and ultrasound-assisted extraction), naringin (19.00 and 8.80 μg/g DW obtained, respectively, by maceration and ultrasound-assisted extraction), and quercetin (3.21 and 2.06 μg/g DW obtained, respectively, by maceration and ultrasound-assisted extraction). On the other hand, naringenin and rutin were present in very low amounts: ~1.2 μg/g DW and < 0.2 μg/g DW obtained, respectively, by maceration and ultrasound-assisted extraction. According to Al-Sayed et al. [41], the main nonvolatile compounds identified in *Eucalyptus* were phenolic compounds that contribute significantly to the antioxidant activities of extracts. In general, several phenolic compounds such as gallic, protocatechuic, and ellagic acids as well as quercetin, quercetin glycoside, naringenin, catechin, epicatechin, rutin, quercitrin, apigenin, and myricetin have been isolated from *Eucalyptus* extracts [41, 42]. Moreover, Santos et al. [40] identified epicatechin, catechin, quercetin glucuronide, ellagic acid rhamnoside, ellagic acid, galloyl-bis-hexa-hydroxyphenyl (HHDP)-glucose, gallic acid, chlorogenic acid, and methyl ellagic acid pentose in *Eucalyptus grandis*, *Eucalyptus urograndis*, and *Eucalyptus maidenii* extracts. In addition, Santos and coworkers [40] identified and quantified the phenolic compounds present in *Eucalyptus grandis, Eucalyptus urograndis*, and *Eucalyptus maidenii* using the HPLC-MS technique. By comparing this study with those obtained by our research team, we can conclude that the phenolic composition present in the *Eucalyptus* species woods and leaves varied significantly. From these results, we concluded that this study could provide useful information for industry to produce the potentially bioactive compound extracted from *Eucalyptus marginata* L. leaves using optimum condition parameters.

4. Conclusion

To the best of our knowledge, this study successfully used RSM in order to optimize the extraction of total phenolic and total flavonoid from *Eucalyptus marginata* L. leaves using maceration and ultrasound-assisted extraction methods with regards to extraction time, temperature, liquid/solid ratio, and ethanol concentration. Therefore, the optimum maceration condition's should be as follows: $t = 88$ min, $T = 74.42°C$, liquid-to-solid ratio = 40 ml/g, and the percentage of ethanol was equal to 59.65%, while the best conditions for ultrasound were obtained as follows:

$t = 49.9$ min, $T = 74.9^{\circ}$C, ratio solvent-solid = 39.5 ml/g, and percentage of ethanol was 58.48%. The levels of phenolic compounds found by RSM were, respectively, ~150 mg GAE/g DW and ~210 mg GAE/g DW for maceration and ultrasound-assisted methods.

Thus, the proposed method meets the terms of green process definition, since it reduces process time, allows use of alternative solvents (aqueous ethanol) and renewable natural products, and ensures a safe and high-quality extract/product.

Data Availability

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

Additional Points

Practical applications: *Eucalyptus marginata* L. leaves are treated using the "green" technique by elaboration of an efficient alternative protocol in order to obtain a phenolic rich extract. Therefore, a response surface methodology was used as a new tool for optimization of ultrasound and maceration methods process parameters including extraction time (min), temperature (°C), liquid-to-solid ratio (ml/g), and ethanol concentration (%). The wide range of phenolic compounds discovered in *Eucalyptus marginata* provides abundant natural health-promoting agents for further applications in medicine and functional food.

Conflicts of Interest

The authors report that they have no conflicts of interest.

Authors' Contributions

Ghayth Rigane and Hanene Ghazghazi contributed equally to this work. Soumaya Hasni and Hajer Riguene performed practical experiences and wrote, followed, and checked the obtained results. Moufida A. Oueslati, Olfa Khedher and Amir Bouallegue performed some practical experiences, coordinated all the analyses, and calculated the results and statistics. Hanene Ghazghazi, Ghayth Rigane, and Ridha Ben Salem supervised the scientific study.

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