© 2021 Journal of Pharmacy & Pharmacognosy Research, 9 (6), 921-936, 2021
ISSN 0719-4250
http://jppres.com/jppres

Original Article

Phenolic fraction concentrate of *Phoenix dactylifera* L. seeds: A promising antioxidant and glucose regulator

[Concentrado de fracción fenólica de semillas de *Phoenix dactylifera* L.: Un prometedor antioxidante y regulador de glucosa]

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Abstract

Context: The beneficial effects of natural polyphenolic compounds are increasingly emerging as powerful antioxidants and digestive boosters. *Phoenix dactylifera* seeds are recently identified as a healthy functional food with rich amounts of phenolic compounds.

Aims: To analyzing the phenolic profile obtained from Moroccan variety of date seeds and evaluating the inhibitory capacity against digestive enzymes and the antioxidant potential of phenolic fraction concentrate (PFC) of date seeds as a promising antihyperglycemic agent.

Methods: Lineweaver–Burk plot analysis was performed to evaluate PFC inhibitory effect against digestive enzyme activity in the presence of different doses of a substrate. Wistar rats were recruited to determine the acute and sub-acute oral toxicity of PFC. Finally, the optimal dose of PFC was selected and its effects on metabolic, hematological and biochemical parameters were determined.

Results: High-performance liquid chromatography (HPLC) analysis revealed that PFC contains abundant amounts of phenolic acid: Caffeic acid, chlorogenic acid, 3,4-dihydroxybenzoic acid, p-hydroxybenzoic acid, and 3-hydroxybenzoic acid. PFC showed powerful antioxidant activity as determined by scavenging and reducing assays. PFC significantly inhibited alpha-amylase and alpha-glucosidase enzymes by 87.3% and 78.7%, respectively, via mixed manipulation of Km and Vmax. Acute toxicity examinations revealed PFC to be practically non-toxic with LD_{50} > 5000mg PFC/kg for both sexes of rats. The no-observed-adverse-effect-level (NOAEL) and lowest observed adverse effect level (LOAEL) for oral administration of PFC were 1000 and 2500 mg/kg for both sexes. Finally, the sub-acute study showed that a PFC dose of 50 mg/kg did not cause any changes in metabolic, biochemical and hematological parameters and was considered to be the optimal healthy dose for rats. Consequently, it induces improvement in glucose concentration in rats.

Conclusions: The rational use of phenolic date seeds fraction presents powerful antioxidant and blood glucose regulating nutraceutical that could aid in diabetes mellitus management.

Keywords: acute and sub-acute toxicity; antioxidant activity; digestive enzyme; Moroccan date seeds; phenolic fraction concentrates; Wistar rats.

Resumen

Contexto: Las semillas de *Phoenix dactylifera* se identificaron recientemente como un alimento funcional saludable con grandes cantidades de compuestos fenólicos.

Objetivos: Analizar el perfil fenólico de la variedad marroquí de semillas de dátiles y evaluar la capacidad inhibidora frente a las enzimas digestivas y el potencial antioxidante del concentrado de la fracción fenólica (PFC) como agente antihiperglucémico prometedor.

Métodos: Se realizó un análisis del diagrama de Lineweaver-Burk para evaluar el efecto inhibidor de la PFC contra la actividad de las enzimas digestivas en presencia de diferentes dosis de sustrato. Se reclutaron ratas Wistar para determinar la toxicidad oral aguda y subaguda de la PFC. Finalmente, se seleccionó la dosis óptima de PFC y se determinaron sus efectos sobre parámetros metabólicos, hematológicos y bioquímicos.

Resultados: El análisis de cromatografía líquida de alta resolución (HPLC) reveló que el PFC contiene abundantes cantidades de ácido fenólico: ácido cafeico, ácido clorogénico, ácido 3,4-dihidroxibenzoico, ácido p-hidroxibenzoico y ácido 3-hidroxibenzoico. El PFC mostró una potente actividad antioxidante determinada mediante ensayos de eliminación y reducción. PFC inhibió significativamente las enzimas alfa-amilasa y alfa-glucosidasa en 87,3 y 78,7%, respectivamente mediante la manipulación mixta de Km y Vmax. Los exámenes de toxicidad aguda revelaron que el PFC fue prácticamente no tóxico con LD_{50} > 5000 mg PFC/kg para ambos sexos de rata. El nivel sin efectos adversos observados (NOAEL) y el nivel mínimo de efectos adversos observados (NOAEL) para la administración oral de PFC fueron respectivamente 1000 y 2500 mg/kg para ambos sexos. Finalmente, el estudio sub-agudo mostró que la dosis de PFC 50 mg/kg no provocó ningún cambio en los parámetros metabólicos, bioquímicos y hematológicos y se consideró la dosis óptima en ratas. En consecuencia, induce una mejora en la concentración de glucosa en ratas.

Conclusiones: El uso racional de fracción fenólica de semillas de dátiles presenta un potente nutracéutico antioxidante y regulador de la glucosa en sangre que podría ayudar en el manejo de la diabetes mellitus.

Palabras Clave: actividad antioxidante; concentrado fracción fenólica; enzima digestiva; ratas Wistar; semilla dátiles marroquíes; toxicidad aguda y subaguda.

ARTICLE INFO
Received: May 19, 2021.
Received in revised form: July 14, 2021.
Accepted: July 19, 2021.
Available Online: July 27, 2021.

INTRODUCTION

For so long, natural plant products held an important place in traditional medicine (Katiyar et al., 2012; Keter and Mutiso, 2012). They present a major source of functional bioactive compounds. Almost 80% of the populations in developing countries use medicinal plants for therapeutic purposes against several diseases, such as cardiovascular disorders, obesity, diabetes and neurological disorders (Akah, 2008; Zengin et al., 2015). Lately, there has been a growing interest in nutraceuticals rich in polyphenolic compounds for their therapeutic potential.

The *Phoenix dactylifera* L. (family *Arecaceae*), commonly known as date palm, is one of the most potent products cultivated in North Africa and Middle-East regions (Akasha et al., 2016). The valuable effects of dates palm are not only restricted to fruit use but also to their seeds. Several phytochemical studies identified a variety of functional bioactive molecules such as the polyphenolic compounds; gallic, vanillic, caffeic, protocatechuic and p-hydroxybenzoic acids (Al-Farsi and Lee, 2008). The phenolic compounds present in date seeds exhibit an excellent source of natural antioxidants (El-Mergawi et al., 2016; Bouhlali et al., 2017), making this by-product a promising alternative to synthetic or semi-synthetic antioxidants. Moreover, increasing evidence indicates that date seeds possess anti-inflammatory (Saryono et al., 2020), immuno-stimulant (Saryono et al., 2019), and hepatoprotective properties (Khalid et al., 2017).

Numerous preclinical studies suggest that date seeds extract has a significant anti-diabetic activity through inhibiting digestive enzymes, which intervene in the hydrolysis and absorption of carbohydrates (Khan et al., 2016; Khalid et al., 2017). Also, they have been reported to stimulate endogenous insulin secretion and repair pancreatic cells (El-Fouhil et al., 2013; Saryono, 2019). Hence, date seeds are considered a good source of anti-hyperglycemic molecules that can aid in the management of diabetes mellitus (Djaoudene et al., 2019). However, till now, the use of date seeds is still relatively limited, where they are discarded or used generally as a complementary food for animals or as soil fertilizer (Habib and Ibrahim, 2009; Baliga et al., 2011).

To the best of our knowledge, no previous studies have evaluated the beneficial health effects of Moroccan date seeds phenolic fraction concentrates. Nevertheless, no research has been conducted to explore its potential toxicity. Therefore, the present study aimed at (I) prepare phenolic fraction concentrate (PFC) of Moroccan date seeds to maximize the beneficial fraction of seeds; (II) analyze the phenolic profile and the antioxidant activity of PFC; (III) investigate PFC inhibitory effect and mechanism on digestive enzymes; (IV) evaluate the acute and sub-acute oral toxicity of PFC on Wistar rats, to select the optimal dose and evaluate its effects on the various metabolic, hematological, and biochemical parameters.

MATERIAL AND METHODS

Plant material

Freshly harvested *Phoenix dactylifera* (dates) were collected from the Ouarzazate region (30°55′23″ N, 6°54′15″ W). The species was identified by Professor Youssef Aboussaleh and a specimen was deposited in the herbarium of University Ibn Tofail, Faculty of Science, Kenitra, Morocco, under number: DASE-215/2020. The seeds were separated from dates, washed with water, and stored in a dark place until used. The date seeds used in this study (*Bousthammi,* Moroccan variety) were selected based on the high content of phenolic compounds identified by Bouhlali et al. (2017).

Extraction and preparation of phenolic fraction concentrate

Extraction of phenolic fraction concentrate from date seeds (PFC) was conducted according to the protocol described by Al-Farsi and Lee (2008), with slight modifications. Briefly, date seeds were dried in the oven (Oven BIOBASE) for 48 hours at 50°C. Then, finely ground using an electronic hammer mill, until powder of diameter (1-1.5 mm) was obtained. The powder was then macerated

with 50% acetone and 2% acetic acid (to dissolve the phenolic compounds) (Tabart et al., 2007) in a solvent/sample ratio (10:1). The macerate was subjected to extraction at a stirring speed of 120 rpm via a magnetic stirring plate (Magnetic stirrer Hot Plate) for one hour at 45°C. The extract obtained was centrifuged and filtered on Whatman No. 4 filter paper (to separate and remove the particles). Then, the resulting extract was evaporated to dryness at 60°C under vacuum using a rotary evaporator (Buchi Rotavapor R-300 V). N-butanol was used to purify the phenolic compounds from the crude extract obtained using funnel separation (500 mL). Then, the obtained fraction was evaporated at 60°C. The phenolic fraction produced was dried in an oven at 60°C and ground into powder. The yield of phenolic concentrates was measured using the following formula [1]:

$$\text{Yield (\%)} = (W_{PFC}/ W_{SP}) \times 100 \quad [1]$$

Where W_{PFC}: weight of the phenolic fraction concentrate "powder", and W_{SP}: weight of the seeds powder (100 g).

Spectrophotometric assessment of phenolic contents

Total phenolic content

The total phenolic content (TPC) was evaluated according to the method of Folin-Ciocalteu (Singleton and Rossi, 1965), using gallic acid (0-100 µg/mL) as standard. Briefly, 5 mL of Folin-Ciocalteu reagent was diluted with distilled water (1:10) and mixed with 1 mL of sample, then 4 mL of sodium carbonate solution (7.5% w/v) was added. The mixture was homogenized and incubated in a water bath at 45°C for 30 min. Absorbance was determined at 765 nm using a spectrophotometer. The total polyphenols content was determined via a standard calibration curve of 6 points (from 0 to 100 µg/mL) using equation [2] and repeated in triplicates. The content is the average value of triplicates and was expressed as mg of gallic acid equivalents per g of sample on a wet weight basis (GAE mg/g PFC).

$$y = 12.22x + 0.036 \qquad R^2 = 0.99 \quad [2]$$

Total flavonoids and tannins content

Aluminum trichloride colorimetric method (Dewanto et al., 2002) was used to assess total flavonoid content (TFC), using quercetin (0-40 µg/mL) as standard. About one milliliter of the dissolved sample was placed in a volumetric flask filled with 5 mL of distilled water. 0.3 mL of 5% sodium nitrite was then added, then 0.3 mL of 10% aluminum trichloride was added after 5 minutes. After 6 min, 2 mL of 1 M sodium hydroxide was added, then the volume was adjusted to 10 mL with distilled water. The solution was mixed and allowed to stand for 30 min. The solution absorbance was recorded at 510 nm against blank. The experiment was repeated in triplicates. Flavonoid content was expressed as milligram of quercetin equivalents per grams of sample on a wet weight basis (mg QE/g PFC) using a standard calibration curve of 9 points (from 2.5 to 40 µg/mL) and equation [3]. The flavonoid content was expressed as the average value of the 3 replicates.

$$y = 0.0634x - 0.0133 \qquad R^2 = 0.996 \quad [3]$$

Furthermore, the condensed tannin content (CTC) was evaluated by spectrophotometric analysis using the method described by Julkunen-Tiitto (1985) with catechin (0-300 µg/mL) as standard**.** The content of condensed tannins was expressed as milligram of catechin equivalents per grams of sample on a wet weight basis (mg CE/g PFC). A standard calibration curve of 6 points (from 0 to 300 µg/mL) and equation [4] were used for calculation, and CTC was expressed as the average value of the 3 replicates.

$$y = 0.0331x - 0.0015 \qquad R^2 = 0.98 \quad [4]$$

Phytochemical HPLC analysis

Identification and quantification of phenolic compounds were performed using an HPLC-DAD-MS system. This system consisted of an automatic sampler (Agilent Technologies, G1330B) coupled to a mass spectrometer under-equipped with an electrospray ionization source (MS; ESI; Micromass Quattro Micro, MA, United States), and a binary pump (Agilent Technologies, G1312A). HPLC separation was performed on an

Agilent Zorbax C18 column (100 mm × 2.1 mm × 1.7 µm). The MS was operated in negative ion mode with the following parameters: extractor 2 V; cone voltage 20 V and capillary voltage 3.0 kV. Cone gas flow was 30 L/h, and desolvation gas flow was 350 L/h, with a source temperature was 100°C, and desolvation temperature was 350°C. The mobile phase elements were (A): 0.1% formic acid and (B): acetonitrile with 0.1% formic acid. The mobile phase conditions were: 0 min, 90% (A); 0 to 18 min, 30% (A); 18 to 20 min, 30% (A); 20 to 23 min, 30% (A); 23 to 25 min, 90% (A) and 25 to 30 min, 90% (A). The flow rate of the mobile phase and the injection volume was 0.5 mL/min and 10 µL, respectively, at a column temperature of 35°C. The phenolic compounds were identified and quantified based on their retention times. Results were expressed in milligrams per kilograms of the sample (mg/kg).

Spectrophotometric assessment of antioxidant activity

DPPH scavenging assay

The DPPH method (1,1-diphenyl-2-picryl-hydrazil) was used to evaluate the free radical elimination activity of the extract of date seeds, based on the protocol described by Huang et al. (2011). A DPPH solution (0.2 mM) in methanol was prepared, and 0.5 mL of this solution was then added to 2.5 mL of date seed extract. The contents are then allowed to stand for 30 minutes at room temperature. Absorbance was recorded at 517 nm against blank. Results were presented as a percentage reduction in DPPH radical compared to a control, and IC_{50} (mg/mL) was calculated by the formula [5].

$$\text{DPPH reduction (\%)} = [(As-Ai)/As] \times 100 \quad [5]$$

Where As: absorbance of DPPH alone, Ai: Absorbance of DPPH in the presence of sample on a wet weight basis.

Determination of ferric reducing/antioxidant power (FRAP)

To measure the reducing capacity of seed extracts, a ferric ion (Fe^{3+}) reducing antioxidant power method was used based on the protocol described by Oyaizu (1986) with slight modification. Different concentrations of extract from the stock solutions and ascorbic acid as standard were mixed with 2.5 mL of 1% potassium ferricyanide (w/v) and 2.5 mL of phosphate buffer (0.2 M). After incubating the mixture at 50°C for 20 min, (2.5 mL) of 10% trichloroacetic acid was added to the reaction mixture. The mixture was centrifuged at 1000×g for 10 min. Then, 2.5 mL of the top layer of the solution was mixed with 0.5 mL of 0.1% ferric chloride (w/v) and 2.5 mL of deionized water. The absorbance was measured at 700 nm at the reaction time of 30 min. The result was expressed as milligram of ascorbic acid equivalents per grams of sample on a wet weight basis (mg AAE/g PFC).

Trolox equivalent antioxidant capacity (TEAC)

The antioxidant capacity of the extract from date seeds to trap the radical cation 2,2′-azino bis (3-ethylbenzothiazo-line-6-sulfonate) ($ABTS^+$) was evaluated according to the protocol described by Re et al. (1999) with slight modifications. Briefly, the cation radical $ABTS^+$ was produced by the reaction between 100 µL of 70 mM potassium persulfate and 10 mL of 2 mM ABTS in H_2O while kept in the dark at room temperature for 24 h. Before analysis, the $ABTS^+$ solution was diluted to obtain an absorbance of 0.70 at 734 nm and equilibrated at 30°C. Then, the samples were prepared in triplicate by adding 200 µL of extract in 2 mL diluted $ABTS^+$ solution. The absorbance was measured 1 min after the initial mixing at 734 nm. Trolox was used as a reference standard ($R^2 = 0.99$). The result was expressed in (mg TE/g PFC) "Defined as the concentration of standard Trolox with the same antioxidant capacity as sample on a wet weight basis".

Spectrophotometric determination of digestive enzymes inhibition and kinetics

Alpha-amylase inhibition test was determined according to the protocol described by Kee et al. (2013), with some modifications reported by Marmouzi et al. (2017). A volume of 250 µL of the sample (PFC) was mixed with 250 µL of alpha-amylase enzyme (240 U/mL) in 0.02 M phosphate

buffer. Then the reaction mixture was incubated at 37°C for 10 min. A volume of 250 µL of 1% soluble starch (substrate) (in 0.02 M phosphate buffer) was then added to the reacting mixture. After incubating the mixture at 37°C for 25 min, 250 µL of dinitrosalicylic acid reagent (DNS) were added. To stop the reaction, the mixture was incubated in a boiling water bath for 10 min. Absorbance was read at 540 nm after dilution of the reaction mixture with 2 mL of sodium phosphate buffer.

For the alpha-glucosidase inhibition test, p-nitrophenyl β-D-glucopyranoside (pNPG) was used as the substrate, based on the protocol reported by Marmouzi et al. (2017). Briefly, pNPG (1 mM) and alpha-glucosidase enzyme (0.1 U/mL) were dissolved in sodium phosphate buffer (0.1 M, pH = 6.7), while the sample (PFC) was dissolved in distilled water. The reaction mixture containing (100 µL) of the enzyme α-glucosidase dissolved in PBS (0.1 M) with the sample (150 µL) were pre-incubated at 37°C for 10 min. After pre-incubation, (200 µL) of the substrate (pNPG, 1 mM) was added to the reaction mixture. The reaction mixture was incubated at 37°C for 30 min. Then, 1 mL of sodium carbonate (1 M) was added. Absorbance was measured at 405 nm.

Acarbose was used as a positive control for both tests. All samples were analyzed in triplicates with increasing concentrations (the concentrations were selected according to their inhibition percentages) to evaluate the IC_{50} values. The percentage of inhibition (%) was calculated according to the formula [6] for both assays (Marmouzi et al., 2017).

$$\text{Inhibition (\%)} = [(AC - ACb) - (AS - ASb)/(AC - ACb)] \times 100 \quad [6]$$

Where ACb (control blank), AC (control), AS (sample) and ASb (sample blank).

Inhibition mode of PFC, against alpha-amylase and alpha-glucosidase activities, were determined with increasing concentrations of the substrates (pNPG and starch) in absence or presence of PFC (inhibitor) in two concentrations (250 and 500 µg/mL) for alpha-amylase and (500 and 750 µg/mL) for alpha-glucosidase. PFC concentrations used were selected based on the results from the inhibitory activity assay obtained. Lineweaver-Burk plot analysis was obtained. According to Michaelis-Menten kinetics, the data calculated from the results allowed us to determine the inhibition mode of PFC.

Acute and sub-acute oral toxicity studies of PFC

Animal model and their maintenance

Wistar rats weighing about 200-220 g provided by the laboratory of nutrition, health and environment, Faculty of Science Kenitra, Ibn Tofail University, Morocco, were used for the study. The rats were kept in standard laboratory conditions. Rats were acclimatized through an adaptation period of two weeks before the beginning of the experiment. Rats were kept in a 12-hour light-dark cycle, with a constant temperature of 22 ± 2°C and relative humidity of 55%, with unlimited access to both water and standard food (KUWTK 62, Tamara, Morocco).

Ethics approval was obtained from Ibn Tofail University in Kenitra, under the responsibility of the Laboratory of Nutrition, Health and Environment at the Faculty of Science, Kenitra (E511/2020). In this study, we used a minimized number of laboratory rats to limit the suffering of animals, and the experiments following the standards and principles outlined in the "Guide for the care and use of laboratory animals".

Acute toxicity in rat models

To evaluate acute oral toxicity, different doses of PFC were administered by gavage to eight groups of rats (7 males and 7 females) at doses: 500; 1000; 2000; 5000; 7500; 10,000; 12,500 and 14,000 mg/kg body weight (another group for each sex was kept as normal control). PFC was dissolved in a physiological solution (0.9% NaCl adjusted to 10 mL/kg per dose). Rats were observed continuously for four hours (first day) after treatment, then monitored three times daily for abnormal behavior, symptoms of toxicity, mortality, and the mortality latency for two weeks following treatment. LD_{50} ± standard deviation (with 95% confidence interval) values were calculated according to the method of Miller and Tainter

(Randhawa, 2009). Briefly, the 0 and 100% of dead rats were corrected before the determination of probit using the formula: for 0% dead: 100 × (0.25/n) and for 100% dead: 100 × (n-0.25/n) (where n was the number of rats in each group), then the correction for dead rats at each concentration was transformed to probit. While the lowest-observed-adverse-effect level (LOAEL) and the no-observed adverse effect level (NOAEL) were determined according to Alexeeff (2002).

Sub-acute toxicity in rat models

After determining LD_{50}, the sub-acute oral toxicity of PFC was evaluated. Thirty-two male Wistar rats were used in this study. Animals were divided into four groups (eight animals for each group). Group 1: as control. Groups 2, 3 and 4 were treated with PFC at doses of 50, 100 and 200 mg/kg of body weight, respectively (doses were selected based on the results of the acute toxicity study described above and the human dose in traditional medicine). PFC (dissolved in a physiological solution adjusted to 10 mL/kg per dose) was administered orally daily for 28 days. Rats were observed daily during the treatment period for toxic manifestations and behavioral symptoms. During the study, the following parameters were determined at the end of each two weeks for each rat: body weight (using electronic balance), daily water and food intake (using metabolic cages) and the rectal temperature (electronic thermometer).

Determination of biochemical and hematological parameters

At the end of the experiment, the rats were sacrificed under anesthesia, and blood samples were taken from the jugular vein and collected in EDTA tubes for hematological analysis. Hematological analysis was performed using an automated analyzer (Architect c8000, Clinical Chemistry System) and included the following: (number of red blood corpuscles, white blood corpuscles, total hemoglobin level, platelet count and the number of lymphocytes, neutrophils and eosinophils). Meanwhile, heparinized blood was centrifuged immediately at 4000 rpm for 10 min at 4°C. The biochemical parameters (glucose, cholesterol, triglyceride, HDL and LDL cholesterol, creatinine levels, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using specific diagnostic kits and following manufacturers' protocols (DiaSys, GmbH, Germany). Also, LDL/HDL ratio was used to calculate the atherosclerosis index (AI) (Marmouzi et al., 2017).

Statistical analysis

All data were presented as mean ± standard deviation (SD). Analysis of variance (ANOVA One Way) followed by a *post-hoc* (Tukey test) was used to determine the significant differences among the studied groups. Also, a t-Test was used to determine the differences between IC_{50} of PFC *versus* acarbose. In all tests, $p<0.05$ was significant. Statistical analysis was performed using GraphPad Prism 8.0 (USA) software for Windows.

RESULTS

Phytochemical analysis

The yield, phenolic contents and antioxidant activity of PFC obtained by fractionation procedure are shown in Table 1. As observed in this table, the extract exhibited a remarkably high total phenolic, with a yield of phenolic fraction equivalent to 5.97%. Table 1 also shows the antioxidant capacity of PFC, as evaluated by three *in vitro* antioxidant methods DPPH, FRAP and TEAC.

Table 2 presents the list and quantities of the major phenolic compounds in PFC using HPLC-MS. It was found that caffeic acid constitutes the major compound in PFC, followed by chlorogenic acid. HPLC analysis also showed considerable amounts of p-hydroxybenzoic acid, luteolin, 3,4-dihydroxybenzoic acid, 3-hydroxybenzoic acid, naringenin, vanillic acid and 4-hydroxycinnamic acid (Fig. 1).

Table 1. Fractionation yield, phenolic compounds, and antioxidant activity of the phenolic fractions concentrate obtained from date seeds.

Yield (%)	Phenolic compound			Antioxidant capacity		
	TPC (mg GAE/g PFC)	TFC (mg QE/g PFC)	CTC (mg CE/g PFC)	DPPH IC_{50} (mg/mL)	FRAP (mg AAE/g PFC)	TEAC (mg TE/g PFC)
5.97	152.02 ± 2.85	25.14 ± 0.24	14.55 ± 1.49	0.21 ± 0.02	16.53 ± 0.77	4.15 ± 0.14

*Values are represented as mean ± SD (n=3). PFC: Phenolic fractions concentrate produced from date seeds. TPC: Total phenolic content; TFC: Total flavonoids content; CTC: condensed tannin content.

Table 2. Phenolic composition identified in phenolic fraction concentrate.

Compound	Retention time (min)	Retention factor (k)	PFC (mg/kg)
Pyrogallol	0.73	0.404	0.17 ± 0.08
3,4-dihydroxybenzoic acid	1.01	0.942	193.72 ± 11.10
Chlorogenic acid	1.26	1.423	584.10 ± 23.12
3-Hydroxybenzoic acid	1.37	1.634	115.65 ± 6.81
Caffeic acid	1.59	2.058	597.37 ± 20.99
Pyrocatechol	1.60	2.077	2.18 ± 0.16
Vanillic acid	1.64	2.154	20.34 ± 2.75
Gallic acid	1.83	2.519	4.67 ± 0.32
p-Hydroxybenzoic acid	1.88	2.615	173.40 ± 7.76
4-hydroxycinnamic acid	2.26	3.246	18.96 ± 1.28
Coumaric acid	2.27	3.365	6.52 ± 0.81
3-hydroxycinnamic acid	2.58	3.961	2.27 ± 0.12
Ferulic acid	2.69	4.173	0.58 ± 0.04
Quercetin	3.64	6.019	1.48 ± 0.07
Luteolin	5.04	8.692	49.27 ± 3.60
Naringenin	5.99	10.519	34.87 ± 3.10
Hesperetin	6.31	11.134	2.86 ± 0.18

*PFC: Phenolic fractions concentrate produced from date seeds. Values are represented as mean ± SD (two replicates).

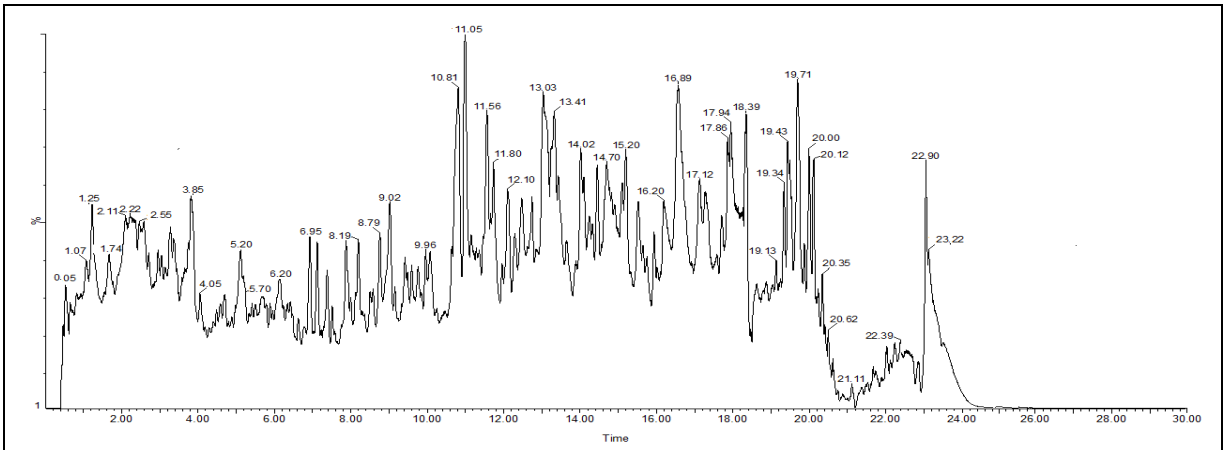

Figure 1. HPLC chromatogram of phenolic compounds in phenolic fraction concentrate.

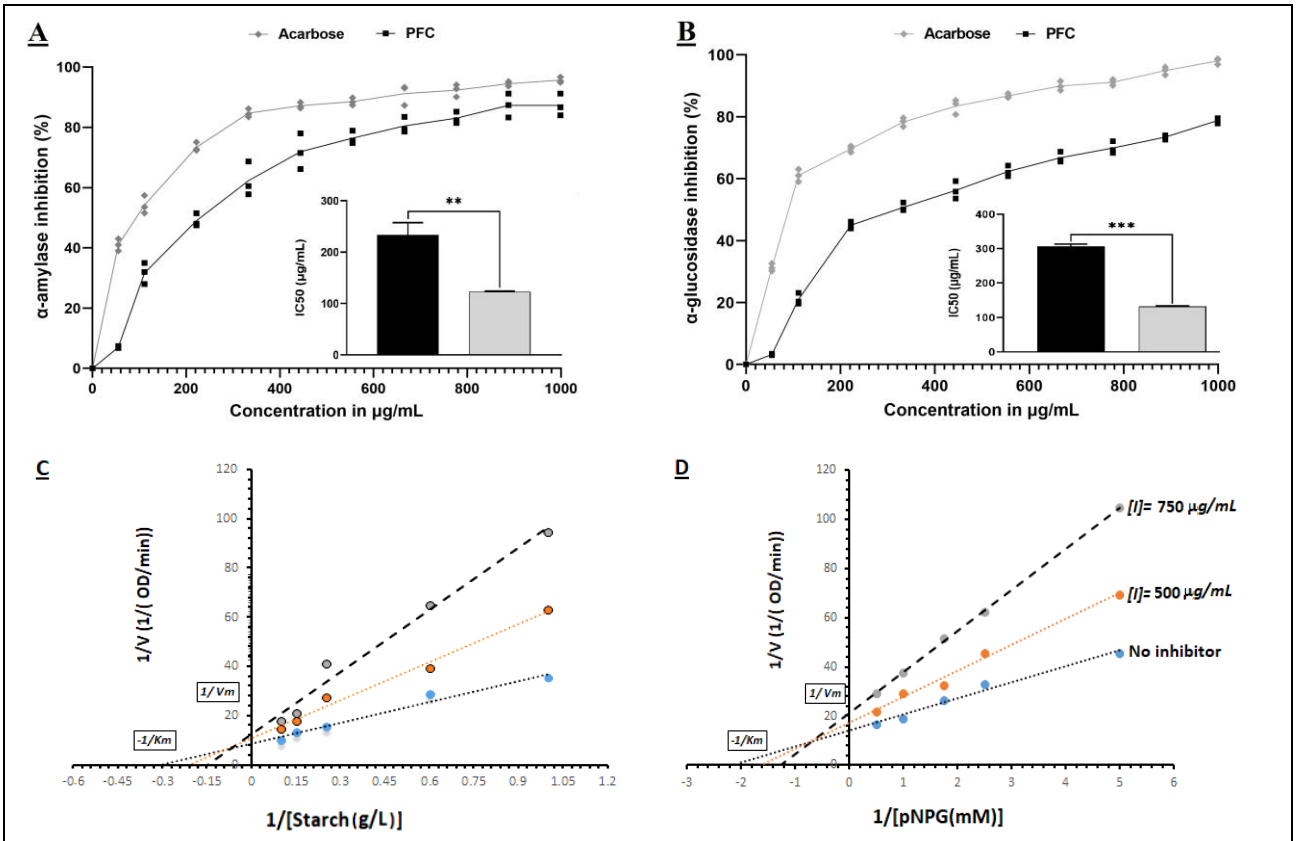

Figure 2. Inhibitory activity and kinetics analysis of phenolic fraction concentrate (PFC) produced from date seeds.

(A) alpha-amylase inhibitory activity of PFC (with IC_{50} value), **(B)** alpha-glucosidase inhibitory activity of PFC (with IC_{50} value), **(C)** Lineweaver-Burk plot of the PFC of alpha-amylase inhibition, **(D)** Lineweaver-Burk plot of the PFC of alpha-glucosidase inhibition. Values are reported as mean ± SD (n=3). **p< 0.01, ***p< 0.001 (PFC *versus* acarbose).

Inhibition of digestive enzymes (*in vitro*)

In vitro inhibitory activity of PFC, on digestive enzymes (alpha-amylase and alpha-glucosidase), are indicated in Fig. 2A-B. The results obtained were compared with the standard inhibitor acarbose. The results demonstrated that PFC inhibited alpha-amylase activity and alpha-glucosidase activity in a dose-dependent manner. t-Test analysis showed significant differences ($p<0.01$) between IC_{50} of PFC and acarbose IC_{50} for both enzymes Fig. 2A-B.

Furthermore, Lineweaver-Burk double reciprocal plot of enzymes activity in absence or presence of PFC (Fig. 2C-D) offered a useful visual aid to identify the mode of inhibition of PFC. Values of the kinetic parameters, Km (Michaelis constant) and Vmax (maximum rate), were altered with increasing PFC concentrations: where Km increased while Vmax decreased (Table 3). Additionally, the lines intersected in the second quadrant of the graph (Fig. 2C-D). Consequently, PFC can be classified as a mixed inhibitor against both (α-amylase and α-glucosidase) enzymes.

Acute and sub-acute oral toxicity

Acute oral toxicity

Table 4 presents the signs of toxicity and mortality percentage due to oral administration of PFC at different doses. The NOAEL and LOAEL for oral PFC administration were 1000 mg/kg and 2500 mg/kg, respectively, for both sexes. However, the death rate of 0% up to a concentration of 2500 mg/kg and a gradual increase to 100% was observed at 14,000 mg/kg for both sexes. While the main toxic symptom noticed immediately after administration of PFC and persisted until death

was hyperactivity. Other side effects were also observed after 24 h of gavage, such as weight loss and anorexia in both sexes. LD_{50} values of PFC are also illustrated in (Fig. 3). As can be seen on this plot, LD_{50} ± SD (CI 95%) of the PFC corresponds to 6606.9 ± 1866.4 (4740.5-8473.3) for male rats and 7516.2 ± 1954.4 (5561.8-9470.6) for female rats. These values allowed us to classify this fraction in the category of practically non-toxic products (5000 mg/kg > LD_{50} < 15000 mg/kg) for both sexes of rat according to the Hodge and Sterner toxicity scale.

Sub-acute oral toxicity

Water, food intake, body weight and rectal temperature of animals

Table 5 presents the effect of daily oral administration of PFC on various parameters in rats. Compared to control group (G I), rats in group 4 (G IV), which were treated with PFC dose of 200 mg PFC/kg showed a significant increase in daily water intake ($F_{3, 28}$ = 24.836; p = 0.000), associated with a significant reduction in body weight ($F_{3, 28}$ = 17.878; p = 0.000). While food intake increased significantly ($F_{3, 28}$ = 6.305; p = 0.002) at the end of the experiment in the same group compared to the control group. Furthermore, no significant difference in rectal temperature was observed in treated groups of rats compared to the control group ($F_{3, 28}$ = 1.090; p = 0.369).

Biochemical and hematological analysis

In order to determine the effect of the PFC produced from date seeds on physiological parameters, the biochemical and hematological parameter were evaluated. The results obtained are presented in Table 6. Statistical analysis did not show any significant difference in the serum level of lipid parameters such as total cholesterol ($F_{3, 28}$ = 2.618; p = 0.071), triglycerides ($F_{3, 28}$ = 2.427; p = .086), HDL ($F_{3, 28}$ = 1.511; p = 0.233), and LDL ($F_{3, 28}$ = 0.885; p = 0.461) cholesterol between the evaluated groups. In addition, the atherosclerosis index (LDL/HDL ratio) did not show any significant difference ($F_{3, 28}$ = 1.007; p = 0.404) in all groups. On the other hand, a significant reduction of glucose concentration was observed in all treated rats with the PFC compared to control group rats ($F_{3, 28}$ = 14.286; p = 0.000). In addition, oral administration of PFC had no significant effect on hepatic enzymes AST ($F_{3, 28}$ = 2.965; p = 0.050), ALT ($F_{3, 28}$ = 2.072; p = 0.127) nor renal creatinine ($F_{3, 28}$ = 2.944; p = 0.051). (Table 6). Meanwhile, analysis of blood constituents showed a slight fluctuation without significant difference between treated and control rats. Only red blood cells were significantly higher in treated PFC group with dose (100 mg PFC/kg) compared to control group ($F_{3, 28}$ = 8.713; p = 0.015).

Table 3. Kinetic parameters of α-amylase and α-glucosidase inhibition by phenolic fraction concentrate.

Kinetic parameters	Alpha-amylase			Alpha-glucosidase		
	No inhibitor	[I]= 250 µg/mL	[I]= 500 µg/mL	No inhibitor	[I]= 500 µg/mL	[I]= 750 µg/mL
K_m	3.367	4.807	6.629	0.490	0.628	0.888
V_{max}	0.124	0.094	0.076	0.072	0.059	0.047
K_m / V_{max}	27.15	51.13	87.22	6.805	11.55	18.89
Type of inhibition	Mixed			Mixed		

Table 4. Acute toxicity study of phenolic fractions concentrates administered orally to rats.

Doses (mg/kg)	Log-dose	Sex	Dead (%)	Corrected (%)	Probit	Mortality latency	Toxic symptoms
0	-	M	0	-	-	-	None
		F	0	-	-	-	None
500	2.698	M	0	3.6	3.18	-	None
		F	0	3.6	3.18	-	None
1000	3	M	0	3.6	3.18	-	None
		F	0	3.6	3.18	-	None
2500	3.397	M	0	3.6	3.18	-	Hypoactivity
		F	0	3.6	3.18	-	Hypoactivity
5000	3.698	M	28.6	28.6	4.44	48> h <72	Hypoactivity
		F	14.3	14.3	3.94	72> h <96	Hypoactivity
7500	3.874	M	57.1	57.1	5.18	24> h <120	Hypoactivity, weight loss, anorexia
		F	42.8	42.8	4.81	24> h <96	Hypoactivity, weight loss, anorexia
10,000	4	M	71.4	71.4	5.56	12> h <96	Hypoactivity, weight loss, anorexia
		F	71.4	71.4	5.56	12> h <120	Hypoactivity, weight loss, anorexia
12,500	4.096	M	85.7	85.7	6.07	12> h <72	Hypoactivity, weight loss, anorexia
		F	85.7	85.7	6.07	12> h <96	Hypoactivity, weight loss, anorexia
14,000	4.146	M	100	96.4	6.80	12> h <48	Hypoactivity, weight loss, anorexia
		F	100	96.4	6.80	12> h <72	Hypoactivity, weight loss, anorexia

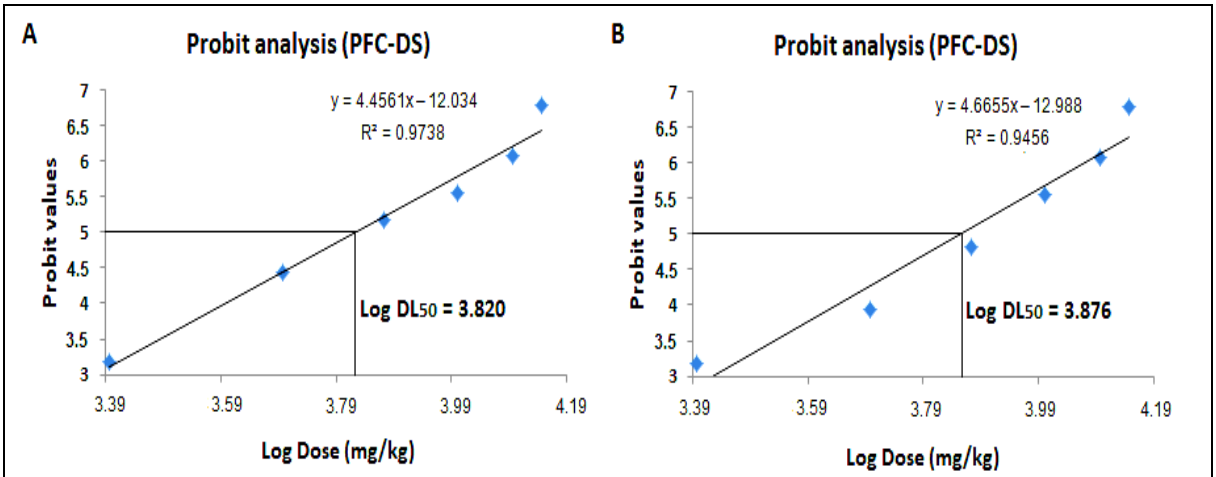

Figure 3. Plot of log-doses *vs.* probits to determine LD_{50} values of the phenolic fraction concentrates obtained from date seeds (PFC-DS) administered orally to rats. **(A)** males, and **(B)** females.

Table 5. Changes in water, food intake, body weight and rectal temperature.

Parameters	Treatment (days)	Groups (G)			
		G I (Control)	G II (50 mg PFC/kg)	G III (100 mg PFC/kg)	G IV (200 mg PFC/kg)
Water intake (mL/rat/day)	D1	20.12 ± 2.53	18.62 ± 1.40	22.87 ± 2.23	22.25 ± 2.71
	D14	19.50 ± 2.26	20.12 ± 2.74	19.62 ± 1.99	22.37 ± 1.99*
	D28	21.38 ± 1.68	20.75 ± 1.98	22.25 ± 2.49	28.66 ± 2.44***
Food intake (g/rat/day)	D1	17.37 ± 1.40	17.01 ± 1.30	16.50 ± 1.60	17.75 ± 1.90
	D14	17.01 ± 1.30	17.50 ± 2.20	16.87 ± 2.16	16.22 ± 1.99
	D28	16.33 ± 1.59	15.05 ± 2.12	14.95 ± 1.03	12.63 ± 1.99**
Body weight gains (g/rat)	D14	10.25 ± 1.48	8.75 ± 1.03	9.37 ± 1.18	6.50 ± 1.19***
	D28	18.62 ± 1.50	17.12 ± 1.12	16.05 ± 2.11*	13.75 ± 1.48***
Rectal temperature (C°)	D1	36.04 ± 0.51	36.44 ± 0.96	36.52 ± 0.64	35.64 ± 0.58
	D14	36.48 ± 0.79	36.05 ± 0.74	36.35 ± 0.52	36.57 ± 0.69
	D28	35.98 ± 0.62	36.53 ± 0.78	36.42 ± 0.55	36.26 ± 0.57

Values are represented as mean ± SD (n= 8). *p<0.05, ***p<0.001 (statistically significant *versus* Control). PFC: phenolic fraction concentrate.

Table 6. Effects of phenolic fractions concentrate on biochemical and hematological parameters.

Parameters	Groups (G)			
	G I (Control)	G II (50 mg PFC/kg)	G III (100 mg PFC/kg)	G IV (200 mg PFC/kg)
Neutrophils (10^3/µL)	17.36 ± 0.92	16.78 ± 0.84	17.93 ± 1.21	17.99 ± 0.81
Eosinophils (10^3/µL)	2.13 ± 0.16	2.24 ± 0.40	2.32 ± 0.29	2.51 ± 0.38
Lymphocytes (10^3/µL)	58.20 ± 3.25	60.84 ± 4.63	59.50 ± 4.87	61.87 ± 5.76
Total hemoglobin (g/dL)	12.67 ± 0.63	13.02 ± 0.87	11.99 ± 1.03	12.47 ± 1.01
WBC (10^3/µL)	5.28 ± 0.41	5.59 ± 0.31	5.83 ± 0.50	5.96 ± 0.58
RBC (10^6/µL)	6.22 ± 0.07	6.04 ± 0.43	6.78 ± 0.23*	6.68 ± 0.40
Platelet count (10^3/µL)	543.4 ± 49.47	556 ± 34.74	522.7 ± 27.51	541.2 ± 38.07
Glucose (g/L)	1.23 ± 0.14	0.96 ± 0.13***	0.91 ± 0.09***	0.92 ± 0.06***
Cholesterol (g/L)	0.50 ± 0.05	0.46 ± 0.04	0.49 ± 0.02	0.52 ± 0.05
Triglycerides (g/L)	0.33 ± 0.03	0.29 ± 0.04	0.31 ± 0.04	0.28 ± 0.02
HDL (g/L)	0.23 ± 0.02	0.24 ± 0.03	0.22 ± 0.04	0.25 ± 0.03
LDL (g/L)	0.22 ± 0.03	0.20 ± 0.02	0.20 ± 0.09	0.21 ± 0.05
Atherosclerosis index	0.94 ± 0,19	0.84 ± 0.16	0.90 ± 0.13	0.83 ± 0.13
AST (IU/L)	107.6 ± 5.75	112.5 ± 16.04	113.1 ± 6.33	109.0 ± 7.25
ALT (IU/L)	45.75 ± 6.01	43.50 ± 5.52	49.01 ± 4.56	43.53 ± 4.10
Creatinine (mg/L)	3.99 ± 0.14	4.15 ± 0.16	4.06 ± 0.13	4.18 ± 0.14

Values are represented as mean ± SD (n= 8). *p<0.05, ***p<0.001 (statistically significant *versus* Control). PFC: phenolic fraction concentrate.

DISCUSSION

Natural plant products have a significant place in traditional medicine. They constitute a good source of secondary metabolites, including phenolic compounds. Date seeds are by-products that have shown to be an inexpensive natural source of phenolic compounds (Al-Farsi and Lee, 2008; Bouhlali et al., 2017). However, optimizing their benefits can be significantly improved through optimizing the extraction methods. In this study, we optimized the extraction and fractionation of the phenolic compounds in attempt to get the highest phenolic concentrates. Besides, the presented results go in line with previous studies (El-Mergawi et al., 2016; Thouri et al., 2017), which have shown that date seeds have significant amounts of total flavonoids content and condensed tannins. It is here worth mentioning that the extraction method, the number of extractions steps and the polarity of solvents used play a significant role in increasing the solubility and the fractionation of phenolic compounds (Bashash et al., 2014). Therefore, the extraction method used in our study greatly increased the amount of phenolic acids extracted from the date seeds. Consequently, the results of HPLC analysis spotted the presence of five major phenolic acids in PFC, which agreed with our assessment of total phenolic contents determined spectrophotometrically.

Phenolic acids are characterized by their significant antioxidant properties (Robards et al., 1999). The PFC showed significant antioxidant potential assessed *in vitro* through three tests: DPPH, FRAP, and TEAC. Regarding the result of FRAP assay, PFC displayed a significant ferric reducing antioxidant power. These results are in accordance with the findings of Bouhlali et al. (2017), who reported a high reducing antioxidant power of date seeds extract, whereas phenolic compounds can act as metal chelators, which prevent oxidation induced by reactive hydroxyl radicals (Perron and Brumaghim, 2009). Also, PFC demonstrated a significant ability to reduce DPPH radicals. In line with this finding, a recent research conducted by Thouri et al. (2017) showed that DPPH capacity of two Tunisian date seeds variety was remarkably lower than our result with half the maximal inhibitory concentration. Differences in antioxidant activity could be attributed to the extraction conditions, the quality and quantity of the functional compounds present in the sample. We hypothesize that the antioxidant capacity presented by the PFC could be attributed in part to the increased amounts of phenolic acids identified by HPLC analysis, such as caffeic, chlorogenic and dihydroxybenzoic acids.

Till date, diabetes mellitus is a serious public health problem in the world. One of the therapeutic strategies for hyperglycemia control is the inhibition of digestive enzymes, which intervene with carbohydrates hydrolysis (Manaharan et al., 2011). Currently, little research has described the relationship between natural phenolic compounds and digestive enzymes activity involved in blood glucose (Ramkumar et al., 2009). Biological actions of polyphenols have been classified into two groups according to their biological mechanisms: specific mechanisms dependent on the chemical and structural composition of the active polyphenol, and general mechanisms depending essentially on the presence of phenolic groups (Fraga et al., 2010). To the best of our knowledge, there are no published studies that evaluated the inhibitory concentrations and the inhibitory kinetics of PFC date seeds. The present study results revealed that PFC has significant inhibitory properties against enzymes linked to hyperglycemia. The percentage of inhibition of these enzymes increases with increasing concentrations of PFC. Lineweaver-Burk plot showed that PFC had an intermediate mode between the uncompetitive and competitive inhibition (mixed-mode) in both enzymes. Therefore, we suggest this mode of inhibition was probably due to the presence of various phenolic compounds in the PFC, such as caffeic acid.

Prolonged treatment with acarbose is reported to cause several side effects (Andrade et al., 1998; Apostolidis et al., 2007). Consequently, the use of medicinal plants with anti-diabetic effects can present a safe means to manage hyperglycemia. In addition, several studies have demonstrated the anti-diabetic effect of date seeds extract on diabetic animal models (Hasan, 2016; El-Mousalamy et al.,

2016). In the present study, caffeic acid constituted the major compound in PFC. Moreover, Jung et al. (2006) have reported that caffeic acid improves glycemia by attenuating hepatic glucose output and enhancing insulin secretion.

Herbal medicine remains a promising and less expensive strategy for preventing and treating several diseases (Hosein et al., 2020). Currently, vegetable food supplements safety is a major concern (Eddleston and Persson, 2003). Therefore, some natural plants are recommended after rigorous verification of safety and effectiveness of these plants to determine the optimal dose to consume. However, up to date, oral LD_{50}, NOAEL and LOAEL of phenolic fraction concentrate obtained from date seeds are not available in the literature. For this reason, investigating any possible toxicity is necessary to determine the adequate dose (Farsi et al., 2013). Acute oral toxicity of PFC was firstly investigated in a rat model. Consequently, the results obtained showed that PFC belongs to the category of products, which is practically non-toxic. In agreement, one study reported that the LD_{50} of the aqueous extract of *P. dactylifera* seeds tested in rats was greater than 5 g/kg. Therefore, the extract is considered to be non-toxic (Fakhri et al., 2018).

While the acute toxicity study does not predict the dose-response effect of PFC, it provides a significant guideline to select the appropriate doses for sub-acute or sub-chronic studies to determine the overall toxicity of this by-product. Additionally, a long-term study with appropriate doses of PFC will be required to rule out any adverse effects.

In the current study, the sub-acute toxicity study was carried out to identify and evaluate the effect of the oral administration of PFC at different doses on important indices of the physiological and pathological status of rats (Adeneye et al., 2006). In this study, except for a significant increase in red blood cells in the treated group with the middle PFC dose (100 mg PFC/kg), there was no significant difference in other blood elements in treated groups compared to the control group. These insignificant fluctuations are an important indication of the non-toxicity of PFC. In this context, a similar report about sub-chronic toxicity findings was also observed in a recent study conducted by Fakhri et al. (2018), which demonstrated non-significant alterations in the hematological elements in male and female rats that received an oral dose of 1000 mg/kg of lyophilized aqueous extract of date seeds. Additionally, sub-acute oral administration of PFC did not affect lipid, liver or renal parameters in all treated groups compared to the control group. In support of our findings, Plant products with LD_{50} greater than 2g/kg are considered free of any toxicity (Horn, 1956).

Most importantly, a significant improvement in blood glucose level was observed in the treated groups compared to the control group. This improvement may be due to the ability of PFC to inhibit digestive enzymes. Evaluation of blood glucose levels in Wistar normal rats confirmed the results obtained concerning the capacity of PFC to inhibit digestive enzymes. In agreement, Emerging reports show that date seeds compounds can decrease hyperglycemia through stimulating endogenous insulin secretion, inhibiting digestive enzymes linked to hyperglycemia and repairing pancreatic cells (El Fouhil et al., 2013; Saryono, 2019). Additionally, previous experimental studies demonstrated that phenolic content supplementation such as curcumin could control hyperglycemia and hypercholestrolemia due to their powerful antioxidant effect, digestive enzymes regulations and ability to repair pancreatic cells (Pandey and Rizvi, 2009; Abuelezz et al., 2020). The results of our study spot the promising ability of PFC of date seeds as a natural safe blood glucose regulator.

CONCLUSIONS

The present study suggests that the phenolic concentrate produced from date seeds possesses interesting phenolic compounds diversity and has significant antioxidant properties. PFC displayed significant *in vitro* inhibitory activity against alpha-amylase and alpha-glucosidase. Taken together this study highlights, the promising potential of phenolic date seeds fraction as a new sup-

plement for the management of diabetes mellitus. It also spots the possible protective effects of PFC of date seeds against oxidative stress. We highly recommend further studies to fully unleash the effects of date seeds PFC on diabetic animal models.

CONFLICT OF INTEREST

The authors declare no conflicts of interests.

ACKNOWLEDGMENTS

Bikri Samir wishes to thank Kaidi Amin for advice and help with dissection, also Malak Rhioui laboratory for extraction and fractionation. Many thanks to Oussama Chouiyakh for digestive enzymes protocols. Authors would also like to thank Bikri Sanae for help in the experimental section and Boutalaka Moulay Chrif for HPLC analysis.

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Contribution	Samir B	Aboussaleh Y	Abuelezz N	Benmhammed H	Berrani A	Louragli I	Ahami A
Concepts or ideas	x	x					x
Design	x	x					x
Definition of intellectual content	x	x					
Literature search	x		x	x		x	
Experimental studies	x	x		x	x		
Data acquisition	x					x	
Data analysis							
Statistical analysis	x	x			x		
Manuscript preparation	x	x	x	x	x	x	
Manuscript editing	x	x	x	x			
Manuscript review	x	x	x	x	x	x	x

Citation Format: Samir B, Aboussaleh Y, Abuelezz N, Benmhammed H, Berrani A, Louragli I, Ahami A (2021) Phenolic fraction concentrate of *Phoenix dactylifera* L. seeds: A promising antioxidant and glucose regulator. J Pharm Pharmacogn Res 9(6): 921–936.