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Research Article

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Optimization and Extraction of Phenolic Compounds from Capcicum annuum Using Response Surface Methodology

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Abstract—

The interest in the consumption of Peppers (Capsicum annum) is to a large extent due to its content of bioactive compounds and their importance as dietary antioxidants. Peppers are used as a colorant, flavourant, and as a source of pungency. Green chilli is good source of vitamin C, beta-carotene. Flavonoids, organic and phenolic acids are act as antioxidants. The antioxidant activity in (Capsicum annum), the free radical scavenging abilities of GCP (green chilli paste) determined by Ferric-Reducing Antioxidant Power (FRAP) assay method. Natural antioxidants are preferred because synthetic antioxidants are considered carcinogenic. In the present day, effect of solvent extraction on recovery of phenolic compounds from GCP was investigated. Operational parameters of solvent extraction were optimized using response surface methodology (RSM) on total phenolic content and antioxidant activity. GAE was used as standard in measurement by UV/Visible Spectrophotometer.

Keywords— Capsicum annum, anti oxidants, Phenolic compounds, Solvent extraction, Response surface methodology,

I. INTRODUCTION

The planet earth that we are living on is a unique planet in the Solar system, it is full of lives. One of the most important features, which make Earth different from the others, is that the atmosphere contains approximately 20% oxygen. Organisms had been evolving long time ago to utilize oxygen so as to increase energy production from food. Oxygen is vital but it is also oxygen itself that puts the organisms in danger. Oxygen is an active element that causes oxidation to most substances and this oxidation do occur in living organisms. Among the oxygen inhaled in a day, about 90-95% is converted to water molecules, whereas the remaining 5-10% will undergo univalent and divalent reduction during the normal aerobic cellular metabolisms that generate oxidative stress [1].

Although our body has developed powerful antioxidant systems in the defense against the harmful effect of oxidative stress, under certain circumstances, our body can no longer cope with the excessive free radicals produced and disease will result over time. The protection against oxidation is the use of specific additives, which inhibit or retard the reaction. These oxidation inhibitors are generally known as "antioxidants". Antioxidants represent a class of substances that vary widely in chemical structure and have varied mechanisms of action. Antioxidant activity depends on many factors, including lipid composition, antioxidant concentration, temperature, oxygen, pressure, and the presence of other antioxidants and food constituents. Antioxidants were first used prior to World War II as food preservatives [13].

II. SOLVENT METHOD USED FOR EXTRACTION OF ANTIOXIDANT

Antioxidant compounds are usually present in rather low amounts in natural materials. Therefore, large additions of antioxidant-containing material would be required to obtain a significant improvement in stability against oxidation, which may be accompanied by a negative effect on the flavor or functional properties of the product. The easiest way to prepare more concentrated materials is to remove water by a suitable drying procedure. The next most optimal procedure is extraction. The choice of solvent is of crucial importance [1]. Conventional methods to extract natural antioxidants from plants are generally based on the employment of organic solvents, which may generate residue issues and have detrimental effects In the last two decades, numerous studies have shown that the human body is in great danger from the cumulative harmful effects of oxidative stress which then contribute to the development of many diseases including diabetes mellitus [12].

2.1. Biological Application of Antioxidant

In diabetes mellitus, the high plasma levels of glucose, free fatty acids (FFA) and sometimes insulin, favor the formation of free radicals which aggravate the situation and lead to potentially fatal complications such as cardiovascular disease. Controlling only the blood glucose level was proven to be insufficient in controlling the disease. It is also necessary to control the associated oxidative stress. To boost up the antioxidant defense systems, modifying the diet with increased intake of food rich in antioxidants is important. Green chillis have long been used by practitioners of traditional Chinese medicine for its anti-aging and sometimes hypoglycemic properties. Scientists have recently found that the 3 green chili is indeed rich in various kinds of antioxidants. Therefore it might be of useful in alleviating the oxidative stress in diabetes and its complications [13].

2.2. Measurement of Antioxidant Activity

For monitoring antioxidant activity in a food, potential measurements include PV, thiobarbituric acid value, iodine value, free fatty acid content, polymer content, viscosity, absorption at 232 and 268 nm, color, fatty acid composition and role of unsaturated to saturated fatty acids. Physiological activity can be assessed by *in vitro* measurements such as the susceptibility of isolated LDL to oxidation. Alternatively, the immunological response to antigenic lipid oxidation product can be measured [1]. Several analytical strategies are

1) Measurement at a fixed time point
2) Measurement of reaction rate
3) Lag phase measurement
4) Integrated rate measurement

Ethanol has exhibited antioxidant activity in certain circumstances and this must be considered when measuring the antioxidant activity of alcoholic beverages or when lipophilic compounds have to be added as ethanolic solutions to be test substrate [1].

2.3. Response Surface Methodology for Optimization

RSM was used to determine the optimal conditions for extraction. RSM was performed using the Design-Expert software (Trial Version 7.1.6, Stat-Ease Inc., and Minneapolis, USA). A central composition design (CCD) was used to investigate the effects of three independent variables (solvent concentration, extraction temperature and extraction time) at three level on the dependent variables (TPC and FRAP). CCD uses the method of least-square regression to fit the data to a quadratic model. The resulting regression model indicated that a quadratic polynomial model was best suited for spectrophotometrically determined total phenolic, whereas individual HPLC-measured phenolic and antioxidant activity of the extract were best described by a series of linear models. Solvent concentration and time had significant effects on total phenolic content [11].

III. MATERIALS AND METHODS

3.1. Preparation of Ethanolic Extracts

Lyophilized peppers of *Capsicum annum* (Green chilli) were collected from local market and washed in distilled water three times. *Capsicum annum* was homogenized with ethanol using homogenizer and the mixture was not filtered with filter paper [17].

3.2. Extraction Of Phenolic Antioxidants

The extraction process is one of the most important unit operations in the agro-food industry. Compounds obtained from the process may be used as food additives or as nutraceuticals. Extraction of antioxidants required to be cost effective and efficient. The yield of antioxidant compounds from plant material based on the extraction process carried out, one must optimize the extraction process [2].

The phenolic antioxidants were extracted using 30%, 60%, and 90% (v/v) ethanol in water. Ethanol (40mL) was added to 5g of GCP and placed in water bath. Samples were heated to temperature of 30, 50, and 70^{0}C for 10, 25, and 40min, respectively. The crude extracts were cooled to room temperature before centrifugation at 10,000 rpm to 15 min. The supernatant was collected and placed in a 50 mL volumetric flask for further analysis. Each solvent extraction was carried out in triplicate [13].

3.3. Determination Of Total Phenolic Compounds

The total phenolic content (TPC) was determined according to the Folinciocalteu method. A 50µL sample of the solution was transferred into 10mL volumetric flask and mixed with 6mL of distilled water. To each sample, 0.5 mL of Folin-ciocalteu reagent (50% v/v) was added and mixed. After 5 min, 1ml of Na_2CO_3 (5%, m/v) was added to the mixture and adjusted to 10mL with distilled water. After standing for 60 min at room temperature, the absorbance was measured at 760nm. Gallic acid was used to construct the standard curve. TPC was expressed as mg gallic acid equivalents (GAE)/g of wet paste mixture (WPM) [14, 18].

Table 1: Concentration of total phenolic compounds (mg GAE/g WPM) at 765nm

Concentration of solvent	**30^{0}C for 10 minutes**	**50^{0}C for 25 minutes**	**70^{0}C for 40 minutes**
30% of ethanol	5.576	6.176	6.776
60% of ethanol	11.176	12.176	14.576
90% of ethanol	14.676	15.376	16.776

3.4. Determination of Antioxidant Activity

Antioxidant activity was determined using a ferric-reducing antioxidant power (FRAP) assay. The working FRAP reagent was prepared by mixing 10 vol of 300 Mm acetate buffer (pH 3.6) with 1 vol TPTZ(10 mM) in HCl (40mM) and adding 1 vol of $FeCl_3$ (20mM). The freshly prepared FRAP reagent was warmed at 37^{0}C, and a reagent

blank reading was taken at 593 nm. Subsequently, 20µL of the sample was mixed with 480 µL of distilled water and added to the FRAP reagent (4.5mL). A second reading at 593 nm was performed after 8 min. To determine the FRAP value of the sample, the initial blank reading with the FRAP` reagent alone was subtracted from the sample, the initial blank reading with the FRAP reagent alone was subtracted from the final reading of the FRAP reagent plus the sample. A standard curve was prepared using a range of the FRAP reagent alone was subtracted from the final reading of the FRAP reagent plus the sample. A standard curve was prepared using a range of concentrations (25 to 1500mM) of $FeSO_4$. The FRAP value of the extracts was expressed as mM $FeSO_4$/ of WPM [17, 18].

3.5. Quantification Of Antioxidants

Quantitative as well as qualitative variations have been observed in the content of green chilli paste .HPLC analysis of different parts of green chilli (C. annum) revealed the presence of a number of phenolic acids, namely, tannic, gallic, Caffeic, vanillic, ferulic, chlorogenic, and cinnamic acids. Gallic acid was found to be the major phenolic acid in all part of green chilli. Highest amount of gallic acid was recorded in green chilli (8.5µg/g). Caffeic acid was highest in collar region (83.64µg/g), Ferulic acid was recorded in all parts of green chilli except in pulp, but the concentration does not exceeds above 1.00µg/g fresh weight at any case. Chlorogenic acid was found in pulp (0.83µg/g) while cinnamic acid in pulp (0.54µg/g). In green chilli seeds, tannic acid constituted almost 53.76% of total phenolics while as high as 75.45 and 67.03 % of caffeic acid was in root and collar region of chilli respectively [19].

HPLC system equipped with a tertiary pump, refrigerated auto-sampler and a UV/visible wavelength detector was used for sample analysis. Phenolic materials were separated using a reverse phase C-18, HPLC Gemini-NX (5 µm, 100 mm × 4.6 mm) column. Acetonitrile – CH3COOH 2% (6:4) as mobile phase, flow rate 1.0 ml/min, injection volume 10 µl and using UV detector at 280 nm. Retention time and area under the curve of each sample was recorded [16, 19].

Table 2: Codes levels of independent variables used in the RSM design

Independent variables	**Coded symbols**	**Levels**		
		-1	0	1
Extraction time (min)	X_1	10	25	40
Extraction temperature (^{0}C)	X_2	30	50	70
Solvent concentration (%, v/v)	X_3	30	60	90

TABLE: 3 Experimental Design Ad Response Values

Run number	**Time (min)**	**Temperature (°C)**	**Solvent concentration (%,V/V)**	**TPC(mg GAE/g WPM)**	**FRAP value (FėSO4)**
1	10	30	90	34	1500
2	25	50	60	62	1400
3	40	30	30	37	1470
4	10	30	90	44	1590
5	25	70	30	57	1610
6	25	50	30	82	1530
7	10	70	90	82	1590
8	40	70	30	51	1540
9	25	70	60	63	1510
10	25	50	60	152	1610
11	25	25	50	88	1460
12	25	25	30	50	1250
13	40	40	30	24	1420
14	10	10	70	49	1430
15	25	25	50	54	1250
16	40	50	50	47	1240
17	25	25	50	63	1310
18	10	10	70	44	1420
19	10	10	50	57	1380
20	25	25	50	105	1350

IV. RESULTS AND DISCUSSIONS

4.1. OPTIMUM TPC RECOVERY CONDITIONS

ANOVA analysis of the quadratic regression model for TPC demonstrated the model to be significant ($p<0.05$) with an F-value of 4.56. The R^2 of the model was 0.8039, and no significance was found in the lack of fit ($p> 0.05$). This indicated that the accuracy of the polynomial model was adequate. The second- order polynomial model was expressed by the following quadratic equation:

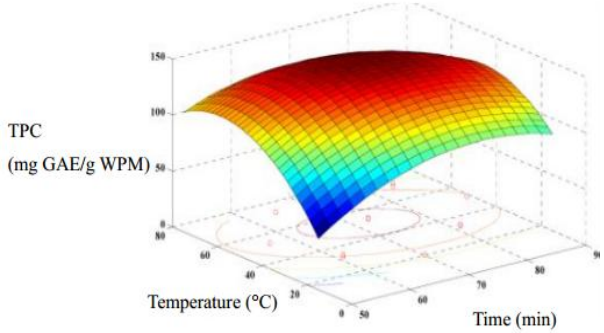
$$Y=61.02-6.20X1+10.10X_2+4.70X_3-2.25X1X_2-7.75X1X_3+3.75X_2X_3-19.55X1^2-15.05X_2^2+21.95X_3$$

Table 4: ANOVA for the Effect of Time, Temperature and Solvent Concentration on the FRAP Value, Using a Quadratic Response Surface Model.

Source	Sum of square	DF	F-value	P-value
Model	2.624E+005	9	4.88	0.0105 significant
A	23619.60	1	3.95	0.0749
B	3168.40	1	0.53	0.4833
C	3097.60	1	0.52	0.4881
AB	180.50	1	0.030	0.8655
AC	31250	1	5.23	0.0453 significant
BC	840.50	1	0.14	0.7155
A^2	255.36	1	0.043	0.8404
B^2	16151.11	1	2.70	0.1312
C^2	52095.36	1	8.72	0.0145 significant
Residual	59773.84	10		
Lack of Fit	59773.84	5		Not significant
R^2	0.8145			

A, B, and C were the variables of extraction time, extraction temperature and extraction concentration, respectively.

To determine the optimum condition for recovery of TPC from green chilli paste, three-dimensional surface plots were constructed. The influence of extraction time and temperature on TPC at a fixed ethanol concentration of 90% is shown. TPC increased rapidly as the temperature increased from 30°C and 60°C, and moderately thereafter to higher temperatures of approximately 70°C. While at the range of a longer extraction time (10 min), decreased TPC was observed with increasing time after it reached the maximum. The impact on TPC of temperature was found to be similar to that of time.



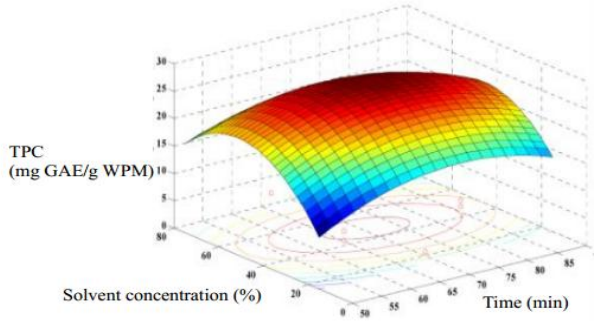


Figure 4.1 Response surface plots of total phenolic contents (TPC) for green chilli extracts as affected by extraction time, extraction temperature and solvent concentration (ethanol/aqueous, v/v). (A) Extraction temperature and time (solvent concentration set as constant); (B) Solvent concentration and extraction time (extraction temperature set as constant).

The effect of extraction time and ethanol concentration on TPC at a fixed extraction temperature of 50°C is presented in Figure (4.1 B). Initially, TPC increased slowly with increasing ethanol concentration until a maximum was reached and subsequently decreased rapidly. A clear quadratic effect on TPC of ethanol concentration at a fixed temperature was also observed. Based on the model, the maximum predicted yield of TPC of 84 mg GAE/g WPM was obtained under the optimum recovery conditions of 90%, 70^0C, and 10min for ethanol concentration, temperature, and time, respectively. Validation experiment carried out under the optimal conditions found the yield to be 82.75 mg GAE/g WPM, which was not significantly different from the predicted value ($p<0.05$). These results indicated that the quadratic model was reliable.

From the prospective of potential industrial applications, low cost and high recovery efficiency are preferable criteria for large-scale production. Compared to the other reported results, the TPC yield (84 mg GAE/g WPM) obtained from green chilli under the optimized conditions in the present study were considerably higher than those extracted previously from food processing wastes, such as grape seed (2.72 mg GAE/g dry material), citrus peel (19.12 mg GAE/g dry material), potato peel (3.95 mg GAE/g dry material) and apple peel (35.22 mg GAE/g dry material) and apple pomace (5.8 mg GAE/g dry material). The yield in the current study is however, lower than that obtained from peanut skin (118 mg GAE/ g dry material). From the obtained results, it is concluded that green chilli can be treated as a phenolic-enriched resource for potential industrial development.

4.2. OPTIMUM FRAP EXTRACTIONCONDITIONS

FRAP is a measure of the antioxidant effect of a substance in a reaction medium in term of its reducing ability, which is the ability of a natural antioxidant to donate electrons. As shown in Table(5.2), the highest (1610 mM $FeSO_4$/g WPM) and lowest (1240 mM $FeSO_4$/g WPM) FRAP values were observed in experimental runs 5 and 16, with the conditions of 30% ethanol concentration, 70°C, and 25 min, and 60% ethanol concentration,50°C, and 40min, respectively. ANOVA analysis revealed that the model was adequate, and that the antioxidant activity of the extracts was significantly ($p< 0.05$) affected by the linear terms of extraction time, extraction temperature and ethanol concentration, and by the quadratic terms of extraction time and ethanol concentration in Table (5.2). The second- order polynomial model expressed by following quadratic equation:

$$\mathbf{Y=1274.15-4860X_1+17.80X_2-17.60X_3+4.75X_1X_2-62.50X_1X_3-10.25X_2X_3+9.64X_1^2+76.64X_2^2+137.64X_3^2}$$

Table 5: ANOVA for the Effect of Time, Temperature and Solvent Concentration on FRAP Value, Using a Quadratic Response Surface Model

Source	**Sum of square**	**DF**	**F-Value**	**P-Value**
Model	4918.72	9	4.56	0.0133 significant
A	384.40	1	3.20	0.1037
B	1020.10	1	8.50	0.0154 significant
C	220.90	1	1.84	0.2046
AB	40.50	1	0.34	0.5741

AC	480.50	1	4.01	0.0732
BC	112.50	1	0.94	0.3557
A^2	1050.57	1	8.76	0.0143 significant
B^2	622.51	1	5.19	0.0459 significant
C^2	1325.51	1	11.05	0.0077 significant
Residual	1199.48	10		
Lack of Fit	1199.48	5		Not Significant
R^2	0.8039			

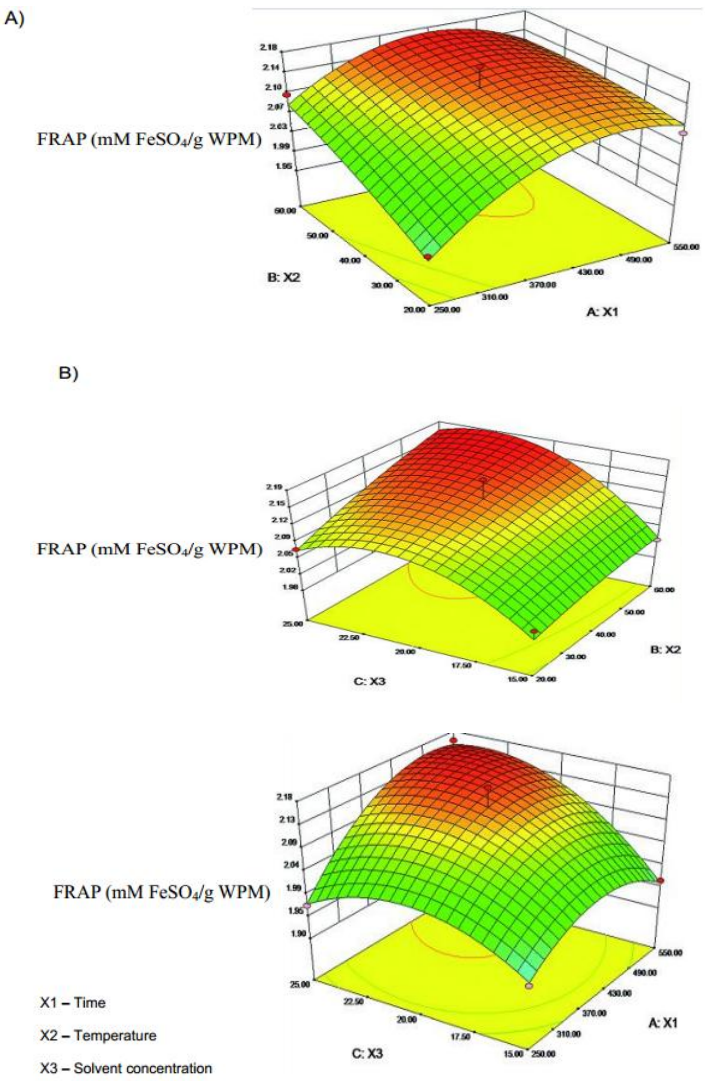


Figure 4.2 Response surface plots of total phenolic contents (FRAP) of green chilli extracts as affected by extraction time, extraction temperature and solvent concentration (ethanol, v/v). (A) Extraction temperature and time (solvent concentration set to 60%); (B) Solvent concentration and extraction time (extraction temperature set to 50°C); (C) Solvent concentration and extraction temperature (extraction time set to 25min).

The three-dimensional plots for FRAP values are presented in Figure (4.2). A linear increase in the FRAP value with increasing extraction time at a fixed temperature was observed. A similar increase in the FRAP value with increasing extraction temperature at a constant time was also observed Figure 4.2(A). As shown in Figure 4.2(B), the FRAP value initially increased with increasing ethanol concentration and then declined rapidly when ethanol concentration increased further at a fixed extraction time. A similar effect of 25 ethanol concentration on the FRAP value at a fixed extraction temperature was observed Figure 4.2(C). The influences of extraction time and extraction temperature on the FRAP value at the fixed ethanol concentrations were also similar, as illustrated in Figure 4.2(B and C). Based on results, the optimum recovery conditions for the antioxidant activity were found to be 90%, 30°C, and 10 min for ethanol concentration, temperature and time, respectively. It should be noted that these conditions, yielding a maximum predicted FRAP value of 499 mM FeSO4/g WPM, are very similar to the optimum conditions for TPC recovery. In addition, validation experiments carried out under these optimal conditions found the FRAP value was 498±23 mM FeSO4/g WPM, which was not significantly different from the predicted value ($p<0.05$).

4.2. ANALYSIS OF ANTIOXIDANTS

Quantitative as well as qualitative variations have been observed in the content of green chilli paste. HPLC analysis of different parts of green chilli (C. annum) revealed the presence of a number of phenolic acids, namely, tannic, gallic, Caffeic, vanillic, ferulic, chlorogenic, and cinnamic acids. Gallic acid was found to be the major phenolic acid in all part of green chilli. Highest amount of gallic acid was recorded in green chilli (8.5µg/g). Caffeic acid was highest in collar region (83.64µg/g), Ferulic acid was recorded in all parts of green chilli except in pulp, but the concentration does not exceeds above 1.00µg/g fresh weight at any case. Chlorogenic acid was found in pulp (0.83µg/g) while cinnamic acid in pulp (0.54µg/g). In green chilli seeds, tannic acid constituted almost 53.76% of total phenolic compound while as high as 75.45 and 67.03 % of caffeic acid was in root and collar region of chilli respectively [17, 20]. Green chilli which constituted almost 81.11 and 53.76% of total phenolic was determined. Capsaicinoid and their phenolic intermediates in various lines of C.annum. Vitamin C, flavonoids, carotenes and organic acids present in green chilli has the activity of antioxidants. The phenolic compounds present in the samples were identified by comparing retention time (Rt) of the standards and by co-injection. Contents of phenolic compounds were calculated by comparing the peak areas of reference compound with those in the samples run under similar elution conditions [4].

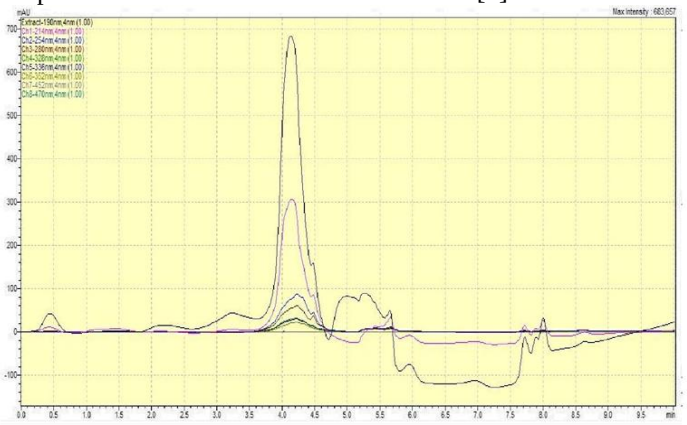


Figure 1: HPLC chromatogram of green chilli extract for all compounds present in experimental run of 18.

V. CONCLUSIONS

The maximum predicted TPC (84 mg GAE/ g WPM) was obtained from GCP under optimized conditions (90% ethanol, 70^0C, and 10 min) and the maximum predicted FRAP (499 mM FeSO4/g WPM) was obtained under the optimized conditions (90%ethanol, 30^0C, and 10min). HPLC analysis of different parts of green chilli (C. annum) revealed the presence of number of phenolic acids namely, tannic, gallic, caffeic, vanillic, ferulic, chlorogenic, and cinnamic acid. The phenolic compounds present in the samples were identified by comparing retention time (Rt) of the standards and by co-injection. Contents of phenolic compounds were calculated by comparing the peak areas of reference compound with those in the sample run under similar elution conditions. These results indicated that the quadratic model was reliable. From the point of view of industrial production, ethanol extraction has been presented as an effective method for the recovery of phenolic antioxidants from green chilli, although full characterization of the profile of different phenolic in green chilli requires further study.

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