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Abstract:

Polysaccharides were extracted from a wild species of Ornithogalum by using three methods: maceration, ultrasound-assisted extraction, and combination of maceration and ultrasound. Extraction conditions were optimized by using response surface method (RSM) with a central composite design (CCD). Four parameters were considered in the optimization method, *i.e*. total extraction time, extraction temperature, ratio of water volume to raw material mass, and time percentage of ultrasound treatment in the extraction process. The optimal extraction yield was 81.7 %, 82.5 % and 85.7%, and the optimal polysaccharides yield was 74.7 %, 75.7 %, and 82.8% under the optimum conditions of maceration, ultrasound-assisted extraction and combined extraction, respectively. These results indicate that the combination method significantly improves the extraction and polysaccharides yields compared to traditional extraction methods. The combination method also allows reducing the time of ultrasound treatment and thus its adverse effects on polysaccharides. In addition, these results well corroborate with the theoretically predicted values. The NMR (^{1}H,^{13}C, HSQC, HMBC, and COSY) analysis shows that the extract is composed of fructo-polysaccharides with a backbone of (2→6)-linked β-d-fructofuranosyl (Fruf)and (2→1)-linked β-d-Fruf branched chains, and terminated with glucose and Fructose residues. The antioxidant activities of the extract were evaluated from ABTS radical scavenging activity, total antioxidant capacity, metal-chelating power and β-carotene bleaching test. Data show that the extract presents outstanding antioxidant activities.

Keywords: Ultrasonic-assisted extraction; optimization; polysaccharides; anti-oxidant activity.

1. Introduction

Synthetic antioxidant compounds such as butylated hydroxyanisole (BHA) are extensively employed as preservatives in pharmaceuticals, cosmetic and food industry. However, some studies have shown that BHA causes adverse effects on cells [1], and thus could have negative

impact on human health. Therefore, naturally occurring antioxidants have been attracting more and more interest as they actually represent an alternative to synthetic ones.

More than 90% of the carbohydrate mass in nature is in the form of polysaccharides. Many studies reported that various polysaccharides present remarkable antioxidant activities, and could be used as healthy food additives [2]. Ornithogalum is a genus of perennial plants with various species, including *Ornithogalum arabicum, saundersiae, caudatum Ait*, *dubium*, *nutans*, *pyrenaicum*, *thyrsoides* and *umbellatum*. Polysaccharides extracted from *Ornithogalum caudatum Ait* present outstanding antioxidant, anticancer, antimicrobial and anti-inflammatory activities [3]. They have been used for the treatment of hepatitis, parotitis, and cancers [3]. *Ornithogalum billardieri* is a non-edible wild plant widespread in Lebanon which could be a potential source of valuable polysaccharides with promising bioactive properties. To the best of our knowledge, this plant has never been studied, so far.

The extraction of polysaccharides represents an important issue for their applications in pharmaceutical and agri-food industries [4]. Maceration is the most commonly used method to extract polysaccharides due to its simplicity. Nevertheless, the major inconvenience of maceration is the low extraction yield [5], long extraction time, and large energy consumption. Ultrasound (US) treatment is a time and energy saving method which is widely used for extraction of various polysaccharides [6]. It allows to reduce the particle size, to disrupt the cell wall, and to improve the mass transfer from solid to liquid phase due to the intense shear forces, thus leading to higher extraction yield [7]. Compared to maceration, US treatment presents many advantages such as reduced extraction time, lower temperature, and in some cases less water consumption [8]. Importantly, US extraction allows to improve the purity of extracted polysaccharides, and their antioxidant activity [9]. However, US treatment can provoke chain cleavage and compositional changes of polysaccharides due to the cavitation effects [9]. Side reactions could also happen and lead to the formation of carbonyl and hydroxyl radical groups.

The aim of this work was to implement and optimize a new extraction process combining both US and maceration in order to improve the extraction yield while reducing the adverse effect of US treatment. Different combinations of maceration and US treatment were tested to extract polysaccharides from *Ornithogallum billardieri* using the Surface Response Methodology (RSM) applied to an experimental model such as Box-Behnken design (BBD) and central composite design (CCD) [10]. The studied operating parameters include the total duration of the extraction process (time), the extraction temperature, the ratio of added water

volume to raw material mass, and the time ratio of US treatment in the total process (% US). The extracted polysaccharides were characterized by using ^{1}H,^{13}C, HSQC, HMBC, and COSY NMR analysis.

The antioxidant properties of the polysaccharides extracted under optimal conditions were investigated, including ABTS radical scavenging activity, total antioxidant capacity (TCA), metal-chelating power, and β-carotene bleaching test so as to evaluate their potential for applications in the agro-food industry as alternative to synthetic antioxidants.

2. Experimental

2.1. Materials and reagents

The onion plant Ornithogalum was collected in the region of Bekaa in Lebanon. The plant was carefully washed, cut into small pieces, and freeze dried. The dried plant was manually crushed, and sieved at 0.6 mm to obtain a homogeneous powder. Sulfuric acid, phenol, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ascorbic acid, potassium peroxydisulfate, sodium phosphate, ammonium molybdate, α-tocopherol, iron dichloride, ferrozine, diethylenediamine (EDTA), phenol, anhydrous ethanol, butanol, and chloroform of analytical grade were purchased from Sigma Aldrich, and used as received.

2.2. Extraction of polysaccharides

Before extraction, the powder was purified at 70°C using a Soxhlet for 2 days with ethanol as solvent. This preliminary step allowed to eliminate all pigments, polyphenols, oligosaccharides, simple oses and amino acids, and also to inactivate enzymes. The powder recovered from the Soxhlet cartridge was vacuum dried overnight at 40°C, and then crushed and sieved at 0.6 mm. The powder was added in ultrapure water, and homogenized for 30 sec at 3500 rpm in a Speed mixer (Hauschild DAC 150.1 FVZ-K). Extraction then proceeded at a given temperature (25-65°C) for predetermined time periods (10-60 min). The extraction temperature was controlled (± 0.2°C) using a thermo-cryostat (Vacuo-Temp P, Selecta) and a thermostat cell. The ratio of the water volume to the powder mass varied in the range of 10 to 40 mL/g. Ultrasound treatment was performed at fixed frequency (35 KHz) and power (120 W). The time ratio of ultrasound treatment in the total extraction process (%US) varied between 0% and 100%. After extraction, the aqueous solution was immediately centrifuged at 6000 rpm for 15 min three times. The supernatant was lyophilized using a freeze-dryer (Freezone 4.5 Labconco). Finally, the obtained powder was washed with ethanol, and vacuum dried up to constant weight.

The extraction yield (%) was calculated using Eq. 1.

$$\text{Extraction yield (\%)} = [(\text{Weight of dried product} / \text{Weight of crude powder})] \times 100 \quad \text{(Eq. 1)}$$

2.3. Total carbohydrates content

The total carbohydrate content (TCC), also known as the polysaccharides yield, was determined according to Dubois method [11]. The method allows to quantify all carbohydrate species, including mono-, di-, oligosaccharides, their methyl derivatives and polysaccharides. A calibration curve was first established from standard glucose solutions at concentrations from 0 to 100 µg/mL. 1 mL of standard solution was added to 5 mL of concentrated sulfuric acid, followed by addition of 1 mL of 5% (v/v) phenol solution. After 10 min stirring at 100 °C, the solution was cooled down to room temperature away from light. Finally, the absorbance was measured using a spectrophotometer at 490 nm (λ max). The absorbance was then plotted against glucose concentration to obtain a calibration curve. The samples are analyzed using the same procedure to obtain the concentration of polysaccharides in samples. The total carbohydrate content (%) of samples is determined using the following equation:

$$\text{Total carbohydrate content (\%)} = [(\text{Weight of polysaccharides} / \text{Weight of sample})] \times 100 \quad \text{(Eq. 2)}$$

2.4. Extraction optimization

2.4.1 Single-factor experiments

A series of preliminary experiments were first performed in order to determine the intervals of the 4 parameters (time, temperature, volume to mass ratio, and US%), and the range of extraction yield and polysaccharides yield. For each experiment, a single parameter was varied while keeping the others constant: the time was varied from 10 to 60 min, the temperature from 20°C to 80°C, the volume to mass ratio from 10 to 50 mL/g, and the US% from 0 to 100%. The results obtained from single factor analysis were used to build a model based on RSM to identify the interactions between parameters.

2.4.2 Experimental design

The Surface Response Methodology (RSM) is applied to the central composite design (CCD) for the adaptation of a second order polynomial by the least square technique. Eq. 3 is used to determine the effects of test variables to the searched responses (extraction yield and polysaccharides yield) and the correlation between variables.

$$Y = \beta\kappa_0 \sum_{i=1}^{4} \beta\kappa_i \, X_i + \sum_{i=1}^{4} \beta\kappa_{ii} \, X_i^2 + \sum_{i<j=2}^{4} \beta\kappa_{ij} \, X_i \, X_j \quad \text{(Eq. 3)}$$

Where Y is the predicted response, $\beta\kappa_0$ the intercept, and $\beta\kappa i$, $\beta\kappa ii$, and $\beta\kappa ij$ the coefficients of linearity, quadratic and interaction, respectively. And X_i and X_j are the coded independent variables. The response function was also related to the coded variables (X_i, i = 1, 2, 3) and i≠j, by a second-degree polynomial using the method of least squares.

The CCD consists of 2^n factorial points and 2^n axial points, with n = number of factors studied and Nc central points (replicates in the center of the experimental domain). Four parameters have been taken into account in this study, i.e. Time X_1 (min), Temperature X_2 (°C), Volume X_3 (volume to mass ratio, mL/g), and US percentage X_4 (%). The CCD is thus composed by 16 factorial points, 8 axial points and 5 central points, giving a total of 29 experiments. The axial points are placed at a distance α=2 from the center, that makes the design rotatable. The range and levels of the variables are given in Table 1 according to the actual and coded values.

The regression coefficients of individual linear, quadratic and interaction terms were determined using Design-Expert (Version 11) software. They were then used to make statistical calculations, which generate dimensional and contour maps from the regression models.

Table 1 Independent variables and their levels used in the response surface design

Coded value	α = -2	-1	0	+1	α = +2
	Real Value				
Time (min)	10	22.5	35	47.5	60
Temperature (°C)	25	35	45	55	65
Volume to mass ratio (mL/g)	10	20	30	40	50
% US (%)	0	25	50	75	100

2.5 Structural characterization

2.5.1 Fourier transform infrared (FT-IR)

FT-IR spectra were recorded using an IR-TF Nicolet IS50 Fourier counter exchange absorption infrared spectrometer (Bruker, Germany) over a range of 400–4000 cm^{-1}. The samples were analyzed as KBr pellets.

2.5.2 Size-exclusion chromatography (SEC)

Size-exclusion chromatography (SEC) was carried out using HPLC (DW-LC1620A) equipped with TSK gel PW5000 + PW3000 columns and refraction index and ultraviolet detectors. The temperature of the columns and detectors was 20 and 35 °C, respectively. A pH 6 phosphate

buffer at 10 mg/mL was used as eluent at a flow rate of 1 mL/min. Calibration was realized using pullulan standards with molar masses from 500 to 25000 Da. The results were processed using OmniSEC software.

2.5.3 NMR Spectroscopy

^{1}H, ^{13}C, HSQC, HMBC, and COSY NMR spectra were recorded using a Bruker Avance III spectrometer operating at 600 MHz and 150 MHz, respectively. D_2O was used as solvent, and the chemical shifts were expressed in ppm. The spectra were treated using Mestre Nova 12.0.0. software package.

2.6 Antioxidant activities

2.6.1. ABTS radical scavenging activity

2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) stock solution was prepared by dissolving 7 mM ABTS with 2.45 mM $K_2S_2O_8$ in ultra-pure water, followed by stirring in the dark for 12 h. The solution was diluted with ethanol to obtain an absorbance of 0.7 (± 0.02) at 734 nm at 30 °C. 0.1 mL of sample at concentrations from 0.02 to 10 mg/mL was mixed with 3.9 mL of the diluted solution of $ABTS^{\cdot+}$. After 20 min incubation at 30 °C, the absorbance was measured at 734 nm. Ultrapure water was used as blank sample [12], and ascorbic acid as positive control. The ABTS radical scavenging activity was calculated from the following equation:

$$\text{Scavenging rate (\%)} = [A_0 - (A_e - A_{ie})]/A_0 \times 100 \qquad \text{(Eq. 4)}$$

Where A_0, A_{ie} and A_e are the absorbance of the blank sample, the positive control, and the samples, respectively.

2.6.2 Total antioxidant capacity (TCA)

100 µL of samples at concentrations ranging from 0.025 to 10 mg/mL were mixed in a tube with 1 mL of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The mixture was incubated at 95°C for 90 min. A phosphomolybdenum complex was formed after cooling down to room temperature. Absorbance of the solution was measured at 820 nm. Ultrapure water was used as control [13]. The antioxidant activity is presented in terms of absorption.

2.6.3. Metal-chelating power

100 µL of samples at concentrations ranging from 0.2 to 10 mg/mL were mixed with 50 µL of $FeCl_2$ (2 mM), and vigorously stirred for 5 min. Then 100 µL of Ferrozine (5 mM) were added together with 2.75 mL of ultrapure water. After 10 min at room temperature, the absorbance at 562 nm was measured.

The blank sample was prepared without addition of ferrozine, while the negative control was prepared without addition of test sample. Ethylenediaminetetraacetic acid (EDTA) was used as positive control [14].

The antioxidant activity was calculated from the following equation:

$$\text{Antioxidant activity \%} = [(A_{control} - A_{sample}) / A_{control}] \times 100 \qquad \text{(Eq. 5)}$$

Where $A_{control}$ and A_{sample} are the absorbance of the negative control and the sample, respectively.

2.6.4 β-carotene bleaching test

The inhibition of β-carotene bleaching was evaluated according to the method described by Koleva *et al*. [15]. 0.5 mg of β-carotene was dissolved in a mixture of 1 mL of chloroform, 25 µL of linoleic acid and 200 µL of Tween-80. The solvent was evaporated in a rotary evaporator at 45°C. 100 mL of ultrapure water was then added to the suspension under stirring. 2.5 mL of freshly prepared suspension were transferred to a tube containing 0.5 mL of test samples at concentrations from 0.05 to 5 mg/mL. The mixture is incubated for 2 h at 50 °C after homogenization. Ultrapure water was used as control. Absorbance was then measured at a wavelength of 470 nm. The antioxidant activity was calculated from the following equation:

$$\text{Antioxidant activity (\%)} = [1 - (A_0 - A_{120})_{test} / (A_0 - A_{120})_{control}] \times 100 \qquad \text{(Eq. 6)}$$

Where A_0 and A_{120} are the absorbance of the sample or the control before and after 120 min incubation.

2.7 Statistical analysis

The data processing is based on the statistical analysis performed by using the ANOVA Test (Analysis of Variance). A value of $p < 0.05$ is considered statistically significant. The data are expressed as mean ± standard deviation (SD) for three replicates. The lack-of-fit test, F value, determination of coefficient (R^2), adjusted determination coefficient (R_{adj}^2), coefficient of

variation (C.V.%) calculated from Design Expert were used to evaluate the adequacy of the models.

3 Results and discussion

The challenge of this work is to find a compromise between the positive and negative effects of US extraction and temperature on the extraction and polysaccharides yields, in order to maximize the mass transfer without degrading the quality of the product of interest.

3.1 Optimization of the extraction

3.1.1 Single-factor experiments

Single-factor experiments were first performed to determine the effect of the 4 parameters, i.e. extraction time, temperature, volume to mass ratio, and US %, on both of the total extraction yield and polysaccharides yield. The results are presented in Fig. S1 (Supporting information). The same trend is observed in all cases, i.e. an increase of the yields to reach a maximum followed by a decrease.

Based on the results of single factor experiments, it appears that both the extraction and polysaccharides yields are largely dependent on the extraction conditions, and the 4 parameters are inter-dependent. Opposite effects of the parameters are clearly evidenced. In fact, hydrolysis of polysaccharides occurs under harsh conditions. It is thus essential to understand the effect of each parameter on the extraction and polysaccharides yields and the relationship between the parameters by applying the RSM, an efficient statistical technique for optimizing complex processes. The following intervals are selected for the optimization study: time from 22.5 to 47.5 min, temperature from 35 to 55°C, volume to mass ratio from 20 to 40 mL/g, and %US from 25 to 75%.

3.1.2 RSM statistical analysis and models validity

Based on the results obtained from single factor analysis, optimization was carried out by using RSM to study the effect of four independent variables [X_1: time, X_2: temperature, X_3: volume (ratio of volume to mass), X_4: %US]. The preliminary results pave the way to determine the interactions between parameters and their influence on the investigated responses. Table 2 shows the matrix of variables in real values and the obtained responses. The predicted response Y can be correlated to the variables by applying multiple regressions analysis method. The fitting of response functions Y_1 (extraction yield) and Y_2 (polysaccharides yield) with the

experimental data gives a second-order polynomial equation. The analysis of variance table is generated by the Design Expert software (Version 11) used in this study.

The extraction and polysaccharides yields obtained in the 29 experiments vary from 76.2 to 86.1% and from 70.1 to 82.9%, respectively. Such relatively high values in narrow ranges could be explained by the fact that large amounts of polysaccharides can be easily extracted from the superficial layer of the particles. However, harsher extraction conditions are required to extract species located well inside the particles.

Table 2 RSM centrale composite design and results for extraction yield (Y_1) and polysaccharides yield (Y_2).

RUN	X_1	X_2	X_3	X_4	Y_1	Y_2
1	22.5	35	20	25	76.2	70.1
2	47.5	35	20	25	77.2	71.0
3	22.5	55	20	25	76.5	71.6
4	47.5	55	20	25	76.9	72.2
5	22.5	35	40	25	78.7	72.2
6	47.5	35	40	25	83.5	77.4
7	22.5	55	40	25	79.4	72.8
8	47.5	55	40	25	83.0	77.3
9	22.5	35	20	75	81.9	75.2
10	47.5	35	20	75	78.1	72.3
11	22.5	55	20	75	80.0	73.4
12	47.5	55	20	75	76.6	71.0
13	22.5	35	40	75	82.7	75.0
14	47.5	35	40	75	83.3	76.5
15	22.5	55	40	75	78.8	72.8
16	47.5	55	40	75	80.7	74.3
17	10	45	30	50	76.7	71.1
18	60	45	30	50	80.1	72.9
19	35	25	30	50	78.7	74.3
20	35	65	30	50	80.2	73.1
21	35	45	10	50	76.7	70.1
22	35	45	50	50	80.8	77.3
23	35	45	30	0	78.5	72.5
24	35	45	30	100	82.0	76.1
25	35	45	30	50	85.3	82.6
26	35	45	30	50	86.1	82.9
27	35	45	30	50	85.8	81.7
28	35	45	30	50	84.6	82.9
29	35	45	30	50	85.2	82.0

In order to highlight the significance of models, statistical indicators such Analysis of Variance (ANOVA) and correlation coefficients (R^2, adjusted R^2 and predicted R^2) are determined, as shown in Table 3.

The ANOVA results show that both quadratic models developed for Y_1 and Y_2 are highly significant with F values (Fisher coefficient) of 25.1 and 121.3, respectively. Considering also the p-values well below 0.05 ($P<0.0001$) and the negligible pure errors towards the quadratic model, the lack of fit of these models is insignificant. The correlation coefficient R^2, adjusted R^2 and predicted R^2 are 0.950, 0.912, and 0.780 for the extraction yield, and are 0.989, 0.981, and 0.959 for the polysaccharides yield, respectively. These values are reasonably close to 1,

showing a high degree of correlation. On the other hand, the Adeq. Precision is used to measure the signal to noise ratio, and a ratio above 4 is considered as acceptable. The obtained Adeq Precision is 15.5 and 32.3 for the extraction and polysaccharides yields, respectively, indicating a very high degree of precision and a good reliability of the experimental data. Further analysis of the statistical indicators in Table 3 also shows that the model developed for the polysaccharide yield presents slightly better precision than the one for the extraction yield.

Table 3 Analysis of variance (ANOVA) for Response Surface Quadratic Model of extraction and polysaccharides yields

Source	Extraction yield					Polysaccharides yield				
	Sum of Squares	df	Mean Square	F Value	p-value	Sum of Squares	df	Mean Square	F Value	p-value
Model	263.55	12	21.96	25.13	< 0.0001	448.3	12	37.36	121.3	< 0.0001
Residual	13.99	16	0.87	-	-	4.93	16	0.31	-	-
Lack of Fit	12.65	12	1.05	3.15	0.1388	3.72	12	0.31	1.02	0.5444
Pure Error	1.34	4	0.33	-	-	1.21	4	0.3	-	-
C.V.%	1.16					0.74				

3.1.3 Regression equations and significant terms analysis

ANOVA tests are performed to evidence the significance of the studied parameters on the responses. The insignificant terms are removed in order to refine the developed models. As shown in Fig. 1, the experimental data are scattered around the theoretical line (Predicted vs Actual values), which indicates that the results obtained from the model well agree with the experimental data.

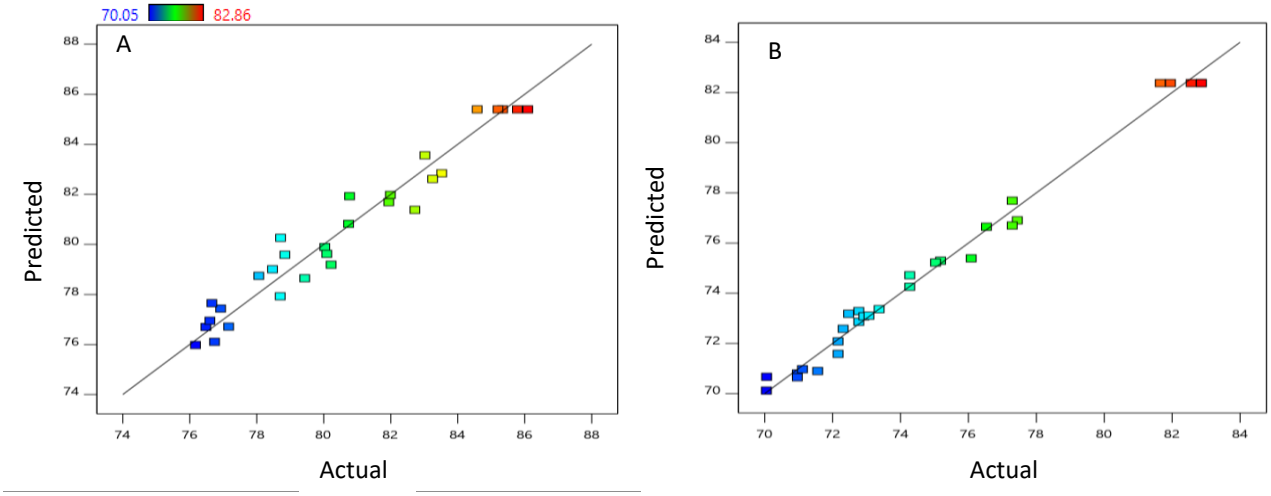


Fig. 1 Diagnostic plot (predicted vs actual values) for the adequacy of proposed model for extraction yield (A) and polysaccharides yield (B).

The simplified equations for the extraction yield Y_1 and polysaccharides yield Y_2 are presented in terms of coded factor as follows:

$Y_1 = 85.40 + 0.49\ X_1 - 0.27\ X_2 + 1.46\ X_3 + 0.74\ X_4 + 1.04\ X_1X_3 - 0.92\ X_1X_4 - 0.63\ X_2X_4 - 0.56\ X_3X_4 - 1.69\ X_1^2 - 1.42\ X_2^2 - 1.60\ X_3^2 - 1.23\ X_4^2$ (Eq. 7)

$Y_2 = 82.38 + 0.53\ X_1 - 0.29\ X_2 + 1.51\ X_3 + 0.55\ X_4 + 1.04\ X_1X_3 - 0.85\ X_1X_4 - 0.68\ X_2X_4 - 0.51\ X_3X_4 - 2.59\ X_1^2 - 2.17\ X_2^2 - 2.17\ X_3^2 - 2.02\ X_4^2$ (Eq. 8)

The coefficients associated to the parameters or the interactions give a precise idea of how they affect the corresponding responses (positively or negatively). Firstly, it appears that the effect of the parameters and interactions follow the same trend for both Y_1 and Y_2. Regarding the coefficients, the ratio of volume to mass (X_3) is the most important factor affecting the extraction process, followed by the US % (X_4) and the extraction time (X_1). The extraction temperature (X_2) is the less significant parameter. It is well known that temperature generally enhances the polysaccharide solubility and mass transfer, but it is also a destructive factor as the US treatment. Raza *et al*. reported similar adverse effect of temperature [16]. Thus, opposite effects could happen, minimizing the overall effect on the response. Regarding the interactions, the most important one is referred to the synergistic (positive) effect between the treatment time and the volume to mass ratio (X_1X_3) which enhances the polysaccharide extraction, in agreement with literature [16]. Indeed, both factors (X_1 and X_3) are non-destructive parameters. When the ratio of volume to mass is higher, the overall driving force is higher, thus accentuating the effect of the extraction time.

It is also noticed that US has systematically a negative effect when combined with another factor. This means that US has a positive effect under mild extraction conditions, i.e. all other parameters at low level. But as soon as the extraction conditions become harsher, the destructive effect becomes predominant. This phenomenon is more accentuated with the treatment time (X_1X_4), which makes sense as US (percentage of X_1) is directly dependent on the treatment time.

3.1.4 Response surface analysis and optimization

The two responses Y_1 and Y_2 follow the same trend as indicated above. Therefore, the same effects and interactions of the parameters are expected for both responses. Only the polysaccharides yield was investigated by using the Design-Expert to construct a three-dimensional surface according to Eq. 8. The RSM was used to determine the relationship between extraction parameters and responses, as shown in Fig. 2. Two variables are continuously varied while keeping the two others constant.

Fig. 2A presents the effect of interaction between volume to mass ratio and extraction time on the polysaccharides yield, while the extraction temperature is fixed at 35°C and US% at 25%. The highest polysaccharides yield of 78.2% is observed at 40 min and 36 mL/g. In fact, the increase of volume to mass ratio results in increase of the polysaccharides yield. But in the meantime, the extraction time required to obtain the maximum of polysaccharides increases due to the dilution effect [17].

Fig. 2B presents the effect of temperature and US % while keeping the time fixed at 22.5 min and volume to mass ratio at 20 mL/g. At low temperature (35°C), the polysaccharides yield increases from 70.1 to 75.3% with US% increasing from 25 to 75. It appears that there is little destructive effect of US under mild extraction conditions.

Fig. 2C presents the effect of volume to mass ratio and US% on polysaccharide yield at fixed time (47.5 min) and temperature (55°C). Under these harsh conditions, the US treatment above 42% leads to a decrease by 8.2% of the maximum of polysaccharides yield.

Fig. 2D presents the effect of time and US% on polysaccharides yield while keeping the volume to mass ratio at 30 mL/g and the temperature at 45°C (level 0). A remarkable increase of the polysaccharides yield is detected with extraction time. However, an extraction time longer than 37.1 min leads to decrease of the polysaccharides yield. In fact, prolonged extraction time could lead to cleavage of polysaccharide chains, resulting in decrease of the polysaccharides yield [18]. The polysaccharides yield increases with the increase of US% up to a maximum at 51.7%. Beyond, a decrease of the polysaccharides yield is observed. Additionally, the extraction time required to obtain the maximum of polysaccharides yield becomes shorter with increase of US%. These results evidence the effect of the main parameters and their interactions, which allows to better understand the extraction process. In particular, it is clearly shown that the US is definitely promoter of extraction because of improved mass transfer. But US also has a destructive effect in combination with other parameters such as high extraction temperature.

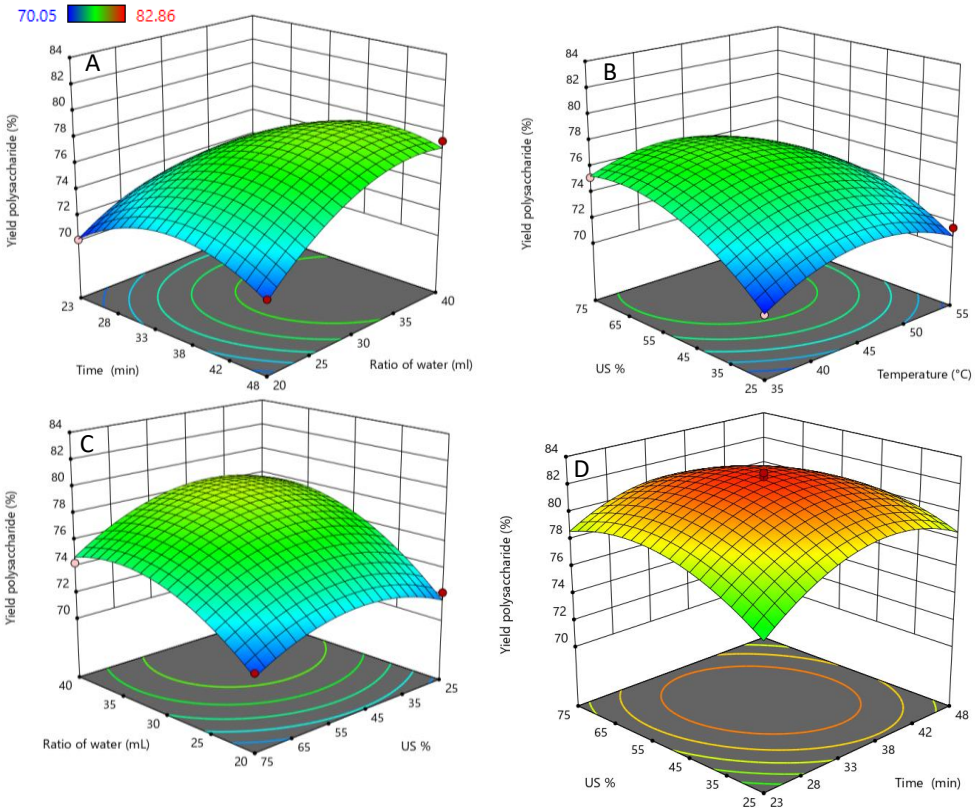


Fig. 2 3D response surface plots showing the interactive effects of (A) time and volume to mass ratio at (-1, -1), (B) temperature and US% at (-1, -1), (C) volume to mass ratio and US % at (+1, +1), and (D) time and US % at (0, 0).

3.1.5 Validation of predictive models

The optimal extraction conditions to obtain the highest extraction and polysaccharides yields are given below: time (38.5, 37.1 min), temperature (43.5, 44.0°C), volume to mass ratio (35.4, 33.8 ml/g), and US% (52.8, 51.7%). Under these conditions, the theoretical extraction and polysaccharides yields are 85.9% and 82.7% respectively.

Extraction was repeated three times under the optimal conditions in order to validate the two models for the extraction and polysaccharides yields. The obtained extraction yield is 85.7 ± 0.2 %, and the polysaccharides yield is 82.8 ± 0.1%. These values are very close to the theoretical ones, thus validating the models for evaluation of the effects of the 4 parameters on the extraction and polysaccharides yields.

In order to evidence the ambivalent effects of the US treatment, additional experiments were conducted at either 0% US (only maceration) or 100% US. The experimental results are then compared with the theoretical values obtained from the developed models, as shown in Table 4. For the extraction yield, the first test was performed at 0% US under the predicted optimal conditions (time: 47.49 min; temperature: 47.84°C; volume to mass ratio: 40 mL/g), giving a maximum predicted extraction yield of 81.7%. The average extraction yield of three repeated experiments at 0% US was 81.7% ± 0.1%. The second test was performed at 100% US, which

399 allowed to obtain a maximum predicted extraction yield of 82.7% under the optimal conditions
400 (time: 30.03 min; temperature: 39.62°C; volume to mass ratio: 30.02 mL/g). The average
401 extraction yield of three repeated experiments at 100% US was 82.5% ± 0.1%.
402 In the case of the polysaccharides yield, at 0% US the maximum predicted polysaccharides yield
403 was 74.8% under the optimum conditions (time: 42.12 min; temperature: 47.1°C; volume to
404 mass ratio: 37.2 mL/g). The average yield of three repeated experiments was 74.7 ± 0.1%. At
405 100% US, the maximum predicted polysaccharides yield was 75.8% under the optimum
406 conditions (time: 32.41 min; temperature: 41.2°C; volume to mass ratio: 30.8 ml/g). The
407 average polysaccharides yield of three repeated experiments was 75.7 % ± 0.1%.
408 The model was then used to achieve the yield maximum under the mildest conditions. A
409 theoretical extraction yield of 83.0% was predicted with the following conditions: 23.7 min,
410 39°C, 22.6 mL/g and 69.5 % US. The experiences gave an average yield of 83.0 ± 0.1%, which is
411 nearly the same as the predicted value. This technique leads to significant decrease in time by
412 6.3 min, temperature by 0.7°C and volume to mass ratio by 7.4 mL/g, while a small increase in
413 extraction yield by 0.4% is obtained as compared to 100% US extraction (Table 4). Similarly, an
414 optimal polysaccharide yield of 76.3% is predicted under the following conditions: 23.1 min,
415 35°C, 21.3 mL/g and 65.3% US. Extraction performed three times under these conditions gave
416 a polysaccharide yield of 76.1% ± 0.1%. Comparison between the combined system and
417 extraction with 100% US indicates that the former allows a small increase in yield by 0.4%,
418 while decreasing significantly the time, temperature, and volume to mass ratio by 9.3 min,
419 6.2°C, and 9.5 mL/g, respectively (Table 4).
420 It is also of interest to compare the purity of the extract which is defined as the ratio of the
421 polysaccharides yield to the total extraction yield. Under the optimum conditions of
422 maceration, 100% US, and combined system, the purity of polysaccharides was 91.4 ± 0.1 %,
423 91.8 ± 0.2 %, and 96.6% ± 0.1%, respectively. Thus, the combination of maceration and US
424 allows improving the purity of the extract.
425 Chen *et al*. reported an optimized polysaccharide yield of 36.8 ± 1.8% for extraction from
426 *Ornithogalum Caudatum Ait* under the following conditions: frequency 40 kHz, temperature 60
427 °C, extraction time 60 min, ultrasound power 500 W, solvent to raw material 30 mL/g, 3 times
428 extraction [26]. The yields obtained in the present study (frequency 35 kHz, ultrasound power
429 120 W) are significantly higher, in agreement with successful optimization of ultrasound
430 assisted extraction. The combined extraction process allowed to reduce the sonication time,
431 and thus to prevent excessive polysaccharide degradation.
432

Table 4 Comparison of parameters, predicted and experimental values for optimization of extraction yield (Y1) and polysaccharides yield (Y2) by mixed system, maceration and US treatment

X_1 (min)	X_2 (°C)	X_3 (mL/g)	X_4 (%)	Predicted (%)	Experimental (%)
			Y1		
38.5	43.5	35.3	52.8	85.9	85.7 ± 0.2%
47.5	47.8	40	0	81.7	81.7 ± 0.1%
30.0	39.6	30.0	100	82.6	82.5 ± 0.1%.
23.7	38.9	22.6	69.5	83.0	83.0 ± 0.1%.
			Y2		
37.1	44.2	33.8	51.7	82.7	82.8 ±0.1%
42.1	47.1	37.1	0	74.8	74.7 ± 0.1%
32.4	41.2	30.8	100	75.8	75.7 ± 0.1%
23.1	35	21.3	65.3	76.3	76.1 ± 0.1%

The extract with optimal purity of polysaccharides, namely OP%US, was obtained under the following conditions: time 37.1 min, temperature 44.2°C, volume to mass ratio 33.8 mL/g, and US% 51.7%. OP%US was used for further analyses.

3.2 Structural characterization

3.2.1 FT-IR analysis

The FT-IR spectrum of OP%US is presented in Fig. S2 (Supporting information). The large band at 3405 cm^{-1} is assigned to the stretching vibration of O–H groups, and the band at 2941 and 2896 cm^{-1} were assigned to the stretching of $-CH_3$ and $-CH_2$ groups respectively. The band in the range of 1420 cm^{-1} belongs to the deformation vibration of C–H bond. The monosaccharide of OP%US has pyranose rings as evidenced by two strong absorption bands of C-O-C asymmetric stretching at 1132 cm^{-1} and 1027 cm^{-1}. The band at 931.2 cm^{-1} s attributed to the symmetric stretching of furan rings. And the band detected at 599 cm^{-1} is assigned to the presence of the skeletal -CH_2 of pyranose rings. An α-configuration in the polysaccharide is confirmed by the presence of characteristic absorptions at 818 cm^{-1}.Therefore, OP%US was an α-configuration polysaccharide and consisted of pyranoside and funanside rings[19,20].

3.2.2 Size-exclusion chromatography analysis

From the results obtained in the RSM experiments, it appears that chain cleavage of polysaccharides occurred to some extent during extraction. GPC analysis was then performed for the corners of the design expert model in order to figure out the effect of extraction parameters on the molar masses of the extracts, as summarized in Table 5. The effect of extraction time is evidenced from the comparison between runs 17 and 18 since different extraction times were used while keeping the three other parameters constant. Similarly, the effect of extraction temperature and US % is evidenced from the comparison between runs 19 and 20, and runs 23 and 24, respectively.

In runs 17 and 18, the average molar mass (Mw) decreases from 5190 to 2940 Da when the extraction time is raised from 10 to 60 min at fixed temperature (45°C), volume to mass ratio (30 mL/g), and US% (50%). In the meantime, the dispersity (*Đ* = Mw/Mn) slightly increases from 1.41 to 1.53. The negative effect of ultrasonic time on the molar mass of schizophyllan has been also reported by Zhong *et al.* [21].

In runs 19 and 20, with the extraction temperature increasing from 25 to 65°C, the Mw of the extract slightly decreases from 3180 to 2830 Da, whereas the dispersity remains almost unchanged.

Finally, in runs 23 and 24, with increase of US treatment from 0% to 100%, the Mw slightly decreases from 3350 to 3030 Da, and the dispersity remains almost unchanged. These results suggest that long extraction time, high extraction temperature, and ultrasound treatment provoke chain cleavage and molar mass decrease of the extract, which could affect the extract yield as shown in the RSM experiments.

Table 5 Molar mass (Mw) and dispersity (Đ) of the extract obtained in selected runs

Run	Time (min)	Temperature (°C)	% US (%)	Ratio (mL/g)	Mw (Da)	*Đ*
17	10	45	50	30	5 190	1.41
18	60	45	50	30	2 940	1.53
19	35	25	50	30	3 180	1.49
20	35	65	50	30	2 830	1.52
23	35	45	0	30	3 350	1.50
24	35	45	100	30	3 030	1.52

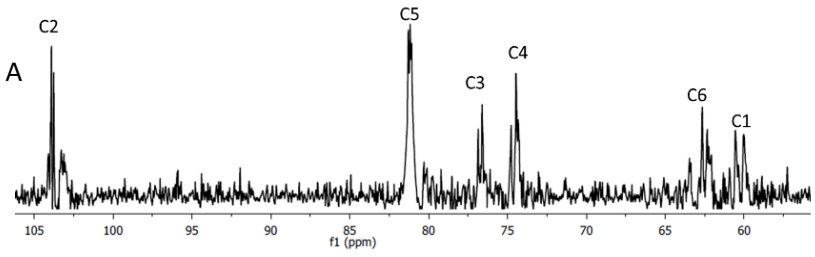
3.2.3 NMR Spectroscopy

Various NMR analyses, including ^{1}H ,^{13}C, HSQC, HMBC, and COSY NMR were performed to determine the composition of the extract.

The ^{13}C NMR in Fig. 3A presents signals in the region of 59.0-61.48, 73.3-75.15, 57.3-77.77, 79.05-82.5, and 103.0-104.2 related to the C_1, C_6, C_4, C_3, C_5, and C_2 of fructose rings respectively. Additionally, four anomeric C_2 carbon signals are detected (103.11, 103.76, 103.90, and 103.95 ppm), which indicates the presence of four fructose residues, *i.e*. β-d-Fruf-(2→, →1,6)-β-d-Fruf-(2→, →1)β-d-Fruf-(2→, and →2)β-d-Fruf-(6→ respectively respectively designated as residues B, C, D, and E.

Fig. 3B presents the ^{1}H NMR spectrum of the extract. Small signals are detected around 2.4, 2.5 and 2.7 ppm, which could be assigned to the presence of amino acids, carboxylic acids, alcohols or phenols, in agreement with literature [22]. The signals at 3.8-3.7, 4.27, 4.13, 3.89, and 3.86-

493 3.68 ppm belong to the protons of fructose ring from H_1 to H_6, whereas the signals at 3.50,
494 3.59, and 3.98 ppm are attributed to the protons H_4, H_2 and H_5 of glucose, respectively. The
495 signal at 5.45 ppm indicates the presence of a proton on α of anomeric carbon. This proton
496 belongs to glucose named as residue A as fructo-ketose has no anomeric proton, which is in
497 agreement with FTIR results [23]. The chemical shifts for H_1, H_3, H_4 of residues B, C, D, and E of
498 fructose presented in Table 6 were determined from ^1H, HSQC, ^1H–^1H COSY spectra, and the
499 assignment of H_5 and H_6 realized from the ^1H–^1H COSY and HMBC spectra.
500
501 The HQSC spectrum fig. 3C of the extract presents typical signals at 81.08/3.9 and 80.28/3.98
502 ppm assigned to the C_5 of →2) β-d-Fru-(1→ and →2) β-d-Fru-(6→, respectively [24]. The
503 signals of H_1, H_3, H_4, H_5 and H_6 of fructose are related to the carbon signals at 60.46, 76.62,
504 74.46, 81.08, and 63.37 ppm, respectively. Meanwhile, the signals at 3.51, 3.59, 3.79, 3.98, 3.9-
505 3.7, and 5.45 ppm are linked to C_4 69.32, C_2 71.18, C_3 72.52, C_5 71.67, C_6 61.9, and C_1 of anomeric
506 carbon 92.15 ppm for glucopyranose ring, respectively [25].
507
508 The sequence among residues and the linkage sites were achieved using the HMBC spectrum
509 which provides signals correlations between protons and carbons (Fig. 3D). The various signals
510 are assigned as follows. C_2 (103.11 ppm) for residue B and H_1 (3.91 ppm) for residue D
511 (BC_2/DH_1), suggesting that C_2 of residue B is linked to O-1 of residue D. Similarly, cross signals
512 at 103.11/3.89 ppm are assigned to BC_2/EH_6, 103.76/3.73 ppm to CC_2/CH_6, 103.93/3.71 ppm to
513 DC_2/CH_1, 103.93/3.91 ppm to DC_2/DH_1, 103.95/3.73 ppm to EC_2/CH_6, and 103.96/3.89 ppm to
514 EC_2/EH_6. Thus, the results obtained from HMBC suggest the following sequences: BC_2→ EO_6,
515 CC_2→ CO_6, DC_2→ CO_1, DC_2→ DO_1, EC_2 → CO_6, and EC_2→ EO_6. Finally, the COSY spectrum in Fig.
516 3S (Supporting information) shows cross signals between H_3/H_4; H_4/H_5 and H_5/H_6, in agreement
517 with literature [26].



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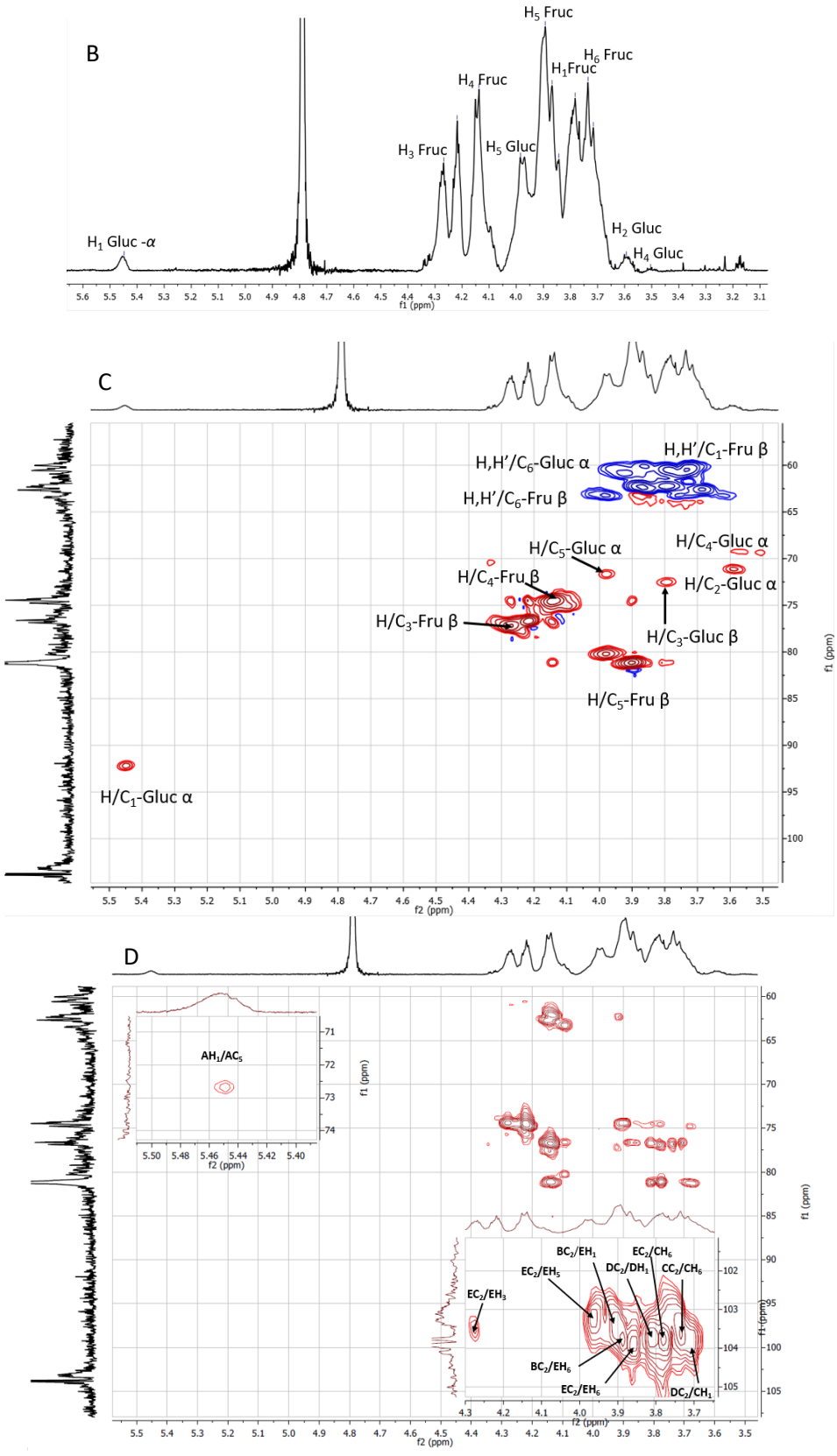

Fig. 3 ^{1}H (A), ^{13}C (B), HSQC (C), and HMBC(D) NMR spectra of OP%US, sample obtained with optimal purity of polysaccharides.

Table 6 ^{1}H and ^{13}C NMR chemical shifts of OP%US

	C_1/H_1	C_2/H_2	C_3/H_3	C_4/H_4	C_5/H_5	C_6/H_6
-d-Glcp-(1 →residue A	92.15 / 5.45	71.18/3.59	72.52/3.79	69.32/3.51	71.67/3.98	61.9/3.74
β-d-Fruf-(2 →residue B	60.55/3.78	103. 11/-	76.57/4.28	74.42/ [a]4.12	81.28/3.92	62.65/3.8
→1,6) β-d-Fruf-(2 →residue C	57.25/3.71	103.76/-	76.98/4.28 [a]	74.34/4.14	80.31/3.88	62.19/3.73
→1) β-d-Fruf-(2 →residue D	60.46/3.91	103.93/-	76.62/4.27	74.46/4.15[a]	81.08/ 3.9	63.37/3.74
→2) β-d-Fruf-(6 →residue E	60.04/3.8	103.95/-	76.85/4.16 [a]	74.77/4.01	80.28/ 3.98	62.31/3.89

[a]Unresolved from other signals.

3.3 Antioxidant activity

3.3.1 ABTS radical scavenging activity

Fig. 4A presents the ABTS radical scavenging activity changes of OP%US as a function of concentration, in comparison with ascorbic acid as a reference. The antioxidant activity of OP%US increases with concentration to reach a maximum of 96.4% at 10 mg. The half maximal inhibitory concentration (IC50) is 1.28 ± 0.13 mg/mL. In the case of ascorbic acid, a very sharp increase of the antioxidant activity is observed with concentration to reach 100% at 0.1 mg/mL. Luo et al. reported a maximum scavenging activity of 28.0% and 82.6% for different fractions of polysaccharides extracted from *Dendrobium nobile Lindl* against $ABTS^{\cdot+}$ at 2 mg/mL [27]. Hu et al. studied the $ABTS^{\cdot+}$ scavenging activity of different fractions of polysaccharides extracted from *Flammulina velutipes.* The maximum activity was 36.7%, 42.4%, and 61.8% at a concentration of 3 mg/mL, with an IC50 value of 2.8 mg/mL [28]. Comparison with literature data suggests that OP%US has a good scavenging activity against $ABTS^{\cdot+}$.

3.3.2 Total antioxidant capacity (TCA)

Mo (VI) is able to combine with proteins at the metal-binding site and to cause DNA and protein damage [29], based on the reduction of Mo (VI) to Mo (V) by the formation phosphate/Mo (V) complex at acidic pH. Fig. 4B presents absorbance changes of OP%US as a function of concentration compared to BHA and α-tocopherol, two commonly used food additives. High absorbance indicates high antioxidant activity. When the concentration increases from 0.025 to 5 mg/mL, the absorption increases from 0.14 to 2.04, from 0.02 to 0.9 and from 0.01 to 1.4 for α-tocopherol, BHA and OP%US respectively. It is noticed that the absorption of OP%US becomes higher than that of BHA beyond 4 mg/mL. These results suggest that OP%US presents a remarkable TCA capacity.

3.3.3 Metal-chelating power

The chelating agents are reported as secondary antioxidants because they can reduce the redox potential, thereby stabilizing the oxidized form of the metal ion [30]. The ferrous chelating capacity was evaluated from absorption measurement at 562 nm. Fig. 4C presents the chelating capacity changes of OP%US as a function of concentration, using EDTA as a reference. The chelating activity of OP%US gradually increases with increasing concentration, reaching a maximum of 79.7 % at 10 mg/mL. The IC50 of OP%US is 3.7 mg/mL. In contrast, the chelating activity of EDTA rapidly increases with concentration to reach 100% at 0.4 mg/mL.

Nobre et al. investigated the chelating activity of various polysaccharides. The authors obtained a maximum chelating activity of 69.9, 57.8, 46.1, and 43.3% for polysaccharides extract from *C. Prolifera, C. Sertularioides*, D. *Cervicornis*, and *D. Mertensis* at 2 mg/ml, respectively [31]. Qi *et al*. determined the chelating activity of sulfated polysaccharides extracted from *Ulva pertusa.* Data show that the maximum activity is 36% and 10% for highly or less sulfated polysaccharides at 2 mg/mL, respectively [32]. Comparison with literature data indicates that OP%US exhibits an acceptable Fe^{2+} chelating activity.

3.3.4 β-carotene bleaching test

Linoleic acid generates peroxide radicals that will oxidize highly unsaturated β-carotene, known as provitamin A which turns from red color to transparency. Antioxidant compounds are able to neutralize free radicals, and thus preventing the oxidation and bleaching of β-carotene [33]. This test is of major importance for human safety as it allows to determine the ability of samples to neutralize lipophilic free radicals that can easily enter human cells causing serious damage to DNA [34]. Two other samples, namely OP M (only maceration) and OP 100%US (only ultrasound), were prepared under the same conditions as OP%US but with or without ultrasound.

Fig. 4D presents the β-carotene bleaching data of the three samples as a function of concentration in comparison with BHA as reference. The results show that the antioxidant activity of OP M, OP%US and OP 100%US are enhanced with increasing concentration from 0.05 to 5 mg/mL. A maximum capacity of 62.5% ,98.1%, and 82.8% are obtained at 5 mg/mL for OP M, OP%US and OP 100%US, respectively. BHA exhibits slightly higher activity to β-carotene bleaching than OP%US in the 0.05 to 0.5 mg/mL concentration range. No significant difference is noticed between OP%US and BHA in the range from 1.0 to 5.0 mg/mL. The IC50 of OP M, OP%US and OP 100%US are 0.82 ± 0.05, 0.131 ± 0.03 and 0.29 ± 0.03 mg/mL, respectively. Chen *et al.,*Guo *et al.* and Shang *et al.* reported that ultrasound treatment enhances the antioxidant activity [18][35]]. Tang *et al.* observed that the antioxidant activity of polysaccharide from *Cyclocarya paliurus* slightly increases with US treatment from 55.5% and

59.0% at 500 µg/mL [36]. OP 100%US presents higher activity compared to OP M, whereas the combined system presents the highest activity compared to the traditional extraction systems.

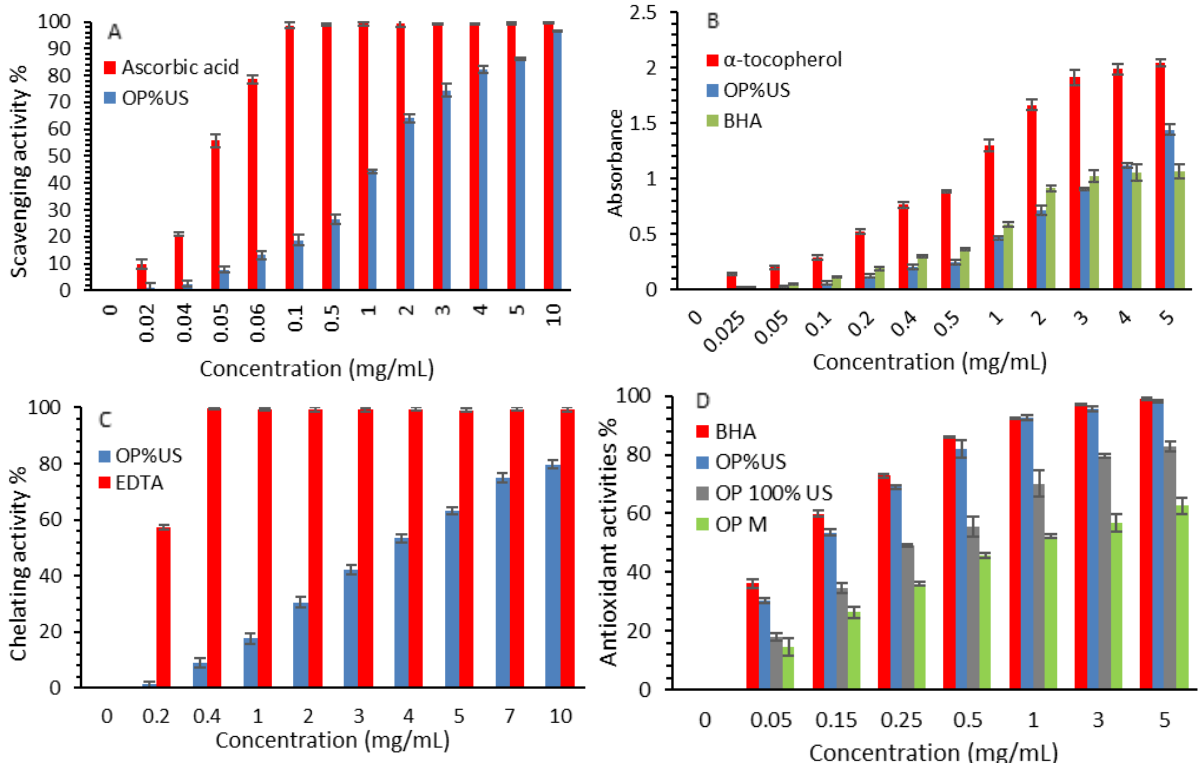

Fig. 4 Antioxidant activity of OP%US against: ABTS radical (A), total antioxidant capacity (B), metal-chelating power (C), β-carotene bleaching (D).

The inhibition activity of different polysaccharides on β-carotene bleaching has been studied by many researchers. Major difference was observed between the reported values. The highest bleaching inhibition rate (82.3%) was reported by Han *et al.* for polysaccharides from *Plantago depressa* at 3.0 mg/mL [37]. Khatua *et al.* reported a maximum inhibition activity of 53% for polysaccharides from *Russula senecis Elicits* at 0.5mg/ml, and an IC50 value of 0.49 mg/mL [38]. In contrast, a value of 13.0 % and 19.3% was obtained by Rukiye *et al.* for polysaccharides from *tragacanth gum* at 20 mg/mL and from locust bean gum at 10 mg/mL, respectively [39]. And Li *et al.* reported an IC50 of 0.14 mg/mL for polysaccharides from *Lycium barbarum fruits* [40]. Interestingly, in comparison with the literature, the extract obtained in the present work displays a more efficient antioxidant activity against lipophilic radicals compared to hydrophilic radicals, suggesting that it could have an anti-cancer activity [34].

4 Conclusions

Optimization of polysaccharides extraction was carried out for the first time via combination of two extraction methods, i.e. maceration and ultrasound treatment by using the Surface

Response Methodology. Four influencing parameters were considered, namely total extraction time, extraction temperature, ratio of water volume to raw material mass, and time percentage of US treatment in the extraction process.

The interaction between parameters and their effects on the extraction yield and polysaccharides yield were investigated. The models to predict the optimal conditions have been validated by additional experiments.

The combination of both extraction methods improves the extraction and polysaccharides yields. The combined system allows to significantly reduce the time of extraction, the temperature and volume to mass ratio, and to improve the purity which is of major importance for potential applications. Finally, the optimum extraction process consists of a maximum duration of 20 min maceration and 20 min US treatment at relatively low temperature (ca. 44°C) using only water as solvent.

The extract is identified as fructo-polysaccharides the RMN analysis indicate that the OP%US possessed a backbone of (2→6)-linked β-d-fructofuranosyl (Fruf), with (2→1)-linked β -d-Fruf branched chains, and terminated with glucose and fructose residues. It presents good antioxidant activities as evidenced by ABTS radical scavenging activity, reducing activity of molybdate, metal-chelating power, and β-carotene bleaching tests. The RSM allowed designing an efficient process to extract polysaccharides from *Ornithogalum billardieri* with remarkable anti-oxidant properties. Additionally, the combined system enhances the anti-lipophilic radical activity of the extract. These natural compounds could be promising as alternative to synthetic antioxidants like BHA for applications in agro-food and pharmaceutical industries.

Conflicts of interest

There are no conflicts of interest to declare.

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References

[1] V. Labrador, P. Fernández Freire, J.M. Pérez Martín, M.J. Hazen, Cytotoxicity of butylated hydroxyanisole in Vero cells, Cell Biol. Toxicol. 23 (2007) 189–199. https://doi.org/10.1007/s10565-006-0153-6.

[2] J.K. Yan, Y.Y. Wang, H. Le Ma, Z. Bin Wang, Ultrasonic effects on the degradation kinetics, preliminary characterization and antioxidant activities of polysaccharides from

646 Phellinus linteus mycelia, Ultrason. Sonochem. 29 (2016) 251–257.
647 https://doi.org/10.1016/j.ultsonch.2015.10.005.
648 [3] R. Chen, F. Meng, Z. Liu, R. Chen, M. Zhang, Antitumor activities of different fractions of
649 polysaccharide purified from Ornithogalum caudatum Ait, Carbohydr. Polym. 80 (2010) 845–
650 851. https://doi.org/10.1016/j.carbpol.2009.12.042.
651 [4] Z. Persin, K. Stana-Kleinschek, T.J. Foster, J.E.G. Van Dam, C.G. Boeriu, P. Navard,
652 Challenges and opportunities in polysaccharides research and technology: The EPNOE views
653 for the next decade in the areas of materials, food and health care, Carbohydr. Polym. 84 (2011)
654 22–32. https://doi.org/10.1016/j.carbpol.2010.11.044.
655 [5] A.I. Gray, Natural Products Isolation, Second, Humana Press Inc., 2006.
656 [6] Z. Hrom, Ultrasonic extraction of plant materials — investigation of hemicellulose
657 release from buckwheat hulls, Ultrason. Sonochem. 10 (2003) 127–133.
658 https://doi.org/10.1016/S1350-4177(03)00094-4.
659 [7] Z. Ying, X. Han, J. Li, Ultrasound-assisted extraction of polysaccharides from mulberry
660 leaves, Food Chem. 127 (2011) 1273–1279. https://doi.org/10.1016/j.foodchem.2011.01.083.
661 [8] Y. Wang, A. Cornea, C. Wang, M. Guo, Effects of Ultrasound Treatment on Extraction
662 and Rheological Properties of Polysaccharides from, Molecules. 24 (2019) 939.
663 https://doi.org/10.3390/molecules24050939.
664 [9] A. Ebringerová, Z. Hromádková, An overview on the application of ultrasound in
665 extraction , separation and purification of plant polysaccharides, Cent. Eur. J. Chem. 8 (2010)
666 243–257. https://doi.org/10.2478/s11532-010-0006-2.
667 [10] S. Wang, X. Dong, J. Tong, Optimization of enzyme-assisted extraction of
668 polysaccharides from alfalfa and its antioxidant activity, Int. J. Biol. Macromol. (2013) 1–10.
669 https://doi.org/10.1016/j.ijbiomac.2013.09.029.
670 [11] M. DuBois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric Method for
671 Determination of Sugars and Related Substances, Anal. Chem. 28 (1956) 350–356.
672 https://doi.org/10.1021/ac60111a017.
673 [12] D. Tang, S. Yu, Y. Ho, B. Huang, G. Tsai, H. Hsieh, Food Hydrocolloids Characterization of
674 tea catechins-loaded nanoparticles prepared from chitosan and an edible polypeptide, Food
675 Hydrocoll. 30 (2013) 33–41. https://doi.org/10.1016/j.foodhyd.2012.04.014.
676 [13] S.A. Mohamed, J.A. Khan, Antioxidant capacity of chewing stick miswak Salvadora
677 persica, Complement. Altern. Med. 19 (2013) 40. https://doi.org/10.1186/1472-6882-13-40.
678 [14] L. Sutharson, P.K. Kar, L.K. Nath, Shila.E.Besra, R.V.S. Joseph, Free radical scavenging
679 activity of leaves of BISCHOFIA JAVANICA BLUME AND FRAXINUS FLORIBUNDA WALLICH,
680 Pharmacologyonline. 1332 (2009) 1324–1332.

681 [15] I.I. Koleva, T.A. Van Beek, J.P.H. Linssen, A. De Groot, L.N. Evstatieva, Screening of Plant
682 Extracts for Antioxidant Activity : a Comparative Study on Three Testing Methods, Phytochem.
683 Anal. 13 (2002) 8–17. https://doi.org/10.1002/pca.611.
684 [16] A. Raza, F. Li, X. Xu, J. Tang, Optimization of ultrasonic-assisted extraction of antioxidant
685 polysaccharides from the stem of Trapa quadrispinosa using response surface methodology,
686 Int. J. Biol. Macromol. 94 (2017) 335–344. https://doi.org/10.1016/j.ijbiomac.2016.10.033.
687 [17] P.F. Jean LEYBROS, Extraction solide-liquide.Aspects théoriques, Tech. l'ingenieur. 3
688 (2019) 2–21.
689 [18] R. Chen, Y. Li, H. Dong, Z. Liu, S. Li, S. Yang, X. Li, Optimization of ultrasonic extraction
690 process of polysaccharides from Ornithogalum Caudatum Ait and evaluation of its biological
691 activities, Ultrason. Sonochem. 19 (2012) 1160–1168.
692 https://doi.org/10.1016/j.ultsonch.2012.03.008.
693 [19] Q. Chen, S.Z. Zhang, H.Z. Ying, X.Y. Dai, X.X. Li, C.H. Yu, H.C. Ye, Chemical characterization
694 and immunostimulatory effects of a polysaccharide from Polygoni Multiflori Radix Praeparata
695 in cyclophosphamide-induced anemic mice, Carbohydr. Polym. 88 (2012) 1476–1482.
696 https://doi.org/10.1016/j.carbpol.2012.02.055.
697 [20] X. Wang, R. Sun, J. Zhang, Y. Chen, N. Liu, Structure and antioxidant activity of
698 polysaccharide POJ-U1a extracted by ultrasound from Ophiopogon japonicus, Fitoterapia. 83
699 (2012) 1576–1584. https://doi.org/10.1016/j.fitote.2012.09.005.
700 [21] K. Zhong, Q. Zhang, L. Tong, L. Liu, X. Zhou, S. Zhou, Molecular weight degradation and
701 rheological properties of schizophyllan under ultrasonic treatment, Ultrason. Sonochem. 23
702 (2015) 75–80. https://doi.org/10.1016/j.ultsonch.2014.09.008.
703 [22] G.L. Ailiesei, M. Ciobanu, M. Balan, C. Stavarache, L. Barbes, A. Nicolescu, C. Deleanu,
704 NMR detected metabolites in complex natural fluids. Quinic acid in apple juice, Ovidius Univ.
705 Ann. Chem. 26 (2015) 51–56. https://doi.org/10.2478/auoc-2015-0009.
706 [23] J. Liu, A.L. Waterhouse, N.J. Chatterton, Proton and carbon NMR chemical-shift
707 assignments for [β-d-Fruf-(2 → 1)]3-(2 ↔ 1)-α-d-Glcp (nystose) and [β-d-Fruf-(2 → 1)]4-(2 ↔
708 1)-α-d-Glcp (1,1,1-kestopentaose) from two-dimensional NMR spectral measurements,
709 Carbohydr. Res. 245 (1993) 11–19. https://doi.org/10.1016/0008-6215(93)80056-K.
710 [24] C. Wang, D. Hua, C. Yan, Structural characterization and antioxidant activities of a novel
711 fructan from Achyranthes bidentata Blume, a famous medicinal plant in China, Ind. Crops Prod.
712 70 (2015) 427–434. https://doi.org/10.1016/j.indcrop.2015.03.051.
713 [25] L. Zhang, N. Reddy, C. Soo Khoo, S. Rao Koyyalamudi, C.E. Jones, Antioxidant and
714 Immunomodulatory Activities and Structural Characterization of Polysaccharides Isolated from

715 Lobelia chinensis Lour, Pharmacologia. 9 (2018) 157–168.
716 https://doi.org/10.5567/pharmacologia.2018.157.168.
717 [26] J. Chen, K.L. Cheong, Z. Song, Y. Shi, X. Huang, Structure and protective effect on UVB-
718 induced keratinocyte damage of fructan from white garlic, Carbohydr. Polym. 92 (2013) 200–
719 205. https://doi.org/10.1016/j.carbpol.2012.09.068.
720 [27] A. Luo, X. He, S. Zhou, Y. Fan, A. Luo, Z. Chun, Purification , composition analysis and
721 antioxidant activity of the polysaccharides from Dendrobium nobile Lindl ., Carbohydr. Polym.
722 79 (2010) 1014–1019. https://doi.org/10.1016/j.carbpol.2009.10.033.
723 [28] L. Cell, Characterization and Antioxidant Activities of Yellow Strain Flammulina velutipes
724 (Jinhua Mushroom) Polysaccharides and Their E ff ects on ROS Content in, Antioxidants. 8
725 (2019) 1–15. https://doi.org/10.3390/antiox8080298.
726 [29] William Pratt, Transformation of Glucocorticoid and Progesterone Receptors to the
727 DNA-Binding State, J. Cell. Biochem. 68 (1987) 51–68.
728 [30] K. Pavithra, S. Vadivukkarasi, Evaluation of free radical scavenging activity of various
729 extracts of leaves from Kedrostis foetidissima (Jacq .) Cogn ., Food Sci. Hum. Wellness. 4 (2015)
730 42–46. https://doi.org/10.1016/j.fshw.2015.02.001.
731 [31] L.S. Costa, L.T.D.B. Nobre, G.P. Fidelis, S.L. Cordeiro, R.M. Oliveira, D.A. Sabry, R.B.G. Ca,
732 M.S.S.P. Costa, E.H.C. Farias, E.L. Leite, H.A.O. Rocha, Biological activities of sulfated
733 polysaccharides from tropical seaweeds, Biomed. Pharmacother. 64 (2010) 21–28.
734 https://doi.org/10.1016/j.biopha.2009.03.005.
735 [32] H. Qi, Q. Zhang, T. Zhao, R. Chen, Q. Huimin, Z. Quanbin, Z. Tingting, C. Rong, Gz. Hon,
736 N. Xizhen, L. Zhien, Antioxidant activity of different sulfate content derivatives of
737 polysaccharide extracted from Ulva pertusa (Chlorophyta) in vitro, Int. J. Biol. Macromol. 37
738 (2005) 195–199. https://doi.org/10.1016/j.ijbiomac.2005.10.008.
739 [33] L. Unten, M. Koketsu, M. Kim, Antidiscoloring Activity of Green Tea Polyphenols on -
740 Carotene, J. Agric. Food Chem. 8561 (2012) 1–3.
741 [34] G. Amandine, Plantes médicinales et antioxydants, TOULOUSE III PAUL SABATIER, 2016.
742 [35] X. Guo, X. Shang, X. Zhou, B. Zhao, J. Zhang, Ultrasound-assisted extraction of
743 polysaccharides from Rhododendron aganniphum: Antioxidant activity and rheological
744 properties, Ultrason. Sonochem. 38 (2017) 246–255.
745 https://doi.org/10.1016/j.ultsonch.2017.03.021.
746 [36] W. Tang, L. Lin, J. Xie, Z. Wang, H. Wang, Y. Dong, M. Shen, M. Xie, Effect of ultrasonic
747 treatment on the physicochemical properties and antioxidant activities of polysaccharide from
748 Cyclocarya paliurus, Carbohydr. Polym. 151 (2016) 305–312.
749 https://doi.org/10.1016/j.carbpol.2016.05.078.

[37] N. Han, L. Wang, Z. Song, J. Lin, C. Ye, Z. Liu, J. Yin, International Journal of Biological Macromolecules Optimization and antioxidant activity of polysaccharides from Plantago depressa, Int. J. Biol. Macromol. 93 (2016) 644–654. https://doi.org/10.1016/j.ijbiomac.2016.09.028.

[38] S. Khatua, K. Acharya, Water Soluble Antioxidative Crude Polysaccharide From Russula senecis Elicits TLR Modulated NF- κ B Signaling Pathway and Pro-inflammatory Response in Murine Macrophages, Front. Pharmacol. 9 (2018) 1–11. https://doi.org/10.3389/fphar.2018.00985.

[39] R. Boran, A. Ugur, N. Sarac, Investigation of Hyaluronidase, Collagenase and Elastase Inhibitory Potentials and Comparative Evaluation of the Antimicrobial, Antioxidant and Homeostatic Activities of Two Natural Polysaccharides, J. Nat. Appl. Sci. 22 (2018) 1182–1189. https://doi.org/10.19113/sdufenbed.471994.

[40] X.M. Li, X.L. Li, A.G. Zhou, POLYMER Evaluation of antioxidant activity of the polysaccharides extracted from Lycium barbarum fruits in vitro, Eur. Polym. J. 43 (2007) 488–497. https://doi.org/10.1016/j.eurpolymj.2006.10.025.