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Research Article

Effect of pH Dependent Extraction in Anti-Inflammatory and Antioxidant Activity of Leaf Extract from *Phoenix dactylifera* L

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ABSTRACT
Leaf of *Phoenix dactylifera* L is a rich source of natural bioactive compounds, which serve many functions in human body and prevent food from oxidation processes. The study aimed to optimize the pH extraction medium of polyphenol from *Phoenix dactylifera* L using different system for pH 3 to pH 7 was carried out. In addition, influence of pH medium extraction on phenolic content, proanthocyanidins, anthocyanin, antioxidant and anti-inflammatory activities. The total phenolic content measured by Folin-Ciocalteu method was as well conducted. Antioxidant efficacies of different pH extracts were estimated by their abilities to scavenge ABTS and FRAP. It was observed that phenolic content increased from pH 5 at 6, and the pH effects on the composition extraction process were illustrated. For the antioxidant activity, similar results are observed. The high anti-inflammatory activity founded for weak acid (pH 5 and 6). The optimum pH value of medium aqueous extraction it is 6.

Keywords: pH, Medium extraction, Polyphenols, Antioxidant, *Phoenix dactylifera* L, anti-inflammatory.

INTRODUCTION

Phoenix dactylifera L. has a high nutritional value, which has raised its ranking as one of the best fruits in the world. In fact, next to its high contents of fiber, carbohydrate, vitamins, and minerals at the fully mature stage, it also contains significant levels of secondary metabolites[1-3]. The polyphenolic compounds exert a strong antioxidant enzymes such as manganese super-oxide dismutase and catalase[4,5]. Polyphenols can also regulate mitochondrial activity and energy homeostasis. During ageing, polyphenolic compounds such as flavonoids play an important role in the reduction of oxidative stress levels and maintenance of mitochondrial homeostasis. Polyphenolic treatment confers overexpression of genes for oxidative phosphorylation and mitochondrial biogenesis[6] and therapeutic benefits derive from their modulatory role in cell signaling and anti-inflammatory activity. In addition, the polyphenols may be important in the prevention of multiple diseases, cardiovascular and neurodegenerative diseases, atherosclerosis, type II diabetes, and cancer[7]. Moreover, such direct radical scavenging activity or reducing power of polyphenols is observed only at concentrations significantly higher than the physiological levels found in vivo. Increasing evidence shows that the in vivo anti-inflammatory effects of polyphenols arise from their ability in modulating cellular signaling transductions. Recent studies have established that these compounds do have significant modulatory effect on cellular biomarkers related to inflammation, which lead to reduced risk of many chronic diseases. The extraction of polyphenols is demanding due to their chemical structure and their interaction with other food components. Many factors, such as pH, type of the solvent, nature and preparation of material to be extracted, chemical structure of phenolic compounds, temperature, extraction time, solid-liquid ratio, extraction method employed and possible presence of interfering substances. Solvent extraction, i.e. solid-liquid extraction, is commonly used for the isolation of phenolic compounds from plant material[8,9]. The selection of pH of aqueous medium extraction is one of the most important steps in the extraction process. The present study was designed to investigate the influence of pH of aqueous medium extraction on amount of phenolic content, condensed tannins and total anthocyanin content. In addition, the effect in antioxidant and anti-inflammatory activities of leaf extract from *Phoenix dactylifera* L. The results help to find the preferred aqueous phase pH to have higher antioxidant and anti-inflammatory activities.

MATERIALS AND METHODS

Plant material: leaf from *Phoenix dactylifera* L were collected from south east of Algeria, state of El Oued on November 2014. The leaves were thoroughly washed, reduced into small pieces and dried in the laboratory at room temperature. The dried leaves powdered into particles. 20 g of the powder was extracted with 120 ml of water (60 °C) for 24 h using classical method in different pH (7; 6 ;5 ;4 ;3). Where the pH values were fixed by adding a solution of acetic acid to increase the acidity of pH either to lose, we added a solution of NaOH. At the end of the extraction, each extract was passed through

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Whatman No.1 filter paper. The filtrate obtained was concentrated in vacuum using evaporator and re-dissolved in the same extraction solvent (water) to prepare the required concentration for the subsequent essays.

Total phenolic content

The total phenolic contents in all organs were determined by the folin-Ciocalteu method[10]. Briefly, 100 µL of both the sample and the standard (gallic acid) of known concentrations were made up to 2.5 mL with water. In addition, mixed with 0.25 mL of 1N Folin-ciocalteu reagent. After 5 min, 2.5 ml of sodium carbonate aqueous solution (2%, w/v) was added to the mixture and was completed the reaction for 30 minutes in darkness at room temperature. The absorbance was read at 765 nm using a UV-visible spectrophotometer (Shimadzu UV-1800, Japan). For the blank the same protocol was used but the extract was replaced by solvent. The concentration of total polyphenols in the extracts was expressed as mg gallic acid equivalent (GAE) per g of dry weight using UV-Visible (Shimadzu UV-1800, Japan). All results presented are means (±SEM) and were analyzed in three replications.

Quantitave determination of condensed tannins (proanthocyanidins)

Determination of proanthocyanidins content was determined using a spectrophotometric method[11]. A volume of 0.5 ml of extract or standard (catechin) was added to the mixture of 3 ml of 4% vanillin- methanol (4%, v/v), 1.5 ml of hydrochloric acid and then vortexed. The resulting mixture was allowed to stand for 15 min at room temperature; the absorbance of each was measured at 500 nm using spectrophotometer (Shimadzu UV-1800, Japan). Total proanthocyanidin content was calculated as mg catechin equivalent (mg CTE/g).

Total anthocyanin measurement using pH differential

The total anthocyanin content of the leaf extract of *Phoenix dactylifera* L was evaluated using spectrophotometer (Shimadzu UV-1800, Japan) by the pH-differential method[12,13]. Two buffer solution: KCl buffer, pH 1.0 (0.025 M) and sodium acetate buffer, pH 4.5 (0.4 M). A volume of 3.6 ml for corresponding buffers added to 0.4 ml of different extract or standard (cyanidin-3-glucoside) and read against a blank at 510 and 700 nm. Absorbance (A) was calculated using the following equation: $\Delta A = (A510 - A700)_{pH\ 1.0} - (A510 - A700)_{pH\ 4}$. Monomeric anthocyanin pigment concentration in the extract was estimated as cyanidin-3-glucoside (mg/l) = A · MW · DF · 1000/ (MA · 1) where A: absorbance; MW: molecular weight (449.2); DF: dilution factor; MA: molar absorptivity (26,900). The total anthocyanin was expressed as milligrams of cyanidin-3-glucoside per g of dry Weight (mg/g).

Measurement of ferric reducing power

The reducing power was determined by using FRAP assay[14]. Briefly, the FRAP reagent contained 2.5 mL of 10 mM tripyridyltriazine (TPTZ) in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3$ and 25 mL of 0.3M acetate buffer (pH 3.6), was freshly prepared. A volume 0.2 mL, of extract or standard was mixed with 1.8 mL of freshly prepared FRAP reagent. The absorbance of each sample solution was subsequently measured at 595 nm. For the calibration curve, $FeSO_4$ was prepared in same solvent extraction in the range of 100–700 µM and Querecetin was used as positive controls. The results were expressed as mg/ml of Fe(II), using the equation obtained from the calibration curve of $FeSO_4$: $Y = 6.908x$, $R^2 = 0.998$.

ABTS assay (2,20-azinobis[3-ethylbenzothiazoline-6-sulfonate])[15]

The ABTS scavenging assay was carried out in triplicate, ABTS reagent was prepared by 10 mL of 7 mM ABTS solution and 178 µL of 140 mM potassium persulfate aqueous, the mixture was incubated at room temperature in darkness for for 13 h before use. 2 µL ethyl acetate extracts or standard was added to 1.588 µL diluted ABTS solution to react in the dark at room temperature for 10 min, and the absorbance was measured at 732 nm. The percentage inhibition of ABTS radical by the extract and BHT as calculated and compared following the equation: ABTS radical scavenging activity=[$(Abs_{control}-Abs_{sample})/Abs_{control}$] x100 (1), Where, $Abs_{control}$:Is the absorbance of ABTS radical + water, Abs_{sample} : Is the absorbance of ABTS radical + aqueous extract or standard.

β-Carotene linoleic acid bleaching assay[16]

The antioxidant capacity is determined by measuring the inhibition of the production of volatile organic compounds and the formation of conjugated diene hydroperoxides arising from linoleic acid oxidation. β-Carotene was prepared by dissolving 2 mg of this reagent in 10 mL of chloroform. In the round-bottom flask are added 2 mL of β-Carotene solution, 40 mg of linoleic acid and 400 mg of Tween 80, after this preparation, the chloroform is removed at 40 °C using the rotary evaporator). The resulted mixture was added to 100 mL of distilled water (aerated) under vigorous shaking and protection from the light. A 4.8 mL of the last solution was transferred into different tube containing 0.2 mL of each extracts in ethyl acetate with different concentrations. A control sample was prepared of 0.2 mL ethyl acetate and 4.8 mL of β-Carotene reagent. The tubes were incubated at 50 °C for 2 h. the absorbance at 470 was measured, using UV-Visible spectrophotometer. The essay was carried out in triplicate and the results were provided as 50 % inhibition (EC_{50} µg/ml). The antioxidant activity was calculated using the following equation (1).

Anti-inflammatory activity

Soybean lipoxygenase inhibition assay[17]

The assay is based on measuring the formation of the complex Fe3þ/xylenol orange in a spectrophotometer at 560 nm. 15-Lipoxygenase (15-LOX) was incubated with extracts or standard inhibitor at 25 °C for 5 min. Then linoleic acid (final concentration, 140 mM) in Tris-HCl buffer (50 mM, pH 7.4) was added and the mixture was incubated at 25 °C for 20 min. The assay was terminated by the addition of 100 mL of FOX reagent consisting of sulfuric acid (30 mM), xylenol orange (100 mM), iron (II) sulfate (100 mM) in methanol/water (9/1). For the control, only LOX solution and buffer were pipetted into the wells. Blanks (background) contained the enzyme LOX during incubation, but the substrate (linoleic acid) was added after the FOX reagent. The lipoxygenase inhibitory activity was evaluated by calculating the percentage of the inhibition of

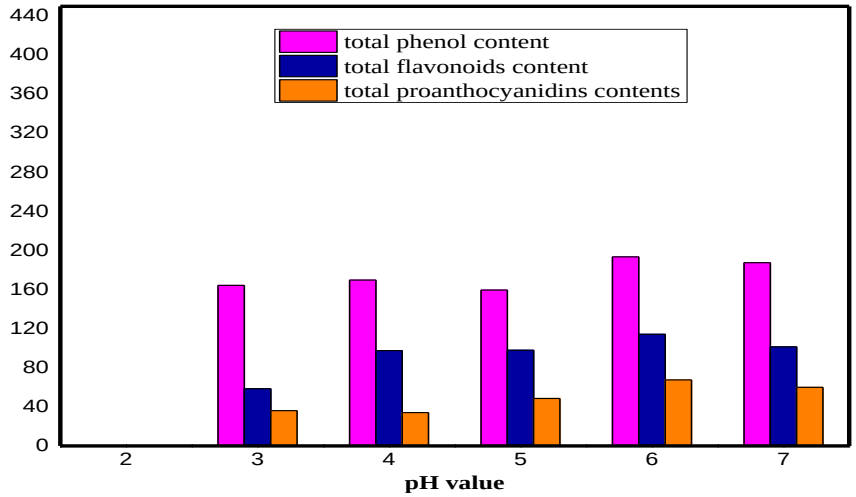

Figure 1: Total phenol content, total condensed tannin and Total proanthocyanidins of leaves extract of *Phoenix dactylifera* L obtained by different pH.

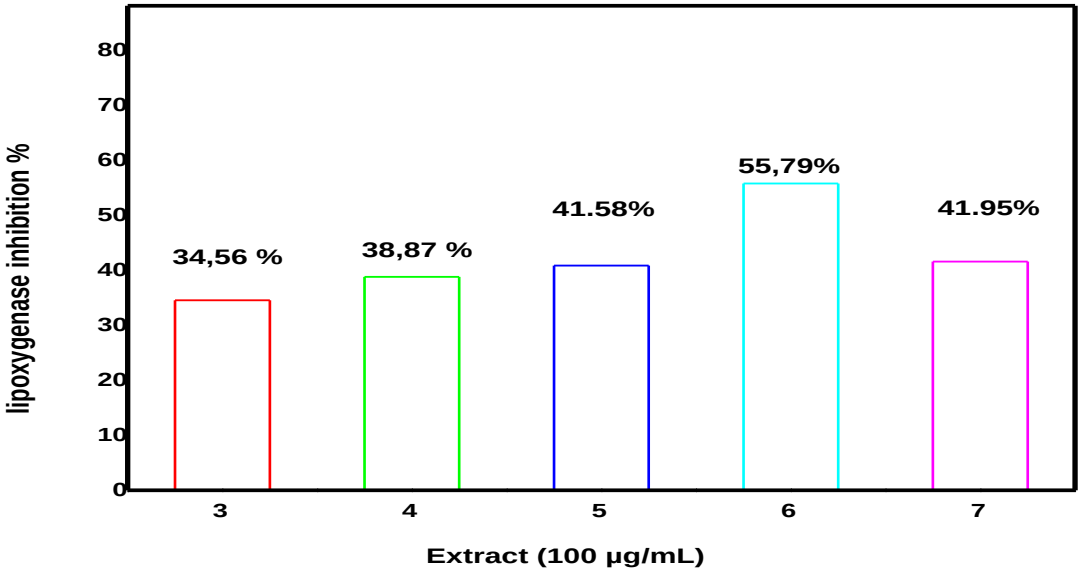

Figure 2: Percentage inhibition of LOX of different pH extract from *Phoenix dactylifera* L.

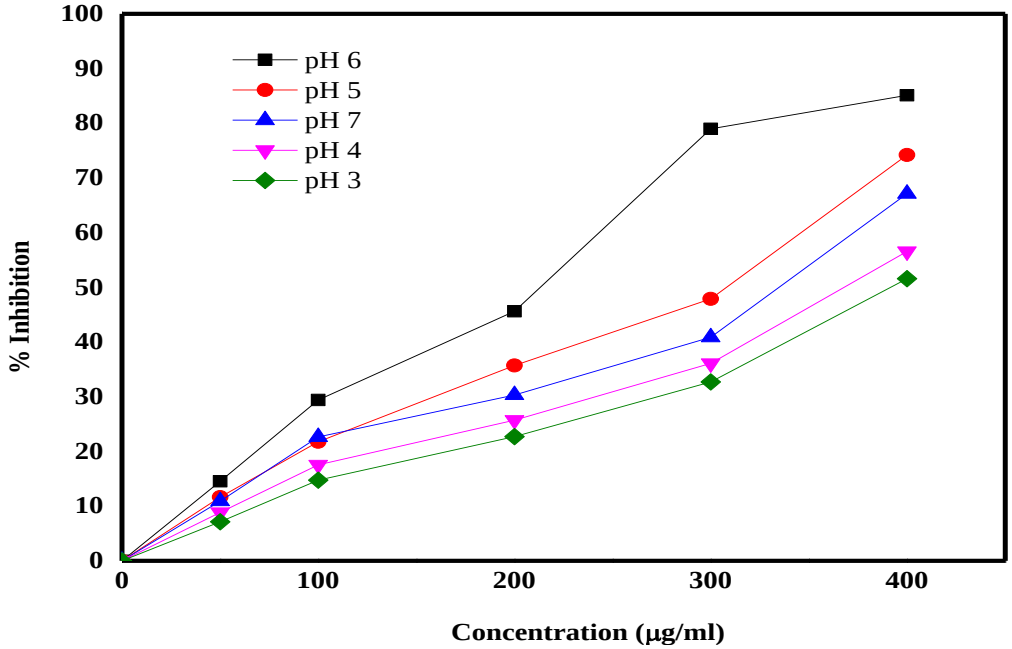

Figure 3: Nitric oxide scavenging activities of different pH extract of from *Phoenix dactylifera* L.

Table 1: Scavenging activity of FRAP and ABTS of *Phoenix dactylifera* L extracts obtained by different extraction pH value. Antioxidant activity was expressed as % inhibition IC_{50} values (µg/ml).

pH	FRAP (IC_{50}= µg/ml)	ABTS (IC_{50}= µg/ml)
3	156.96±0.30	75.92±0.60
4	164.73±4.48	68.34±0.71
5	168.04±1.86	63.62±0.69
6	178.31±5.15	44.12±1.08
7	169.27±1.77	52.81±0.73

hydroperoxide production from the changes in absorbance values at 560 nm after 30 min at 25 °C.

Nitric oxide generation and determination by Griess reagent

Nitric oxide was produced from sodium nitroprusside. It interacts with oxygen to produce nitrite ion and determined by the use of Griess reagent[18,19]. A volume of 2 mL of sodium nitroprusside prepared in saline phosphate buffer (pH= 7.4) was added to 0.5 mL of different concentrations of extracts, BHT and querecetin. The mixture was set at 25 °C for 150 min. 0.5 mL of each sample from above solutions were added to 0.5 mL of Griess reagent (1% sulphanilamid, 2% H_3PO_4 and 0.1% ACS reagent) and allowed to stand for 30 min. The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamid and subsequent coupling with naphtylethylene diamine was measured at 546 nm.

Statistical Analysis

Experimental values are given as means ± standard error (SEM) of three replicates. Statistical significance was determined by one way variance analysis (ANOVA).

RESULTS AND DISCUSSION

Total phenolic content: Figure 1 shows the TPC (mg GAE/ g of extract), Total proanthocyanidins contents (mg catechin E/g of extract) and total anthocyanin cyanidin-3-glucoside. Results of the analysis of polyphenols indicated that the pH=6 contained the highest concentration of polyphenols, condensed tannin proanthocyanidins (193.48±6.87, 114,28±5.75, 67,6±3.25 respectively), following by pH 7, pH 4, pH 5, pH 3 and the lowest value for pH value 2. Most plants including lettuce show maximum phenolic compounds activity at or near neutral pH values[20,21], pH is a determining factor in the expression of enzymatic activity. It alters the ionization states of amino acid side chains or the ionization of the substrate. One reason for the loss of antioxidant activity at pH 7 could be higher activity of phenolic compounds at this pH resulting in higher phenol degradation. The extraction amount of phenolic compounds from *Phoenix dactylifera* L increased as the pH values increased from 2 to 6, but it decreased as the pH values higher than 6 were used, which indicated that the extraction pH value significantly affected the extraction of phenolic compounds. The increased extraction of phenolic content under the low pH conditions could be due to the inhibition of the enzymatic oxidation of phenolics and/or the maintenance of the extracted[22].

FRAP assay

The antioxidant properties of a given compound depends not only on its chemical structure but also on the type of the generated radical, it can neutralize. For this reason, we tested the antioxidant potential of *Phoenix dactylifera* L extracts obtained by different extraction pH value (3, 4, 5, 6 and 7) against more than one radical type. The percentage inhibition of scavenging activities, at different pH extracts for ferric reducing, was shown in Table 1. The best antioxidant capacity founded for pH 6 (IC_{50}=178.31±5.15 µmol Fe II /g FW), while the pH 3 shows the least (IC_{50}=156.96±0.30 µmol Fe II /g FW). Phenolic and flavonoids have been reported to be the most important phytochemicals responsible for the antioxidant capacity. Plant-derived polyphenols display characteristic inhibition pat-terns toward the oxidative reaction in vitro and in vivo[23]. The higher FRAP activity of the extracts could be attributed to the high amount of polyphenolic content in the different pH extracts of *Phoenix dactylifera* L.

ABTS scavenging activity

The extracts of different pH value of leaves from *Phoenix dactylifera* L also measured and compared for their free radical scavenging activity against the ABTS radical. Table 1 shows that all extracts used in this study had significant ABTS radical scavenging activities. The IC_{50} values ABTS radical scavenging activities of extraction pH value 3, 4, 5, 6 and 7 were in the range of 52.81±0.73 µg/ml to 75.92±0.60 µg/ml. The highest ABTS radical scavenging activities was found in extract pH value 6 (IC_{50}=52.81±0.73 µg/ml, the medium pH 4 (68.34±0.71 µg/ml), pH 5 (63.62±0.69 µg/ml), pH 7 (52.81±0.73 µg/ml) and the lowest value in pH extract 3 75.92±0.60 µg/ml).

β-Carotene

In β-Carotene linoleate model system free radical arises from oxidation of linoleic acid, attacked by the highly unsaturated β-Carotene molecules and causing decrease in absorbance at 470 nm. The presence of different antioxidants can hinder the extent of β-Carotene blanching by neutralization of the linoleate-free and other free radicals formed in the system[24]. The results of inhibition of different pH extracts were showed in Table 2, the highest value obtained at pH 6 (IC_{50}= 343.53± 8.23 µg/mL), pH 5 (IC_{50}= 388.32± 7.03 µg/mL), pH 7 (IC_{50}= 395.53± 9.73 µg/mL) and pH 4 (IC_{50}= 429.72± 9.85 µg/mL). The lowest inhibition found for pH 3 (IC_{50}= 494.28 ± 11.35 µg/mL). The interaction of a potential antioxidant with β-Carotene depends on organ extracts. The results indicated and supported that the presence of phenolic content with high concentration in the extracts of can moderately prevent the degradation of β-Carotene caused by radical reactions. Thus, consumption of such underutilization of the antioxidant can protect the oxidation and degradation of cellular macromolecules due to free-radical attacks.

Soybean lipoxygenase assay

The 15-lipoxygenase inhibiting activity was measured using the 96-well micro plate based ferric oxidation of xylenol orange (FOX) assay. In a preliminary screening to select samples for dose-response study, extracts were tested at a single concentration of 100 µg/mL. The results presented in Figure 1 show that all the pH extracts

Table 2: β-Carotene bleaching activities of different pH extract from of *Phoenix dactylifera* L. Antioxidant activity was expressed as % inhibition IC_{50} values (µg/ml).

pH	Samples (µg/ml)	concentrations	Reaction time (min)		Antioxidant activity IC_{50}= (µg/ml)
			30	120	
		100	16.56 ± 0.50	9.35 ± 0.11	343.53± 8.23
6		200	31.25 ± 1.27	20.35 ± 0.73	
		500	70.32 ± 3.68	69.97 ± 1.43	
		50	12.37 ± 0.32	08.40 ± 0,29	388.32± 7.03
5		200	23.55 ± 2.01	18.60 ± 1.02	
		500	68.21 ± 2.12	59.41 ± 1.85	
		50	13.21 ± 0.02	06.92 ± 0.06	395.53± 9.73
7		200	20.61 ± 0.21	12.31 ± 0.73	
		450	58.23 ± 1.05	56.09 ± 1.05	
		50	11.45 ± 0.31	04.14 ± 0.15	429.72± 9.85
4		200	15.65 ± 1.06	13.86 ± 0.42	
		500	56.12 ± 1.45	53.34 ± 1.65	
		50	08.45 ± 0.31	03.14 ± 0.15	494.28 ± 11.35
3		200	10.65 ± 1.06	11.86 ± 0.42	
		500	46.12 ± 1.45	51.34 ± 1.65	

investigated had a high level of 15-lipoxygenase inhibitory effect. For the concentration at 100 µg/mL, the percentage of inhibition of pH 3, 4, 5, 6 and 34.56±1.53%, 38.87±1.42%, 41.58±1.23%, 55.79.82±1.07% and 41.95±1.38% respectively. Similar for the FRAP test, the highest inhibition at pH 6. LOX are the key enzymes in the biosynthesis of leukotrienes that play an important role in several inflammatory diseases such as arthritis, asthma, cancer and allergic diseases[25]. These results indicate that *Phoenix dactylifera* L has high anti-inflammatory effect, which can be presumably related to polyphenolic content and antioxidant property of extract[26].

Nitric oxide generation

The scavenging activity of the extracts against nitric oxide was calculated. All different pH extracts down-regulated NO production with IC_{50} < 380 µg/mL. The strongest effect was observed for the pH 6 with an IC_{50}= 224.67 ± 8.42 µg/mL. Regarding the other extracts, pH 5 IC_{50}= 277.53 ± 7.25 µg/mL, pH 7 IC_{50}= 298.77±7.25 µg/mL, pH 4 IC_{50}= 335.87 ± 7.25 µg/mL and pH 3 IC_{50}= 373.64 ± 7.15 µg/mL, the results were shown in figure 2. Both oxidative stress and inflammation can cause pathogenesis of chronic diseases and metabolic disorders, and anti-inflammatory effects of phenolics can essentially affect similar biomarkers. In the last five years, numerous in vivo and in vitro studies have shown that dietary phenolic compounds have protective effects on inflammation through modulating NLRP3 activation. The propolis extracts rich in cinnamic acids such as p-coumaric acids inhibited inflammasome mediated secretion of IL1b and activation of caspase (caps)-1 from ex vivo inflamed mouse macrophages[27]. Flavonoids such as apigenin and procyanidin B2 were also able to inhibit inflammasome activation and IL-1b secretion in LPS-in- duced human macrophages[28].

CONCLUSION

In this study, phytochemical investigation, in vitro antioxidant and anti-inflammatory activities of different pH extracts (3, 4, 5, 6 and 7) of leaves from *Phoenix dactylifera* L have been evaluated. The results indicated that the extract obtained by pH 6 exhibited highest phytochemical compounds, strongest antioxidant and anti-inflammatory activities. The contents of polyphenols extract were significantly higher than other extraction pH value, which were possibly responsible for higher antioxidant activities. From the results, we can draw the conclusion that not only the more bioactive components obtained but also the extract has better free radical and reactive oxygen species scavenging activities through extract pH value 6. These findings further illustrate that extraction of pH 6 has a bright prospect for extracting active ingredients from plant materials than the other extraction pH value 3, 4, 5 and 7.

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