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Optimizing conditions for antioxidant extraction from Sea Buckthorn leaf (*Hippophae rhamnoides* L.) as herbal tea using response surface methodology (RSM)

Touseef Ahmed Wani, *Wani, S. M., Shah, A. G. and Masoodi, F. A.

Department of Food Science and Technology, University of Kashmir, Srinagar, India-190006

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Abstract

Response Surface Methodology (RSM) was employed to optimize the conditions for antioxidant potential and polyphenols from sea buckthorn (*Hippophae rhamnoides* L.) leaf powder using two variables: time (20, 30, 40, 50, 60 and 70 min.) and temperature (70, 80, 90 and 100°C). The results showed that antioxidant potential and total polyphenols in the experiments varied from 76.44 to 88.82% and 67.91 to 88.69 GAE/g respectively. The F-values for antioxidant potential and total polyphenols were 16.96 and 0.72 respectively, with the respective coefficient of determination (R^2 values) of 0.8249 and 0.1661. Under the optimum conditions of 37.02 minutes and 74.20°C the values for antioxidant potential and total polyphenols were 85.34% and 72.13 GAE/g respectively. These conditions can be used to produce herbal extracts from sea buckthorn leaves.

Introduction

Sea buckthorn (*Hippophae rhamnoides* L., family: Elaeagnaceae) growing in North-West Himalayas at high altitude, 7000–15,000 feet, is a dwarf to tall (3–15 feet) nitrogen fixing, thorny, deciduous shrub, found in Europe, central Asia and temperate regions of South Asia, India and China, that has been domesticated in several countries (India, China, Nepal, Pakistan, Myanmar, Russia, Britain, Germany, Finland, Romania, France, etc.) (Rousi, 1971). Against their synthetic counterparts that pose a threat of carcinogenesis, bioactive compounds with antioxidant properties present in spices and different herbs have gained interest in the recent past and among them sea buckthorn is one of the most important versatile nutraceutical crop with diverse uses, from controlling soil erosion to being a source of horse fodder, nutritious foods, drugs, and skin-care products (Rodriguez-Meizoso *et al*., 2006; Fan *et al.*, 2007).

All parts of the plant i.e. fruits and leaves are considered to be good source of large number of bioactive substances like vitamin (A, C, E, riboflavin, folic acid and K), carotenoids (α, β, ð-carotene and lycopene), and flavonoids (isorhamnetin, quercetin, myricetin, kaempferol and their glucoside compounds), organic acids (malic acid and oxalic acid), sterols (ergosterol, stigmasterol, lanosterol and amyrins) and some essential amino acids (Hakkinen *et al.*, 1999; Upendra *et al.*, 2008). The leaves of the plant are rich in flavonoids, tannins and triterpenes, and they have been used in some countries to make extracts, tea, animal feed, pharmaceuticals and cosmetics (Beveridge *et al*., 1999; Kallio *et al.*, 2002). Sea buckthorn has been shown to have many other benefits, including preventive effects against influenza infection, cardiovascular disease, mucosal injuries, skin disorders and the adaption to the effects of stress (Eccleston *et al*., 2002; Saggu *et al*., 2007). In particular, extracts from sea buckthorn leaves exhibited antioxidant, anti-bacterial, anti-viral, anti-tumor and immunomodulatory properties (Tsybikova *et al*., 1983; Ganju *et al*., 2005).

Gallic and ellagic acids of sea buckthorn leaf extracts are anti-diabetic and radio-protective (Pandurangan *et al*., 2011). The aqueous and hydroalcoholic extracts of Sea buckthorn leaves are reported to have marked cytoprotective activities (Nitin *et al*., 2010). The phenolic rich fraction of sea buckthorn leaves has hepatoprotective action against oxidative damage (Maheshwari *et al.*, 2011). Taken together, sea buckthorn leaf extracts have significant potential as natural antioxidants and in disease prevention.

As only a specific quantum of antioxidants are available in a given food sample, the fact that antioxidant activity is dependent on the polyphenols released in solution and the released polyphenols being sensitive to heating, it becomes imperative to know the optimum time and temperature to achieve the optimum extraction avoiding unnecessary boiling

*Corresponding author.
Email: *wanisajad82@gmail.com*
Tel.: +91 9858445878

of the food and the wastage thereof. The objective of present work was to optimize the conditions including temperature and time for aqueous extraction of antioxidants and estimation of polyphenols thereof from sea buckthorn leaves.

Materials and Methods

Plant material

Fresh leaves of sea buckthorn were collected from the hilly regions of Ladakh, (Jammu and Kashmir) India in the month of September, in which the plant grows widely under natural condition. The leaves were reduced to powder using electric grinder and stored in a dark place at room temperature before use.

Bulk density

It was calculated for the leaves by dividing the mass of a quantity of leaves by its volume, which was measured by using a constant volume cylinder.

$Bd = W/V$

Where:

Bd = Bulk density of leaves in kg/m^3,

W= Mass of the same quantity of leaves in kg and

V= Volume of the same quantity of leaves in m^3.

Coefficient of friction

The static coefficient of friction of sea buckthorn leaves was determined against the surfaces of plywood, cardboard, newspaper and polyethylene. A wooden stand with an inclinable surface was used for this purpose. The inclinable surface was raised gradually until approximately 75% of the sea buckthorn leaves slided down. By measuring the angle of surface (θ) at this state, static coefficient of friction was considered as tanθ.

Angle of repose

The angle of repose is the angle with the horizontal at which the material will stand piled. The angle of repose of dry sea buckthorn leaves (moisture content 8.6 ± 0.29%) was determined by Digimizer software.

True density

The leaves were reduced to powder using an electric grinder and true density was calculated by dividing the mass of a quantity of leaf powder by its volume, which was measured by using a constant volume cylinder.

$Td = W/V$

Where:

Td: True density of leaf powder in kg/m^3,

W: Mass of the same quantity of leaf powder in kg and

V: Volume of the same quantity of leaf powder in m^3.

Extraction procedure

(1:5 w/v) of sea buckthorn leaves powder was heat extracted in water to the requisite time temperature combination using a water bath. The extract was filtered through Wattman No. 4 filter paper and then centrifuged at 10000 rpm for 10 minutes at 4°C. The extract was analyzed fresh every time.

Determination of total phenol content

Total phenol content of extract was determined according to the method of Thaipong *et al*. (2006). 150 µL of extract, 2400 µL of nanopure water and 150 µL of 0.25N Folin-Ciocalteu reagent were combined and then mixed well by shaking. The mixture was allowed to react for 3 minutes then 300 µL of 1N Na_2CO_3 solution was added and mixed well again by shaking. The solution was incubated at room temperature in the dark for 2 hours. The absorbance was measured at 725 nm using a spectrophotometer and the results were expressed as milligram of Gallic acid equivalents (GAE) per 1 gram of extract using standard curve prepared from Gallic acid (0.1 mg/ml) solution.

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The antioxidant activity of sea buckthorn leaf was determined according to the method of Matthus (2002). The DPPH radical scavenging assay is regularly used for the relatively rapid evaluation of the antioxidant activity. DPPH is a stable free radical, even at room temperature, and shows strong absorbance at 515 nm. The DPPH radical accepts an electron or hydrogen radical to become a stable diamagnetic molecule with a different colour. Thus the degree of its discoloration from purple to yellow is attributed to the hydrogen donating ability of the added compound, which is indicative of its radical scavenging potential.

80 µL of the sample was mixed with 200 µL of 0.05% DPPH in a total volume of 4ml methanol and allowed to react in the dark for 30 minutes. The results were expressed as percent inhibition using the relation

% inhibition= (Absorbance of control-Absorbance of sample)/Absorbance of control×100

Experimental design and statistical analysis

Design-expert software (Trial Version 8.0.7.1,

Stat-Ease Inc.) was used for the experimental design and statistical analysis of the data. User defined design (UDD) was used to examine the effects of the two variables: time (X_1) and temperature (X_2), as presented in Table 2. A total of 24 experimental runs were completed. The properties of sea buckthorn leaves expressed as the dependent variables were determined: Percent Inhibition (%) (R_1) and Total phenols (GAE/g) (R_2). These variables were expressed individually as a function of the independent variables. The quadratic model for predicting the optimal point was expressed according to the equation

$$Y = \beta_0 + \sum_{i=1}^{2} \beta_i x_i + \sum_{i=1}^{2} \beta_{ii} x_i^2 + \sum_{i<j}^{2} \beta_{ij} x_i x_j$$

where: β_0 is the value for the fixed response at the central point of the experiment and β_i, β_{ii} and β_{ij} are the linear, quadratic and cross-product coefficients, respectively. The statistical significance of the backward regression coefficients was determined by Student's t-test, the second order model equation was determined by Fischer's test and the proportion of variance explained by the model was given by the multiple coefficient of determination, R^2. The optimum concentrations of the variables were obtained by the graphical and numerical analyses using the Design Expert program, based on the criterion of desirability.

Results and Discussion

Physical properties of sea buckthorn leaves

Dry sea buckthorn leaves having a moisture content of 8.6 ± 0.29% were analysed for the physical properties- bulk density, true density, angle of repose and coefficient of friction (plywood, cardboard, newspaper, polyethylene), the average values of which along with the standard deviations are shown in Table 1. The highest value of coefficient of friction was observed for cardboard 46.31° while the lowest value was observed for plywood sheet 26.50°. The angle of repose for the leaf powder being 47.31°. These properties play an important role in designing of various equipments and significantly influence extraction rate.

Analysis of response surface

The water extract obtained from (1:5 w/v) of sea buckthorn leaves powder was analysed for its antioxidant potential and the corresponding total poly phenols following 24 different combinations of two independent variables, viz. time and temperature as per the experimental design (Table 2).

The results showed that antioxidant potential and total polyphenols in the experiments varied from 76.44 to 88.82% and 67.91 to 88.69 GAE/g respectively. The dependent variables were analysed to obtain a regression equation that could predict the responses under the given range. The quadratic models obtained from regression analysis for antioxidant potential Y_1 and total polyphenols Y_2 were as follows:

$$Y_1 = 88.64 + 2.80\ X_1 + 1.84\ X_2 - 3.10\ X_1X_2 - 3.05\ X_1^2 - 0.089\ X_2^2$$

$$Y_2 = 71.40 + 0.29\ X_1 - 1.86\ X_2 - 0.97\ X_1X_2 - 1.98\ X_1^2 - 0.15\ X_2^2$$

Where X_1 and X_2 are the coded variables for time and temperature respectively.

The Analysis of Variance (ANOVA) indicated the model F-value of 16.96 and 0.72 (Table 3) for antioxidant potential and total polyphenols respectively at a 5% level of significance, which implies that the model is significant for antioxidant potential but not significant for total polyphenols, which is also the case for the values of the R^2 for

Table 1. Physical properties of sea buckthorn leaves

Physical property	Mean Values
Bulk density	0.08 ± 0.01g/ml
True density	0.20 ± 0.00 g/ml
Angle of repose	47.31^0 ± 0.75
Coefficient of friction (Plywood)	26.50^0 ± 1.18
Coefficient of friction (Cardboard)	46.31^0 ± 1.55
Coefficient of friction (Newspaper)	45.18^0 ± 1.57
Coefficient of friction (Polyethylene)	42.05^0 ± 1.10

Table 2. Experimental design for antioxidant potential and total polyphenols

Runs	Time (minute) X_1	Temperature (°C) X_2	Percent Inhibition (%) Y_1	Total Phenols (GAE/g) Y_2
1	60	70	88.6 ± 1.10	68.6 ± 1.11
2	50	80	88.8 ± 1.10	72.1 ± 0.96
3	30	80	84.5 ± 0.85	71.2 ± 1.73
4	40	100	88.6 ± 1.30	68.7 ± 0.73
5	60	80	88.6 ± 1.20	72.7 ± 2.11
6	40	70	86.1 ± 1.80	69.0 ± 1.10
7	50	70	88.6 ± 0.50	88.6 ± 2.11
8	20	90	86.8 ± 0.10	70.7 ± 3.11
9	30	100	88.8 ± 1.10	68.6 ± 1.11
10	40	90	88.8 ± 1.01	68.4 ± 1.73
11	50	100	88.6 ± 1.50	69.0 ± 1.31
12	70	80	88.6 ± 1.31	72.3 ± 1.79
13	70	100	88.6 ± 1.20	69.1 ± 2.10
14	50	90	88.8 ± 1.20	68.3 ± 2.19
15	20	100	88.8 ± 1.11	69.0 ± 1.20
16	70	90	88.6 ± 0.13	68.3 ± 2.13
17	30	70	83.4 ± 1.10	67.9 ± 1.95
18	60	100	88.8 ± 1.10	68.2 ± 1.06
19	60	90	88.6 ± 1.10	68.3 ± 2.11
20	40	80	88.6 ± 1.08	72.3 ± 1.90
21	70	70	88.5 ± 1.03	68.4 ± 2.90
22	20	80	76.4 ± 0.81	71.8 ± 3.31
23	30	90	88.6 ± 0.50	68.5 ± 1.11
24	20	70	77.3 ± 0.77	68.2 ± 2.13

Table 3. Analysis of Variance (ANOVA) for surface quadratic model

Response	Source of variation	Sum of squares	Degree of freedom	Mean square	F – value	p-value Prob > F
	Model	228.27	5	45.65	16.96	< 0.0001* significant
Antiox.	Residual	48.46	18	2.69		
Potentiol	Total	276.73	23			
		R^2 = 0.8249		R^2 (Adj) = 0.7762		
	Model	67.84	5	13.57	0.72	0.6190*
Poly						not significant
Phenols	Residual	340.69	18	18.93		
	Total	408.52	23			
		R^2 = 0.1661		R^2 (Adj) = -0.6656		

*Significant at $p < 0.05$

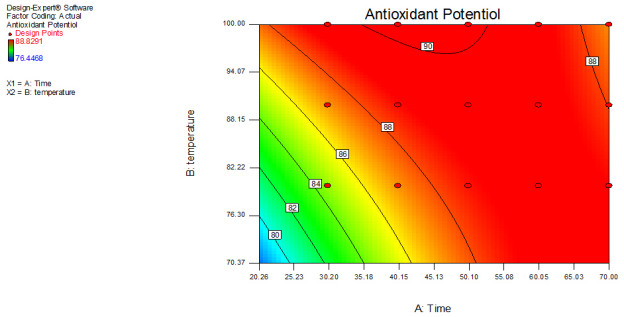

Figure 1. Effect of time and temperature on antioxidant potential (contour)

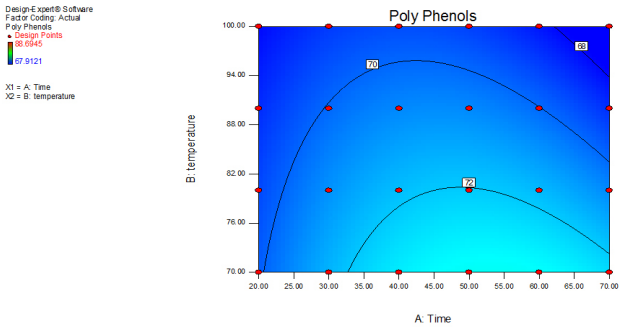

Figure 2. Effect of time and temperature on total polyphenols (contour)

antioxidant potential and total polyphenols. The R^2 values for antioxidant potential and total polyphenols were 0.8249 and 0.1661 respectively, which means that the calculated model was able to explain 82.49 and 16.61% of the results in the case of antioxidant potential and total polyphenols respectively. The adjusted R^2 values for antioxidant potential and total polyphenols were 0.7762, and -0.0656 respectively, out of which only antioxidant potential was found in agreement with the R^2 value. Therefore, the model was found to be adequate in representing the response data of the antioxidant potential only but not total polyphenols and can be further used for analysis and prediction purposes for the same response.

The significant effects of independent variables and their mutual interaction on the antioxidant potential and total polyphenols can be seen on the contour plots shown from Fig. 1-2. The plots were generated by plotting the response, using the z-axis against two independent variables. Fig. 1 show the interaction between time and temperature on the antioxidant potential. Increase in time from 20–45.13 minute with increase in temperature from 70–94.05°C enhanced the antioxidant potential, while increasing time over 45.13 minute and temperature over 94.05°C did not show a significant variation of the antioxidant potential.

Fig. 2 indicates the effect of time and temperature total polyphenols. Increase in time from 20–56.96 minute with increase in temperature from 70–89.34°C enhanced the total polyphenols, while increase time over 56.96 minute and temperature over 89.34°C did not show a significant variation of the total polyphenols.

Conclusion

Sea buckthorn leaves showed the highest and the lowest value of the coefficient of friction for cardboard and plywood respectively. The water extract obtained from sea buckthorn leaf powder (1:5 w/v) was analysed for its antioxidant potential and the corresponding total polyphenols following 24 different combinations of two independent variables, viz. time and temperature as per the experimental design. F-value of 16.96 implies that the model is significant for antioxidant potential with R^2 value of 0.8249. F-value of 0.72 implies that the model is non significant for total polyphenols with a low R^2 value of 0.1661. The antioxidant potential in sea buckthorn leaf can be brought to the desired level by a simple combination of time and temperature. Using Response Surface Methodology (RSM), the optimum condition of time and temperature were obtained. These optimum conditions were 37.02 minutes at 74.20°C at which the values for antioxidant potential and total polyphenols were 85.34 % and 72.13 GAE/g respectively. These conditions can be used to produce herbal tea extracts from sea buckthorn leaves.

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