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Original Article

Antibacterial activity and principal analysis of chemical composition and antioxidant activity of Tunisian date palm (*Phoenix dactylifera* L.) fruit during ripening

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Abstract

In this study, we investigated how the degree of ripeness affects the contents of total phenolic, total flavonoid, condensed tannins and soluble solids, the titratable acidity and the concentrations of polyphenolic acids of two categories of date fruit; soft (Alig and Deglet Nour) and dry (Horra). The antioxidant activity evaluated by the DPPH and ABTS scavenging activity and the reducing power assays and the antibacterial activity evaluated using microplate dilution test were also determined. Results show that the concentrations of the phytochemical compounds decreased as the fruit ripening progressed from besser to tamr stage, followed by a decrease in the antioxidant and the antibacterial activities. A strong correlation between the phytochemical compounds and the antioxidant activity was found.

Mots clés :
Dattes
Stage de maturation
Composition phytochimique
Activité antioxydante
Activité antibactérienne

Résumé

Evaluation de l'activité antibactérienne et l'analyse de la composante principale de la composition chimique et l'activité antioxydante des dattes (*Phoenix dactylifera* L.) tunisiennes durant la maturation. Le but de cette étude est d'évaluer l'effet du stade de maturation sur la teneur en polyphénol totaux, en flavonoides, en tanins condensés, en solides solubles et en composés polyphénolique, et sur l'acidité titrable de deux catégories des dattes ; molles (Alig et Deglet Nour) et sèche (Horra). L'activité antioxydante évaluée par les tests de DPPH, d'ABTS et de pouvoir réducteur ainsi que l'activité antibactérienne évaluée par la méthode de dilution sur microplaque de ces cultivars ont été également déterminées. Les résultats ont montré que les teneurs en composés phytochimique et l'acidité titrable ainsi que l'activité antioxydante et l'activité antibactérienne de ces cultivars diminuent progressivement et significativement durant la maturation. De plus une forte corrélation entre la composition phytochimiques et l'activité antioxydant a été trouvée.

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INTRODUCTION

Date palm (*Phoenix dactylifera* L.) fruit play an important role in the life of the people living in arid and semiarid regions. Dates are an ancient and precious fruit; they were mentioned in holy books like the Quran. It has been cultivated in the Middle East since at least 6000 BC (Al-Qarawi *et al*., 2003).

The dates could be consumed as fresh fruit at *besser* and *rutab* stages (short shelf life), or at *tamr* stage (good storability), or may be processed into various products such as date paste, syrup or powder which are used as ingredients in cookies or cake manufacturing. It has been shown that a regular consumption of dates is beneficial in alleviating many diseases like; rheumatism, nephropathy, gastropathy, bronchitis and sexual debility (Selvam, 2008), and due to their high amount of potassium and low content of sodium these fruit are also suitable for hypertensive persons (Al-Hooti *et al*., 2002). Recent studies have indicated that date has antioxidant, antimutagenic, antibacterial, antifungal anti-tumoral and anticancer activities (Baliga *et al*., 2011). These activities could be attributed to its good source of carbohydrates, fiber, minerals and vitamins and to its high content of polyphenolic compounds as important dietary constituents, beside to its little fat and protein contents (Al-Shahib and Marshall, 2003; Mansouri *et al*., 2005; Al-Turki *et al*., 2010).

Recently, researchers and food manufacturers are interested in phytochemical compounds such as phenolic acids, flavonoids and tannins, because of their strong antioxidant properties, abundance in the human diet, and their probable role in the prevention of various diseases associated with oxidative stress, such as cancers, cardiovascular diseases and inflammation (Chung *et al*., 1998; Ghasemzadeh and Ghasemzadeh, 2011). The content of these compounds in fruit can vary depending on numerous factors such as pre-harvest environmental conditions, post-harvest storage conditions, genotype and processing (Shahidi and Naczk, 2004). The ripening process is another important factor that influences the compositional quality of fruit. During fruit ripening, several biochemicals, physiological and structural modifications happen and these changes determine the attributes of the fruit quality and could have an effect on the properties of date fruits. For this reason the aim of this study is to evaluate the influence of the ripening stage on the individual phenolic content, on several quality attributes of dates, such as total phenolic, total flavonoids, condensed tannins and total soluble solides contents, and also on their biological properties.

MATERIAL AND METHODS

Plant material

Fresh fruit samples used in these experiments consisted of three date cultivars 'Alig', 'Deglet Nour' and 'Horra', were procured from Kébili – southern Tunisia from the 2008 harvest season at three different stages: *besser*, *rutab* and *tamr* stages. Immediately after harvesting, at each maturation stage, date palm fruit were selected for freedom from defects and color uniformity, washed with distilled water, dried with filter papers and stored at -20 °C until analysis. For all the analysis, three replicates were carried out and 10 fresh dates were used for each replicate for each type of date.

Determination of total soluble solids and titratable acidity

Fresh date palm fruit were cut and the pulp portion was homogenized with water (1:2, w/v). The produced homogenate was filtered by a filtering cloth and then centrifuged at 2907*g* for 15 min. Total soluble solids (°Brix) were determined using ABBE mark 11-digital refractometer (Cambridge Instrument Inc., Buffalo, NY, USA) at 20 °C. Titratable acidity (TA), expressed as the percentage of citric acid, was measured by 0.1 mol/L sodium hydroxide solution (phenolphthalein as indicator) until the appearance of a pink color (Bhattarai and Gautam, 2006).

Estimation of phytochemical contents

Methanolic-water (50/50, v/v) fruit extracts for total phenolic content (TPC) and antioxidant activity (AA) analysis were prepared according to the method of Al-Farsi *et al.* (2005). TPC in date palm fruit extracts was estimated using Folin-Ciocalteu reagent as described by Al-Farsi *et al.* (2005). Measurements were carried out in triplicate and calculations based on a calibration curve obtained with gallic acid. The TPC was expressed as mg of gallic acid equivalents (GAE) per 100 g of fresh weight (FW). Total flavonoid content (TFC) was estimated using the aluminum chloride reagent, as described by Liu *et al.* (2009). The results were expressed as milligrams catechin equivalents (CE) /100g FW. Condensed tannins content (CTC) was determined according to the procedure as described by Liu *et al.* (2009). The results were expressed as mg CE/100g FW.

Identification and quantification of phenolic compounds

Preparation of samples for HPLC analysis: Dates dissolved in HPLC grade MeOH, to make 10 mg/mL sample solution, was shacked overnight at room temperature. After centrifugation, all samples were filtered with a 0.45 μm membrane filter before HPLC analysis.

HPLC analysis: The phenolic compounds' analysis was carried out using the same method as described in our previous work (EL Arem *et al.*, 2012). Peaks were identified at 280 nm by congruent retention times compared to standards (gallic, protocatechuic, syringic, 3-hydroxybenzoic,

isovanillic, chlorogenic, caffeic, *p*-coumaric, *m*-coumaric, *o*-coumaric, ferulic, cinnamic, apigenic, catechin, hydroxyphenylacetic and phenylacetic acids).

Antioxidant activity

Antioxidant activity (AA) was determined by the DPPH procedure proposed by Brand-Williams *et al.* (1995) and described by Saafi *et al.* (2009). Absorbance was measured at 515 nm. The antioxidant capacity was expressed as antiradical efficiency ($AE = 1/EC_{50}$). The ABTS method described by Saafi *et al.* (2009) was also used. Absorbance was measured at 734 nm and the results were expressed as mmol of Trolox equivalents (TE) per 100 g FW from a trolox standard curve. For the reducing power assay, the capacity of date extracts to reduce Fe^{3+} was assessed by the method of Oyaizu (1986). Absorbance against blank was determined at 700 nm. Four concentrations of trolox (0.125, 0.25, 0.5 and 1mg/ml) were used for calibration curve and the reducing power activity was reported as mmol TE per 100 g FW.

Antibacterial activity

Extraction*:* Before extraction, each cultivar at the appropriate maturation stage was washed with sterile distilled water. Then, fifty grams of stone-free fresh dates were separately blended (for 1 min at a low speed and for further 1 min at a high speed) with 250 ml of absolute methanol. The mixtures were then left, in the dark, at 4° C for 24 h prior to filtration (Whatman no. 1) and centrifugation at 3864 *g* for 20 min at 5 °C. The clear extracts were membrane filtered (0.45 µm ø), followed by concentration under a vacuum using rotovap at 40 °C until reaching a constant weight.

Microorganisms*:* Eight reference strains were chosen for the antibacterial investigation: Gram-positive bacteria: *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* CIP 106510, *Bacillus cereus* ATCC 11778, *Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* ATCC 19115, and Gram negative bacteria: *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* LT2 DT104.

Determination of minimal inhibitory and minimal bactericidal concentrations*:* The minimal inhibitory concentration (MIC) that prevents a visible bacterial growth was measured by the broth dilution method, following the procedure of Gulluce *et al.* (2007). Active cultures for the MIC determination were prepared by transforming a loopful of cells from stock cultures to flasks inoculated in Muller-Hinton (MH) medium and incubated at 37 °C for 24 h. The cultures were diluted with fresh MH broth to achieve optical density of 1×10^7 CFU/ml for the test organisms at 600 nm by UV/Vis Spectrophotometer. All extract stock solutions were prepared by dissolution in 10% dimethylsulfoxyde (DMSO). The tested date extracts' concentrations ranged from 0.04 to 25 mg/ml in MH broth medium. The MIC of each extract was defined as the lowest concentration which inhibited bacterial growth, after the incubation at 37 °C between 18 and 24 h. The minimal bactericidal concentration (MBC) was determined by a subculture on nutrient agar at 37 °C between 18 and 24 h. Ampicillin and tetracyclin (1mg/ml sterile distilled water) were used as antibacterial positive controls and DMSO 10% was used as negative control.

Statistical analysis

Data were reported as means ± SD per 100 g FW. Three replicates were carried out and 10 dates were used for each replicate for each type of dates. Differences between groups were tested by one way ANOVA. P-values <0.05 were considered significant. Standardized traits mean values were used to perform the principal component (PCA) and the hierarchical cluster (HCA) analyses using XLSTAT 2010 (software version 3.06). The correlation between variables was evaluated using Pearson's correlation coefficient.

RESULTS AND DISCUSSIONS

Total soluble solids (TSS) and titratable acidity (TA)

Total soluble solids are an important quality factor attribute for many fresh fruit during ripening (Lu, 2004). The solids include soluble sugar; sucrose, glucose and fructose as well as acids. In the present experiment, the TSS content of the date cultivars varied significantly in fruit of different stage increasing in average from 29.77 at besser stage to 41.66 °Brix at tamr stage (Table I). Meanwhile, titratable acidity was reduced in average from 0.45 to 0.28% citric acid. Kulkarni and Aradhya (2005) suggested that a slow decrease in acidity, concomitant with an increased TSS and total sugar content, is an intrinsic process during ripening of fruit to impart the flavor. The increase in the TSS of these cultivars was correlated with the increase in their total sugar content, previously reported in other works (EL Arem *et al.*, 2011). Our results showed that at the *besser* stage the highest value of soluble solids was found in 'Horra' reaching 31° Brix, and the lowest in 'Alig' with 28.33° Brix. At the *tamr* stage 'Deglet Nour' had the highest soluble solids value and the lowest percentage of titratable acid. The increase of TSS (°Brix) in these three cultivars during ripening was in accord with those reported by Al-Yahyai and Al-Khaurusi (2012).

Table I: Titratable acidity (TA), total soluble solids (TSS) and phytochemical contents of three Tunisian date palm cultivars during ripening.

Cultivars	Ripening stage	TPC[a] (mg GAE/100g fw)	TFC (mg CE/100g fw)	CTC (mg CE/100g fw)	TSS (Brix°)	TA (% citric acid)
Alig	*Besser*	907.04 ± 1.79c	450.63 ± 1.76c	333.33 ± 1.44c	28.33 ± 0.33a	0.44 ± 0.02a
	Rutab	459.77 ± 1.60c	270.88 ± 1.19c	234.70 ± 0.72c	34.00 ± 1.15b	0.37 ± 0.00a
	Tamr	375.64 ± 0.90c	189.87 ± 1.19c	146.06 ± 1.44c	40.00 ± 0.66a	0.28 ± 0.00a
Deglet Nour	*Besser*	709.55 ± 1.28a	322.78 ± 0.11b	293.38 ± 0.72b	30.00 ± 0.00a	0.43 ± 0.00a
	Rutab	440.68 ± 0.44a	239.24 ± 0.73b	203.49 ± 1.88b	36.00 ± 0.00b	0.38 ± 0.00a
	Tamr	332.59 ± 0.43a	153.16 ± 1.65b	104.86 ± 0.72b	44.00 ± 0.57b	0.27 ± 0.00a
Horra	*Besser*	864.15 ± 1.13b	267.08 ± 1.62a	244.69 ± 1.44a	31.00 ± 0.00a	0.47 ± 0.00a
	Rutab	456.08 ± 0.65b	112.65 ± 0.73a	172.28 ± 0.72a	35.00 ± 0.57ab	0.38 ± 0.00a
	Tamr	343.17 ± 1.13b	92.40 ± 1.19a	94.88 ± 0.72a	41.00 ± 0.57a	0.29 ± 0.00b
Average	*Besser*	826.91	346.83	290.47	29.77	0.45
	Rutab	452.18	207.59	203.49	35.00	0.38
	Tamr	347.02	145.14	115.27	41.66	0.28

Data are reported as the mean ± SD of three replicates of ten fruits each per stage. At each ripening stage, values of the same column, followed by the different letters, mean significant difference between cultivars at $p<0.05$ by Duncan's test. TPCa: Total phenolic content ; TFC: Total flavonoid content ; CTC: Condensed tannins content.

Phytochemical evaluation of date palm fruit during ripening

The analysis of the contents of total phenolic, total flavonoid and condensed tannins of date fruit indicates that all the parameters decreased significantly ($p<0.05$) during ripening.

The highest levels of these phytochemical constituents were detected in 'Alig' and the lowest ones in 'Horra' cultivar (Table I). The gradual decrease in the TPC could be connected with an increase in the polyphenol oxidase (PPO) activity (Parr and Bolwell, 2000). Many works showed that the TPC of date decreased as the fruit reached full ripening (Tafti and Fooladi, 2005; Amoros *et al.*, 2009). However those works have reported different amounts of TPC than those found in the present study. This difference may be due to various factors, such as variety, growing and environmental conditions during fruit development and to experimental conditions (storage, analytical procedures and chemical standard).

The TFC followed the same trend as for the TPC, decreasing with ripeness. The pattern of flavonoid accumulation and the subsequent decline during ripening suggests the degradation of flavonoïds and their utilization in the biosynthesis of other compounds and/or association with other cellular compounds by stable covalent links. In comparaison with four common Tunisian cultivars 'Gondi, Gasbi, Khalt Dhahbi, and Rtob Ahmar' reported in our previous work (EL Arem *et al.*, 2012) we have found that Alig and Deglet Nour contained an important level of TF during ripening. Moreover, the TFC of these two cultivars was more important than those estimated by Benmeddour *et al.* (2013) in ten matured Algerian date cultivars (11.52–225.77 mg QE/100 g FW) and those given by Chaira *et al.* (2009) for different matured Tunisian date cultivars (6.41 to 54.46 mg quercetin equivalent/100 g FW). These differences may be due to the cultivar, climate, cultivation practices and extraction procedure.

Alig and Deglet Nour cultivars are also characterized with the higher level of CT especially at *besser* stage. It's worth to noting that the CTC of these two cultivars during all ripening stages was more important then those found in Horra cultivar and in four common Tunisians cultivars 'Gondi, Gasbi, Khalt Dhahbi, and Rtob Ahmar' reported in our previous study (EL Arem *et al.*, 2012). This important level of CTC of these two cultivars characterized them with an astringent taste especially during *besser* and *rutab* stages. The decrease in the CTC was also investigated by Awad *et al.* (2011), regardless of the analytical method employed. Nonetheless, these authors reported level of CT lower than those found in our study. These differences may be due to cultivar type, geographical origin, growing and environmental conditions (mainly light and temperature) during fruit development, harvest time, and experimental and storage conditions.

In general, phenolic compound metabolism varies greatly according to the physiological stage of the fruit, and its biochemical production rates rely on the participation of many key enzymes, which are

also limited by the availability of precursors. Thus, it is expected that each class of phenolic compounds shows a quite different content evolution during ripening (Raffo *et al.* 2004), and consequently may explain the differences found between cultivars during ripening.

Characterization of phenolic compounds

The profile of phenolic compounds of the investigated date cultivars at different stages of ripening is shown in Table II. From the phenolic compound groups analyzed, the hydroxycinnamic acids were the most predominant ones at all the stages of ripening. This was observed even when the concentrations decreased in the ripened dates. In this group, caffeic (CFA) and ferulic (FA) acids were found as the major ones, followed by *p*-coumaric (*p*-CMA), and chlorogenic (CGA) acids, whereas, cinnamic acid (CAN) was detected as the minor one. In contrary, the concentration of hydroxybenzoic acids especially gallic (GA) and 3-hydroxybenzoic acids increased from *besser* to *rutab* stages and then decreased at *tamr* stage. In comparing the individual phenolic profile of these cultivars with those of four common Tunisian date 'Gasbi, Gondi, Khalt Ahmar and Rtob Ahmar' cultivars (EL Arem *et al.*, 2012) we found that the three cultivars were very rich on gallic, chlorogenic, caffeic, *m* and *o*-coumaric acids, catechin, hydroxyphenylacetic and phenylacetic acids. During ripening, the drop of the hydroxycinnamic acids content was also observed in other fruit; Highbush blueberries (Castrejon *et al.*, 2008). Gu *et al.* (2003) demonstrated that mature 'Deglet Nour' contains proanthocyanidins of type B consisting exclusively of (epi) catechin. This finding agrees with our results; catechin was detected in all the three cultivars, with a mean content decreasing from 4.34 to 3.72 mg/100g FW as date ripened. Detailed phenolic profile in mature Tunisian date palm fruit was published by Regnalut-Roger *et al.* (1987). They identified several phenolic acids; gallic acid, protocatechuic, *p*-hydroxybenzoic (*p*-HBA), syringic (SA), vanillic, caffeic, *p*-coumaric and ferulic acids as major phenolic acids in date palm fruit. In addition to these phenolic acids, Mansouri *et al.* (2005) demonstrated the presence of sinapic acid, 5- *o*-caffeoylshikimic acid and its three isomers, xantoxylin, hydrocaffeic acid, and coumaroylquinic acid in seven varieties of ripe Algerian dates. Furthermore, Duke (2001) showed that date palm fruit also contains chlorogenic acid. In our study, the mean amount of this compound decreased from 4.36 to 3.66 mg/100 g FW during ripening, where 'Deglet Nour' cultivar contained the highest level until full ripeness. Phenylacetic acid (PAA), the major metabolites of quercetin-3-rutinoside, was detected in the three date cultivars with an appreciable amount. Hydroxyphenylacetic (HPAA) acid was absent only in 'Horra' cultivar at *besser* and *rutab* stages. In comparing dates with other fruit and berries (Mattila *et al.*, 2006) we can considered it a phenolic acid rich fruit.

Antioxidant activity

In this study, the free radical and free caution scavenging activities and the reducing power tests were used to evaluate the antioxidant capacity of date palm fruit during ripening. A high AA was observed at *besser* stage, which decreased significantly at *rutab* and *tamr* stages with significant differences ($p<0.05$) among cultivars. Figures 1A and 1B show the AA, expressed as mmol TE/100 g FW and AE, present in the three cultivars at different ripening stages evaluated withe ABTS and reducing power, and DPPH tests, respectivly. The lowest AA was observed in 'Horra' at *tamr* stage and the highest one in 'Alig' at *besser* stage. The three cultivars contained an important AA (mmol TE/100 g FW) as compared to four common date cultivars (EL Arem *et al.*, 2012). Regardless of the analytical method used, the decrease in the AA of date palm fruit during ripening is in agreement with those found by Allaith (2008), Amoros *et al.* (2009) and Awad *et al.* (2011). The results of the AA found in our cultivars are different to those found by those authors. These differences can be explained by the origin and the genetic variability of date palm fruit and the environmental and experimental conditions.

The determination of the AA in fruit should take into account the overall concentration and composition of diverse antioxidants, because the total antioxidant capacity is in function of the combined activities of diverse antioxidants. Several studies have reported positive correlations between the AA and the total phenol content in dates (Mansouri *et al.*, 2005; Amoros *et al.*, 2009; Awad *et al.*, 2011). In this study, the AA evaluated by ABTS, reducing power (RP) and DPPH tests correlated with total phenolic, total flavonoid and condensed tannins contents (Table III). The AA also correlated significantly with caffeic acid. An important significant correlation was also found with *o*-coumaric and cinnamic acids. Chlorogenic and catechuic acids correlated significantly only with the AA evaluated by ABTS and RP tests, and fairly correlated with the DPPH test (Table III).

Table II: Phenolic acids contents (mg/100g FW) of three Tunisian date palm cultivars at three ripening stages.

	Alig			Deglet Nour			Horra		
	Besser	***Rutab***	***Tamr***	***Besser***	***Rutab***	***Tamr***	***Besser***	***Rutab***	***Tamr***
GA	2.36±0.18c	2.60±0.17a	2.25±0.00c	2.26±0.20b	2.74±0.83b	1.72±0.10a	nd[A]	2.58±0.36a	1.92±0.20b
SA	nd	nd	nd	nd	nd	2.24±0.00b	2.07±0.00b	nd	1.91±0.03a
IVA	nd	nd	nd	nd	nd	4.01±0.00b	1.94±0.05b	nd	2.30±0.12a
3HBA	nd	nd	nd	2.09±0.27c	3.69±0.28c	1.59±0.19b	1.79±0.19b	3.30±0.52b	2.57±0.52c
CFA	6.69±0.20b	5.89±0.06c	5.73±0.20c	6.76±0.40b	5.06±0.46a	4.91±0.30a	6.26±0.11a	5.48±0.15b	5.14±0.32b
CGA	4.31±0.37a	3.89±0.23a	3.75±0.62b	4.56±0.42b	4.05±0.60b	3.85±0.12b	4.21±0.24a	3.76±0.08a	3.19±0.18a
PCA	5.90±0.49b	6.24±0.67a	7.18±0.15b	7.26±0.31c	7.08±0.59c	7.38±0.57c	4.60±0.95a	6.59±0.85b	4.94±0.25a
FA	5.35±0.39b	4.59±0.00a	4.47±0.26c	4.72±0.31a	5.61±0.32b	3.82±0.18b	5.31±0.09b	4.63±0.29a	3.48±0.18a
***p*-CMA**	4.64±0.21a	3.72±0.02a	2.63±0.02a	6.35±0.08c	4.63±0.22b	4.56±0.09c	5.34±0.26b	4.23±0.27b	3.69±0.29b
***m*-CMA**	3.40±0.05b	2.70±0.22b	2.96±0.31b	3.38±0.17b	2.94±0.27c	2.84±0.13b	1.55±0.29a	1.69±0.24a	2.10±0.12a
o-**CMA**	4.44±0.16c	3.14±0.02b	3.20±0.07b	4.01±0.07b	3.33±0.07b	3.09±0.04b	3.01±0.34a	1.97±0.31a	1.95±0.00a
CNA	3.30±0.25b	1.10±0.13b	nd	1.76±0.13a	1.49±0.49c	0.64±0.00b	1.81±0.00a	nd	nd
CAT	6.06±0.16c	5.56±0.48c	4.64±0.30c	4.25±0.43b	4.23±0.32b	3.93±0.04b	3.29±0.18a	2.21±0.15a	2.29±0.27a
PAA	2.63±0.20b	2.10±0.04a	1.32±0.00a	2.64±0.63b	2.54±0.30b	2.51±0.00c	2.33±0.07a	2.16±0.39a	2.04±0.14b
HPAA	2.87±0.20b	2.25±0.30b	2.13±0.16b	3.60±0.05c	3.21±0.09c	1.47±0.33a	nd	nd	2.06±0.25b

Data are reported as the mean ± SD of three replicates of ten fruits each per stage. At each ripening stage, values in the same line followed by the different letters mean significant difference between cultivars at p<0.05 by Duncan's test. nd: not detected ; GA: Gallic acid ; SA: syringic acid ; 3HBA: 3-Hydroxybenzoic acid ; IVA: Isovanillic acid ; CGA: Chlorogenic acid ; PCA: Protocatechuic acid ; CFA: Caffeic acid ; p-CMA: p-coumaric acid ; FA: Ferulic acid ; m-CMA: m-coumaric acid ; o-CMA: o-coumaric acid ; CNA: Cinnamic acid ; CAT: Catechin ; PAA: Phenylacetic acid ; HPAA: Hydroxyphenylacetic acid.

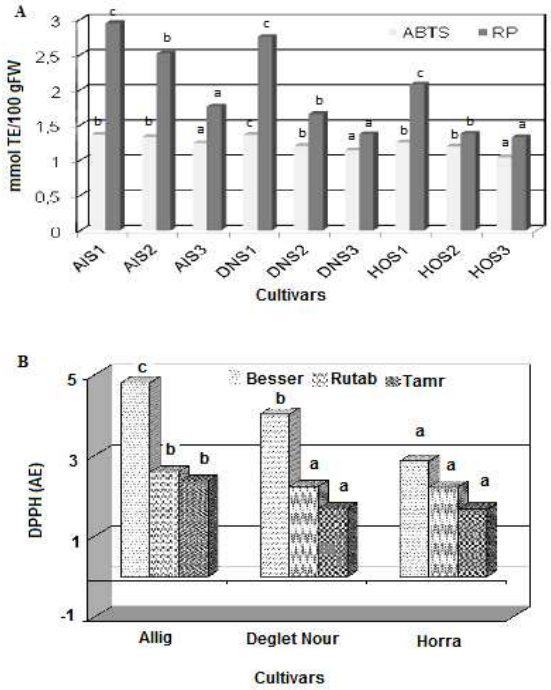

Figure 1. Antioxidant activity of date palm cultivars during ripening stage evaluated by ABTS and reducing power tests (**A**) and by DPPH tests (**B**). AL: Alig. DN: Deglet Nour. HO: Horra. *Besser* stage (S1), *rutab* stage (S2), and *tamr* stage (S3). Data are reported as the mean ± SD of three replicates of ten fruits each per stage. (a–c) Mean significant difference between ripening stage at $p < 0.05$ by Duncan's test.

Antibacterial activity of date palm fruit at three ripening stages

The *in vitro* antibacterial activity of dates' extracts against the microorganisms employed and their potential activities were quantitatively assessed by the minimal inhibitory (MIC) and minimal bactericidal (MBC) concentration values. According to the results given in table IV, the date palm fruit extracts have a great potential of antibacterial activity against all tested bacteria, being more active against Gram-positive bacteria (especially *S. aureus*, *B. cereus*, *E. faecalis* and *L. monocytogenes*) than Gram negative bacteria (*E. coli*, *P. aeruginosa* and *S. typhimurium*). These dates' extracts possessed significantly a higher antibacterial activity at the *besser* stage than that at both *rutab* and *tamr* stages. Amongst the three cultivars, Deglet Nour at the *besser* stage was found the most active on the majority of bacterial strains such as *P. aeruginosa*, *B. cereus*, *S. aureus*, *L. monocytogenes* and *E. faecalis*. Moreover, this variety at both *besser* and *rutab* stages inhibit the growth of the most resistant germ to ampicillin the *pseudomonas aeruginosa.* However, 'Alig' extracts were found more active on *E. coli* and *L. monocytogenes* at the three maturation stages. Alig extracts were also found more active to inhibit the growth of *Listeria monocytogenes* than that of ampicillin (Table IV).

Table III: Pearson correlation between the titratable acidity, total soluble solids, phytochemical contents, phenolic compounds and the antioxidant activity.

Variables	TPC	TFC	CTC	TSS	TA	DPPH	ABTS	RP	GA	SA	IVA	3HBA	CFA	CGA	FA	*p*-CMA	*m*-CMA	*o*-CMA	CNA	CTA	PAA	HPAA
TPC	**1**																					
TFC	**.815**	**1**																				
CTC	**.873**	**.932**	**1**																			
TSS	**-.882**	**-.772**	**-.907**	**1**																		
TA	**.891**	**.675**	**.859**	**-.857**	**1**																	
DPPH	**.792**	**.893**	**.833**	**-.776**	.575	**1**																
ABTS	**.674**	**.860**	**.901**	**-.769**	.651	**.704**	**1**															
RP	**.760**	**.927**	**.932**	**-.872**	**.670**	**.820**	**.927**	**1**														
GA	-.407	.019	.020	.112	-.290	.103	.137	.078	**1**													
SA	-.096	-.404	-.500	.362	-.229	-.387	-.623	-.480	**-.754**	**1**												
IVA	-.252	-.436	-.580	.512	-.405	-.405	-.634	-.524	-.567	**.949**	**1**											
3HBA	-.225	-.484	-.291	.278	.065	-.448	-.521	-.547	.026	.150	.117	**1**										
CFA	**.853**	**.809**	**.881**	**-.933**	**.734**	**.776**	**.850**	**.909**	-.156	-.367	-.481	-.461	**1**									
CGA	**.757**	**.810**	**.856**	-.657	**.747**	.621	**.834**	**.760**	-.101	-.350	-.358	-.148	**.740**	**1**								
FA	.654	**.676**	**.746**	-.519	**.761**	.506	.627	.511	-.022	-.482	-.584	.032	.466	**.713**	**1**							
***p*-CMA**	.625	.442	.568	-.519	**.696**	.327	.371	.427	-.292	.094	.052	.346	.478	**.747**	.369	**1**						
***m*-CMA**	.086	.586	.386	-.126	-.093	.510	.499	.529	.533	-.476	-.295	-.407	.299	.449	.151	.111	**1**					
***o*-CMA**	.614	**.915**	**.769**	-.517	.421	**.771**	**.789**	**.808**	.100	-.377	-.318	-.489	.665	**.818**	.551	.416	**.806**	**1**				
CNA	**.859**	**.944**	**.882**	**-.722**	**.739**	**.856**	**.694**	**.802**	-.118	-.195	-.243	-.272	**.689**	**.783**	**.695**	.580	.446	**.832**	**1**			
CTA	.344	**.799**	.608	-.360	.182	.637	**.747**	**.742**	.293	-.475	-.386	**-.743**	.478	.519	.421	-.030	**.765**	**.843**	.642	**1**		
PAA	.497	.473	.501	-.365	.552	.418	.247	.354	-.051	.097	.140	.329	.226	.584	.356	**.833**	.229	.431	**.680**	.132	**1**	
HPAA	.019	.479	.337	-.138	-.052	.369	.342	.457	.563	-.463	-.361	-.162	.220	.302	.130	.142	**.885**	.635	.376	.586	.239	**1**
PCA	-.402	-.026	-.082	.256	-.360	-.104	.187	-.040	.615	-.471	-.210	.020	-.178	.236	-.008	-.011	.615	.294	-.159	.275	.010	.419

Bold values are different from 0 at significance level alpha = 0.05. TPC: Total phenolic content. TFC: Total flavonoids content. CTC: Condensed tannins content. TA: Titratable acidity. TSS: Total soluble solids. RP: reducing power. GA: Gallic acid. SA: syringic acid. 3HBA: 3-Hydroxybenzoic acid. IVA: Isovanillic acid. CGA: Chlorogenic acid. CFA: Caffeic acid. *p*-CMA: *p*-coumaric acid. FA: Ferulic acid. *m*-CMA: *m*-coumaric acid. *o*-CMA: *o*-coumaric acid. CNA: Cinnamic acid. CTA: Catechuic acid. HPAA: Hydroxyphenylacetic acid. PAA: Phenylacetic acid. PCA: Protocatechuic acid.

Table IV: Antibacterial activity of the methanolic extracts of three Tunisian date palm cultivars at three ripening stages.

		E. coli		*S. typhi*		*P. aeruginosa*		*B. cereus*		*St. aureus*		*St. epidermidis*		*Li. monocytogenes*		*E. faecalis*	
		MIC[a]	**MBC[b]**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Alig	***RS1***	0.05	0.1	>25	>25	>25	>25	6.25	12.50	0.10	0.10	>25	>25	0.05	0.05	0.80	1.60
	RS2	0.10	0.20	25	25	>25	>25	12.50	25	3.13	6.25	>25	>25	0.1	0.1	6.25	12.50
	RS3	12.50	25	>25	>25	>25	>25	12.50	25	25	25	>25	>25	0.20	0.20	25	25
Deglet Nour	***RS1***	0.10	0.20	25	>25	1.56	3.13	3.13	6.25	0.05	0.10	1.56	3.13	0.05	0.10	0.10	0.20
	RS2	0.4	0.80	12.50	25	12.50	25	12.50	25	3.13	6.25	25	>25	6.25	12.50	12.50	25
	RS3	25	>25	>25	>25	>25	>25	25	25	12.50	25	>25	>25	12.50	25	6.25	12.50
Horra	***RS1***	25	>25	>25	>25	>25	>25	25	25	25	>25	>25	>25	25	>25	25	>25
	RS2	>25	>25	>25	>25	>25	>25	25	>25	25	>25	>25	>25	25	25	25	>25
	RS3	>25	>25	25	>25	>25	>25	25	>25	25	25	>25	>25	25	25	25	>25
Ampicillin (µg/ml)		15	30	2	4	>500	>500	62	125	2	4	2	4	250	500	15	30
Tetracyclin (µg/ml)		15	30	4	8	62	125	75	15	2	4	4	8	8	16	15	30
DMSO 10%		na[c]	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na

Results are the mean of three determinations.
a: minimal inhibitory concentration (mg/ml).
b: minimal bactericidal concentration (mg/ml).
c: na: not active
E. coli: Escherichia coli ; S. thyphi: Salmonella typhimurium ; P. aeruginosa: Pseudomonas aeruginosa ; B. cereus : Bacillus cereus ; St. aureus: Staphylococcus aureus ; St. epidermidis: Staphylococcus epidermidis ; Li. monocytogenes: Listeria monocytogenes ; E. faecalis: Enterococcus faecalis.

While Horra extracts were found moderately active on the majority of strains with MIC of 25mg/ml until *tamr* stage. The inhibitory effects of the dates' extracts could be attributed to their polyphenolic composition. Recent works have been published on the antibacterial effects of dates (Ayachi *et al.*, 2009; Nazari and Weiss, 2010) and their results provide evidence for the presence of antibacterial compounds in the extracts of date palm fruit. According to the existing literature there are several phenolic acids, such as chlorogenic, caffeic, *p*-coumaric, ferulic, *p*-hydroxy-benzoic, vanillic, protocatechuic, Syringic and gallic (Aziz *et al.*, 1998; Wen *et al.*, 2003), as well as some other phenolic compounds like quercetin, hydroxytyrosol, resveratrol (Chan, 2002; Aziz *et al.*, 1998) identified to have antimicrobial activities.

Principal component analysis

To obtain a broad view on the phytochemical and the AA changes that occurs during dates ripening, all parameters set of TSS content, TA, TP, TF and CT contents, phenolic acid compounds and AA evaluated with DPPH, ABTS and RP methods were analyzed by principal component analysis (PCA). PCA yielded 4 components that have an eigenvalue of 12.31, 4.25, 2.15 and 2.04, respectively. Only the two first components were used which accounted for 53.54 % and 18.48 % of the total variance, respectively. The influence of the ripening process on the phytochemical composition and the AA of fruit can be shown from the correlation matrix (Table III). Figure 2A shows the PCA, based on the correlation mentioned above, obtained from our data; the lines intersecting at (0, 0) represent the experimental variables; the length of each vector is proportional to its contribution to the principal components. The first main component is closely related, negatively to the TSS, 3HBA and isovanillic acid (IVA), and positively to the, TA, TPC, TFC, CTC, CFA, CGA, FA, *p*-CMA, *o*-CMA, CNA, CTA and PAA contents and to the AA tested by DPPH, ABTS and RP tests. The second main component is related to the GA, PCA, SA, *m*-CMA and HPAA contents. So the first main component represents the variables that could define the ripening stage in date palm fruit. The position of each variable in the loading plot (Figure 2A) describes its relationship to the other variables. Variables which are close have high correlations. Variables on the same side of the origin (0,0) are positively correlated and those on the opposite side of the origin are negatively correlated. Not surprisingly, total antioxidant activity measured by DPPH, ABTS and RP are clustered together on the right hand side of the loading plot. These parameters are significantly correlated as proved by their Pearson correlation coefficients. 3HBA, SA and IVA are found in opposition to all other parameters (Figure 2A), while TSS occupied a unique location at the left and at the upper part of PC1. The last parameter was an important index of date palm fruit ripening. The clear separation of the three different stages was observed in the PCA scores plot where each coordinate represents a sample (Figure 2B). Three clusters were found according to the ripening stage of date cultivars. Firstly, all the three cultivars at *besser* stage are located in right position of the plot, 'Alig' and 'Deglet Nour' on the positive side of PC1 and 'Horra' on the negative side of PC2. Secondly, at *rutab* stage, the three cultivars are located in an intermediate position of the plot. Interestingly, at the both stages, 'Horra' is located quite some distance away from the two other cultivars, indicating that its composition, in terms at least of some of the measured parameters, differs significantly from the two other cultivars. However, at the *tamr* stage, all the cultivars are located at the left position of the plot. In this cluster, 'Alig' cultivar was positively supported by PC2 and is located quite some distance away from the two other cultivars due to the absence of 3HBA and their higher content of GA. By using the plots (Figures 2A, B), we can detected that the total solid soluble content (°Brix) is a very important variable for fruit at *tamr* stage. The obtained clusters allow classifying each ripening stage according to their physicochemical properties and confirming that they are statistically differentiable.

Hierarchical cluster analysis

In the hierarchical cluster analysis (HCA), samples are grouped on the basis of similarities, without taking into account the information about the class membership. Cluster analysis uses less information (distances only) than PCA. It is interesting to observe what kind of classification can be made on the basis of distances only. The results obtained following HCA are shown as a dendrogram (Figure 3) in which four well-defined clusters are visible. A group of samples (C1) consists of 'Horra' at *rutab* stage (S2) alone. This is in agreement with the results of the PCA in which 'Horra' sample lay at some distance from the two other cultivars.

A second cluster (C2) consists of 'Horra' at *besser* (S1) and *tamr* (S3) stages, respectively. A third cluster (labeled C3) includes 'Alig' and 'Deglet Nour' at *rutab* stage (S2). These cultivars are associated with high antioxidant activity as measured by DPPH, RP, ABTS, and also with higher phytochemical contents. While C4 cluster is clearly discernible, this is composed of 'Deglet Nour' and 'Alig' cultivars at both *besser* and *tamr* stages. It is possible that cluster C1 and cluster C4 are well separated due to

variations in total antioxidant activity and individual polyphenolic antioxidant compounds. This classification showed that 'Horra' cultivar is entirely different from 'Alig' and 'Deglet Nour' cultivars in terms of the selected parameters during the three ripening stages.

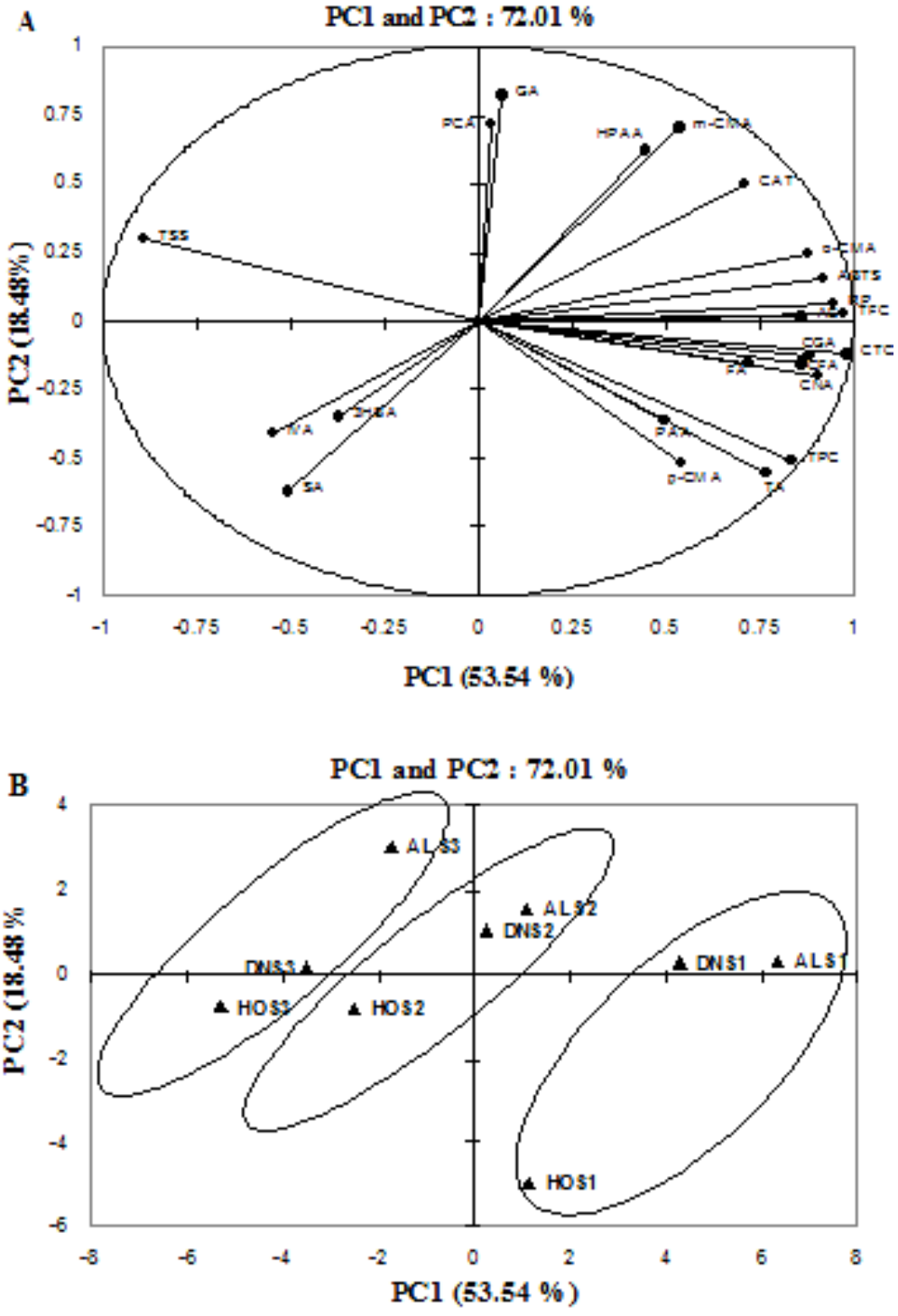

Figure 2. A) Principle component analysis results of different parameters measured at different ripening stages in all cultivars. **B**) Principal component analysis results of distinct ripening stage, *besser* stage (S1), *rutab* stage (S2) and *tamr* stage (S3) as measured in the three cultivars (AL; Alig. DN; Deglet Nour. HO; Horra). GA: Gallic acid. PCA: Protocatechuic acid. SA: syringic acid. 3HBA: 3-Hydroxybenzoic acid. IVA: Isovanillic acid. CGA: Chlorogenic acid. CFA: Caffeic acid. p-CMA: p-coumaric acid. FA: Ferulic acid. m-CMA: m-coumaric acid. o-CMA: o-coumaric acid. CNA: Cinnamic acid. CTA: Catechuic acid. HPAA: Hydroxyphenylacetic acid. PAA: Phenylacetic acid.

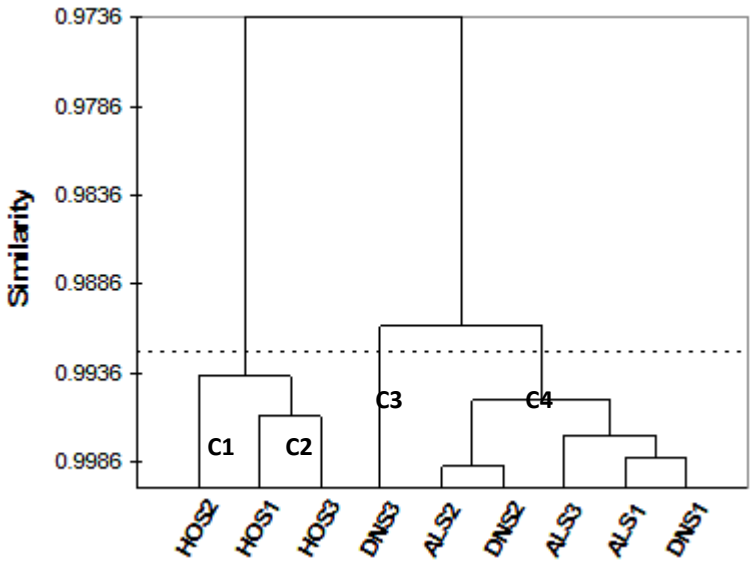

Figure 3. Dendrogram of hierarchical cluster analysis of the phytochemical composition, TSS, TA and the antioxidant activity of date cultivars during ripening. HO: Horra. AL: Alig. DN: Deglet Nour. *Besser* stage (S1), *rutab* stage (S2), and *tamr* stage (S3). C1, C2, C3 and C4: Cluster number.

CONCLUSION

The results clearly demonstrated that the chemical properties and the antioxidant and the antibacterial capacities of date fruit are affected by ripening stages. The besser stage had the highest antioxidant and antibacterial capacities. Moreover, significant variability was found for overall total phenolic, total flavonoid, condensed tannins and total soluble solids contents, titratable acidity, polyphenolic compounds and antioxidant capacity during ripening. The total phenolic, total flavonoid and condensed tannins contents affected the antioxidant capacity.

The observed results claim further analysis to explore specific details of this trend.

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