Available online on www.ijcpr.com
International Journal of Current Pharmaceutical Review and Research; 7(2); 84-89

ISSN: 0976 822X

Research Article

Influence of Extraction Method on Phytochemical Composition and Antioxidant Activity from Leaves Extract of Algerian *Phoenix dactylifera* L.

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Available Online:17th January, 2016

ABSTRACT

The genus Phoenix is one of the most widely cultivated groups of palms around the world. In this study, we have evaluated the antioxidant activity and total phenolic compounds by using various in vitro systems and analysis of marker compounds by High performance liquid chromatography (HPLC) of leaves extracts of *Phoenix dactylifera* L obtained by classical, ultrasonic assisted and Soxhlet method. The total phenolic content, flavonoids and tannin were determined by a spectrophotometric method. Total antioxidant activity expressed by reducing Mo (VI) to Mo (V) and DPPH radical-scavenging methods were applied to test the antioxidant activities. The results indicated that the extract obtained by the ultrasonic assisted method extraction showed the highest total contents of phenolic. The antioxidant activities also found to be highest with the ultrasonic assisted method extract followed by other extraction techniques (Soxhlet and classical method). The results suggest that the leaves of the *Phoenix dactylifera* L can be considered as a good source of natural antioxidant, that we can use these natural extracts as food additives in replacement of synthetic compounds

Keywords Antioxidant activity, DPPH, technique extraction, phenolic, *Phoenix dactylifera* L, HPLC.

INTRODUCTION

Free radicals are chemically unstable atoms that cause damage to lipid cells, proteins and DNA as a result of imbalance between the generation of reactive oxygen species (ROS) and the antioxidant enzymes[1]. They are known to be the underlying cause of oxidative stress which is grossly implicated in the pathogenesis of various diseases such as cancer, diabetes, cardiovascular diseases, aging and metabolic syndrome. Examples of these radicals include superoxide anions, hydroxyl, nitric oxide and hydrogen peroxide radicals[2]. An antioxidant, which can quench reactive free radicals, can prevent the oxidation of other molecules and may have health-promoting effects in the prevention of degenerative diseases[3]. The interest in antioxidants has been increasing because of their high capacity in scavenging free radicals related to various diseases[4]. Many plants contain substantial amounts of antioxidants and can be utilized to scavenge the excess free radicals. The protection offered by different edible plants against oxidative stress in several diseases has been attributed to various antioxidants and vitamins. Potential antioxidant properties of the dietary phenolic compounds and flavonoids present in various fruits and vegetables have recently been recognized in a number of investigations[5,6]. The date palm (*Phoenix dactylifera* L., Arecaceae) is one of mankind's oldest cultivated plants. It is highly popular worldwide, particularly in the Middle East and North Africa[7]. Different extraction techniques, such as classical, Soxhlet and ultrasonic extraction, are widely used for obtaining extractive substances from *Phoenix dactylifera* L. As a novel technique, ultrasonic extraction has recently been shown to be very promising and effective for obtaining bioactive substances. The main benefits of the use of ultrasound are the increase of the extraction yield, a faster process and even the improved quality of the extracts[8]. The aim of present works is to study in vitro antioxidant activities of the acetone extracts of *Phoenix dactylifera* L obtained by different methods extraction, using PPM and DPPH radical scavenging assays. In addition, the total phenolic content, flavonoids and condensed tannin from plant extracts were also measured.

MATERIALS AND METHODS

Origin of plant material

The *Phoenix dactylifera* L were collected from southeast of Algeria, state of El Oued on December 2013. The leaves then separated from each other, washed and dried at room temperature. All these organs were ground to a powder with a basic electric grinder and stored in the dark at room temperature before use.

Extraction of plant material

Classical extraction

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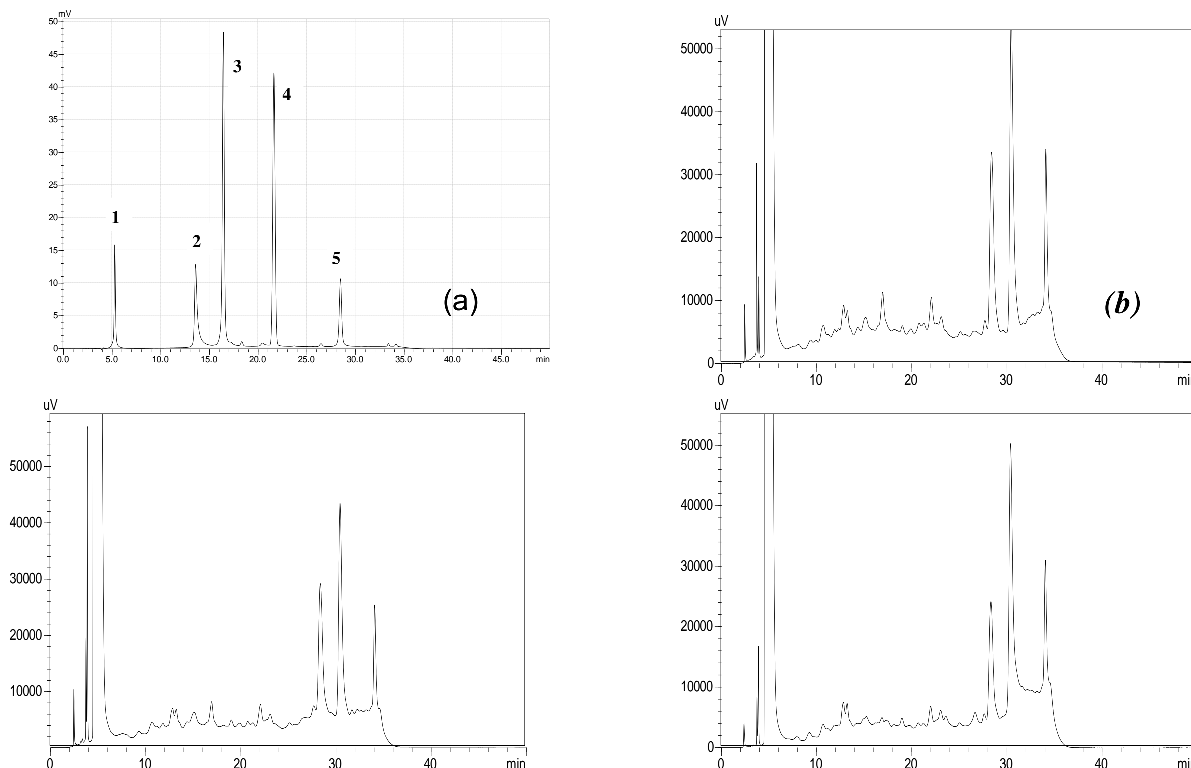

Figure 1: Chromatograms of standard mi), classical extraction (b), ultrasound extraction (c) and extraction (d). Peak 1: Ascorbic acid; Peak 2 ogenic acid; Peak 3: Caffeic acid; Peak 4: Vanillin; Pea in.

Ground plant material (15 g) and 70% Acetone (90 ml) were put in a series of the Erlenmeyer flasks (150 ml), and the ratio of plant material mass (g) to solvent volume (ml) was 1:8. No additional stirring was applied. The extraction was performed at 25°C for 24h. At the end of the extraction cycle the liquid extract was separated from the solid residue by vacuum filtration. The solid residue was washed twice with fresh solvent (20 ml). The filtrates were collected and the solvent was evaporated in a rotary vacuum evaporator at 40°C. Extracts were stored at +4 °C in dark until use.

Ultrasound extraction

Ultrasonic apparatus from Branson (40 kHz, 1500W, dimension 49 cm×14 cm×15 cm) was used for accelerated extraction. A beaker was partially submerged in an isothermal water bath to maintain the extraction temperature at 25°C. Fifteen grams (15 g) leaves were then extracted with 70% Acetone (90 ml) for 60 min. The extract was filtered through Whatman paper on a Büchner funnel by vacuum; the solids were washed with an additional 60 ml of ethanol. The filtrate was rotary-evaporated under vacuum at 40◦C to dryness. The crude extracts were dried in a vacuum oven at 40 ◦C. Extracts were stored at +4°C in dark until use.

Soxhlet extraction

The ground plant material (15 g) and 70% Acetone (90 ml) were taken into the Soxhlet apparatus, and were extracted for 6 hours. Previously, we found that the yield of extractive substance was not increased after that time. The liquid extract was evaporated under vacuum at 40 °C to constant mass. Extracts were stored at +4 °C in dark until use.

Evaluation of antioxidant activity

Determination of total antioxidant activity

The total antioxidant activity of the extracts was estimated by the phosphomolybdenum method. This method was based on the reduction of MO (VI) to MO (V) by the extract and the formation of a green phosphate/MO (V) complex at acid pH[9]. An aliquot of 0.2 mL of the sample solution was mixed with 2.0 mL of the reagent solution (600 mM sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The reaction mixture was incubated at 95°C for 60 minutes and absorbance was measured at 695 nm against a blank contained 2 mL of reagent solution. Total antioxidant activity was expressed equivalent to gallic acid[10].

DPPH radical scavenging activity

The radical scavenging activity using free-radical DPPH assay was carried out using the spectrophotometric method[11]. An aliquot (1mL) of each extract was added to 2 mL of daily prepared acetone DPPH solution (0.1mM). The mixture was shaken gently and left to stand at room temperature in the dark for 15min. Thereafter, the absorbance was read at 515 nm. The scavenging activity was measured as the decrease in absorbance of the samples versus DPPH standard solution. Results were expressed as radical scavenging activity percentage (%) of the DPPH radical according to the formula:

$$\%\text{DPPH radical scavenging} = [(A_0 - As)/A_0] \times 100$$

Where A_0 is the absorbance of the control and As is the absorbance of the sample. The effective concentration having 50% radical inhibition activity (IC_{50}), expressed as mg extract/ mL, was determined from the graph of the free radical scavenging activity (%) against the extract

Table 1: Total phenolic, flavonoid and condensed tannin contents of different extract of *Pheonix dactylifera* L

phytochemical	Method		
	Classical extraction	Ultrasound extraction	Soxhlet extraction
phenols	484.44 ± 4.38	625.17 ± 11.82	528.81 ± 29.55
flavanoids	205.94 ± 2.39	139.8 ± 0.45	102.06 ± 1.90
tannins	246.19 ± 21.34	240.79 ± 20.37	269.94 ± 18.24

Values are expressed as means ± SD of triplicate measurements.

Table 2: Total antioxidant activity and capacity (PPM and DPPH assays) of different extract of *Phoenix dactylifera* L

Method	Antioxidant activity	
	PPM (mg AAE/g DW)	DPPH. IC_{50}= (µg/ml)
Classical extraction	445.77 ± 14.43	0.0144 ± 0.0002
Ultrasound extraction	608.34 ± 21.32	0.0104 ± 0.0001
Soxhlet extraction	531.43 ± 16.83	0.0132 ± 0.0004

Values are expressed as means ± SD of triplicate measurements.

concentration[12]. All results are means ±SD.

Phytochemical investigation

Determination of total phenolic content (TPC)

The total phenolic contents in all organs were determined by the Folin-Ciocalteu method[13]. Diluted acetone extracts (0.2 ml) was mixed with 1 ml of (10%) Folin-Ciocalteu reagent. After 5 min, 0.8 ml of 7.5% sodium carbonate solution was added to the mixture and incubated for 30 min at the 27±2 °C. Absorbance was measured at 765 nm on spectrophotometer (Shimadzu UV-1800, Japan). Total phenolic content was standardized against gallic acid and expressed as milligrams of gallic acid equivalents (GAE) per gram of the sample[14].

Determination of total flavonoid content (TFC)

The total flavonoid content in extracts was determined spectrophotometrically[15]. Briefly, 1 mL of 2% aluminum trichloride (AlCl3) acetone solution was mixed with the same volume of extract solution. The absorbance values of the reaction mixtures were determined at 415 nm after 10 min duration against a blank. Rutin was used as the standard and the total flavonoids content of the extracts was expressed as mg rutin equivalents per gram of extract (mg RE g^{-1} extract)[16].

Determination of condensed tannin content (CTC)

CTC was determined by a miniaturised procedure[17]. Firstly, a 10 µl acetone solution of samples, catechin or blank were introduced in wells. After wards a 4% vanillin acetone solution (120 µl) was added, followed by addition of concentrated HCl (60 µl). The red mixture was allowed to stand for 15 min and the absorbance was measured at 500 nm using a microplate spectrophotometer (Shimadzu UV-1800, Japan). Results were ex pressed in mg of catechin equivalents/g of dry extract (mg CE/g). All samples were analysed at least three times[18].

HPLC analysis

The composition of the extracts was analyzed by high performance liquid chromatography (HPLC)[19]. We used a Shimadzu (LC 20A, Japan). System comprised of a 2LC-10AT pump (A**&**B), a CTO-20A column oven, a SPD-20A UV-DAD detector and CBM-20A interface. A LC-18 column (150 mm x 4.6 mm i.d. x 5 mm) was employed. Samples were injected. The components of the samples were separated by gradient elution at at 30 °C. The mobile phases were: A, 98:2 (v/v) acetic acid, and B, acetonitrile and the elution gradient was: 0–5 min, 5% B; 10 min, 10% B; 11 min, 20% B; 20 min 20% B; 30 min 40% B; 40 min 50% B; 50 min 80% B. The flow rate was 1 ml/min and the detection wavelength was 300 nm. Phenolic compound standards Ascorbic acid, Chloregenic acid, Vanillin, Caffeic acid and Rutin were dissolved in solvents extraction and used for identification of the phenolic acids present in different extracts of *Phoenix dactylifera* L. Peak identification in HPLC analysis was achieved by comparison of retention time and UV spectra of reference standards. Quantification of individual phenolic compounds in the extracts was done using the peak area of reference compounds and reported as mg/g of extract.

Statistical analyses

The data obtained in this study were expressed as the mean of three replicate determinations plus or minus the standard deviation (SD). Statistical comparisons were made with Student's test. *P* values <0.05 were considered to be significant.

RESULTS AND DISCUSSION

In the present study, three extraction methods were used to evaluate the total phenolic contents, total flavonoids, condensed tannin contents and antioxidant activity of leaves extracts from *Phoenix dactylifera* L. Among the different solvent extraction methods, successive Soxhlet extraction could provide comparable or even better results than the maceration and ultrasonic assisted for extracting phenolic compounds and showed a significant antioxidant activity over the other two methods. Nowadays, several novel extraction techniques such as sonication and microwave-assisted have been developed and employed along with conventional extraction techniques for the extraction of bioactive compounds and nutraceuticals from plants[20].

Total phenolic, flavonoid and condensed tannin contents

Phytochemicals are increasingly accepted as health promoting, maintaining and repairing agents in cells, tissues, or the whole human body. The phytochemicals that are frequently associated with human health are polyphenols, carotenoids and tocopherols. Polyphenols are the major secondary metabolites in most plants reported to possess antioxidant and free radical scavenging activity. There are several studies on dietary polyphenol use to

Table 3: Constituents content analysed by HPLC.

Method	Ascorbic acid (µg/mg)	Chlorogenic acid (µg/mg)	Caffeic acid (µg/mg)	Vanillin (µg/mg)	Rutin (µg/mg)
Classical extraction	190	1.62	1.86	0.53	10.40
Ultrasound extraction	190	1.11	1.44	0.36	8.37
Soxhlet extraction	200	0.52	0.079	0.23	6.44

Values are mean±standard deviation of three separate determinations (n=3). All results are significantly at $p<0.05$

decrease the risk of coronary heart disease[16]. Phenolic compounds, tannins and flavonoids have been reported to have multiple biological effects, including antioxidant and anti-inflammatory properties[21].Recent evidences suggest that diets rich in phenolic compounds play a significant role against oxidative stress related disorders because of their antioxidant activities[22]. The total phenolic, flavonoid and tannin contents in the *Pheonix dactylifera* L extracts are summarized in Table 1.

Results of the total phenolic content showed that maximum phenolic content was obtained with ultrasound extraction (625.17 mg GAE/g of fw) and the minimum phenolic content was obtained with Classical extraction (484.44 mg GAE/of fw).

Total flavonoid content (TFC) of different extracts was measured using aluminum chloride colorimetric methods (Table 1). The results showed that the TFC of *Phoenix dactylifera* L varied considerably from 102.06 to 205.94 mg in terms of rutin equivalents/ g fw of sample. The total flavonoid in increasing order was: CE > UAE > SE. Flavonoids are considering as phenolic compounds with highest antioxidant activity due to their chemical structure .Plant flavonoids are an important part of the diet because of their effect on human nutrition [23]. Even though it is apparent that the flavonoids were an important phenolic compounds contributing to the antioxidant activity of date palm, it is also possible that other phenolic compounds could also contribute to the antioxidant properties of these plant [3]. In the case of both *Phoenix dactylifera L* the highest amounts of the total phenolic compound and flavonoids are found in the extracts obtained by ultrasound extraction and classical extraction respectively, and the lowest one is obtained by the Soxhlet extraction. This may be explained by oxidation and degradation of these bioactive compounds under higher extraction temperature and the much longer extraction time of the Soxhlet extraction[24]. The plant species and the extraction method have a statistically significant influence on the total phenolic and flavonoid content in the extracts.

The analysis of the results of tannins condensed consigned in table 1, reveals that the Soxhlet extraction is more effective for the extraction of the tannin (269.94 mg Ca/g of fw) that the other techniques extraction (classical extraction and ultrasound extraction respectively). The increase in the temperature supports on the one hand the diffusion and the solubility of the extracted substances; on the other hand, it destroys certain substance fragile[25]. This increase in the contents of tannin condensed in this extract can be explained by the destruction by the heat of the polyphenols oxidases (PPO which lower the content polyphenols; thus, the rupture of connections between polyphenols and other substances (proteins, polysaccarides...) driving with accessibility with this active ingredient can explain its share this abundance[26].

Antioxidant activity

For evaluating the effectiveness of antioxidants, different methods specific to their chemical properties have been used. In this study two complementary methods were followed to evaluate the antioxidant activity due to their simplicity, stability and accuracy.

Total antioxidant activity

The phosphomolybdenum method is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compounds and the formation of green Mo (V) complexes with a maximal absorption at 695 nm[27,28]. Using this method, the result indicated that the UAE extract of *Phoenix dactylifera* L had the highest antioxidant capacity with a value of 608.34±21.32 mg ascorbic acid equivalent/g dry weight; this activity may be due to the presence of phenolic compounds[29]. The SE and CE extracts showed lower activity with values of 531.43±16.83 and 445.77±14.43mg ascorbic acid equivalents/g dried extract, respectively (Table 2).

DPPH radical-scavenging activity assay

The DPPH is a stable free radical and has been widely accepted as a tool for estimating free radical scavenging activities of antioxidants[30]. Results in Table 2, demonstrated that all samples exhibited dose-dependent DPPH radical scavenging activities. The interaction of a potential antioxidant with DPPH depends on the extraction technique and the plant species[8]. For the plant The UAE exhibited the strongest antioxidant activities against DPPH radicals IC_{50}= 0.0105 µg/ml, followed by SE IC_{50}= 0.0115µg/ml and the lowest value in CE IC_{50}= 0.0145 µg/ml.

In previous investigations of *Phoenix dactylifera* L varieties, evaluation of the antioxidant activity and the presence of polyphenol compounds were reported[31].

HPLC analysis

HPLC fingerprinting provides the chemical characterization of the crude extract. It is known that the polyphenols and flavonoids, being secondary metabolites, are present in several plants and can serve as markers of the crude extract. Therefore, phytochemical analysis of the crude extract was assessed by analysis[32].

The constituents in the different extracts were analyzed by HPLC. Figure 1 showed the chromatograms of three extracts sample and standard markers mixture. Peaks 1, 2, 3, 4 and 5 were ascorbic acid, chlorogenic acid, caffeic acid, vanillin and rutin, respectively. The contents of these components in different extracts were determined according to the calibration curves of ascorbic acid $y = 2260.81x$ ($r^2 = 0.998$), chlorogenic acid $y = 39728x-18813$ ($r^2 = 0.996$), caffeic acid $y = 75728x$ ($r^2 = 0.986$), vanillin

y = 82773x-14238 (r^2 = 0.99) and rutin y = 24113x-10605 (r^2 = 0.996), where y was the peak area and x was the concentration of analytes. The quantitative results are summarized in Table 3. As shown, ascorbic acid was the most dominant constituent and similar in the different extracts. All The extracts obtained by different techniques are contains five compounds.

CONCLUSION

In this study, in vitro antioxidant activities, phenolic, flavonoids and tannin contents of leaves extracts from *Phoenix dactylifera* L obtained by classical, Soxhlet and ultrasonic assisted methods have been evaluated. The results indicated that ultrasound extract exhibited strongest antioxidant activities and the contents of polyphenols. The highest content of flavonoids was obtained by classical extract than those by other extraction methods. This study explained the advantage of the ultrasound, compared to the conventional extraction methods both for polyphenols, was similar since polyphenols yield obtained with a lower solvent consumption and a shorter extraction time.

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