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Optimization protocol for the extraction of antioxidant components from *Origanum vulgare* leaves using response surface methodology

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Abstract In the present work, the response surface methodology (RSM) based on a central composite rotatable design (CCRD), was used to determine optimum conditions for the extraction of antioxidant compounds from *Origanum vulgare* leaves. Four process variables were evaluated at three levels (31 experimental designs): methanol (70%, 80%, and 90%), the solute:solvent ratio (1:5, 1:12.5, 1:20), the extraction time (4, 10, 16 h), and the solute particle size (20, 65, 110 micron). Using RSM, a quadratic polynomial equation was obtained by multiple regression analysis for predicting optimization of the extraction protocol. Analysis of variance (ANOVA) was applied and the significant effect of the factors and their interactions were tested at 95% confidence interval. The antioxidant extract (AE) yield was significantly influenced by solvent composition, solute to solvent ratio, and time. The maximum AE was obtained at methanol (70%), liquid solid ratio (20), time (16 h), and particle size (20 micron). Predicted values thus obtained were closer to the experimental value indicating suitability of the model. Run 25 (methanol:water 70:30; solute:solvent 1:20;

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extraction time 16 h and solute particle size 20) showed highest TP contents (18.75 mg/g of dry material, measured as gallic acid equivalents) and DPPH radical scavenging activity (IC_{50} 5.04 μg/mL). Results of the present study indicated good correlation between TP contents and DPPH radical scavenging activity. Results of the study indicated that phenolic compounds are powerful scavengers of free radical as demonstrated by a good correlation between TP contents and DPPH radical scavenging activity.

1. Introduction

The *Origanum* is one of the main genuses of the family *Lamiaceae* and comprises more than 38 species of annual, perennial and shrubby herbs, most of which are native to or restricted to the eastern part of the Mediterranean area, Europe, Asia and North Africa (Hussain et al., 2011). This genus includes some important culinary herbs and medicinal plants, including *Origanum vulgare* L. (common name 'oregano') (Esen et al., 2007). The use of the *O. vulgare* plant for the treatment of various diseases was in practice, being antiseptic and stimulant (Özcan and Chalchat, 2009). Besides their commercial importance, such plants have been used, for long, as condiments and spices for foods like salads, soups, sausages and meats (Hussain et al., 2011; Matsuura et al., 2003). The antioxidant and other biological properties of the *Origanum* extracts and essential oil have recently been of great interest in both academia and food industries because of their antioxidant and radical scavenging potentials (Hussain et al., 2011; Matsuura et al., 2003).

Quantity and quality of plant extracts are dependent on extraction protocol (Hussain et al., 2012; Sultana et al., 2009). Many factors including type of solvent, the temperature, the pH, the number of extraction steps, liquid-to-solid ratio and the particle size of the solute contribute to the efficacy of the extraction process (Mafart and Béliard, 1992). The extraction parameters selected must be ensured to complete extraction of the compounds of interest, and it must avoid their chemical modification (Wu et al., 2011). Extraction techniques are employed taking into account the chemistry and uneven distribution of phenolic antioxidants in the plant matrix. Depending upon the stability and nature of the phenolic compounds different extracting solvent/procedures are used for extraction purposes. Polar solvents like methanol, ethanol are frequently employed for the recovery of polyphenols from different plant matrices (Anwar et al., 2009a; Sultana et al., 2007). However, a single solvent may not be able to extract maximum phenolic compounds from all types of plant materials. Combinations of aqueous-organic solvents are more efficient in recovering antioxidants than corresponding pure solvents (Sultana et al., 2009).

In classical optimization experiments only one factor is variable at a time and this method is called as one-factor-at-a-time approach. This technique is tedious, expensive, consuming and failed to elaborate the interaction effects between variables. Response surface methodology (RSM) is a useful method to evaluate the effects of multiple factors and their interactions on one or more response variables. RSM can effectively be used to find a combination of factor levels that produce an optimum response. One of the main advantages of this method is that it generally requires fewer experimental runs than what is needed in traditional full factorial designs, while providing statistically acceptable results (Silva et al., 2007). It has been used for optimization of various food processing methods such as milling, extraction, and fermentation (Baş and Boyacı, 2007; Quanhong and Caili, 2005).

The objective of this study was to use the RSM approach to optimize the conditions (solvent concentration, solute:solvent ratio, extraction time and solute particle size) for the extraction of antioxidant compounds from *O. vulgare* leaves and to measure total phenolic contents and free radical scavenging capacity of obtained extracts.

2. Materials and methods

2.1. Collection of sample

Leaves of the *Oreganum vulgare* (*O. vulgare*) plant were collected from the Gattwala Forest Park, Faisalabad, Pakistan. The specimens were further identified and authenticated from Dr. Muhammad Naeem, Department of Botany, Government College University Faisalabad (Voucher No. 24953, University of Agriculture Faisalabad, Pakistan). Samples were dried at room temperature and ground into a semi powder using (LG BL 999SP) and finally passed from the vibratory sieve shaker (Octagon sieve (OCT-DIGITAL 4527-01) to gain different mesh sizes (20, 65 and 110 micron). These samples were stored in air tight polythene bags at 4 °C for the extraction process.

2.2. Chemicals and reagents

Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid and Folin–Ciocalteu reagent were obtained from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals and solvents used in this study were purchased from Merck (Darmstadt, Germany), unless stated otherwise.

2.3. Optimization of parameters for extraction of bioactive components

A protocol for the extraction of bioactive components from *O. vulgare* leaves was established by response surface methodology (RSM) which was employed to determine the best combination of variables for optimum extraction yield and antioxidant activity. RSM was used to analyze the influence of four extraction process variables, namely, the methanol/water ratio, solute/solvent ratio, the extraction time, and the solute particle size, on the yield of antioxidant extracts.

Factorial levels were chosen by considering the requirements of the protocol. Methanol/water ratio, solvent/solute ratio, extraction time and solute particle size were varied in the ranges 90:10, 80:20, 70:30; 1:5, 1:12.5, 1:20 (g/mL); 4, 10, 16 h; 20, 65, 110 micron, respectively, according to previous work. The response variable used to build the model corresponded to the yield (Y) of *O. vulgare* extracts that was obtained in each experiment. The regression equation and analysis of variance (ANOVA) were obtained using MINITAB® 11.12 software. ANOVA was used to summarize the results obtained under all the experimental conditions. Confidence interval of 95% was set to test the significant effect of the factors and their interaction. The *F* statistic test was used to evaluate whether the regression model was adequate to describe the observed data. The percentage of variability of the optimization parameter was analyzed by *R* squared statistics. Additionally, the normal probability plots of the residuals and the plots of the residuals versus the predicted response were utilized to evaluate the adequacy of the model.

2.4. Preparation of antioxidant extracts

Antioxidant extracts were prepared using an orbital shaker using all the combinations suggested by RSM. Ground samples were taken in 500 mL conical flasks followed by the addition of solvent and the flasks were put on an orbital shaker for optimum hours. Then extracts were separated from solids by filtering through Whatman No. 1 filter paper. The extracts were concentrated under reduced pressure using a rotary evaporator. Viscous extracts were stored at −4 °C until testing and analyzed (Hussain et al., 2012).

2.5. Determination of total phenolic (TP) contents

Amounts of TP from all the isolated extracts were assessed using the Folin–Ciocalteu reagent, following the method reported earlier (Hussain et al., 2012). Briefly, 50 mg of crude extract was mixed with 0.5 mL of Folin–Ciocalteu reagent and 7.5 mL deionized water. The mixture was kept at room temperature for 10 min, and then 1.5 mL of 20% sodium carbonate (w/v) was added. The mixture was heated in a water bath at 40 °C for 20 min and then cooled in an ice bath. Absorbance was measured at 755 nm using a spectrophotometer (Bio Tek Instrument, Inc., VT, USA). Amounts of TP were calculated using a gallic acid calibration curve (0.195–3.125 mg/mL) (Fig. 1) and reported in mg/g of dry plant material, measured as gallic acid equivalent (GAE).

2.6. DPPH radical scavenging assay

2,2-Diphenyl-1-picrylhydrazyl radical (DPPH) assay was carried out to measure the free radical scavenging activity as described previously (Hussain et al., 2008). *O. vulgare* extract concentrations in methanol (1–100 μg/mL) were mixed with an equal volume of 90 μM methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read at 517 nm. Butylated hydroxyl toluene (BHT) and butylated hydroxyl anisol (BHA) were used as the positive control for comparison and 90 μM DPPH solution was taken as the blank. The percent scavenging was calculated by the following formula;

$$\text{Scavenging}\,(\%) = 100 \times (A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}})$$

where A_{blank} is the absorbance of the DPPH solution and A_{sample} is the absorbance of the extract solution. Extract concentration providing 50% scavenging (IC_{50}) was calculated from the graph-plotted inhibition percentage against extract concentration.

2.7. Statistical analysis

Three different samples of *O. vulgare were* analyzed individually in triplicate. Data were reported as mean of six determinations. Response surface methodology (RSM) was employed with a central composite rotatable design (CCRD) to investigate the effect of different solvent concentrations, time of extraction, solute to solvent ratio and particle mesh size. The experimental plan was designed and the results obtained were analyzed using Minitab 11.12 (Minitab Inc., State College, PA, USA) software to build and evaluate models and to plot the contours and three dimensional (3D) response surface curves.

3. Results and discussion

3.1. Optimization

Response surface methodology (RSM) is a mathematical and statistical technique which can be used to study and optimize multivariable systems by finding the true relationship between the response and the set of independent variables. In this study four operational variables (solvent percentage, liquid solid ratio, extraction time and particle size) were optimized for maximum antioxidant extract (AE) yield by using RSM. Table 1 shows the template of the central composite rotatable design (CCRD). Initially, influential factors like solvent concentration, particle size, liquid solid ratio and extraction time were investigated separately to determine the extraction yield. By using RSM the AE yield varied from 5.32 to 14.65 g/100 g (Table 3). The maximum AE yield was obtained at solvent (70%), liquid solid ratio (20), time (16 h), and particle size (20 micron).

Many reports are available in the previous literature regarding the Response Surface Methodology. The results highlighted the effects of different factors like solvent concentration, particle size, liquid solid ratio and extraction time on antioxidant component yields of some plant extracts. (Badwaik et al., 2012; Radojković et al., 2012; Mukherjee et al., 2012).

Table 2 shows the analysis of variance (ANOVA) results based at 95% confidence interval. Regression models were evaluated by using *F* statistic and lack of fit test however; *R*-squared statistics was analyzed for the percentage of variability of the optimization parameter. Normal probability plots of the residuals and the plots of the residuals versus fitted values were used for the adequacy of the model (Fig. 1a and b). To check the performance of the model, the predicted values were compared with the experimentally observed values (Table 1). Finally, surface plots were used to show how AE yield (g/100 g) relates to two factors based on a model equation.

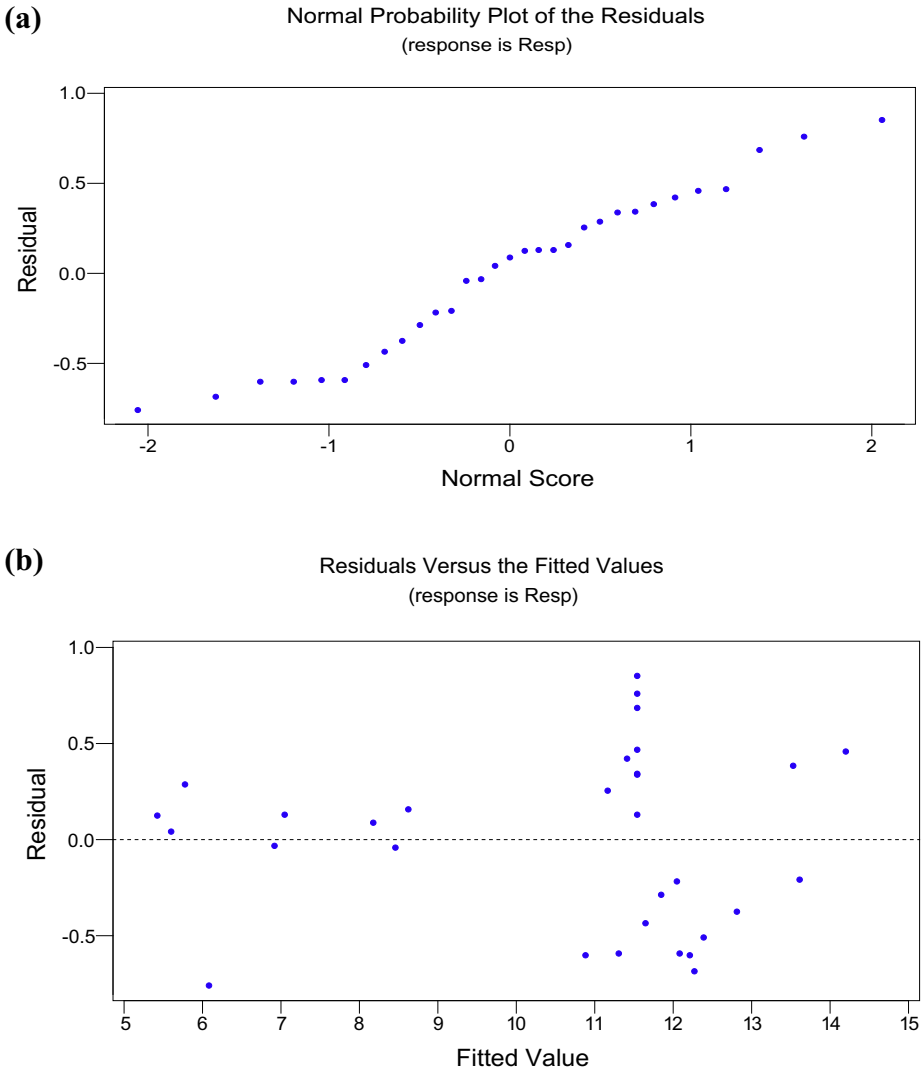

Figure 1 (a) Normal probability plot of the residuals for antioxidant extract yields. (b) Plot of residuals versus fitted values for antioxidant extract yields.

The linear and quadratic effect of the independent variables, their interaction and regression coefficients on the response variables were analyzed as shown in Table 2. The goodness of fit of the model to justify its robustness was evaluated by the coefficient of determination (R^2).

By using experimental data (Table 1), second degree polynomial model for the AE yield (g/100 g) was regressed and obtained by using actual values of the predictors, Methanol (A), Solute (B), Time (C), and Particle (D) (Fig. 2a–f)

$$\text{AE yield (g/100 g)} = 36.70 - 1.11A + 2.71B + 0.40C - 0.07D + 0.01A^2 - 0.05B^2 - 0.01C^2 - 0.01AB - 0.01BC.$$

3.2. Antioxidant activity

3.2.1. Total phenolics (TP) contents

Data regarding the effect of extraction parameters on the amount of total phenolic contents are presented (Table 3). The amount of TP, extracted from *O. vulgare* ranged from 5.76 to 18.75 mg/g of dry plant material, measured as gallic acid equivalent (GAE). Run 25 (methanol:water 70:30; solute:-solvent 1:20; extraction time 16 h and solute particle size 20) showed highest TP contents while run 31 (methanol:water 80:20; solute:solvent 1:5; extraction time 10 h and solute particle size 65) showed the lowest TP contents. The effects of extraction parameters exercised significant ($P \leqslant 0.05$) effects on the extraction of phenolic compounds from *O. vulgare* leaves. The above results show that the higher TP contents can be extracted from plant materials with solvents having higher polarity. It is considered that the phenolic compounds contribute to overall antioxidant activities of extracts (Sultana et al., 2007). Several studies showed excellent linear correlation between the "total phenolic content" and antioxidant activity (Anwar et al., 2009b; Hussain et al., 2012; Shahidi and Wanasundara, 1992). Methanol is efficient and most widely used to prepare the phenolic/antioxidant extracts due to the fact that the methanol–water mixture has a high dielectric constant/polarity and can be categorized as

Table 1 Experimental design for the extraction of antioxidants from *Origanum vulgare* leaves using response surface analysis.

Run	Process variables			
	Ratio Methanol:Water	Ratio (g:mL) Solute:Solvent	Extraction time (hours)	Solute particle size (micron)
1	80:20	1:12.5	10	110
2	70:30	1:5	16	110
3	70:30	1:20	16	110
4	90:10	1:5	16	110
5	90:10	1:20	16	110
6	70:30	1:5	4	110
7	90:10	1:12.5	10	65
8	90:10	1:20	4	20
9	80:20	1:12.5	10	65
10	80:20	1:12.5	10	65
11	80:20	1:12.5	4	65
12	70:30	1:12.5	10	65
13	80:20	1:12.5	10	65
14	80:20	1:12.5	10	65
15	90:10	1:5	16	20
16	80:20	1:12.5	10	20
17	90:10	1:5	4	110
18	70:30	1:20	4	20
19	80:20	1:12.5	10	65
20	80:20	1:12.5	16	65
21	70:30	1:5	16	20
22	90:10	1:20	4	110
23	70:30	1:20	4	110
24	80:20	1:20	10	65
25	70:30	1:20	16	20
26	90:10	1:5	4	20
27	80:20	1:12.5	10	65
28	90:10	1:20	16	20
29	80:20	1:12.5	10	65
30	70:30	1:5	4	20
31	80:20	1:5	10	65

Table 2 Analysis of variance for response surface quadratic model.

Source	Degree of freedom	Sum of squares	Mean square	*F*	*P*-Value
Regression	14	194.74	13.91	35.79	0
Linear	4	140.83	35.21	90.57	0
Square	4	33.85	8.46	21.77	0
Interaction	6	20.06	3.34	8.60	0
Residual Error	16	6.22	0.39		
Lack of fit	10	5.80	0.58	8.38	0.009
Pure error	6	0.42	0.69		
Total	30	200.96			

CV = 0.62%, R^2 = 0.97, R^2_{Adj} = 0.94, Predicted R^2 = 0.89.

the most suitable extracting solvent (Siddhuraju and Becker, 2003; Sultana et al., 2007). The extraction protocol for the preparation of antioxidant extracts has been found to be the key factor toward the antioxidant performance of extracts.

Table 3 Total phenolic contents and DPPH free radical scavenging activity of different *Origanum vulgare* extracts.

Run order	Extract yields (g/100 g)	Total phenolic contents (mg/g of dry plant material, measured as gallic acid equivalent)	DPPH scavenging IC_{50} (μg/mL)
1	11.49	16.31	5.57
2	6.07	7.43	12.12
3	12.43	14.16	7.69
4	8.41	11.20	9.40
5	11.84	14.84	7.28
6	5.64	5.94	13.17
7	11.64	11.82	9.02
8	11.43	15.47	6.94
9	11.89	13.63	8.88
10	11.88	15.46	6.97
11	10.28	13.23	8.98
12	11.59	14.95	7.48
13	12.40	14.72	7.22
14	12.31	14.11	7.26
15	8.79	11.93	9.29
16	11.61	13.00	8.41
17	8.27	9.96	10.01
18	13.41	14.23	7.68
19	12.23	13.30	8.67
20	10.72	13.20	8.04
21	6.88	8.01	10.73
22	11.83	13.99	8.70
23	13.92	16.67	5.67
24	11.22	14.43	7.48
25	14.65	18.75	5.04
26	7.18	7.96	11.15
27	11.68	14.25	7.12
28	11.55	13.63	8.56
29	12.02	15.51	6.17
30	5.55	7.86	10.93
31	5.32	5.76	15.00
Mean values	10.52	12.76	8.60
Median values	11.59	13.63	8.41
Maximum values	14.65	18.75	15.00
Minimum values	5.32	5.76	5.04
Correlation coefficient		−0.97	

3.2.2. DPPH radical scavenging activity

Free radical scavenging capacity of *O. vulgare* extracts increased in a concentration dependent manner (data not shown) and the extract concentration providing 50% scavenging (IC_{50}) is listed in Table 3. Overall all the *O. vulgare* extracts showed excellent antioxidant activity ranging from 5.04 to 15.00 μg/mL. *O. vulgare* extracts obtained with run 25 showed the best radical scavenging activity with the smallest IC_{50} value (5.04 μg/mL). However, run 31 showed a relatively weaker free radical scavenging activity (IC_{50} value 15.00 μg/mL). The mean IC_{50} value of all the prepared extracts was 8.41 μg/mL. The good correlation between total phenolic contents and the IC_{50} value was found with a correlation coefficient −0.97 (Fig. 3). Generally extract samples that contained more TP contents showed better radical

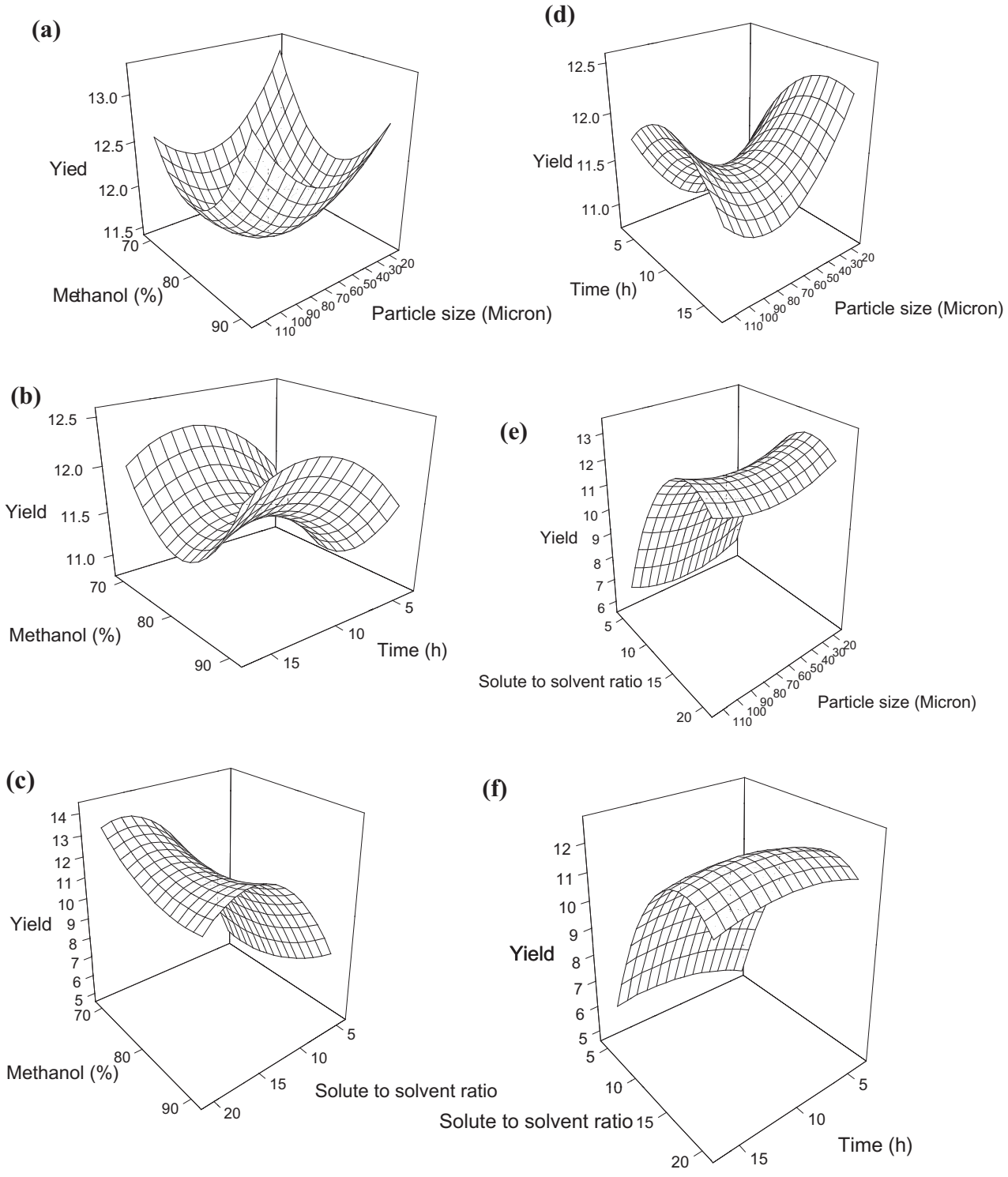

Figure 2 (a) Response surface plot showing the effect of methanol and particle size on antioxidant extract yields. (b) Response surface plot showing the effect of methanol and time on antioxidant extract yields. (c) Response surface plot showing the effect of methanol and solute to solvent ratio on antioxidant extract yields. (d) Response surface plot showing the effect of time and particle size on antioxidant extract yields. (e) Response surface plot showing the effect of solute to solvent ratio and particle size on antioxidant extract yields. (f) Response surface plot showing the effect of solute to solvent ratio and time on antioxidant extract yields.

scavenging activity (less IC_{50}). When IC_{50} values were plotted against TP contents, a linear regression curve was found with an R^2 value of 0.9325. The synthetic antioxidants, BHT and BHA showed good radical scavenging activity with IC_{50} values of 5.39 ± 0.26 and 5.35 ± 0.24 μg/mL, respectively (data not shown).

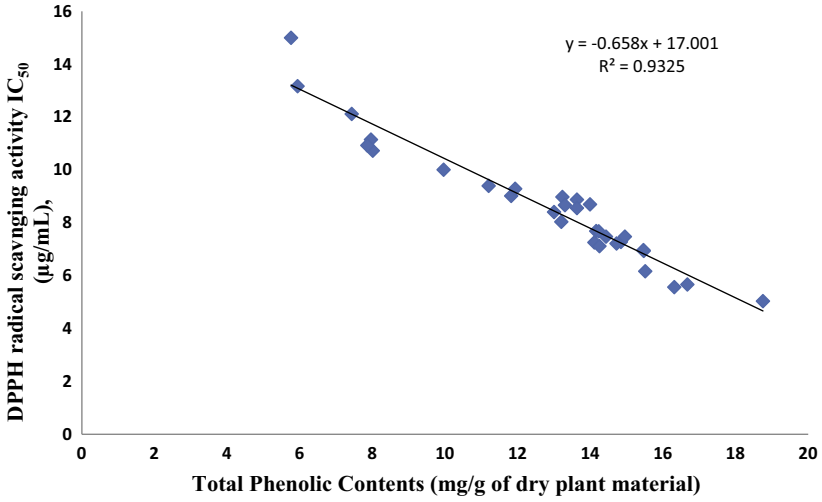

Figure 3 Linear regression line between total phenolic contents (TPC) and DPPH radical scavenging activity (IC_{50}).

The results of the present study indicated that phenolic compounds are powerful scavengers of free radicals as demonstrated by a good correlation between TPC and DPPH radical scavenging activity. Some reports in the literature depicted the strong correlation between TP contents and antioxidant activity. The antioxidant activity of essential oils and extracts may be attributed to the presence of phenolic compounds (Hussain et al., 2011; Sultana et al., 2007). There are some reports available in the recent literature on the antioxidant activity of *O. vulgare* essential oils (Hussain et al., 2011).

4. Conclusion

The response surface methodology was successfully employed to optimize the extraction of phenolic antioxidants from *O. vulgare* leaves. The second-order polynomial model gave a satisfactory description of the experimental data. An optimized condition for maximum extraction of antioxidant extracts was determined. Solvent ratio methanol, liquid solid ratio and time were the most important factors affecting extraction of phenolic compounds. Results of the present study indicated the good correlation between total phenolic contents and DPPH radical scavenging activity. This study can be useful in the development of industrial extraction processes, including further studies concerning the optimal number of sequential steps to enhance the efficacy of a large-scale extraction system.

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References

Anwar, F., Ali, M., Hussain, A.I., Shahid, M., 2009a. Antioxidant and antimicrobial activities of essential oil and extracts of fennel (*Foeniculum vulgare* Mill.) seeds from Pakistan. Flavour Frag. J. 24, 170–176.

Anwar, F., Hussain, A.I., Sherazi, S.T.H., Bhanger, M.I., 2009b. Changes in composition and antioxidant and antimicrobial activities of essential oil of fennel (*Foeniculum vulgare* Mill.) fruit at different stages of maturity. J. Herbs, Spices Med. Plants 15, 187–202.

Badwaik, L.S., Prasad, K., Deka, S.C., 2012. Optimization of extraction conditions by response surface methodology for preparing partially defatted peanut. Int. Food Res. J. 19, 34–46.

Baş, D., Boyacı, İ.H., 2007. Modeling and optimization I: usability of response surface methodology. J. Food Eng. 78, 836–845.

Esen, G., Azaz, A.D., Kurkcuoglu, M., Baser, K.H.C., Tinmaz, A., 2007. Essential oil and antimicrobial activity of wild and cultivated *Origanum vulgare* L. subsp. hirtum (Link) letswaart from the Marmara region, Turkey. Flavour Frag. J. 22, 371–376.

Hussain, A.I., Anwar, F., Hussain Sherazi, S.T., Przybylski, R., 2008. Chemical composition, antioxidant and antimicrobial activities of basil (*Ocimum basilicum*) essential oils depends on seasonal variations. Food Chem. 108, 986–995.

Hussain, A.I., Anwar, F., Rasheed, S., Nigam, P.S., Janneh, O., Sarker, S.D., 2011. Composition, antioxidant and chemotherapeutic properties of the essential oils from two *Origanum* species growing in Pakistan. Rev. Bras. Farmacogn. 21, 943–952.

Hussain, A.I., Chatha, S.A.S., Noor, S., Khan, Z.A., Arshad, M.U., Rathore, H.A., Sattar, M.Z., 2012. Effect of Extraction Techniques and Solvent Systems on the Extraction of Antioxidant Components from Peanut (*Arachis hypogaea* L.) Hulls. Food Anal. Methods 5, 890–896.

Mafart, P., Béliard, E., 1992. Génie Industriel Alimentaire, Tome II: Techniques Séparatives.

Matsuura, H., Chiji, H., Asakawa, C., Amano, M., Yoshihara, T., Mizutani, J., 2003. DPPH radical scavengers from dried leaves of oregano (*Origanum vulgare*). Biosci. Biotechnol. Biochem. 67, 2311–2316.

Mukherjee, S., Bandyopadhayay, B., Basak, B., Mandal, N., Dey, A., Mondal, B., 2012. An improved method of optimizing the extraction of polyphenol oxidase from potato (*Solanum uberosum* L.) peel. Not. Sci. Biol. 4, 98–107.

Özcan, M.M., Chalchat, J.C., 2009. Chemical composition and antimicrobial properties of the essential oil of *Origanum saccatum* L. J. Food Safety 29, 617–628.

Quanhong, L., Caili, F., 2005. Application of response surface methodology for extraction optimization of germinant pumpkin seeds protein. Food Chem. 92, 701–706.

Radojković, M., Zeković, Z., Jokićb, S., Vidović, S., 2012. Determination of optimal extraction parameters of mulberry leaves using Response Surface Methodology (RSM). Rom. Biotechnol. Lett. 17, 7295–7308.

Shahidi, F., Wanasundara, P.K., 1992. Phenolic antioxidants. Critic. Rev. Food Sci. Nutr. 32, 67–103.

Siddhuraju, P., Becker, K., 2003. Antioxidant properties of various solvent extracts of total phenolic constituents from three different agroclimatic origins of drumstick tree (*Moringa oleifera* Lam.) leaves. J. Agric. Food Chem. 51, 2144–2155.

Silva, E.M., Rogez, H., Larondelle, Y., 2007. Optimization of extraction of phenolics from *Inga edulis* leaves using response surface methodology. Sep. Purif. Technol. 55, 381–387.

Sultana, B., Anwar, F., Ashraf, M., 2009. Effect of extraction solvent/technique on the antioxidant activity of selected medicinal plant extracts. Molecules 14, 2167–2180.

Sultana, B., Anwar, F., Przybylski, R., 2007. Antioxidant activity of phenolic components present in barks of *Azadirachta indica, Terminalia arjuna, Acacia nilotica,* and *Eugenia jambolana* Lam. trees. Food Chem. 104, 1106–1114.

Wu, X., Yu, X., Jing, H., 2011. Optimization of phenolic antioxidant extraction from Wuweizi (*Schisandra chinensis*) pulp using Random-Centroid optimization methodology. Int. J. Mol. Sci. 12, 6255–6266.