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Optimization of polysaccharide extraction from olive leaves and evaluation of its antioxidant and rheological properties

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Abstract

Central composite design response surface methodology was used to optimize polysaccharide extraction from olive leaves. Effect of three independent variables [extraction time (3- 7 hours), extraction temperature (60- 100°C) and water-to-raw material ratio (5-25 mL/g)] on extraction yield were studied. Extracted polysaccharide was evaluated for antioxidant properties, total phenolic and flavonoid content and its structure and functional groups were studied using FTIR. Rheological properties and flow behavior of polysaccharide were determined by fitting to power law model. The most important parameter in experimental ranges was temperature and the lowest effect was seen in extraction time. Highest extraction yield was obtained at extraction time of 2 hours, extraction temperature of 80.96°C and water-to-raw material ratio of 17.94 mL/g. Antioxidant properties of extracted polysaccharide were measured using DPPH radical at 517 nm that showed notable antioxidant properties. Rheological property of extracted polysaccharide was studied at 1, 2.5 and 5% concentration. Results showed that at high concentration, polysaccharide shows shear thinning behavior. One of the most important obstacles in native polysaccharide applications is their extraction yield. Extract of olive leaf polysaccharide is highly affected by extraction temperature. Extracted polysaccharide showed good antioxidant properties comparing to BHT and phenolic extract of olive leaf. Moreover it could be used for increasing solution viscosity at higher concentrations.

Keywords: Olive leaf polysaccharide, optimization, response surface methodology, FTIR, antioxidant properties

Introduction

Bioactive compounds have gained more attention especially in the last decade due to their health benefits and functional properties. Bioactive compound extraction usually is done using maceration techniques. Recently some supplementary techniques such as ultrasonic assisted, microwave assisted, supercritical extraction and superheated liquid are used to reduce extraction time and increase bioactive substance extraction yield (Ahmad-Qasem *et al.*, 2013).

Bioactive polysaccharides from natural sources are recently gained attention in biochemistry and pharmacology due to their beneficial activities including antioxidant, anti-tumor/ anticancer, anticoagulant and immune stimulating properties (Tadayoni *et al.*, 2015; Tahmouzi and Ghodsi, 2014). They may have several applications in food industry including, increasing viscosity in different concentrations or synergistic effect with other native biopolymers.

Olive (*Olea europaea* L.) is an evergreen tree which is native to Mediterranean area. Olive fruit is mostly used for preparing oil and table olives (Galanakis, 2011). Olive oil due to presence of functional bioactive compounds like tocopherols, carotenoids and phenolic compounds is considered as health promoting compound. Phenolic compounds of olive leaves have showed antioxidant, anti-hypertensive and anti-inflammatory properties (Ahmad-Qasem *et al.*, 2016). Olive leaf extract is traditionally used for treating fever, malaria, colic, alopecia, paralysis, rheumatism, gout, sciatica, hypertension, arrhythmia, diabetes and cancer (Kamran *et al.*, 2015).

Water soluble polysaccharide extraction usually is done by extracting soluble parts using warm or hot water and finally precipitating extracted polysaccharide using alcohol.

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Response surface methodology (RSM) is a collection of mathematical and statistical techniques that used for understanding a complex system performance and optimization of process output (Mazarei *et al*., 2017). In addition to mathematical optimization, RSM reduces number of experimental runs and consequently decreases time and costs of experiments. In RSM, central composite rotatable design, central composite design and Box- Behnken design using a least square technique, fit a second order polynomial equation to estimate effect of independent variables on output (Samavati and Manoochehrizade, 2013).

In this study at first stage, the effects of three independent variables of temperature, time and water to raw material ratio on extraction yield of olive leaf soluble polysaccharides were evaluated. At the second stage, antioxidant properties of the extracted polysaccharide and its rheological properties were determined.

Materials and Methods

Olive leaves were collected from Khuzestan Agricultural sciences and Natural Resources University garden (Mollasani, Iran). 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from *Sigma* Chemical Company (St. Louis, MO, USA). Analytical grade ethanol and methanol were used for decolorization, polysaccharide sedimentation and DPPH test. All other chemicals including Folin Ciocalteau reagent, Aluminum Chloride, Gallic Acid and Quercetin were purchased from Merck Chemical Co (Darmstadt, Germany)

Polysaccharide extraction

Olive leaves were cleaned and dried in a drying oven at 105°C for 3 hours and after cooling to room temperature, the dried leaves were kept in plastic bags at -18°C until the day of experiments. Dried leaves were ground to 0.05 mm particles. 80% ethanol was added to dried leaf and the mixture was stirred for 8 hours at room temperature to remove fat and color. The mixture was then filtered through nylon filters and residues on filter were washed three times using ethanol and the filtrate was dried at 50°C.

Defatted powder was used for polysaccharide extraction by hot water at different temperatures, times and water to dried matter ratios based on RSM design. To remove suspended particles, the extract was centrifuged at 6000 rpm for 15 minute. Extracted soluble polysaccharide was sedimented by adding 3 volume 96% ethanol and keeping for 48 hours at 4°C. Precipitated polysaccharide was removed by centrifugion at 6000 rpm for 20 minutes and the pellet was dried at 50°C. Polysaccharide Extraction yield (%w/w) was calculated using following formula:

$$\text{Yield Extraction \%} = (W_{DP} / W_{DR}) \times 10 \qquad (1)$$

W_{DP} is dried extracted polysaccharide and W_{DR} is dried raw material.

Experimental design

After pretests the range of three extraction variables including X_1, extraction temperature; X_2 extraction time and X_3, water-to-raw material ratio were introduced to the software (Design Expert 9) based on central composite design (Table 1). Variables were coded according to following equation:

$$x_i = (X_i - X_o/\Delta X_i) \qquad (2)$$

Where x_i is coded value of variable; X_i actual value of variable; X_0 actual value at the center point and ΔX_i is the step change of actual value

20 experiments were designed using the software that based on non-linear quadratic model as given by equation 3

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 \square b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 \qquad (3)$$

While (Y) is extraction yield (b_0) is polynomial coefficient represent by constant term, (b1, b2 and b_3) are coefficient of linear effects, (b11, b22 and b_{33}) are coefficient of quadratic effect and interactions (b_{12}, b_{13} and b_{23}).

Regression coefficient (R^2), adjusted-R^2

(R2adj), adequate precision (AP), the prediction error sum of squares (PRESS) and coefficient of variation (CV) were used to evaluate adequacy of model.

Table 1. Independent variables and their levels

Independent variable	Factor levels				
	-2 (-α)	-1	0	1	2 (+α)
Extraction temperature	60	70	80	90	100
Extraction time	3	4	5	6	7
Water ratio	5	10	15	20	25

Olive leaf composition

Fresh leaves of olive were used to determine moisture, ash, protein, fat and total carbohydrate.

Antioxidant activity

Radical scavenging of extracted polysaccharide was measured based on its ability to scavenge DPPH radicals. Briefly 3 mL of different concentrations of polysaccharide was added to 1 mL methanolic solution of DPPH (0.004%) and kept 30 minute in dark place at room temperature. Aliqout absorbance was measured at 517 nm and radical scavenging activity was obtained as follow (Shen *et al.*, 2014):

$$RSA\% = \frac{Abs\ of\ blank - Abs\ of\ sample}{Abs\ of\ blank} \times 100 \quad (4)$$

Results were compared with ethanolic extract of olive leaf and BHT as synthetic antioxidant.

Total phenolic content

Total phenolic content was measured using Folin Ciocalteu colorimetric method. One mL of 0.1 mg/mL of polysaccharide solution was mixed with 2.5 mL Folin Ciocalteu reagent (1:10 dilution) and kept at room temperature for 2 minute. Two mL of sodium carbonate solution (7.5% in deionized water) was then added to the mixture and after 1 hour incubation at room temperature, absorbance was measured at 725 nm using UV-Vis spectrophotometer and result expressed as Gallic acid equivalent (GAE) (Scherer *et al.*, 2013).

Flavonoid content

Total flavonoid content was measured based on formation of flavonoid compounds and aluminum complex. One mL of 0.1 mg/mL polysaccharide solution diluted with 2.5 mL distilled water and 75 µl of 5% sodium nitrite was then added to diluted solution. After 6 minute, 150 µl Aluminum chloride (10%, pH=5.8) was added and after 5 minute, 1 mL NaOH (1M) added to the mixture and the absorbance was measured at 510 nm. Results expressed as Quercetin Equivalent (QE) (Hossain and Rahman, 2011).

Fourier transform infrared spectroscopy (FTIR)

FTIR spectrometer was used to perform infrared scanning on extracted polysaccharide to determine functional groups. Infrared scan preformed in range of 4000-400 cm^{-1} using KBr pellet method with sample to KBr ratio of 1:100.

Rheological properties of polysaccharide

To evaluate rheological properties of extracted polysaccharide, its flow properties was measured. Rheological properties of three concentrations of 1, 2.5 and 5% were measured at room temperature using rheometer equipped with spindle no. 18 (LVDV Pro II, Brookfield Engineering Laboratories, USA)

Statistical analysis

All measurements were done in three replicates and their mean values were compared using Duncan test at 5% significant levels. Statistical analysis was performed using SPSS 16.

Results and discussion

Chemical composition olive leaf

Results of chemical composition of olive leaf are shown in Table 2.

The average moisture content of olive leaves was 55% and it could be categorized as an intermediate moisture product. In other researches olive leaf moisture content was reported 39-64%. Total carbohydrate was measured by subtracting other chemical constituents from leaves weight. Reported total carbohydrate for different varieties was 11-43% (Boudhrioua *et al.*, 2009; Cavalheiro *et al.*, 2015; Kamran *et al.*, 2015).

Table 2. Average composition of olive leaf

Constituent	composition (g/100g)
Moisture	55.17± 0.99
Ash	6.12± 0.11
Fat	3.18± 0.075
Protein	8.21± 0.32
Water soluble carbohydrate	8.58± 0.397
Insoluble carbohydrate	18.74± 0. 53
Total carbohydrate	27.32± 0.86

Single factor effect on olive leaf polysaccharide extraction

To evaluate the effect of single factors of temperature, time and water-to-raw material ratio on polysaccharide extraction yield, two factors were kept constant and at the middle point and the effect of one factor was determined.

Results (Fig. 1) showed that temperature and water-to-raw material ratio had the highest influence on polysaccharide extraction and the lowest effect belonged to extraction time. By increasing temperature (Fig. 1A) from 60-100°C, at fixed time of 5 hours and water-to-raw material ratio of 15, extraction yield increased 59%.

In water-to-raw material range of 5 to 25 (Fig. 1C), at fixed point of 80°C and extraction time of 5 hours, extraction yield increased 50% but in 3-7 hours extraction (Fig. 1B) at fixed temperature of 80 °C and water-to-raw material ratio of 15, extraction yield just increased 11.8%. Increasing polysaccharide extraction by increasing temperature may be due to higher solvation and increasing diffusion coefficient. For industrial use it is important to find a balance between high temperature causing higher cost for extraction and other extraction parameters. Highly positive effect of water-to-raw material ratio may be due to higher concentration gradient of polysaccharide which improves polysaccharide extraction. The most important point is the weak effect of extraction time on polysaccharide extraction that could be very precious in industrial extraction of polysaccharide.

Predicted model and statistical analysis

Response surface methodology advantageous over single parameters or factorial design is saving time and costs. Twenty runs containing 5 central point were designed by software in a central composite rotatable design. Experiment variables and final data of extraction yield are showed Table 3.

Software using experimental results introduced a quadratic equation based on multiple regression analysis.

$$Y = +18.56 - 0.4\,X_1 + 0.64\,X_2 - 0.33X_3 - 0.039X_1X_2 + 0.004\,X_1X_3 + 0.04\,X_2X_3 + 0.003\,X_1^2 + 0.21X_2^2 - 0.003X_3^2 \qquad (5)$$

To evaluate model adequacy and determine significant factors, analysis of variance (ANOVA) was performed (Table 4). The important thing is that lack of fit model was significant which may be due to some noises but models R^2 and adjusted R^2 was 0.94 and 0.88 respectively that means it reasonably fits on data. Adequate precision shows signal to noise ratio and ratio greater than 4 is desirable. Adequate precision for the model was 16.53. CV of model was 5.91 that indicates high degree of model precision. All statistical parameters except lack of fit show reliability of model to predict data trend

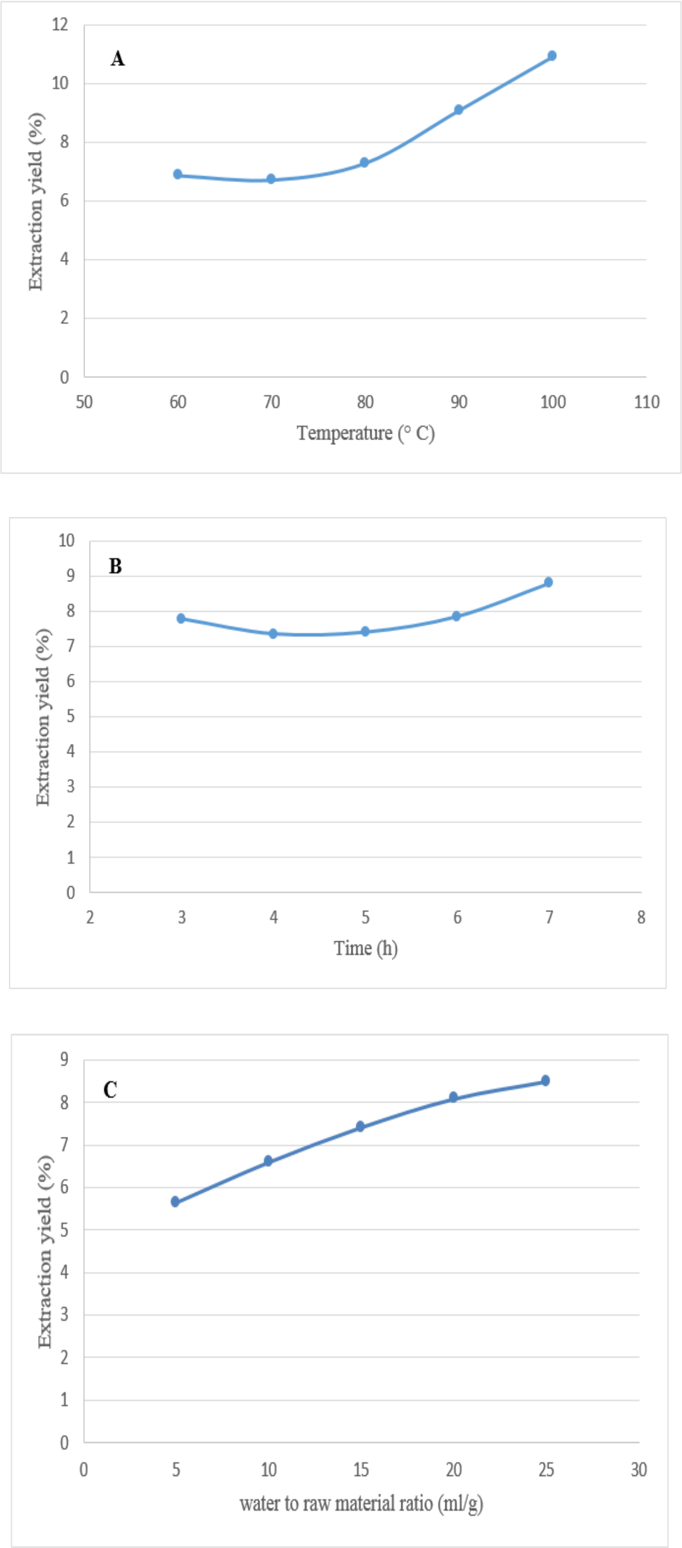

Fig. 1. Effects of temperature, time and water-to-raw material ratios on extraction yield

Table 3. Response surface central composite design and extraction yield

Std order	Run order	X1 (°C)	X2 (h)	X3 (ratio)	Extraction yield (%)		
					Actual	predicted	Residual
8	1	90	6	20	10.32	10.00	0.32
11	2	80	3	15	7.28	7.80	-0.52
18	3	80	5	15	7.32	7.42	-0.10
12	4	80	7	15	8.58	8.75	-0.17
7	5	70	6	20	8.25	8.23	0.019
14	6	80	5	25	8.37	8.55	-0.18
20	7	80	5	15	7.41	7.42	-0.014
1	8	70	4	10	6.36	6.00	0.36
17	9	80	5	15	7.29	7.42	-0.13
15	10	80	5	15	7.1	7.42	-0.32
2	11	90	4	10	9.09	8.42	0.67
3	12	70	6	10	7.2	6.85	0.35
10	13	100	5	15	10.63	11.11	-0.48
5	14	70	4	20	6.92	6.56	0.36
4	15	90	6	10	8.02	7.70	0.32
16	16	80	5	15	7.32	7.42	-0.10
19	17	80	5	16	7.42	7.42	-4.091E-003
6	18	90	4	20	10.23	9.90	0.33
9	19	60	5	15	6.71	6.92	-0.21
13	20	80	5	5	5.18	5.69	-0.51

Table 4. Analysis of variance of fitted model

Source	Sum of square	df	Mean Square	F- Value	Probability>F
Model	33.95	9	3.77	17.54	< 0.0001
A-temp	17.58	1	17.58	81.75	< 0.0001
B-time	0.90	1	0.9	4.18	0.0683
C-ratio	8.17	1	8.17	37.97	0.0001
AB	1.24	1	1.24	5.77	0.0372
AC	0.42	1	0.42	1.95	0.1931
BC	0.34	1	0.34	1.58	0.2370
A^2	3.96	1	3.96	18.43	0.0016
B^2	1.13	1	1.13	5.26	0.0448
C^2	0.15	1	0.15	0.69	0.4262
Residual	2.15	10	0.22		
Lack of Fit	2.08	5	0.42	31.19	0.0009
Pure Error	0.067	5	0.013		
Core total	36.10	19			

An important part of optimization is deleting insignificant parameters from model which can lower time and cost of the method. P-value is used to evaluate significance of model terms. In model proposed for extraction yield of polysaccharide linear terms of temperature and ratio were significant and time of extraction with p-value of 0.0683 was insignificant that shows its slight effect on extraction yield. For quadratic terms temperature and time highly influenced extraction model and were significant, but ratio was insignificant. For interaction terms just temperature × time was significant.

Contour plots shows combination effect of independent variables on extraction yield. It is clear that extraction time of 2- 7 hour has the lowest effect on extraction yield and extraction temperature has the highest effect. Low effect of extraction time may be due to high solubility of olive leaf polysaccharide and consequently exiting major parts within 2 hours.

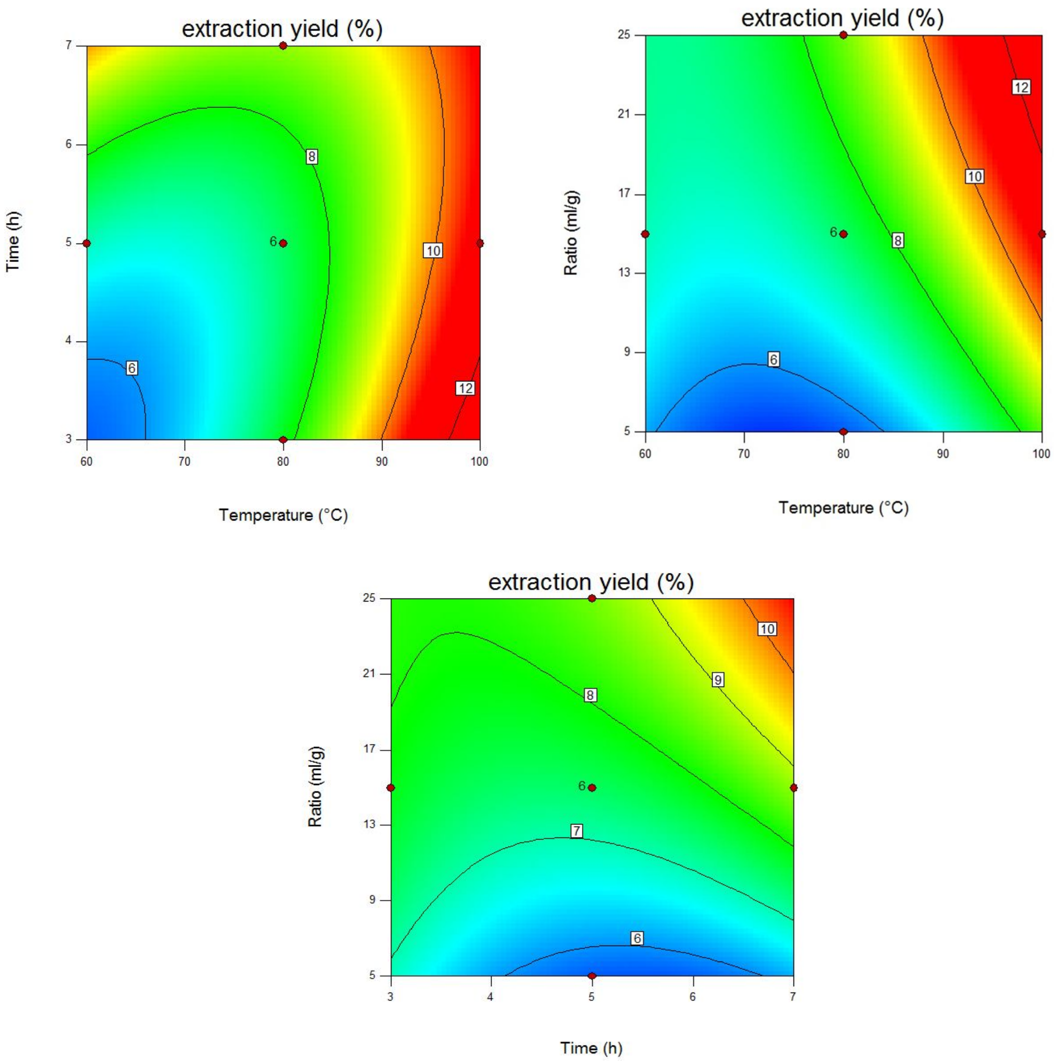

Fig. 2. Contour plots of independent variables interaction

Verification of model

To verify model ability to maximize the response, in optimization section of software two conditions of temperature and water-to-raw material ratio were introduced to evaluate their effect and importance (Table 5). In first scenario, temperature and ratio were defined in range, time set on minimum and our goal was defined for maximizing the yield with highest importance.

In second scenario ratio was defined in range and temperature and time were set on minimum and the goal was defined to be minimized with highest importance.

Table 5. Optimization conditions and predicted extraction yield

	Optimized conditions			Extraction yield (%)	
	Temperature (°C)	**Time (h)**	**ratio**	**predicted**	**actual**
1st scenario	89.87	2	19.37	11.43	9.74± 0.27
2nd scenario	80.96	2	17.94	8.88	8.27± 0.15

Results showed that based on optimization conditions, proposed model can predict extraction parameters to maximize extractions. At high temperature and water-to-raw material ratio predicted extraction was 11.43%, but in experimental conditions extraction yield was 9.74%.

Antioxidant properties of extracted polysaccharide

DPPH radical was used to monitor antioxidant of extracted polysaccharide. Ability of polysaccharide for donating electron to free radical of DPPH and decreasing violet color of DPPH solution to yellow color was compared to synthetic antioxidant of BHT and ethanolic extract of olive leaf (Figure 3).

Results showed good antioxidant properties and concentration dependent activity of extracted polysaccharide. IC_{50} for extracted polysaccharide was 250 µg/mL, comparing to 130 and 53 µg/mL for ethanolic extract and BHT respectively. Acceptable scavenging activity of polysaccharide is due to high proton donating activity that may be attributed to polyphenol or tannin extraction with polysaccharide (Tadayoni *et al.*, 2015).

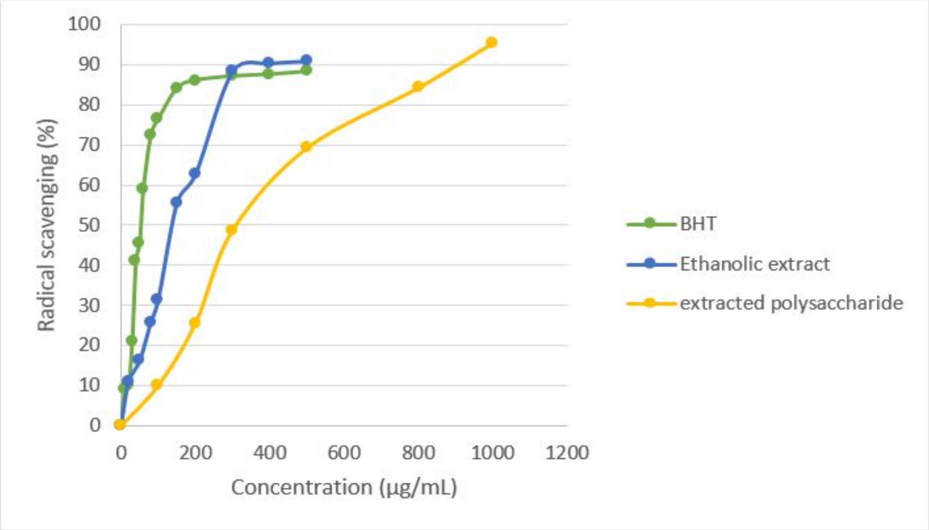

Fig. 3. Radical scavenging activity of extracted polysaccharide

Total phenol and flavonoid content

Total phenolic content of extracted polysaccharide and ethanolic extract of olive leaves are in compatible with their antioxidant properties. Total phenol and flavonoid contents were 57.9 and 36.66 mg GAE/mL and 1127 and 926 µg QE/mL for ethanolic extract and extracted polysaccharide respectively. Results showed that in extraction process of polysaccharide, some parts of phenolic compounds were entered in extracted polysaccharide.

Olive leaf polysaccharide structure by FTIR

Extracted polysaccharide structure and functional group was studied by FTIR spectroscopy based on vibrations at molecular state. In FTIR spectrum (Fig. 4) of polysaccharides C-H stretching was seen at 2925 cm^{-1} band. Observed band at 3376 cm^{-1} attributed to O-H group that usually is seen at 3200- 3500 cm^{-1}. Absorption peak at 890 cm^{-1} could be attributed to β-D-Glucose or β-D-Galactose (Yang and Zhang, 2009). Broad peaks in the 950-1200 cm^{-1} range indicate polysaccharide as major component in the extract (Azmi *et al.*, 2012). Bands at 1415 and 1272 cm^{-1} belong to COH bending groups and Absorption at 1077 cm^{-1} indicate pyranose form of sugar (Luo *et al.*, 2010).

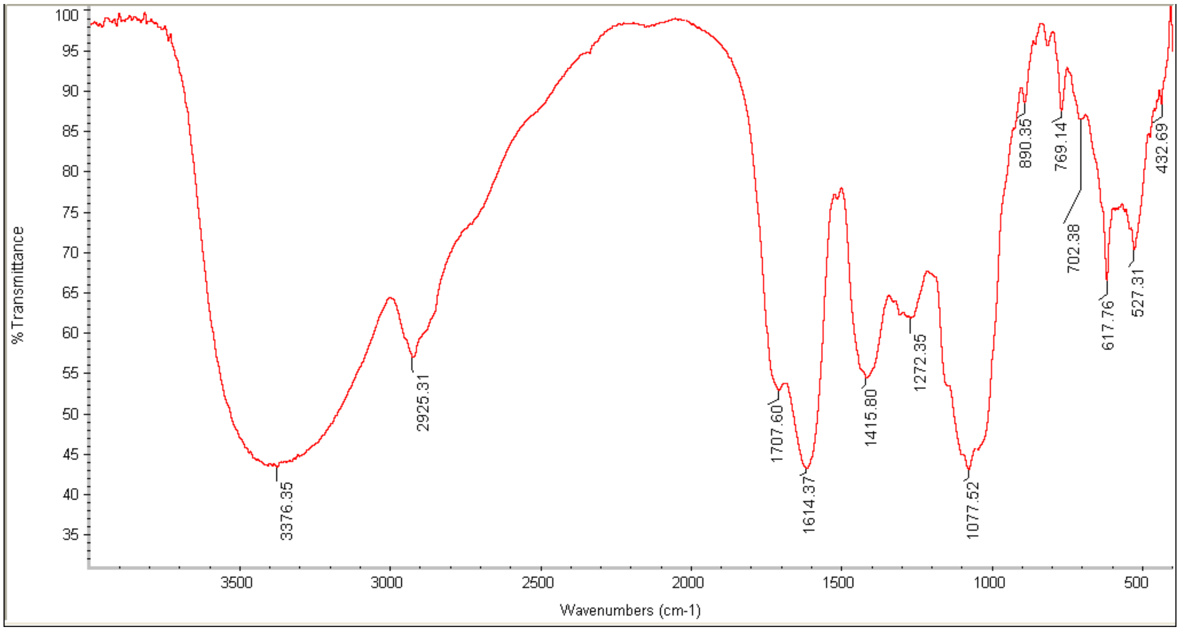

Fig. 4. FTIR spectra for olive leaf polysaccharide

Rheological properties

One of the most used applications of extracted polysaccharides is its usage in increasing solutions viscosity. At low concentrations of polysaccharide, it showed mostly Newtonian behavior (Fig 5) but at higher concentration it showed slightly shear thinning behavior. Flow behavior of extracted polysaccharide was evaluated in 1, 2.5 and 5% concentrations by fitting power law model on shear rate, shear stress data. Results showed that consistency coefficients were 1.44, 2.52 and 3.11 Pa.s and flow behavior indices were 0.985, 0.898 and 0.89 for 1, 2.5 and 5% concentrations respectively. Flow behavior index less than 1 in all concentrations shows shear thinning behavior of polymer solutions (Mehrnia *et al*., 2017).

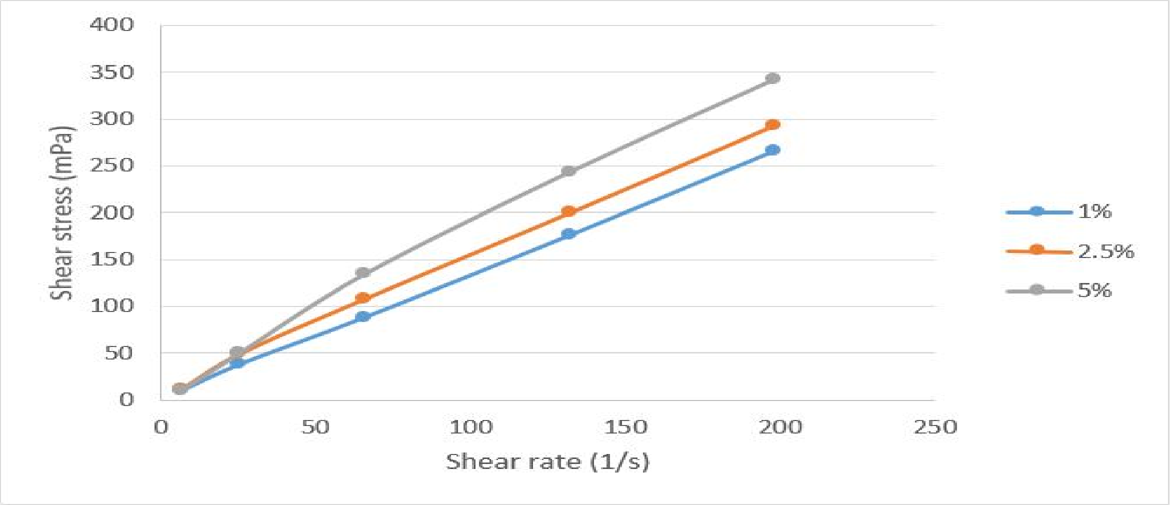

Fig. 5. Rheological properties of olive leaf polysaccharide

Conclusions

Native and extracted polysaccharides could be used as good substitutes for commercial polysaccharide. In this research the effects of temperature, time and water-to-raw material ratio on extraction yield were evaluated. Results showed that temperature has the highest and extraction time the lowest effect on extraction yield. Antioxidant properties of polysaccharide were notable comparing synthetic antioxidant of BHT and compatible with total phenol and flavonoid content of polysaccharide. Rheological properties and flow behavior of extracted polysaccharide showed shear thinning behavior at high concentration. It is concluded that extracted polysaccharide of olive leaf could be used as low cost biopolymer with high antioxidant and good rheological properties.

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بهینه‌سازی استخراج پلی‌ساکارید از برگ زیتون و ارزیابی ویژگی‌های رئولوژیکی و آنتی‌اکسیدانی آن

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چکیده

طرح مرکب مرکزی روش سطح پاسخ برای بهینه‌سازی استخراج پلی‌ساکاریدهای برگ زیتون مورد استفاده قرار گرفت. اثر سه متغیر مستقل زمان استخراج (7-3 ساعت)، دمای استخراج (60- 100 درجه سانتی‌گراد) و نسبت آب به ماده خشک (5- 25 ml/g) برر روی بازده استخراج مورد بررسی قرار گرفت. پلی‌ساکارید استخراج شده برای ویژگی‌های آنتی‌اکسیدانی، میزان کل ترکیبات فنلی و فلاوونوئیدی و ساختار و گروه‌های فعال با FTIR بررسی شد. ویژگی‌های رئولوژیکی و رفتار جریان با برازش بر روی مدل توان تعیین شد. مهمترین پارامتر در دامنه‌های مورد آزمایش دما و کم اثرترین در مورد زمان استخراج مشاهده شد. بالاترین بازده استخراج در شرایط 2 ساعت، دمای 80/96 درجه سانتی‌گراد و نسبت آب به ماده خشک 94/17 ml/g مشاهده شد. ویژگی‌های آنتی‌اکسیدانی پلی‌ساکارید استخراج با استفاده از رادیکال DPPH در طول موج 517 نانومتر بررسی شده که نشان‌دهنده ویژگی‌های آنتی‌اکسیدانی قابل توجه نمونه بود. ویژگی‌های رئولوژیکی پلی‌ساکارید استخراجی در غلظت‌های 1، 2/5 و 5% بررسی شد. نتایج نشان داد که در غلظت‌های بالا رفتار از نوع شل‌شونده می‌باشد. یکی از مهم‌ترین مشکلات در ارتباط استخراج پلی‌ساکاریدهای بومی بازده استخراج آنها می‌باشد. پلی‌ساکارید استخراجی ویژگی‌های آنتی‌اکسیدانی خوبی در مقایسه با BHT و عصاره فنلی برگ زیتون نشان داد. علاوه بر این پلی‌ساکارید استخراجی می‌تواند برای افزایش ویسکوزیته محلول در غلظت‌های بالا مورد استفاده قرار گیرد.

واژه‌های کلیدی: پلی‌ساکارید برگ زیتون، بهینه‌سازی، روش سطح پاسخ، FTIR، ویژگی‌های آنتی‌اکسیدانی

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