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ORIGINAL ARTICLE

RSM based optimized enzyme-assisted extraction of antioxidant phenolics from underutilized watermelon (*Citrullus lanatus* Thunb.) rind

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Abstract Enzyme assisted solvent extraction (EASE) of phenolic compounds from watermelon (*C. lanatus*) rind (WMR) was optimized using Response Surface Methodology (RSM) with Rotatable Central Composite Design (RCCD). Four variables each at five levels i.e. enzyme concentration (EC) 0.5–6.5 %, pH 6–9, temperature (T) 25–75 °C and treatment time (t) 30–90 min, were augmented to get optimal yield of polyphenols with maximum retained antioxidant potential. The polyphenol extracts obtained under optimum conditions were evaluated for their in-vitro antioxidant activities and characterized for individual phenolic profile by RP-HPLC-DAD. The results obtained indicated that optimized EASE enhanced the liberation of antioxidant phenolics up to 3 folds on fresh weight basis (FW) as compared to conventional solvent extraction (CSE), with substantial level of total phenolics (173.70 mg GAE/g FW), TEAC 279.96 mg TE/g FW and DPPH radical scavenging ability (IC_{50}) 112.27 mg/mL. Chlorogenic acid (115.60–1611.04), Vanillic acid (26.13–2317.01) and Sinapic acid (113.01–241.12 μg/g) were major phenolic acid found in EASEx of WMR. Overall, it was concluded that EASE might be efficient and green technique to revalorize under-utilized WMR into potent antioxidant phenolic for their further application in food and nutraceutical industries.

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Introduction

Watermelon [*Citrullus lanatus* (Thunb.)]; a widely cultivated fruit crop of Mediterranean basin, belongs to *Cucurbitaceae* family (Tlili et al. 2011a) and provides a wide variety of dietary phytonutrients and minerals (Rimando and Perkins-Veazie 2005). Watermelon is consumed almost all over the world due to its aesthetic taste and nutritious compositions resulting annual production of 104,472,354 tons (FAO 2011). Watermelon rind (WMR) constitutes almost 30 % of whole fruit by weight and has been explored for value added products e.g. an ingredient in pickle, candy, vadiyam, cheese, etc., and biosorbent for the removal heavy metals (Liu et al. 2012; Al-Sayed and Ahmed 2013). Unfortunately, more than 90 % of WMR is still discarded indiscriminately constituting environmental challenges.

Recently, awareness about diverse health benefits of eating antioxidant ingredients has attracted food chemists to explore more and more resources of these valuable natural bioactives. The literature survey confirms scarce studies focusing watermelon antioxidant compounds. Al-Sayed and Ahmed (2013) and Tlili et al. (2011b) explored that watermelon and watermelon rind contains substantial level of phenolic antioxidants like caffeic, vanilline, syrinigic, chlorogenic and sinapic acid. Epidemiological reports (Tarazona-Diaz et al. 2013) further attribute the miscellaneous health benefits of watermelon regarding hypertension, blood pressure, age related health disorder and degenerative diseases to non-nutritive phytochemicals (Figueroa et al. 2011).

WMR is mainly structured by combination of celluloses, hemicelloses, pectins and lignins with entrapped sugars, lycopene, carotenoids, citroline and phenolics (Rimando and

Perkins-Veazie 2005). In conventional extraction techniques, solvent system used might not be able to completely distribute itself through compact cellulosic composite. Acid and alkaline hydrolysis can rupture cell wall and produce pretty good yield but the resultant extracts lack in their respective antioxidant activities since both cause deterioration of potent phenolic compounds (Bener et al. 2013). Presently, enzymes have been incorporated in many extraction processes because of their target specific maceration power (Gaur et al. 2007; Liu et al. 2009; You et al. 2013). In this conjunction, present work was designed to revalorize under-utilized watermelon rind (WMR) into phenolics of high antioxidant and medicinal value by enzyme cocktail. Response Surface Methodology (RSM) was used to get most suitable experimental conditions for optimal response of parameters investigated (Bezerra et al. 2008).

Material and methods

Materials and chemicals

Watermelon *Citrullus lanatus* (Thunb.) var. Sugar baby was purchased from local market of Faisalabad and authenticated from Department of Botany, University of Agriculture, Faisalabad. Enzyme cocktail "Kemzyme" Kemine, Germany, composed of pectinase, *endo-1,3 (4)-beta-glucanase, alpha-amylase, endo-1,4-beta-xylanase and bacillolysine (protease*) with a guaranteed minimum enzyme activity of 2,350, 18,000, 400, 35,000, and 1,700 μg/g, respectively was kindly provided by Ghazi Brothers, Pakistan. All the reagent including 2,2-azinobis (3-ethylbenzothiazoline- 6-fulfonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (TROLOX), butylated hydrxytoluence (BHT), Folin-Ciocalteu and standards comprising 3, 4-dihydroxy benzoic, *p*-hydroxy benzoic, gallic, linoleic, vanillic, caffeic, *p*-coumaric, ferulic, syringic and sinapic acids were purchased from Sigma, ST. Louis whereas chemical including Ammoniun Thiocyanate, Potassium persulfate, Potassium Ferrocyanide, Na_2CO_3, and acetic acid were procured from Merck (Darmstadt, Germany).

Experimental design

Enzymatic maceration parameters i.e. enzyme concentration (E), pH, temperature (T) and incubation time (t) predominantly influencing enzymatic hydrolysis were investigated at five levels ($-\alpha$, −1, 0, +1, $+\alpha$) using rotatable central composite design (RCCD; α=1.682) as expressed in Table 1. A total of 21 experimental runs with eight runs for each at axial and factorial points and five replicate runs at center points were augmented to estimate major effects and pure error using Design Expert version 8.0.7.1. All the responses obtained including, extract yield (yield), total phenolic contents (TPC), Trolox equivalent antioxidant capacity (TEAC) and DPPH radical scavenging capacity were fitted into second order polynomial equation give below:

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

Where Y denotes response to be optimized; b_0 intercept; $\sum_{i=1}^{k} b_i X_i$, linear effect of variables; $\sum_{i=1}^{k} b_{ii} X^2$, quadratic effect and $\sum_{i>1}^{k} \sum_{j}^{k} b_{ij} X_i X_j$, interaction between different parameters. The statistical significant of estimates, fitness of model applied and proportion of variance were determined by student's *t*-test, lack of fit test and multiple coefficient determination (R^2), respectively.

Table 1 The factors (actual and coded) and their levels (actual and coded) in randomized run order investigated for EASE using CCD design

Treatments		E (%) (A)	pH (B)	T (°C) (C)	t (Min) (D)
3	Axial	3(0)	7.5(0)	50(0)	30(+α)
9	Axial	3(0)	7.5(0)	50(0)	90(−α)
11	Axial	3(0)	9(+α)	50(0)	60(0)
13	Axial	3(0)	6(−α)	50(0)	60(0)
14	Axial	3(0)	7.5(0)	25(−α)	60(0)
16	Axial	0.5(−α)	7.5(0)	50(0)	60(0)
17	Axial	3(0)	7.5(0)	75(+α)	60(0)
18	Axial	6.5(+α)	7.5(0)	50(0)	60(0)
1	Factorial	5(+1)	6.5(−1)	70(+1)	75(+1)
2	Factorial	1(−1)	8.5(+1)	30(−1)	75(+1)
7	Factorial	5(0)	8.5(+1)	30(−1)	45(−1)
8	Factorial	1(−1)	6.5(−1)	70(+1)	45(−1)
10	Factorial	1(−1)	6.5(−1)	30(−1)	45(−1)
12	Factorial	1(−1)	8.5(+1)	70(+1)	75(+1)
20	Factorial	5(+1)	8.5(+1)	70(+1)	45(−1)
21	Factorial	5(+1)	6.5(−1)	30(−1)	75(+1)
4	Center	3(0)	7.5(0)	50(0)	60(0)
5	Center	3(0)	7.5(0)	50(0)	60(0)
6	Center	3(0)	7.5(0)	50(0)	60(0)
15	Center	3(0)	7.5(0)	50(0)	60(0)
19	Center	3(0)	7.5(0)	50(0)	60(0)

Extraction procedure

Accurately weighed 10 g of fresh (FW) watermelon rind (WRM) sample was taken in 250 mL Erlenmeyer flask, diluted with 10 mL of phosphate buffer of required pH and blended with observed concentration of enzyme (Table 1). After enzymatic treatment the enzyme cocktail was deactivated by heating at 90 °C for 5 min. The contents were degassed in ultrasonic reactor (UTECH, Albany, New Yark, USA), shaken in an orbital shaker (Pamico, Pakistan) with 100 mL 80 %

aqueous methanol and filtered through 0.22 μm filter paper under pressure (Gallenkamp, UK). The extracts obtained were concentrated using Rotary Evaporator (EYELA, N-N series, Tokyo, Japan) under reduced pressure and weighed to calculate the extraction yield.

Total phenolic contents (TPC)

TPC in extracts obtained by EASE were assessed using Folin-Ciocalteu reagent as described by Sulaiman et al. (2011) with modifications. In this assay, 50 mg of extract was diluted with 7.5 mL deionized water, mixed with 0.5 mL of Folin-Ciocalteu reagent and incubated at room temperature for 10 min. Sodium carbonate (20 % w/v, 1.5 mL) was added to this mixture, kept at 40 °C for 20 min and chilled in an ice bath. 200 μl of the contents were transferred to 96-well micro plate and absorbance was measured at 755 nm (Biotek-MQX-200, Biotek Ind., Highland park, USA). Gallic acid at different concentration was used as positive control and TPC were calculated using gallic acid calibration curve within range of 10–100 ppm (R^2=0.9986).

HPLC profile of phenolic compounds

The extracts of WMR obtained against the optimum conditions for EASE were authenticated and quantified for individual phenolic compounds using reverse phase high performance liquid chromatography coupled with diode array detector (RP-HPLC-DAD) as previously reported by Abadio Finco et al. (2012). Briefly, 50 mg of extracts was refluxed at 95 °C in 5 mL of 1 % acidified methanol containing 0.5 mg /mL BHT as preservative antioxidant. The extracts were cooled and centrifuged at 5,000 rpm for 10 min. The upper layer was separated, sonicated for 2 min to remove any air bubbles present, filtered through a 0.45-μm filter (Millipore) and injected into Shimdadzu LC-10A HPLC system equipped with Shim-Pack CLC-ODS C-18 (250 mm×4.6 mm×5 μm i.d.) column (Merck KGaA, 64271 Darmstadt, Germany) and DAD (G1315B DAD) detector thermo stated at 25 °C). Gradient mode mobile phase comprising A (H_2O:CH_3COOH 94:6) and B (acetonitrile 100 %) was used at 1.0 mL/min during 0–15 min (15 % B), 15–30 (45 % B) and 30–45 min (100 % B). The data obtained was processed using CSW32 (data apex) Chromatography Station.

Trolox equivalent antioxidant capacity assay (TEAC)

Trolox Equivalent antioxidant capacity (TEAC) was assessed following in-vitro assay documented earlier by Arts et al. (2004) with slight modification. To generate $ABTS^{+}$, 7 mM ABTS and 2.45 Mm potassium per sulphate (0.5/1; v/v) were mixed and incubated in dark for 8 h. The resultant solution was diluted with 80 % ethanol until it produced an absorbance of 0.700±0.050 at 734. Now, 10 μL of extract containing 50 mg/mL was added to 190 μL of diluted $ABTS^{\bullet+}$ in a 96-well microplate and allowed to stand for 6 min and absorbance was measured at 734 nm (Biotek-MQX-200, Biotek Ind., Highland park, USA). Trolox was considered as positive control and antioxidant potential was expressed as mg Trolox Equivalent (TE)/g WMR extract.

DPPH scavenging assay

DPPH radical scavenging activity (IC_{50}) of WMR extracts was appraised following a previously documented procedure (Chen et al. 2013). Briefly, 100 μL of freshly prepared DPPH (1 mg/mL) was mixed with 110 μL of each concentration of 1, 0.1, 0.01 and 0.001 mg/mL of extract. After 15 min incubation at room temperature absorbance was measured at 517 nm using a spectrophotometer. The effective dose of extract for 50 % inhibition of DPPH° (IC_{50}) was obtained from a plot of percentage inhibition verses extract concentration. Butylated hydroxyl toluene (BHT) was used as positive control.

Results and discussion

Experimental design robustness

The enzyme concentration, incubation time, temperature and pH were optimized by using Response Surface Methodology (RSM) to get maximum yield of phenolic compounds from under-utilized watermelon rind (WRM) with substantial level of retained antioxidant activities. The analysis of variance (ANOVA) results obtained appraised the quality and suitability of model applied (Table 2). The significance of the regression used to foresee extraction yield was significantly controlled by linear, interaction and quadratic effects (Fig. 1) and hence can be predicted by quadratic equation.

The Model F-value of 20.25 with probability (p) 0.0007 implies that the model is significant and there are only 0.07 % chances that this large F-value could occur due to noise. Parameters having values of probability (p) less than 0.0500 indicate model terms are significant. In this perspective A(EC), B(pH), C(T), D(t), AB, AC, BC, CD, A^2, B^2 and C^2 were found to be significant. Similarly, "Lack of Fit F-value" of 0.08 implies that model selected has good fit. Higher value of R^2 (0.9793) showed that quadratic polynomial expression selected predict well the extraction of polyphenol under given experimental conditions. The value of adjusted R^2 (0.9309) proved good agreement between actual and observed extraction yield of WMR. Coefficient of variation (CV) 4.52–7.96 further authenticates that results obtained are quite reliable (Ravikumar et al. 2006).

The interaction can be understood from three dimensional quadratic profiles for yield (Fig. 2a–f). The look at Fig. 2

Table 2 Analysis of estimates of extraction yield, TPC, TEAC and their significance

	Extract yield (g/100 g FW)			TPC mg GAE/g extract of WMR			TEAC mg TE/g FW WMR		
Source	Mean Square	F-ratio	*p*-value[a]	Mean Square	F-ratio	*p*-value[a]	Mean Square	F-ratio	*p*-value[a]
Model	54.02	20.25	0.0007	1907.16	24.33	0.0004	3882.88	21.52	0.0006
A	20.48	7.68	0.0324	512.00	6.53	0.0431	1223.64	6.78	0.0404
B	22.45	8.41	0.0273	2888.00	36.85	0.0009	9721.76	53.89	0.0003
C	42.24	15.83	0.0073	736.82	9.40	0.0220	2430.55	13.47	0.0104
D	32.00	12.00	0.0134	1682.00	21.46	0.0036	257.19	1.43	0.2775
AB	26.04	9.76	0.0205	35.16	0.45	0.5279	356.15	1.97	0.2096
AC	16.82	6.31	0.0458	318.40	4.06	0.0904	236.10	1.31	0.2962
AD	5.53	2.07	0.20000	1553.74	19.83	0.0043	6518.77	36.14	0.001
BC	75.65	28.36	0.0018	1803.30	23.01	0.0030	320.05	1.77	0.2312
BD	0.59	0.22	0.6545	109.56	1.40	0.2818	20.92	0.12	0.7451
CD	58.32	21.86	0.0034	2466.48	31.47	0.0014	5974.34	33.12	0.0012
A^2	158.06	59.25	0.0003	4252.92	54.27	0.0003	11008.79	61.03	0.0002
B^2	223.92	83.94	<0.0001	6439.86	82.17	0.0001	9673.81	53.63	0.0003
C^2	113.61	42.59	0.0006	4220.90	53.86	0.0003	10152.79	56.28	0.0003
D^2	6.73	2.52	0.1632	624.56	7.97	0.0302	1588.05	8.80	0.0251
Lack of Fit	0.081434	0.9233		0.25	0.7932		0.83	0.5002	
R^2	0.9793		0.9827		0.9805				
Adj R^2		0.9309		0.9423		0.9349			
Pred R^2		0.8816		0.8686		0.3884			
CV		7.96		6.80		4.52			

[a] The *p* values were used as a tool to check the significance of each coefficient. Very small *p* values for the linear and quadratic terms indicate the significant contribution of these predictors in the fitted model

revealed the presence of plateau in (a), (c) and (d) confirming strong interaction between enzyme concentration, reaction temperature and pH. The curvature in Fig. 2 (b) and (f) indicated the incubation time does not markedly affect the extraction within studied experimental conditions. At the same time Fig. 2(e) indicated the incubation lesser than 30 min will reduce the extraction yield. Figure 3(a) showed that the levels of the yield predicted from the fitted empirical model are in line agreed with the observed values under the observed experimental conditions, with a sensibly high value

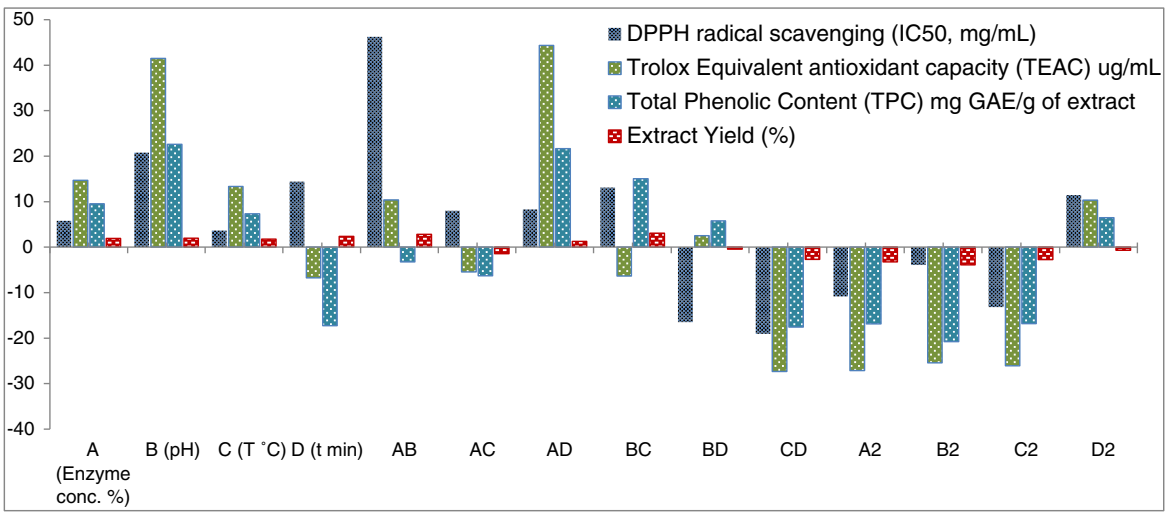

Fig 1 Importance of enzymatic maceration parameters (coded) towards extract yield (%), Total phenolic content (TPC), Trolox Equivalent Antioxidant Capacity (TEAC, μg/mL) and DPPH radical scavenging potential (IC_{50}, μg/mL)

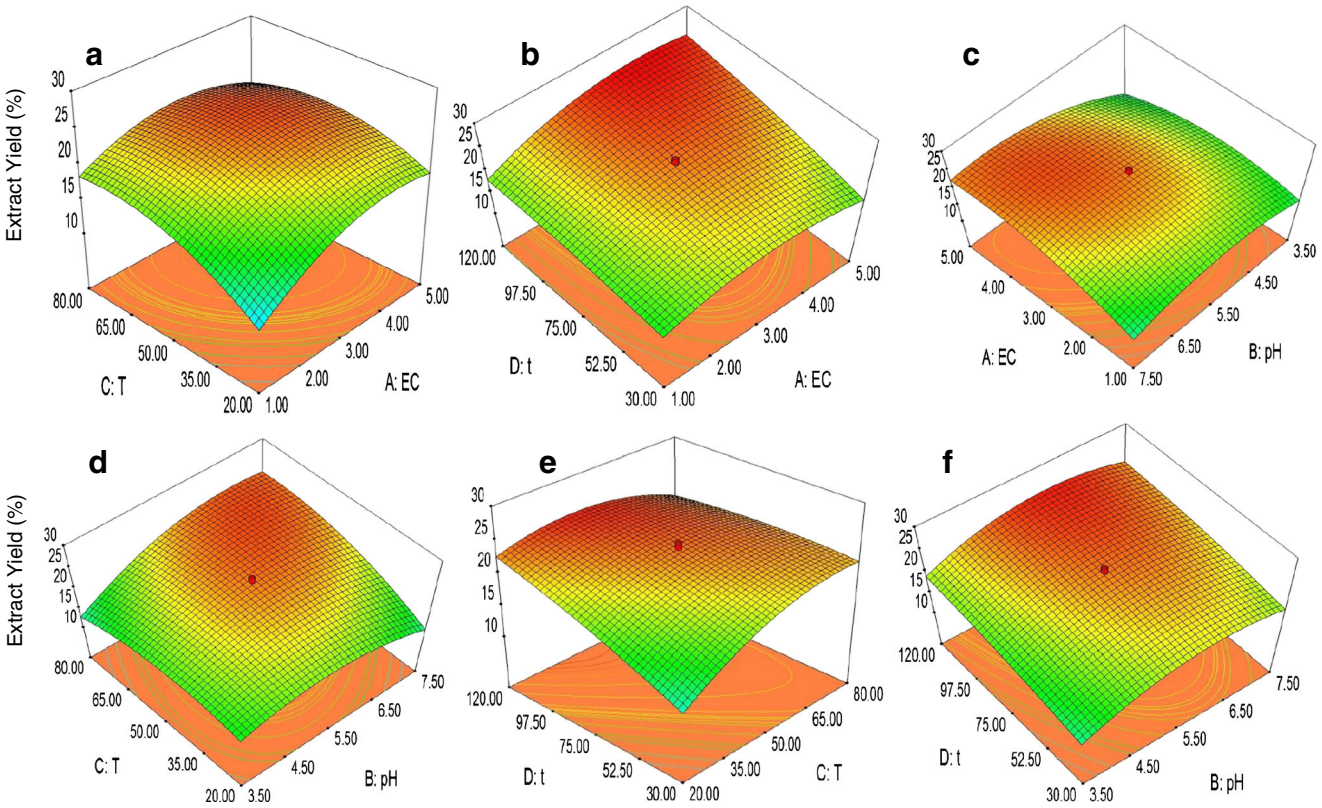

Fig 2 Three dimensional representation of interaction between factors affecting EASE of phenolics from WMR **a** Temperature and enzyme concentration, **b** Enzyme concentration and incubation time, **c** Enzyme concentration and pH, **d** pH and Temperature, **e** Temperature and incubation time, **f** pH and Incubation time

of the coefficient of determination of 0.9793 (R^2) (Table 2). The straight line obtained for normality plot (Fig. 2b) robust the validation of design fitted and resulted ANOVA (Gunst and Mason 2009).

Determination of the optimal conditions

A single response optimization by RSM is relatively an easy and simple task. However, analytical chemists mostly encounter the situation when predictors control almost all the responses to be observed. This difficulty can be overwhelmed by the use visual display and multi-criteria methodology (Wei et al. 2009). In present study multi-responses i.e. extraction yield, total phenolic content, Trolox Equivalent antioxidant capacity and DPPH free radical scavenging potential were optimized simultaneously. The measured individual responses were further transformed into a dimensionless scale "desirability" covering value 0–1. In this context, six solutions were obtained with desirability value 0.65–0.70. The desirability value 0 showed a completely undesirable experimental design and that of 1 indicated a fully desirable design. The advocated solution with maximum desirability (Fig. 4) for extraction of phenolics from WMR was achieved by applying 2.24 % enzyme cocktail for 30 min at 51.8 °C and 6.58 pH producing an extract of 18.93 g/100 g FW of WMR with substantial level of total phenolics (173.70 mg GAE/g FW), TEAC (279.96 mg TE/g) and DPPH radical scavenging (112.27 mg/mL IC_{50}). Overall trends observed during the present attempt were in agreement with the study conducted by Zhang et al. (2013). The higher extraction yield of phenolic compounds observed under optimum conditions might be attributed enzyme pretreatment. Fully ripened WMR is composite of cellulosic micro cemented together by lignin and shield with pectin. Most of phenolic compounds are bound to polysaccharides of cell wall via covalent linkage. The presence of *alpha-amylase, glycosidase units and bacillolysine (protease*) hydrolyze plant cell wall releasing the entrapped phenolic compounds.

Phenolics in watermelon rind

Phenolics compounds have showed wide range of cumulative biological affects including anti-inflammatory, antibacterial, vasodilator actions, ant carcinogenic, antiviral, antithrombotic, antiallergic, and hepatoprotective affects (Haggag et al. 2011). The biochemical, chemical, epidemiological and clinical evidences support the chemo protective effects of phenolic substances against oxidative stress facilitated disorders (Turner et al. 2005; Del Bano et al. 2006; Jayaram and Dharmesh 2011). Recent awareness regarding their anti-cancer potential built up a pressure on food and pharmaceutical industries to

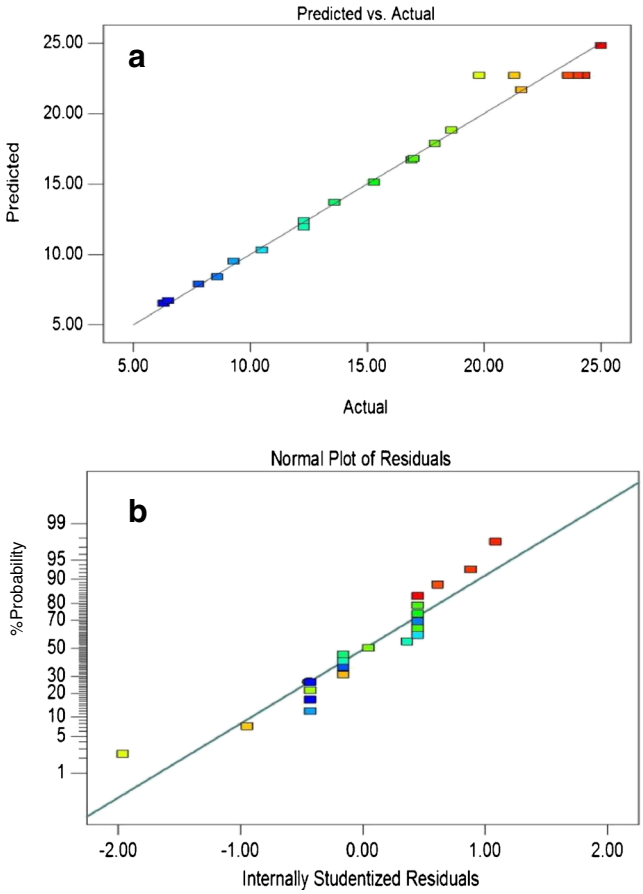

Fig 3 Actual vs. predicted values **a**, and normal plot of probability **b** for extract yield (%)

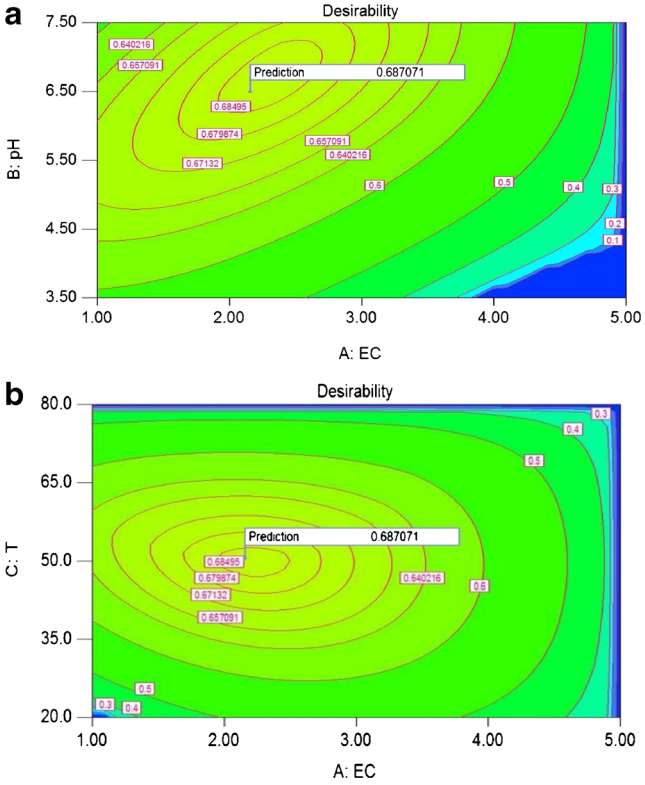

Fig 4 Contour plot of desirability for various solutions for **a** pH and Enzyme concentration, and **b** Enzyme concentration and incubation temperatur suggested during statistical analysis

explore more and more phenolic resource and innovate efficient extraction techniques.

The total phenolic contents in Enzyme assisted solvent extracts (EASEx) of WMR were estimated by Folin-Ciocalteu reagent (FCR) using gallic acid as calibration standard (R^2 0.9976). Overall, total phenolic content in watermelon rind (WRM) were found to be 110.85±8.85 mg GAE/g of fresh WRM extracts. The FCR-based assay commonly known as the total phenols (or phenolic) assay actually measures a sample's reducing capacity. The exact chemical nature of the FCR is not known, but it is believed to contain heteropolyphosphotunstates-molybdates which undergo one or two-electron reduction reaction leading to the formation of blue species, possibly of $(PMoW11O_{40})^{4-}$. Obviously, the FCR is nonspecific to phenolic compounds as it can be reduced by many nonphenolic compounds. Hence, it will more reliable to determine phenolic compounds using more sophisticated analytical technique. For this purpose the extracts obtained under optimal conditions were characterized by RP-HPLC-DAD for individual phenolic compounds. The major phenolic compounds found in watermelon rind were: vanillic, Sinapic, *p*-coumaric, chlorogenic, and *p*-hydroxybenzoic acid (Table 3).

The presence of potential phenolic acids in WMR extracts endorsed that enzyme assisted extraction protocol is potential candidate for the recovery of phenolic compounds from underutilized agro residues. The higher availability of phenolic compounds might be attributed to the compositional profile

Table 3 Phenolics compounds authenticated in watermelon rind extracts obtained using EASE by HPLC-DAD

Sr. No	Retention Time (min)	Phenolics	Concentration (μg/g of extract)
1	4.51	Gallic acid	3.21–5.12
2	2.99	Quercitin	4.69–171.27
3	12.44	Caffeic acid	18.01–135.42
4	13.07	Vanillic acid	26.13–2317.01
5	14.95	Myricetine	16.18–135.13
6	15.22	Chlorogenic acid	115.60–1611.04
7	16.25	Syringic acid	ND*
8	17.97	*p*-coumaric acid	0.50–2.51
9	20.51	*m*-coumaric acid	1.81–14.96
10	22.92	Ferulic acid	ND*
11	27.22	Sinapic acid	113.01–241.12

[a] The phenolic compounds (μg/g FW) determined by HPLC-DAD in extracts obtained against optimal conditions, * not detected

Table 4 Antioxidant activities of fresh watermelon rind extract obtained EASE optimized using RSM

	TEAC[W]				DPPH antiradical capacity[X]			
	Observed	Predicted	Residual	Cook's distance	Observed	Predicted	Residual	Cook's distance
R1	229.92	228.80	1.12	0.05	119.52	118.67	0.85	0.08
R2	175.97	170.98	4.99	1.04	111.87	111.18	0.69	0.05
R3	159.49	158.37	1.12	0.05	46.81	45.96	0.85	0.08
R4	161.15	156.16	4.99	1.04	57.82	57.13	0.69	0.05
R5	189.55	184.56	4.99	1.04	87.68	86.99	0.69	0.05
R6	234.84	233.72	1.12	0.05	88.03	87.18	0.85	0.08
R7	127.52	126.40	1.12	0.05	37.47	36.62	0.85	0.08
R8	133.86	128.87	4.99	1.04	84.66	83.97	0.69	0.05
R9	136.00	140.32	−4.32	1.09[a]	63.52	64.61	−1.09	0.19
R10	185.47	189.79	−4.32	1.09[a]	82.99	84.08	−1.09	0.19
R11	95.82	100.14	−4.32	1.09[a]	57.93	59.02	−1.09	0.19
R12	235.26	239.58	−4.32	1.09[a]	127.65	128.74	−1.09	0.19
R13	136.74	145.66	−8.92	0.14	60.67	61.56	−0.89	0.00
R14	190.82	190.54	0.28	0.00	72.52	73.81	−1.29	0.01
R15	278.00	282.32	−4.32	1.09[a]	111.86	112.95	−1.09	0.19
R16	255.32	259.64	−4.32	1.09[a]	160.29	161.38	−1.09	0.19
R17	235.02	241.82	−6.80	0.00	112.98	104.88	8.10	0.02
R18	235.87	241.82	−5.95	0.00	94.82	104.88	−10.06	0.03
R19	256.92	241.82	15.10	0.02	117.80	104.88	12.92	0.05
R20	260.65	241.82	18.83	0.04	97.99	104.88	−6.89	0.01
R21	230.78	241.82	−11.04	0.01	103.34	104.88	−1.54	0.00

Predicted and observed values of [W], Trolox equivalent antioxidant capacity (TEAC), mg TE/g FW WMR, [X] DPPH radical scavenging activity (IC_{50}mmol/g), and [a] indicates mild outlier

of enzyme cocktail especially presence of pectinase, protease and α-amylase and β-glycosidase units. The level of major phenolic compounds in watermelon rind e.g., Sinapic acid, chlorogenic and vanillic acid were comparable with the findings of Al-Sayed and Ahmed (2013) and higher than agro-wastes investigated by Sultana et al. (2012).

Antioxidant activities watermelon rind extract obtained by EASE

The extracts of WMR obtained by EASE were characterized for their potential antioxidant activities using model in-vitro assays. Various methods have been reported regarding the assessment of antioxidant character of plant derived and synthetic materials. Most frequently applied assays utilize chromogen substances having radical nature that accelerate the reductive oxygen species. Antioxidants react and make the radical chromogens to disappear. One of such most frequently used in-vitro assay is Trolox equivalent antioxidant capacity (TEAC). This method involves ABTS radical cation oxidant, produced by the reaction of ABTS using potassium persulphate. The ABTS radical cations being scavenged by the extracts were quantified spectrophotometerically at 734 nm (Sashidhara et al. 2011). Trolox (Vitamin E) was used

Table 5 Validation results observed against most desirable experimental conditions (d>0.5)

Sr. No.	Variable investigated				Responses observed			
	E (%) (A)	pH (B)	T (°C) (C)	t (Min) (D)	Extract yield[l]	TPC[M]	TEAC[N]	DPPH[O]
1	2.24	6.58	51.8 °C	30 min	19.03	175.70	270.26	115.54
2	2.24	6.58	51.8 °C	30 min	19.89	176.55	278.33	124.25
3	2.24	6.58	51.8 °C	30 min	19.54	174.23	274.45	110.78
Average					19.49	175.71	274.35	116.86
Results predicted with maximum desirability					18.93	173.70	279.96	112.27

[L, M, N] and [O] are the values in g/100 g FW, mg GAE/g FW, mg TE/g FW and mg/mL (IC_{50}), respectively

as positive control and values obtained are expressed as mmol of Trolox equivalent (TE) /g FW extract. The observed and predicted values of TEAC are incorporated in Table 4. The maximum observed antioxidant activity 278.00 mg TE/g of WMR extract was a mild outlier and very close to most suitable predicted TEAC value 279.96 mg TE/g under optimal conditions. Hence, enzyme cocktail comprising pectinase, protease and α-amylase and β-glycosidase units released the phenolic compounds from watermelon rind (WMR) with retained antioxidant activity under 2.24 % EC (A), 6.58 pH (B), 51.8 °C T (C) for 30 min (D).

The DPPH radical is another widely used reliable tool to measure free-radical scavenging activity of plant materials (Zheng et al. 2010). DPPH; a deep violet colored and relatively stable organic free radical produces absorption maxima within 515–528 nm range. Protonation of DPPH by hydrogen donor species, specifically phenolic compounds, causes loss of its chromophoric group which appears in yellow coloration. The DPPH radical scavenging potential increase with the increase in phenolic compounds or their respective degree of hydroxylation (Kedare and Singh 2011). The higher sensitivity of DPPH free radical towards hydrogen donors species facilitate this assay up to very low concentration of phenolic compounds (Martysiak-Zurowska and Wenta 2012).

The tabulated data regarding DPPH radical scavenging capacity (IC_{50} mg/ml) of fresh watermelon rind (WRM) were in a range of 36.62–160.62 mg/mL. All the values observed were in line agreed with those of predicted (Table 4). The optimal predicted response of DPPH radical scavenging was observed at 2.33 % EC, 6.72 pH, 50.06 °C and 30.0 min incubation time.

Validation of optimized conditions

Table 5 summaries the results of validation experiments conducted for the conditions supposed most suitable during statistical analysis. The data indicates the results observed under suggested conditions are quite agreement with those supposed by the design analysis report. Furthermore, HPLC-DAD analysis of extracts obtained under optimum conditions revealed the presence of major phenolic acids i.e. Sinapic, *p*-coumaric, chlorogenic acid, and hydroxybenzoic acid and its derivate.

Conclusions

Nutritional, medicinal and functional food potential of plant phenolics; wastage volume of under-utilized watermelon rind (WRM) and lack of efficient extraction strategy endorsed us to optimize enzyme assisted solvent extraction (EASE) protocol of potent phenolic from WRM. The observed results revealed that EASE enhanced extraction yield of phenolics up to 3 folds. The validation results, HPLC-DAD and antioxidant characterization of extracts of fresh WMR revealed that extracts contained substantial amount of phenolic acids with ample level of retained antioxidant character. Hence, Kemzyme cocktail comprising pectinase, protease, α-amylase and β-glycosidase can be optimistically used for industrial scale extraction of polyphenols to value add food and pharmaceutical industry.

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