Antioxidant and antiglycated activities of polysaccharides from Tunisian date seeds (*Phoenix dactilyfera L.*)

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Abstract: The extraction and purification of polysaccharides from date seeds (*Phoenix dactilyfera L.)*, as well as the evaluation of their antioxidant and antiglycated activities *in vitro* were investigated.
Aqueous and oxalate extracts (PPW and PPO) were obtained by sequential extraction from date seeds powder. Sugar contents and macromolecular characteristics were estimated by colorimetric assays and size exclusion chromatography (SEC/MALS/VD/DRI).
The best yield was obtained for aqueous extract PPW (1.9%). These experiments showed that all extracted samples were polysaccharides having a moderately high molecular weight (1680 and 1200 kDa for PPW and PPO, respectively). Preliminary antioxidant and antiglycated tests indicated that PPW showed even better ability in the scavenging of DPPH• and NO• radicals and the inhibition of lipid peroxidation and protein glycation.
These findings suggest that water-soluble polysaccharides from date seeds could be explored as free radical scavengers and protein glycation inhibitors.

Keywords: *Phoenix dactilyfera L.*, date seeds, polysaccharide, antioxidant activitiy, antiglycated activity.

INTRODUCTION

The date palm is a suitable species to the Saharan climate [1]. It has always played an important part in the economic and social lives of the people of arid and semi-arid regions of the world [2].
In Tunisia, the production was estimated to be equal to 7.6 million tons in 2014 and 755 thousand tons of date seeds were approximately produced and could be used as alternative source of polysaccharide [3]. Generally, the pits date could be used as a soil organic addition, a feed for various livestock [4] and an additive to animal feed to enhance growth. Moreover, they are a suitable wastewater biosorbents under specific conditions [5]. The seeds powder is also used in some traditional medicines and has been investigated for human potential health benefits [6].
Date seeds could be used in cosmetics (manufactured into soap) [7], pharmaceuticals [8] and food [9] such as mayonnaise [10] and coffee powder [11].
In date pits, the percentage of non-reducing sugars, glucose and fructose are 3.82%, 1.68% and 1.53%, respectively [11]. In terms of dry weight, it has been reported that date pits contain: 5-10% moisture, 1-2% ash, 5-7% protein, 7-10% oil, 10-20% crude fiber and 55-65% carbohydrates (cellulose, hemicelluloses, water soluble polysaccharide…) [12]. Studies indicate that the aqueous extracts of dates have potent antioxidant activity [13]. The antioxidant activity is attributed to the wide range of phenolic compounds in dates including p-coumaric, ferulic and sinapic acids, flavonoids and procyanidins [14-16]

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Synthetic antioxidants are included in various foods; however their safety can be doubted. For this reason, the demand of natural antioxidants has grown over the recent years. Therefore, the importance of replacing synthetic antioxidants with natural compounds has greatly increased [17].

Over the past few years, the contribution of advanced glycation end products for diabetes and aging has received considerable attention. The modification of proteins by glucose through the glycation process leads to the formation of advanced glycation end products (AGEs). The pathologic effects of AGEs are related to their ability to generate reactive oxygen species in addition to auto-oxidation reactions yielding radicals and other reactive intermediates. Therefore, more attention has been paid to polysaccharides with antioxidant potentials that may retard the process of AGEs formation by preventing further oxidation of glycation products [18,19].

Date seeds contain substantial amounts of secondary metabolites, mainly phenolic compounds [20]. Given the lack of studies establishing the identity of the constitutive aqueous compounds of date pits. In fact, the purpose is to improve the valorization of date pits, and prove it as a potential tool for botanical and geographical discrimination, to determine the aqueous content and to evaluate the potential antioxidant and antiglycated activities of the date pits extracts.

The purpose of the present investigation is to study the structural proprieties of polysaccharides extracted from date seeds such as molecular features and FT-IR spectroscopy and to elucidate their antioxidant and antiglycation activities.

MATERIALS AND METHODS

1. Preparation of seeds date materials

The seeds date was supplied by Horchani date in Tozeur Tunisia during the month of july 2015. After washing and cleaning steps (in order to eliminate external impurities), the seeds date were oven dried at 60°C during 24 hours. The resulting materials were milled and sieved. The particle size distribution of seeds date fragments was investigated by laser diffraction using a Mastersizer Malvern and the granulometry was found to be around 50 µm. Thereafter, the dried pits powder was submitted to refluxing in a Soxhlet apparatus for 24 hours with a toluene-ethanol mixture (38:62 v/v) in order to remove oils, fats and waxes.

2. Extraction of polysaccharides

Polysaccharides were sequentially extracted (Figure 1) from defatted pits powder by mechanical stirring in water (2×2 hours at 80 ºC) and aqueous ammonium oxalate solution (100 mM, pH 5) (2×2 hours at 80 ºC) [21].

The purification of the extracts was performed by dialysis for three days against deionized water using dialysis tubing with molecular weight cut off 14 kDa. The recovered dialysates were concentrated in deionized water, frozen overnight and freeze-dried.

Finally, we obtained two extracts: a water-soluble polysaccharide (PPW) and a chelating-soluble polysaccharide (PPO).

3. Colorimetric assays

Total sugars were determined by phenol-sulphuric acid assay using galactose as standard [22].

The content of galacturonic acid in the extracts is an important parameter in the quantitative and structural analysis of this polysaccharide. Based on Bitter's method [23], we can estimate the quantity of uronic acids.

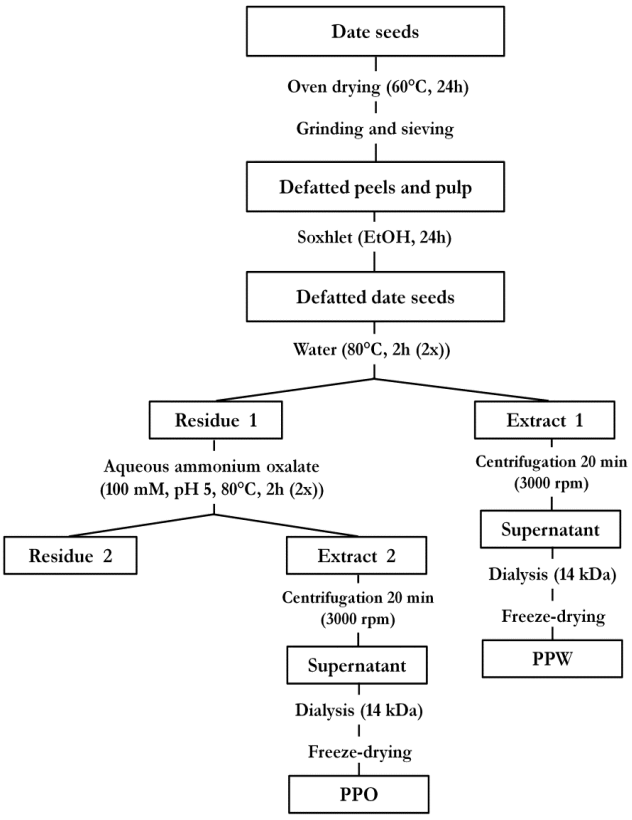

Figure 1: Polysaccharide's extraction from date seeds.

4. Fourier Transform-Infrared Spectroscopy (FTIR)
The infrared spectrums of extracted samples were determined using a Fourier transform infrared spectrophotometer (Perkin-Elmer BX FTIR system spectrometer).

5. Determination of esterification degree (DE)
It has been reported that the esterification degree (DE) is proportional to the ratio of the area of the band at 1748 cm^{-1} (corresponding to the esterified carboxylic groups) over the sum of the areas of the bands at 1748 and 1640 cm^{-1} (corresponding to the total carboxylic groups) [24]. DE was estimated using the following equation (1):

$$DE = [(A1748/A1748 + A1640) + 0.107] \times 100 \quad (1)$$

A1748: Areas of the bands at 1748 cm^{-1}
A1640: Areas of the bands at 1640 cm^{-1}

6. Size exclusion chromatography (SEC)
Analyzes of various samples was performed using size exclusion chromatography (SEC) equipped with a triple detection: multi-angle light scattering (MALS) (Down HELEOS II, Wyatt Technology, Ca, USA), viscometer detector (VD) (Viscostar II, Wyatt Technology, Ca, USA) and differential refractive index (DRI) (RID 10 A Shimadzu, Japan). The samples were dissolved in the eluent ($LiNO_3$ 0.1 mol/L) at 2 g/L. The analyzes from MALS were performed by a data processing Zimm "order 1" using angles from 34.8° to 142.8° [25].
The Astra 6.0.1.7 software package was set to collect and extrapolate data with the goal to obtain, for each elution volume, the molecular weight (M_w), the hydrodynamic radius (R_H) and the intrinsic viscosity ([η]).

7. Antioxidant activity
7.1. DPPH radical scavenging assay
The free radical scavenging effect of PPW and PPO was estimated according to the method of Qiao et al. [26] with slight modification. Briefly, 1 mL of each sample was mixed with 0.2 mL of DPPH methanolic solution (400 µM). Then, 2.0 mL of water was added. The reaction mixture was vortexed and incubated for 30 min in room temperature. The absorbance of the solution was measured at 517 nm and the DPPH inhibitory percentage was calculated using the following equation (2):

$$DPPH\ radical\ scavenging\ activity\ (\%) = (1 - Abssample / Abscontrol) \times 100 \quad (2)$$

Abs_{sample} : Absorbance of the sample
$Abs_{control}$: Absorbance of the control

7.2. Nitric oxide radical scavenging assay
The procedure is based on the principle that, at physiological pH, sodium nitroprusside in aqueous solution spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent [27]. Scavengers of NO• compete with oxygen, leading to reduced production of nitrite ions. 2 mL of each sample was mixed with 4 mL of sodium nitroprusside (10 mM, in PBS pH 7.4), and incubated at room temperature for 150 min. Then, 0.5 mL of Griess reagent was added. The absorbance of the formed chromophore was measured at 546 nm. The NO• scavenging activity was calculated using the following equation (3):

$$Nitric\ oxide\ radical\ scavenging\ activity\ (\%) = (1 - Abssample / Abscontrol) \times 100 \quad (3)$$

7.3. Linoleic acid peroxidation with TBARS assay
The reaction mixture contained 500 µl of linoleic acid (20 mM), 500 µl of Tris HCl (100 mM, pH 7.5), 100 µl of $FeSO_4.7H_2O$ (4 mM) and a varying concentration of each sample. Linoleic acid peroxidation was initiated by the addition of 100 µl of ascorbic acid (2 mM). The mixture was incubated for 30 min at 37 °C. The peroxidation was achieved by the addition of 2 mL of trichloroacetic acid (10%). Therefore, 1 mL of the mixture was added to 1 mL of thiobarbituric acid (1% in 50 mM NaOH), followed by heating for 10 min at 95 °C. The mixtures were centrifuged at 3500/g for 10 min and the absorbance of thiobarbituric acid-reacting substances (TBARS) in the supernatant was measured at 532 nm [28]. Ascorbic acid was used as standard. The percentage of antioxidant activity was determined using the following equation (4):

$$Linoleic\ acid\ peroxidation\ inhibition\ (\%) = [(Ac - As) / (Ac - An)] \times 100 \quad (4)$$

Ac = Absorbance of control (without extract)
As = Absorbance of extract
An = Absorbance of blank (without extract and $FeSO_4.7H_2O$).

Table I: Polysaccharide's extraction yields and sugar determination

Samples	Yield [a] (%)	Total sugars (%)	Gal acid (%)	Un-Gal acid [b] (%)	DE [c] (%)
PPW	1.9	58	16.1	6.7	58.3
PPO	0.9	42	10.9	6.1	43.8

[a] *Yield from dry matter*
[b] *Un-Gal acid: un-esterified galacturonic acid*
[c] *DE: Degree of esterification determined by FTIR*

8. BSA-Galactose glycation model

The inhibition of protein glycation on the BSA-Galactose model (BSA-Gal) was determined by the method of Yang et al. [29]. Bovine serum albumin (20 mg/mL, in 20 mM PBS, pH 7.4) containing 0.1% (w/w) sodium azide (in PBS, pH 7.4) was preincubated with samples or aminosalicylic acid for 10 min at room temperature. 1 M galactose solution was added to the reaction mixture. The solutions were incubated in dark at 60 °C for 72 hours. The reaction mixture without D-galactose was used as a blank solution and aminosalicylic acid was used as standard [30]. Fluorescence intensity was measured using a fluorometer (Versa Fluor spectrofluorometer, Bio Rad), with an excitation wavelength of 370 nm and an emission wavelength of 450 nm. The percentage of inhibition was calculated using the following equation (5):

Inhibition of protein glycation (%) = [((1 - *FBSA* + *galactose* + *sample*)) / *FBSA* + *galactose*] x 100 (5)

9. Data Treatment

Each value in figure 3 was carried in triplicate and presented as mean ± standard deviation (SD; $n = 3$). All experiments were carried out in triplicate and the results were expressed as mean values ± SD ($n = 3$). Antioxidant activity measurements were compared in terms of mean values.

RESULTS AND DISCUSSION

1. Extraction yield and sugar determination

Extraction yield, total sugars and galacturonic acid rates of extracted samples (PPW and PPO) are shown in Table I.

From the extraction data, it appears that the aqueous extract PPW presents a higher carbohydrate's amount (1.9%) than the extract in chelating medium PPO (0.9%). In addition, we notice that the two extracts are polysaccharides since they contain high amounts of total sugars (42 to 58 %) and galacturonic acid (10.9 to 16.1%).

2. FT-IR analysis

The FT-IR spectra of native PPW and PPO are shown in Figure 2.

The four samples displayed a broad stretching intense characteristic peak at around 3407 and 3417 cm^{-1} for the stretching vibration of hydroxyl group. Each particular polysaccharide has a specific band in the 1150-1012 cm^{-1} regions. This region is dominated by ring vibrations overlapped with stretching vibrations of (C–OH) side groups and the (C–O–C) glycosidic bond vibration. The stretching signals at 1050 cm^{-1} suggested the presence of C–O bonds. In addition, signals at 1640 cm^{-1} were due to the asymmetric stretch vibration of C=O of galacturonic acid. The signals at 2926 cm^{-1} and 1411 cm^{-1} were attributed respectively to the stretch vibration of C–H and the symmetric stretch vibration of COO^- of

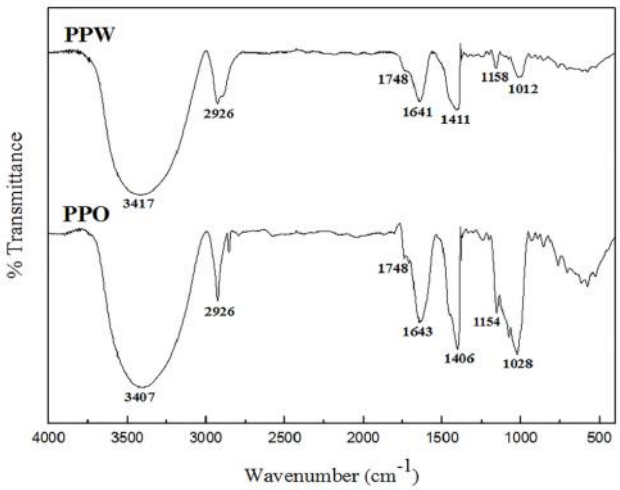

Figure 2: FT-IR spectrums of PPW and PPO

galacturonic acid and the stretch vibration of C–O within COOH [30].

Moreover, a relatively high DE was found for the water soluble extracts PPW and the chelating soluble extract PPO (58.3% and 43.8%, respectively).

Esterification degrees determined from the bands areas of infrared spectrums (DE) are noted in the Table I.

The obtained values of DE for PPW and PPO are in agreement with the extraction method. In fact, it has been reported that extraction in a neutral medium gives highly esterified pectin, whereas the extraction with chelating agents (ammonium oxalate solutions) leads to low esterified pectin [31].

3. Macromolecular characteristics

PPW and PPO analyzed by Size Exclusion Chromatography with the three detectors MALS, VD and DRI on line. The knowledge of molecular weight (M_w), hydrodynamic radius (R_H) and intrinsic viscosity ([η]) permit to obtain information on polymer conformation.

Table II: Results of size exclusion chromatography

Samples	M_w (kDa)	Đ [a]	R_H (nm)	[η] (mL/g)
PPW	1680	3.8	51.8	67.6
PPO	1200	4.1	29.6	63.5

[a] *Đ: Polydispersity index (Đ = $\overline{M_w}$ / $\overline{M_n}$)*

The average characteristics for PPW and PPO are summarized in the Table II.

From these data, we can notice that, although the intrinsic viscosity is almost the same for the two extracts, PPW shows higher values of M_w (1680 kDa) and R_H (51.80 nm) than PPO (1200 kDa and 29.60 nm, respectively). Consequently, it can be deduced that PPO is more rigid than PPW.

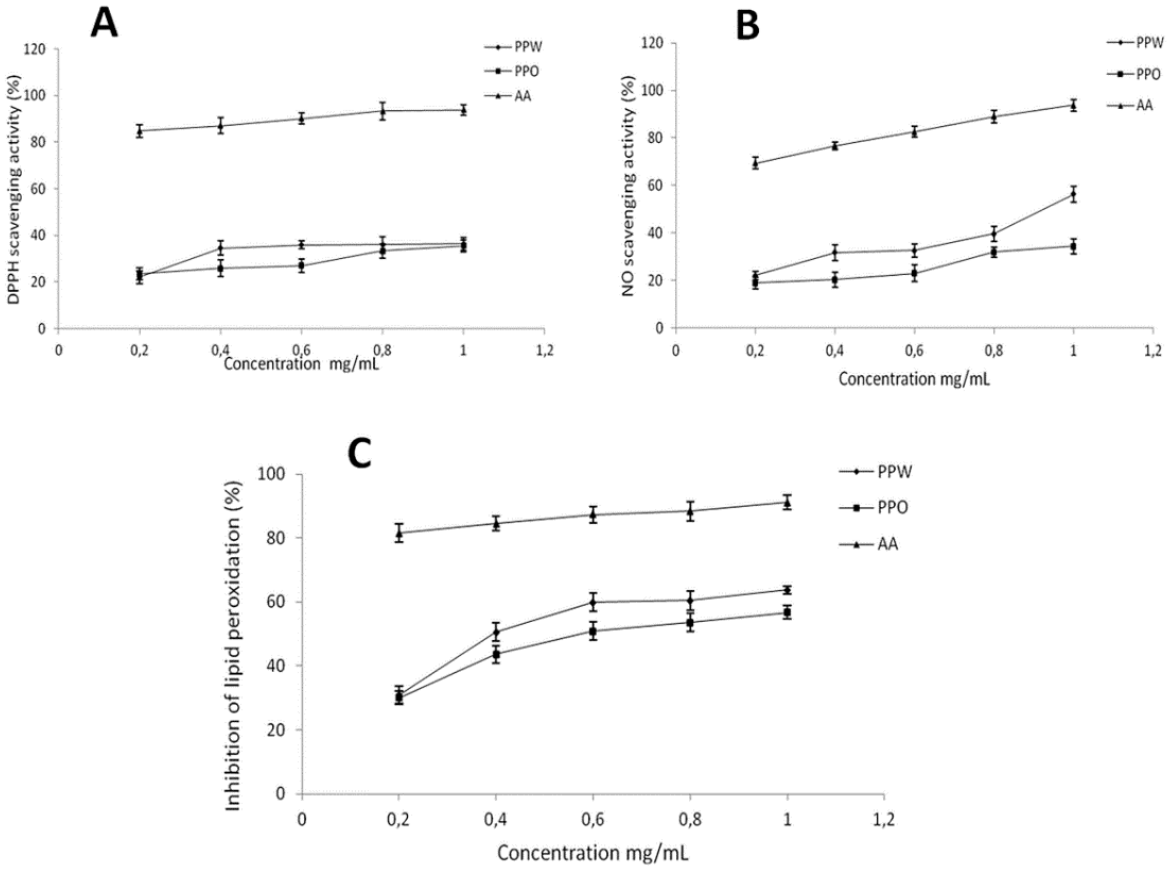

Figure 3: DPPH (a), NO (b) radical-scavenging capacity and inhibition of lipid peroxidation (c) of PPO and PPW extracted from date seeds. Acid ascorbic (AA) was used as standard. Each value is presented as mean ± SD (n = 3).

4. Antioxidant Activity

DPPH has been used extensively as a free radical to evaluate the reduction of substances in various foods, and estimate the free radical scavenging activities of antioxidants [32]. DPPH radical scavenging activity of PPO, PPW and AA was shown in Figure 3a.

The highest DPPH radical scavenging activities were 35.5%, 36.3% and 93.9% for PPO, PPW, and AA respectively, at the concentration of 1 mg/mL.

Concerning nitric oxide scavenging activity, the procedure is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of NO compete with oxygen, leading to reduced production of nitrite ions [33]. Scavenging activities of PPO, PPW and AA against NO were presented in Figure 3b. Notably, PPW showed higher scavenging activity than PPO. The scavenging effects of PPO, PPW and AA increased with the increase of sample concentration ranging from 0.2 to 1 mg/mL. The scavenging ability of PPO, PPW and AA was 34.2%, 56.2% and 93.7% respectively, at the concentration of 1 mg/mL.

Oxidative stress is one of the most frequent causes of tissue and cell injury and the consequent lipid peroxidation is the main manifestation of free radical damage. It has been found to play an important role in the evolution of cell death [34]. The effect PPO, PPW and AA on peroxidation was shown in Figure 3c. In the linoleic acid lipid peroxidation system, PPO, PPW and AA inhibited lipid peroxidation. The highest inhibitory effect of lipid peroxidation by PPO, PPW and AA were 56.7%, 63.7% and 91.1%, respectively at a concentration of 1 mg/mL, indicating that PPO and PPW possessed moderate lipid peroxidation at the range of 0.2-1 mg/mL.

In this study the antioxidant activity of PPO and PPW was compared. The results confirm that the PPW has a slightly higher activity than PPO. Thereafter, these findings indicated that aqueous extract presents a higher antioxidant activity than the extract in chelating medium PPO, and may be an effective method to enhance polysaccharides date seeds free radical scavenging capacity and antioxidant activity in vitro.

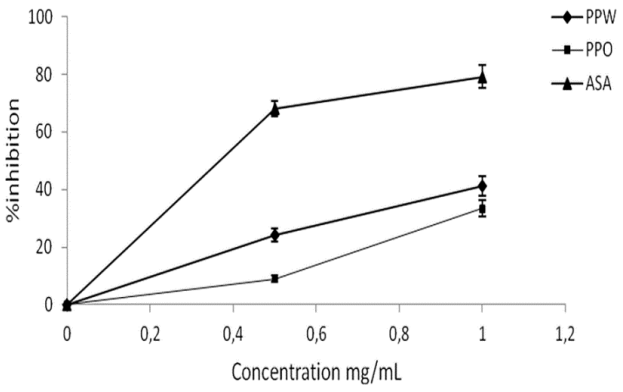

Figure 4: The effects of PPO and PPW extracted from date seeds on fluorescence formation in Galactose-glycated BSA. Each value is presented as mean ± SD (n = 3).

5. Inhibition of AGEs formation

Glycation is the non-enzymatic modification of proteins through reaction with sugars and their metabolized intermediates. It occurs both *in vitro* and *in vivo* through a process called the Maillard reaction and leads to the irreversible formation of AGEs, which may alter the functions of protein and cause protein degradation [35]. In this study, antiglycation activity of PPO, PPW and ASA was investigated, and their results were depicted in Figure 4.

PPO, PPW and ASA showed antiglycation activity and the inhibition was reached to 33.4%, 41.1% and 79.2 at the concentration of 1 mg/mL, respectively, after 72 hours of incubation. Animal studies indicated that supplementation with *Achyranthes bidentata* and *Lycium barbarum* polysaccharides inhibits the non enzymatic glycation in D-galactose induced mouse aging models *in vivo* [17]. Furthermore, *in vitro* studies showed inhibition of the formation of AGEs by polysaccharides from *Punica granatum* [18]. In addition, these results are coherent to previous studies which reported that the biological activities of natural polysaccharides are heavily influenced by several structural features (monosaccharide composition, molecular weight, degree and the size of the branch points, degree and pattern of substitution groups, conformation of the polysaccharide…) [34].

Currently, several biochemical mechanisms of anti glycation reaction that can delay or prevent the glycation process have been proposed [37]. Especially, antiglycation strategies are involved in scavenging of free radicals in the early stages of glycation. Our results indicated that the PPW showed a better scavenging capacity towards the

free radical. Therefore, PPW could react with free radicals, converting them to more stable products, decrease oxidation damage and prevent the glycation of BSA. Additional studies are needed to investigate the ingestion effect of polysaccharides from aqueous extract in the non enzymatic glycation in vivo.

CONCLUSION

Our study was focused on the effect of extraction medium on the antioxidant and antiglycated capacities of polysaccharides extracted from seeds date. Initially, and for comparison reasons, we investigated the effect of aqueous and oxalate extract on the extraction yield. PPW was the richest in polysaccharide (1.9%). SEC/MALLS/ VD/DRI and FTIR analysis revealed that both extracted samples have almost similar macromolecular features and they presented the same functional groups, whereas sugar assays showed that water soluble extract (PPW) possess the highest amount of galacturonic acid (16.%). The antioxidant and antiglycated *in vitro* assays showed that water soluble polysaccharide (PPW) exhibited slightly better potential on scavenging DPPH and nitric oxide radicals and inhibiting linoleic acid peroxidation and protein glycation than chelating-soluble extract (PPO). This result was mainly due to its significant galacturonic acid (16.1%).

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