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RSM/ANN based optimized recovery of phenolics from mulberry leaves by enzyme-assisted extraction

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Abstract: Recovery of phenolics from *Morus alba* leaves (MAL) and extraction into the solvent was optimized using enzyme-assisted extraction. The influence of four parameters, including enzyme concentration (*EC*), temperature (*T*), incubation time (*t*) and pH were investigated using rotatable central composite design (RCCD). Two factors, namely enzyme concentration and pH, exhibited significant effect on extraction efficacy yield of extractable phenolics from MAL. Furthermore, artificial neural network (ANN) model was executed to predict the relationship between dependent and independent variables. Among enzyme complexes (kemzyme dry-plus, natuzyme and zympex-014) employed for extraction, zympex-014 assisted extract depicted maximum amount of phenolic bioactives from MAL. Morphological changes in the cell wall of MAL residue were elucidated by scanning electron microscopy (SEM). The main phenolic compounds identified and quantified by gas chromatography mass spectrometry (GC/MS) in MAL extract were found to be quercetin, gallic acid, *m*-coumaric acid, cinnamic acid, syrinigc acid and vanillic acid.

Keywords: artificial neural network; GC/MS; *Morus alba* leaves; response surface methodology; SEM

Pakistan has a various and rich flora distributed across several agro-climatic regions of the country. There are huge reserves of medicinally important plants with potential for isolation of natural bioactive compounds (NBCs) and development of nutraceuticals/phyto-medicines. One of the very important regions, The Pothohar Plateau, in north eastern Pakistan, contains plenty of wildly grown food and medicinal plants (Lucock *et al*. 2004).

Mulberry (*Morus alba* L.) is one of the valuable food plants with high medicinal value grown in Pothoharic region of Punjab, Pakistan. Being a multipurpose agro-forestry plant, *M. alba* is used in medicine for treating several diseases such as fever, hypertension, arthritis, liver and disorders. Moreover, it has been described in the literature that *M. alba* roots, leaves and fruits exhibit multiple biological activities including antioxidant, antimicrobial, anticancer, hypolipidemic, neuroprotective, antidiabetic and antihypertensive effects (Ghareeb *et al.* 2016).

A wide array of plant bioactives have been isolated and identified in plants that provide an excellent pool of molecules, which have broad range of functionalities to develop therapeutics and natural products. Among different phytochemicals such as lipids, polyphenols, terpenoids and colouring pigments, the plant phenolics are of vital importance and have gained greater recognition as natural antioxidants

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bioactive compounds with substantial health benefits (You *et al*. 2013).

In perspective of cost-effectiveness and green methodology, enzyme-assisted extraction holds its uniqueness as a potential bioprocess for effective recovery of phenolics with high antioxidant activity. A particularly useful application of enzyme lies in its ability to effectively hydrolyse the cell wall by releasing maximum compounds into extraction medium and thereby reducing the amount of solvent. Enzymes are extensively used for cell wall degradation and thereby improving bioactives extractability (Latif *et al*. 2011).

So far, no detailed work has been compiled on the RSM/ANN based enzyme-assisted extraction of phenolic bioactives from *M. alba* leaves grown in Pothohar region, Pakistan. The main aim of the current research work was to explore application of selected enzyme combinations towards effective recovery of phenolics antioxidants from *M. alba* leaves. Scanning electron microscopy was used to elucidate ultrastructure and morphological changes in the control and enzyme-assisted residues. Systematic characterization of individual phenolic compounds in the extracts produced was made by GC/MS.

MATERIAL AND METHODS

Morus alba leaves (MAL) were collected from village Khai Kotli district Jhelum, Punjab in Pakistan. After drying, the ground powder was stored at – 4°C until analysed (Qadir *et al*. 2018). Commercial enzyme mixtures, such as kemzyme dry-plus (β-glucanase, xylanase, cellulase, α-amylase and protease) (Kemin Industries, Pakistan), natuzyme (xylanase, cellulase, β-glucanase, pectinase, protease, phytase and alpha-amylase) (Mehrban poultry services, Pakistan). While zympex-014 (xylanase, β-glucanase, β-mannase, σ- galactosidase, amylase and acid protease) was obtained from BA Traders (Pakistan).

Response surface methodology (RSM). After preliminary screening experiments, four independent variables, enzyme concentration (*EC*), temperature (*T*), incubation time (*t*) and pH were investigated at axial points (eight runs), centre points (five runs) and factorial points (eight runs) with different levels (– α, – 1, + 1, and + α) using central composite design as shown in Table 1. Extract yield (response) was modelled through 2nd order polynomial equation using Design Expert Software as follow:

$$Y = b_0 + \sum biXi + \sum biiXi^2 + \sum bijXiXj \quad (1)$$

where: Y – response; b_0 – intercept; $\sum biXi$ – linear effect; $\sum biiXi^2$ – quadratic effect; $\sum bijXiXj$ – interaction between variables

Artificial neural network (ANN). ANN is a more valuable tool to interpret the relationship between the input and output data of augmented experimentations. In particular, ANN is an efficient algorithm to identify any function with limited number of discontinuities (Akbar *et al*. 2012).

Enzyme-assisted extraction pf phenolic components/extracts. Enzymatic pretreatment was carried out under pre-optimized conditions for each enzyme mixture. Briefly, fresh MAL sample powder (10.00 g) was diluted with 15 ml of phosphate buffer (pH 6–9) in a flask and blended with selected multi enzyme complex (0.5–6.5 g) for 30–90 minutes. Followed by enzymatic treatment, the selected enzyme cocktail was deactivated by heating at 100°C for 10 min and sample was extracted in an orbital shaker with 100 ml of 80% aqueous methanol and filtered through 0.22 µm filter paper under the pressure (Heidolph, Germany). The extracts were then concentrated using a rotary evaporator and weighed to estimate the yield of extract. Five gram of crude concentrated extract (CCE), dissolved in 100 ml water, was purified using liquid-liquid partitioning (*n*-hexane, ethyl acetate) to get phenolic-rich fractions.

Study of morphological changes in enzymatically hydrolysed MAL residue. Scanning Electron Microscopy (SEM) was employed for detecting ultrastructure and morphological changes in the MAL residue hydrolysed by zympex-014.

Derivatization and GC-MS analysis. Ethyl acetate extract (44.1 mg) of *M. alba* leaves was taken in 1 ml sample vial and further addition of 5 mg 3-hydroxy benzoic acid (as an internal standard), along with mixture of 300 µl pyridine + 50 µl trimethylchlorosilane (TMCS) + 150 µl *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) was executed. After derivatization of the sample, anaylsis of TMCS derivatives was carried out using GC-MS (Agilent Technologies 7890A GC equipped with Agilent 5975 MSD). The carrier gas (helium) was used at a flow rate of 0.5 ml per min. The quadropole triple axis MS detector was set at 250°C (transfer line). GCMS was analysed using electron impact ionization at 70 eV and data was evaluated using total ion count (TIC) for compound identification and quantification (Ahmad *et al*. 2016).

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RESULTS AND DISCUSSIONS

RSM/ANN model treatments and prediction of extract yield. Through response surface methodology (RSM), effects of enzyme concentration (*EC*), temperature (*T*), incubation time (*t*) and pH were optimized at different levels to analyse the enhanced recovery of extractable compounds such as phenolics from mulberry leaves. The experimental design along with the extract yields of complex enzymes is shown in Table 1. In current RSM design, maximum extract yield (30.00 g/100 g) was obtained at optimized conditions such as 5.0% enzyme concentration, 8.5 pH, 70°C temperature and 45 min reaction time employing zympex-014. The response (extract yield) observed for zympex-014 was found to be higher than kemzyme plus-dry (25.00 g/100 g) and natuzyme (24.50 g/100 g).

In another study, ANN modelling was also applied to predict the extraction yield by taking parameters such as enzyme concentration (*EC*), temperature (*T*), incubation time (*t*) and pH as input variables and extract yield as output variable. Neurons in hidden layer were optimized and impact of neurons was developed by calculating weights. Number of neurons in input layer was four, in hidden layer nine and one in target output layer *i.e.* extract yield. Among ANN techniques, Multilayer Perceptron (MLP) method was found to be more precise, accurate and reliable due to less training error (0.007571) and test error (0.006278), with test performance (0.998956) near to one. The predicted results obtained through MLP were quite in close agreement with the experimental values.

Among enzyme complexes tested, zympex-014 recovered maximum extract yield from mulberry leaves (Table 1) that may be attributed to the presence of xylanase, β-glucanase, β-mannase, σ- galactosidase, amylase and acid protease in it, which effectively ruptured MAL cell wall by releasing bound phenolic moieties (Saeedeh *et al.* 2011).

RSM and ANN based predicted results for zympex-014 MAL extract, , were very close to the experimental values which demonstrate the applicability of

Table 1. Extract yield (g/100 g) of enzyme complexes and prediction through RSM and ANN

Run	Point type	Independent variables (actual)				Enzyme complex			Predicted results for Zympex-014	
		A	*B*	*C*	*D*	Kemzyme Dry-Plus	Natuzyme	Zympex-014	RSM	ANN
		EC (%)	(pH)	*T* (°C)	*t* (min)					
1	**axial**	3 (0)	7.5 (0)	50 (0)	30 (– α)	13.00	14.00	18.00	17.27	17.60
2		3 (0)	7.5 (0)	50 (0)	90 (+ α)	14.00	14.50	19.00	19.73	23.13
3		3 (0)	9 (+ α)	50 (0)	60 (0)	17.70	20.00	23.00	23.97	22.10
4		3 (0)	6 (– α)	50 (0)	60 (0)	17.00	19.00	22.00	21.03	23.17
5		3 (0)	7.5 (0)	25 (– α)	60 (0)	24.30	17.00	19.20	19.93	23.30
6		0.5 (– α)	7.5 (0)	50 (0)	60 (0)	11.40	14.00	17.00	18.35	24.36
7		3 (0)	7.5 (0)	75 (+ α)	60 (0)	21.00	22.00	24.00	23.27	22.80
8		6.5 (+ α)	7.5 (0)	50 (0)	60 (0)	21.10	23.40	26.00	25.31	23.19
9	**factorial**	5 (+ 1)	6.5 (– 1)	70 (+ 1)	75 (+ 1)	11.00	11.50	13.00	13.00	22.82
10		1 (– 1)	8.5 (+ 1)	30 (– 1)	75 (+ 1)	23.00	21.80	26.00	24.82	22.98
11		5 (+ 1)	8.5 (+ 1)	30 (– 1)	45 (– 1)	19.50	23.00	25.50	25.77	22.80
12		1 (– 1)	6.5 (– 1)	70 (+ 1)	45 (– 1)	21.00	21.50	25.00	25.00	22.97
13		1 (– 1)	6.5 (– 1)	30 (– 1)	45 (– 1)	20.60	21.00	24.00	24.00	22.85
14		1 (– 1)	8.5 (+ 1)	70 (+ 1)	75 (+ 1)	17.80	18.00	25.00	24.77	23.27
15		5 (+ 1)	8.5 (+ 1)	70 (+ 1)	45 (– 1)	23.90	24.00	30.00	31.18	23.01
16		5 (+ 1)	6.5 (– 1)	30 (– 1)	75 (+ 1)	18.80	20.00	23.00	23.00	22.69
17	**centre**	3 (0)	7.5 (0)	50 (0)	60 (0)	22.40	22.00	26.00	26.17	25.01
18		3 (0)	7.5 (0)	50 (0)	60 (0)	24.50	24.00	27.00	26.17	24.80
19		3 (0)	7.5 (0)	50 (0)	60 (0)	25.00	24.50	28.00	26.17	24.80
20		3 (0)	7.5 (0)	50 (0)	60 (0)	21.00	22.50	25.00	26.17	24.80
21		3 (0)	7.5 (0)	50 (0)	60 (0)	23.50	21.00	25.50	26.17	24.80

EC – enzyme concentration

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these models as given in Table 1. As reported in our previous study for enzyme-assisted extraction of phenolics from *Momordica balsamina* fruit (Qadir *et al*. 2019), response surface methodology and neural network modelling can be successfully employed to predict the extract yield from MAL by incorporating experimental data (Guine *et al.* 2015).

Fitting the RSM model and three dimensional graphical representation. Robustness/significance of the model applied was verified by analysis of variance (ANOVA) as shown in Table 2. ANOVA was examined by synergizing the effect of linear, quadratic and interaction coefficients on the response factor. In this case, *A*, *AB*, *AC*, *BC*, *BD*, *CD*, A^2, B^2, C^2, D^2 are significant model terms. The model *F* (8.99) and *P* (0.0064) also indicate the siginifcance of model terms (> 0.005). Values greater than 0.1000 indicate that model terms are not significant. Insignificant lack of fit value such as low *F* (3.22) and high *P* (0.1466), relative to the pure error also proved the accuracy of model applied. The determination coefficient (R^2) of 0.9183, and the adj. R^2 of 0.8448 indicated a satisfactory correlation between responses and independent variables. Coefficients of variation at 5.79 further authenticated that the results obtained are quite reliable (Ravikumar *et al*. 2006).

The 3D response plot was applied on the data obtained from experimental results and effect of parameters including time, pH and temperature was monitored to observe extract yield. According to the Figure 1A, B and F, the response value (extract yield) elevated with the increasing extraction time up to 65 min, but after that it began to decrease. Likewise, temperature increase up to 80°C resulted in an increased extract yield. Enzyme concentration exerted a strong influence on the response value (Figure 1C and D), while it could also be observed that pH caused almost similar effect on response value as can be seen in Figure 1D, E and F. Enzyme concentration and pH have been found as major factors involved in affecting extract yield. Combining the results of ANOVA as in Table 2 and the response surfaces plots, conclusion could be drawn that enzyme concentration exerted more significant influence on the response value (extract yield) than other variables i.e. temperature, pH and time.

Effect of zympex-014 on MAL surface. MAL surface was analysed by SEM before and after enzymatic treatment at 2 μm resolution. As depicted in the Figure 2A, initial materials consisted of integral and intact parts whereas after enzymatic treatment, wrinkles and fragments appeared on surface of cell

Table 2. ANOVA of the fitted quadratic model

Source	Sum of squares	*MS*	*F*	*P*
Model*	317.59	22.69	8.99	0.0064
A (*EC*)*	33.66	33.66	13.33	0.0107
B (pH)*	1.89	1.89	0.7504	0.4196
C (*T*)*	0.0983	0.0983	0.0389	0.8501
D (*t*)*	8.16	8.16	3.23	0.1223
*AB**	77.56	77.56	30.72	0.0015
*AC**	67.70	67.70	26.82	0.0021
*AD**	6.47	6.47	2.56	0.1606
*BC**	102.84	102.84	40.74	0.0007
*BD**	28.44	28.44	11.27	0.0153
*CD**	22.55	22.55	8.93	0.0244
A^{2*}	36.62	36.62	14.51	0.0089
B^{2*}	19.28	19.28	7.64	0.0327
C^{2*}	29.90	29.90	11.85	0.0138
D^{2*}	84.27	84.27	33.38	0.0012
Residual^{ns}	15.15	2.52		
Lack of fit^{ns}	9.35	4.67	3.22	0.1466

A (*EC*) – enzyme concentration; *A*, *B*, *C*, *D* – linear terms; *AB*, *AC*, *AD*, *BC*, *BD*, *CD* – interaction terms; A^2, B^2, C^2, D^2 – quadratic terms; *significant model terms; ns – non significant

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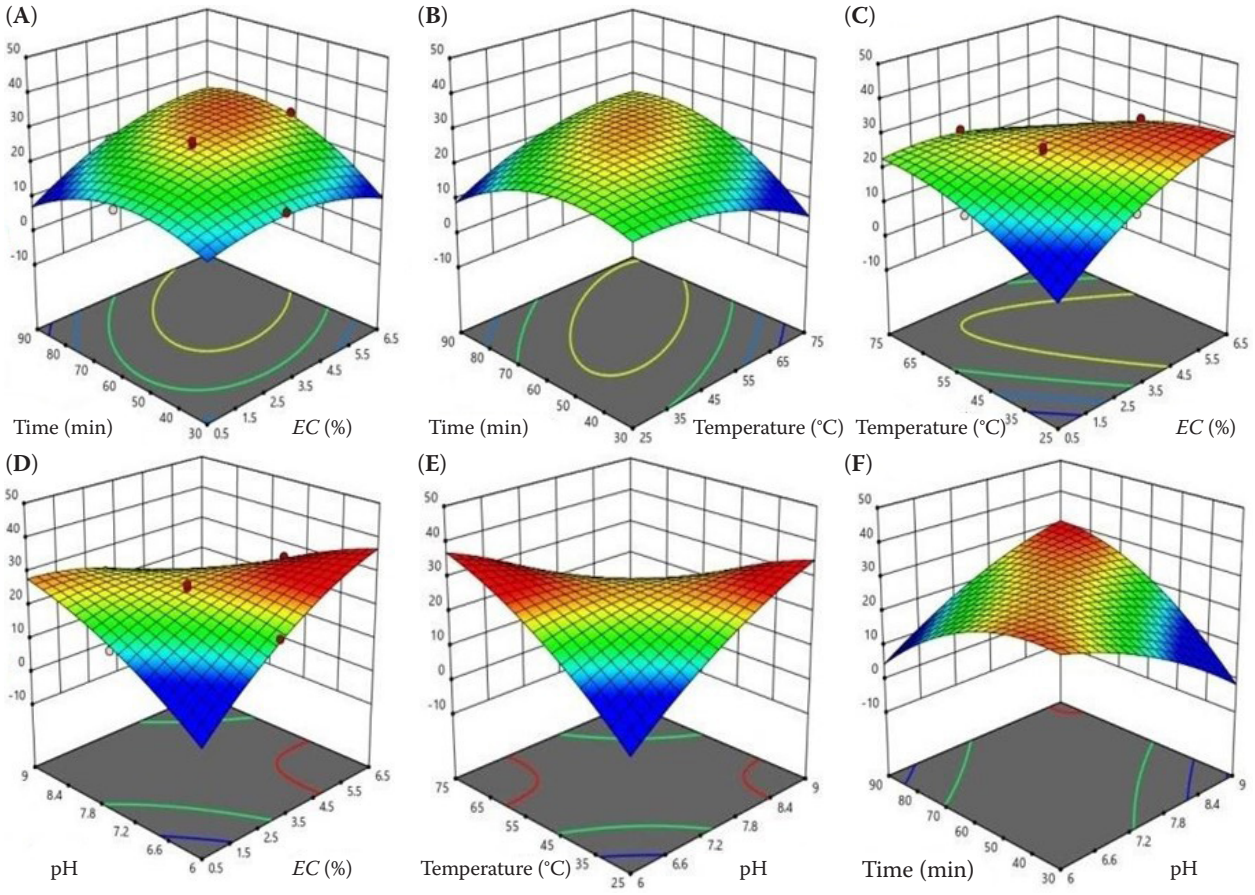

Figure 1. Surface plots showing the interaction between factors affecting enzyme-assisted extraction of phenolic compounds from *M. alba* leaves

EC – enzyme concentration

wall as shown in Figure 2B. The ruptured cell wall assisted in liberation of bound phenolic moieties from cell wall followed by liberation into the extracting solvent.

GC/MS analysis of MAL extract. Plant bioactives present in essential oils are highly suited to GC/MS analysis without derivatization due to their volatility. However, plant extracts usually require chemical derivatization to facilitate the separation by GC by minimizing the polarities of metabolites. The phenolic contents of MAL may be affected by various agro-climatic factors. However, the polyphenols found in mulberry leaves/fruits can induce healthiest effects with substantial strong antioxidant activities. These health effects are mainly imparted due to preventing measures against degenerative diseases, namely cardiovascular diseases

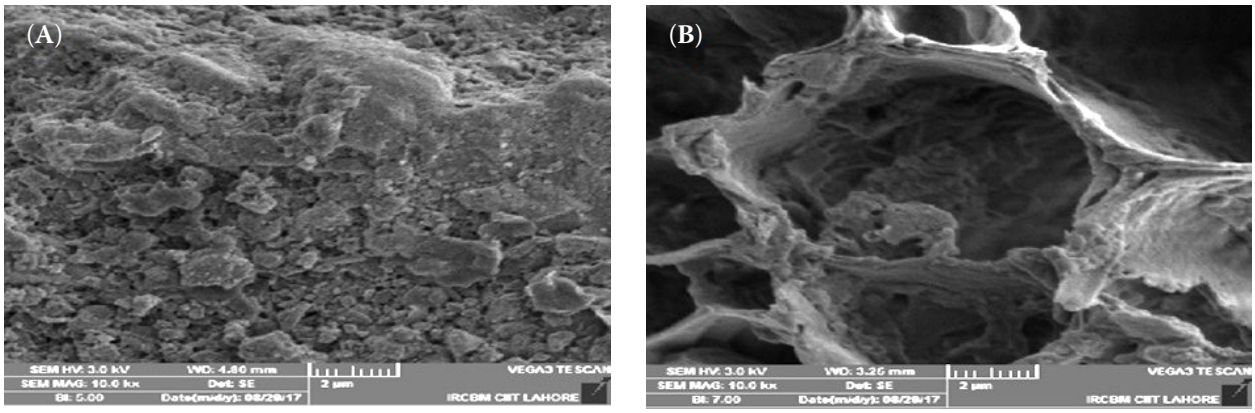

Figure 2. Hydrolytic changes in *M. alba* leaves before (**A**) and after enzymatic treatment (**B**) at 2 µm

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and cancers. Therefore, the identification of phenolic components in the MAL enzyme-assisted extract could support understanding its strong antioxidant capacity (Qader *et al*. 2011; Ahmad *et al*. 2016).

Since MAL purified enzyme-assisted extract was subjected to individual phenolics analysis by GC/MS that revealed distribution of different phenolics. The total ion chromatogram of the sample obtained under the optimal conditions is shown in Figure 3. Calibration was performed using 3-hydroxy benzoic acid as an internal standard. Seven phenolic components were identified (Table 3). The data given in the table authenticated the improved recovery of phenolic moieties that may be linked to an effective hydrolysis and enhanced recovery of phenolics in due part to the enzymatic pre-treatment.

Major phenolics obtained were quercetin, gallic acid, *m*-coumaric acid, cinnamic acid, syrinigc acid and vanillic acid, respectively (Butkhup *et al*. 2013). Their consumption has been reported to exert positive impact on human health. The high content of phenolic compounds in mulberry leaves may support the key role of phenolic phytochemicals in health-enhancing features related to the supplementation of MAL enzyme-assisted extract (Chung *et al*. 2013).

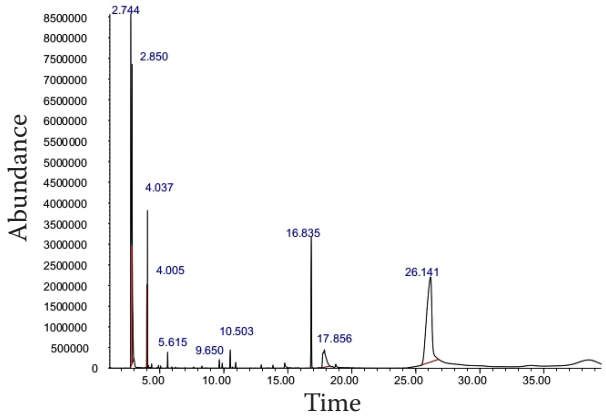

Figure 3. GC/MS analysis of MAL zympex-014 assisted extract after derivation

Table 3. Major phenolics identified in zympex-014 assisted extracts of MAL

No.	Phenolics	*RT* (min)	Concentration (µg/ml)
1	quercetin	2.74	1.55 ± 0.13
2	gallic acid	4.03	1.00 ± 0.15
3	*m*-coumaric acid	5.61	0.06 ± 0.01
4	cinnamic acid	10.50	0.25 ± 0.09
6	syringic acid	16.83	0.76 ± 0.26
7	vanillic acid	17.85	0.23 ± 0.08
IS	3-hydroxybenzoic acid	26.14	5.00 ± 0.65

CONCLUSIONS

An enhanced recovery of phenolics from enzyme pre-treated MAL extract clearly suggests the use of enzyme-assisted extraction as an efficient green extraction method. RSM and ANN models significantly predicted the optimum extract yield and elaborated the relationship between dependent and independent variables. Rupturing and hydrolysis of MAL cell wall by enzyme cocktail, zympex-014, comprising of xylanase, β-glucanase, β-mannase, σ- galactosidase, amylase and acid protease, liberated bound phenolics moieties that resulted in a higher yield of antioxidant phenolics. GC/MS characterization depicted the presence of an appreciable amount of phenolics in the enzyme-assisted MAL extract. The present findings support that enzyme-assisted extraction can be explored as a viable bioprocess for improved phenolics recovery from MAL extract leading to the revalorization of this underutilized agro-material.

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