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Research Article

Reduced-Pressure Boiling Extraction of Oleuropein Coupled with Ultrasonication from Olive Leaves (*Olea europaea* L.)

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Oleuropein was extracted from *Frantoio* olive leaves using reduced-pressure boiling extraction coupled with ultrasound-assist (URPE). Four important factors, extraction temperature, ultrasonic power, ethanol concentration, and the ratio of solid to liquid, were selected to carry out the response surface methodology (RSM) for seeking optimal conditions on high oleuropein extraction yield at different levels. Box-Behnken design was employed to investigate the effects of the four factors on it. The results showed that the ratio of solid to liquid was the most significant factor of all on oleuropein yield. The optimal operation conditions were obtained as follows: ethanol concentration 75% (v/v), extraction temperature 53°C, ultrasonic power 600 W, and the ratio of solid to liquid 1 : 31. Under these optimal conditions, oleuropein extraction yield was 7.08%, which was close to the predicted value 7.121%. The scanning electron microscope (SEM) images of olive leaves after extraction were provided as well. It was seen that, compared with the untreated leaves, URPE could effectively break cells within the olive leaves.

1. Introduction

The olive trees (*Olea europaea*, Oleaceae) are widely distributed in China, Spain, Italy, Greece, and so forth [1]. The fruits of the trees could be squeezed into olive oil for edibility. It was considered as the main resource of dietary fat in Mediterranean countries and the most popular edible oil in the world [2]. However, full advantages of the leaves from the olive trees were not taken in the oil extraction process from olive fruit in most of oil mills. Olive leaves were burned or directly thrown away from the mills as environmentally detrimental waste products. In fact, it was discovered that olive leaves could be used as a folk remedy to treat fever and other diseases, such as malaria [3]. Beside olive oil, recent studies showed that many bioactive components such as oleuropein, hydroxytyrosol, verbascoside, rutin, and olive biophenols (OBPs), were found in oil leaves [4]. For OBPs indicated a few of pharmacology properties for healthy benefits, that is, antioxidant [5–10], anti-inflammatory [11], antimicrobial [12–16], antitumor [17], anticancer [18], protection of cardiovascular disease [9, 19], and antidiabetics [20]. Nowadays, bioactive components, that is, oleuropein, have also been seen in cosmetics and pharmaceutics field. The chemical structure of oleuropein was shown in Figure 1 [21, 22]. Moreover, it was observed that oleuropein content in olive leaves was greater than that in olive oil, and also the highest of all the different parts of the same olive tree, that is, olive fruit, bud and bark [23]. Therefore, it was very important for the researcher to find a highly effective and timely extraction method with the low cost in the processing of olive leaves.

It is known that, in the plant extraction field, conventional solvent extraction (CSE) was at a disadvantage of being time consuming, with a low extraction yield [24]. Ultrasonication

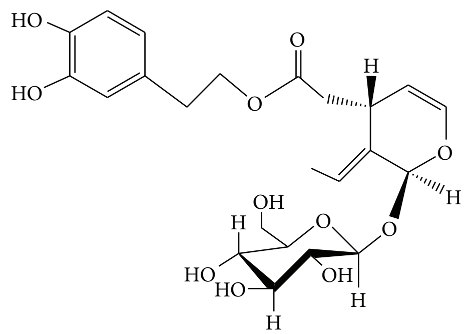


Figure 1: Chemical structure of oleuropein.

assisted extraction (UAE) could significantly improve the process due to oscillating and acoustic cavitation [25–27]. Additionally, reduced-pressure boiling extraction (RBE) was regarded as a relatively new extraction method, with process advantages of relatively low temperature requirements and high extraction effectiveness. Therefore, a combination of RBE and UAE (ultrasound-assisted extraction) was coupled for the extraction of oleuropein. To the best of our knowledge, ultrasound-assisted boiling extraction at reduced pressures (URPE) is being proposed for the first time and has not been investigated in the extraction of oleuropein from olive leaves.

In this paper, four important parameters, solvent, temperature, power, and the ratio of solid to liquid, were selected for optimization using response surface methodology (RSM) on the basis of a Box-Behnken design. In addition, visual evidence of the disruption of the cell way by URPE was provided by SEM images obtained by a scanning electron microscope.

2. Materials and Methods

2.1. Materials. Olive leaves (*Frantoio*) were purchased from Wudu County, Gansu, China, and dried in their natural condition without sunlight. They were milled (40–60 mesh) by high-speed drug crusher and stored in the dry and dark location at room temperature. All chemical reagents were purchased by Nanjing Chemical Reagents Co., Ltd., in China. A 1200 W, 20 kHz ultrasonic generator (model FS-1200N, Shanghai Sonxi Co., Ltd., China) assembled with a probe transducer and a flat tip of 13.0 mm was used in the URPE experiments.

The HPLC equipment of Shimadzu Prominence LC-20A (Japan) was purchased from Shanghai Shimadzu Co., Ltd., in China. The HPLC system included a LC-20AT Solvent Delivery Unit, LC-20AT pump, 0–10 mL/min, a SPD-20A UV-VIS Detector, CBM-20Alite System Controller, LCsolution Single, Ver1.24 Workstation, and a 7725i Manual Sample Injector (Position Sensing Switch Incorporated) with a 20 μL sample loop, Hamilton 702SNR (100 μL). SEM was used to view surface structure of samples (model S-3400N, Hitachi High Technologies Corporation, Japan).

2.2. Methods

2.2.1. Reduced-Pressure Boiling Extraction Oleuropein Coupled with Ultrasound-Assist. In URPE experiments, a standard ultrasonic probe with a flat tip of 13.0 mm in diameter was directly inserted into the mixture of olive leaves powder and extraction solvent and used as an ultrasonic source. The distance from flat top to flask bottom was controlled to about 3–5 mm. A small fraction of artificial zeolite was put into a three-mouth flask with the samples and solvent. The specimens were boiled and irradiated under a vacuum degree at 75 kPa and continuous ultrasonic waves for 3 min at a frequency of 20 kHz with different levels (0–1200 W) of output power. Meanwhile, the temperature was varied by the experiment and was controlled within ±1°C. The treatment of the extract on oleuropein content was detected by HPLC.

2.2.2. Extraction Yield Equation. Consider

$$\text{Yield (\%)} = \frac{W_o}{W_t} \times 100\%, \tag{1}$$

where W_o is the content of oleuropein obtained from one extraction trial and W_t is the weight of the total raw mass of olive leaves.

2.2.3. HPLC Analysis. The HPLC conditions were set on the basis of Xie's method for the determination of oleuropein [28]. Isocratic elution was implemented with methanol-water solution of 40 : 60 (v/v) at a flow rate of 1 mL/min and the runs could be achieved in 15 min under 232 nm detection. The retention time for oleuropein was about 11.5 min. The method was precise, sensitive, and reproducible. Calibration for oleuropein was determined using the range from 1.06 to 10.6 μg in six intervals; and the equation developed was linear and could be expressed by the following formula:

$$Y = 163365 + 1305700X \quad \left(R^2 = 0.9992\right), \tag{2}$$

where X is the amount of standard used in calibration experiments and Y is the relevant peak area in HPLC chromatographs. R^2 is very close to 1, which means that there is a linear relationship between Y and X, and the equation is adequate for quantitative analysis.

2.2.4. SEM Imaging. Samples particles treated by URBE were placed into a silicon wafer special in SEM equipment and subsequently analyzed with the microscope. The samples were first sputtered and then coated with a thin layer of conductive gold at a thickness of 50–100 nm. The shape and surface characteristics of the samples were observed and digitally recorded.

2.2.5. Trials of RSM. Optimization trials of extracting oleuropein from olive leaves were carried out using RSM, which is used as a method of optimization incorporating interaction effect between factors. For this purpose, a four-factor and three-level Box-Behnken design consisting of 29 experimental runs was employed including 5 center point replicates [29]. Four independent variables of the design were temperature (X_1, °C), ultrasonic power (X_2, W), ethanol concentration (X_3, % (v/v, ethanol/water)), and the ratio of solid to liquid (X_4, g/mL), and the response variable was the oleuropein extraction yield. In addition, the levels of the select factors

Table 1: Design of factors and levels coded table.

Independent variables	Units	Symbol	Coded levels		
			−1	0	1
Temperature	°C	X_1	40	50	60
Ultrasonic power	W	X_2	480	600	720
Ethanol concentration	% (v/v)	X_3	65	75	85
Ratio of solid to liquid	g/mL	X_4	1 : 20	1 : 30	1 : 40

Table 2: Design and results of the RSM.

Number	X_1 Extraction temperature	X_2 Ultrasonic power	X_3 Ethanol concentration	X_4 Ratio of solid to liquid	Yield/%
1	−1	−1	0	0	6.71
2	1	−1	0	0	6.77
3	−1	1	0	0	6.64
4	1	1	0	0	6.79
5	0	0	−1	−1	6.03
6	0	0	1	−1	5.95
7	0	0	−1	1	6.46
8	0	0	1	1	5.93
9	−1	0	0	−1	6.29
10	1	0	0	−1	6.39
11	−1	0	0	1	6.55
12	1	0	0	1	6.68
13	0	−1	−1	0	6.65
14	0	1	−1	0	6.81
15	0	−1	1	0	6.59
16	0	1	1	0	6.63
17	−1	0	−1	0	6.61
18	1	0	−1	0	6.59
19	−1	0	1	0	6.88
20	1	0	1	0	6.93
21	0	−1	0	−1	6.01
22	0	1	0	−1	5.91
23	0	−1	0	1	6.59
24	0	1	0	1	6.39
25	0	0	0	0	7.09
26	0	0	0	0	7.13
27	0	0	0	0	7.22
28	0	0	0	0	6.99
29	0	0	0	0	7.08

and the coding results were shown in Table 1. The design arrangement and the experimental results of the optimization design were displayed in Table 2. A second-order polynomial equation was used to fit the experimental data given in Table 2. The generalized model proposed for the response was given in the following equation:

$$\begin{aligned} Y_i = {} & \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 \\ & + \beta_{11} X_1{}^2 + \beta_{22} X_2{}^2 + \beta_{33} X_3{}^2 + \beta_{44} X_4{}^2 \\ & + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 \\ & + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4, \end{aligned} \tag{3}$$

where Y_i is considered as predicted response value, β_0 is the value of fitting response at the center point of the design, β_i's are the linear coefficients, $\beta_i{}^2$'s are the quadratic coefficients, and β_{ij}'s are the interaction coefficients, respectively. The

Table 3: The variance analysis and significance test table of regression coefficient of response surface design.

Source of variation	Degree of freedom	Sum of squares	Mean squares	F-test	P value
X_1	1	0.018	0.018	0.60	0.45
X_2	1	0.0019	0.0019	0.062	0.81
X_3	1	0.0048	0.0048	0.16	0.70
X_4	1	0.34	0.34	11.16	0.0049**
X_1X_2	1	0.0020	0.0020	0.066	0.80
X_1X_3	1	0.0012	0.0012	0.040	0.84
X_1X_4	1	0.0002	0.0002	0.0074	0.93
X_2X_3	1	0.0036	0.0036	0.12	0.74
X_2X_4	1	0.0025	0.0025	0.082	0.78
X_3X_4	1	0.051	0.051	1.66	0.22
$X_1{}^2$	1	0.026	0.026	0.85	0.37
$X_2{}^2$	1	0.34	0.34	11.32	0.0046**
$X_3{}^2$	1	0.52	0.52	17.21	0.0010**
$X_4{}^2$	1	2.69	2.69	88.36	<0.0001**
Model	14	3.41	0.24	7.99	0.0002**
Residual	14	0.43	0.03		
Lack of fit significant	10	0.40	5.72	0.053	0.0537
Pure error	4	0.028	0.0070		
Corrected total	28	3.83			

** Significant at 0.01.

computational software for this study was Design-Expert, version: 8.0.5.0, by Stat-Ease, Inc.

2.2.6. Verification of the Optimal Conditions. To check the validity of the model, the predicted value of the oleuropein yield was compared with the practical value under the optimal conditions, obtained by the second-order polynomial model of RSM.

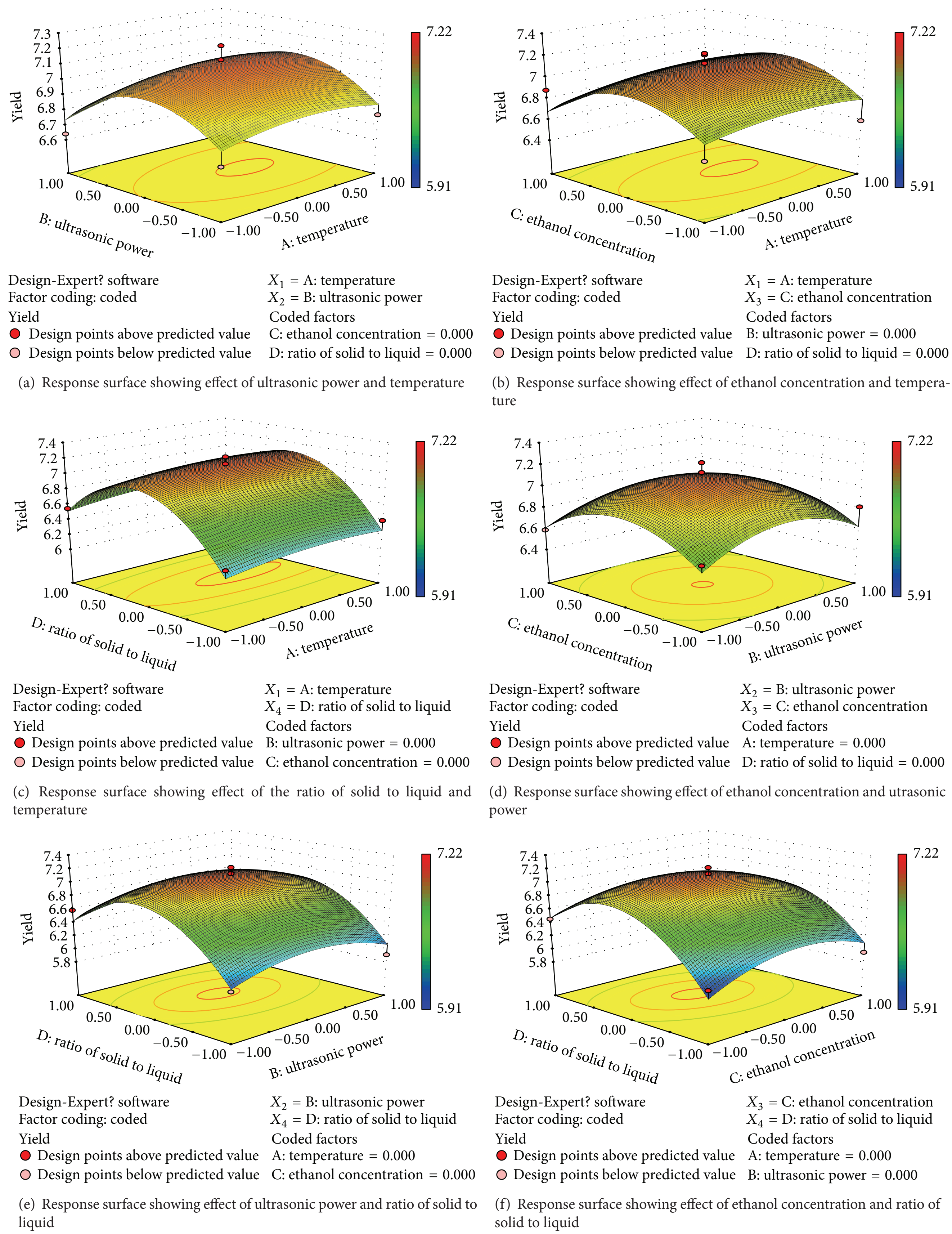
3. Results and Discussion

3.1. Design and Results of RSM Experiments. Four important factors, temperature, ultrasound power, ethanol concentration, and the ratio of solid to liquid, as well as the ranges of these four factors were selected to take the experiments of RSM to simulate a mathematic relationship about the four factors and oleuropein yield. The different variances of regression and significance were shown in Table 3. As it was shown in Table 3, X_4, $X_2{}^2$, $X_3{}^2$, and $X_4{}^2$ were significant at level $P_{0.01}$, and the $F_{0.05}$ value for lack of fit indicated that the lack of fit was not significant. The model itself was significant at level $P_{0.01}$, which implied that the model was dependable. Thus, the fitted model was appropriate for describing the response surface. According to Table 3, a second-order polynomial equation for extraction yield of oleuropein was obtained for the factors coded as shown in the following equation:

$$\begin{gathered} Y = 7.10 + 0.039 \quad X_1 - 0.012 \quad X_2 - 0.020 \\ X_3 + 0.17 \quad X_4 + 0.022 \quad X_1X_2 + 0.018X_1X_3 + 0.0075 \\ X_1X_4 - 0.03 \quad X_2X_3 - 0.025 \quad X_2X_4 - 0.11 \\ X_3X_4 - 0.063 \quad X_1{}^2 - 0.23 \quad X_2{}^2 - 0.28 \\ X_3{}^2 - 0.64 \quad X_4{}^2 \\ \left(R^2 = 0.89\right). \end{gathered} \tag{4}$$

By means of F-test and P value, as it was shown in Table 3, it was seen that the quadratic term of the ratio of solid to liquid ($X_4{}^2$) had the most significant effect on the extraction yield of oleuropein, followed by the quadratic term of ethanol concentration ($X_3{}^2$), the quadratic term of ultrasonic power ($X_2{}^2$), and the ratio of solid to liquid (X_4).

3.2. Analysis of RSM Experiments. As it was shown in Figure 2, six group graphs from the four factors were depicted by three-dimensional plots in order to illustrate the RSM problem. Two factors changed, while the other two were kept constant at zero, also called the central point. Figure 2(a) showed that the interactive effect between temperature and ultrasonic power was not significant and extraction yield increased when the temperature increased for all ultrasonic powers, while this law was not the same as ultrasonic power for all the temperatures. Optimal ultrasonic power 600 W could be the best of extraction yield for all the temperatures. Figure 2(b) displayed the interactive effects of temperature and ethanol concentration, and they also were not significant. When temperature was constant, extraction yield obtained 7.1% when ethanol concentration was 75%. The relationship of extraction temperature and the ratio of solid to liquid with extraction yield of oleuropein was shown in Figure 2(c). The results showed that extraction yield increased with the



(a) Response surface showing effect of ultrasonic power and temperature

(b) Response surface showing effect of ethanol concentration and temperature

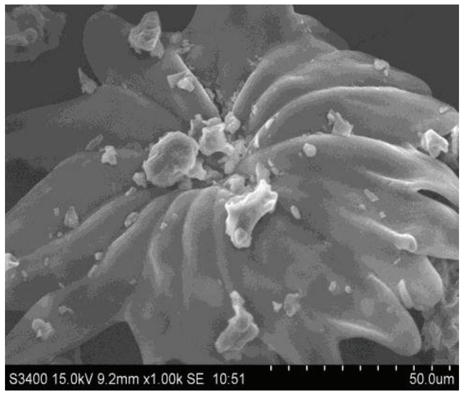
(c) Response surface showing effect of the ratio of solid to liquid and temperature

(d) Response surface showing effect of ethanol concentration and ultrasonic power

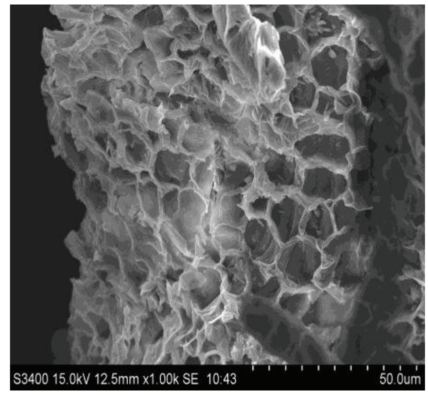
(e) Response surface showing effect of ultrasonic power and ratio of solid to liquid

(f) Response surface showing effect of ethanol concentration and ratio of solid to liquid

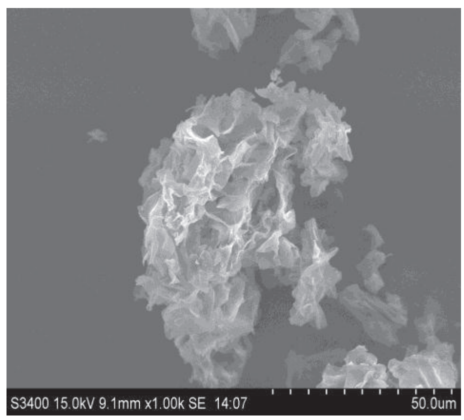
FIGURE 2: Response surface plot optimization of extraction yield between factors.



(a) Untreated samples



(b) Samples treated by URPE



(c) Samples treated by RBE

Figure 3: Displaying samples after being treated by URPE and RBE, compared with untreated ones.

increasing of the ratio up to 1 : 30, followed by decrease afterwards. From the graph it is also determined that the factor of the ratio of solid to liquid had a profound effect on the extraction yield. The interactive relationship between ultrasonic power and ethanol concentration was displayed in Figure 2(d). As the graph showed, extraction yield improved with the increase of ethanol concentration, followed by a slight decrease. The same situation was found with the ultrasonic power. Thus, ultrasonic power 600 W and 75% of ethanol concentration were optimal conditions for the extraction. Figure 2(e) showed the interactive relationship between ultrasonic power and the ratio of solid to liquid. The extraction yield almost formed a linear relationship with ultrasonic power and was not significant, while the yield displayed a quadratic relationship with the ratio of solid to liquid and was extremely significant. The relationship of ethanol concentration and the ratio of solid to liquid was shown in Figure 2(f). The effect of the ratio of solid to liquid was also significant as mentioned above and the ethanol concentration had an important influence on the extraction yield. From the graph, the optimal conditions were 75% ethanol concentration and a 1 : 30 solid to liquid ratio, respectively.

According to the calculation of software Design-Expert version 8.0.5.0, optimal conditions of extraction were obtained when temperature was at 53.1°C, ultrasonic power 599.8 W, ethanol concentration 74.5%, and the ratio of solid to liquid 1 : 31.4. Considering the practical operation, the final optimal conditions were revised to temperature: 53°C, ultrasonic power: 600 W, ethanol concentration: 75%, and the ratio of solid to liquid: 1 : 31.

3.3. Verification of the Results. Predicating optimal response values of the model for the suitability was tested by recommending the optimal conditions. The set of optimal conditions decided by RSM optimization method, together with the other two central points, extraction time 3 min, and vacuum degree 75 Kpa, were tested experimentally compared with the predicted value of extraction yield and with these optimal conditions, the predicted value of extraction yield of oleuropein was 7.121%, while the experimental value was 7.08% which was close to predicted value.

3.4. SEM of Olive Leaves after URPE. From the SEM, compared with Figure 3(a), the samples cell walls processed by URPE and RBE were broken, just as Figures 3(b) and 3(c) showed. The graphs obviously observed that the damage degree of the sample processed by URPE was stronger than that of RBE. One possible reason for the broken cell walls broken by URPE was that extraction solvent first infiltrated the cell wall and then produced boiling bubbles inside the cell when the outside conditions reached the boiling point of the solvent. The continuous bubbling caused a cracking of cell walls. Also, UAE could create this physical effect in the cell wall, when coupled with the other extraction methods, where the samples were broken by the ceaseless shock.

4. Conclusions

In this paper, four important factors (i.e., extraction temperature, ultrasonic power, ethanol concentration, and the ratio of solid to liquid) were selected to carry out experimental optimization using Box-Behnken design. Based on the F-test analysis and P value results of RSM, the quadratic term of the ratio of solid to liquid ($X_4{}^2$) had the largest effect on oleuropein extraction yield. The optimization conditions were determined as follows: extraction temperature 53°C, ultrasonic power 600 W, ethanol concentration 75%, and the ratio of solid to liquid 1 : 31. With these optimized conditions, the experimental value of oleuropein extraction yield (7.08%) was very close to the predicted value of 7.121%. A second-order polynomial equation for extraction yield of oleuropein was obtained by expounding the following equation:

$$\begin{gathered} Y = 7.10 + 0.039 \quad X_1 - 0.012 \quad X_2 - 0.020 \\ X_3 + 0.17 \quad X_4 + 0.022 \quad X_1X_2 + 0.018 \\ X_1X_3 + 0.0075 \quad X_1X_4 - 0.03 \quad X_2X_3 - 0.025 \\ X_2X_4 - 0.11 \quad X_3X_4 - 0.063 \quad X_1{}^2 - 0.23 \\ X_2{}^2 - 0.28 \quad X_3{}^2 - 0.64 \quad X_4{}^2 \\ \left(R^2 = 0.89\right) \end{gathered} \tag{5}$$

which fits the data well. In addition, the SEM images have provided excellent visual evidence of the effectiveness of URPE. Consequently, it is determined to be a feasiable, efficient, and promising extraction technology for the extraction of bioactive ingredients from natural plants.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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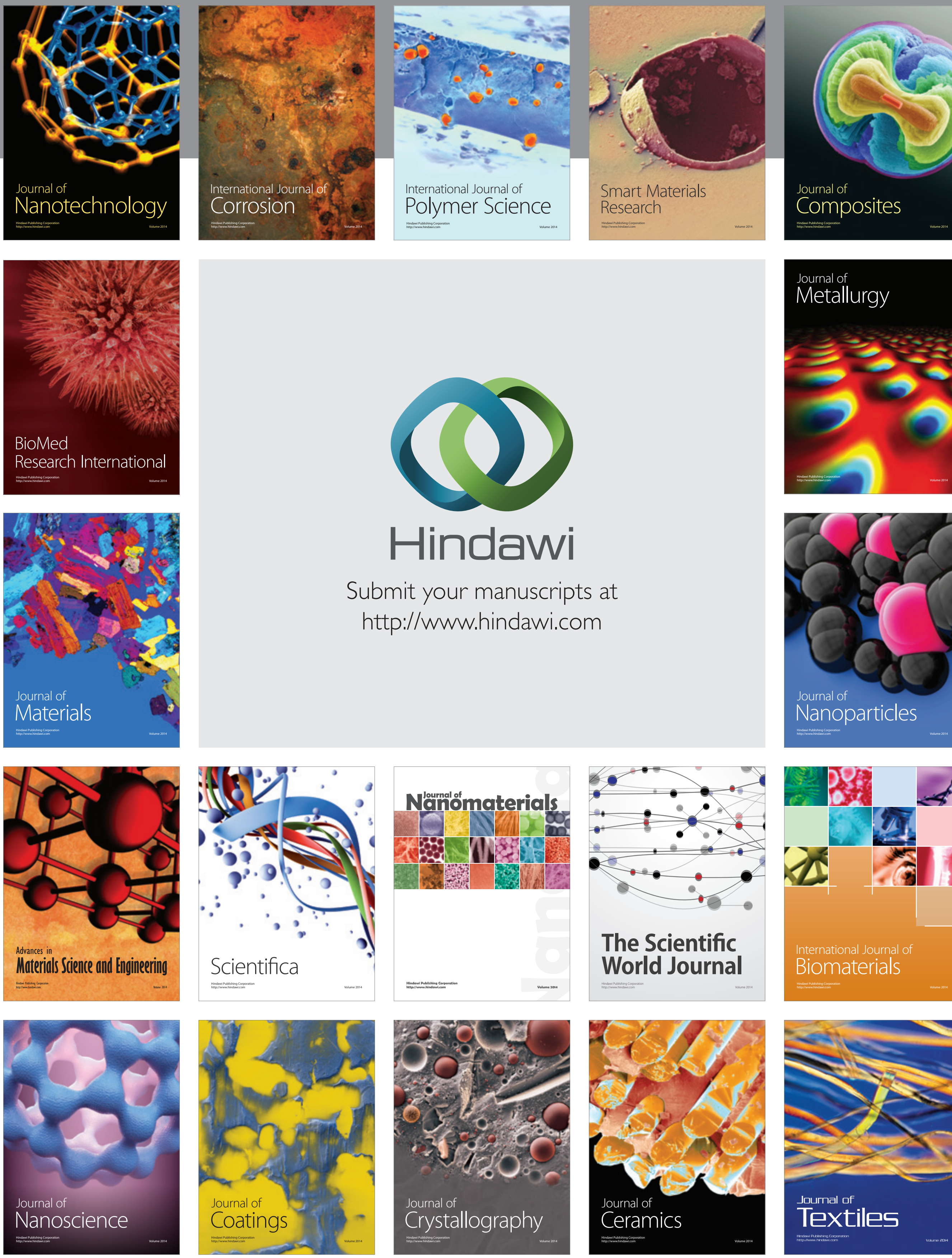
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