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Research Article

In Vitro Free Radical Scavenging Activity and total Phenolic and Flavonoid Content of Spathe Extracts from 10 Cultivar Varieties of *Phoenix dactylifera* L.

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ABSTRACT

This research work deals with the study of antioxidant activity and evaluation of total phenol and flavonoid content of spathes ethanolic extracts of various *P. dactylifera* L. cultivar varieties including Mazafati, Piarom, Khasooei, Shakri, Hanaei, Hajghanbari, Shahani F (female tree), Shahani M (male tree), Halileh and Zahedi. Total phenolic content (TPC) was determined by Folin-Ciocalteu test and total flavonoids content (TFC) by Dowd method. The antioxidant activity evaluation was conducted using various free radical assays including DPPH (2, 2-diphenyl-1-picrylhydrazyl), NO (Nitric Oxide) and ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid). Ferric Reducing Antioxidant Power (FRAP) assay was also performed as a measure of antioxidant capacity of the extracts. Results declared highest phenol and flavonoid contents for Shahani M variety, 38.23 mg gallic acid equivalent (GAE)/g and 1.12 mg quercetin equivalent (QE)/g of dry spathe respectively when compared to other cultivars. This variety exhibited significant antioxidant activity against DPPH free radical. Strong scavenging ability was recorded for Mazafati extract (IC_{50}, 19±0.02 µg/mL) against ABTS free radicals while the extracts of six varieties surpassed quercetin in manifestation of nitric oxide inhibitory properties. The results also showed highest FRAP value for Shahani M extract (IC_{50}, 78±0.32 µg/mL). Despite minor variations observed in phenol and flavonoid contents and the antioxidant activity among different varieties, these *P. dactylifera* spathe extracts can be considered as potential sources of natural antioxidants.

Keywords: *Phoenix dactylifera* L. spathe, phenol, flavonoid, antioxidant

INTRODUCTION

Free radicals are mediators of a wide variety of clinical disorders and strongly connected to various diseases like atherosclerosis, crcinogenesis and aging[1,2]. During past two decades there has been increasing interest in herbal antioxidants due to their specific role in protecting health and preventing diseases. Therefore, to achieve new sources of antioxidants many efforts have been devoted to biological screening of various plant extracts. Antioxidants have been defined defined as "any substance that, when present at low concentrations compared to those of an oxidisable substrate, significantly delays or inhibits oxidation of that substrate"[3]. Various studies have demonstrated that many plant derived compounds are more powerful than vitamin E or C in manifestation of antioxidant activity[4,5]. *Phoenix dactylifera* L. commonly known as date palm is a member of Arecaceae family which is widely cultivated in southern Iran due to its nutritional and economic values[6]. Date fruit is considered as a complete diet and used in the control and prevention of several diseases such as renal dysfunction, limb and face paralysis and rheumatic diseases in traditional Persian medicine[7]. The fruits and seeds of *P. dactylifera* L. are rich sources of phytochemical compounds like phenolic acids, carotenoids, flavonoids, anthocyanins and carbohydrates[8-10]. The antioxidant activity of date fruit has been reported in the literature[11,12]. Spathe is the inflorescence sheath of *phoenix dactylifera* commonly called "Tarooneh" in Persian which is removed from male and female date palm trees during pollination and insemination. It has a specific fragrance particularly when it is fresh and is utilized in the large scale production of Tarooneh hydrodistilled water in Iran.This hydrosol has been used for the treatment of skin disorders, peptic ulcers and connective tissue diseases in folk medicine[13]. We have previously reported the characterisation of volatile constituents of spathes of various *P.dactylifera* varieties[14]. Results of a careful review of literature prompted us to conduct a further study on the antioxidant capacity of date spathe which is reported herein. The main objective of this research was to evaluate the *in vitro* free radical scavenging activity of various date spathes ethanolic extracts against different free radicals. Meanwhile the total content of major chemical components of the extracts like phenols and flavonoids were determined in the present study.

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MATERIALS AND METHODS

Chemicals and Reagents

Folin-Ciocalteu, Ethanol 96%, Methanol, Aluminum chloride, Sodium carbonate (Na_2CO_3), Acetate buffer, Ferric (III) chloride ($FeCl_3.6H_2O$), Hydrochloric acid, and Ammonium persulphate were purchased from Merck (Darmstadt, Germany). Quercetin, Galic acid, DPPH, NO and ABTS and TPTZ (2, 4, 6-tripyridyl-s- triazine) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plant material and sample preparation

The spathes of ten cultivars of *P. dactylifera* L. were collected from Jahrom region at flowering stage in May 2014. Plant samples were *authenticated* by the plant toxonomist of Jahrom Agricultural Research Center and a voucher specimen of each spathe variety (MRCH-91-54-63) were prepared and deposited at the herbarium of Medicinal Plants Processing Research Center, Shiraz University of Medical Sciences. The plant material was dried in shade and powdered separately. Each sample (30 g) was extracted with Ethanol (96%) for 3 hours using a soxhlet apparatus. The extracts were concentrated under reduced pressure at 40°C on a rotary evaporator. All extracts were further concentrated in a speed vaccuum concentrator and freeze dried in a Christ Alpha 1- 4 LD Freeze dryer (Martin Christ, Germany) and stored at 2 °C pending analysis.

Determination of total phenol

Total phenolic content of the spathes ethanolic extracts were determined using a Folin-Ciocalteu spectrophotometric method[15]. To develop a standard calibration curve, 0.5 mL of gallic acid solutions of various concentrations ranging from 6.25-200 mg/L were mixed with 5 mL of 10% Foiln-Ciocalteu reagent and 4 ml of 1.0 M sodium carbonate solution. Proper dilutions of the extracts were oxidized with Folin–Ciocalteu reagent and neutralized by sodium carbonate solution as given for standard. The absorbance of solution was recorded at 765 nm after 15 min against Folin-Ciocalteu reagent as blank using a PG instrument T90 UV spectrophotometer. Total phenolic content was expressed as mg of gallic acid equivalent(GAE)/g of dry plant material using the expression from the calibration curve ($Y=0.00571x - 0.0843$, $R^2 = 0.9985$). Where Y is the absorbance and x is the gallic acid equivalent.

Determination of total flavonoid content

Total flavonoid content of the extracts was determined by an aluminum chloride colorimetric assay as described previously[16]. This colorimetric procedure is based on an aluminum (III) complex formation reaction. For preparation of calibration curve 5 ml of 2% aluminum chloride solution in methanol was mixed with the same volume of quercetin (0-80 mg/L) as a standard. Absorption was measured at 415 nm on a PG-T90 UV spectrophotometer after 10 min. For quantitative determination of total flavonoid content, a solution of 300 mg/L of each extract in methanol was prepared and the analysis was performed using the above procedure. For the control, *methanol* was used instead of the *extract* while *the blank* contained 2 ml of extract and 2 mL of methanol. Total flavonoid content was expressed as mg of quercetin equivalent (QE)/g of dry plant material using the equation obtained from the calibration curve ($Y = 0.02985\ x + 0.04811$, $R^2 = 0.9978$). Where Y is the absorbance and x is the quercetin equivalent.

DPPH Free Radical Scavenging Assay

DPPH radical scavenging activity of the spathe extracts and quercetin were determined using a previously reported procedure[17]. Twenty microliters of various concentration of the methanolic solution of extracts (6.25-200 µg/mL) were mixed with 200 µL solution of 100 mM DPPH in methanol. The mixture was left in the dark for 30 min and the absorbance was measured at 490 nm using a Biotek, ELX800 microplate reader. A sample containing 20 µL of methanol and 200 µL of DPPH solution served as control while a solution of equal amount of extract in methanol was used as the blank. All tests were conducted in triplicate. Percentage inhibition of extracts against DPPH were calculated using the following equation:

$$\%\ \text{Inhibition} = 100 - [(A_{sample} - A_{blank} / A_{control}) \times 100]$$

Nitric Oxide Scavenging Assay (NO)

Nitric oxide assay was carried out following a slightly modified method of Lee et al[18] used in our earlier study. Sodium nitroprusside, SNP (10 mM) in phosphate buffer saline (pH =7.4) was mixed with various concentrations of extracts (6.25-3200 µg/mL) in ethanol and incubated at 25°C for 150 minutes and further mixed with 100 µL of Griess reagent. The absorbance of chromophores formed was read at 542 nm after shaking using a BioTek Epoch microplate spectrophotometer and referred to the absorbance of ascorbic acid used as a positive control. Blank contained 50 µL of extract while control consisted of 50 µL of SNP, 50 µL of ethanol and 100 µL of Griess reagent. Each assay was performed in triplicate. In this assay aqueous solution of SNP at physiological pH spontaneously generates NO which interacts with oxygen to produce nitrite ions that can be estimated by Griess Reagent. Scavengers of NO compete with oxygen leading to reduced production of NO[19]. The nitric oxide inhibitory capacity of the test samples was calculated using the following equation:

$$\text{Percentage of inhibition} = \frac{\text{Abs. control} - \text{Abs. test}}{\text{Abs. control}} \times 100$$

ABTS Radical Scavenging Activity

$ABTS^+$ radical scavenging assay were conducted using the method described by Re et al[20]. $ABTS^+$ was freshly prepared by adding 5mL of Ammonium persulphate solution (4.9 mM) to 5 mL of ABTS solution (14 mM). The mixture was kept in the dark for 16 h and then filtered and diluted with ethanol 96% (1:1) to obtain an absorbance of 0.7±0.02 at 734 nm. The solution of $ABTS^+$ radical (950 µL) was added to 50 µL of various concentrations of the extracts (6.25-500 µg/mL) and vortexed for 10 sec. The reaction mixture was stored at room temperature for 6 min and the absorbance was measured at 734 nm in a microplate spectrophotometer (Epoch BioTek, USA). A mixture of 950 µL of ABTS solution and 50 µL ethanol 96% served as control. Percentage inhibition of $ABTS^+$ free radical was calculated on the basis of the following

Table 1: Yiled of Extracts, total phenol and flavonoid contents and *free radical scavenging* activity of spathes extracts of *P.dactylifera* L. varieties

Spathe /STD	Yield (W/W%)	Total Phenol Content[b]	Total Flavonoid Content[c]	IC50 (µg/mL) DPPH	NO	$ABTS^+$	FRAP (FE mM)
Mazafati	11.93	26.38±0.27	0.60±0.01	82.00±0.21	84.36±2.87	19.01±0.32	2.26±0.09
Piarom	13.40	9.21±0.09	0.40±0.02	206.0±0.63	38.00±1.75	172.0±0.57	1.12±0.05
Khasooei	10.83	15.31±0.26	0.88±0.04	122.0±0.57	55.00±1.78	63.07±0.03	4.17±0.08
Shakri	7.50	21.10±0.48	0.23±0.07	79.00±0.57	59.08±0.79	56.06±0.05	3.04±0.06
Hanaei	7.08	21.08±0.16	0.49±0.09	89.00±0.19	40.68±2.75	83.05±0.55	2.56±0.11
Hajghanbari	5.76	17.11±0.21	0.53±0.04	65.00±0.40	85.74±0.76	82.02±0.61	3.20±0.07
Shahani F	8.96	22.32±0.46	0.50±0.05	63.00±0.19	63.00±1.25	84.04±0.55	5.42±0.10
Shahani M	11.96	38.23±0.17	1.12±0.14	66.00±0.57	68.00±1.5	54.80±0.57	7.36±0.12
Halileh	11.90	34.72±0.25	0.75±0.12	79.00±0.20	76.09±2.1	58.20±0.04	3.29±0.08
Zahedi	10.66	22.30±0.35	0.82±0.23	139.0±0.57	55.00±1.32	56.24±0.01	2.32±0.04
Quercetin	-	-	-	26.00±0.06	68.73±1.59	26.38±0.02	6.85±0.06

[a]All values are the means of three measurements ($p<0.05$) and given as mean ± SD. [b]Total phenol content were expressed as mg GAE /g of dry plant. [c]Total flavonoid content were expressed as mg Quercetin /g of dry plant. FE represents the ferrous ion equivalent (mmole/g).

formula:

% inhibition = [Abs. $_{test}$ /Abs. $_{control}$] × 100

Ferric Reducing Antioxidant Power Assay

The FRAP assay was performed according to a procedure described by Benzie and Strain[21]. Solutions of acetate buffer 300 mM pH 3.6, Ferric chloride (III) 20 mM and TPTZ 10 mM (in 40 mM HCl) were separately prepared. The FRAP reagent was freshly prepared by mixing the above solutions including acetate buffer, TPTZ and $FeCl_3$ at the ratio of 10:1:1 (v/v/v) respectively. Briefly 20 µL of each extract was mixed with 180 µL of FRAP reagent and the absorbance was measured at 593 nm after 10 min incubation at 37 ºC using an Epoch microplate spectrophotometer. Control contained 20 µL of methanol and 180 µL of FRAP reagent without extract. A sample containing extract and the solvent served as blank. Quercetin was used as antioxidant standard and positive control. The absorbance of tested samples was compared to a FeSO4 standard curve and the values were expressed as Ferrous Equivalent (FE), i.e. the concentration of extract which shows the same absorbance as 1 mmol ferrous ion (Fe^{+2}). An antioxidant compound or extract causes reduction of Fe^{3+} to Fe^{2+} ion leading to an increase in the absorption at 593 nm due to the formation of a blue Fe^{2+}/TPTZ complex.

RESULTS

Table 1 represents extracts yield, total phenol and flavonoid contents and antioxidant activity determined in spathe extracts of ten *P. dactylifera* varieties. The extracts yield varied from 5.76 % for Hajghanbari to 13.40% (w/w) for Piarom variety. However, the yield was also relatively high for Shahani M, Mazafati and Halileh and were found to be 11.96, 11.93 and 11.90% respectively. According to our results total phenol content of various samples showed variation ranging from 9.21±0.09 for the lowest phenol containing Piarom to 38.23±0.17 mg GAE/g for Shahani M, a phenol rich variety (Table 1, Fig. 1). The other phenol rich extracts in descending order were Halileh, Mazafati, Shahani F and Zahedi containing 34.72±0.25, 24.38±0.27, 22.32±0.46 and 22.30±0.35 mg GAE/g of dry plant (Table 1, Fig. 1). Total flavonoid content of spathe extracts ranged from 0.23±0.07 for Shakri to 1.12±0.14 mg QE /g for Shahani M variety. Hence Shahani M was found to exhibit high contents of both phenol and flavonoid when compared with other varieties. However relatively high flavonoid content was detected in Khasooei, Zahedi and Halileh spathe extracts and the values were 0.88±0.04, 0.82±0.23 and 0.75±0.12 mg/g respectively (Table 1). The results of antioxidant screening tests and the IC_{50} values from DPPH, NO, and $ABTS^+$ scavenging assays and the FRAP values obtained for the spathe extracts. Shahani F, Shahani M and Hajghanbari varieties respectively with IC_{50} values 63±0.19, 65±0 and 66±0.57 µg/mL declared significant degrees of inhibition against DPPH as compared with quercetin, a known standard antioxidant, IC_{50} 27 ±.05 µg/mL (Table 1). Halileh, Shakri and Hanaei varieties with IC_{50} values 79±0.00, 79±0.57 and 89±0.00 µg/mL showed moderate DPPH inhibitory properties while few varieties were found to exhibit weak anti DPPH effects (Table 1). The results from Nitric oxide assay declared highest NO radical inhibition capacity for Piarom spathe extract (IC_{50} = 38.00±1.75 µg/mL) whereas Hajghanbari demonstrated the lowest potency against NO radical (IC_{50} = 85.74±0.76 µg/mL) among the tested extracts (Table 1). As presented in Table 1 all extracts except Halileh, Mazafati and Hajghanbari surpassed quercetin in NO free radical inhibitory properties. FRAP values of the spathe extracts varied from 7.36±0.12 for Shahani M to 1.12±0.05 mM for Piarom variety and expressed as ferrous equivalent (FE) antioxidant power (Table 1). According to the results Shahani M extract exhibited the highest reducing ability at the concentration of 7.36±0.12 mM FE respectively (Table 1). Based on the results of the present study, the inhibitory properties of *P. dactylifera* spathe extracts of different varieties against $ABTS^+$ radical in terms of IC_{50} values, varied from 19.01±0.32 µg/mL for highly active Mazafati to 172±0.57

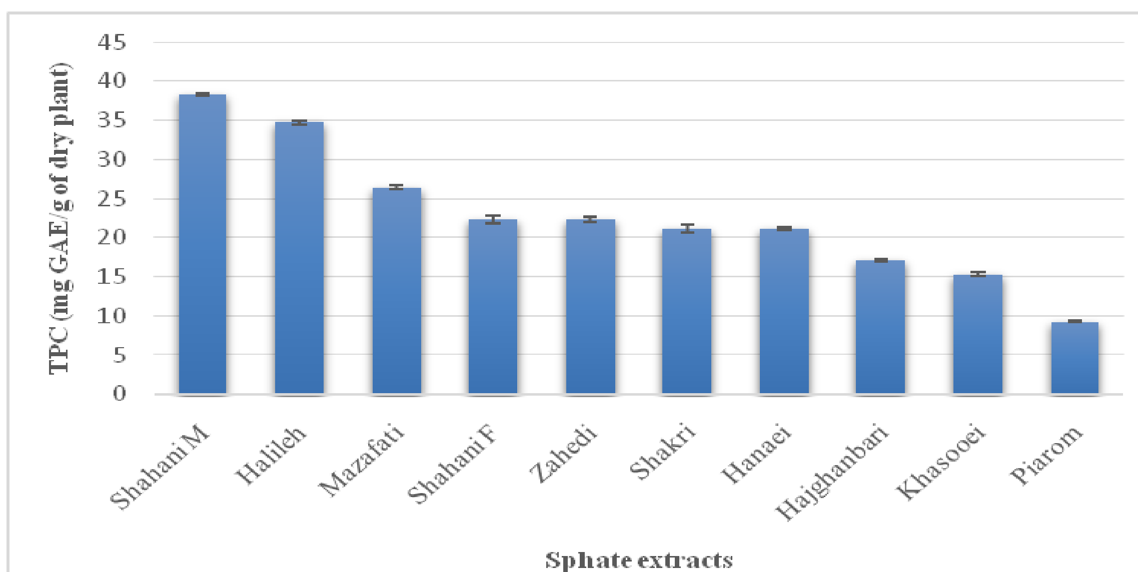

Figure 1: Total phenolic content (TPC) of various ethanolic spathe extracts *P. dactylifera*.

µg/mL for the Piarom variety (Table 1). These values indicated that all spathe ethanol extracts except Piarom exhibited potential $ABTS^+$ radical inhibitory activity. More interestingly, Mazafati extract showed stronger inhibition against ABTS+ radical when compared to quercetin (IC_{50}= 26±0.02 µg/mL). Moreover, a noteworthy correlation was found between phenol content and $ABTS^+$ free radical inhibition capacity declared by Shahani M, Halileh, Mazafati, Hanaei and Zahedi spathe extracts.

DISCUSSION

Date palm Spathe is largely accumulated annually during polination of date palm trees and used in bulk production of spath water distillate, a product widely consumed as beverage in Iranian folk medicine. Characterisation of the volatile components of this spath hydrosol has been the subject of our previously reported study as described earlier in this paper. However, the present study deals with the screening of phenolic and flavonoid content and the free radical inhibition capacity of *P. dactylifera* spathe ethanolic extracts. The results clearly indicated that most of the extracts obtained from different cultivar varieties contain relatively high amounts of phenolics. The presence of structurally diverse bioactive phenolic compounds such as flavonoids, tannins, phenolic acids and proanthocyanidins, the polymers of catechins, which has been reported earlier in some varieties of *P. dactylifera,* may make a large contribution to their free radical inhibitory properties[22]. Following statistical analysis of the results, a significant correlation was observed between phenol content and DPPH free radical inhibition for Shahani F, Shahani M, Halileh and Mazafati spathe extracts. The DPPH inhibiton activity showed by *P. dactylifera* spathe extracts revealed their potential in controlling the unwanted impacts of this free radical.

The flavonoid contents of the spathe extracts were found to be at a moderate level among different varieties. The relationship between phenol and flavonoid contents and free radical scavenging activity have so far been the subject of many studies describing the roles of both these groups of phytoconstituents in manifestation of free radical inhibition properties of herbal extracts[23-25]. Phenolic compounds exert their radical quenching effect mainly through their redox properties and hence act as singlet oxygen quenchers, hydrogen donators and reducing agents and additionally have metal-chelating properties[26,27]. The results of present study clearly indicated that the spathe extracts have significant nitric oxide scavenging activity while some of the extracts preceded quercetin in no inhibition. It has already been reported that chronic expression of nitric oxide radical is associated with various inflammatory conditions such as cancer, multiple sclerosis, coronary heart disease and diabetes[28]. The tested extracts may therefore play a preventive role against nitric oxide free radical which merits further attention. FRAP, ferric reducing antioxidant power is a useful analytical assay for assessment of antioxidants by measuring their oxidation-reduction potential. The spathes ethanolic extracts displayed higher reducing abilities in comparison to quercetin, indicating the presence of compounds with potential reducing capacity in the extracts. In FRAP assay antioxidant agents or extracts can reduce the ferric-tripyridyl triazine (Fe^{+3}-TPTZ) complex formed during this test to an intense blue ferrous form (Fe^{+2}-TPTZ) at low pH conditions. Ferrous (Fe^{2+}) is capable of generating free radicals from peroxide which is implicated in many diseases. Therefore, reduction of Fe^{2+} levels in the Fenton reaction would protect against oxidative damage and hence the reducing ability of an extract or compound may serve as a significant indicator of its potential antioxidant activity[29]. Comparison of data obtained from other antioxidant assays showed that the free radical scavenging ability of date spathe extracts may be partly due to their iron chelating capacity. This interpretation gained support from the previous studies reporting the second position for date fruit extract in demonstration of antioxidant activity among 28 fruits using FRAP assay[30]. Decolorization of ABTS+ by these spathe extracts reflects the capacity of

their chemical components to donate electrons or hydrogen atoms to inactivate this radical cation. Many studies have so far reported the correlation between phenol content and antioxidant activity in various plant products[26,31]. However the pulp of date fruit have been reported to contain phytochemicals like phenolics, sterols, carotenoids, anthocyanins, procyanidins and flavonoids[32]. These classes of phytochemicals may occur in other parts of the plant like spathe and contribute to its antioxidant properties. But the ratio and concentrations of these constituents depend on the variety, stage of maturation, location and soil conditions[33]. Furthermore, antioxidant compounds may exert their effect through various mechanisms such as prevention of chain initiation, decomposition of peroxides, reducing capacity and radical scavenging[34]. The antioxidant activity observed for *P. dactylifera* spathe extracts may be attributed to the type of chemical constituents, their possible synergistic effects and the specific structural features required for the activity such as the position of substituents like hydroxyl groups on the phenolic and flavonoid structures[35]. The hydroxyl groups in the flavonoid skeleton may exist in glycosylated or methylated form in the extracts of various date cultivars. This may significantly affect the overall free radical scavenging activity as reported earlier[36]. Meanwhile the mechanism of antioxidant effect of flavonoids has been reported to be through scavenging or chelation ability[37].

CONCLUSION

Current investigation demonstrated that the ethanolic spathe extracts of various *P.dactylifera* varieties which contain large amounts of phenolic compounds, exhibit remarkable antioxidant and free radical scavenging activities in DPPH, NO and ABTS assays. Additionally, the spathe extracts exhibited significant ferric reducing antioxidant power. The *in vitro* antioxidant ability of *P. dactylifera* spathe ethanolic extract may remain helpful in producing herbal alternaive products for prevention and treatment of oxidative stress-associated diseases. It is therefore worthy of further investigation to isolate and characterise the active volatile and/or non-volatile constituents responsible for the free radical scavenging activity manifested by *P. dactylifera* spathe extracts.

STATISTICAL ANALYSIS

The IC_{50} values (µg/mL) were calculated for all tested samples using probit test in SPSS software. Data were expressed as Mean±SD. Statistical differences between treatments were identified using one-way ANOVA followed by Tukey posttest and significant differences between controls and samples mean values determined using comparison test at a level of $p < 0.05$.

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