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Full Length Research Paper

Extraction, optimization and characterization of crude polysaccharides from *Artemisia Mongolica*

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In this study, the extraction conditions of polysaccharides of *Artemisia mongolia* (PAM) were optimized and their molecular weight, weight distribution, composition and antioxidant activities of the polysaccharides investigated. The degree of polymerization of fructan-oligosaccharides 2-8 was separated by thin layer chromatography (TLC) with good results. Polysaccharides were extracted from the leaves of *A. mongolia* and the effects of the extraction parameters (extraction temperature, extraction time and water to raw material ratio) were optimized under optimal extraction combinations. Based on the study, the maximum yield of polysaccharides (17.38%) was obtained at an extraction temperature of 76.90°C, extraction time of 1.33 h and water-to-raw material ratio of 4.00 ml/mg. The yield of polysaccharides was largely dependent on the extraction parameters. Four isolated fractions of the polysaccharides were further characterized using high-performance liquid chromatography. Using TLC analysis, the degree of polymerization of fructan-oligosaccharides 2-8 was successfully separated. High *A. mongolia* antioxidant polysaccharide with antioxidant activity of 351.3 sc/g was obtained at optimized incubation temperature of 70.37°C, extraction time of 1.45 h and water-to-raw material ratio of 2 ml/mg.

Key words: *Artemisia Mongolia*, crude polysaccharide, antioxidant activity, response surface methodology, box-Behnken design

INTRODUCTION

Artemisia plant, known locally in Chinese Mandarin Language as “mengguhao” and scientifically as *Artemisia mongolica*, was used in this study. *A. mongolica* leaf is a

traditional medicinal herb in China with an early history dating back to the “Nei Meng Gu Zhong Cao Yao” era. The genus *Artemisia* belongs to the family composite

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Arteraceae, which is mainly distributed in the northeast grasslands of China, Korea, Japan, Mongolia and Russia. The leaves of *A. mongolica* are commonly used in folk medicine for the treatment of many diseases, including fever, sore throat, tonsillitis, headache, wounds and hepatitis (Moerman, 1998; Hong et al., 2004).

A number of studies have been conducted on *Artemisia* polysaccharides. Xie et al. (2008) worked on fractionation and characterization of biologically active *Artemisia tripartite* polysaccharides, where five monosaccharides (xylose, glucose, arabinose, galactose and galactosamine) were also isolated. Zhang et al. (2011) screened the chemical characterization of *Artemisia* seed polysaccharides, with specific emphasis on the analysis of monosaccharide components of *Artemisia* seed polysaccharides (ASP). In the study, gas chromatography (GC) was used to determine the monosaccharide components, atomic force microscopy to capture monosaccharide images and scattering method was used to obtain molecular weight.

Polysaccharides are polymeric carbohydrate structures formed by repeated units joined together by glycosidic bonds. Polysaccharides are widely investigated because of their chemical properties and biological activities (Sun et al., 2010; Wang et al., 2009). Particularly, polysaccharides of traditional medicinal herbs have medical applications and are reported to possess a wide range of pharmacological properties such as anti-tumour, anti-oxidant, anti-diabetic and adjuvant activities. To practicalize the promising applications of polysaccharides, there is the need to study the bio-functional properties of polysaccharides from *A. Mongolica* leaves. However, little effort has been devoted to the extraction of *A. mongolica*-based polysaccharides.

The hot-water reflux extraction technique (requiring long extraction time and high temperature) is the most common method used to extract plant-based polysaccharides. However, this extraction method has always had a low efficiency. To obtain high yields of plant-based crude polysaccharides, the extraction process must be optimized by mathematical models (Zhong and Wang, 2010). A good example of such model is the response surface model (RSM), which is based on the Box-Behnken Design (BBD). Additionally, the use of 96-well microplate technique in conjunction with existing chemical methods can efficiently determine the total polysaccharides of a large number of plant samples. This technique not only saves reagents, but also time and sample materials. The microplate-based method of determination of carbohydrates that uses phenol-tetraoxosulphate (VI) acid procedure has also been reported by Masuoka et al. (2006). Then, Tian et al. (2011) noted that the microplate-based method was a more convenient way of optimizing the extraction processes of crude polysaccharides from *A. mongolica* plant. A number of merits make this method preferable

over others, including cheap costs and low reagent/material use.

Thus far, there is little application of BBD and antioxidant of *Artemisia* plant in the investigation of 96-well format. Thus, BBD was used to optimize the process parameter ratios of water to raw-material, extraction temperature and extraction time of crude polysaccharides. Then, the antioxidant properties of the crude polysaccharides were evaluated and assayed in terms of antioxidant activities by testing the scavenging abilities on 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radicals.

MATERIALS AND METHODS

Instruments and reagents

The leaves of *A. mongolica* were collected in March 2011 from Songnen grassland in Jilin Province, China, and identified by Professor Yifei Yang of Northeast Normal University, Changchun, China. The raw samples were rinsed in distilled water to remove impurities such as dust. The leaves were immediately separated from the plant and the former lyophilized and milled. The derived powder was sieved (through 60 mm mesh screen) and stored at 4°C until use. Inulin, purchased from Sigma Chemical Co. (China, Hongkong) as standard, was dissolved in pure water (10 mg/ml as stock solution). The pure water was obtained from Milli-Q Academic A₁₀ water purification system (Millipore Corporation, USA) and EDTA-Ca and TFA purchased from Beijing Shiji. Also, a 20 × 20 cm silica-gel coated glass plate of 250 µm depth (SI 250 JT) was used in the study. The other materials and their origins used in the study included Baker Phillipsburg from NY USA, HP-1050 from US, SHIMADZU-RID-10A HPLC from JAPAN and SHIMADZU UV-2201 from JAPAN.

Absorbance measurement was done in flat-bottomed 96-well format (Thermo Life Sciences, Hampshire, UK) and EDP-plus™ micropipette (Oakland, CA, USA). Then a 25, 250 and 1000 µl liquid-handling robotics was used to dispense solution into the 96-well format. For accuracy and precision, they pipette were respectively 0.3 and 1% or better. All chemicals used in the experiment were of analytical grade and were purchased from Sigma-Aldrich (Malaysia).

Extraction procedure

To remove any color-masking substances, the *A. mongolia* leaves (200 g) were added into ethanol (400 ml) of 80°C water bath for 2.5 h. After oven-drying at 60°C, each pretreated sample was extracted by water at designed extraction temperature, extraction time and water-to-raw-material ratio. The water-extracted solutions were separated from insoluble residues by centrifugation (10,000 rpm for 25 min) and then precipitated by the addition of ethanol. The precipitate was filtered and oven-dried at 60°C for 12 h. The dried crude polysaccharides were refluxed three times with acetone and chloroform to remove lipids. The resultant product was extracted in hot water and then filtered, and the combined filtrate precipitated using ethanol again. The content of the polysaccharides was measured by using the phenol-sulfuric acid method (Dubois et al., 1956).

Analysis of samples

This procedure used to analyze samples in this study was based on

the method proposed by Tian et al. (2011), but with some modifications as follows: 150 μl of concentrated sulphuric acid were added to each well of 96-plate, pre-loaded with 20 μl standard solution (100 mg l^{-1}) — manufacturer's sample solution and blank. 60 μl of 6% aqueous phenol (w/w) were then added to each 96-well plate before incubation at 97°C in an oven for 10 min. Subsequently, the absorbance was measured at 490 nm in a microplate multi-scan reader- measurements were made in triplicates. The response was compared to a inulin-based standard curve and the soluble fructans content expressed as mg l^{-1} of inulin. The purity (%) of fructans was calculated as the sugar content of extraction divided by the weight of dried plant materials.

Polysaccharide extraction and temperature

The test tube was respectively labeled 10, 20, 40 and 60°C and each was repeated three times. It was then placed in a test tube holder and *A. mongolica* leaves weighed by analytical beam balance. Distilled water was added to 10.02–10.08 mg sample weight of the 10, 20, 40 and 60°C test tubes and immerse in 70°C water-bath box for 30 min. The test tubes were then removed from water box and allowed to cool. Next, 2 ml of distilled water was added to 8 ml of the sample and filled to 10 ml. Then different volumes of distilled water, tetraoxosulphate (VI) acid, H_2SO_4 , phenol and standard solution were respectively pipetted into the castor box. Using 20 μl of distilled water as blank, 60 μl of phenol and 150 μl of H_2SO_4 were respectively added to different concentrations of 20 μl standard solution (0.2, 0.4, 0.6, 0.8 and 0.10 ml) and 20 μl sample solution, and stirred and placed in oven for 10 min. The solution was placed in a SPECTRAMax® 190-microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, USA) with scanning monochromator for data collection.

Polysaccharide extraction and time

The test tube was respectively labeled 10, 20, 40 and 60 min and each repeated three times. It was then placed in a test tube holder and *A. mongolica* leaves weighed by analytical beam balance. Distilled water was added to 10.02–10.08 mg sample weight of the 10, 20, 40 and 60 min test tubes and immerse in 70°C water-bath box for 30 min. The test tubes were then removed from water box and allowed to cool. Next, 2 ml of distilled water was added to 8 ml of the sample and filled to 10 ml. Then, different volumes of distilled water, tetraoxosulphate (VI) acid, H_2SO_4 , phenol and standard solution were respectively pipetted into the castor box. Using 20 μl of distilled water as blank, 60 μl of phenol and 150 μl of H_2SO_4 were respectively added to different concentrations of 20 μl standard solution (0.2, 0.4, 0.6, 0.8 and 0.10 ml) and 20 μl sample solution, and stirred and placed in oven for 10 min. The solution was placed in a SPECTRAMax® 190-microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, USA) with scanning monochromator for data collection.

Raw material-to-water extraction ratio

The test tube was respectively labeled 10, 20, 40 and 60% and each repeated three times. It was then placed in a test tube holder and *A. mongolica* leaves weighed by analytical beam balance. Distilled water was added to 10.02–10.08 mg sample weight of the 10, 20, 40 and 60% test tubes and immerse in 70°C water-bath box for 30 min. The test tubes were then removed from water box and allowed to cool. Next, 2 ml of distilled water was added to 8 ml of the sample and filled to 10 ml. Then, different volumes of distilled

water, tetraoxosulphate (VI) acid H_2SO_4 , phenol and standard solution were respectively pipetted into the castor box. Using 20 μl of distilled water as blank, 60 μl of phenol and 150 μl of H_2SO_4 were respectively added to different concentrations of 20 μl standard solution (0.2, 0.4, 0.6, 0.8 and 0.10 ml) and 20 μl sample solution, and stirred and placed in oven for 10 min. The solution was placed in a SPECTRAMax® 190-microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, USA) with scanning monochromator for data collection.

Experimental design and RSM optimization

Based on single-factor polysaccharides production, the extraction temperature, proper ranges of extraction time and water-to-raw-material ratio were determined. A 3-level, 3-variable BBD SAS of SAS Institute, Cary, NC, USA (Aslan and Cebeci, 2007) was used to determine the best combination of extraction variables for the production of polysaccharides. Based on the single-factor experiment, the variables considered were extraction temperature, extraction time and water-to-raw-material ratio. The independent and dependent variables used in the design are listed in Table 1. Then, Table 2 gives the definitions and coding levels used to develop the model. Each experiment was repeated three times and the average extraction yield of the polysaccharides was taken as the final response.

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{i=1}^k \sum_{j=1+1}^k \beta_{ij} X_i X_j \quad (1)$$

$$X_i = \frac{X_i - X_0}{\Delta X} \quad i = 1, 2, 3 \quad (2)$$

TLC analysis of polysaccharides

The supernatant of the water extract was freeze-dried for 72 h and removed from the lyophilizing machine. Crystal polysaccharides were put into Petri dishes and placed in desiccators. Then, 20 mg weight of the substance was put into test tube and 2 ml distilled water was added to it for TLC analysis. 0.5, 1.2 and 20 μl of the substance were drawn for fructan-oligosaccharide determination. Schleicher and Schuell F-1500 ready foils, developed three times in 1-butanol, 2-propanol and water, were used as mobile phase in water in of ratio of 2:8:40. After three times development, it was dried and placed in oven for 10 min to visualize the different levels of oligosaccharides. The position of sucrose and fructans was ascertained by using urea-phosphoric acid reagent (Wise et al., 1955). Then, using sucrose fructose maltose, etc. as the standard, the polysaccharides were analyzed by paper chromatography (PC) as reported by Santoiani et al. (1993).

Antioxidant activity determination

DPPH-free radical scavenging activity of each sample was determined as described by Liu et al. (2009). The extract was reconstituted with distilled water and pre-diluted 20 times. Aliquots of each sample (1 ml) were added to 3 ml of methanolic DPPH solutions (0.1 mM). Discolorations were measured at 516.3 nm after incubation for 30 min at 30°C in the dark.

$$\% \text{DPPH}_{\text{sc/g extract}} = \{(A_{\text{count}} - A_{\text{sample}}) / A_{\text{count}}\} \times 100 / W_{\text{EAP}} \quad (3)$$

Table 1. BBD results for observed and predicted values of yield of *Artemisia mongolica* polysaccharide (%) and %DPPHsc/g extract.

Run	Coded variables			Observed values (Y ₁)	
	X ₁ (Temperature)	X ₂ (Time)	X ₃ (Ratio)	Yield (%)	%DPPHsc/g extract
1	70	80	40	9.43	171.24
2	90	80	40	7.07	148.68
3	70	160	40	9.70	143.34
4	90	160	40	17.38	104.28
5	70	120	20	4.57	351.30
6	90	120	20	3.47	287.28
7	70	120	60	11.86	107.22
8	90	120	60	9.21	91.74
9	80	80	20	6.23	331.08
10	80	160	20	10.76	288.24
11	80	80	60	16.02	104.82
12	80	160	60	14.37	99.72
13	80	120	40	8.70	150.48
14	80	120	40	8.21	149.46
15	80	120	40	8.71	149.34
16	80	120	40	8.31	151.92
17	80	120	40	8.11	162.18

RESULTS AND DISCUSSION

Phenol-tetraoxosulphate (VI) acid/sulphuric acid procedure in 96-well format

About 150 µl of concentrated tetraoxosulphate (VI) acid were added to each 96-well format containing 20 µl of standard solution (1000 mg/l), manufacturer's sample solution and the blank. Then 60 µl of 6% aqueous phenol (w/w) were added to each well of micro-plate before incubation at 80°C in oven for 10 min. Subsequently, the absorbance was immediately read at 490 nm in a micro-plate multi-scan reader. Also, all measurements were done in triplicate. The response was compared with the inulin-based standard curve and the soluble polysaccharide content expressed in g/l of inulin. The purity (99%) of polysaccharides was calculated as the sugar content of extraction divided by the weight of dried materials as plotted in Figure 1.

Effect of extraction temperature on polysaccharide yield

Different extraction temperatures set respectively at 50, 60, 70, 80 and 90°C were used to investigate the effect of temperature on the extraction of crude polysaccharides from *A. mongolica* while all the other reaction conditions were held constant (water-to-raw-material ratio of 40 and extraction time of 2.5 h). Figure 2 shows that the

maximum extraction yield of crude polysaccharides in terms of temperature is 70-90°C. There was no increase in yield with further increase in extraction temperature beyond 90°C. Thus 70-90°C was adopted as the optimal extraction temperature in this experiment.

Effect of extraction time on polysaccharide yield

The effect of extraction time on the extraction yield of *A. mongolica* polysaccharides is shown in Figure 3. In the first step, the extraction time was set respectively at 80, 100, 120, 140 and 160 min while other extraction parameters were held constant (water-to-raw-material of 40 and extraction time of 70°C). It was noted that the extraction yield increased with increasing extraction time (80-120 min), with the peak yield occurring at 120 min. For times longer than 120 min, no further increase was noted in *A. mongolica* polysaccharide extraction yield.

Effect of water-to-raw-material ration on polysaccharide yield

The effect of different ratios of water-to-raw-material (10, 20, 40 and 60) on the extraction yield *A. mongolica* polysaccharides is shown in Figure 4. For the plot (Figure 4), the other extraction factors (extraction temperature and extraction time) were held respectively at 70°C and 30 min.

Table 2. List of ANOVA results for response surface models, including estimated regression model for the relationship between response variables (yield and %DPPHsc/g) and independent variables (X_1 , X_2 , X_3).

Source	Sum of squares	DF	Mean square	F-value	p-value
Yield (%)^a					
Model	153.23	9	17.03	260.46	<0.0001
X_1	6.00	1	6.00	91.85	<0.0001
X_2	3.06	1	3.06	46.78	0.0002
X_3	78.26	1	87.26	1334.93	<0.0001
X_1^2	18.63	1	18.63	284.95	<0.0001
X_2^2	25.56	1	25.56	391.05	<0.0001
X_3^2	4.02	1	4.02	61.44	0.0001
X_1X_2	0.58	1	0.58	8.94	0.0202
X_1X_3	0.60	1	0.60	9.15	0.0192
X_2X_3	9.51	1	9.51	145.45	<0.0001
Residual	0.46	7	0.065		
Lack of fit	0.14	3	0.047	0.59	0.6544
Pure error	0.32	4	0.079		
Total	153.68	16			
%DPPHsc/g extract (%g)^c					
Model	1.120E+005	9	12447.26	282.48	<0.0001
X_1	2489.36	1	2489.36	56.49	0.0001
X_2	1801.80	1	1801.80	40.89	0.0004
X_3	91288.37	1	91288.37	2071.75	<0.0001
X_1^2	57.89	1	57.89	1.31	0.2894
X_2^2	211.24	1	211.24	4.79	0.0647
X_3^2	15369.32	1	15369.32	348.80	<0.0001
X_1X_2	68.06	1	68.06	1.54	0.2539
X_1X_3	589.03		589.03	13.37	0.0081
X_2X_3	352.69		352.69	8.00	0.0254
Residual	308.44	7	44.06		
Lack of Fit	191.25	3	63.75	2.18	0.2335
Pure Error	117.19	4	29.30		
Total	1.123E+005	16			

From Figure 4, it is clear that the extraction yield of *A. mongolica* polysaccharides increased sharply and peaked at 5.26% for an extraction ratio of 4 ml/mg. Thereafter, the extraction yield of *A. mongolica* polysaccharides decreased after the water-to-raw-material ratio exceeded 4 ml/mg (Figure 4).

BBB response surface analysis

The extraction yields of *A. mongolica* polysaccharides were investigated in the study. The parameters were chosen after preliminary analysis with the highest yield of polysaccharides at desired antioxidant activity. The results of 17 runs of BBD in Table 2 include the design

and observed responses. There was a close agreement between field-observed and predicted values. The maximum yield (17.38%) was noted under the experimental conditions of $X_1 = 76.90$ °C, $X_2 = 1.33$ h and $X_3 = 4$ ml/mg. On the other hand, the range of antioxidant property (%DPPHsc/g extract) was 91.74–351.32 %/g. The highest %DPPHsc/g extract (351.32 %/g) was observed under the experimental conditions of $X_1 = 70.37$ °C, $X_2 = 1.45$ h and $X_3 = 2$ ml/mg. Note that the conditions changed with required responses.

Model fit

Table 3 presents the results of the model fits (quadratic

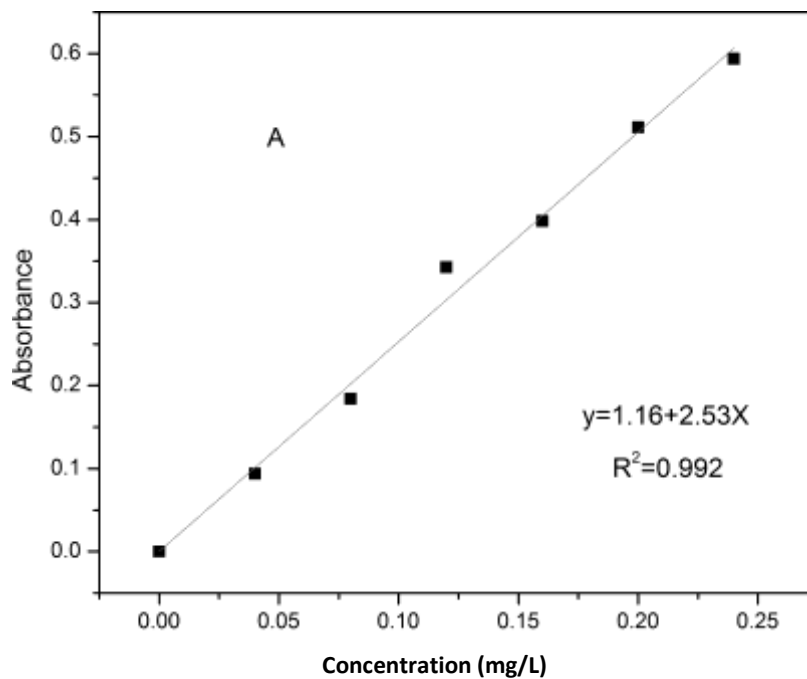


Figure 1. *Artemisia mongolica* polysaccharide calculation based on inulin-based standard curves and the relative absorbances.

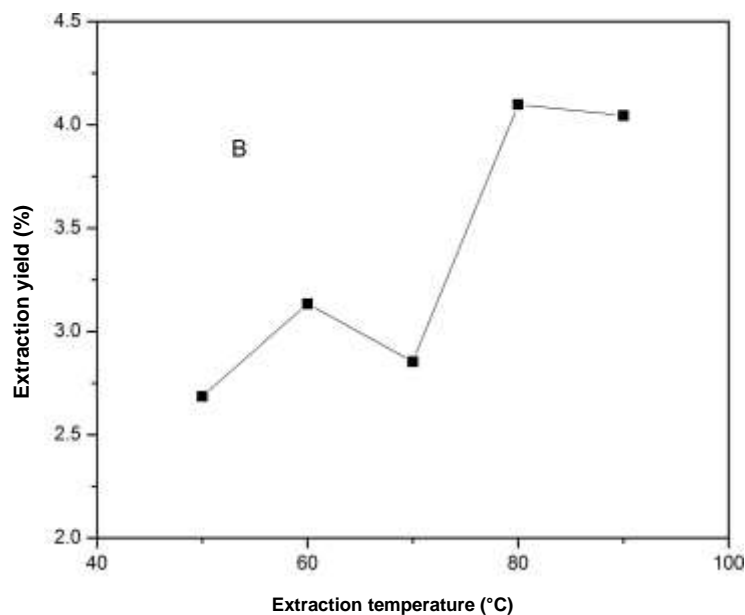


Figure 2. Effect of temperature on extraction yield of *Artemisia mongolica* polysaccharides.

and linear) to observed data. The results of analysis of variance (ANOVA) suggest that the quadratic model significantly explained the responses of the extraction

yields and antioxidant activities. The fitted quadratic models for extraction yield and %DPPHsc/g extract in the coded variables are quantified respectively in Equations 4

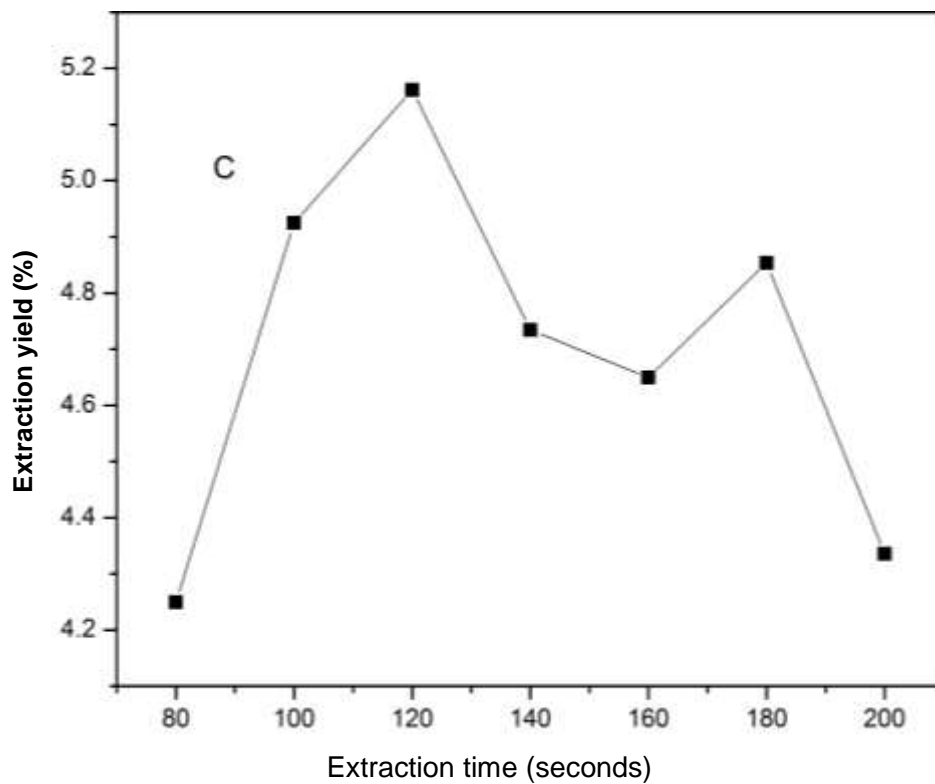


Figure 3. Effect of time on extraction yield of *Artemisia mongolica* polysaccharides.

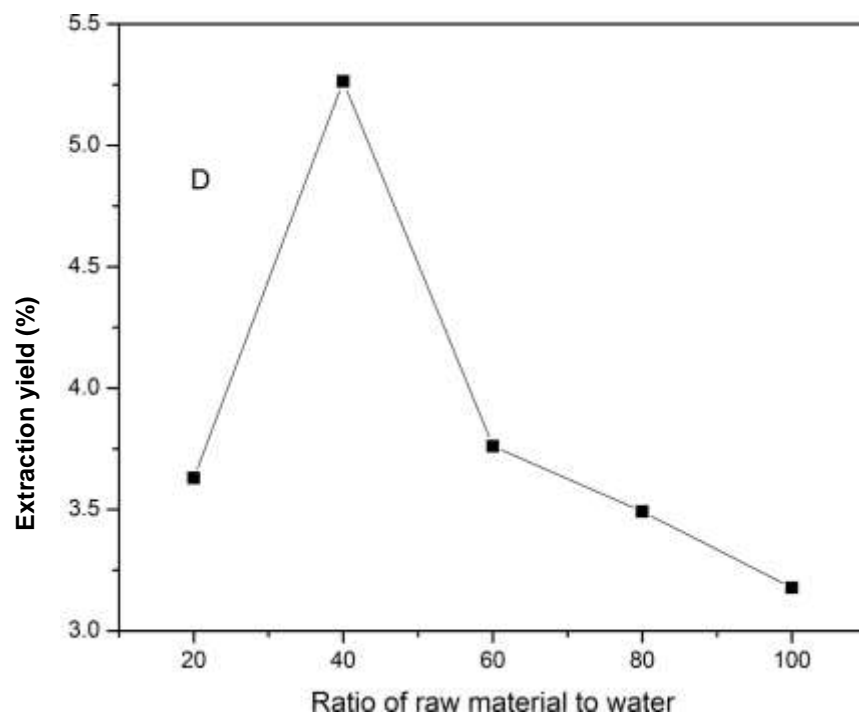


Figure 4. Effect of water-to-raw-material ratio on extraction yield of *Artemisia mongolica* polysaccharides.

Table 3. A list of fit statistics for dependent variable Y.

Y variable		Master model	Predictive model
Yield	RMSE	9.03	9.03
	R-square	99.70	99.70
	Adjusted R-square	99.32	99.32
	Coefficient of variation	2.83	2.83
%DPPHsc/g	RMSE	176.03	176.03
	R-square	99.73	99.73
	Adjusted R-square	99.37	99.37
	Coefficient of variation	3.77	3.77

Table 4. Fit statistics of Y.

Y variable		Master model	Predictive model
Yield	RMSE	9.03	9.03
	R-square	99.70	99.70
	Adjusted R-square	99.32	99.32
	Coefficient of variation	2.83	2.83
%DPPHsc/g	RMSE	176.03	176.03
	R-square	99.73	99.73
	Adjusted R-square	99.37	99.37
	Coefficient of variation	3.77	3.77

and 5. The significance of each coefficient was determined using the F-test and p-value in Table 4. The corresponding variables can be more significant if the

absolute F-value increases greater and the p-value decreases (Atkinson and Donev, 1992):

$$EY = 8.40 - 0.87x_1 + 0.62x_2 + 3.3x_3 + 0.38x_1^2 - 0.39x_2^2 - 1.54x_3^2 - 2.1x_1x_2 + 2.46x_1x_3 - 0.98x_2x_3 \quad (4)$$

$$AP = 152.68 - 17.64x_1 - 15.010.62x_2 - 106.82x_3 - 3.71x_1^2 - 7.08x_2^2 + 60.42x_3^2 - 4.13x_1x_2 + 12.14x_1x_3 + 9.39x_2x_3 \quad (5)$$

where EY is extraction yield and AP antioxidant property

$$\left(\frac{\% \text{ DPPHsc}}{\text{g extract}} \right)$$

Extraction yield: The term with the largest effect on polysaccharide extraction yield was a linear (X_1 , X_2 , X_3), followed by quadratic (X_1X_2 , X_1X_3 , X_2X_3) and then the interaction (X_1X_2 , X_1X_3 , X_2X_3) terms (Table 3). The results in Table 3 suggested that only the changes in extraction temperature, extraction time and in water-to-raw material ratio had significant effects ($p < 0.0001$) on the yield of extracted polysaccharides. The coefficient of determination (R^2) of the model predicted responses was

0.9970 with p -value of 0.59. These values gave a relatively good fit to the mathematical model in Equation 4.

%DPPHsc/g extract antioxidant activity: In terms of antioxidant activity, linear (X_1 , X_2 , X_3) and quadratic (X_1 , X_2) terms of polysaccharide extraction parameters had the largest effect ($p < 0.0001$), followed by the interaction (X_1X_3 and X_2X_3) terms. However, quadratic (X_3) and the interaction terms of the extraction parameters were not significant ($p > 0.05$). The coefficient of determination (R^2) of the model predicted response was 0.9972 with p -value of 2.29. This suggested that there was an excellent fit to the mathematical model in Equation 5. Thus, the responses were sufficiently explained by the models.

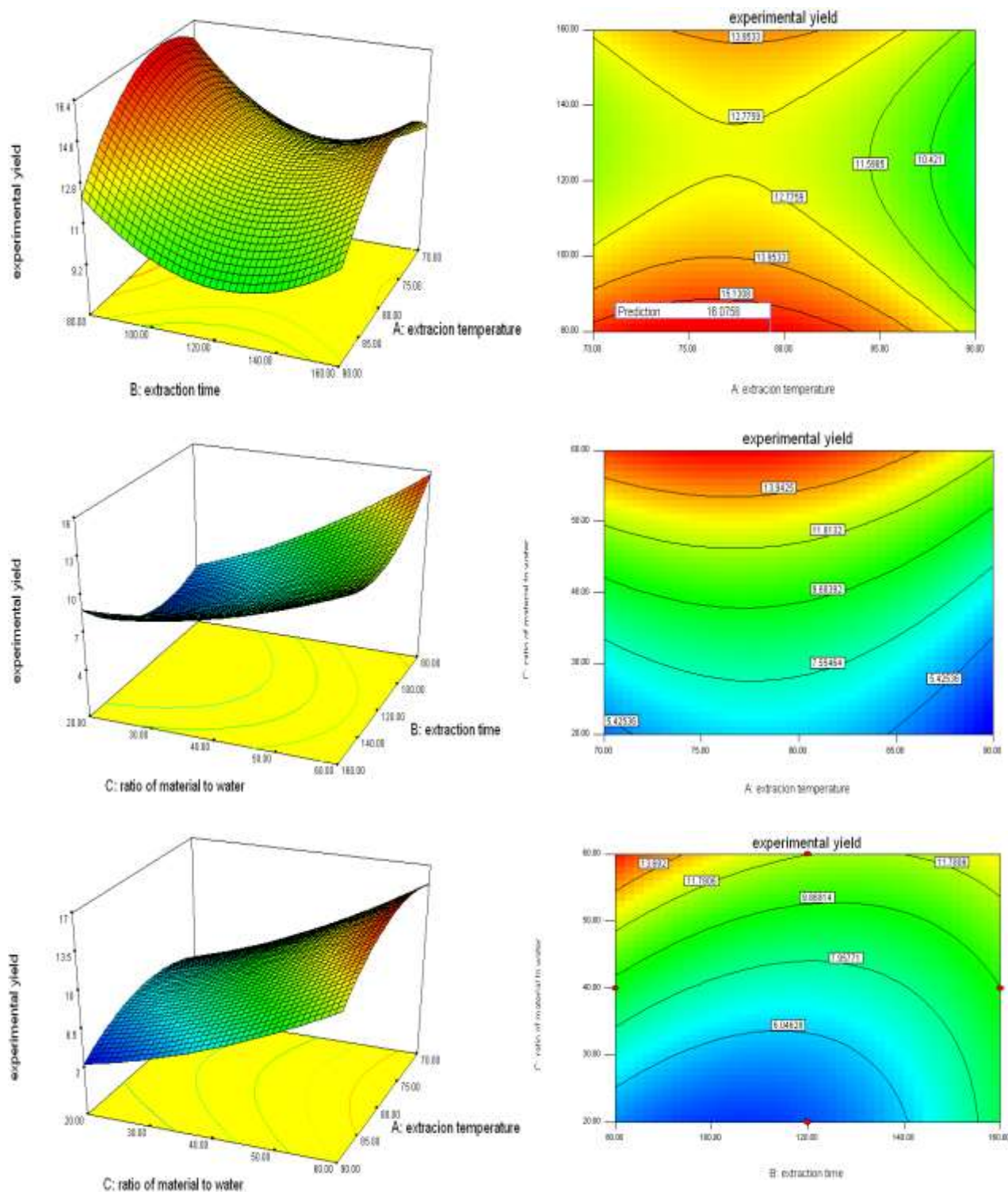


Figure 5. Three-dimensional response surfaces and contours of extraction yields of *Artemisia mongolia* polysaccharides.

Response surface model and contour plot

Extraction yield explanation: Three-dimensional (3D)

and contour plots of the polysaccharide extraction yields are given in Figure 5. The result in Table 3 showed that all the extraction parameters significantly ($p < 0.05$) or

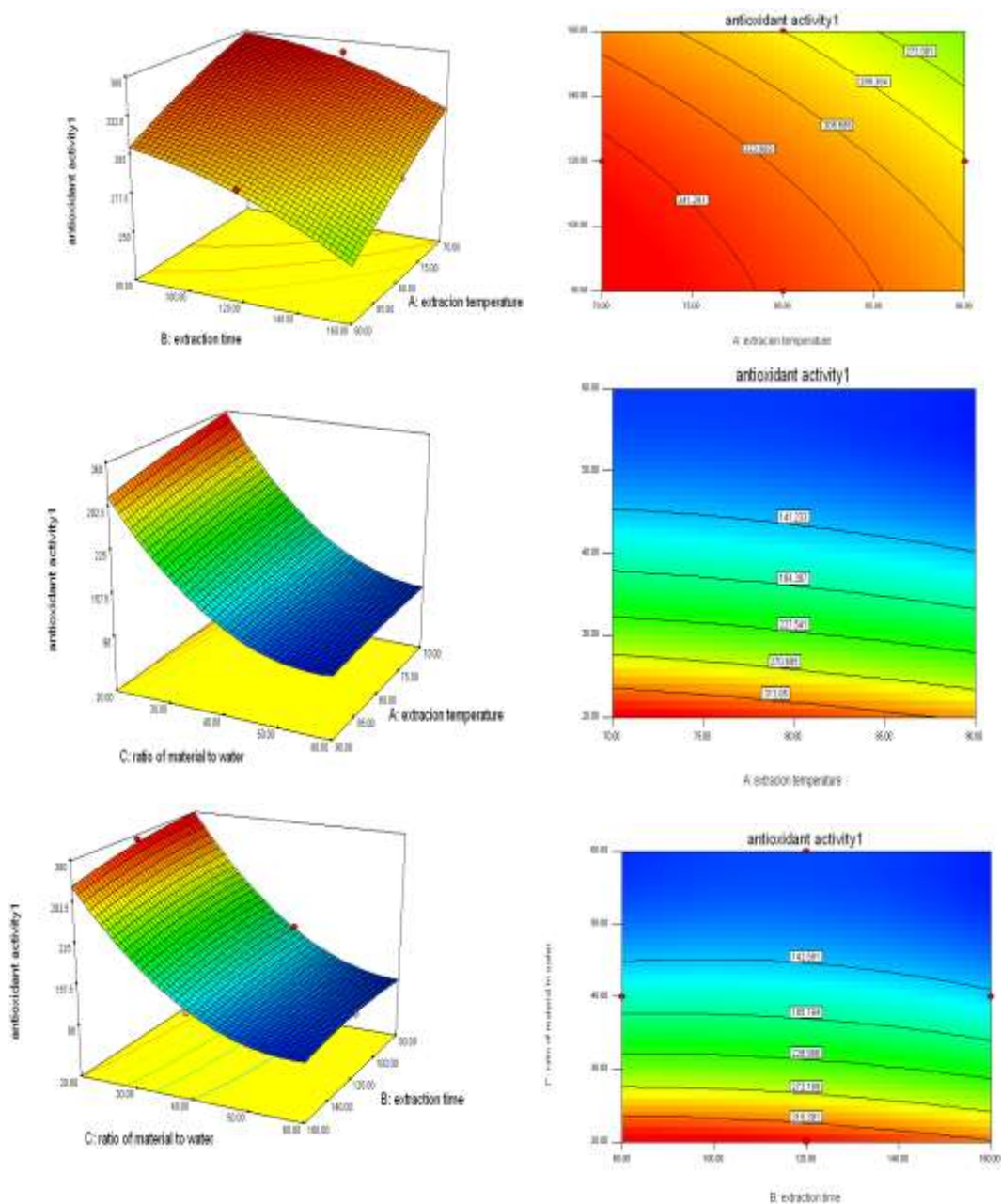


Figure 6. Three-dimensional response surfaces and contours of antioxidant activities (%DPPHsc/g extract) of *Artemisia mongolia* polysaccharides.

highly significantly ($p < 0.0001$) contributed to the extraction response. The maximum yield of the polysaccharides (17.38%) was obtained at an extraction temperature of 76.90°C, extraction time of 1.33 h and water-to-material ratio of 4 ml/mg.

Antioxidant activity (%DPPHs/g extract)

The 3D response surfaces and contours of %DPPHsc/g

extract are given in Figure 6. It was apparent that the polysaccharides possessed antioxidant activity by scavenging DPPH free radicals. It was also observed that with 65–70°C extraction temperature or 80–160 min extraction time, there was increased antioxidant activity. Extract of water-to-raw material ratio of 2.5 or less seemed to have higher antioxidant activity when compared with water-to-raw material ratio higher than 3.0. At extraction temperature of 70.37°C (Figure 6a) and extraction time of 160 min (Figure 6b), %DPPHsc/g

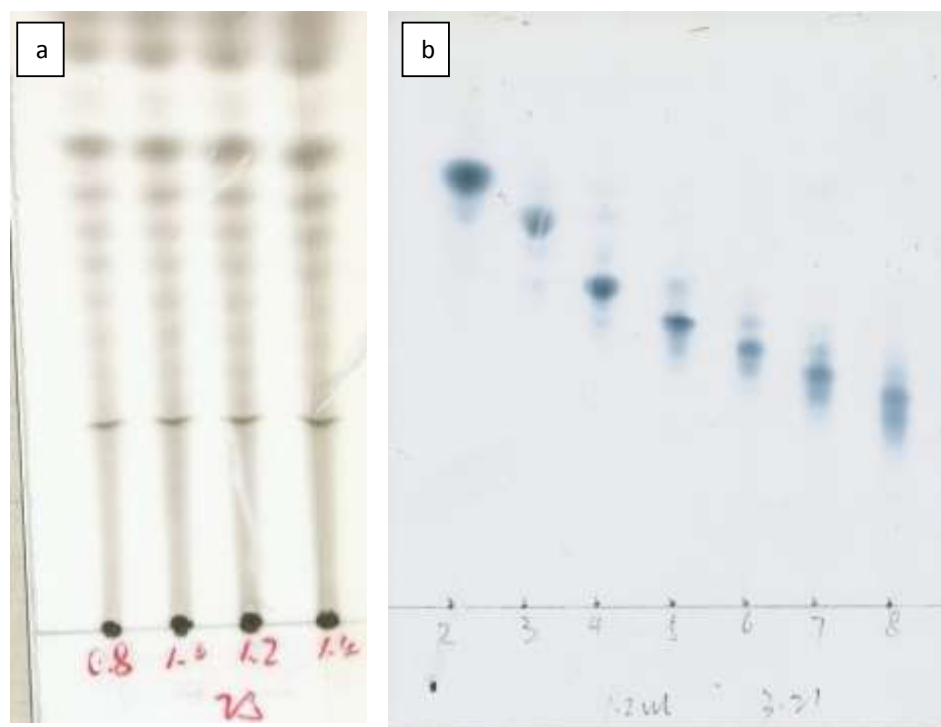


Figure 7. Plots of fructan-oligosaccharide in *Artemisia mongolica* plant 2-8 (a), depicting the mobilities of fructan-oligosaccharides DP 2–8 degree of polymerization (b). Note that each contains fructan equivalent in ethanol sample value.

extract reached 351.3.

Predictive model verification

Based on the above findings, an optimization analysis was done to evaluate the optimal operating conditions for the extraction of high yields of polysaccharides and antioxidant activities. Table 4 has two optimum conditions based on the combination of all the responses. These optimal conditions were: 1) extraction temperature of 76.90°C, extraction time of 1.33 h and water-to-material ratio of 4 ml/mg; and 2) extraction temperature of 70.37°C, extraction time of 1.45 h and water-to-raw material ratio of 2 ml/mg. The corresponding optimum condition of the polysaccharides was 16.02% and that of %DPPHsc/g extract was 351.3%. Only small deviations were noted between the actual and predicted values. Thus, the model was applicable in optimizing the processes of *A. mongolica* polysaccharides extraction.

TLC analyses

The purification of fructan-oligosaccharide polysaccharides with high molecular weight was qualitatively

assessed at each stage of the protocol after ascending thin layer chromatography. The extracted samples were applied to silica-gel coated origin 20 × 20 cm glass plate. Sample were developed in triplicate in butan-1-ol/pro-2-ol/water of 3:12:4 ratio (V/V/V) at room temperature. Then qualitative analysis of fructo-oligosaccharides by TLC method (Figure 7) showed the existence of low DP members of EDTA-Ca series (DP 2-8) in the fractions, although changes in the relative proportions varied with treatment. For plants watered every 30 days, fructose, sucrose and other components of the series were more concentrated; reflecting the observed increase in total fructose in the oligosaccharide fraction. This increase occurred simultaneously with the reductions in polysaccharide fractions in Figures 7a and b.

The purification of fructan-oligosaccharide polysaccharides with high molecular weight was qualitatively assessed at each stage of the process after ascending TLC extracted samples were applied to silica-gel coated origin 20 × 20 cm glass plate. The samples were developed in triplicate in butan-1-ol/pro-2-ol/water with ratio of 3:12:4 (v/v/v) at room temperature. TLC-based qualitative analysis of fructo-oligosaccharides (Figure 8) showed the existence of low DP members of the EDTA-Ca series (DP 2- 8) in this fractions, although changes in



Figure 8. Plots of fructan-oligosaccharide in *Artemisia mongolica* plant 2–8 (a), with mobilities of fructan-oligosaccharides of DP 2–8 degree of polymerization (b).

the relative proportions varied with treatment. In plants watered every 30 days, fructose, sucrose and the other components of the series were more concentrated, reflecting the observed increase in total fructose in the oligosaccharide fractions. This increase occurred simultaneously with reduction in polysaccharide fractions in Figure 8a and b.

Conclusions

The single-factor experiments and BBD together with RSM simulations were used to determine the optimum process parameters with high extraction yield and antioxidant activity of *Artemisia mongolica* polysaccharides. Based on ANOVA analysis, the effects of extraction temperature, extraction time and extraction water-to-material ratio were significant. Quadratic models were fitted to the responses of extraction yield and antioxidant activity. Two optimal conditions were determined: 1) extraction temperature of 76.90°C, extraction time of 1.33 h and extraction water-to-raw material ratio of 4 mL/mg; and 2) extraction temperature of 70.37°C, extraction time of 1.45 h and water-to-raw material ratio of 2 mL/mg. This optimum condition for the polysaccharides was 17.38% and that for %DPPHsc/g extract was 351.3%. Thus, the model was applicable in the optimization of the processes of *A. mongolica* polysaccharide extraction. Preliminary identification of the polysaccharides showed the potential for the use of antioxidant in medicine or health-care foods. The purification of fructan-oligosaccharide polysaccharides was possible after ascending TLC and triple plate development by DP 2-8 fructan-oligosaccharide analyses.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effect of pre-harvest chitosan foliar application on growth, yield and chemical composition of Washington navel orange trees grown in two different regions

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The present study was carried out during 2012 and 2013 seasons to study the effect of pre-harvest foliar application of chitosan (a natural beta-1-4-linked glucosamine polymer) at two concentrations 250 and 500 ppm on vegetative tree growth, fruit yield and quality as well as leaves chemical composition of Washington navel orange trees grown under two locations. As for growth parameters (shoot length, leaves number, and leaves area), the results revealed that chitosan treatments had insignificant effect. Meanwhile, it had a significant improvement on most of the studied fruit characters and leaf chemical constituents, that is, pigments, sugars, total soluble phenols, total free amino acids, endogenous plant hormones "IAA, ABA and GA₃" as well as leaf nutritional status "N, P, K, Zn, Ca, B and Si". Generally, pre-harvest chitosan applications mostly had pronounced positive effects on improving navel orange quality, that is, fruit weight, firmness and T.S.S.%, especially at the rate 500 ppm.

Key words: Citrus, chitosan, growth characters, fruit quality, total chlorophyll, sugar, total soluble sugar (TSS).

INTRODUCTION

Citrus is the most economically important fruit crop in the world. It is considered as one of the main sources of Vitamin C., carotenoids and an extensive array of secondary compounds with pivotal nutritional properties such as vitamin E, pro-vitamin A, flavonoids, limonoids, polysaccharides, lignin, fibers, phenolic compounds and

essential oils (Iglesias et al., 2007). Navel orange is a popular fresh fruit for (i) its seedless fruits, flavor and aroma, and (ii) yield are in important source of early season income for citrus growers at some commercial citrus areas of the world (Wardowski et al., 1985).

Trees production is erratic and usually low in some

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Table 1. Physical and chemical analysis of El-Qalubia and El-Sadat orchards soil.

Physical properties	El- Qalubia	El-Sadat
Sand (%)	19.39	85
Clay (%)	63.64	5
Silt (%)	16.97	10
Texture	Clay loam	Loamy sand
Chemical properties		
pH (Extract 1/2.5 H ₂ O)	8.10	7.74
EC 20°C (dsm ⁻¹)	0.29	0.305
Available elements (mg/kg)		
N	538.4	60
P	22	43.68
K	278	1.2
Ca	5	32.4
Mg	3.5	6.8
Na	38.8	8.0
Zn	39	8.26
Mn	7	19.93
Fe	9.2	68.93
Cu	5	< 2.50

regions; these may be due: (i) to lack functional pollens; (ii) rarely produce viable ovules and (iii) weakly parthenocarpic (Krezdorn, 1965). Moreover, flowers and fruits drop of navel orange occurred at three phases (Villafane et al., 1989).

Chitosan is a polysaccharide resulting from the deacetylation of chitin, the linear polymer of (1-4)- β -linked N-acetyl-D-glucosamine. It is obtained from the outer shell of crustaceans such as crabs and shrimps (Ruiz-García and Gómez-Plaza, 2013; Sandford and Hutchings, 1987; Sandford, 1989). Chitin and chitosan are polysaccharides, chemically similar to cellulose differing only by the presence or absence of nitrogen (Freepons, 1991). The positive charge of chitosan confers to this polymer numerous and unique physiological and biological properties with great potential in a wide range of industries such as cosmetology (lotions, hair additives, facial and body creams) (Lang and Clausen, 1989), food (coating, preservative, antioxidant, antimicrobial) (Sapers, 1992; Pennisi, 1992; Fang et al., 1994; Roller and Covill, 1999; Benjakul et al., 2000; Shahidi et al., 2001), biotechnology (chelator, emulsifier and flocculent) (Hirano, 1989; Sandford, 1989) pharmacology and medicine (fibers, fabrics, drugs, membranes and artificial organs) (Muzarelli, 1989; Kulpinsky et al., 1997; Nishimura, 1997; Liu et al., 2001) and agriculture (soil modifier, films, fungicide, elicitor) (Hoagland and Parris, 1996; Lafontaine and Benhamou, 1996; Makino and Hirata, 1997; Ren et al., 2001).

Chitosan has been widely used for stimulation of plant

defense (Bautista-Baños et al., 2003). Chitosan oligomers enter most regions of the cell, and subsequently induced changes in: Cell membranes, chromatin, DNA, calcium, MAP kinase, oxidative burst, reactive oxygen species (ROS), pathogenesis related (PR) genes/proteins, and phytoalexins (Hadwiger, 2013). Pre-harvest chitosan applications have been noted to be effective in controlling postharvest fungal infection in strawberries (Reddy et al., 2000). Moreover, plants treated with chitosan may be less prone to stress evoked by un-favorable conditions, such as drought, salinity and low or high temperature (Lizarraga-Pauli et al., 2011; Jabeen and Ahmad, 2013).

Therefore, this experiment was conducted to investigate the effect of pre-harvest foliar spray of chitosan (250 and 500 ppm) on tree growth and leaves composition as well as fruit-quality and production of navel orange grown in two different regions.

MATERIALS AND METHODS

The present study was carried out during the two successive seasons (2012 and 2013) at two private citrus orchards; (I) Kalube centr El-Qalyobia Governorate, Egypt. Washington navel orange trees (*Citrus sinensis* lin, Osbek) 40 years- old budded on sour orange rootstock (*Citrus aurantium*) grown on clay loam soil at 5 x 5 m. (II) Cairo-Alex. desert road "El-Sadat City region-El-Monofia Governorate, Egypt. The trees were about 11 years - old budded on Sour orange rootstock (*Citrus aurantium*) grown in reclaimed soil at 4 x 6 m. Soil samples were collected from the two orchards at depths (0-30 cm), physical and chemical properties (Table 1) were

Table 2. Chemical analysis of irrigation water of El-Qalubia and El-Sadat regions.

Chemical analysis	El-Qalubia	El-Sadat
EC mmhos/cm at 25°C	288	541.64
pH	7.10	7.87
Soluble ions (meq/l)		
Cations Ca ⁺²	1.10	1.75
Mg ⁺²	0.80	1.10
Na ⁺	2.50	2.27
K ⁺	0.10	0.14
Anions Cl ⁻	3.80	1.76
SO ₄ ⁻²	0.30	0.47

analyzed according to Piper (1950). The 1st orchard, trees were under basin irrigation system and received about 5000 to 6000 m³ of irrigated water/fed/year. While the 2nd orchard, trees were under drip-irrigation system and received about 3500 to 4000 m³ of irrigated water/fed/year. In both orchards, chemical composition of used water, that is, pH, EC, Ca⁺² and Mg⁺², Cl⁻ and SO₄⁻² concentrations were determined (Table 2).

Environmental factors such as air temperature (°C) (max. and min.), relative humidity (R.H. %) and evapotranspiration rate (mm.) were collected and analyzed (Table 3) for the two regions beside the El-Nubaria region "which consider the best area for citrus production in Egypt" as a control. Fertilization and pests control programs for the two regions were applied as recommended from the Ministry of Agriculture, Egypt. In the two experimental seasons, three pre-harvest foliar treatments were used as follows:

1. Control treatment sprayed with 0.5% acetic acid.
2. Chitosan foliar treatment at the rate of 250 ppm dissolved in acetic acid (0.5 %) according to (Bautista-Baños et al., 2006).
3. Chitosan foliar treatment at the rate of 500 ppm dissolved in acetic acid (0.5 %) according to (Meng et al., 2010).

Pre-harvest foliar spray were applied twice: at one month before the beginning of fruit color break (the 1st week of September) and the 2nd at one month before harvest (the 3rd week of November).

A complete randomized block design was used. Each treatment was replicated three times with one tree for each replicate.

1. Tree growth parameters: At September for new developed twigs of spring cycle; the following growth characters were tabulated:

- a. Twig length (cm)
- b. Number of leaves/ twig
- c. Leaf area (cm²) which estimated by leaf area meter (model CL-203 area meter CID, Inc., USA).
- d. Flowering and fruit characters.
- e. The total number of flowers.
- f. Fruit set percentage (%) = (Number of fruits/ Total number of flowers) × 100
- g. Number of fruits/ tree
- h. Fruit drop (%) = (Total number of fruits at petal-fall stage – number of fruits in late July) / Total number of fruits × 100

Leaves chemical constituents

1. Leaf pigment contents: Sample of fresh leaves at the 1st of

September were extracted with dimethyl formamide to determine chlorophylls a, b and carotenoids concentrations according to Moran (1982) formula.

Ethanol extract of leaves was used for the determination of total sugar (Dubois et al., 1956), total free amino acids (Moore and Stein, 1954) and total soluble phenols concentrations (Swain and Hillis, 1959).

For hormones analysis, leaves of navel orange were extracted twice, each 3 h, with 80% methanol and again twice with 40% methanol, each 2 h (Sadeghian, 1971). The aqueous fraction was adjusted to pH 2.6 by the addition of 1 N HCl and was partitioned three times with ethyl acetate. Gibberellic acid (GA₃), indole-3-acetic acid (IAA) and abscisic acid (ABA) were measured using HPLC according to the method described by Müller and Hilgenberg (1986).

2. Leaf mineral contents: Digestion of plant materials were carried out using sulphuric and per-chloric acids as described by Piper (1950).

3. Nitrogen (%) was determined by the micro-kjeldahl as described by Schouwenburg and Walinga (1978).

4. Phosphorus (P %) was determined colorimetrically as described by King (1951).

5. Potassium (K %) was determined by using flame photometer (Corning 410).

6. Calcium (%), zinc (ppm) and boron (ppm) were determined by using atomic absorption spectrophotometer (Thermo-Jarrellash, AASCANI).

7. Silicon (Si %) was determined according to Schuffelen et al. (1961).

8. Yield, fruit physical and chemical characters: At the end of November, yield of each tree as Kg and number of fruits / tree were estimated as well as the following fruit physical characters were taken as follows:

- a. Fruit weight (g)
- b. Fruit size (cm³): it was measured by water displacement in graduate jar.
- c. Fruit shape index: Fruit length and diameter (cm) were measured by a Vernier caliper and fruit shape index (length/ diameter ratio) was calculated.
- d. Fruit firmness: Fruit firmness of the skin was recorded by LFRA texture analyzer instrument model TS-091000 stainless steel needle, using penetrating cylinder of 1 mm of diameter to a constant distance 5 cm inside the skin to the flesh by a constant speed 2 mm/s. The results were expressed as the resistance force to the penetrating tester in fruits of pressure g/cm² (Harold, 1985).
5. Fruit juice %.
6. Peel thickness (mm): it was were measured by a Vernier caliper.
7. Fruit chemical properties: that is, T.S.S. %, titratable acidity (mg of citric acid/100 ml juice). Vitamin C (mg/100 ml juice).

Statistical analysis

A complete randomized block design was used. The obtained data were subjected to the analysis of variance according to Snedecor and Cochran (1972). Differences between treatments means were compared using the L.S.D. at 0.05 level.

RESULTS AND DISCUSSION

Vegetative growth

Data in (Table 4) indicated that spraying of both chitosan

Table 3. Air temperature (°C), relative humidity (%) and evapotranspiration (mm) in El-Qalubia, El- Sadat and El-Nubaria regions during season 2013.

Location	Month	Air temperature (°C)			Relative humidity (%)	Evapo-transpiration (mm)
		Max.	Min.	Average	Average	Average
El-Qalubia	January	15.7	4.7	10.2	56	1.1
	February	18.9	9.9	14.4	55	1.5
	March	20.7	15.2	17.95	57	2.2
	April	25	16.8	20.9	49	3.0
	May	32.1	19.5	25.8	49	3.6
	June	35.9	22.7	29.3	52	3.8
	July	39.8	25.8	32.8	55	4.2
	August	40.9	26.9	33.9	55	3.4
	September	33.3	23.2	28.25	53	2.5
	October	25.9	15.9	20.9	60	1.8
	November	20.2	12.8	16.5	72	1.3
	December	17.5	7.2	12.35	74	1.0
El-Sadat	January	11.5	1.9	6.7	57	0.1
	February	13.8	3.8	8.8	59	1.3
	March	18.9	9.5	14.2	58	3.7
	April	38.5	11.8	25.2	46	6.9
	May	37.6	25.2	31.4	47	8.0
	June	40.4	26.6	33.5	48	8.4
	July	45.3	28.9	37.1	54	8.1
	August	47.7	28.7	38.2	50	8.4
	September	38.9	22.8	30.9	54	6.2
	October	35.7	10.1	22.9	65	4.0
	November	18.6	8.7	13.7	77	2.2
	December	12.6	3.1	7.9	79	1.5
EL-Nubaria (Behera)	January	17.5	9.6	11.8	81	1.5
	February	19.9	9.2	13.5	75	2.3
	March	23.4	12.0	16.6	69	3.6
	April	24.1	13.0	18.3	69	4.4
	May	28.4	17.8	23.4	69	5.0
	June	29.8	20.5	24.9	70	5.4
	July	29.5	22.3	27.7	75	5.8
	August	30.8	23.1	30.2	80	6.1
	September	29.3	21.0	22.5	75	5.6
	October	26.0	17.4	15.0	72	2.5
	November	24.4	15.5	14.5	69	2.2
	December	19.0	10.4	12.8	70	2.0

*Central Laboratory For Agricultural Climate, Agricultural Research Center, Egypt.

concentrations had non-significant effect on most of the studied growth characters in the two successive seasons as well as in the two different orchards. This result was obtained also by El Hadrami et al. (2010), who found that foliar application of chitosan did not affect maize or soybean height, leaf area and total dry mass.

On the other hand, a contradict results were obtained by Mahdavi (2013) who mentioned that length and weight

of roots and shoots were increased in Isabgol (*Plantago ovata* Forsk) plants pretreated with chitosan under salt stress. Also, El-Miniawy et al. (2013) working on strawberry plants (*Fragaria x ananassa* Duch.) revealed that all tested foliar applications of chitosan increased all vegetative growth characteristics.

In this respect, Bittelli et al. (2001) suggested that chitosan might be an effective as anti-transpiring to

Table 4. Effect of pre-harvest chitosan spray on growth characters of Navel orange tree grown in El-Qalubia and El-Sadat orchards in 2012 and 2013 seasons.

Location	Chitosan Conc. (ppm)	El-Qalubia		El-Sadat	
		2012	2013	2012	2013
Twig length (cm)	Control (0.0)	5.08	8.60	9.10	7.63
	250	5.27	9.25	9.17	7.90
	500	5.50	9.67	9.07	8.00
	L.S.D. _{0.05}	n.s.	0.03	n.s.	n.s.
Number of leaves / twig	Control (0.0)	5.12	6.90	6.13	5.37
	250	5.48	7.02	6.23	6.02
	500	5.42	7.17	6.51	6.05
	L.S.D. _{0.05}	n.s.	n.s.	n.s.	0.92
Average leaf area (cm ²)	Control (0.0)	6.62	11.06	5.91	10.18
	250	6.95	11.67	6.20	10.52
	500	7.72	12.18	6.40	10.87
	L.S.D. _{0.05}	n.s.	0.08	1.74	0.36

preserve water resources used in agriculture.

Flowering and fruit set

From the obtained results in Figure 1, it could be noticed that increase of total number of flowers /tree over control was non-significant in the first season but was significant in the second one as sprayed with both concentrations of chitosan in El-Qalubia region. In El-Sadat orchard, there was a significant effect at the first season but was not in the second one.

In this concern, Ohta et al. (1999) found that flower number of *Eustoma grandiflorum* was greatest in plants grown in chitosan treated. A stimulating effect of chitosan on the number of flowers was observed in plants such as gerbera (Wanichpongpan et al., 2001) and gladioli (Ramos-Garcia et al., 2009). Salachna and Zawadzińska (2014) working on 'Gompey' freesia, reported that the chitosan-treated plants (0.5%) had more leaves and flowered earlier as well as had higher relative chlorophyll content.

Concerning fruit set%, it was found non-significant effect of chitosan in the first season and significant one in El-Qalubia orchard in the second season. Meanwhile, a significant increase in fruit set % was found with the increase concentration of chitosan as compared with control for the two successive seasons in El-Sadat orchard (Figure 1).

In this concern, Ghoname et al. (2010) observed that foliar application of chitosan on sweet pepper significantly increased the number of fruits per plant and the mean weight of fruit, as well as fruit quality characteristics.

Regarding the effect of chitosan on the drop of navel orange fruits %, it was found significant decrease in the

second one with either chitosan concentration in both orchards (Figure 1).

In this respect, it could suggest that chitosan might alter the hormonal balance in ways that are in harmony with observed decreases in fruit abscission. However, the data of fruit yield showed a non-significant effect under foliar application of both chitosan treatments as compared with non-sprayed control trees in both regions (Figure 1).

Leaf chemical constituents

Leaf pigments

Data concerning chlorophyll a, b and total chlorophyll as well as total carotenoids of navel orange leaves in both orchards indicated that total chlorophyll, especially chl. a showed a significant increase by chitosan application as compared to the control in both gardens, especially the higher chitosan concentration. On the contrary, the total carotenoids concentrations were decreased in leaves of both orchards (Table 5).

These results are consistent with El-Tantawy (2009) reported that application of chitosan on tomato plant increased photosynthetic pigments thereby the net photosynthesis increased. Again, Mondal et al. (2012) reported that chlorophyll content was increased in leaves of chitosan applied okra plants (100 ppm) than control.

On the other hand, a reverse trend was detected by El-Miniawy et al. (2013) who reported that there was no significant effect for the chitosan treatments on leaf of strawberry chlorophyll content. Therefore, it could suggest that exogenous chitosan might alleviate abiotic stresses between both regions by increment chlorophyll

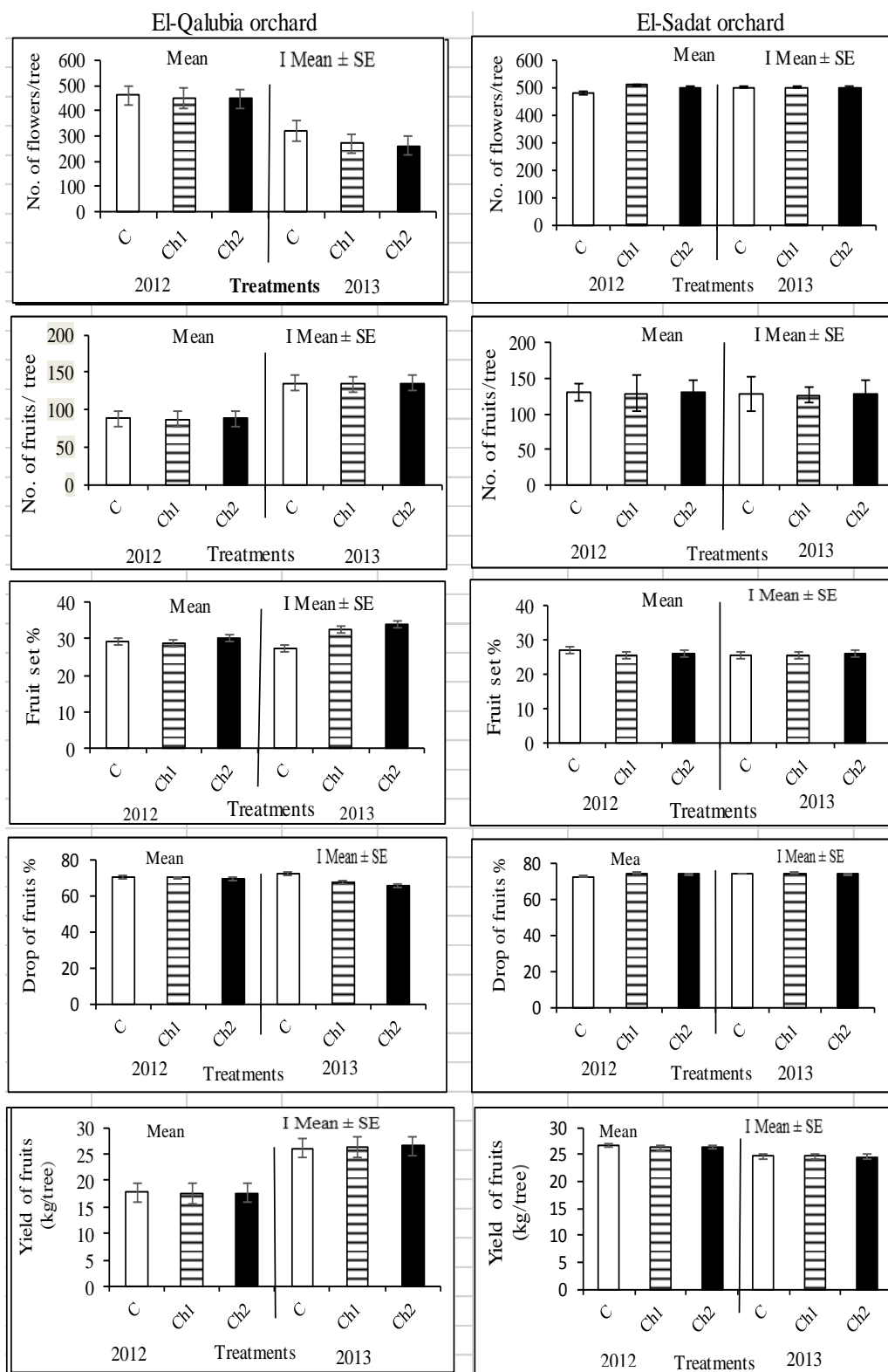


Figure 1. Effect of different chitosan rate on total no. of flower and no. of fruit per tree, fruit set%, fruit drop and yield (kg/tree) at El-Qalubia and El-Sadat orchards in 2012 and 2013 seasons. Control, ; chitosan (250 ppm) and chitosan (500) (data are the mean \pm standard error of nine replicates).

Table 5. Effect of both pre-harvest chitosan rates on plant pigments (chl. a, chl. b, total chls. and total carotenoids) concentrations (mg/g f.w.) in navel orange leaves of El-Qalubia and El-Sadat orchards during 2013 season.

Location	Chitosan Conc. (ppm)	Plant pigment (mg/gf.w.)			
		Chlorophyll a	Chlorophyll b	Total chlorophylls	Total carotenoids
El-Qalubia	Control (0.0)	1.25	0.48	1.73	0.35
	250	1.45	0.50	1.95	0.22
	500	1.60	0.58	2.18	0.30
	L.S.D. 0.05	0.12	0.07	0.20	0.02
El-Sadat	Control (0.0)	1.11	0.41	1.52	0.51
	250	1.68	0.58	2.26	0.20
	500	1.62	0.75	2.37	0.18
	L.S.D. 0.05	0.13	0.07	0.19	0.08

Table 6. Effect of both pre-harvest chitosan rates on total sugar, total free amino acids, total soluble phenols (mg/g f. wt.) and plant hormones concentrations (GA₃, ABA and IAA) as µg/100 g f. wt. in navel orange leaves of El-Sadat and El-Qalubia orchards during 2013.

Location	Chitosan conc. (ppm)	Total sugar (mg/g f. wt.)	Total free amino acids (mg/g f. wt.)	Total soluble phenols (mg/g f. wt.)	Plant hormone (µg/100 g f. wt.)		
					GA ₃	ABA	IAA
El-Qalubia	Control (0.0)	3.68	1.88	1.95	4.06	2.68	1.01
	250	3.78	2.20	1.88	6.15	3.17	1.20
	500	4.06	2.74	2.05	7.08	4.01	1.40
	L.S.D. 0.05	0.06	0.11	0.06	-	-	-
El-Sadat	Control (0.0)	4.53	2.29	2.02	4.57	3.24	1.22
	250	4.25	2.87	1.90	5.05	3.65	1.56
	500	5.30	4.18	2.17	7.85	4.60	1.82
	L.S.D. 0.05	0.22	0.20	0.06	-	-	-

concentration, decreasing the stomatal and non-stomatal transpiration as well as improve water use efficiency.

Organic components

The data in Table 6 revealed that there were significant increases in total sugar, total free amino acids and total soluble phenols concentrations in leaves of navel orange trees sprayed by chitosan, especially at higher concentration in both orchards as compared with control trees.

These results are in harmony with No et al. (2003) who reported that application of chitosan increased carbohydrates in soybean leaves. Cai et al. (2014) reported that chitosan enhanced the production of phenolic acids by 1.5 to 2.0-folds after 3 days of cell suspension cultures of *Malus x domestica* Borkh. El-Miniawy et al. (2013) reported that total carbohydrates of strawberry were increased as a result of chitosan spraying. Also, Mathew and Sankar (2014) mentioned

that chitosan foliar application increased phenolic compounds as well as antioxidant activity in plants.

It appears that chitosan increased the concentration of simple organic molecules such as, sugar, free amino acids and total soluble phenols, playing a role in regulation of plant osmosis and consequently better plant growth and yield under un-favorable environmental conditions recorded in both orchards locations (Table 3). Furthermore, chitosan might play an important role in scavenging the free radicals thus lead to mitigate the adverse impact of stress and improve growth, productivity and quality of plants.

Earlier reports showed that chitosan triggering highest total phenolic content in cell cultures (Chakraborty et al., 2009); low concentration of chitosan (50 mg/l) was found to trigger the highest secondary metabolite content in *O. gratissimum* (Mathew and Sankar, 2014).

Application of chitosan to soybean leaf tissues have been reported to cause an increase activity of phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL); the key enzymes of phenylpropanoid

Table 7. Effect of different pre-harvest chitosan rates on nutrients concentrations in navel orange leaves in El-Qalubia and El-Sadat orchards during season 2013.

Location	Chitosan conc. (ppm)	N%	P%	K%	Ca%	Zn (ppm)	B (ppm)	Si (mg/gd.wt.)
El-Qalubia	Control (0.0)	2.03	0.95	1.20	0.80	25.21	17.45	16.62
	250	2.13	1.04	1.96	0.97	37.15	10.72	18.05
	500	2.30	1.14	2.0	1.08	40.00	12.06	19.77
	LSD _{0.05}	0.23	0.02	0.19	0.16	1.69	0.81	0.97
El-Sadat	Control (0.0)	2.27	1.07	1.67	0.70	12.30	10.46	13.00
	250	2.75	1.39	2.13	0.97	11.80	9.62	16.45
	500	2.89	1.46	2.97	1.10	12.25	10.05	17.06
	LSD _{0.05}	0.15	0.10	0.23	0.10	0.24	0.32	0.63

pathway (Khan et al., 2003). The products of PAL and TAL are modified through phenylpropanoid metabolism to precursors of secondary metabolites including lignin, flavonoid pigments, and phytoalexins, all of which play key roles in a range of plant-pathogen interactions (Morrison and Buxton, 1993).

The results of plant hormones (Table 6) showed an increase in GA₃, ABA and IAA concentrations with the foliar application of both concentrations of chitosan comparing with the control plants. The highest concentrations of GA₃ and ABA might refer to the effect of chitosan on induction of terpenoids formation; GA₃ and ABA are among compounds belong to terpenoids formed in plants.

In this connection, Uthairatanakij et al. (2007) mentioned that chitosan might induce a signal to synthesize plant hormones such as gibberellins as well as signaling pathways related to auxin biosynthesis.

Also, those might refer to stomatal closure which reduces transpiration and transport of solutes to the aerial-parts of the plant. Iriti et al. (2009) reported that chitosan was able to reduce transpiration in bean plants and this might refer to an increase in ABA content in the treated leaves. Increasing endogenous plant hormones (ABA, GA₃ and IAA) as well as osmoprotectants compounds such as sugar, free amino acids and soluble phenols might improve plant tolerance to unfavorable environmental conditions prevailing in both different regions.

Mineral elements

The data in Table 7 revealed significant increases in N, P, K, Ca and Si concentrations of Washington navel orange leaves with chitosan foliar application as compared to control treatment in both orchards. In El-Qalubia orchard, Zn concentration was significantly increased by both chitosan treatments, whereas it was significantly decreased in leaves grown in El-Sadat

orchard. Meanwhile, a significant decrease in B concentration of navel orange leaves of both regions as compared to control tree. In this respect, Shehata et al. (2012) found that foliar spray of chitosan significantly increased N and P concentrations as well as some micro-nutrients (Fe, Zn, Cu and Mn) contents in cucumber leaves. El-Miniawy et al. (2013) mentioned that nitrogen content of strawberry leaves recorded a significant increase for the tested treatments of chitosan as compared with the control plants.

Saif Eldeen et al. (2014) illustrated that receptacle contents of N, P, total sugars % and protein % of globe artichoke were greatly affected by chitosan treatments as compared to the control. Farouk and Abd El Mohsen (2011) showed that pronounce and highly significant increase in nitrogen, phosphorous and potassium percentages in the shoot due to exogenous application of chitosan (250 mg/l).

Concerning the low B concentration detected in leaves of navel orange trees sprayed with chitosan might explain the increase in total soluble phenols, total free amino acids and auxins concentrations in leaves.

In this respect, Mengel and Kirkby (1979) pointed out that when B is present the activity of the pentose phosphate pathway is decreased in favor of glycolysis.

On the other hand, when boron is deficient the pentose phosphate pathway is favored and consequently induces the accumulation of shichemic acid metabolites; among which phenolic compounds and amino acid tryptophan which act as a precursor for auxin synthesis. Similar discussion was reported by Hanafy Ahmed et al. (2008) on wheat plants.

Fruit physical and chemical qualities

The data in Table 8 revealed that both chitosan treatments had a significant increase on fruit weight of navel orange grown in both regions as compared with control.

Table 8. Effect of both pre-harvest chitosan rates on fruit quality in El-Qalubia and El-Sadat orchard of 2013 season.

Location	Chitosan conc. (ppm)	Fruit quality								
		Fruit weight (g)	Fruit shape index	Fruit size (cm ³)	Fruit firmness (g/cm ²)	Peel thickness (mm)	Juice %	T.S. S. %	Vit.C. (mg/100 ml juice)	Acidity (mg/100 ml juice)
El-Qalubia	Control (0.0)	206.2	1.02	360.0	179.1	0.35	60.37	7.89	53.99	1.08
	250	221.2	0.97	382.5	187.5	0.34	55.71	8.04	52.32	1.10
	500	218.3	1.00	396.9	190.7	0.37	57.40	8.47	50.28	0.99
	L.S.D. _{0.05}	0.73	0.04	2.32	2.14	0.01	0.77	0.11	0.63	n.s.
El-Sadat	Control (0.0)	214.5	1.02	388.3	186.5	0.35	65.61	8.90	49.80	1.24
	250	226.0	1.00	390.1	188.7	0.35	59.41	9.58	48.10	1.27
	500	224.8	0.99	396.5	191.5	0.40	62.71	10.15	47.19	1.27
	L.S.D. _{0.05}	0.63	n.s.	2.18	1.86	0.01	0.74	0.10	0.73	n.s.

In this concern, Reddy et al. (2000) reported that chitosan spray significantly maintained the keeping quality of strawberry fruits as compared with control.

Data concerning the fruit shape index presented in Table 8 revealed that foliar spray by chitosan showed a non-significant effect on fruit shape index in El-Sadat garden. Meanwhile, there was a significant decrease in fruit shape index with 250 ppm chitosan and control in El-Qalubia garden. Saif Eldeen et al. (2014) showed that foliar spraying with chitosan was responsible for significant improvement on head quality of Globe artichoke.

The results in Table 8 indicated that foliar application of chitosan at 500 ppm produced the highest significant increase in fruit size as compared to the other treatments in the both locations.

This result was in agreement with those reported by Mondal et al. (2012) who revealed that okra fruit size was increased with increasing chitosan concentration until 25 ppm.

Pre-harvest spray of chitosan showed a significant increase in fruit firmness with increasing the concentration of chitosan (Table 8).

The beneficial effect of the elevated chitosan concentration on firmness has been reported for peach, Japanese pear, Kiwifruit (Du et al., 1997). Reddy et al. (2000) indicated that fruits from chitosan sprayed strawberry fruits were firmer and ripened at a slower rate as indicated by anthocyanin content and titratable acidity.

On the other hand, El-Miniawy et al. (2013) revealed that chitosan spraying did not affect strawberry fruit firmness.

The results in Table 8 indicated that the pre-harvest spray of chitosan showed a significant increase in fruit peel thickness, and this increase was enhanced with increasing the concentration of chitosan. This was accompanied by a significant decrease in fruit juice %.

Concerning, the effects of pre-harvest chitosan spray

on T.S.S. % of navel orange fruits; it was found that the highest recorded values were obtained by chitosan at concentration 500 ppm in both regions.

This result was consistent with Saif Eldeen et al. (2014) who showed that foliar spraying with chitosan was responsible for significant improvements on total soluble solids.

Abdel-Mawgoud et al. (2010) found that T.S.S. of strawberry fruits showed a tendency to increase in response to chitosan application. However, a reverse result was obtained by El-Miniawy et al. (2013) who found that there was no significant difference in fruit soluble solids content between chitosan spray and control.

As for the effect of the foliar application of chitosan on the total acidity, there was no significant effect among treatments on orange fruits in both orchards as shown in Table 8.

Conclusions

Generally, it could suggest that the significant increase in fruit quality obtained by chitosan foliar applications might be attributed to its roles on improving water retention, nutrients uptake and increasing osmoprotectants; sugars, total free amino acids, total soluble phenols as well as enhancing plant hormones biosynthesis of citrus trees grown under unfavorable environmental conditions recorded in both regions. Finally, further studies are needed to evaluate the effect of pre-harvest chitosan application on navel orange fruits quality after harvesting under different storage temperatures.

Conflicts of interests

The authors have not declared any conflict of interests.

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