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RESPONSE SURFACE METHODOLOGY FOR THE EXTRACTION OF POLYPHENOL CONTENTS AND HPLC PROFILING OF CUCUMIS SATIVUS PEELS

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Abstract:

Response surface methodology was used to optimize the extraction yield of total polyphenol contents from the Curcumis sativus peels. The experiment design software 13.01 was used for the optimization by selecting three independent design variables; solvent composition (40-100%), extraction temperature (21-54 ºC) and extraction time (17-43, minutes). The most suitable extraction conditions evaluated using mathematical model were at 70% methanol composition, 37º C extraction temperature and 30 minutes time. The extraction rate of TPC (Total Phenolic Content) was 22.055 at these optimal conditions. The profiling of methanolic peel extract at these optimized conditions was carried out by HPLC analysis. The compounds identified by HPLC included caffeic acid, gallic acid, protocatechuic acid, kampferol, chlorogenic acid and vannilic acid. **Abbreviations:** DPPH (2, 2-diphenyl-1-picrylhydrazyl), RSM (Response surface methodology), HPLC (High performance liquid chromatography), ROS (reactive oxygen species), ND (not detected).

Keywords: Cucumis sativus, Lipase inhibition, ROS, Antioxidant potential, HPLC, Glucosidase inhibition, RSM.

1. INTRODUCTION

Bioactive ingredients and compounds from various plants are commonly used as natural antioxidants in nutraceutical [1] and pharmaceutical products as well as in functional foods [2]. The efficacy of such natural products depends upon the quality, type and

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quantity of constituent components [3] which in turn solely a function of extraction process. The extraction of bio actives from plants is very important step in phytochemistry [4]. The extraction process is influenced by various factors such as temperature, solvent composition, extraction time, pH, particle size etc. [5]. Individual study of these factors is very tedious and time consuming. Therefore optimization of all these factors at one time remained an issue of key concern among scientists. This critical problem was resolved by a novel method known as response surface methodology which involved mathematical and statistical tools for optimizing independent factors affecting the extraction process in a given set of experiments at once [6]. The objective of RSM is to determine the best solvent composition and extraction conditions of independent factors (temperature, time, and solute to solvent ratio) to secure maximum amount of polyphenolic compounds. Recent advances in computation tools like RSM is being frequently used to optimize the extraction yield and polyphenol recovery [7].

In this regard we have selected Cucumis sativus L. is usually called melon, muskmelon, or melon. It has a place with the family Cucurbitaceae, known as the gourd family or by the more commonly name cucurbits. The genus Cucumis comprises of 32 species [8]. C sativus is a widely cultivated vine in Pakistan. The fruits of C. sativus are cylindrical in shape and commonly used as vegetables and in salad. C. sativus peels have many important polyphenolic compounds. These polyphenolic compounds possess strong antioxidant potential against cancer, cardiovascular diseases [9], bacterial [10] and viral diseases [11]. The RSM design was utilized to proceed for optimum extraction conditions for high polyphenol recovery and HPLC analysis was carried out for profiling the optimized methanolic extract.

2. Material and Methodology

2.1 Collection of plant material

C.Sativus fruits were collected from different markets in Lahore. Peels were removed from the fruit of C.Sativus and identified by department of Botany Govt.College University Lahore against voucher number Gc.Herb.Bot 2536.The peels of fruit were washed and dried in the shade for a week, then ground into a fine powder. The powder samples were freeze dried for further studies.

2.2 Chemicals

DPPH (Diphenyl Picryl Hydrazyl), Galli acid. Folin-phenol reagent, methanol,, washing soda (Na_2CO_3), Aluminum chloride ($AlCl_3$), Vannilic acid, kaempferol, catectechuic acid, ascorbic acid, querecitin, chlorogenic acid, a buffer solution tris-HCl and gum were utilized.

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2.3 Extract Preparation

The extract was prepared according to well-known reported method by mixing 10 g of peel powder with 100 mL of methanol of various composition in triplicate. The mixture was kept in the dark for 72 hours and sonicated under soniprep 150 for 30 minutes. The extra solvent was removed on a rotary evaporator under vacuum to get dried sample [12].

2.4 Experimental Design

The effect of three independent variables namely extraction temperature, extraction time and solvent composition on total polyphenolic contents (TPC) recovery were optimized using design expert (software 13.01 version), central composite design was selected to for optimization [13]. The levels of independent variables are given in Table 1 along with their codes.

Table 1: Levels of Independent Variables

Code	Variable	Minimum	Mid	Maximum
A	Extraction Time	17	30.00	43.00
B	Temperature	21	37	54
C	Solvent Composition	30	70.00	100

According to the method of central composite design, the methanol composition [14], extraction temperature and extraction time were selected as design variables and rate of TPC extraction as response value. The complete design is randomized and comprised of 17 runs with three replicates at a central point. A total of 17 experimental runs with three central points were augmented to estimate the effect of independent variables as expressed in table 2.

Table 2: The Plan and Results for RSM by Central Composite Design

		Factor 1	Factor 2	Factor 3	Response 1
Std	Run	A:Methanol composition	B:Extraction time	C:Extraction temperature	TPC extraction rate
		%	mint	Celsius	%
1	1	40	20	25	20.223
11	2	70	17	37	21.332
10	3	100	30	37	19.344
4	4	100	40	25	19.35
3	5	40	40	25	19.878
5	6	40	20	50	19.866
2	7	100	20	25	19.001
6	8	100	20	50	19.002
13	9	70	30	21	21.866
8	10	100	40	50	19.5
12	11	70	43	37	21.883
7	12	40	40	50	19.553
16	13	70	30	37	22.780
14	14	70	30	54	22.001
15	15	70	30	37	22.757
9	16	30	30	37	19
17	17	70	30	37	22.777

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The following second degree polynomial equation was used for the analysis of experimental data [15]. The model equation applied is given as

$$Y = \beta_0 \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i \neq i>1}^{n} \beta_{ij} X_i X_j \quad (1)$$

Y represents the response to be optimized (rate of TPC extraction), $\sum_{i=1}^{n} \beta_i X_i$ represents the linear effect, $\sum_{i=1}^{n} \beta_{ii} X_i^2$ represents quadratic effect, $\sum_{i \neq i>1}^{n} \beta_{ij}$ and $X_i X_j$ represents the interaction between different variables. The statistical significance of model, fitness of model were determined by F-test, t-test lack of fit test and multiple coefficient determination (R^2).

2.5 In vitro antioxidant assay

Spectrophotometric analysis was performed to evaluate the antioxidant activity of peel extracts for DPPH scavenging assays [11]. In this method color of DPPH reagent disappers in the presence of polyphonic compounds [12].The change in color was measured at 517 nm wavelength using spectrophotometer. 0.2mM solution of DPPH was prepared, 3 mL of this solution was mixed with 2 mL of methanolic extract of peels. The mixture was placed in dark for 30 minutes and absorbance of the sample was measure at 517 nm wavelength using BHT as reference standard. The antioxidant potential is measured as IC50 (µg/mL). The following expression was used to calculate the antioxidant potential

Scavenging activity% = (control absorbance-sample absorbance)/control absorbance.

2.6 Determination of Total Phenol Content

The total phenol content of peel extracts was determined by a well-known method [13] with some modifications.

A mixture 1 mL of each peel extract was mixed with 2 mL Folin-phenol reagent, and 2 .5 mL of 15% Na_2CO_3 solution. The obtained mixture was left for 80 minutes and then absorbance was measured at a wavelength of 750 nm [14]. The result of TPC is expressed as gallic acid equivalent per gram of dry weight (mgGAE/gmDWE).

2.7HPLC of polyphenolic contents

HPLC analysis for the identification and quantification [16] of polyphenolic contents was carried out with following HPLC system LC 20 AT series Shimadzu and compounds were detected using UV detector (Detector, SPD-20A) at 254 nm. The stationary phase used was GIST C18 and mobile phase was a mixture of methanol and water (80:20) phase. 10 mL of each extract was concenterated to 1 mL and then each sample was placed in GIST C18 column (diameter 4.6 mm length 250 mm, 5 u particle size). The separation of components was carried out by a reported method with minor modifications the solvent was pumped by LC-20AT pump at the rate of 1ml/mint with isocratic mode. The

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separation was conducted for 15 minutes and components were detected at 254 nm by UV detector [16].

3. RESULTS

The results of extract yields %, DPPH and TPC are given Table 3. The statistical comparison indicated that extract yield given by hydro methanolic solution (70%) was significantly higher than other solvent used in previous studies ($\rho<0.05$) [17]. The results of HPLC analysis are given in table.

Table 3: Extract Yields, DPPH and TPC

Extracting Solvent	TPC (mg GA/g PE)	DPPH Activity %
Methanol 70	21.82 ± 0.10	90.15 ± 0.12

Table 4: Compounds identified in the methanolic extract of C. sativus fruit peels

Sr No	Retention time	Compound identified	Concentration (µg/g)	Reference
1	2.458	Caffeic acid	0.298	Standard
2	2.683	ND		
3	2.2952	Protocatechuic acid	0.866	Standard
4	3.666	Gallic acid	0.7137	Standard
5	4.086	Kampferol	1.764	Standard
6	5.287	Chlorogenic acid	0.689	Standard
7	7.797	Vannilic acid	1.667	Standard

4. Discussions

The antioxidant potential of plants extract depends upon polyphenolic contents, these compounds have ability to scavange the free radicals by donating hydrogen atom to exihibit antioxidant potential. The methanol extract of C. sativus peels exhibit maximum antioxidant potential due the presence important polyphenolic compouds [18] as confirmed by HPLC analysis in current investigation

The model developed by using RSM methodology was significant $p<0.05$ All variables had significant effect on extraction rate of polyphenols. The solvent composition and time had linear and quadratic effect on extraction rate. The best optimized conditions evaluated were at methanol composition 70%, time 30 minutes and temperature 37.5º C. Under these conditions maximum rate of extraction was obtained as compared to convention extraction as revealed by previous studies [19].

The analysis of variance (ANOVA) results revealed quality and suitability of the model as expressed in table 5. The model F value 40.86 probability (p) < 0.0001 suggested the model is significant and there is only 0.01% chance that an F-value large occur due to noise. The factors A, B and A^2 have p values less than 0.05 indicating that solvent

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composition and time had significant effect on the rate of extraction of polyphenols. Similarly lack of fit value of value of 0.0980 suggested that model has good fit. High value of R^2 0.9813 suggested that selected polynomial expression predict well the extraction of TPC under experimental conditions. The adjusted R^2 value 0.9513 was close to R^2 value which proved the agreement between predicted and observed rate of polyphenol extraction.

4.1 ANOVA for Quadratic model

Response 1: TPC Extraction Rate

Table 5: ANOVA for Quadratic Model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	33.62	9	3.74	40.86	< 0.0001	significant
A-Methanol composition	0.0826	1	0.0826	4.67	0.0375	
B-Extraction time	0.0730	1	0.0730	0.7990	0.0401	
C-Extraction temperature	0.0109	1	0.0109	0.1187	0.7405	
AB	0.2831	1	0.2831	3.10	0.1218	
AC	0.0867	1	0.0867	0.9488	0.3625	
BC	0.0041	1	0.0041	0.0448	0.8384	
A^2	26.97	1	26.97	295.00	< 0.0001	
B^2	2.83	1	2.83	31.01	0.0008	
C^2	1.47	1	1.47	16.12	0.0051	
Residual	1.2669	10	0.0914			
Lack of Fit	1.2599	8	0.1280	9.2674	0.0980	Not significant
Pure error	0.0244	2	0.0126			
Core total	8.7657	28				

The Final equation in terms of coded factors is given below:

$$\text{TPC extraction rate} = 22.7754 + -0.192831 * A + 0.0797713 * B + -0.0307512 * C + 0.188125 * AB + 0.104125 * AC + 0.022625 * BC + -2.0723 * A\text{^}2 + -0.671867 * B\text{^}2 + -0.484414 * C\text{^}2$$

The negative value of A and C coefficients showed that negative change in these factors can bring negative change in the response value. The positive value of B indicated that positive change of B value can bring positive change in response value. By using parameters optimization on the basis of built mathematical model, the following experimental optimal conditions were obtained; solvent composition 70%, extraction temperature 37.5 ºC and extraction time 30 minutes.

4.2 Interaction between variables

4.2.1 Effect of methanol composition on the extraction rate of polyphenols

A linear increase in rate of polyphenol extraction was observed for certain level (up to 70%) then the rate of polyphenol extraction decreases with solvent composition as shown in figure 2 [20] A because polyphenols are mainly dissolved in vacuoles of cells low amount of water and methanol have access to the cells. But high level of methanol

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concentration can denature the protein and decreases its rate of extraction [21]. The best optimum composition for the extraction of polyphenols is 70% solvent composition as evaluated using 3D surface plot at 37.5 C temperature with 30 minutes time

4.2.2 Effect of temperature on the extraction rate of polyphenols

As it is obvious from 3 D surface the rate of extraction of polyphenols increases with increase in temperature up to certain level then it starts decreasing as shown in figure 2 B [22]. High temperature can weaken the cell wall so solvent has more access to the polyphenols [23]. But very high temperature may result in loss of solvent and poor rate of extraction in addition very high temperature may cause the oxidation of polyphenols [24]. The optimum temperature calculated using RSM was 37.5 ºC

4.2.3 Effect of extraction time on the extraction of polyphenols

From 3D surface plot it is clear that time is also an important factor influencing the rate of extraction of polyphenol? The rate of extraction increase with the time because it will prolong the contact of solvent with polyphenols [25]. But after 30 minutes the extraction rate starts decreasing because prolong duration may cause oxidation of polyphenols as shown in figure 3 C. The optimum extraction time evaluated by RSM was 30 minutes.

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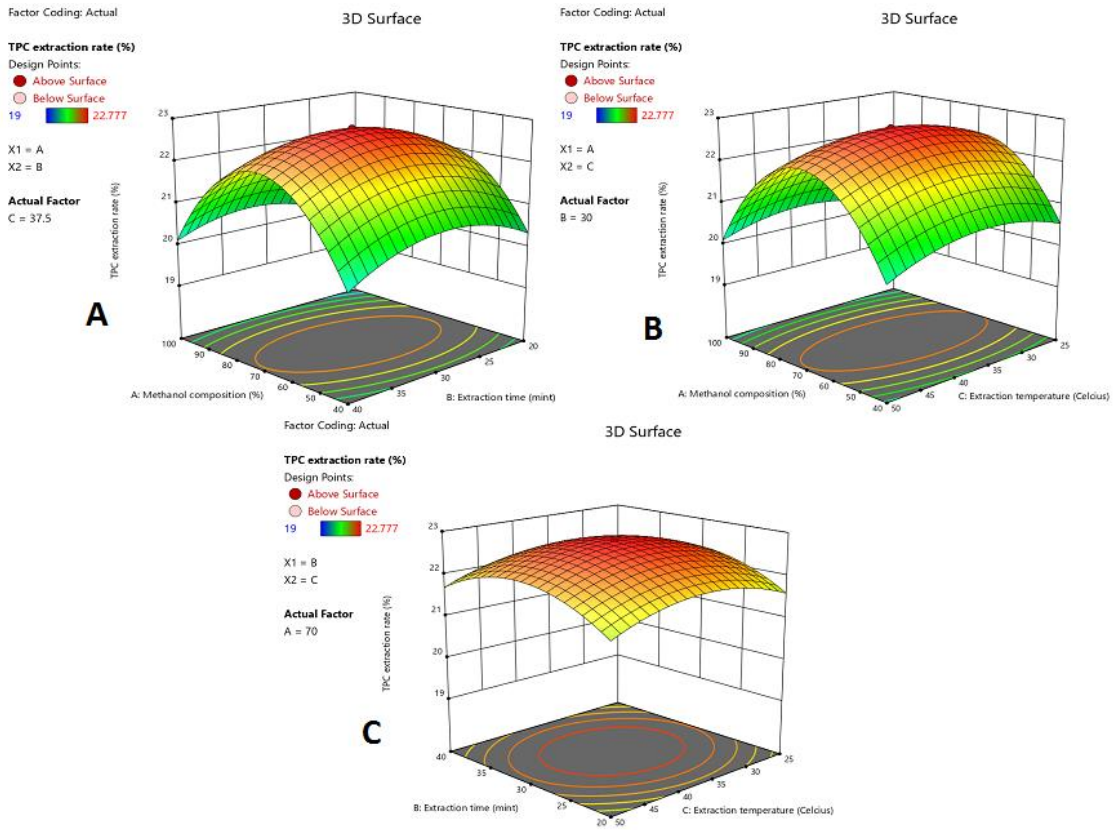

Figure 1: Correlative Effect of Methanol Composition A, Extraction Temperature B, Extraction Time C. on Extraction Rate of TPC.

4.2.4 HPLC Profiling of C. Sativus Peels

The HPLC analysis of methanolic extract of C.sativus peels at 254 nm had shown the presence of various compounds such as caffeic acid, protocatechuic acid, gallic acid, kamferol, chlorogenic acid and vannilic acid at different retention times 2.458, 2.2952, 3.666, 4.086, 5.287 and 7.797 respectively. These compounds were identified by comparing the peak position and retention time with their available standards [26] . The HPLC chromatogram of sample is shown in figure 3 and amount of these compounds was calculated on the basis of % peak area [27] and is presented in the table 4. Vannilic acid had shown the highest retention 7.797 min. The concentration of identified compounds was presented in µg/g of extract. Kampferol was present in highest amount and caffeic acid was present in lowest amount.

The structures of these identified comounds are given below.

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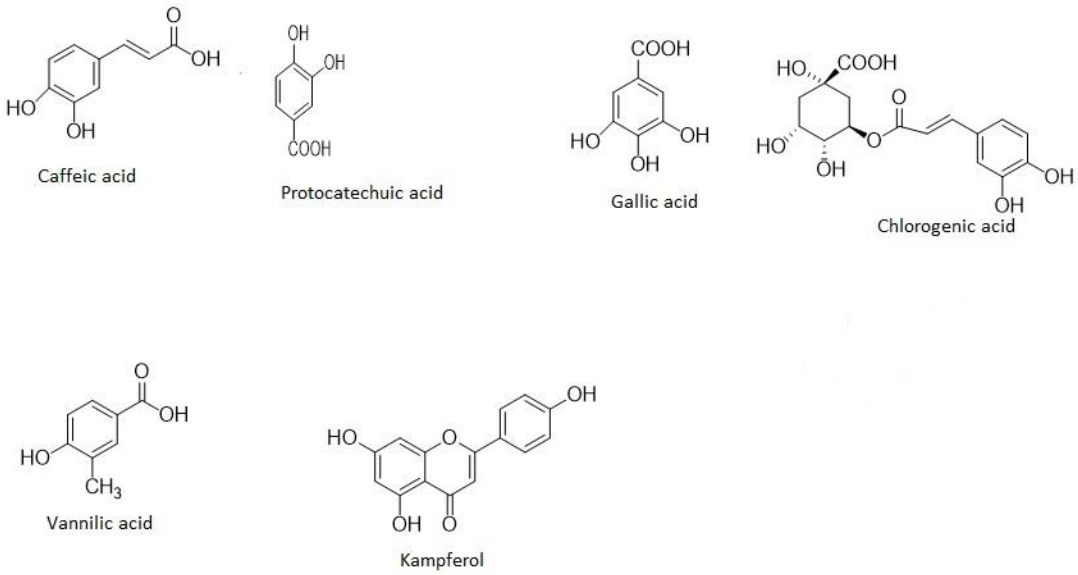
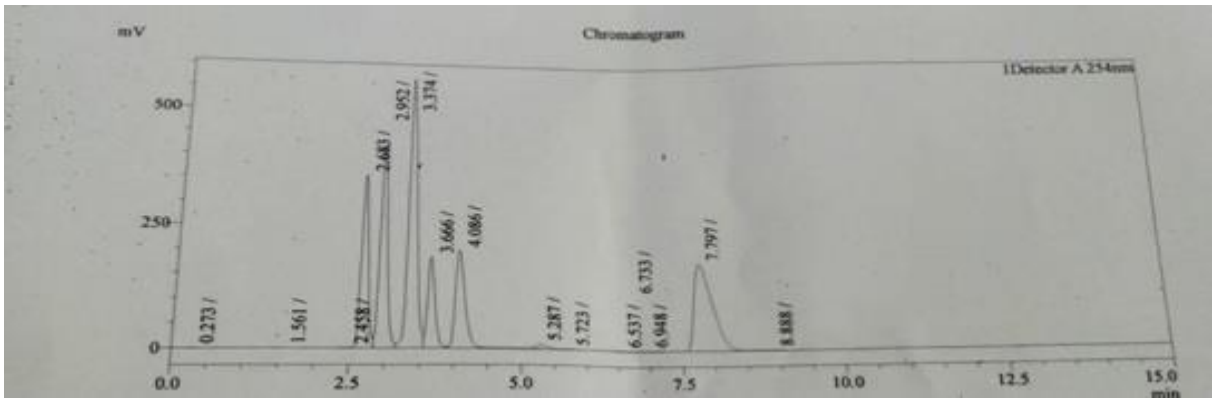


Compounds identified in C. sativus peels by HPLC analysis



HPLC chromatogram of C.sativus

4.3 Verification of predictive model

The RSM programme model was further verified by carrying out the experiment using RSM based optimal conditions i.e solvent composition 70%, extraction temperature 37.5 , extraction time 30 minutes the rate of extraction of polyphenol was 22.77. The experimental condition are given in the table 6. The experimental values were consistent with predicted values. Therefor the extraction conditions optimized by RSM were accurate and reliable.

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Table 6: **result of experimental verification**

Analysis	Methanol Composition	Extraction Temperature	Extraction Time	Extraction Rate of TPC
Predicted by RSM	70%	37.5	30	22.77
Experiment	70%	37.5	30	21.80

5. CONCLUSION

The current study suggested the methanol as most suitable solvent for extraction of secondary metabolites like polyphenolics. The methanolic extract exhibited excellent in vitro antioxidant activity due to the presence of important secondary metabolites as confirmed by HPLC analysis. The three independent factors extraction time, temperature and solvent composition proved remarkable effects on the extraction yield of TPC, DPPH. According to mathematical model the RSM based optimized conditions were at 70 % methanol composition, Extraction temperature 37.5 ºC and 30 minutes time the extraction rate of polyphenoic contents was 22.055 which is much better than any previous reported single factor analysis.

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