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Date (*Phoenix dactylifera* L.) Fruit Soluble Phenolics Composition and Anti-atherogenic Properties in Nine Israeli Varieties

Hamutal Borochov-Neori,*[,†] Sylvie Judeinstein,[†] Amnon Greenberg,[†] Nina Volkova,[‡] Mira Rosenblat,[‡] and Michael Aviram[‡]

[†]Southern Arava Research and Development, M. P. Hevel Eilot 88820, Israel

[‡]The Lipid Research Laboratory, Rappaport Faculty of Medicine and the Rappaport Family Institute for Research in the Medical Sciences, Technion-Israel Institute of Technology, Rambam Medical Center, Haifa 31096, Israel

ABSTRACT: Date (*Phoenix dactylifera* L.) fruit soluble phenolics composition and anti-atherogenic properties were examined in nine diverse Israeli grown varieties. Ethanol and acetone extracts of 'Amari', 'Barhi', 'Deglet Noor', 'Deri', 'Hadrawi', 'Hallawi', 'Hayani', 'Medjool', and 'Zahidi' fruit were analyzed for phenolics composition by RP-HPLC and tested for anti-atherogenicity by measuring their effects on LDL susceptibility to copper ion- and free radical-induced oxidation, and on serum-mediated cholesterol efflux from macrophages. The most frequently detected phenolics were hydroxybenzoates, hydroxycinnamates, and flavonols. Significant differences in phenolics composition were established between varieties as well as extraction solvents. All extracts inhibited LDL oxidation, and most extracts also stimulated cholesterol removal from macrophages. Considerable varietal differences were measured in the levels of the bioactivities. Also, acetone extracts exhibited a significantly higher anti-atherogenic potency for most varieties. The presence of soluble ingredients with anti-atherogenic capacities in dates and the possible involvement of phenolics are discussed.

KEYWORDS: *anti-atherogenicity, antioxidants, cholesterol removal, date fruit, LDL oxidation, phenolics, Phoenix dactylifera L*

INTRODUCTION

The date fruit has been cultivated and utilized as an important staple food in the Arabian Peninsula, Middle East, and North Africa for over 5,000 years.[1] The nutritional benefits and ethnomedical uses of the fruit were extensively reviewed in recent publications.[2−4] Dates constitute a high-energy diet due to the high sugar content, and they are rich in dietary fibers, valuable macro- and microminerals, including potassium, calcium, magnesium, iron and zinc, and phenolic compounds. Dates are considered as general tonic in the traditional Indian medicine and are used in treatment for various diseases in Middle Eastern folk medicine, probably due to the fruit immunomodulatory,[5] antibacterial,[6] and antifungal[7] properties. Furthermore, hydrophilic extracts of date fruit from various varieties grown in different regions exhibited potent antioxidant and free radical scavenging capacities with magnitudes depending on cultivar and location.[8−14] The antioxidant properties were mostly attributed to the wide range of phenolic compounds in the dates, including phenolic acids (such as gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, *p*-coumaric, *o*-coumaric, syringic, ferulic, and caffeic acid) and flavonoids (such as flavonols, flavanones, and procyanidins).[8−12,15,16]

Atherosclerosis, the leading cause of morbidity and mortality among people with a western life style, develops as a result of various risk factors. Macrophage cholesterol accumulation and foam cell formation initiate atherogenesis,[17] and oxidative stress enhances the development and progression of the disease.[18] Phenolic compounds, especially flavonoids, are highly effective nutritional antioxidants,[19] as they inhibit LDL oxidation, secondary to their ability to scavenge free radicals and to chelate transition metal ions.[20] Indeed, epidemiological studies have demonstrated that increased dietary intake of antioxidant rich foodstuff was associated with reduced morbidity and mortality from coronary artery disease.[21] We have demonstrated the anti-atherogenic beneficial effects of the consumption of polyphenolics rich fruit, such as pomegranate and marula, by atherosclerotic patients, and also by healthy subjects.[22,23]

Considering its high content and diversity of phenolic compounds, the date fruit is a potential candidate as an anti-atherogenic food. Indeed, in our recent *in vivo* study on regular consumption of two date varieties ('Medjool' and 'Hallawi') by healthy human subjects,[12] we have found that the levels of serum triglycerides decreased significantly. Notably, the basal serum oxidative status and the susceptibility of serum to AAPH-induced lipid peroxidation also decreased significantly after the consumption of 'Hallawi' but not 'Medjool' dates. These two varieties largely differed in the fruit soluble phenolics composition. The above varietal dependent health effects could be associated with the diverse bioactivities exerted by different phenolic compounds, but the involvement of other components in the date fruit cannot be excluded.

The superiority of 'Hallawi' dates in improving serum oxidative status is of a special interest, since it is a local variety whereas the 'Medjool' fruit is worldwide recognized and appreciated. Of the many available cultivars of dates, only a few ('Deglet Noor', 'Medjool', and 'Barhi') have become preeminent in the world market. Underutilized date varieties may gain commercial significance if established as health beneficial.

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The present study examined the soluble phenolics composition and anti-atherogenic activities of nine Israeli grown date varieties, including renowned ('Medjool', 'Deglet Noor', and 'Barhi') and local ('Amari', 'Deri', 'Hadrawi', 'Hallawi', 'Hayani', and 'Zahidi') varieties. Ethanol and acetone extracts were prepared from commercial fruit and analyzed for phenolics profile, inhibition of LDL oxidation, and *in virto* stimulation of serum-mediated cholesterol efflux from macrophages.

MATERIALS AND METHODS

Date Varieties. Commercial date fruit of nine varieties, 'Amari', 'Barhi', 'Deglet Noor', 'Deri', 'Hadrawi', 'Hallawi', 'Hayani', 'Medjool', and 'Zahidi', were supplied shortly after harvest by *Hadiklaim*, Israel Date Growers Cooperative Ltd. 'Amari', 'Deglet Noor', 'Deri', 'Hadrawi', 'Hallawi', 'Medjool', and 'Zahidi' dates were semidry tree ripened ("tamr" stage) fruit. 'Hayani' fruit were harvested in the "khalal" stage (mature unripe) and postharvest subjected to freeze/thaw pack-house practices to soften the fruit and remove its astringency. 'Barhi' fruit were harvested as "khalal". The fruit were stored at −20 °C until further processing.

Materials. Caffeic acid, catechin, catechin gallate, chlorogenic acid, *o*-coumaric acid, *p*-coumaric acid, epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate, ferulic acid, gallic acid, gallocatechin, gallocatechin gallate, hydrocaffeic acid, phenyl acetate, pyrogallol, protocatechuic acid, sinapic acid, syringic acid, vanillic acid, tannic acid, and Folin-Ciocalteau reagent were all purchased from Sigma-Aldrich Co., Israel. Ellagic acid, 2-hydroxybenzoic (salicylic) acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, kaempferol-3-glucoside, procyanidin B2, and quercetin-3-β-glucoside were acquired from Fluka. Cyanidin-3,5-diglucoside, delphinidin-3,5-diglucoside, pelargonidin-3,5-diglucoside, and malvidin-3-glucoside chloride were purchased from Apin Chemicals. Cyanidin-3-glucoside, delphinidin-3-glucoside, and pelargonidin-3-glucoside were obtained from Polyphenols Laboratories AS. 2,2′-Azobis-2-amidinopropane hydrochloride (AAPH) was from Wako, Japan. Acetonitrile HPLC grade was purchased from Merck, and phosphoric acid was purchased from Frutarum, Israel. [^{3}H]-Labeled cholesterol was acquired from Amersham. Dulbecco's modified Eagel's medium (DMEM) and fetal calf serum (FCS) were purchased from Biological Industries, Beit Haemek, Israel.

Water Content Determination. Ten gram samples of thinly sliced pitted date fruit were arranged in a single layer on drying plates and placed in a laboratory air circulating oven (Heraeus Instruments, model UT6, Germany) set at 60 °C. After 24 h the plates were weighed twice daily and removed from the oven after the same weight was recorded in two consecutive measurements (up to a total of 48 h). Moisture content was calculated as follows:

$$\text{water content (\%)} = \frac{\text{init wt} - \text{final wt}}{\text{init wt}} \times 100$$

Extract Preparation. Forty pitted fruit (350−800 g, depending on date variety) were minced with Moulinex HV8 mincer ME60514A. Single-cycle extracts from 20 g of minced fruit were prepared in triplicate. *Ethanol extracts*: minced dates were homogenized in cold 50% ethanol using a mortar and pestle. The extraction solution was added stepwise to a final fruit to solvent ratio of 1:2 (w/w). The homogenate was centrifuged (10,000 rpm for 10 min at 4 °C, Sorvall Instruments RC5C, rotor number SS-34), and the clear supernatant was collected. *Acetone extracts*: minced dates were homogenized as described earlier in cold 70% acetone containing 0.5% acetic acid. The final fruit to solvent ratio was 1:3 (w/w). After centrifugation, the clear supernatant was collected, and acetone was removed at 40 °C by a rotary evaporator (Rotavapor model R114, BUCHI Labortechnik AG, Switzerland). Ethanol and double distilled water (DDW) were added to the acetone-free fraction to resume the original extract volume at a final solvent composition of 50% ethanol.

Total Soluble Phenolics Content. Total soluble phenolics content in date extracts was measured colorimetrically with Folin-Ciocalteau 2N phenol reagent following Singleton and Rossi.[24] Extracts were diluted 10- and 20-fold with DDW and assayed in triplicate. Aliquots of 100 μL were added to a 900 μL reaction solution consisting of 200 μL of freshly prepared 10-fold diluted Folin-Ciocalteau reagent, 100 μL of 20% Na_2CO_3, and 600 μL of DDW. Calibration curves were constructed with gallic acid (0, 5, 10, 25, 50, 75, and 100 μg mL^{-1}). The absorbance at 765 nm was measured after 1 h of incubation, and the results were expressed in terms of gallic acid equivalents (GAE).

RP-HPLC Analysis of Phenolics. Date fruit extracts were filtered through a 0.45 μm filter before injection. Samples were analyzed with a LaChrom Merck Hitachi HPLC system, consisting of a Pump L7100, a column oven L7350, and a mixer-degasser L-7614, coupled with a diode array detector with a 3D feature (Multiwavelength Detector, Jasco MD-2010 Plus), an interface (Jasco LC-Net II/ADC), and scientific software (EZChrom *Elite* Client/Server version 3.1.6 build 3.1.6.2433) that provides real time data acquisition and postrun data manipulation and integration capabilities. Extract (20 μL) was injected using a manual injector (Rheodyne, Rohnert Park, CA) and loaded onto a PurospherStar RP-18 end-capped column (250 mm × 4 mm LichroCART cartridge, 5 μm particle size) with an end-capped Lichrospher100 RP-18 guard column (4 mm × 4 mm LichroCART cartridge, 5 μm particle size). The binary mobile phase consisted of 0.1% phosphoric acid, pH 2.4 (solution A), and acetonitrile (solution B). Elution was carried out at a flow rate of 1 mL min^{-1} with the following gradient outline: 1−10 min, 5−15% solution B; 10−30 min, 15−30% solution B; 30−40 min, 30−100% solution B. The column was then washed and equilibrated by 10-min postruns with 100% and 5% B, respectively. Each run was monitored in real time by three display modes simultaneously: contour plot, chromatogram display at a chosen wavelength (usually 280 nm), and absorption spectra (200−650 nm). The oven temperature was set at 40 °C, and the pressure was 158 atm. Acetonitrile was HPLC grade (LiChrosolv Merck); column-filtered water was further distilled with a Corning Megapure System, MP-6A, and passed through a 0.20 μm Nylon membrane. Phosphoric acid was of analytical grade.

Phenolics Identification and Quantification. A phenolics standard library was constructed as follows: Each authentic standard (50−100 μg mL^{-1} in methanol) was injected separately, and the data acquired by the photodiode array detector with the 3D feature were incorporated into the system phenolic standard library. The library included catechin; epicatechin; catechin gallate; epicatechin gallate; gallocatechin; epigallocatechin; gallocatechin gallate; epigallocatechin gallate; caffeic, chlorogenic, *o*-coumaric, *p*-coumaric, 3-hydroxybenzoic, 4-hydroxybenzoic, ellagic, ferulic, gallic, hydrocaffeic, protocatechuic, salicylic, sinapic, syringic, vanillic, and tannic acid; kaempferol-3-glucoside; pyrogallol; quercetin-3-β-glucoside; procyanidin B2; 3-mono- and 3,5-diglucosides of delphinidin; cyanidin and pelargonidin; and malvidin 3-glucoside chloride. Peak assignment was performed by the software on the basis of UV/vis absorbance spectra and the retention times of the phenolics standards. Each peak was tested for purity by a three point purity test and for similarity by a library search comparing the peak spectrum to that of the standards. High similarity index and a common retention time with the standard were considered a positive identification; a similar UV/vis absorption spectrum but a different retention time was considered a partial identification (e.g., derivative of the phenolic compound with the similar absorption spectrum). Under the conditions employed in this study, the relative standard deviation for the retention times in three repetitive runs was in the range of 0.2−1.9%.

Individual phenolic compounds were quantified from the corresponding chromatogram peak area calculated by the software and standard calibration curves. Calibration curves (linear, $R^2 = 0.999$) were constructed with authentic standards of caffeic acid, gallic acid, quercetin-3-β-glucoside, and salicylic acid at six concentrations in the range of 1−200 μg mL^{-1} and of cyanidin 3-glucoside at four concentrations in the range of 5−500 μg mL^{-1}. Sample concentrations of gallic acid derivatives were expressed in terms of GAE; monohydroxybenzoic acid derivatives in salicylic acid equivalents; hydroxycinnamic acid derivatives in caffeic acid equivalents; flavonols in quercetin-β-glucoside equivalents; and anthocyanins in cyanidin-3-glucoside equivalents. The concentrations were determined from three repetitive runs.

Table 1. Soluble Phenolics Extraction from Nine Israeli Grown Date Varieties by Ethanol and Acetone Solvents: Extract Concentration and Extraction Yield[a]

	extract phenolics conc (GAE, μmol mL^{-1})		date phenolics extraction yield (GAE, μmol g FW^{-1})	
date variety	ethanol	acetone	ethanol	acetone
Amari	3.44 ± 0.17 a	4.71 ± 0.28* a	6.36 ± 0.32 a	9.42 ± 0.57* b
Barhi	0.86 ± 0.04 c	1.05 ± 0.13 d	2.15 ± 0.11 c	3.16 ± 0.38# d
Deglet Noor	2.41 ± 0.24 ab	2.30 ± 0.21 b	4.21 ± 0.42 b	5.40 ± 0.49# c
Deri	3.01 ± 0.45 a	1.93 ± 0.23# bc	6.03 ± 0.90 a	3.90 ± 0.47# cd
Hadrawi	2.21 ± 0.18 b	1.63 ± 0.25# c	4.19 ± 0.34 b	3.13 ± 0.47# d
Hallawi	2.31 ± 0.14 b	1.44 ± 0.14* cd	4.50 ± 0.27 b	2.61 ± 0.26* d
Hayani	2.20 ± 0.26 b	4.75 ± 0.48* a	5.07 ± 0.61 ab	15.29 ± 1.53* a
Medjool	2.39 ± 0.29 b	1.76 ± 0.09# c	4.31 ± 0.52 b	3.60 ± 0.18 cd
Zahidi	3.28 ± 0.33 a	1.67 ± 0.13* c	5.25 ± 0.52 ab	2.95 ± 0.24* d

[a]Values are mean ± SD (n = 3). Values within a column with the same letter are not significantly different at a 95% confidence interval (Tukey–Kramer posthoc). * and # denote significant differences at $P < 0.01$ and $P < 0.05$, respectively (one-way ANOVA), between acetone and ethanol extracts of the same variety.

LDL Preparation. LDL was isolated from plasma derived from healthy normolipidemic volunteers, by discontinuous density gradient ultracentrifugation.[25] The LDL was washed at d = 1.063 g mL^{-1}, dialyzed against 150 mmol L^{-1} NaCl, 1 mmol L^{-1} Na_2EDTA (pH 7.4) at 4 °C, and then sterilized by filtration (0.45 μm), kept under nitrogen in the dark at 4 °C, and used within 2 weeks. The LDL protein concentration was determined with the Folin phenol reagent.[26] Prior to oxidation, the LDL was dialyzed against EDTA-free, phosphate buffered saline (PBS) solution, pH 7.4, at 4 °C.

LDL Oxidation. *Copper Ion-Induced Oxidation.* LDL (100 μg of protein mL^{-1}) was preincubated for 30 min at room temperature with increasing volume concentrations (0–25 μL mL^{-1}) of the date extracts. Then, 5 μmol L^{-1} of $CuSO_4$ was added and the tubes were incubated for 1.5 h at 37 °C. At the end of the incubation, the extent of LDL oxidation was determined by measuring the generated amount of thiobarbituric acid reactive substances (TBARS). The TBARS assay was performed at 532 nm, using malondialdehyde (MDA) for the standard curve.[27] The experiments were repeated three times.

AAPH-Induced Oxidation. LDL (100 μg of protein mL^{-1}) was preincubated for 30 min at room temperature with increasing volume concentrations (0–25 μL mL^{-1}) of the date extracts. Then, 5 mmol L^{-1} of the free radical generator 2,2′-azobis-2-amidinopropane hydrochloride (AAPH)[28] was added for 1.5 h at 37 °C. At the end of the incubation, the extent of LDL oxidation was determined by the TBARS[27] assay. The experiments were repeated three times.

Serum-Mediated Cholesterol Efflux from Macrophages. J774 A.1 murine macrophage cells were purchased from the American Tissue Culture Collection (ATCC, Rockville, MD). Cells were grown in DMEM containing 5% FCS. The macrophages were preincubated at 37 °C for 20 h with 20 μL mL^{-1} of the various date extracts. The cells were then washed and incubated with [^{3}H]-labeled cholesterol (2 μCi mL^{-1}) at 37 °C for 1 h, followed by cell wash in ice-cold PBS (×3) and further incubation in the absence or presence of 20 μL mL^{-1} of serum for 3 h at 37 °C. Cellular and medium [^{3}H]-labels were quantified, and serum-mediated cholesterol efflux was calculated as the ratio of [^{3}H]-label in the medium/([^{3}H]-label in the medium + [^{3}H]-label in the cells).[29] The experiments were repeated three times.

Statistical Analysis. Results are reported as means ± their respective standard deviations (SD) of three replicates (n = 3). Statistical procedures in IBM SPSS version 17.0 were used to test for significant varietal and solvent related differences. One-way ANOVA and Tukey–Kramer posthoc means comparison tests were employed to verify significant varietal differences ($p < 0.05$). Solvent effects were tested with one-way ANOVA.

RESULTS AND DISCUSSION

The study was conducted on commercial ready-to-consume date fruit of nine varieties grown in Israel. 'Amari', 'Deglet Noor', 'Deri', 'Hadrawi', 'Hallawi', 'Medjool', and 'Zahidi' fruit were semidry ripe ("tamr" stage) and contained 23.3, 21.9, 17.8, 18.9, 14.1, 18.8, and 13.7% moisture, respectively. 'Barhi' and 'Hayani' were harvested as "khalal" (mature unripe fruit) and, thus, contained approximately 50% of the sugar accumulated in ripe dates and higher proportions of moisture, 70.5 and 62.4%, respectively. 'Barhi' fruit were crisp but without their typical slight astringency due to the freeze/thaw cycle included with the in-laboratory handling. 'Hayani' fruit were soft and nonastringent following customary pack-house postharvest practices.

Two solvents of different polarities were employed to extract date fruit phenolics: 50% ethanol and 70% acidic acetone, with dielectric constants of 46 and 36, respectively. Considering the variable polarities of phenolic compounds, rendered by their basic molecular structure, and the nature and placement of substituents, the two solvents are expected to differentially extract date phenolics. Extraction of grape skin with 70% acetone was shown to yield excessively more tannins and higher polymer sizes compared to 50% ethanol.[30] Also, 70% acetone was reported to be more efficient in extracting procyanidins from "khalal" 'Deglet Noor' date fruit.[16] On the other hand, flavan-3-ol monomers in grape seeds were shown to be more soluble in 50% ethanol than in 70% acetone.[31] Thus, ethanol date extracts are likely to be enriched with polar phenolics whereas the acetone extracts are probably richer in the less polar compounds.

Date Fruit Extract Phenolics Composition. *Total Soluble Phenolics Content.* The concentration of soluble phenolics in the date fruit extracts, expressed in micromoles of GAE per milliliter, varied considerably with variety and extraction medium (Table 1). The phenolics content in the ethanol extracts was the highest for 'Amari', 'Deri', and 'Zahidi' (3.0–3.4 μmol GAE mL^{-1}), significantly lower for 'Deglet Noor', 'Hadrawi', 'Hallawi', 'Hayani', and 'Medjool' (2.2–2.4 μmol GAE mL^{-1}), and significantly the lowest for 'Barhi' fruit (0.9 μmol GAE mL^{-1}). In the acetone extracts, the content of phenolics was the highest for 'Amari' and 'Hayani' (4.7 μmol GAE mL^{-1}), significantly lower for 'Deglet Noor', 'Deri', 'Medjool', 'Hadrawi', 'Zahidi', and 'Hallawi' (1.4–2.3 μmol GAE mL^{-1}), and significantly the lowest for 'Barhi' fruit (1.0 μmol GAE mL^{-1}). It should be noted, however, that some of the varietal differences in extracts' phenolics concentration may correspond to the differences in fruit moisture content, especially in 'Barhi' and 'Hayani'. The phenolics concentration in the ethanol compared to acetone extracts was significantly higher in 'Zahidi', 'Hallawi', 'Deri', 'Medjool', and 'Hadrawi' by 94, 64, 58, 37, and 36%, respectively,

comparable in 'Barhi' and 'Deglet Noor', and significantly lower in 'Hayani' and 'Amari' by 54 and 27%, respectively.

The yield of phenolics extraction, expressed in micromoles of GAE per gram of fruit fresh weight (FW), was solvent and variety dependent (Table 1). The ethanol solvent extracted significantly more phenolics from 'Zahidi', 'Hallawi,' 'Deri', and 'Hadrawi' fruit compared to the acetone solvent (by 78, 72, 55, and 34%, respectively), suggesting high proportions of polar phenolics in these varieties. On the other hand, ethanol was significantly less efficient than acetone in extracting phenolics from 'Hayani', 'Amari', 'Barhi', and 'Deglet Noor' fruit (by 67, 33, 32, and 22%, respectively), implying that these varieties contained higher proportions of the less polar phenolics. The extraction yields from 'Medjool' fruit with both solvents were not significantly different, suggesting similar proportions of polar and less polar phenolics or, alternatively, that the majority of the phenolics were of an intermediate polarity.

Evidently, fruit of all the nine date varieties contained considerable amounts of soluble phenolic compounds, in agreement with earlier reports on diverse date varieties from different locations.[8,10−14] However, multicycle consecutive extractions with solvents of different polarities are required to fully assess the soluble phenolics content in the various date varieties.

Phenolics Composition. Date fruit extracts were analyzed by RP-HPLC to tentatively identify and quantify the phenolics constituents. Typical HPLC chromatograms at selected wavelengths and a 10 nm window width are presented in Figure 1 for

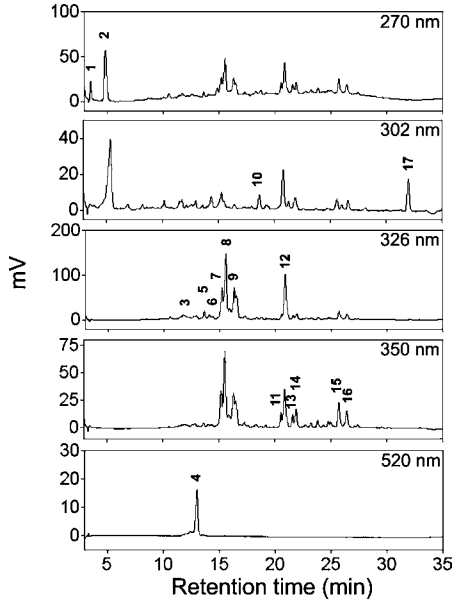

Figure 1. HPLC chromatograms of acetone date extracts at selected wavelengths. The chromatograms at 270, 326, and 350 nm were obtained with 'Amari'; at 302 nm with 'Hallawi'; and at 520 nm with 'Hayani' extract. Window width was set at10 nm. Peaks are numbered in order of ascending retention times.

the acetone extracts of 'Amari' (270, 326, and 350 nm), 'Hallawi' (302 nm), and 'Hayani' (520 nm). Analogous chromatograms were generated for all the date extracts. Seventeen prominent phenolics peaks were frequently detected; the peaks were numbered along the chromatograms (Figure 1) in order of ascending retention times. Ten peaks (1, 3, 5, 7−9, 12, and 14−16) appeared in all the chromatograms, however, at variable intensities, depending on variety and extraction solvent. The incidence and magnitude of the remaining seven peaks were variety and solvent dependent.

On the basis of the authentic standard library, the major phenolic compounds in the extracts could be assigned to five subclasses: gallic acid derivatives (GA) (peaks 1 and 2), monohydroxybenzoic acids (peak 17), hydroxycinnamic acids (HCA) (peaks 3, 5−10, and 12), flavonols (Fl) (peaks 11 and 13−16), and anthocyanins (peak 4). The presence of phenolics of these five subclasses in date fruits was reported earlier.[8−12,16] Three HCA peaks were positively identified, including caffeic, coumaric, and ferulic acid (peaks 6, 10, and 12, respectively), consistent with our earlier report on 'Medjool' and 'Hallawi' methanol extracts[12] and others reports on various date varieties.[13,20] Other HCA peaks (7−9) were partially identified as chlorogenic acid derivatives. Within the Fl subclass, peak 13 was identified as quercetin-3-β-glucoside and peaks 11 and 14−16 were partially identified as kaempferol derivatives. Fl glycosides, including quercetin, were reported earlier in Algerian dates[9] and "khalal" 'Deglet Noor' fruit.[16] Peaks 17 and 4 were partially identified as derivatives of salicylic acid (SA) and cyanidin (Cy), respectively. Anthocyanins were reported in fresh dates, especially of the red color varieties.[10]

It should be noted that only trace proportions of procyanidins, if any, were present in our date extracts. High content of soluble procyanidins was reported for "khalal" 'Deglet Noor' date fruit.[16] Dates at the "khalal" stage are very rich in soluble tannins, but in the ripe fruit only minimal amounts were reported,[13,14] probably due to the formation of insoluble products with other date components and/or chemical breakdown following cell membrane deterioration. 'Barhi' and 'Hayani' fruit in our study were harvested as "khalal" but then subjected to freeze/thaw cycles prior to analysis. Freeze/thaw-induced membrane rupture may have led to loss of soluble procyanidins together with the associated astringency. It is, however, possible that our extraction procedures were not suitable to recover the residual soluble procyanidins.

Table 2 summarizes the date extracts' concentrations of GA (sum of peaks 1 and 2), SA (peak 17), HCA (sum of peaks 3, 5−10, and 12), Fl (sum of peaks 11 and 13−16), and Cy (peak 4), in terms of nanomole per milliliter GAE, salicylic acid, caffeic acid, quercetin-3-β-glucoside, and cyanidin-3-glucoside equivalents, respectively. Considerable varietal differences in phenolics profile were established, consistent with the findings on Algerian[9] and Omani[10] date varieties. In addition, ethanol and acetone extracts of each variety significantly differed in phenolics composition, reflecting the diverse polarities of these compounds in dates.

All date extracts contained phenolics corresponding to the GA, HCA, and Fl subclasses. SA was virtually absent in 'Deglet Noor' and 'Hayani' extracts, and Cy was only detected in 'Hayani' extracts.

The most pronounced difference in phenolics composition between ethanol and acetone date extracts was the level of GA. Ethanol extracts were significantly richer in GA with all varieties but 'Hadrawi', where comparable levels were measured in both solvents. The higher GA concentration in ethanol compared to acetone extracts can be explained by the highly polar nature of the compounds. GA concentrations in ethanol extracts were significantly the highest in 'Medjool', significantly lesser in 'Hadrawi' and 'Zahidi', and yet lower in the remaining varieties (223, 139−146, and 79−104 nmol mL^{-1} GAE, respectively). In the acetone extracts, on the other hand, GA content was

Table 2. Phenolics Composition of Ethanol and Acetone Fruit Extracts from Nine Israeli Date Varieties[a]

		phenolics conc (nmol mL^{-1})				
solvent	date cultivar	GA	SA	HCA	Fl	Cy
ethanol	Amari	96.5 ± 5.3 CD	8.7 ± 1.8 D	198.6 ± 6.4 A	40.8 ± 3.2 A	n.d.
	Barhi	98.8 ± 6.1 CD	11.9 ± 2.4 D	44.1 ± 2.1 CD	1.7 ± 1.0 D	n.d.
	Deglet Noor	104.5 ± 4.0 C	n.d.	186.5 ± 5.2 A	30.7 ± 2.5 B	n.d.
	Deri	96.8 ± 6.2 CD	28.2 ± 2.3 B	35.8 ± 2.8 D	28.2 ± 3.1 B	n.d.
	Hadrawi	146.4 ± 7.5 B	20.8 ± 2.4 C	38.2 ± 4.1 CD	14.1 ± 2.4 C	n.d.
	Hallawi	100.1 ± 5.3 CD	48.7 ± 4.9 A	34.0 ± 2.2 D	15.8 ± 1.9 C	n.d.
	Hayani	78.5 ± 11.4 D	n.d.	34.5 ± 4.4 D	32.5 ± 3.0 B	4.1 ± 1.4
	Medjool	222.7 ± 16.6 A	n.d.	140.5 ± 7.5 B	15.7 ± 1.6 C	n.d.
	Zahidi	138.6 ± 7.3 B	22.9 ± 2.7 BC	51.1 ± 3.8 C	26.1 ± 2.4 B	n.d.
acetone	Amari	40.5 ± 4.8* de	6.7 ± 1.3 d	203.1 ± 15.7 a	40.6 ± 2.7 a	n.d.
	Barhi	67.9 ± 5.3* bc	10.4 ± 1.3 d	41.0 ± 2.3 e	4.8 ± 0.9# e	n.d.
	Deglet Noor	43.9 ± 4.8* de	n.d.	107.6 ± 8.3* c	13.3 ± 1.7* d	n.d.
	Deri	41.1 ± 3.0* de	34.7 ± 2.6# b	50.1 ± 2.7* de	19.1 ± 2.0# cd	n.d.
	Hadrawi	123.1 ± 18.1 a	27.6 ± 2.3# c	45.9 ± 2.7 de	27.4 ± 4.4# b	n.d.
	Hallawi	34.3 ± 4.0* e	68.1 ± 2.1* a	38.8 ± 4.9 e	20.5 ± 1.4# bcd	n.d.
	Hayani	56.8 ± 6.2# bcd	n.d.	38.6 ± 3.1 e	23.2 ± 4.0# bc	16.5 ± 1.9*
	Medjool	70.7 ± 3.9* b	1.5 ± 0.9# e	132.9 ± 9.4 b	15.2 ± 2.3 d	n.d.
	Zahidi	48.7 ± 4.1* cde	29.6 ± 3.0# bc	62.8 ± 4.2# d	24.9 ± 2.0 bc	n.d.

[a]Values are mean ± SD (n = 3). Values within a column with the same uppercase (ethanol extracts) or lowercase (acetone extracts) letter are not significantly different at 95% confidence interval (Tukey–Kramer posthoc). * and # denote significant differences at $P < 0.01$ and $P < 0.05$, respectively (one-way ANOVA), between acetone and ethanol extracts of the same variety. Abbreviations: GA, gallic acid der.; SA, salicylic acid der.; HCA, hydroxycinnamic acid der.; Fl, flavonols; Cy, cyanidin der.; n.d., not detected.

significantly the highest in 'Hadrawi'; significantly lower in 'Barhi', 'Hayani', and 'Medjool'; and significantly the lowest in the remaining varieties (123, 57–71, and 34–49 nmol mL^{-1} GAE, respectively).

The concentration of SA was significantly higher in acetone compared to ethanol extracts in five out of the seven SA-containing date varieties, consistent with SA being the least polar of the major phenolics detected in the date extracts. The significantly highest contents were measured in 'Hallawi' extracts (49 and 68 nmol mL^{-1} salicylic acid equivalents in ethanol and acetone extracts, respectively). Levels significantly lower by 43–59% were measured in extracts of 'Deri', 'Hadrawi', and 'Zahidi' and by 76–90% in 'Amari' and 'Barhi'. A very low concentration of SA was measured in the acetone extract of 'Medjool' (1.5 nmol mL^{-1} salicylic acid equivalents), but none in the ethanol extract.

HCA concentrations in both solvents were comparable for the majority of the date varieties (seven out of nine), possibly reflecting the intermediate polarity of the subclass compounds. 'Amari', 'Deglet Noor', and 'Medjool' extracts contained markedly higher HCA concentrations compared to extracts of the remaining varieties (2- to 6-fold). Both extracts of 'Amari' and the ethanol extract of 'Deglet Noor' contained significantly the highest levels of HCA (198–203 and 186 nmol mL^{-1} caffeic acid equivalents, respectively), followed by 'Medjool' extracts and the acetone extract of 'Deglet Noor' (133–140 and 108 nmol mL^{-1} caffeic acid equivalents, respectively). Significantly lower concentrations were measured in extracts of the remaining varieties (35–63 nmol mL^{-1} caffeic acid equivalents). It should be noted that ferulic acid was the most abundant HCA in all date extracts (30–75% of the HCA subclass, depending on variety).

Fl concentration in ethanol compared to acetone extracts depended on date variety and was significantly higher with 'Deglet Noor', 'Deri', and 'Hayani'; comparable with 'Amari', 'Medjool', and 'Zahidi'; and significantly lower with 'Barhi', 'Hadrawi', and 'Hallawi'. The results may correspond to the diverse varietal Fl composition. Fl level in the ethanol extracts was significantly the highest in 'Amari', followed by 'Deglet Noor', 'Deri', 'Hayani', and 'Zahidi'; significantly lower in 'Hadrawi', 'Hallawi', and 'Medjool'; and significantly the lowest in 'Barhi' (41, 26–32, 14–16, and 2 nmol mL^{-1} quercetin-3-β-glucoside equivalents, respectively). Fl concentration in the acetone extracts was significantly the highest in 'Amari', followed by 'Hadrawi', 'Zahidi', 'Hayani', 'Hallawi', and 'Deri'; significantly lower in 'Medjool' and 'Deglet Noor'; and significantly the lowest in 'Barhi' (41, 19–27, 13–15, and 5 nmol mL^{-1} quercetin-3-β-glucoside equivalents, respectively).

Cy was detected only in 'Hayani' extracts and originated from the fruit peel. Its concentration was higher in the acetone compared to the ethanol extract (16 and 4 nmol mL^{-1} cyanidin-3-glucoside equivalents, respectively). The superior extractability by the acetone solvent may arise from the inclusion of acid in the extraction medium.

In summary, the most abundant phenolic compounds in the fruit extracts from nine Israeli date varieties were derivatives of gallic, salicylic, and chlorogenic acids, quercetin, and kaempferol along with ferulic, caffeic, and coumaric acids. Significant varietal differences in phenolics composition were established in both solvents. Mostly, ethanol extracts were richer in the highly polar gallic acid derivatives and poorer in the least polar salicylic acid derivative compared to acetone extracts. The two types of extracts also significantly differed in flavonol concentration but not in the content of hydroxycinnamic acid derivatives for most of the date varieties.

Anti-atherogenic Properties of Date Ethanol and Acetone Extracts. *Date Extracts Effect on LDL Oxidation.* Increasing volume concentrations (0–25 μL mL^{-1}) of ethanol and acetone extracts were employed to study the effect of the various date extracts on LDL susceptibility to oxidation.

Copper Ions-Induced LDL Oxidation. All the ethanol and acetone date extracts considerably and dose-dependently inhibited copper ions-induced LDL oxidation, as determined by the TBARS assay (Figure 2). Among the ethanol extracts, the

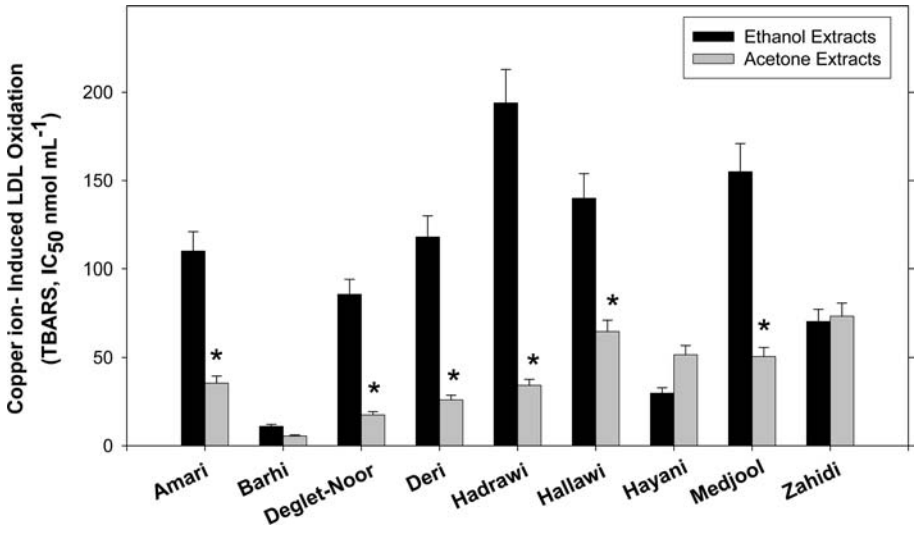

Figure 2. Effect of various ethanol and acetone date extracts on copper ion-induced LDL oxidation. LDL (100 μg protein mL^{-1}) was incubated with increasing concentrations (0−25 μL mL^{-1}) of the date ethanol or acetone extracts in the presence of 5 μmol L^{-1} $CuSO_4$. The extent of LDL oxidation was determined by the TBARS assay. The phenolics concentrations needed to achieve 50% inhibition (IC_{50}) are shown in terms of nmol GAE mL^{-1}. Results are the mean ± SD of three different experiments. * denotes significant difference ($P < 0.01$) between ethanol and acetone extracts of the same date variety.

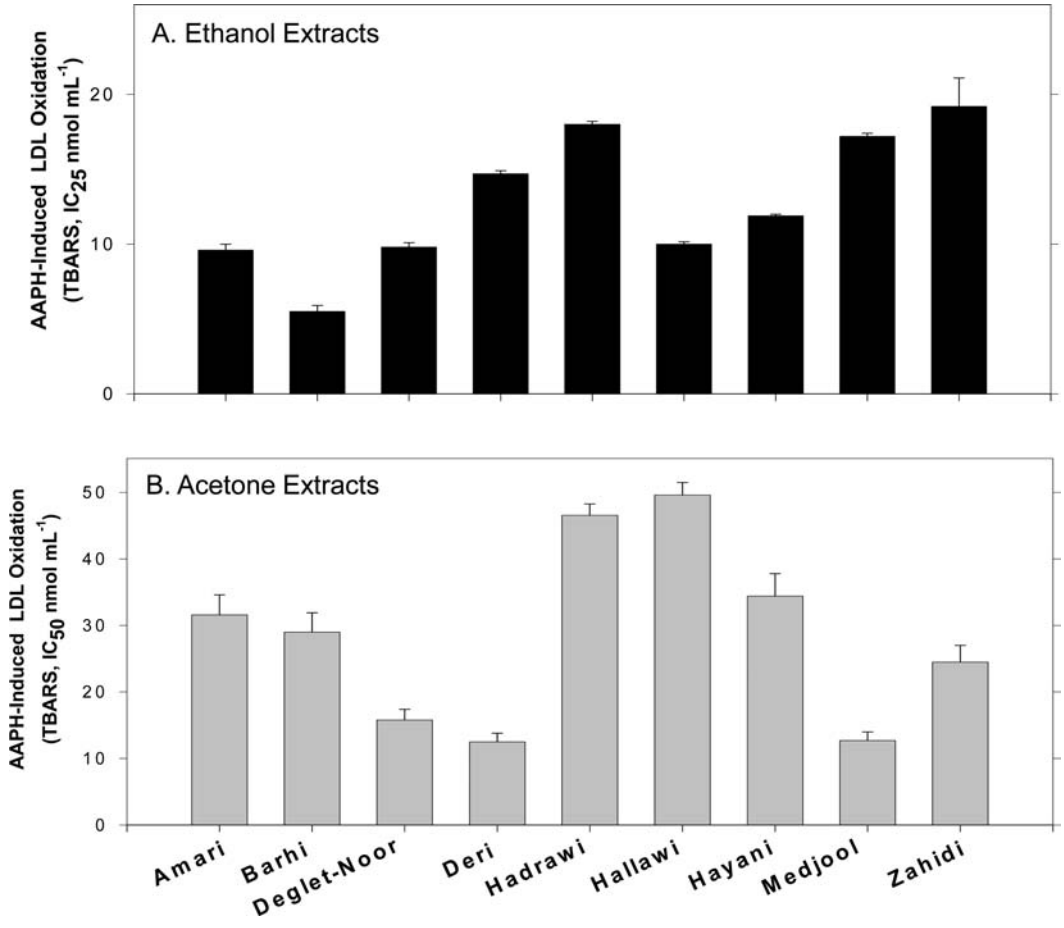

Figure 3. Effect of various ethanol or acetone date extracts on AAPH-induced LDL oxidation. LDL (100 μg protein mL^{-1}) was incubated with increasing concentrations (0−25 μL mL^{-1}) of the date ethanol (A) or acetone (B) extracts in the presence of 5 mmol L^{-1} AAPH. The extent of LDL oxidation was determined by the TBARS assay. The phenolics concentrations needed to obtain 25% inhibition (IC_{25}) and 50% inhibition (IC_{50}) are shown in parts A and B, respectively, in terms of nmol GAE mL^{-1}. Results are the mean ± SD of three different experiments.

most potent were 'Barhi' and 'Hayani', requiring the lowest phenolics concentrations to obtain 50% inhibition (IC_{50}): 11 and 30 nmol GAE mL^{-1}, respectively. 'Zahidi', 'Deglet Noor', 'Amari', and 'Deri' were less potent inhibitors of LDL oxidation, with IC_{50}

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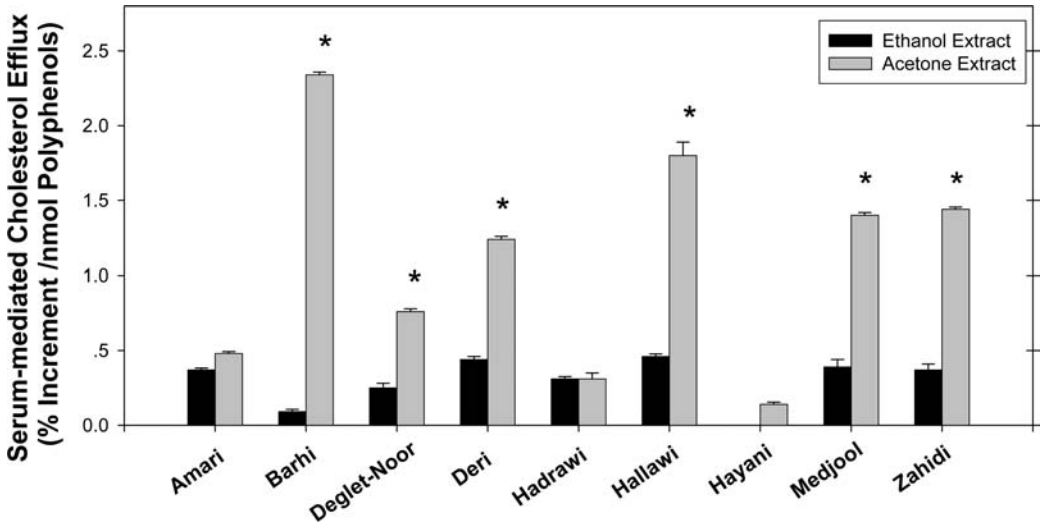

Figure 4. Effect of various ethanol or acetone date extracts on serum-mediated cholesterol efflux from macrophages. J774A.1 macrophages were preincubated with 20 μL mL^{-1} of the date ethanol or acetone extracts for 20 h. After cell wash, the cells were labeled with [^{3}H]-cholesterol and the extent of serum (20 μL mL^{-1})-mediated cholesterol efflux from the cells was determined as described under the Materials and Methods section. Extract effect is shown in terms of percent increase in cholesterol efflux per nanomole of GAE added. Results are the mean ± SD of three different experiments. * denotes significant difference ($P < 0.01$) between ethanol and acetone extracts of the same date variety.

values of 70, 86, 110, and 118 nmol mL^{-1}, respectively. The weakest inhibitors were 'Hallawi', 'Medjool', and 'Hadrawi' with IC_{50} of 140, 155, and 194 nmol mL^{-1}, respectively.

Among the acetone date extracts, 'Barhi' was the most potent inhibitor, with IC_{50} of 5.5 nmol GAE mL^{-1}. 'Deglet-Noor', 'Deri', 'Hadrawi', and 'Amari' were less potent, with IC_{50} values of 17, 26, 34, and 36 nmoL mL^{-1}, respectively. 'Medjool', 'Hayani', and 'Hallawi' were the weakest inhibitors, with IC_{50} values of 51, 52, and 65 nmoL mL^{-1}, respectively. Notably, the acetone extracts were significantly more potent than the corresponding ethanol extracts for most date varieties, requiring much lower phenolics concentrations to obtain 50% inhibition of copper ion-induced LDL oxidation.

AAPH-Induced LDL Oxidation. AAPH is a free radical generator that oxidizes LDL by a different mechanism than that for the divalent copper ion. All date extracts considerably and dose-dependently inhibited AAPH-induced LDL oxidation, as determined by the TBARS assay (Figure 3). The concentrations of date ethanol extract phenolics needed to get 25% inhibition (IC_{25}) are shown in Figure 3A. The order of date extract potency to inhibit AAPH-induced LDL oxidation was as follows: 'Barhi' > 'Amari' ≈ 'Deglet Noor' ≈ 'Hallawi' > 'Hayani' > 'Deri' > 'Medjool' > 'Hadrawi' > 'Zahidi', with IC_{25} values of 5.5, 9.8, 11.9, 14.7, 17.2, 18.0, and 19.2 nmol GAE mL^{-1}, respectively.

The IC_{50} values for date acetone extracts are shown in Figure 3B. The most potent inhibitors were 'Deri', 'Medjool', 'Deglet Noor', and 'Hallawi' with IC_{50} values of 12.5, 12.7, 15.8, and 19.6 nmol GAE mL^{-1}, respectively. 'Zahidi', 'Barhi', 'Amari', and 'Hayani' were less potent, with IC_{50} values of 24.5, 29, 31.6, and 34.4 nmol mL^{-1}, respectively. 'Hadrawi' was the weakest inhibitor of AAPH-induced LDL oxidation with IC_{50} of 46.6 nmol mL^{-1}.

To summarize, all date extracts reduced LDL susceptibility to oxidation. Substantial varietal differences were observed in the potency to inhibit copper ion— as well as AAPH-induced LDL oxidation. Moreover, the extents of the inhibitory effect of a date extract on the two processes of LDL oxidation were often inconsistent. A prominent example is the acetone extract of 'Medjool', which exhibited a low inhibitory effect on copper ions-induced LDL oxidation but was a very efficient inhibitor of AAPH-induced LDL oxidation. The results indicate that the extracts possessed variable copper ion chelating abilities as well as free radical scavenging capacities. The latter is consistent with the diverse phenolics composition in the date extracts. Flavonoid interaction with copper ions and potency to inhibit copper ions-induced LDL oxidation were reported to depend on the molecule structural properties.[32] Also, different hydroxycinnamic acid derivatives varied in their capacity to inhibit LDL oxidation induced by either copper ions or AAPH.[33] Moreover, certain derivatives were better at inhibiting copper ions than AAPH-induced oxidation and *vice versa*. It should be noted, however, that the involvement of solutes other than phenolics cannot be excluded.

Date Extracts Effect on Serum-Mediated Cholesterol Efflux. Reverse cholesterol transport is a major mechanism for the attenuation of atherosclerosis development. We, thus, next analyzed the extent of serum-mediated cholesterol efflux from J774A.1 macrophage cells that were preincubated with the dates extracts.

Enrichment of the cells with 20 μL mL^{-1} ethanol and acetone extracts of most of the date varieties resulted in a considerable increase in the extent of serum-mediated cholesterol efflux from the cells, as shown in Figure 4 in terms of percent increase per nanomole of GAE added. The varietal order of the ability of date ethanol extracts to increase serum-mediated cholesterol efflux was as follows: 'Hallawi' > Deri > 'Medjool' > 'Amari' ≈ 'Zahidi' > 'Hadrawi' > 'Deglet Noor'. The extracts of 'Barhi' and "Hayani', on the other hand, had no effect.

The varietal order of date acetone extracts ability to increase serum-mediated cholesterol efflux from the cells was as follows: 'Barhi' > 'Hallawi' > 'Zahidi' ≈ 'Medjool' > 'Deri' > 'Deglet Noor' > 'Amari'. The extracts of 'Hadrawi' and 'Hayani' were the least effective.

To summarize, supplementing macrophages with most of the date extracts considerably enhanced serum-mediated cholesterol efflux from the cells. These findings suggest that phenolic compounds and/or other constituents in the date extracts may

interact with the macrophage plasma membrane and, consequently, enhance HDL binding to— and cholesterol removal from the cells. In addition, the molecules may affect the expression of transporters involved in serum-mediated cholesterol efflux from macrophage foam cells. Notably, acetone extracts were significantly more potent than the corresponding ethanol extracts for most date varieties. The superior effect of the extracts enriched with the less polar phenolics may pertain to the mode of phenolics interaction with the bilayer, i.e., the partition of less polar compounds in the hydrophobic interior of the membrane, and the formation of hydrogen bonds between phospholipid polar head groups and the more hydrophilic phenolics at the membrane/water interface.[34] The large varietal differences in the magnitude of the bioactivity may as well correspond to the diverse phenolics composition in the extracts of the different varieties.

Taken together, the results of the current study imply that the nine Israeli grown date varieties herein examined contain soluble ingredients that are potent inhibitors of LDL oxidation and stimulators of cholesterol efflux from macrophages: two of the major anti-atherogenic pathways. Our *in vitro* studies bear relevance to *in vivo* anti-atherogenic capacities, given that metabolites of the phenolics subclasses detected in the date extracts maintain the active sites of the parent molecules. Also, date phenolics concentrations used in the studies are in the range of blood phenolics concentrations, nano- to low micromolar.[35] However, *in vivo* studies with the different date varieties are required to establish anti-atherogenic benefits to date fruit consumption. In a recent *in vivo* pilot study, we have shown that 'Medjool' and 'Hallawi' date consumption had beneficial anti-atherogenic effects in healthy human subjects.[12] The results of the present study suggest that other date varieties also contain ingredients that may convey favorable effects on atheroschlerosis risk factors. Such health value, if indeed verified *in vivo* for other date varieties, may benefit the date industry through the expansion to additional market sectors, including health conscious consumers and the functional food industry.

AUTHOR INFORMATION

Corresponding Author

*Tel: +972-8-6355747. Tel: +972-8-6355730. E-mail: hamutalborochov@rd.ardom.co.il.

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ABBREVIATIONS USED

AAPH, 2,2′-azobis-2-amidinopropane hydrochloride; An, anthocyanins; Cy, cyanidin derivative; DDW, double distilled water; DMEM, Dulbecco's modified Eagel's medium; FA, ferulic acid; FCS, fetal calf serum; Fl, flavonols; FW, fresh weight; GA, gallic acid derivatives; GAE, gallic acid equivalents; HCA, hydroxycinnamic acid derivatives; HDL, high-density lipoprotein; LDL, low density lipoprotein; MDA, malondialdehyde; PBS, phosphate buffered saline; RP-HPLC, reversed phase high-performance liquid chromatography; SA, salicylic acid derivative; SD, standard deviation; TBARS, thiobarbituric acid reactive substances

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