



Enzymatically assisted extraction of antioxidant and anti-mutagenic compounds from radish (*Raphanus sativus*)

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ABSTRACT

In addition to being sources of carbohydrates, protein, and fiber, vegetables, and fruits also represent important sources of micro-nutrients, including antioxidants, and anti-mutagens in the form of carotenoids, and polyphenols, such as flavonoids. We describe the enhanced extraction of these bioactive compounds from radish (*Raphanus sativus* L.) by use of an enzymic pre-treatment to break up starch, and the complex mix of polysaccharides, cellulose, hemicellulose, and pectin, which constitute the plant cell wall. The enzymic cocktail included cellulases, pectinases, amylases, glucanases, and hemicellulases. Response surface methodology (RSM) was utilized to maximally explore the yield by estimating extraction conditions, enzyme concentration, incubation time, temperature, pH, and extraction solvents. Extracted antioxidant activity was assayed in terms of total phenolics content (TPC), free radical scavenging (DPPH and ABTS tests), inhibition of linolic peroxidation assay, Reducing potential, and Trolox equivalent antioxidant capacity (TEAC). Two different bacterial mutant strains (TA-98, TA-100, *S. typhimurium*) were used by applying Ames bacterial test. It was concluded that highest desirability was achieved at 3.53% (w/w) of enzyme formulation, at pH of 6.1, incubation time 66.08 min and 46.08 °C temperature produced 19.2 g/100 g FW of crude extract which contains 45.9 mg GAE/g FW total phenolics contents, 0.480 mg/mL Reducing Power, 85.9% LA inhibition and 271.7 mmole/g FW of TEAC in fresh radish root. We found that radish extract was an excellent source of antioxidants that might be further investigated for commercial exploitation.

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1. Introduction

Phytochemicals present in many natural plant sources plays a major role in quenching and neutralize the free radical which is helpful in the cure and to lower the risk of human diseases. Phytochemicals gained an increased importance owing to new valuable findings in the biological systems (Ben-Shabat et al., 2020; R Vasanthi et al., 2012).

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Phytochemicals (carotenoids, vitamins, flavonoids, alkaloids, terpenoids, tannins and polyphenols) are natural antioxidants found in medicinal plants extracted to cure human diseases for thousands of years (Kennedy and Wightman, 2011; Saura-Calixto et al., 2010).

Several studies maximally explored vegetables as an inevitable part of diet owing to pharmaceutical, and nutritional benefits (Soundararajan and Kim, 2018). Antioxidant activities used the mechanism of manifesting free radicals, and impeding the oxidation of lipoproteins (low-density). Historically radish is used in folk medicines to treat several disorders including hepatic inflammation, stomach disorders, cardiac disorders, and urinary infections (Rakhmawati et al., 2009). Radish (*Raphanus sativus* L.) root vegetable is a highly valued member of the *Brassicaceae* family of plants that are grown and consumed throughout the world (Kitashiba et al., 2014). The radish crop represents some 2% of the total world vegetable production (7 million tons/year) and is a rich source of nutrients, and micronutrients including thiamine, pantothenic acid, riboflavin, vitamin C, Ca, Mg, Zn, P, K, and antioxidants, in particular, polyphenols (Arro and Labate, 2019). These nutrients are widely acknowledged for their contribution to the good health of consumers (Sedani et al., 2018; Tungmunthum et al., 2018).

Radish extract has been proven to be useful to treat several diseases and protect from harmful ailments such as cancer, and diabetes, liver infections, and inflammation. Researchers are focused on determining the regulatory pathways, and pharmacokinetic elucidation of bioactive compounds from radish extract. Overall, studies conducted on radish can play a key role in drug discovery with fewer side effects (Baenas et al., 2016; Kobayashi et al., 2021).

Radish composition was found to be mainly consist of flavonoids, phenolic acid and anthocyanins. These antioxidants are suggested to be successfully utilized in cosmetic and nutraceuticals. Researchers have investigated the cytotoxicity, Biogenic amines, and microbiological counts from radish extract (Papetti et al., 2014). Previously the anthocyanin content was investigated by a mixture of acidified water and 50% ethanol. It was concluded that dark storage is favorable to maintain the quality and shelf life while light may deteriorate radish contents (Xiao et al., 2014). Bound and free phenolics which include vanillic acid, coumaric acid, pyrogallol and epicatechin are most commonly found in radish root and leaves (Goyeneche et al., 2015).

Antioxidants are commonly extracted from plants using aqueous methanol (Do et al., 2014). Conventional methods of extraction were time-consuming and require a larger amount of solvent. However, it is not clear how effective this is in the presence of starch and the complex polysaccharides of the plant cell wall (cellulose, hemicellulose, and pectin). Although these polysaccharides can be broken up and dispersed by alkaline and/or acid hydrolysis, such harsh conditions can result in concomitant degradation of the targeted antioxidants (Castro-López et al., 2017; Conde et al., 2013).

With the emergence of non-conventional techniques, extraction becomes easy for us. New techniques require less amount of solvent and are rapid. One of the non-conventional techniques is enzyme-assisted extraction, in which enzymes assist extraction by making phenolic compounds less bound to their cell wall, hence became free (Dzah et al., 2020; Liu et al., 2019). This technique is environmentally friendly and requires relatively less amount of solvent. An alternative, mild approach to breaking up the complex polysaccharide matrix, and to improve the extractive yield of anti-oxidants is to use enzyme assisted extraction technique by hydrolytic enzymes (Chávez-González et al., 2020; Gaur et al., 2007; Ghandahari Yazdi et al., 2018; Liu et al., 2009; You et al., 2013). Such enzymes would include, for example, pectinases, cellulases, amylases, hemicellulases, and glucanases, which leave the anti-oxidants intact (Wang et al., 2017). The key objectives of this work are to maximally exploit the effective recovery of antioxidants by enzyme cocktail and investigating *in vitro* antioxidant, anti-mutagenic potential from Radish (*Raphanus sativus*) extracts. Response surface methodology (RSM) was employed to compare different extraction and maceration conditions for the effective recovery of antioxidants (Said and Amin, 2015; Saifullah et al., 2020; Sedraoui et al., 2020).

2. Material and methods

2.1. Collection of sample and chemicals

Radish (*Raphanus sativus* L.) samples were purchased from the local market used immediately and authenticated at the Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad, Pakistan. Chemicals, methanol, ethanol, sodium hydroxide, hydrochloric acid, 1,1-diphenyl-2-picrylhydrazine (DPPH), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS), potassium ferrocyanide, ferric chloride, ferrous chloride, 3,5-di-tert-4-butylhydroxytoluene (BHT), ammonium thiocyanate, linoleic acid, sodium carbonate, aluminum chloride, potassium persulfate, Folin-Ciocalteu reagent, gallic acid, trichloroacetic acid, acetic acid ascorbic acid, sodium nitrite, and other reference chemicals were acquired from Sigma (St. Louis USA) and Merck (Darmstadt, Germany). The enzyme cocktail includes β -xylosidase, xyloglucanase, pectinases, xylanase, α -amylases, β -glucosidase, and protease (Sigma-Aldrich; St. Louis, USA) were purchased from Ghazi Brothers, Pakistan. Mutant strains (*S. typhimurium* TA98 and *S. typhimurium* TA100) and culture media were acquired from Oxoid Ltd. (Hampshire, UK).

2.2. Experimental design

Temperature, pH, incubation time, and enzyme concentration for sample pre-treatment were optimized with the help of Response Surface Methodology (RSM) using a Small Rotatable Central Composite Design (SRCD) while 21 runs were

reported in this design which includes eight runs for each axial and factorial while five for center points. Each of these key factors were inspected at 5 different statistical levels with ($\alpha = 1.682$) as shown in Table 1. Statistical analysis was performed using Design Expert software (Version, 9.0.7.1) for numerical optimization, robustness and graphical representation. To evaluate the corresponding parameters and their interactions with other parameters a mean was calculated at 95% confidence interval. Six responses involving total phenolic contents (TPC), extract yield, DPPH, TEAC, ferric reducing antioxidant power (FRAP) and % inhibition peroxidation were determined using a polynomial equation of second order given as follows. Y

$$Y = b_0 + \sum_{i=1}^k b_i X_i + \sum_{i=1}^k b_{ii} X_i^2 + \sum_{i>1}^k \sum_j^k b_{ij} X_i X_j$$

where Y represents the factor to be optimized; b_0 is intercept; $\sum_{i=1}^k b_i X_i$ linear variables; $\sum_{i=1}^k b_{ii} X_i^2$ quadratic effect and $\sum_{i>1}^k \sum_j^k b_{ij} X_i X_j$ are interaction factors.

Multiple coefficient determination (R^2) and lack of fit test was applied statistically to check the fitness of the model and to evaluate the proportion of variance. Experimental Design Robustness and Statistical data analysis was performed by RSM to investigate the highest yield from radish root extract with optimization of pH, temperature, incubation time and enzyme concentration (E.C) (Skrypnik and Novikova, 2020).

2.3. Extraction procedure

The extraction procedure was performed by taking 10 g of fresh radish root for each experimental run and pulverized with a mortar pestle. Then put into 250 mL extraction bottles, while 40ml of phosphate buffer of required pH and enzyme of specified concentration was mixed. The reaction mixture flask was covered with aluminum foil and it was kept in a shaker at the temperature as set as per treatment to activate the enzymes. The enzyme was deactivated at 90°C for few minutes. After cooling, the contents were extracted by using an ultrasonic machine (Albany, UTECH, USA), with 80% ethanol (100 mL) using an orbital shaker (Pamico, Pakistan). The extracted flask contents were filtered with a filter paper (Whatman-1, 0.22 μ m) using pressure (Gallenkamp, UK).

After the separation of the residues, the liquid extract was concentrated using rotary evaporator and dried by evaporation using hot plates. The difference between the weights of the empty beaker and the beaker containing the material helped to deduce the net weight of the extract. A small quantity of methanol was added to completely dissolve the extract. Dark bottles were used to store the extract at 4 °C. The formula for calculating yield is given below:

$$\% \text{yield} = \frac{X_1 - X_2}{10}$$

2.4. Total phenolic contents (TPC)

Folin–Ciocalteu reagent utilized to investigate the TPC content of radish extract (Noman et al., 2021). According to the protocol radish extract (50 mg) was added to the deionized water (7.5 ml) and Folin–Ciocalteu reagent (0.5 ml). After keeping the mixture at room temperature (10 min), 1.5 mL of (20%w/v) the sodium carbonate solution was added. Then at 40 °C, the mixture was kept for 20 min and after that the mixture was cooled in the ice bath. The absorbance of the mixture was measured at 755 nm by using a spectrophotometer. The Gallic acid calibration curve was used to calculate the amount of total phenolic compounds within a range of 10–100 ppm (Supplementary Figure 1). TPC results were expressed as GAE (Gallic acid equivalents) per dry matter while samples were investigated in triplicates and analyzed as mean of all values.

2.5. DPPH (1, 1-diphenyl-2-picrylhydrazine) scavenging assay

The Free radical scavenging action of radish extracts was surveyed utilizing the technique depicted by Park and co-workers (Park et al., 2016). 100 μ L of four different extract concentrations (1.0, 0.1, 0.01 and 0.001 mg/mL) were added to the DPPH solution (100 μ L, 1 mg/mL) at room temperature. The absorbance at 515 nm for 0, 0.5, 1, 2, 5, and 10 min was estimated with the spectrophotometer. The rest of the measurements were analyzed from the calibration curve. Radical scavenging action of radish was examined by using the absorbance estimated at the 5th minute. Butylated hydroxyl toluene (BHT) utilized as positive control.

2.6. Trolox equivalent antioxidant capacity assay (TEAC)

ABTS ([2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)]) decolorization assay was used to determine the ABTS radical scavenging action of the test compounds (Dong et al., 2015; Ilyasov et al., 2020). 2.45 mM of potassium persulphate was mixed into the 7 mM ABTS and the mixture was put in the dark for 8–10 h at room temperature. Different amounts of test compounds or extracts were added to the ABTS solution. In the ABTS solution (190 μ L), different concentrations of

Table 1

Rotatable central composite design (RCCD) for several responses observed to estimate maceration conditions.

Treatment	Point type	pH	T °C	t (min)	E.C	Extract yield (g/100 g FW)	TPC (mg GAE/g FW)	DPPH (IC ₅₀ , mg/mL)	Reducing Power (mg/mL)	% inhibition of linoleic acid peroxidation	TEAC (mg TE/g FW)
1	Factorial	7.8(+1)	63.9(+1)	83.9(+1)	3.4(−1)	11.9	40.95	4.13	0.289	92.9	206.8
2	Factorial	7.8(+1)	63.9(+1)	66.0(−1)	4.5(+1)	12.8	67.85	1.82	0.355	87.8	216.9
3	Factorial	7.8(+1)	46(−1)	83.9(+1)	3.4(−1)	11.3	41.96	10.5	0.305	67.4	270.1
4	Factorial	6.1(−1)	63.9(+1)	66(−1)	3.4(−1)	11.4	49.90	9.19	0.405	65.0	279.5
5	Factorial	7.8(+1)	46(−1)	66(−1)	4.5(+1)	12.0	40.27	12.1	0.322	85.2	229.0
6	Factorial	6.1(−1)	46.0(−1)	83.9(+1)	4.5(+1)	10.2	48.24	5.26	0.379	73.5	220.6
7	Factorial	6.1(−1)	63.9(+1)	83.9(+1)	4.5(+1)	10.7	50.92	9.50	0.499	73.6	260.0
8	Factorial	6.1(−1)	46(−1)	66(−1)	3.4(−1)	11.3	58.60	5.29	0.405	98.7	280.8
9	Axial	5.5(−α)	55(0)	75(0)	4(0)	15.6	59.17	2.26	0.402	91.8	271.3
10	Axial	8.5(+α)	55(0)	75(0)	4(0)	14.0	69.29	45.5	0.589	85.5	256.6
11	Axial	7(0)	40(−α)	75(0)	4(0)	14.0	56.58	2.40	0.400	90.7	267.6
12	Axial	7(0)	70(+α)	75(0)	4(0)	12.3	40.34	10.3	0.555	72.5	273.1
13	Axial	7(0)	55(0)	60(−α)	4(0)	16.0	45.29	11.7	0.589	88.0	266.8
14	Axial	7(0)	55(0)	90(+α)	4(0)	13.6	42.58	4.06	0.365	62.5	229.8
15	Axial	7(0)	55(0)	75(0)	3(−α)	15.7	69.19	4.33	0.590	72.8	225.2
16	Axial	7(0)	55(0)	75(0)	5(+α)	14.6	54.17	6.16	0.409	80.9	202.2
17	Center	7(0)	55(0)	75(0)	4(0)	22.5	74.78	2.75	0.599	85.1	222.5
18	Center	7(0)	55(0)	75(0)	4(0)	21.5	65.22	3.60	0.433	72.1	242.7
19	Center	7(0)	55(0)	75(0)	4(0)	19.7	59.42	14.1	0.444	85.3	222.4
20	Center	7(0)	55(0)	75(0)	4(0)	21.2	50.26	1.84	0.589	87.2	253.8
21	Center	7(0)	55(0)	75(0)	4(0)	19.6	49.08	6.28	0.588	75.3	238.1

the test compounds/extracts (10 µL, (50 mg/mL) were added. After six minutes of incubation, at 734 nm absorbance was estimated using a spectrophotometer (Biotek-MQX-200, Highland Park, Biotek Ind., USA). The known antioxidants trolox and ascorbic acid were used as positive controls. This technique gives an estimate of the antioxidant activity of the scope of phenolics, carotenoids, and some plasma antioxidants, formed by the decolorization of the ABTS⁺, through estimating the lessening of the radical cation as the percentage hindrance of absorbance at 734 nm. The ABTS decolorization test is most generally used to estimate the antioxidant power. The TEAC test is frequently used to quantify the antioxidant capacity of drinks nutritional supplements and foods (Sharma and Singh, 2013).

2.7. Determination of antioxidant activity in linoleic acid system

The antioxidant activity of radish extracts was also determined by measuring % of oxidation of the linoleic acid system (Iqbal et al., 2007). Extracts, 1 mg/mL were added to a solution of linoleic acid (0.13 mL), 99.8% ethanol (10 mL) and 10 mL of 0.2 M sodium phosphate buffer (pH 7). The mixture was made up to 25 mL with distilled water and incubated at 40 °C up to 360 h. The extent of color change was observed after each 24-hour interval. Degree of oxidation was measured by peroxide value applying the thiocyanate method as described by Kim (2018). Briefly, 10 mL of ethanol (75% v/v), 0.2 mL of aqueous solution of ammonium thiocyanate (30% w/v), 0.2 mL of sample solution and 0.2 mL of ferrous chloride (FeCl₂) solution (20 mM in 3.5% HCl v/v) added in sequence. After 3 min of stirring, 200 µL was taken from the above-mentioned reaction mixture and transferred into a 96-well microplate and absorption was determined at 500 nm using a spectrophotometer (Hitachi U-2001). Increment in the absorbance values presents a higher extent of linoleic acid oxidation and percent inhibition was calculated with the help of following formula:

$$100 - [(Abs. \text{ increase of sample at } 360 \text{ h} / Abs. \text{ increase of control at } 360 \text{ h}) \times 100]$$

2.8. Determination of reducing power

An important antioxidant activity from radish extract was performed by following the method by Pasau and coworker (Pasau, 2019). According to the method, dry extract (5 mg) was added to potassium ferricyanide solution (5.0 mL, 1.0%) and sodium phosphate buffer (5.0 mL, 0.2 M, pH 6.6) at 50 °C later 10% trichloroacetic acid (5 mL) was added and centrifuged at 980g with centrifuge (CHM-17; Kokusan Denki, Tokyo, Japan) for 10 min at 5 °C. The upper layer was taken out and mixed with ferric chloride (1.0 mL, 0.1%) and distilled water (5 mL). The absorbance was determined at 700 nm. Analysis was performed in triplicate and averaged. A blank was used as a control that consists of all reagents except the sample. Greater the absorbance indicates greater the reducing potential.

2.9. Anti-mutagenic activity

For mutagenic and anti-mutagenic estimation from radish extract a protocol utilized Ames bacterial reverse mutation test in liquid medium with TA98 and TA100 strains of *Salmonella typhimurium* (Abd Razak et al., 2007). A reagent mixture

was created by mixing D-glucose (4.5mL, 40% w/v), L-histidine (0.01% w/v), bromocresol purple (2.3 mL, 0.2% w/v), D-biotin (1.19 mL, 0.01% w/v) and Davis Mingioli (21.6 mL, concentrated 5.5×) in five clean bottles and subsequently added deionized water (17.5 ml) to each of them. Blank contained only reagent (2.5ml) while background and standards contains bacterial strain (5 µL) and standard mutagen (0.1 ml) along with reagent respectively, while sample contains extract (5 µL) mixed with DMSO to the above mentioned reagents. Standard mutagen was added to the TA 100 strain while potassium dichromate (30%) to the TA98 and kept at 37 °C for 2 h. 96-well ELISA plate was utilized for further analysis and wrapped tightly with aluminum foil after transferring 200 µL from each aliquot mixture. After 4 days the blank was first investigated and showed purple coloration. Anti-mutagenic activity was expressed as % inhibition of mutagenicity by following formula:

$$\text{Anti-mutagenicity (\%)} = \frac{1 - \text{positive wells in test sample-SM}}{\text{Positive wells in positive control-SM}} \times 100$$

where SM indicated the spontaneous mutation caused by standard mutagen. When % inhibition is greater than 40% the sample is considered as strong anti-mutagenic while 25%–40% is moderate and less than 25% is considered as weak.

The variable parameters presented above are pH (A), temperature (B), reaction time (C) and enzyme concentration (D) their interaction (AB, AC, AD, BC, BD and CD) and quadratic factors (A^2 , B^2 , C^2 , and D^2) were determined by *F*-ratio and probability (*p*). *Non-significant at $p > 0.05$ and significant at $p < 0.05$.

The variables including pH (b_1), temperature (b_2), incubation time (b_3), and enzyme concentration (b_4), their interaction (b_{12} , b_{13} , b_{14} , b_{23} , b_{24} and b_{34}) and quadratic factors are represented by b_1^2 , b_2^2 , b_3^2 and b_4^2 . Where ns represent non-significant

2.10. Statistical analysis

Statistical analysis was carried out using the design expert (version 9, Stat-Ease, Inc., Minneapolis, USA) to examine the relation between the different responses and their effects. Analysis of variance (ANOVA) was conducted to investigate the significant and non-significant figures from the second-order polynomial equation to plot the 3-dimensional (3D) graphs between any of the two response factors to validate the accuracy of the applied model.

3. Results and discussion

3.1. Effect of variables on extraction yield

The extraction of commercially and nutritional important materials from a plant source depends upon the nature and composition of the source. Enzymes being specific in their activity, can be successfully used for this purpose. Keeping in view the above-mentioned conditions and possibility, an attempt was made in this dissertation to extract the phenolic constituent from radish by using cocktail enzyme with different concentrations and maceration conditions at pre-optimized treatment conditions followed the extraction in 80% ethanol. Statistical significant parameters for extraction yield were determined by using *F*-ratio and *p*-values, as given in table 4.2. *F*-value (28.83) indicates that model is significant, while lack of fit (3.33) exhibit good fitness of modal. Modal terms are considered significant at *F*-ratio less than 5.00×10^{-2} . Different variables including pH (A), temperature (B), time (C) and enzyme concentration (D) and their interaction (AB, AC, AD, BC, BD and CD) and quadratic factors (A^2 , B^2 , C^2 , and D^2) were investigated by analyzing *F*-ratio and *p*-values for all the antioxidant activities examined (Chen et al., 2009). Polynomial equation of second order was used for radish hydrolyzed by enzymes to plot out the responses.

Extraction yield:

$$Y = -197.8 + 34.20A + 5.062B - 0.466C + 20.47D + 0.134AC + 3.716AD - 0.013BC \\ - 0.670 BD + 0.208 CD - 3.041 A^2 + 4.964E - 003C^2 - 2.519D^2$$

In the above expression Y represents the yield while two parameters AB and B^2 are non-significant ($p > 5.0 \times 10^{-2}$) for extraction yield. The co-efficient of determination R^2 for all of three above mentioned responses agreed that polynomial terms are sufficient to express the effective recovery of antioxidants under given experimental conditions. Actual and observed values represents good agreement ($\text{adj } R^2 = 0.933$ (Tables 3, 4)). Response surface methodology was used to elaborate the different maceration conditions for extraction of bioactive compounds from the radish root. It was clear from Fig. 1(a–f) that the pH and E.C are the main factors to affect the extraction and yield is minimum at low pH and less E.C whereas extraction efficiency showed increase with the E.C up to a specified level, after that specified level the yield decreases or remained ineffective. Likewise at higher pH (7.9) and temperature (63.9 °C), the extraction yield declined rapidly. Analysis of normal % probability and actual vs. predicted values depicts that the observed experimental factors and values for predicted yield were in line agreement with the statistically applied empirical model (Fig. 2). Coefficient of determination $R^2 0.9793$ showed that statistical model was efficiently applicable (Table 3). Statistical data represented in Table 1 showed Run # 17 gave the highest yield (22.5 g/100 gm DW) due to optimized conditions of pH, temperature, reaction time and enzyme concentration. Results were comparable to those reported by Kehili et al. (2019) and Kim (2018).

Table 2

Statistical model estimation of extract yield and observed antioxidant activities for TPC, DPPH, TEAC, FRAP and (%) Inh. Linoleic acid.

Response	Model	Main effect				Interaction effect						Quadratic effect			
		A	B	C	D	AB	AC	AD	BC	BD	CD	A ²	B ²	C ²	D ²
Extract Yield ^L (g/100g FW)															
<i>p</i> -value*	0.000	0.006	0.000	0.005	0.000	0.06	0.000	0.000	0.000	0.006	0.000	0.000	0.17	0.000	0.023
F-ratio	0 28.83	10.44	19.71	10.67	166.8	0.27	23.24	19.73	10.16	28.50	24.80	48.23	2.04	20.56	6.5
TPC ^M (mg GAE/g FW)															
<i>p</i> -value*	0.001	0.088	0.004	0.212	0.000	0.026	0.225	0.010	0.884	0.058	0.196	0.000	0.000	0.000	0.000
F-ratio	16.64	4.14	19.12	1.95	2.94	8.52	1.820	13.24	0.022	5.460	2.11	48.21	82.70	38.53	60.47
DPPH ^N (IC ₅₀ mg/mL)															
<i>p</i> -value*	0.000	0.285	0.007	0.006	0.000	0.000	0.000	0.16	0.001	0.15	0.029	0.000	0.000	0.000	0.000
F-ratio	79.43	1.38	40.8	16.86	114.6	76.14	46.94	2.53	32.22	2.70	8.07	123.2	339.6	332.7	178.7
TEAC (mg TAE/g)															
<i>p</i> -value*	0.000	0.044	0.000	0.010	0.027	0.019	0.000	0.002	0.298	0.878	0.001	0.000	0.005	0.600	0.026
F-ratio	21.5	6.78	53.8	13.4	1.43	1.97	1.31	36.1	1.77	0.12	33.1	61.0	53.6	56.2	8.80
Reducing Power ^P															
<i>p</i> -value*	0.001	0.027	0.001	0.005	0.135	0.302	0.155	0.211	0.202	0.038	0.834	0.011	0.001	0.000	0.163
F-ratio	33.89	56.48	4.36	35.25	4.77	0.92	2.95	44.25	0.89	20.2	23.99	39.13	100.2	56.2	116.3
(%) Inh. Linoleic acid ^Q (g/100g)															
<i>p</i> -value*	0.044	0.034	0.009	0.022	0.020	0.527	0.099	0.004	0.113	0.002	0.011	0.022	0.111	0.032	0.302
F-ratio	19.33	6.53	3.58	9.40	21.46	0.45	4.06	1.83	3.01	0.40	10.47	0.54	2.17	3.86	7.97

The variable parameters presented above are pH (A), temperature (B), reaction time (C) and enzyme concentration (D) their interaction (AB, AC, AD, BC, BD and CD) and quadratic factors (A², B², C², and D²) were determined by *F*-ratio and probability (*p*).

*Non-significant at *p* > 0.05 and significant at *p* < 0.05.

Table 3

Statistical data regarding robustness and regression coefficients of experimental model:

Regression coefficients	Yield ^L	TPC ^M	DDPH ^N	TEAC ^O	% Inh. of Linoleic acid ^P	RP ^Q
<i>b</i> ₀	−197.8	−1742	−188.8	+2203	−231.1	−0.386
<i>b</i> ₁	+34.2	ns	ns	−175.8	+171.5	−0.112
<i>b</i> ₂	+5.06	+10.9	+1.600	−1.917	+55.09	ns
<i>b</i> ₃	−0.46	ns	+2.505	−25.24	−29.17	+0.009
<i>b</i> ₄	+20.4	Ns	+31.88	−140.5	+44.87	ns
<i>b</i> ₁₂	ns	−0.62	+0.207	−2.643	ns	ns
<i>b</i> ₁₃	−0.13	Ns	+0.104	+1.737	−3.073	ns
<i>b</i> ₁₄	+3.71	−11.6	−0.576	+11.85	+56.23	+0.053
<i>b</i> ₂₃	−0.01	ns	ns	ns	ns	ns
<i>b</i> ₂₄	−0.67	ns	−0.058	ns	−10.66	−0.003
<i>b</i> ₃₄	+0.20	−0.29	ns	+2.161	+4.114	+0.002
<i>b</i> ₁ ²	−3.04	−6.99	−1.243	+9.898	−33.39	−0.015
<i>b</i> ₂ ²	ns	−0.09	−0.020	ns	ns	+0.092
<i>b</i> ₃ ²	+4.96	−0.06	−0.020	ns	+0.267	−1.546
<i>b</i> ₄ ²	−2.51	−17.6	−3.371	ns	ns	−0.061
R ²	0.966	0.974	0.994	0.899	0.782	0.987
Adj. R ²	0.933	0.916	0.982	0.722	0.892	0.958
Lack of fit	3.33	0.36	1.45	0.70	0.35	2.43
CV	6.32	5.73	3.38	7.66	7.80	4.85

The variables including pH (*b*₁), temperature (*b*₂), incubation time (*b*₃), and enzyme concentration (*b*₄), their interaction (*b*₁₂, *b*₁₃, *b*₁₄, *b*₂₃, *b*₂₄ and *b*₃₄) and quadratic factors are represented by *b*₁², *b*₂², *b*₃² and *b*₄². Where ns represent non-significant.

3.2. Effect of variables on TPC

Total phenolic contents were analyzed by the method described by Chaovanalikit and Wrolstad (2004). *F*-value (16.64) and lack of fit (0.36) implies the significance of model. Different variables and their interaction factors were examined by

Table 4

Extract yield and total phenolic contents (TPC) of fresh radish root extract optimized using RSM predicted and observed values.

Treatments	Extract yield				TPC			
	Observed	Predicted	Residual	Cook's distance	Observed	Predicted	Residual	Cook's distance
R1	11.30	11.52	−0.222	0.395	40.96	41.63	−0.676	0.357
R2	19.77	20.96	−1.19	0.027	67.85	69.65	−1.80	0.006
R3	11.30	11.30	0.002	0.000	41.97	42.43	−0.465	0.169
R4	12.00	12.22	−0.222	0.395	49.90	50.58	−0.676	0.357
R5	12.30	12.14	0.155	0.272	40.27	39.47	0.806	0.717
R6	10.77	10.77	0.002	0.000	48.25	48.71	−0.465	0.169
R7	11.44	11.66	−0.222	0.395	50.93	51.60	−0.676	0.357
R8	16.00	15.58	0.423	0.059	58.61	57.55	1.06	0.036
R9	14.00	13.84	0.155	0.272	59.18	58.37	0.806	0.717
R10	19.66	20.96	−1.30	0.033	69.30	69.65	−0.350	0.000
R11	15.70	15.54	0.155	0.272	56.59	55.78	0.806	0.717
R12	11.94	11.94	0.002	0.000	40.34	40.81	−0.465	0.169
R13	10.22	10.22	0.002	0.000	45.30	45.76	−0.465	0.169
R14	12.88	13.10	−0.222	0.395	42.58	43.26	−0.676	0.357
R15	21.55	20.96	0.593	0.007	69.19	69.65	−0.458	0.000
R16	13.66	13.77	−0.112	0.004	54.17	53.62	0.556	0.010
R17	22.22	20.96	1.26	0.031	74.79	69.65	5.14	0.050
R18	21.22	20.96	0.263	0.001	65.22	69.65	−4.42	0.037
R19	14.00	13.84	0.155	0.272	59.43	58.62	0.806	0.717
R20	15.66	15.50	0.155	0.272	50.27	49.46	0.806	0.717
R21	14.60	14.44	0.155	0.272	49.08	48.27	0.806	0.717

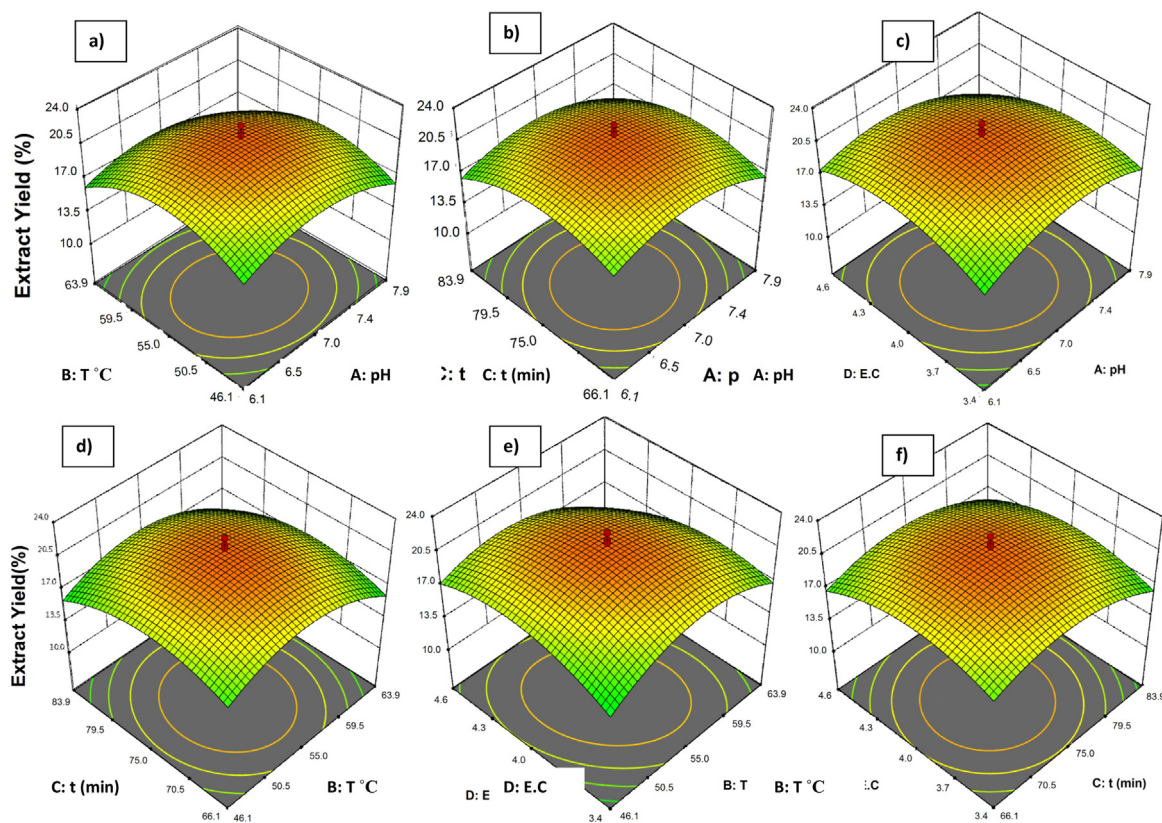


Fig. 1. a–f Three dimensional (3D) plots of extract yield (%) from Radish root observed under different enzyme concentrations (EC), temperature (T), pH and incubation time (t) and their interaction.

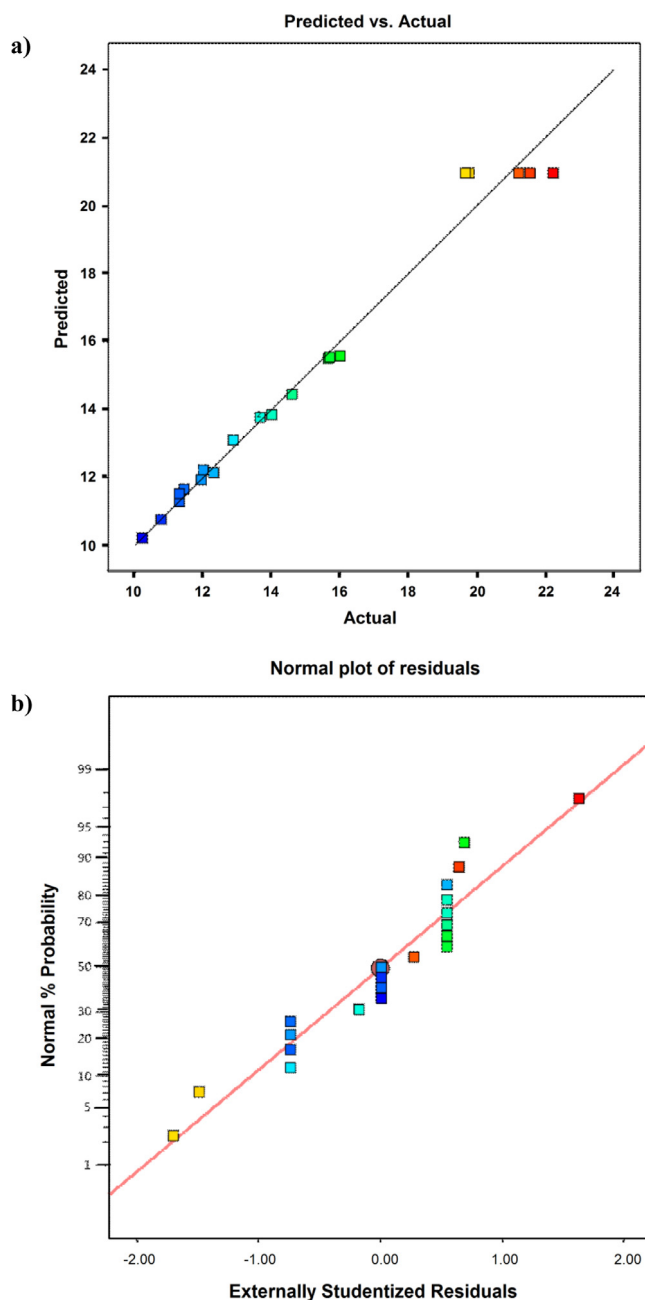


Fig. 2. Actual vs. predicted graph for extract yield (a) normal plot of residuals (b) for (%) normal probability of extract yield. Prediction versus actual plot of the lycopene extraction yields from tomato peels by-product using maceration in refined olive oil according to the quadratic model (1).

(*p*) and *F*-ratio values. It is clear from Table 3 that, the variables including pH(A), incubation time (C), E.C (D), interaction of pH with E.C (AC), temperature with incubation time and, E.C (BC, CD) are non-significant for TPC. The second order polynomial results can be reduced as:

TPC:

$$Y = -1742.7 + 10.96B - 0.624AB - 11.67AD - 6.992A^2 - 0.091B^2 - 0.062C^2 - 17.61D^2 \quad (1)$$

TPC results were represented as gallic acid equivalent (GAE per gram of FW). Amounts of the total phenolic contents in radish extract were maximum for sample 17 (74.78 mg GAE/g) at pH 7, E.C 4% and 75 min of incubation time and

minimum for sample 5 (40.27 mg GAE/g) of radish extract. Results were comparable to those reported by Goyeneche and colleagues (Goyeneche et al., 2015). Statistical results signified in (Tables 1, 2, 3).

3.3. Effect of variables on DPPH radical scavenging assay

DPPH radicals using a widely used model system assessed the radish extract obtained from different enzyme concentrations under different conditions to examine the scavenging characteristics of different bioactive compounds. Color of reaction mixture changed (purple to yellow) on scavenging the DPPH radicals while absorbance decreases at 515 nm. Higher phenolic compounds represent greater DPPH scavenging activity and consequently high antioxidant activity. Significance of model is analyzed by *F*-value (79.43) and lack of fit (1.45) for DPPH.

Variables including pH (A), pH interaction with E.C (AD) and temperature with E.C (BD) are non-significant for DPPH. The polynomial results can be reduced as:

DPPH:

$$Y = -188.80 + 1.600B + 2.505C + 31.88D + 0.207AB + 0.104AC - 8.692BC + 0.065CD \\ - 1.2439A^2 - 0.0206 B^2 - 0.0204C^2 - 3.3712D^2$$

This radical is very sensitive to active ingredients at low concentrations and can accommodate a large number of samples in a very short time; it has been extensively used for measuring the radical scavenging activity of different plant extracts. DPPH scavenging activity in radish extract was maximum for sample 10 (45.5 mg/mL) at 55 °C, 8.5 pH, incubation time 75 min and minimum for sample 2 (1.82 mg/mL) as shown in Table 1. Results were comparable to those reported by Ashraf et al. (2016). Statistical data represented in Tables 1, 2, and 3.

3.4. Effect of variables on reducing power

An important antioxidant activity from radish extract was performed by the method reported in latest studies (Manivannan et al., 2019; Pasau, 2019). In order to analyze the reducing properties ferric ions are reduced to ferrous and color change is also observed from yellow to bluish green (Kulczyński et al., 2020; Pająk et al., 2017). For radish extract the reducing potential increases upon concentration increment. Significance for the variable parameters was evaluated with the *F*-value (33.89) and lack of fit (2.43) and it clarifies the fitness of model ($p > 5.0 \times 10^{-2}$). Table 3 depicts that Temperature (B), E.C (D), interaction of pH with temperature (AB) and time (BC) and pH with time (AC) are non-significant ($p > 5.0 \times 10^{-2}$) factors for reducing potential, So the second order polynomial equations can be reduced as:

Reducing Power:

$$Y = -0.3869 - 0.1123A + 0.009C + 0.0536AD - 0.0036BD + 0.0025CD - 0.0158A^2 - \\ + 0.092B^2 - 1.546C^2 - 0.0614 D^2$$

Overall radish extract produces maximum reducing power for sample 17 (0.599) at 10 mg/ml concentration under 55 °C, 7 pH and 4% enzyme concentration. Results were comparable to those reported by Ashraf and colleagues (Ashraf et al., 2016). Statistical results have been shown in Tables 2, 3.

3.5. Effect of variables on antioxidant activity of linoleic acid system

Antioxidant characteristic was also determined by the inhibition of peroxidation in linoleic acid system using the thiocyanate method (Kızıl et al., 2008). Linoleic acid is a polyunsaturated fatty acid, upon oxidation peroxides are formed which oxidize Fe^{2+} to Fe^{3+} , the later forms complex with SCN, concentration of which is determined spectrophotometrically by measuring absorbance at 500 nm. Higher the absorbance higher will be the concentration of peroxides formed during the reaction, consequently lower will the antioxidant activity. The model analyzes that *F*-value (19.33) and lack of fit (0.80) was significant and proved good fit for LA% inhibition. It is clear from Table 3 that after reducing non-significant terms for LA% inhibition the polynomial equations can be reduced as:

LA% inhibition:

$$Y = -231.115 + 171.518A + 55.0949B - 29.17C + 49.875D - 3.0733AC + 56.232AD \\ - 10.661BD + 4.114CD - 33.392A^2 + 0.267C^2$$

Overall it could be concluded that the Percentage inhibition of linoleic acid for radish extract was highest for sample 8 (98.7%) at 46 °C, pH 6.1 and 66 min incubation time whereas minimum for sample 14 (62.5%) as mentioned in Table 1. Results were comparable to those reported by previous study (Xiaoling et al., 2001). Tables 2, 3 showed statistical data for LA % inhibition.

Table 5

Anti-mutagenic activity of radish extract.

	<i>Salmonella typhimurium</i> TA-98			<i>Salmonella typhimurium</i> TA-100		
	Positive wells/96 wells	%Anti-mutagenic activity	Effect	Positive wells/96 wells	%Anti-mutagenic activity	Effect
Blank ^a	8	–	NA	14	NA	NA
K ₂ Cr ₂ O ₇ ^b	93	–	–	NA	NA	NA
NaN ₃ ^c	NA	NA	NA	0	0	–
Run # 17	18	80.7	+	15	3.2	++
Run #10	17	81.7	+++	14	85.0	+++
Run # 15	16	82.8	+	22	75.3	+
BHT	28	85.47	++	21	91.13	++

Positive control ^{b,c}, negative control^a, +++, ++, + indicates strong, moderate and weak anti-mutagenic properties respectively. – signify mutagenic and - non-mutagenic response.

3.6. Effect of variables on trolox equivalent antioxidant (TEAC) activity

The method described above for TEAC activity analysis provide a great range of antioxidants to be examined by the ABTS⁺ discoloration, radical cation reduction and measuring absorbance at 734 nm. The TEAC assay is often used to measure the antioxidant capacity of foods, beverages, and nutritional supplements. Trolox is synthetic water soluble analogue of vitamin E that displays antioxidant activity, the standard corresponding to trolox equation and calibration curve were used to indicate the antioxidant characteristics of radish extract to express the trolox equivalent antioxidant capacity or TEAC values (Supplementary Figure 2). The Model F-value of 21.5 for TEAC represent the model is significant. Lack of fit for TEAC (0.7) showed a good fit of the model. Incubation time (B²), temperature (C²), enzyme concentration E.C (D²), interaction of temperature with reaction time (BC) and E.C (BD) are non-significant terms for TEAC. So the polynomial equations can be reduced as:

TEAC:

$$Y = +2203.17 - 175.86278A - 1.91758B - 25.24362C - 140.56201D - 2.64356AB \\ + 1.73777 AC + 11.85871AD + 2.16199CD + 9.89826A^2 + 0.12758B^2 - 27.95932D^2$$

Overall it was concluded that maximum antioxidant capacity was observed for sample 8 (280.8 mg TE/g FW) at 46 °C, pH 6.1 and 66.08 min whereas lowest for sample 16 (202 mg TE/g FW) as mentioned in Table 1. TEAC results for radish extract were comparable to those described by Dannehl and colleagues (Dannehl et al., 2009). Statistical data presented in Tables 2, 3.

3.7. Anti-mutagenic activity

Genotoxicity of bioactive compounds should be considered and investigated by different assays available (Verschaeve, 2015). Ames bacterial test was applied to assess the anti-mutagenic characteristics of the radish extract which is carried out by strains *Salmonella typhimurium* TA98 and TA100 strains (López-Romero et al., 2018). The standard mutagen and sodium azide were added for TA100 strain while potassium dichromate (30%) with TA-98 at 37 °C for 2 h. This protocol is most reliable and provides rapid results. Triplicates of each sample were analyzed in all experiments. All of the radish extracts showed a strong anti-mutagenic potential towards *salmonella* strains of TA98 and TA100. An opposite relation was observed between the anti-mutagenic activity and number of positive wells. Strongest anti-mutagenic potential was observed in TA100 as shown in Table 5.

The strongest anti-mutagenic activities of radish extract (80.7% and 83.2% for strain TA-98 and TA-100, respectively) were observed from the treatment (Run #17) having highest TPC content while (81.7% and 85% for bacterial strain TA-98 and TA-100, respectively) were observed from treatment (run #10) relatively high in TPC content. Similarly, (82.8% and 75.3% for TA 98 and TA100, respectively) for treatment (run# 15) having considerable amount of TPC under the experimental conditions of 4% E.C, pH 7, 55 °C and 75 min (Table 5). TA-98 mutant strain showed less anti-mutagenic potential as compared to TA-100. The results obtained were comparable to the standard antioxidant BHT (76.47 and 91.13%). The strongest anti-mutagenic potential directly related to the availability of phenolic compounds extracted from radish extracts.

3.8. Determination and validation of optimized conditions of the optimal conditions

The estimated specific responses were additionally converted into “desirability” which represents values (0–1) on a dimensionless scale. Desirability value 0 exhibited a totally unwanted experimental model whereas 1 depicts the desirable design. The supported solutions which represents highest desirable design for the extraction of bioactive compounds from radish root was achieved by implying 3.5% (w/w) of cocktail enzyme formulation, at temperature of 46°C, pH of 6.1 and incubation time of 66 min (Table 6, Fig. 3).

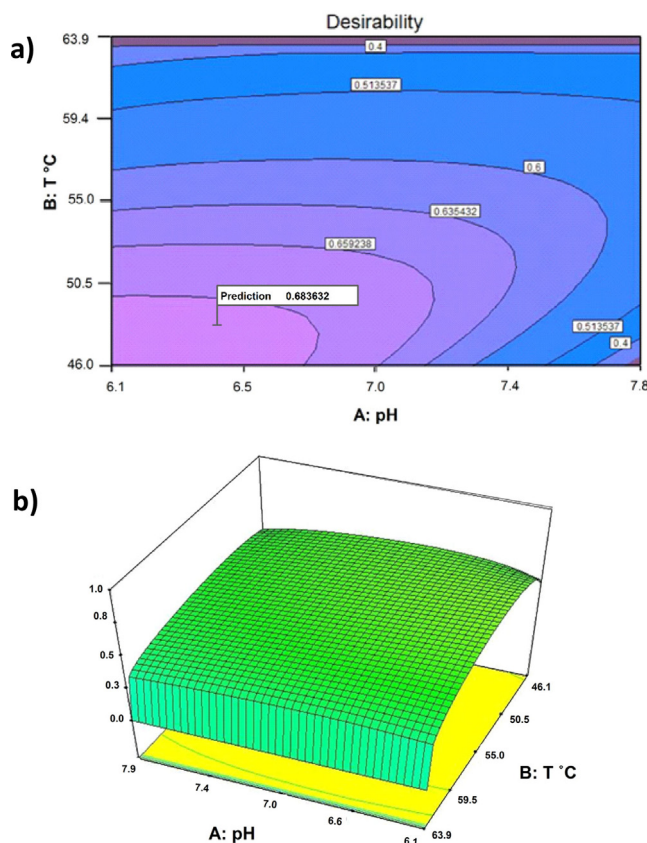


Fig. 3. (a) Represents the contour graph of desirability for different solutions related to temperature and pH and suggested during statistical analysis (b) Typical 3D response of desirability plot.

Table 6

Desirable experimental conditions to validate the observed results ($d > 0.7$).

Sr. No.	Variable investigated				Response observed					
	pH (A)	T (°C) (B)	t(Min) (C)	E.C (%) (D)	Extract Yield (g/100 g FW)	TPC (mg GAE/g FW)	TEAC (mg TE/g FW)	DPPH (mg/mL (IC ₅₀),	Reducing power (mg/mL)	% inh. Linolic acid
1	6.10	46.08	66.08	3.53	19.7	49.2	270.1	14.10	0.489	88.0
2	6.10	46.08	66.08	3.53	19.6	49.1	270.9	14.14	0.486	80.9
3	6.10	46.08	66.08	3.53	18.6	48.5	271.3	14.16	0.483	81.2
Average	6.10	46.08	66.08	3.53	19.3	48.9	270.7	14.13	0.486	83.3
Results predicted with maximum desirability					19.2	45.9	271.7	14.17	0.480	85.9

Table 4 indicated the predicted and observed values of radish extract yield and Total phenolic contents (TPC). The maximum values (observed and predicted) for extract yield were in line agreement in the range of (40.27–74.78). Observed and predicted values regarding TPC indicates that optimal predicted activity (74.78 mg GAE/g) was potentially explored at 4% E.C, pH 7, 50.6 °C and 75 min reaction time. In Table 4, Cook's Distance is used to estimate the impact of a data point with observed and estimated variables when performing a least squares regression analysis.

4. Conclusion

Overall it is concluded that enzyme assisted extraction of radish displayed a remarkable antioxidant activity with maximal extraction of phenolic contents. TPC highest and lowest values of antioxidant activity of all antioxidant capacity tests was observed for highest and lowest yield treatments under different circumstances of incubation time, pH and enzyme concentration. Anti-mutagenic characteristics from radish extract was done in aqueous cultures utilizing the Ames bacterial assay (using specific strains of *S. typhimurium* TA-100 and TA-98). It was observed that TPC and anti-mutagenic potential showed a significant correlation (0.976) as shown in (Table 5), which proves that anti-mutagenic activity and phenolic contents of radish extract are directly related. Significant model analysis were assessed in the

antioxidant viability of radish extracts among all amounts of concentration. It was observed that radish extract displayed maximal of antioxidant capability. Results acquired utilizing response surface methodology displayed that radish extracts could be taken as a powerful antioxidant source and may be utilized for different medicinal and nutraceutical purposes.

CRedit authorship contribution statement

Andleeb Rani: Conceptualization, Methodology, Software, Data curation, Writing - original draft, Writing - review & editing. **Yasir Arfat:** Conceptualization, Methodology, Software, Data curation, Writing - original draft, Supervision, Writing - review & editing. **Rao Salman Aziz:** Visualization, Investigation. **Liaqat Ali:** Visualization, Investigation. **Hammad Ahmed:** Software, Validation. **Shazia Asim:** Software, Validation. **Muhammad Rashid:** Visualization, Investigation. **Charles H. Hocart:** Data curation, Writing - original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary material related to this article can be found online at <https://doi.org/10.1016/j.eti.2021.101620>.

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