OPTIMIZED EXTRACTION OF PHENOLIC COMPOUNDS FROM BARLEY (*HORDEUM VULGARE* L.) SEED AND THEIR RADICAL SCAVENGING PROPERTIES

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ABSTRACT

Extraction of phenolic compounds from barley (*Hordeum vulgare* L.) seed was optimized by using a designed experiment including three process variables, i.e., temperature (45–60C), time (3–6 h) and ethanol concentration (65–80%). The extraction temperature and time were found significant ($P < 0.05$) process variables and maximum of 2.18 mg GAE/g dried seed powder of total phenolic compounds was obtained by extraction at 60C for 6 h using 65% ethanol. Regression analysis and response surface method were used for the prediction of optimum levels of process variables which were 58C temperature, 5.4 h time and 65% ethanol for the maximum amount of total phenolic compounds (2.31 mg GAE/g seed powder) from barley that showed a good experimental validation (2.28 mg GAE/g seed powder of phenolic compounds). All the extracts prepared from barley seed showed good antiradical activities that ranged between 52 and 65.24%; the highest was achieved by the optimized extract.

PRACTICAL APPLICATIONS

Barley is one of the major cereal crops and used mainly as a source of carbohydrates and energy. There is a need to find out bioactive compounds present in barley seed along with other cereals. This study was designed to elaborate a simple, inexpensive and easily reproducible method for the recovery of phenolic compounds from barley seed. Response surface optimization resulted in achieving maximum amount of phenolics and best antioxidant properties in the extract. This study is useful in establishing a process for the recovery of phenolic compounds from barley for their subsequent use in nutraceutical and functional foods.

INTRODUCTION

Different studies indicate that the use of plant materials rich in phytochemicals, particularly phenolics, imparts health protective and disease preventive effects. These effects may include anti-inflammatory, anti-mutagenic, antiviral and antibacterial effects (Senevirathne *et al.* 2006). It has been amply documented that different types of phenolic compounds from plant-based foods can decrease or prevent different health problems due to their antiradical and antioxidant activities (Surh 2002). Antioxidants also reduce rancidity of foods, restrict the toxic products formed due to oxidation reaction, provide nutrition and increase shelf life of foods. There have been various studies about phenolics from different parts of plants such as their leaves, bark, roots, fruits, peels and seeds, and corresponding antioxidant activities (Ghafoor and Choi 2009; Ghafoor *et al.* 2011).

Cereal crops are a very important source of carbohydrate and energy for masses around the globe. A lot of different evidence is being obtained these days whereby researchers study the bioactive compounds potential of different cereal crops (Peterson *et al.* 2001; Sun and Ho 2005). Barley is one of the most ancient cereal crops and widely consumed over the world. Major portions of barley crop are utilized in malt production and animal feed; however, these days, there is increasing interest for using barley as an ingredient of

healthy foods due to the presence of bioactives such as tocols and β-glucans (Peterson 1994). There have been reports that the seeds of barley can be considered as a source of bioactive compounds such as phenolics, and there is a need to find out the best suited method for their extraction (Liu and Yao 2007).

The recovery of bioactive compounds from plant sources is a research area whereby intensive studies are being carried out, because these compounds have potential to become ingredients of special dietary products, nutraceuticals, functional foods, food additives, pharmaceutics and cosmetics. Many types of organic solvent systems and procedures are applied for obtaining bioactives from natural sources (Chavan *et al.* 2001). The amounts of recovered bioactive compounds can vary depending on the extraction method (Goli *et al.* 2005). Hence, these methods should be designed to maximize recoveries with minimal or no chemical changes in the desired products (Zuo *et al.* 2002). Commonly used methods include use of water and various concentrations of organic solvents such as ethanol, methanol, acetone and *n*-hexane, besides different modern but more expensive techniques (Sun and Ho 2005; Azmir *et al.* 2013). There are different process variables, depending on the method, which have high significance and impact on the yield of bioactive compounds and may also include temperature, time, particle size, etc. (Hui *et al.* 2009; Ghafoor *et al.* 2011). Statistical techniques such as response surface methodology are frequently applied for optimizing process variables in food manufacture (Farah *et al.* 2012). Such approach is important for enhancing recovery of biologically important components from different plant matrices along with selection of suitable extraction method.

The objective of carrying out this research was to optimize the ethanol-assisted extraction of total phenolic compounds from barley seed using response surface methods and to evaluate the radical scavenging properties of extracts obtained.

MATERIALS AND METHODS

Materials

Seeds of barley (*Hordeum vulgare* L.) grown in Qassim, Saudi Arabia region, were obtained from local market and their final moisture content was maintained at approximately 6.8% w/w after drying in hot air oven. With intact hulls, they were ground to a powder form using grinder and sieved from 1 mm pore size sieve. Ethanol (96% v/v) was purchased from BDH Laboratory Supplies (Poole, U.K.) and all other analytical chemicals were from Sigma Chemical Co. (St. Louis, MO).

Preparation of Extracts

Seed extracts were prepared in a shaking water bath (Model 1083, Ollman & Co KG, Friedberg, Germany) at a continuous speed of 30 rpm by mixing 2 g of barley seed powder with 100 mL ethanol, filtered through Whatman No. 5 filter paper and evaporated until dry. Ethanol is generally considered as a safe alcohol and may be preferred over methanol and other organic solvents. The percentage yield of barley extracts was obtained as $100 \times DW_{extract}/DW_{sample}$, where $DW_{extract}$ is the weight of dried extract, and DW_{sample} is the dry weight of barley powder. The yield of extracts ranged from 8 to 11%. For analysis, dried extract was stored at 4C before analyses. It was redissolved in ethanol to make a total volume of 100 mL to carry on analytical work.

Experimental Design

The experiments were based on orthogonal array design (OAD) in order to optimize the temperature, time and ethanol concentration used in extraction process for the optimal recovery of phenolics from barley seed powder. The orthogonal design consisted of L_{16} matrix and the count of variables was three, each variable contained four levels and experiments were arranged accordingly as shown in Table 1. The temperature was 45, 50, 55 and 60C, ethanol concentration was 65, 70, 75 and 80%, and the extraction time was 3, 4, 5 and 6 h. These conditions were selected based on a series of preliminary trials whereby we observed that maximum values for dependent variables were achieved when using above conditions and any further increase did not positively affect the response variables. The response or dependent variables were extract yield and total phenolic compounds (being the main response for complete process optimization and experimental verification). The data obtained after quantification of response variables through triplicate measurements were subjected to regression analysis to obtain an experimental second-order polynomial model (Eq. 1) as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

where Y represents the response variable, and X_1, X_2 and X_3 are independent extraction process variables, namely extraction temperature, time and ethanol concentration, respectively. The β_n presents the value of corresponding coefficient obtained after regression analysis.

Analysis of Phenolic Compounds

The analysis of total phenolics obtained from ethanolic extracts of barley was based on Folin–Ciocalteu reagent (FCR) methods as explained by Lim *et al.* (2010). A 200 μL

TABLE 1. ORTHOGONAL ARRAY EXPERIMENTAL DESIGN AND TOTAL PHENOLIC COMPOUNDS OF BARLEY SEED EXTRACTS

No.	Temperature (C)	Time (h)	Ethanol (%)	Yield (%)	Total phenolics (mg GAE/g DW)*
1	45	3	65	7.05 ± 0.67	1.92 ± 0.07
2	45	4	70	7.52 ± 1.11	1.94 ± 0.11
3	45	5	75	8.14 ± 0.87	1.95 ± 0.17
4	45	6	80	8.23 ± 0.42	2.03 ± 0.07
5	50	3	70	8.20 ± 0.58	1.99 ± 0.03
6	50	4	65	8.41 ± 0.38	2.01 ± 0.06
7	50	5	80	8.40 ± 1.01	2.04 ± 0.05
8	50	6	75	8.72 ± 0.84	2.08 ± 0.10
9	55	3	75	8.55 ± 0.48	2.03 ± 0.10
10	55	4	80	9.12 ± 0.62	2.07 ± 0.11
11	55	5	65	9.24 ± 0.52	2.09 ± 0.06
12	55	6	70	9.58 ± 0.47	2.14 ± 0.11
13	60	3	80	9.55 ± 0.35	2.05 ± 0.07
14	60	4	75	9.64 ± 0.42	2.08 ± 0.08
15	60	5	70	9.75 ± 0.53	2.15 ± 0.11
16	60	6	65	9.79 ± 0.55	2.18 ± 0.12

* Analytical results represented by means ($n = 3$) ± SD.

sample of properly diluted barley extract or standard solution was mixed with 400 µL of FCR. The volume of this reaction mixture was made 4.6 mL with the addition of distilled water. The reaction mix was shaken, and held for 10 min at room temperature. Afterward, 1 mL of 10% Na_2CO_3 was added and then mixed thoroughly. After incubation in the dark for 90 min, the absorbance was measured at 760 nm using a spectrophotometer (Ultrospec II 4050, LKB Biochrom, Cambridge, U.K.). The blank solution consisted of all the chemicals except the barley extract or standard compound solutions and prepared following the above described methods. The quantities of total phenolics were presented as milligram gallic acid equivalents per gram of dried barley (mg GAE/g DW), with the help of a calibration curve obtained using gallic acid as standard at varying concentrations.

DPPH Radical Scavenging

The commonly used 1,1-diphenyl-2-picrylhydrazyl or DPPH assay (Ghafoor *et al.* 2011) was used to evaluate the free radical scavenging activity of barley extract containing phenolic compounds. The barley extract sample was diluted fivefold and 1 mL of this diluted sample was added to 2 mL of DPPH radical solution that was obtained by mixing 1 mg DPPH in 100 mL of methanol (99.9). This mixture was shaken well and kept at room temperature for 5 min for radical scavenging reaction of barley antioxidants to complete. The absorbance values (Δ_{517nm}) were obtained using spectrophotometer. The lower absorbance values of the reaction mixture indicate that antioxidants eradicated more free radicals and vice versa. The mechanism of this method is based on a reduction reaction (DPPH radicals are reduced by the antioxidant compounds in a certain plant extract) and the purple color of DPPH solution changes to yellow due to production of diphenyl picrylhydrazine (Ghafoor *et al.* 2010). The control was made by mixing 1 mL of distilled water in 2 mL of DPPH solution and the free DPPH radical scavenging activities (DRA) were calculated using Eq. (2):

$$DRA[\%] = \frac{\left(\Delta_{517\text{ nm control}} - \Delta_{517\text{ nm sample}}\right)}{517\text{ nm control}} \times 100 \qquad (2)$$

Statistical Analysis

The analytical measurements were obtained in triplicate analysis in each case and data were presented as means ± standard deviation ($n = 3$). The data on response variables obtained after performing experiments according to designed experiments were subjected to statistical analysis using multiple nonlinear regression for determination of the coefficients of the second-order polynomial model. The fit of the polynomial model was qualified and presented using the coefficient of determination R^2, the statistical significance of R^2 was assessed through an F-test. The optimization of extraction conditions for barley phenolics was accomplished by plotting the significant variables on response surface plots. The statistical analysis was carried out in Statistical Analysis System software (v 9.1, SAS Institute, Cary, NC). Data were analyzed using analysis of variance and the significance was set at $P < 0.05$.

RESULTS AND DISCUSSIONS

Extraction Process Modeling for Phenolics from Barley Seed

Optimization of phenolic compounds from barley seed was based on OAD experiments as shown in Table 1, which also

TABLE 2. REGRESSION COEFFICIENTS AND ANALYSIS OF THE MODEL FOR EXTRACT YIELD AND TOTAL PHENOLIC COMPOUNDS FROM BARLEY SEED

		Estimate		Standard error		*t* value		*P* value	
Coefficient	df	Extract yield	Phenolics	Extract yield	Phenolics	Extract yield	Phenolics	Extract yield	Phenolics
β_0	1	–4.405	–0.573	10.5134	7.9878	–0.42	–0.02	0.6898	0.9850
β_1	1	0.185	0.621	0.3270	0.2897	0.57	2.14	0.0319	0.0259
β_2	1	1.764	3.285	1.5004	1.1542	1.18	2.79	0.0443	0.0245
β_3	1	0.023	0.028	0.0872	0.1875	0.26	0.23	0.8007	0.6352
β_{11}	1	–0.001	–0.0025	0.0020	0.0389	–0.42	–0.79	0.6915	0.2578
β_{22}	1	–0.023	–0.0639	0.0518	0.0689	–0.42	–0.79	0.6915	0.3589
β_{33}	1	–0.001	–0.0002	0.0010	0.0254	–0.57	–0.25	0.5867	0.8345
β_{12}	1	–0.018	–0.0496	0.0151	0.0280	–1.19	–2.36	0.2796	0.0582
β_{13}	1	0.002	–0.00013	0.0026	0.0108	0.58	–0.07	0.584	0.9854
β_{23}	1	–0.005	–0.0043	0.01307	0.0425	–0.39	–0.39	0.7102	0.7528

presents the analytical values of percent extract yields and total phenolic compounds of barley seed extracts at different sets of experimental variables. The coefficient values are given in Table 2. The outcomes of data analysis for analysis of variance, model adequacy and goodness of fit are presented in Table 3. A Student's *t*-test and *P* values were used to evaluate the significance of regression coefficients as shown in Table 2. The results showed good fitness with Eq. (1), which was acceptable when $P < 0.05$ and passable with satisfactory R^2 values.

Effect of Extraction Variables on the Yield and Total Phenolics in Extract from Barley

The extraction used in this study can be regarded as solid-liquid extraction in which constituents from a solvent matrix migrate to solvent phase. This phenomenon of solutes transfer can be improved by changing the coefficients for diffusion as result of temperature of extraction, concentration of solvent and extraction time (Ghafoor *et al.* 2011). The extract yield was calculated and results are shown in Table 1. It was observed after regression analysis of crude extract yield that temperature, time and linear regression terms of these two variables significantly ($P < 0.05$) affected the yield. Detailed regression analysis data and coefficient values for yield are presented in Tables 2 and 3, and the results are summarized in Eq. (3), which was based on the significant coefficient values as under

$$Y_1 = 0.18512X_1 + 1.76379X_2 \quad (3)$$

where Y_1 is the extract yield, and X_1 and X_2 represent temperature and time, respectively. The model's R^2 value was 0.978, the adjusted R^2 value was 0.985, *F* value was 29.95, and *P* value was 0.0003. Based on Eq. (3), a response surface plot was constructed to show the relationship between temperature and time for the extract yield as shown in Fig. 1.

The main objective of this study was to optimize the extraction of phenolic compounds from barley seed, and the quantities of these compounds obtained under different extraction experiments with variable temperature, time and solvent concentration are presented in Table 1. The optimal recovery of total phenolic compounds (2.18 mg GAE/g DW) from barley seed was possible in the experiment where the extraction was performed at 60C for 6 h using 65% ethanol as solvent. As described before, a multiple regression test of data was performed to estimate the model coefficients and the significance. The effects of temperature (45–60C) and time (3–6 h) of extraction on the total phenolic compounds were significant ($P < 0.05$); however, that of ethanol concentration (65–80%) was nonsignificant. Increase of extraction time or that of temperature allows movement of more soluble compounds from plant matrix

TABLE 3. ANALYSIS OF VARIANCE OF THE SECOND ORDER FOR EXTRACT YIELD AND TOTAL PHENOLIC COMPOUNDS OF THE BARLEY SEED MODEL

		Sum of squares		Mean square		*F* value		*P* value	
	df	Extract yield	Phenolics	Extract yield	Phenolics	Extract yield	Phenolics	Extract yield	Phenolics
Total model	9	9.984	43.159	0.9784	0.975	29.95	47.05	0.0003	<.0001
Linear	3	9.8794	42.369	0.9684	0.982	88.92	152.80	<.0001	<.0001
Quadratic	3	0.0274	0.152	0.003	0.003	0.24	0.49	0.8634	0.6250
Cross product	3	0.0774	0.549	0.008	0.012	0.69	1.89	0.5886	0.1254
Total error	6	0.2224	0.653	0.037	0.633				

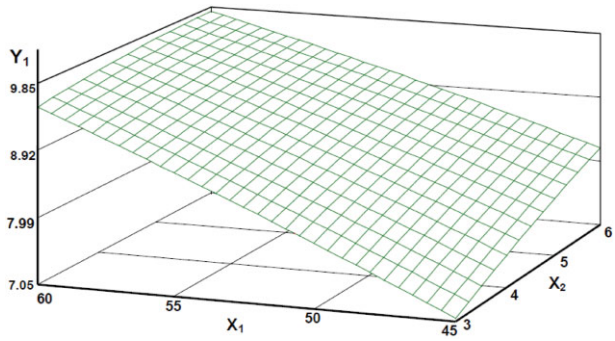

FIG. 1. THE RESPONSE SURFACE PLOTS OF TOTAL EXTRACT YIELD (Y_1) FROM BARLEY SEED EXTRACT AS AFFECTED BY TEMPERATURE (X_1) AND TIME (X_2) DURING THE EXTRACTION PROCESS

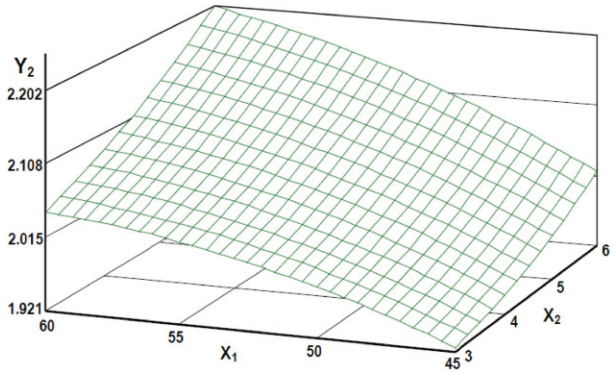

FIG. 2. THE RESPONSE SURFACE PLOTS OF TOTAL PHENOLIC COMPOUNDS (Y_2) FROM BARLEY SEED EXTRACT AS AFFECTED BY TEMPERATURE (X_1) AND TIME (X_2) DURING THE EXTRACTION PROCESS

into extraction medium. The coefficient values as presented in Table 2 were applied to construct an equation (Eq. 4) for prediction. The nonsignificant quadratic and cross product terms were neglected, and the final simple model was given as follows:

$$Y_2 = 0.62114X_1 + 3.28541X_2 \quad (4)$$

where Y_2 represents the response variable (total phenolic compounds), and X_1 and X_2 are significant process variables, i.e., temperature and time for extraction, respectively. Based on this model, the response surface plot showing the linear relation between temperature and time of extraction was constructed, which is presented in Fig. 2. This shows a linear increase in total phenolic compounds by increasing temperature while keeping the time constant or vice versa. On the basis of these findings, the predicted extraction conditions (based on regressing analysis of the experimental data) were 58C temperature, 5.4 h time and 65% ethanol for the maximum possible amount of total phenolic compounds (2.31 mg GAE/g DW) from barley seed. The model's R^2 value was 0.986, the adjusted R^2 value was 0.981, F value was 49.05, and P value was 0.0001. This shows that the model was good in estimating the relationship between variables chosen in this study. The comparison of actual and predicted values was done by performing extraction experiments using predicted conditions for maximal extraction of phenolics. The actual yield of phenolics from barley on predicted conditions was 2.28 ± 0.17 mg GAE/g DW, which was close to that of predicted value. A Student's t-test was used to compare predicted and actual values which showed nonsignificant differences. Hence, a good correlation between these results showed that the model was sufficient to estimate the optimized recovery of phenolic compounds from barley seed. The results of this research can be effectively used in establishing a simple and economical ethanol (safe alcohol) based method for the recovery of phenolics from barley seeds.

Antiradical Activities of Barley Seed Extract

The data about antiradical activities of barely seed extracts (1–16) and one obtained by carrying extraction on optimized conditions (58C, 5.4 h and 65% ethanol, phenolics 2.18 mg GAE/g) by using DPPH radical scavenging assay are presented in Fig. 3. The optimized barley seed extract scavenged 65.24% of the DPPH radicals in this study, which was higher than the other extracts obtained as shown in Table 1. The extracts 10, 11 and 12 had antiradical activities in the range of 61–63%, whereas all other extracts had these activities in the range from 52 to 60%. Antiradical activities

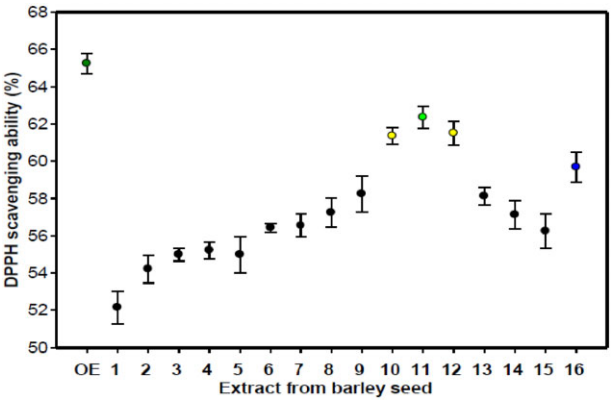

FIG. 3. ANTIRADICAL PROPERTIES OF BARLEY SEED OPTIMIZED EXTRACT (OD) AND THOSE PREPARED ACCORDING TO ORTHOGONAL DESIGN (1–16) AND ASSESSED USING DPPH ASSAY RESULTS ARE SHOWN ALONG WITH STANDARD ERROR OF MEANS ($N = 3$)

of the barley seed extract were in close agreement with those determined by Lee *et al.* (2010), who observed that the DPPH radical scavenging activity of barley seed by-products and milled barley seed was up to 65%. The presence of phenolic compounds can impart various health-promoting properties to foods such as cereal products, and it was observed that the textural characteristics of baked products made with flour fortified with phenolics were acceptable to consumers (Lim *et al.* 2010). It has been previously reported that phenolic compounds are a major group of phytochemicals or bioactive compounds, and there is usually a direct correlation between phenolic compounds and antioxidant properties of plant extracts (Ghafoor and Choi 2009). Therefore, the barley extracts containing the maximum amount of phenolics (optimized extract) showed the highest ability to scavenge DPPH radicals in this study.

The cost of an extraction process and quality of final products can be controlled through optimization of process temperature and time, which are mostly among the main process variables. It is generally agreed that that high temperature may improve the yield of extraction through improved solute solubility and coefficient of diffusion, but very high temperature can affect the structure and, hence, functionality or quality of phenolics (Yilmaz and Toledo 2006). The significance of temperature of extraction for other plant materials has also been previously documented. In one of the study on grape marc (Spigno and De Faveri 2007), it was observed that yield of phenolics was significantly higher at 62C than that obtained at 27C. However, it should be considered that beyond certain temperatures, the extraction yield of polyphenolic compounds may decrease due to heat-induced degradation and/or increase in polymer length which may also result in erroneous quantification (Pinelo *et al.* 2005). Considering these results, we performed our study for optimization of phenolic compounds from barley seed at a temperature range from 45 to 60C. The temperature effects, however, cannot be generalized because they have close relationship with nature of compounds. As such, Cacace and Mazza (2003) observed that temperature in the range from 30 to 35C was sufficient for anthocyanin extraction from ribes when using 85% ethanol. Herodez *et al.* (2003) observed that even less than 30C temperature was sufficient for the best yields of ethanol-assisted extraction phenolic acids from leaves of balm. It is also notable that researchers in these studies used higher concentrations of ethanol that may have reduced the need for higher temperatures. Hence, we can conclude that the temperature of extraction is a very significant factor for recovery of bioactive compounds from plant materials and should be carefully optimized considering the nature of compounds and the type of raw material. Extraction time (1–4 h) was observed to be other significant variable for the extraction of phenolic compounds from barley seed, which is in agreement with the findings of other researchers (Spigno *et al.* 2007) who observed that the yield of phenolics from plant matrix such as grape marc increased significantly with increasing the time of extraction up to 5 h; however, beyond that and up to 25 h, the effect of time was nonsignificant.

Alcohols and acetone solutions in water at different concentrations have been routinely applied in the recovery of phenolics from plant materials such leaves and stems of herbal plants. The selection of an extraction solvent is dependent generally on the extraction objectives, the polar nature of the desired and undesired compounds, the cost of the process, and safety for human and environment (Yu *et al.* 2002). Adding a certain amount of water in ethanol might improve the extraction efficiency, besides the fact that it is a safe alcohol compared with other organic solvents. The solvent used in our study was ethanol at varying concentrations in water (40–70%); however, the effect was observed to be nonsignificant in this range. Ethanol is considered to be a safe alcohol for dietary applications, and even a lower concentration can be used for extraction purposes. Furthermore, higher concentration of ethanol such as above 90% may not be feasible for the extraction of phenolic compounds due to the simultaneous extraction of lipid fractions, which reduces the yield of target phenolics (Wang *et al.* 2008). Reactive oxygen species are formed within the human body due to various chemical reactions, pollution, smoking, stress and various other factors. These generate free radicals that are harmful to living cells and can cause various health disorders such as inflammation, tumor and cancer development. In addition, oxidation reactions in food systems can also result in unwanted changes that reduce the quality and shelf life of foods (Ghafoor and Choi 2009; Casarotti and Jorge 2014). Plant-based functional foods and pharmaceutics may have an important objective of removing these free radicals to promote health and to prevent diseases. This objective is largely fulfilled by the bioactive compounds such a phenolics present in nutraceuticals and pharmaceutics. It was observed in the present study that barley seed phenolics can effectively act as free radical scavengers or antioxidants, and that the extracts obtained after optimization of extraction process for maximum recovery of phenolic compounds were more effective antioxidant sources (Tang *et al.* 2014). There is a need for more studies whereby various other process parameters such as particle size, stage of maturity, moisture, sample preparation, varietal differences, organic solvent types, and conventional and modern extraction methods should be studied to find out the best suitable combination of different variables for optimal recovery of phenolics from barely seeds, and for maximum bioactive potential of such compounds.

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