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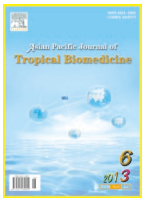

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The optimization of phenolic compounds extraction from cactus pear (*Opuntia ficus-indica*) skin in a reflux system using response surface methodology

Aguirre Joya Jorge[1], De La Garza Toledo Heliodoro[2], Zugasti Cruz Alejandro[1], Belmares Cerda Ruth[1*], Aguilar Cristóbal Noé[1]

[1]*Food Research Department, School of Chemistry, Universidad Autónoma de Coahuila, Saltillo, 25280, Coahuila, México*

[2]*Basic Science Department, Universidad Autónoma Agraria Antonio Narro, Saltillo, 25315, Coahuila, México*

PEER REVIEW

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Dr. Rosa María Rodríguez Jasso, Food Research Department. School of Chemistry, Universidad Autónoma de Coahuila, Unidad Saltillo, Blvd. V. Carranza e Ing. José Cárdenas Valdés, Saltillo, Coahuila C.P. 25001, Mexico.
Tel: 844-4169213,4161238
Fax: 4390511
E-mail: rosy_rjrl@hotmail.com

Comments

This work is a relevant contribution in the study of polyphenols extraction from agroindustrial wastes. The authors evaluated the effect of a reflux system on the antioxidants recovery from *O. ficus-indica* peel. The results showed higher yields and antioxidants activities than that of previous research reports.
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ABSTRACT

Objective: To extract, quantify, and evaluate the phenolic content in *Opuntia ficus-indica* skin for their antioxidant capacity with three different methods (ABTS, DPPH, and lipid oxidation) and to optimize the extraction conditions (time, temperature and ethanol concentration) in a reflux system. **Methods:** The extraction process was done using a reflux system. A San Cristobal II experimental design with three variables and three levels was used. The variables evaluated were time of extraction (h), concentration of ethanol (%, v/v) and temperature (°C). The extraction process was optimized using a response surface methodology. **Results:** It was observed that at higher temperature more phenolic compounds were extracted, but the antioxidant capacity was decreased. The optimum conditions for phenolic compounds extraction and antioxidant capacity mixing the three methods were as follows: 45% of ethanol, 80 °C and 2 hours of extraction. Values obtained in our results are little higher that other previously reported. **Conclusions:** It can be concluded the by-products of *Opuntia ficus-indica* represent a good source of natural antioxidants with possible applications in food, cosmetics or drugs industries.

KEYWORDS

Opuntia ficus-indica, Reflux, Phenolics, Antioxidants, Prickly pear, Response surface methodology

1. Introduction

Nowadays, there has been an increase of research studies about natural antioxidants plant-based extracts to obtain bioactive compounds for food and pharmaceutical industries interes[1]. This has been derived for a number of studies that had correlated in a positively diet based on plant food and a reduced risk of diseases associated with oxidative stress[2,3]. Natural antioxidants obtained from plants included carotenoids, phenolic compounds and polyphenolic compounds. There is evidence that these compounds can exert their antioxidant functions in human health[4]. Some researchers suggest that the main content of antioxidants in plants is in the skin of the fruit where they

*Corresponding author: Belmares Cerda Ruth, Food Research Department, School of Chemistry, Universidad Autónoma de Coahuila, Saltillo, 25280, Coahuila, México.
Tel: +52 844 4161238
Fax: +52 844 4159534
E-mail: ruthy_251m@yahoo.com.mx

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play an important role of protecting against some insects, microorganisms and other predators as well as adverse conditions[5]. Some authors suggest that the importance of the use of natural antioxidants is that these compounds can reduce the risk of age dependent diseases and inhibit the oxidation on food and they have lower toxicity than synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene[6-8].

Opuntia ficus-indica (*O. ficus-indica*, cactus pear) is a cactus well adapted to arid and semiarid conditions[9]. The central part of Mexico hosts the greatest diversity of this cactus in the world. This cactus produces an edible prickly pear that is consumed as a fresh fruit. It has been reported that the outer coating of this fruit is approximated 45% to 50% of the total weight and it is managed as a waste of the agroindustrial process, which means that those by-products can be improved as a natural and economic source of antioxidants[10,11].

The response surface methodology (RSM) has been demonstrated to be a helpful tool that can determine the factors and their interactions, which allows process optimization to be conducted effectively. RSM is the preferred methodology for fitting polynomial model to analyze the response surface of multi-factor combinations and a faster and economical method for gathering research results than classic one variable at a time or full factors in experimentation[12-14]. To our knowledge there is scarce information about phenolic compounds extraction of *O. ficus-indica* skin using reflux. In order to approach the agroindustial wastes as natural sources of antioxidants with application in food industries, the main objective of this study was to optimize the process parameters (time of extraction, concentration of ethanol as a solvent and temperature) for the phenolic compunds extraction from *O. ficus-indica* skin with RSM and to evaluate the antioxidant potential of this extracts.

2. Material and methods

2.1. Reagent and apparatus

2,2-azino bis-3-etilbenzotiazolin-6-sulfonic acid (ABTS), 2,2-diphenil-1-picril-hidracil (DPPH), and gallic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA) and linoleic acid was from Fluka (St. Louis, MO, USA). All the other reagents were of analytical grade.

2.2. Phenolic extraction of cactus pear skin

The *O. ficus-indica* pears were purchased from a local market in Saltillo, Coahuila, Mexico, at their consumption maturity stage and keep at -20 °C until further analysis. The fruits were hand peeled and the skin was weighed and then dehydrated at 60 °C for 24 h. the dehydrated skin was then pulverized in a grinder (pulvex mini 100). The extraction was done in a reflux system at different times (2, 4, 6 and 7 h), temperatures (60, 70, 80 and 93 °C) and ethanol concentration as a solvent for extraction (0, 35, 70 and 82% v/v in water). The relation mass/solvent for the extraction was constant and it was of 1:8 (w/v).

2.3. Determination of phenolic content by micro plate assay

A volume of 20 μL of each dilution were mixed with the same quantity of Folin-Ciocalteau's reagent in each well and let it react for 5 min. After that 20 μL of sodium carbonate (0.01 mol/L) were added, after 5 min 125 μL of water were aggregated and the absorbance was read at 790 nm (Epoch, Biotek industries, Highland park, USA)[15,16].

2.4. ABTS assay

This technique was done following previous literaturewith minimum modifications[17]. Briefly, 1 mL of ethanolic solution of ABTS was mixed with 10 μL of sample in a quartz cell and the absorbance was read at 734 nm in a spectrophotometer (Thermo Spectronic, Biomate 3, Minnesota, USA). The equation 1 was used to determinate the inhibition percent of the samples:

$$\%\ \text{inhibition ABTS} = \frac{(Ac-As)}{Ac} \times 100 \quad (1)$$

Where Ac is the control absorbance and As is the absorbance of the sample.

2.5. DPPH in micro plate assay

DPPH assay was done by using the method described in the literature with some modifications[18]. Briefly, 193 μL of the DPPH solution were mixed with 7 μL of the sample in each well of the micro plate. After 30 min the absorbance was read at 517 nm in an ELISA reader (Epoch, Biotek industries, Highland, USA). The DPPH cation inhibition percent was calculated as follows (equation 2).

$$\%\ \text{inhibition DPPH} = \frac{(Ac-As)}{Ac} \times 100 \quad (2)$$

Where Ac is the absorbance in control and As is the absorbance of the sample.

2.6. Lipid oxidation inhibition assay

In this study linoleic acid was use as a source of lipids according to previous studies[19]. The linoleic acid solution was prepared by diluting 0.56 g of linoleic acid and 1.5 g of Tween 20 in 8 mL of ethanol (96%). Each extract (50 μL) was mixed with linoleic acid solution (100 μL) and 1.5 mL of 0.02 mol/L acetate buffer, pH 4.0. Controls contained water instead of extract. All of the samples were homogenized in a vortex (Labnet International, Edison, NJ) and sonicated in an

ultrasonic bath (Bransonic 2510R-MTH; Branson, Dambury, CT) for 3 min. Obtained emulsions were incubated at 37 °C. After 1 min, 750 μL of $FeCl_2$ (50 mol/L) was added to induce reaction of oxidation. After 1 h 1 mL of 0.1 mol/L NaOH in 10% ethanol was added to 250 μL of the mixture to stop the oxidation process. The same steps are repeated at 24 h of incubation. After mixing, 2.5 mL of 10% ethanol were added, the absorbance was measured at 232 nm and the percentage of antioxidant activity was calculated by the equation 3 with slight modifications[20].

$$\% \text{ LOI} = \frac{(\Delta\text{ODcont} - \Delta\text{ODextr})}{\Delta\text{ODcont}} \times 100 \quad (3)$$

Where ΔODcont is the difference between 24 and 1 h of lipid oxidation in controls and ΔODextr is the difference between 24 and 1 h of lipid oxidation in samples.

2.7. Statistical analysis

After quantification, a San Cristobal II experimental design means were compared with Tukey's procedure in Statistical Analysis System (SAS 9.0). Later a response surface methodology (RSM) was used to optimize the conditions of extraction in a SAS software (SAS 9.0) and the plots were made in a Statistica 7 software. The response function (Y) was portioned into linear, quadratic and interactive components and the experimental data were fitted to the second order regression equation (4). Table 1 shows the experimental matrix with the 12 applied treatments.

$$Y = \beta o + \sum_{i=1}^{2} \beta i Xi + \sum_{i=1}^{2} \beta i Xi + \sum_{i=j=1}^{2} \beta ij Xi Xj \quad (4)$$

Table 1
Experimental matrix obtained by a San Cristobal II design.

Treatment	Percentage of ethanol in wather (v/v)	Temperature (°C)	Time (h)
1	0	60	2
2	0	60	6
3	0	80	2
4	0	80	6
5	70	60	2
6	70	60	6
7	70	80	2
8	70	80	6
9	82	70	4
10	35	93	4
11	35	70	7
12	35	70	4

Treatments evaluated in the reflux system to extract the phenolic compounds from the *O. ficus-indica* skin. Each treatment was evaluated by triplicate.

Where Y is the predicted response, βo is the intercept, βi, βii, βij are the linear, quadratic and interaction coefficients, respectively to the model, and Xi and Xj are the levels of independent variables. The three dimension surface plots were generated showing the relationship between the response and independent variables[21]. The means of the three assays were ranked to evaluate a single response for antioxidant capacity in a RSM as an independent replicates.

3. Results

3.1. Determination of phenolic content by microplate assay

Phenolic compounds were quantified following the Folin-Ciocalteau's reagent method as descried using microplates. In this assay the tungstenic and molybdenum acids were applied as reagents to react with the polyphenols in the sample resulting in a blue-green coloration[15,16]. The gallic acid was used as a standar. Thus the polyphenol concentration is expressed as a gallic acid equivalents (GAE) (Figure 1). The maximum yield of extraction was 457 μg/mg GAE (93 °C, 35% ethanol, 4 h).

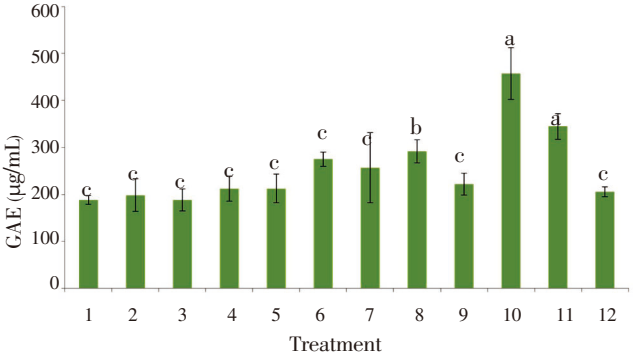

Figure 1. Quantification of gallic acid equibalents (GAE μg/mL of extract).
The axis of y corresponds to the content of polyphenolic compounds expresed as GAE in μg/mL for extracts obtained by each treatment (axis of x). Different letters are statistically different (Tukey's range test alpha=0.01). The assay was performed by triplicate (mean±SD).

3.2. ABTS assay

Figure 2 shows the percentage of inhibition for the ABTS radical. The treatments 5 to 12 do not show statistical difference, these treatments have in common that contain ethanol in water as a solvent of extraction while the treatments 1 to 4 contained only water (Table 1). The average radical inhibition between treatments 5 to 12 was 92%. The higher values were detected using extracts obtained under conditions of treatments 5-12 (Figure 2).

3.3. DPPH in microplate assay

In this experiment the maximum percentage of inhibition was detected using extracts obtained by the treatments number 6 and 8 (Figure 3). The conditions of these extractions were 70% of ethanol, 60 °C, 6 h of extraction and 80 °C, 70% of

ethanol and 6 h, respectively (Table 1).

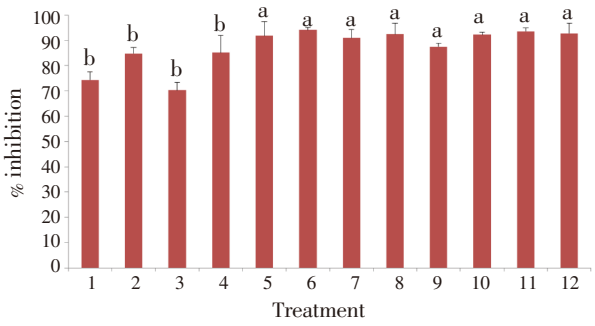

Figure 2. Percentage of the radical ABTS inhibition.
On the axis of y corresponds to the antioxidant capacity of extracts expressed as the percentage of ABTS radical caption inhibition for each treatment (axis of x). Different letters are statistically different (Tukey's range test alpha=0.01). Assay was performed by triplicate (mean±SD).

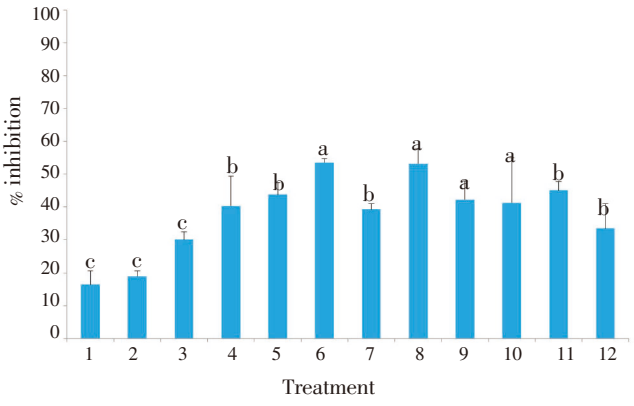

Figure 3. Percentage of the radical DPPH inhibition.
The axis y corresponds to the antioxidant capacity of extracts expressed as the percentage of DPPH radical inhibition for each treatment (defined in axis x). Different letters are statistically different (Tukey's range test alpha=0.01). The assay was carried out by triplicate (mean±SD).

3.4. Lipid oxidation inhibition assay

The maximum inhibition of lipids oxidation was observed using the extracts obtained by the treatments 6 and 12 (Figure 4). The conditions applied in these treatments were 70% ethanol, 60 °C and 6 h and 35% ethanol, 70 °C and 4 h, respectively. The percentages of inhibition of lipid peroxidation were 80% and 60% respectively (Figure 5).

3.5. Statistical analysis

The antioxidant capacity was evaluated by three different methods. So the means of each method for the corresponding treatment were studied like an independent treatment and ranked to obtain a total antioxidant power. The RSM was applied to ranked values to optimize the extraction conditions. Figure 5 shows the ranked values for the antioxidant response.

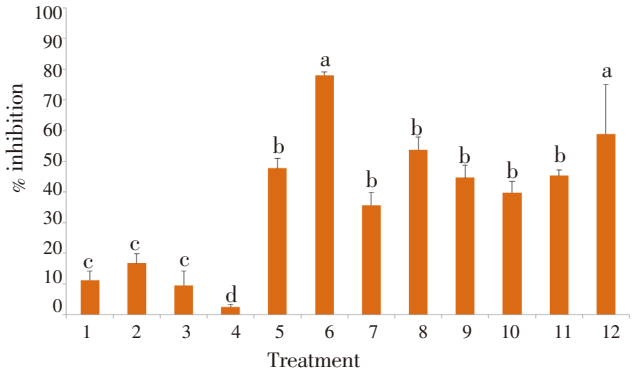

Figure 4. Percentage of the lipid oxidation inhibition.
The axis of y corresponds to the antioxidant capacity of extracts expressed as the percentage of the lipid oxidation inhibition for each treatment (axis x). Different letters are statistically different (Tukey's range test alpha=0.01) results obtained in assay performed by triplicate (mean±SD).

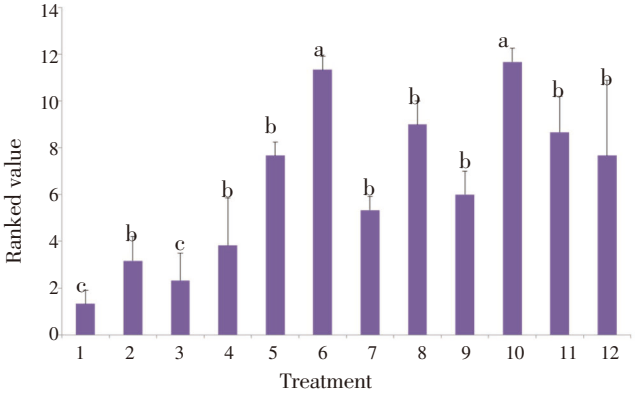

Figure 5. Percentage of net antioxidant activity expressed as ranking results achieved by the three methods applied for each extract obtained under different treatment.
Different letters are statistically different (Tukey's range test alpha=0.01), the assay was performed by triplicate (mean±SD).

3.6. Optimization by RSM for the extraction

Table 2 shows the coefficients of regression and their significance for the process of phenolic compounds extraction. The second order polynomial model was applied. The results of analysis by RSM indicate that the temperature in a quadratic term and temperature in a linear term affects primordially the process of extraction followed by the quadratic effect of ethanol expressed in the quadratic term. Figure 6 shows the response surface plot for phenolic concentration. In the y axis appears the quantification of polyphenols in ppm, in the x the percentage of ethanol and z the time of extraction. The maximum response of phenolic extraction corresponds to conditions: 45% of ethanol and 2 h of extraction at 80 °C (Figure 6).

In the case of the antioxidant determination, evaluated by three different methods, Table 3 shows the regression coefficients and their statistical significance. According to values of quantified coefficients (Table 3), the ethanol and temperature in the linear and quadratic terms shows more affect on the antioxidant capacity followed by the interaction

of ethanol and temperature.

Table 2

Regresion coefficients of the second order polynomial model for the phenolic content.

Factors		Values
Intercept		1957.53[a]
Linear	Ethanol (X1)	2.16
	Temperature (X2)	−50.81[a]
	Time (X3)	−42.71
Quadratic	Ethanol (X1)	−0.04[a]
	Temperatue (X2)	0.37[b]
	Time (X3)	6.96
Interaction	X1×X2	0.02
	X1×X3	0.11
	X2×X3	−0.08

Tukey´s media comparison applied at the response surface methodology; it shows the intercept, linear, quadratic and interaction effects of the factors evaluated in the reflux system for the phenolic compounds extraction. A negative sign indicates an inverse effect, while the positive represents a direct effect. [a]P<0.05 and [b]P<0.01.

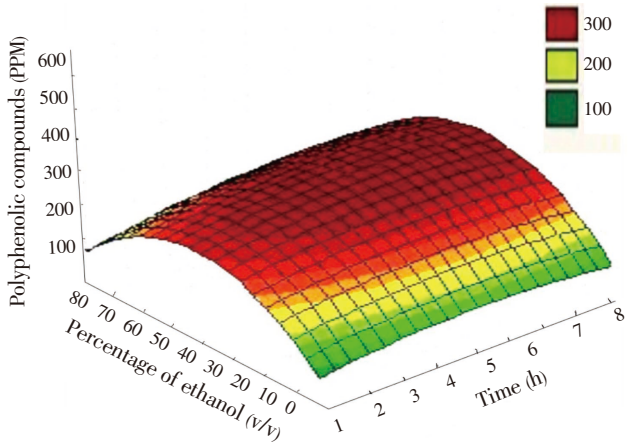

Figure 6. Surface response plot for phenolic compounds concentration as the function of extraction time and ethanol concentration based on the quantification of phenolic compounds in the extracts obtained under different conditions.

Table 3

Regresion coefficients of the second order polynomial model for the antioxidant capacity.

Factors		Values
Intercept		46.289 960[a]
Linear	Ethanol (X1)	0.284 267[b]
	Temperature (X2)	−1.360 561[b]
	Time (X3)	−0.366 115
Quadratic	Ethanol (X1)	−0.001 469[a]
	Temperatue (X2)	0.010 172[b]
	Time (X3)	0.088 406
Interaction	X1×X2	−0.001 786[a]
	X1×X3	0.005 952
	X2×X3	0.002 083

Tukey´s media comparison applied at the response surface methodology results; it shows the intercept, linear, quadratic and interaction effects of the factors evaluated in the reflux system for the phenolic compounds extraction. A negative sign indicates an inverse effect, while the positive represents a direct effect. [a]P<0.05 and [b]P<0.01.

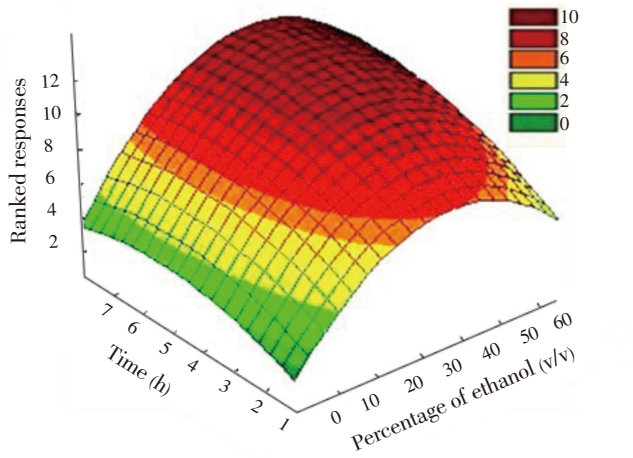

Figure 7. Surface response plot for net antioxidant activity of extracts as the function of extraction time and ethanol concentration. Antioxidant activity (axis of y) is expressed as the ranked values evaluated by three different methods.

Figure 7 shows the response surface plot for the antioxidant capacity, considering ranked values (Figure 5) as ethanol concentration and the time of extraction. Figure 5 shows the ranked values for each treatment. The treatments with higher antioxidant activity considering the three methods were the number 6 and 10, the conditions of each treatment are as follows: 70% of ethanol, 60 °C, 6 h and 35% of ethanol, 93 °C and 4 h, respectively.

4. Discussion

The percentage weight of *O. ficus-indica* skin in some reports is between 33% to 55% of fresh weight[22], but it is a little lower compared with others[23].

At higher temperature more phenolic compounds were extracted. This finding is consistent with the previous report[24]. The treatment with greater phenolic content was the number 10. The conditions applied in this extraction were 35% of ethanol, 93 °C and 4 h of treatment. It was a higher temperature in comparison with that in other performed assays. Likely the high temperature increases the molecular movement that helps in the extraction. Our results are lower than previously reported for a similar process under the conditions 90 °C, 80% ethanol and 6 h of extraction[25]. However, in our study relation mass/solvent was 1:8, while the same parameter applied in previous assay was 25:1, which implicated the using of greater quantity of vegetal material. This may be cause of a greater phenolic content in the extract[24]. On the other hand, our results are greater than (55.6±5.9) μg/mg, reported in the study performed by using the complete fruit without heating[1]. Thus, according to our results and one reported by other authors, heating process is an important aspect for the phenolic compounds extraction.

The analysis of antioxidant capacity values determined by ABTS assay leads to a conclusion that a minimum concentration of ethanol is required to obtain optimal

polarity conditions to extract biological compounds like polyphenols. The inhibition of ABTS radicals formation detected in the present study is superior to that reported by other researchers who achieved only 33% of inhibition using extracts of polyphenols obtained by maintaining the vegetal material in ethanol solution for at least 24 h at room temperature[22]. Greater values of antioxidant capacity indicate that the high temperature facilitates the antioxidant compounds extraction process.

According to results obtained by DPPH in microplate assay, the ethanol concentration and time of extraction are other factors that influenced the extraction of antioxidant compounds. The major percentages of inhibition were found with extracts obtained by treatments 6 and 8, which have in common with the concentration of ethanol and the extraction time.

The results obtained by lipid oxidation inhibition assay indicate that greater extraction time and ethanol concentration lead to higher activity of the extracts, while the second option might be lower temperature and ethanol concentration. Our results are higher than previous report in which the researchers achieved 18% of inhibition using the extract from the edible portion or pulp of cactus pear obtained without heating[25]. It is confirmed that the higher concentrations of polyphenols in most of plants and fruits are found in their skin which plays an important role as a part of defense system; moreover, thermic treatment can help to extract them more efficiently[5].

The extraction of phytochemicals like polyphenols is a mass transfer process of solute from the plant cells to the solvent. The mathematical model for the solid-liquid extraction focusses on the diffusional effects of the phytochemical compounds: increasing the temperature of the solvent in extraction process leads to the increase of diffusion phenomena that helps to extract the polyphenols presented in the plant, probably due to vibratory effects of the wall cell molecules, which facilitates migration of free phytochemicals to the solvent[26]. The results obtained by appling RSM led to conclusion that extraction may be optimized by using of ethanol at 45% and a temperature at 80 °C. The maximum response is observed from 2 h of extraction, and slightly increases with increase of this parameter. We recommend use this condition as optimum to save economic resources in the heating and cooling process. Thus, we postulate that the optimum conditions for the phenolic compounds extraction from skin of *O. ficus-indica* cactus pear are 45% of ethanol, 80 °C and 2 h of extraction.

The values of antioxidant capacity of extracts obtained under applied conditions, which were evaluated by three methods, were higher than previously reported and 1.6, 1.8 and 4.4 times of the results in ABTS, DPPH and lipid oxidation inhibition assays, respectively[24-26]. This suggests that the byproducts of *O. ficus-indica* represent a good source of natural antioxidants. These natural antioxidants can be widely applied in food, cosmetics or drugs industries, decreasing product costs due to use of cheaper raw materials.

In general this is the first report that explores the use of RSM to optimize the polyphenolic compounds extraction from *O. ficus-indica* pear skin in a reflux system. The results obtained show that these agro industrial byproducts represent a cheaper and natural source of polyphenolic compounds with a lower toxicity compared with the synthetic ones.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

The demand of natural antioxidants use in food industry has drastically increased due to the global necessity of eliminate synthetic based additives. *O. ficus-indica* is a native Mexican perennial succulent harvested on summer and autumn, however its commercialization has been limited to traditional markets, producing a high quantity of residues, hence it's a possible alternative to be evaluated as antioxidant source.

Research frontiers

Studies performed in this work has been based on the evaluation of optimal extraction conditions to recover phenolic compounds from *O. ficus-indica* peel using a heat reflux method, and the evaluation of the antioxidant capacity by three different methods.

Related reports

Previous reports related with polyphenols extraction of *O. ficus-indica* showed lower yields and antioxidants activities than the values reported in this work. The reflux extraction optimal conditions are related with solvent effect over phytochemical diffusion with the increase of temperature.

Innovations and breakthroughs

Studies based on natural antioxidants of *O. ficus-indica* are scarce in literature. Moreover reflux system improve the extraction yield of polyphenols compounds with high free radical inhibitory effect.

Applications

The high percentage of peel in *Opuntia* fruit are a natural and economic agroindustrial waste for biotechnology study and application. The optimal conditions obtained by reflux extraction methodology are the preliminary study for future perspective phytochemicals industry.

Peer review

This work is a relevant contribution in the study of polyphenols extraction from agroindustrial wastes. The authors evaluated the effect of a reflux system on the antioxidants recovery from *O. ficus-indica* peel. The results showed higher yields and antioxidants activities than that of previous research reports.

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