Metabolite characterization of different palm date varieties and the correlation with their NO inhibitory activity, texture and sweetness

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Abstract The aim of this study was to examine the variation in metabolite constituents of five commercial varieties of date fruits; Ajwa, Safawi and Ambar which originated from Madinah, the Iranian Bam and Tunisian Deglet Noor. The differences of metabolome were investigated using proton nuclear magnetic resonance (¹H NMR) spectroscopy combined with multivariate data analysis (MVDA). Principal Component Analysis (PCA) revealed clear separation between the date varieties. The Tunisian Deglet Noor demonstrated distinct cluster from the rest of the palm date samples based on the metabolite composition as shown by the pattern observed in Hierarchical Clustering Analysis (HCA) and PCA. Deglet Noor exhibited a significant higher level of sucrose (δ 5.40) and fructose (δ 4.16) in comparison with the other four varieties which can be associated with the distinctive sweet taste of this variety.

Dates originated from Madinah and Tunisia exhibited a contrast manner in the amount of xylose and moisture content. These two aspects may contribute towards the soft texture of Tunisian dates. All Madinah dates were found to contain phenolic compounds which were well established as great antioxidant and anti-inflammatory agent. Ajwa dates exerted greater effect in inhibiting the generation of nitric oxide (NO) from the stimulated RAW264.7 cells at 95.37% inhibition. Succinic acid was suggested to have the most significant correlation with the trend of NO inhibitory shown by the selected date palm varieties.

Keywords *Phoenix dactylifera* · Ajwa dates · ¹H-NMR spectroscopy · RAW cells · Nitric oxide inhibitory activity

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Introduction

Palm dates (*Phoenix dactylifera*), have become a common fruit in many parts of the world because of modern transportation systems. In Malaysia, several varieties originating from different countries are now commercially available in most major cities. In Middle Eastern and North African countries, the fruits of the date palm (*Phoenix dactylifera*) have social and environmental significance, and they have become important economic food item for oasis farmers (Al-Farsi and Lee 2008). Dates are not only utilized as a food resource, but they are also cherished for their medicinal uses. Date palm fruit is an excellent nutritional source, with 70% of its content in the form of carbohydrates, primarily sugars (Al-Farsi and Lee 2008). In Morocco, they are used medicinally for the treatment of hypertension and diabetes, while in Egypt they are used as an ingredient in aphrodisiacs and tonic confections (Tah-raoui et al. 2007). Ayurvedic medical system utilizes dates

for their antitussive, expectorant, demulcent, laxative, diuretic and restorative properties (Khare 2007). A blended form of dry dates with nuts and spices is prescribed as supplementary nutritional food to pregnant women and lactating mothers (Puri et al. 2000).

Currently, more than 2000 different cultivars of date palm are known to exist, but only a few of them have been evaluated for their benefits and qualities (El Arem et al. 2011). Different date varieties receive different acceptability by the consumers and have been priced at the various range on the market. Each variety might possess different nutritive content and pharmacological activities that might be influenced by many factors, including genetic makeup and chemical compositions. Unfortunately, there has not been any scientific method established for the pricing practice other than by basing it on their external observable features. Thus, new approaches that can discriminate the quality and identify the metabolites among varieties are needed.

Previous studies have reported that dates possessed anti-oxidant, anti-inflammatory and anti-microbial properties (Al-Farsi and Lee 2008; Mohamed and Al-Okbi 2004; Zhang et al. 2013). Nitric oxide (NO) is one of the essential components in normal vascular biology and some physiological activities, which plays a crucial part in the immune system. Nonetheless, the excessive accumulation of NO might result in health problems (Kao et al. 2015). In this study, the NO inhibitory effect of date fruit extracts was evaluated, utilizing the murine macrophage RAW 264.7 cells.

Thus, the purpose of this study is to discriminate five different varieties originating from Madinah al-Munawarah (Ajwa, Ambar and Safawi), Tunisia (Deglet Noor) and Iran (Bam), utilizing ^1H NMR-based metabolomics approach and to identify the chemical markers responsible for their discrimination, utilizing multivariate analytical tools. The NO inhibitory activity of these five varieties of date fruits was also determined along with the assessment of correlation between the detected metabolites and NO inhibition.

Materials and methods

Chemicals

Lipopolysaccharide (LPS), phosphate buffered saline (PBS), recombinant murine IFN- γ , curcumin and other standard compounds used including gallic acid, quercetin and kaempferol, were purchased from Sigma-Aldrich Co. (St. Louis, MO). Cell culture media, Dulbecco's Modified Eagle's Medium (DMEM) containing HEPES and L-glutamine for both, with and without phenol red, Penicillin–Streptomycin antibiotic solution, fetal bovine serum (FBS),

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and triple Express enzyme were purchased from Gibco/BRL Life Technologies Inc. (Eggenstein, Germany). Deuterated methanol- d_4 (CD_3OD , D 99.8 at%), deuterated chloroform- d (CDCl_3 , D, 99.8 at%), non-deuterated KH_2PO_4 , sodium deuterium oxide (NaOD) to adjust the pH of the buffer, trimethylsilyl propionic acid- d_4 sodium salt (TSP) and deuterium oxide (D_2O , D 99.9 at%) were supplied by Merck (Darmstadt, Germany).

Sample preparations

Ajwa, Ambar and Safawi varieties originating from Madinah al-Munawarah, Saudi Arabia were purchased from the al-Dabta Farm date shop in Aliah district, Madinah al-Munawarah. Deglet Noor and Bam originating from Tunisia and Iran, respectively, were purchased from the date shop, Manz Delight in Putrajaya, Malaysia. Six replicates of five fruits from each variety, resulting in a total of 150 of date fruits were cleaned from impurities. The fruits were pitted, cut into small pieces and stored in -80°C (overnight) prior to freeze-drying process (3 days). The dried date fruits (1 g) were extracted by stirring with 30 mL of methanol at room temperature for 24 h. The extracts were then filtered using Whatman filter paper No 1 in a Buchner funnel, and the filtrate was concentrated to dryness with a rotary evaporator at 40°C and freeze dried. All samples were stored in the dark at 4°C until further analysis (Abdul-Hamid et al. 2016). Moisture content was clarified based on sample weight loss after the oven drying treatment at 110°C for overnight following AOAC method 920.87 (2005).

NMR measurement

Ten milligrams of the dried extracts was transferred into 2 mL Eppendorf tubes containing 0.375 mL of CD_3OD and 0.375 mL of KH_2PO_4 buffer in D_2O (pH 6.0) containing 0.1% TSP. The tube was vortexed and then ultrasonicated for 10 min at room temperature. The solution was then centrifuged at 13,000 rpm for 10 min. The ^1H NMR spectra were obtained using a 500 MHz Varian INOVA NMR spectrometer (Varian Inc., California, USA) at a running frequency of 499.887 MHz and maintained at 26°C . Suppression of the large water resonance was achieved by applying a PRESAT pulse sequence to the ^1H -NMR data acquisition (Mediani et al. 2012).

Chemometric data analysis and metabolite profiling analysis

All ^1H NMR spectra were phased and base-lined corrected and binned using Chenomx software (v. 5.1, Alberta,

Canada). The spectra were bucketed at δ 0.04 covering the range from δ 0.50–10.00. All spectra were then automatically loaded into ASCII files. The region between δ 3.28–3.32, which correspond to methanol, were excluded, and all the spectral data were normalized to the total spectral area. The averaged binned ^1H NMR data were then subjected to multivariate data analysis (MVDA) performed with SIMCA-P+ version 13.0 (Umetrics AB, Umeå, Sweden). The relative quantification for xylose and fructose was obtained using Chenomx NMR suite version 7.7 (Chenomx Inc, Alberta, Canada) by using the concentration of a known reference signal.

Nitric oxide inhibitory activity

The NO inhibitory activity, measurement of nitrite and cell viability test was determined using the procedures reported by Abas et al. (2006) with some modifications. The RAW 264.7 cells, the mouse macrophage cell line was obtained from American Type Culture Collection (ATCC). Briefly, RAW 264.7 murine macrophages cells were challenged with the triggering agents of 200 U/ml of recombinant murine IFN- γ and 10 $\mu\text{g}/\text{ml}$ lipopolysaccharide, which were seeded into the wells of 96 well tissue culture plates. 50 μL of the prepared extracts were loaded into the wells of cell plates to yield a final concentration of DMSO at 0.4% per well. The analysis was done in six replicates and the cells were incubated for 17 h at 37 °C, 5% CO_2 in humidified incubator. The stable nitric oxide conversion product, nitrite (NO_2^-) was measured using the freshly prepared Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl)-ethylene diamine dihydrochloride, 2.5% H_3PO_4) at room temperature. The absorbance was taken at 550 nm using Spectramax Plus (Molecular Devices) UV/Vis microplate reader.

Cell viability

After the removal of media, 100 μL of complete DMEM was loaded into the wells. Following this, 20 μL of MTT solution (5 mg/ml) in phosphate buffered saline (PBS) 7.2 were added. The cells were incubated at 37 °C in 5% CO_2 for 4 h. The formazan salt was then dissolved in DMSO. The absorbance was read at 570 nm.

Statistical analyses and metabolic pathway construction

Significance differences of the obtained data were tested using ANOVA analysis on GraphPad PRISMTM software (Graph Pad Software, Inc., San Diego, CA, USA). Results are expressed as a mean of six replicates $\pm$ SD. The significance level was set at $p < 0.05$. Quantitative

normalization and heatmap diagram was constructed using Metaboanalyst online analysis. Metabolic pathway was generated according to pathway analysis on Metaboanalyst, according to KEGG metabolic database (Xia et al. 2015).

Results and discussion

Visual inspection of the ^1H NMR spectra

The assignments of the signals were achieved based on comparisons with the chemical shifts of standard compounds, database obtained from Chenomx NMR software suite and comparison with previously published data (Georgiev et al. 2011; Mediani et al. 2012). These are presented in Table 1, representing a wide range of metabolites including amino acid, sugars and phenolic compounds. The representative spectrum of Deglet Noor was shown in supplementary material Fig. S1. The most intense signals are observed in the mid to low field region of δ 3.00–5.00 of the 1D NMR spectrum, representing the carbohydrate constituents with several overlapping signals (Supplementary Material Fig. S1a). This was in agreement with the results of the previous study, which found that simple sugars were the main metabolites detected in dates, where glucose and fructose were accounted for around 97% of total ion chromatogram (TIC) (Al-Farsi et al. 2005). Sugars were identified by their characteristics peaks for sucrose at δ 5.40, anomeric protons of beta glucose at δ 4.60 and alpha xylose at δ 5.20. The high field region of δ 0.80 to δ 3.50 displayed peaks mostly associated with amino acids. Peaks at δ 0.89 and δ 1.90, which may be assigned to steroid and acetate were observed. Meanwhile, the low field area beyond δ 6.00 demonstrated the occurrence of some aromatic compounds. The signal attribute to gallic acid at δ 7.20 was present along with δ 6.62 for fumaric acid. Although the overlapping resonances are quite complex, some signals were distinctive. The difficulty in metabolite identification resulting from overlapping signals in the spectra was overcome by utilizing 2D J-resolved NMR technique (Supplementary Material Fig. S1b).

Pattern recognition analysis of the NMR spectra

The unsupervised PCA scores plot was constructed based on the acquired NMR spectral data as shown in Fig. 1a, which accounted for the total variance of 81.4% ($\text{PC1} = 53.5\%$ and $\text{PC2} = 27.9\%$) from the original data. From this plot, Deglet Noor appeared to be clearly separated from the other four varieties. The Ajwa and Bam samples were also well clustered and separated from one another. The results suggested that a good clustering of date fruits of different varieties and origin was achieved,

Table 1 Assignments of NMR spectral signals attained from different varieties of *P. dactylifera* using CD₃OD as solvent where; (*s* singlet, *d* doublet and *m* multiplet) and identification of the varieties where the compounds were identified

Compounds	Chemical shift	Different varieties of date palm fruits				
		Ajwa	Ambar	Safawi	Deglet Noor	Bam
Steroid	0.79 (<i>s</i>), 0.89 (<i>s</i>)	+	+	+	+	+
Alanine	1.46 (<i>d</i> , <i>J</i> = 7.5)		+	+	+	+
Arginine	1.60–1.75 (<i>m</i>)	+	+	+	+	+
Asparagine	2.73–2.78 (<i>m</i>), 2.77–2.90 (<i>m</i>)	+	+	+	+	+
Proline	1.99–2.09 (<i>m</i>)	+	+	+	+	+
Valine	2.28 (<i>m</i>)			+	+	+
Phenylalanine	7.32–7.33 (<i>m</i>), 7.38 (<i>m</i>)	+	+	+	+	+
Betaine	3.27 (<i>s</i>), 3.85 (<i>s</i>)	+	+	+	+	+
Choline	3.30 (<i>s</i>)	+	+	+	+	+
Inosine	4.22 (<i>m</i>), 4.38 (<i>m</i>), 4.69 (<i>m</i>)	+	+	+	+	+
Glutamine	2.07–2.17 (<i>m</i>), 2.00–2.10 (<i>m</i>)	+	+	+	+	+
Glycine	3.53 (<i>s</i>), 3.57 (<i>s</i>)	+	+	+	+	+
Isoleucine	0.89 (<i>t</i> , <i>J</i> = 7.4)	+	+	+	+	+
Leucine	0.94 (<i>t</i> , <i>J</i> = 6.1)	+	+	+	+	+
Acetate	1.90 (<i>s</i>)	+	+	+	+	+
Acetic acid	2.05 (<i>s</i>)		+		+	
Lactic acid	1.31 (<i>d</i> , <i>J</i> = 6.5)			+	+	+
Linoleic acid	1.25–1.38 (<i>m</i>)	+	+	+	+	+
Glutamic acid	1.92–2.05 (<i>m</i>)	+	+	+	+	+
Pyruvic acid	2.38 (<i>s</i>)	+	+	+	+	+
Succinic acid	2.63 (<i>s</i>), 2.66 (<i>s</i>)	+	+	+	+	+
Ascorbic acid	3.72–3.76 (<i>m</i>), 3.70–3.73 (<i>m</i>)	+	+	+	+	+
Maleic acid	6.13 (<i>s</i>), 6.39 (<i>s</i>)	+	+	+		+
Fumaric acid	6.62 (<i>s</i>)	+	+	+	+	+
Flavonoid	1.38 (<i>s</i>), 1.52 (<i>s</i>), 1.70 (<i>s</i>)	+	+	+		
Quercetin derivatives	6.19 (<i>d</i> , <i>J</i> = 2.0), 6.28 (<i>d</i> , <i>J</i> = 1.8), 6.33 (<i>d</i> , <i>J</i> = 1.5), 6.99 (<i>d</i> , <i>J</i> = 8.6)	+		+		
Kaempferol	6.17 (<i>d</i> , <i>J</i> = 2.0), 6.24 (<i>d</i> , <i>J</i> = 2.0), 6.31 (<i>d</i> , <i>J</i> = 2.0), 6.72 (<i>d</i> , <i>J</i> = 8.6), 6.91 (<i>d</i> , <i>J</i> = 8.0)	+	+			
Epicatechin	6.89–6.91 (<i>m</i>), 6.98–7.01 (<i>m</i>)	+	+	+	+	+
Gallic acid	7.01 (<i>s</i>)	+		+	+	+
Beta Glucose	4.47–4.49 (<i>d</i> , <i>J</i> = 8.0), 4.60 (<i>d</i> , <i>J</i> = 8.0)	+	+	+	+	+
Alpha Xylose	5.20 (<i>d</i> , <i>J</i> = 3.5)	+	+	+	+	+
Sucrose	5.40 (<i>d</i> , <i>J</i> = 3.8), 4.19 (<i>d</i> , <i>J</i> = 8.7)	+	+	+	+	+
Fructose	4.16 (<i>d</i> , <i>J</i> = 8.6)	+	+	+	+	+

with clear discrimination between some clusters. The Iranian Bam dates were distinguishable from Deglet Noor by PC2, whereas the Madinah Ajwa was separated from Deglet Noor and Bam by PC1 (Fig. 1a). Among the Madinah dates, well clustering are shown by the Safawi and Ajwa which separated by PC2, while the Ambar samples are more dispersed.

The PCA loadings plot indicated that the Deglet Noor samples are clustered in the bottom left quadrant and

separated from other varieties, were influenced by the fructose and sucrose contents. Other metabolites including epicatechin, fumaric acid, quercetin, kaempferol, maleic acid, alpha xylose, ascorbic acid, asparagines, succinic acid, pyruvic acid, glutamine, acetate, arginine, alanine, lactic acid, leucine and steroid are located in the right quadrant, hence contribute to the separation of Ajwa from Deglet Noor by PC1 (Fig. 1b).

Fig. 1 **a** PCA scores plot for varieties of *Phoenix dactylifera*; Deglet Noor (dn), Ambar (am), Ajwa (aj), Safawi (as) and Bam (bm). **b** The PCA loadings plot **c** HCA dendrogram: 1, gallic acid; 2, fumaric acid; 3, asparagine; 4, glutamine; 5, acetate; 6, arginine; 7, alanine; 8,

lactic acid; 9, steroid; 10, sucrose; 11, alpha xylose; 12, beta glucose; 13, fructose; 14, choline; 15, glycine; 16, leucine; 17, pyruvic acid; 18, succinic acid; 19, ascorbic acid; 20, maleic acid; 21, flavonoid; 22, quercetin derivatives; 23, kaempferol; 24, epicatechin

The PCA scores plot offers adequate information about the clustering pattern of date samples on the basis of the metabolite constitution. Nevertheless, PCA did not demonstrate the extent of similarities between the clusters. Hierarchical cluster analysis (HCA) was carried out to observe this trend the model was developed using Ward linkage method (Madala et al. 2014). Comparable clustering pattern to those of PCA is observed in the resulting HCA showing the extent of similarities (or difference) between different date samples. As shown in Fig. 1c, HCA dendrogram clearly revealed the formation of two major cluster groups (1) and (2). The first cluster group (1) is mostly dominated by the Deglet Noor, suggesting the distant relationship between these dates from other varieties. This was in agreement with the cluster shown in PCA scores plot, where the Deglet Noor cluster is found in the bottom left quadrant of the PC1, and separated farther from the other clusters (Fig. 1a). In the other cluster group (2), two sub-groups are formed, one being largely dominated by the Bam and the other by the Ajwa varieties. The Safawi and Ambar clusters are more dispersed and overlap with the Ajwa and Bam.

Characteristics of compounds from *Phoenix dactylifera* varieties

The metabolite profile of the five dates samples are listed in Table 1. All Madinah dates were found to contain phenolic compounds. Cultivation practice and environmental condition, for instance temperature, UV intensity, sun exposure time and rainfall contribute effectively to the metabolic course and the resulting metabolite profiles of plants (Kim et al. 2011). Previous study has shown that the metabolite compositions of cabbages originating from Korea are different from those of China (Kim et al. 2013).

The relative concentrations of metabolites in individual dates variety are represented in Fig. 2. Analysis of variance (ANOVA) suggested that the level of metabolites in different date varieties from different origins were statistically significance. The Madinah dates, especially the Ajwa contained a higher amount of most metabolites, except for sucrose (Fig. 2d). However, Deglet Noor showed a remarkably higher amount of fructose as compared to others, while all the Madinah dates are lacking in alpha xylose (the cell wall non-cellulosic sugar) (Table 2). Earlier study has shown that the metabolism of this compound, which was one of the products of pectin degradation, plays a role towards the softening of fruit (Nikolic and Mojovic 2008). The higher amount of xylose results in the softer fruit texture. It is, therefore, plausible to suggest that the variation of textures in different date varieties could be associated with the level of xylose present. The high concentration of xylose in Deglet Noor and Bam was probably

contributed towards the softer textures in these two varieties. This was further supported by the same trends displayed for moisture content in different date varieties (Table 2). Deglet Noor showed higher moisture content compared to the other four varieties with the Madinah dates being the least. The same trend was also observed in the earlier study where the hardness of wheat samples decreased with the increment of moisture content (Katz et al. 1961). Therefore, based on the amount of xylose and moisture content presented here, Deglet Noor was indeed having the softest texture among these five date varieties. It has been demonstrated that Deglet Noor was among date varieties that having higher sensitivity to rainfall and humidity (Abul-Soad et al. 2013). The softness and the high moisture content might be the possible reason for Deglet Noor to be susceptible to injury due to those two circumstances.

Since fructose is known to be sweeter than glucose or sucrose, the higher sweetness level displayed by Deglet Noor could, therefore, be associated with the higher level of this sugar (Bray 2007). With respect to texture, the trend of results was in association with those clusters formed in PCA score plot (Fig. 1a). It can be observed that the soft texture of dates is decreasing from left to the right quadrant of the respective PCA score plot.

NO inhibitory activity

The Ajwa dates demonstrated the highest NO inhibitory effect at 95.37% inhibition as summarized in Table 2. In contrast, Deglet Noor exhibited the lowest inhibition at 46.54% inhibition. Curcumin which served as the positive control was found to inhibit the production of NO by 97% at 0.1 mg/mL. All date extracts showed high percentage of cell viability, as presented in Table 2 hence, the observed results were not due to the cytotoxicity effect. The potential efficacy of medicinal plants can be associated with the biochemistry of plant species, which might differ owing to external factors mentioned earlier. Therefore, the observed NO inhibitory activity could be explained by taking into accounts the origins of the date fruits (Taylor and van Staden 2001).

Correlations between metabolite variation and biological activity

The partial least square (PLS) biplot (Fig. 3a) demonstrated the association between the metabolite contents (peak area) assigned as the X variable, and NO inhibitory activity, assigned as the Y variable. The model developed from the spectral and biological data is statistically acceptable with the values of the total sum of variation in Y explained by the model (R^2Y) at 0.88, and goodness of

Fig. 2 a–d Relative quantification of the identified metabolites on the basis of the mean peak area of the ^1H -NMR signals, where Ajwa (aj), Safawi (as), Ambar (am), Deglet Noor (dn) and Bam (bm); 1, gallic acid; 2, fumaric acid; 3, asparagine; 4, glutamine; 6, arginine; 7,

alanine; 8, lactic acid; 9, steroid; 10, sucrose; 12, beta glucose; 16, leucine; 17, pyruvic acid; 18, succinic acid; 21, flavonoid; 22, quercetin derivatives; 23, kaempferol

Table 2 The variation in nitric oxide (NO) inhibitory activity, texture and sweetness level obtained from different varieties of *Phoenix dactylifera*; Deglet Noor (dn), Ambar (am), Ajwa (aj), Safawi (as) and Bam (bm)

Samples	NO		Texture		Sweetness
	NO inhibition (%)	Cells viability %	Xylose content (mM)	Moisture content (%)	Fructose content (mM)
<i>Different varieties of date palm fruits</i>					
Aj	95.37 $\pm$ 1.27 ^a	72.33 $\pm$ 2.62 ^a	29.39 $\pm$ 3.85 ^a	19.31 $\pm$ 0.40 ^a	10.63 $\pm$ 2.29 ^a
Am	77.76 $\pm$ 3.65 ^b	75.65 $\pm$ 0.26 ^a	32.47 $\pm$ 1.96 ^a	18.21 $\pm$ 0.23 ^a	18.73 $\pm$ 2.71 ^b
As	87.59 $\pm$ 1.55 ^c	85.16 $\pm$ 0.26 ^b	25.06 $\pm$ 3.35 ^a	17.13 $\pm$ 0.08 ^a	11.20 $\pm$ 1.20 ^a
Dn	46.54 $\pm$ 3.31 ^d	76.69 $\pm$ 0.96 ^a	39.00 $\pm$ 3.33 ^b	22.64 $\pm$ 1.32 ^b	19.67 $\pm$ 0.26 ^c
Bm	58.05 $\pm$ 3.19 ^c	60.47 $\pm$ 0.13 ^c	39.91 $\pm$ 2.84 ^b	20.47 $\pm$ 0.56 ^c	18.60 $\pm$ 1.21 ^c

Value = mean $\pm$ SD. Value in the same column with different superscript letter differ significantly at 5% probability level

prediction Q^2 at 0.85. The PLS biplot showed good separation of the samples into two groups with the samples from Madinah were positioned in the right quadrant, while Deglet Noor and Bam were clustered in the left quadrant.

Metabolites that contribute to the separation of clusters are also presented in the same plot, which indicates the presence of alpha xylose, choline, linoleic acid, succinic acid, lactic acid, some amino acids (proline, glutamine, arginine, leucine, isoleucine, asparagines), and phenolic

Fig. 3 **a** The PLS biplot **b** Heatmap visualization: 1, gallic acid; 2, fumaric acid; 3, asparagine; 4, glutamine; 6, arginine; 7, alanine; 8, lactic acid; 9, steroid; 10, sucrose; 11, alpha xylose; 13, fructose; 14,

choline 15, glycine 16, leucine; 17, pyruvic acid; 18, succinic acid; 21, flavonoid; 22, quercetin derivatives; 23, kaempferol

compounds (kaempferol, quercetin derivatives, gallic acid) (Fig. 3a), thus suggesting that these metabolites contribute to the activity of the respective samples. These metabolites are located nearer to the profile of the Ajwa. On the other hand, sucrose and fructose were positioned in the opposite

direction of the plot, hence suggesting the negative correlation with NO inhibitory activity.

Variable importance in the projection (VIP) values was generated to assess the association between the X and Y variables in the PLS model. The VIP values of the major contributing compounds for the observed discrimination in

the scores plot derived from PLS were larger than 0.7, thus suggesting that the respective compounds prominently contributing to the observed NO inhibitory activity (Supplementary Material Fig. S2). The succinic acid was having the highest VIP value hence suggesting the most significant correlation between the identified metabolite and NO inhibitory activity. Previously, Chien et al. (2008) reported that succinic acid derivatives inhibited the production of inflammation mediator in the LPS stimulated RAW cells. Besides that, succinic acid has also been used as one of the formulations in developing newly combined drug to treat inflammation related diseases (Leleka et al. 2016). These may explain the observed correlation between succinic acid and the tested biological activity in the current study. However, the mechanism of action for succinic acid as the anti-inflammatory agent has yet to be proven. The PLS model was cross-validated using a permutation test, which gave the Y-intercept of R^2 and Q^2 of 0.05 and -0.30 , respectively, indicating a good validity of the model (Supplementary Material Fig. S3) (Eriksson et al. 2006). The PLS model also demonstrated that it fits well based on the values of regression coefficient at 0.88 for the NO activity (Supplementary Material Fig. S4). These findings support the implication that the constructed PLS model based on NMR data can be used in predicting the NO inhibitory activity of new date samples.

Previous study has reported that phenolic compounds exhibited anti-inflammatory properties in various experimental models (Hsu et al. 2013; Joo et al. 2014). Thus, it is not surprising that all the Madinah date samples exhibited strong NO inhibition activity due to the presence of kaempferol, quercetin derivatives and gallic acid, particularly by the Ajwa variety. Earlier studies also demonstrated that a rich sucrose diet could lead to obesity in mice exhibiting a higher expression of inflammatory markers in adipose tissue (Ma et al. 2011). Additionally, the fructose fed rats can also be distinguished by higher plasma NO level, indicating elevated production of nitric oxide (Buserolles et al. 2002). On the basis of this, the higher sucrose and fructose contents in the Deglet Noor and Bam dates could contribute to the weak NO inhibitory activity.

The observed inhibition coincides with results of the *in vivo* study on white albino rats performed by Mohamed and Al-Okbi (2004), which showed that the methanolic extracts from an edible portion of date fruits possessed the anti-inflammatory property. The study revealed that the oral administration of date extracts caused a significant reduction in the hind paw inflammation throughout the experiment. The Ajwa dates has been the most cherished date variety cultivated in Madinah, and has been sold at the price three times higher than its next best competitor (Zhang et al. 2013). The results obtained from this study

support the consistency of higher pricing of the Ajwa dates due to its medicinal and nutritional values.

The correlation between NO inhibitory activity and metabolites content was also assessed by generating heatmap using Ward's method, emphasizing the metabolites obtained from previous PLS analysis (Fig. 3b). The significance of metabolites and NO inhibition were displayed in red for high, and green for low abundances (Xia et al. 2015). The resulting heatmap generated from the biological and spectroscopic data demonstrated that the Ajwa dates, which exhibited strongest NO inhibitory activity, contained a higher prevalence of quercetin, kaempferol and gallic acid. Both Ajwa and Safawi showed the brightest green color for sucrose and fructose in contrast to the high intensity of red color shown by Deglet Noor. The dendrogram (vertical axis) displayed closer relationship of NO with these two metabolites hence further suggesting that they are contributing more towards the low NO inhibition of Deglet Noor.

Identification of impacted metabolite pathways

To determine the metabolic pathways implicated to affect the variation in metabolite profiles of different date varieties, the metabolites with VIP scores > 0.7 were mapped as presented in Supplementary Material Fig. S5. The utilized metabolite list is considerably limited and offers not more than five metabolite hits for each pathway, yet a ranking of relative importance was acquired (Alam et al. 2012). The pathway analysis of the corresponding pathways displayed by p value on the Y axis and the impact values along the X-axis. The size of round spots implies the pathway impact, estimated on the basis of the impact of every identified metabolite in the specified pathway. The round spot color is classified based on its p value from pathway enrichment analysis (Yang et al. 2015). The alanine, aspartate and glutamate metabolisms were found as having the highest $-\log(p)$ values for these five date varieties. Glycolysis and gluconeogenesis are also indicated as comparatively essential followed by the starch and sucrose metabolisms. Aspartate is a precursor of asparagine, while the pyruvic acid and succinic acid can be synthesized through alanine metabolism and 2-ketoglutaric acid leads to the synthesis of glutamine as presented in Fig. 4. Another matched metabolite; fumaric acid was also synthesized from aspartate metabolism. Based on Fig. 4 it can be seen that Ajwa showed higher accumulation of the previously mentioned metabolites except for succinic acid (Fig. 4).

Fig. 4 Metabolic pathway of alanine, aspartate and glutamate metabolism as generated by Metaboanalyst software: red bars indicate the Ajwa, green for Ambar, dark blue for Safawi, light blue bars for Bam and light purple for Deglet Noor

Conclusion

¹H-NMR fingerprinting coupled with MVDA was effectively used to differentiate five commercial palm dates samples obtained from different countries into fairly distinct clusters. Deglet Noor can be well separated from the other four varieties because of the higher level of sucrose and fructose. There were 34 metabolites were identified where phenolics can be suggested to be the compounds responsible towards the discrimination of Ajwa from the other date varieties. The higher presence of phenolics including quercetin, kaempferol and flavonoid might be responsible for the valuable properties of the Madinah dates. In this study, the Ajwa dates were the strongest NO inhibitor compared to the other date varieties with Deglet Noor being the least. The generated PLS model revealed that succinic acid was the metabolite with the most significant correlation with the NO inhibitory activity. Meanwhile, sucrose and fructose were negatively correlated with the NO inhibition of Deglet Noor. The high

content of these two sugars may lead to the weak NO inhibition of Deglet Noor dates. Furthermore, Deglet Noor also appeared to demonstrate the softest texture and higher sweetness level compared to the Iranian and Madinah dates as suggested by the amount of xylose and fructose, respectively. The Ajwa dates demonstrated the high level of most metabolites and the variation of the amount of metabolites in the generated alanine, aspartate and glutamate metabolisms. The Madinah Ajwa dates, which has received a high preference among consumers, was indeed having better NO inhibition and also variation in term of metabolites compositions. The findings described here can serve as the groundwork for selection of date palm varieties especially in conducting further detailed experimental study in the development of functional food.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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