Antioxidant and Anti-inflammatory Assays Confirm Bioactive Compounds in Ajwa Date Fruit

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ABSTRACT: Ajwa, a variety of date palm *Phoenix dactylifera* L., produces the most expensive date fruits. Percentages of seed, moisture, fructose, glucose, soluble protein, and fiber in Ajwa dates were 13.24, 6.21, 39.06, 26.35, 1.33, and 11.01, respectively. The ethyl acetate, methanolic, and water extracts of Ajwa dates, active at 250 μg/mL in the MTT assay, inhibited lipid peroxidation (LPO) by 88, 70, and 91% at 250 μg/mL and cyclooxygenase enzymes COX-1 by 30, 31, and 32% and COX-2 by 59, 48, and 45% at 100 μg/mL, respectively. Bioactivity-guided purifications afforded compounds **1–7**, in addition to phthalates and fatty acids. Compounds **1–3** showed activity at 100 μg/mL in the MTT assay; inhibited COX-1 enzyme by 59, 48, amd 50% and COX-2 enzyme by 60, 40, amd 39% at 50 μg/mL; and inhibited LPO by 95, 58, amd 66% at 100 μg/mL, respectively. The soluble protein fraction was also very active in both antioxidant and anti-inflammatory assays.

KEYWORDS: *sugars, flavonoid glycosides, triterpenoids, triglycerides, steroids, phthalates, fatty acids*

INTRODUCTION

Numerous varieties of date palm, *Phoenix dactylifera* L. (Palmaceae), are grown in the Middle East, North Africa, South Asia, and the United States. Major date palm varieties grown in the United States are Deglet Noor and Medjool. Ajwa date fruits, soft and dry, are from a date palm variety cultivated in the Al Madinah region of western Saudi Arabia. This date variety is ascribed as having great medicinal value. Reference about Ajwa dates was made in "Hadith" and Islamic historical literature because it is believed that eating this variety will cure many chronic ailments. The Ajwa date fruit is one of the most popular and expensive dates, fetching 3 times the price of the next best variety, and belongs to the holy city of Al Madinah Al Munawara and its adjoining areas in Saudi Arabia.

Date fruit is consumed as a staple food or as an important component of the diet in the Middle East region. This fruit is considered to be highly nutritional because of its rich sugar content in the form of fructose and glucose, dietary fiber, vitamins, and minerals.[1,2] An overall composition including functional and nutritional quality of a variety of date palm fruits has been also reported.[3,4] For example, the aqueous extract of date fruit showed antioxidant and antimutagenic activities, which was attributed to the presence of compounds with free radical scavenging activity.[5] Several varieties of date fruits from Saudi Arabia and Algeria showed antioxidant activity due to their phenolic content.[6−9] The carotenoid profile of some Algerian date fruit varieties has also been investigated.[10] The flavonoid glycoside and procyanidin composition of Deglet Noor dates from California was determined using liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI/MS/MS).[11] A recent paper described the chemical constituents and biological activity of date seeds.[12] Although limited nutritional, chemical, and bioactivity studies are available on date fruits, this is the first attempt of a bioassay-guided evaluation of constituents in dates and characterization of pure and active isolates from it.

In this study, antioxidant and anti-inflammatory activities of hexane, ethyl acetate, methanolic, and water extracts of Ajwa date fruits were determined using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT),[13−15] lipid peroxidation (LPO),[14−18] and cyclooxygenase enzymes (COX-1 and -2) inhibitory[14−18] assays as per published studies from our laboratory. Also, we report the purification, structure elucidation, and bioactivity studies of pure isolates as a measure to determine their functional food quality.

MATERIALS AND METHODS

Safety. There are no safety concerns.

General Experimental Procedures. All solvents used for isolation and purification were of ACS reagent grade (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). Merck silica gel (60 mesh size, 35−70 μm) with particle size of 60 μm was used for preparative medium-pressure liquid chromatography (MPLC). Silica gel plates (250 and 500 μm; Analtech, Inc., Newark, DE, USA) were used for preparative thin-layer chromatography (TLC). TLC plates were viewed under UV light at 254 and 366 nm in a Spectroline CX-20 ultraviolet fluorescence analysis cabinet (Spectroline Corp., Westbury, NY, USA) and sprayed with 10% sulfuric acid solution. NMR spectra were recorded on a 500 MHz (Varian Unity ±500, ^{1}H NMR) and 125 MHz (Varian Unity ±500, ^{13}C NMR) VRX instruments. The mass spectrum was recorded on a Waters Quattro micro API LC/MS/MS spectrometer. 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), *tert*-butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), aspirin, naproxen,

and ibuprofen were purchased from Sigma-Aldrich Chemical Co. Similarly, the nonsteroidal anti-inflammatory drug (NSAIDs) Celebrex was a physician's professional sample provided by Dr. Subhash Gupta, Sparrow Pain Center, Sparrow Hospital, Lansing, MI, USA. COX-1 and -2 enzymes were prepared in our laboratory from ram seminal vesicles (Oxford Biomedical Research, Inc., Oxford, MI, USA) and insect cells cloned with human PGHS-2 enzyme, respectively. Arachidonic acid was purchased from Oxford Biomedical Research, Inc. 1-Stearoyl-2-linoleoyl-sn-glycerol-3-phosphocholine (SLPC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). The fluorescent probe 3-(p-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid was purchased from Molecular Probes (Eugene, OR, USA). All enzymes and reagents were stored in the Bioactive Natural Products and Phytoceuticals Laboratory at Michigan State University (East Lansing, MI, USA). MTT antioxidant activity was tested on a Bio-Tek Elx800 universal microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). COX assays were performed in an Instech micro oxygen chamber and electrode (Instech Laboratories, Plymouth Meeting, PA, USA) attached to a YSI model 5300 biological oxygen monitor (Yellow Springs Instrument, Inc., Yellow Springs, OH, USA). LPO assay was tested on a Turner model 450 fluorometer (Barnstead/Thermolyne Corp., Dubuque, IA, USA).

Extraction and Isolation. Ajwa fruit samples were procured from a farm located in the Madinah region. This farm cultivates only Ajwa date palms. From five palms, 1 kg of fruits was drawn and mixed in a bag. From this, 1 kg aliquots of fruit sample were packed in cardboard boxes and sent to Riyadh for onward shipment to Michigan State University. At the time of harvest, the fruit was in "Tamar" or ripened stage, and identification was based on the local knowledge of the farmer and as per information provided by the Ministry of Agriculture, Saudi Arabia.[19]

Ajwa dates (740 g) were pitted to remove seeds (98 g). The pit-free fruit (642 g) was blended with 1.5 L of water and the puree lyophilized to yield powdered fruit (596 g). The dry powder (596 g) was packed in a glass column and eluted sequentially with hexane (2 L), ethyl acetate (2 L), and methanol (2 L). The evaporation of organic solvents under vacuum at 35 °C afforded hexane (40 mg), ethyl acetate (480 mg), and methanolic extracts (348.5 g), respectively. The residue left in the column was removed, blended with 2 L of water, centrifuged, and lyophilized (supernatant and residue separately) to afford water-soluble (170 g) and -insoluble (fibrous, 77 g) fractions.

The hexane extract was combined with the ethyl acetate extract on the basis of TLC profiles. An aliquot of this combined extract (500 mg) was fractionated by silica gel vacuum liquid chromatography (VLC) and eluted under gradient conditions using hexane/acetone (10:1, 3:1, 1:1, v/v), followed by CHCl$_3$/MeOH (10:1, 3:1, 1:1, v/v). The fractions collected were A, 126 mg; B, 85 mg; C, 44 mg; D, 60 mg; and E, 185 mg. An aliquot of fraction A (115 mg) was purified by preparative TLC (hexane/acetone, 15:1, v/v; and CHCl$_3$/MeOH, 200:1, v/v) to yield compounds **2** (10.7 mg), **3** (5 mg), **4** (3.6 mg), and **7** (4.7 mg), bis(2-ethylhexyl) terephthalate (3 mg), and bis(2-ethylheptyl) phthalate (7 mg). An aliquot of fractopm B (68 mg) was purified by preparative TLC (hexane/acetone, 10:1; and CHCl$_3$/MeOH, 100:1) to afford a fatty acid mixture containing equal amounts of linoleic, oleic, and stearic acids (21.5 mg). An aliquot of fraction C (30 mg) was purified by preparative TLC (CHCl$_3$/MeOH, 15:1) to yield **5** (8 mg). Similarly, fraction D (20 mg) was purified by preparative TLC (CHCl$_3$/MeOH, 10:1) to afford **6** (6.4 mg).

An aliquot of the methanolic extract (25 g) was fractionated by MPLC (C18 column) and eluted with MeOH/H$_2$O (gradient elution, 1:9, 3:7, 5:5, 7:3, 9:1, v/v) and finally with MeOH (100%) to yield fractions F, 24.8 g; G, 95 mg; and H, 101 mg. Major compounds in fraction F were determined as β-D-glucopyranose (22.9%), α-D-glucopyranose (16.7%), β-D-fructopyranose (45.3%), and β-D-fructofuranose (13.4%) on the basis of their NMR spectral data and TLC profiles with authentic samples. Compound **1** (1.8 mg) was isolated from fraction G by preparative TLC (CHCl$_3$/MeOH/H$_2$O, 4:1:0.1, v/v). Fraction H was identical to the ethyl acetate extract based on TLC and hence was not purified further.

Tituration of a portion of the water extract (5 g) with MeOH (50 mL × 3) yielded MeOH-soluble (I, 4.58 g) and -insoluble (J, 421 mg) fractions. Analysis of fraction I revealed that it contained sugars (99.5%), primarily fructose and glucose, on the basis of TLC profiles with authentic samples of fructose and glucose. Insoluble fraction J was dissolved in water (15 mL) to yield water-soluble fraction (K, 289 mg) and residue (L, 132 mg). Preliminary NMR study suggested that **K** was proteinaceous in nature. This fraction was not studied further because the scope of it was beyond this study.

Chrysoeriol-7-O-(2,6-dirhamnosyl)-glucoside 1: yellow powder; ^{1}H NMR (500 MHz, CD$_3$OD) δ 7.55 (1H, dd, J = 9.0, 2.0 Hz, H-6′), 7.51 (1H, d, J = 2.0 Hz, H-2′), 6.93 (1H, d, J = 9.0 Hz, H-5′), 6.83 (1H, d, J = 2.0 Hz, H-8), 6.69 (1H, s, H-3), 6.47 (1H, d, J = 2.0 Hz, H-6), 5.28 (1H, J = 1.5 Hz, H-1′′′), 5.20 (1H, d, J = 8.0 Hz, H-1″), 4.57 (1H, br s, H-1′′′′), 3.96 (3H, s, H-7′), 3.30–3.95 (14H, m, H-2″, 3″, 4″, 5″, 6″, 2′′′, 3′′′, 4′′′, 5′′′, 2′′′′, 3′′′′, 4′′′′, 5′′′′), 1.32 (6H, d, J = 5.5 Hz, H-6′′′, 6′′′′).[20]

Lup-20(29)-en-3-one 2: white powder; ^{1}H NMR (500 MHz, CDCl$_3$) δ 4.70 (1H, br d, J = 2.0 Hz, H-29a), 4.58 (1H, br t, J = 2.0 Hz, H-29b), 2.35–2.55 (3H, m, H$_2$-2 and H-19), 1.85–1.95 (2H, m, H-1a and H-21a), 1.69 (3H, s, H$_3$-30), 1.08 (6H, s, H$_3$-23 and H$_3$-26), 1.04 (3H, s, H$_3$-24), 0.97 (3H, s, H$_3$-27), 0.94 (3H, s, H$_3$-25), 0.81 (3H, s, H$_3$-28).[21,22]

Lupeol 3: white powder; ^{1}H NMR (500 MHz, CDCl$_3$) δ 4.70 (1H, br d, J = 2.0 Hz, H-29a), 4.58 (1H, br dd, J = 1.5, 1.0 Hz, H-29b), 3.19 (1H, dd, J = 11.5, 5.0 Hz, H-3), 2.38 (1H, m, H-19), 1.95 (2H, m, H$_2$-21), 1.69, 1.04, 0.98, 0.95, 0.84, 0.80, 0.77 (each 3H, s, CH$_3$ × 7).[23]

1,2-Dilinoleoyl-3-stearin 4: colorless oil; APCI-MS, m/z 883 [M + H]+; ^{1}H NMR (500 MHz, CDCl$_3$) δ 5.3–5.4 (8H, m, 9″, 10″, 12″, 13″, 9′′′, 10′′′, 12′′′, 13′′′), 5.27 (1H, m, H-2), 4.30 (2H, dd, J = 12.0, 4.3 Hz, H-1a, 3a), 4.15 (2H, dd, J = 12.0, 6.0 Hz, H-1b, 3b), 2.78 (4H, m, H$_2$-11″, 11′′′), 2.32 (6H, m, H$_2$-2′, 2″, 2′′′), 2.05 (8H, m, H$_2$-8″, 14″, 8′′′, 14′′′), 1.62 (6H, m, H$_2$-3′, 3″, 3′′′), 1.2–1.4 (56H, m, H$_2$-4′, 5′, 6′, 7′, 8′, 9′, 10′, 11′, 12′, 13′, 14′, 15′, 16′, 4″, 5″, 6″, 7″, 15″, 16″, 17″, 4′′′, 5′′′, 6′′′, 7′′′, 15′′′, 16′′′, 17′′′), 0.89 (9H, m, Me-18′, 18″, 18′′′); ^{13}C NMR (125 MHz, CDCl$_3$) δ 173.2, 172.8 (C-1′, 1″, 1′′′), 130.2, 130.0, 128.0, 127.8 (C-9″, 10″, 12″, 13″, 9′′′, 10′′′, 12′′′, 13′′′), 68.9 (C-1), 62.1 (C-2, 3), 34.2, 34.0, (C-2′, 2″, 2′′′), 31.9, 31.5 (C-16′, 16″, 16′′′), 29.0–29.7 (C-4′, 5′, 6′, 7′, 8′, 9′, 10′, 11′, 12′, 13′, 14′, 15′, 16′, 4″, 5″, 6″, 7″, 15″, 16″, 4′′′, 5′′′, 6′′′, 7′′′, 15′′′, 16′′′), 27.2, 27.1 (C-8″, 14″, 8′′′, 14′′′), 25.6 (C-11″, 11′′′), 24.8 (C-3′, 3″, 3′′′), 22.6, 22.5 (C-17′, 17″, 17′′′), 14.1, 14.0 (C-18′, 18″, 18′′′).[24]

β-Sitosteryl-3β-glucopyranoside-6′-O-palmitate 5: colorless oil; ^{1}H NMR (500 MHz, CDCl$_3$) δ 5.37 (1H, m, H-6), 4.50 (1H, dd, J = 11.7, 4.5 Hz, H-6′a), 4.39 (1H, d, J = 7.4 Hz, H-1′), 4.27 (1H, br d, J = 11.7 Hz, H-6′b), 3.3–3.7 (5H, m, H-3, 2′, 3′, 4′, 5′), 1.02 (3H, s, Me-19), 0.93 (3H, d, J = 6.3 Hz, Me-21), 0.89 (3H, t, J = 7.1 Hz, Me-16″), 0.85 (3H, t, J = 7.5 Hz, Me-29), 0.84 (3H, d, J = 6.8 Hz, Me-27), 0.82 (3H, d, J = 6.8 Hz, Me-26), 0.69 (3H, s, Me-18); ^{13}C NMR (125 MHz, CDCl$_3$) δ 174.7 (C-1″), 140.3 (C-5), 122.2 (C-6), 101.2 (C-1′), 79.6 (C-3), 76.0 (C-3′), 74.0 (C-5′), 73.6 (C-2′), 70.1 (C-4′), 63.5 (C-6′), 56.8 (C-14), 56.1 (C-17), 50.2 (C-9), 45.9 (C-24), 42.3 (C-13), 39.8 (C-12), 38.9 (C-4), 37.3 (C-1), 36.7 (C-10), 36.2 (C-20), 34.2 (C-2″), 34.0 (C-22), 31.9 (C-7, 8, 14″), 29.2–29.7 (C-2, 4″–13″), 28.3 (C-16), 26.1 (C-23), 25.0 (C-3″), 24.3 (C-15), 23.1 (C-28), 22.7 (C-15″), 21.1 (C-11), 19.8 (C-27), 19.4 (C-19), 19.0 (C-26), 18.8 (C-21), 14.1 (C-16″), 12.0 (C-29), 11.9 (C-18).[25]

Bis-(2-ethylhexyl) terephthalate (PH1): colorless oil; APCI-MS, m/z 391 [M + H]+; ^{1}H NMR (500 MHz, CDCl$_3$) δ 8.11 (4H, s, H-3, 4, 6, 7), 4.28 (4H, m, H$_2$-1′, 1″), 1.75 (2H, m, H-2′, 2″), 1.30–1.50 (16H, m, H$_2$-3′, 3″, 4′, 4″, 5′, 5″, 7′, 7″), 0.96 (6H, t, J = 7.5 Hz, H$_3$-8′, 8″), 0.92 (6H, t, J = 7.1 Hz, H$_3$-6′, 6″); ^{13}C NMR (125 MHz, CDCl$_3$) δ 165.9 (C-1, 8), 134.2 (C-2, 5), 129.4 (C-3, 4, 6, 7), 67.7 (C-1′, 1″), 38.9 (C-2′, 2″), 30.5 (C-3′, 3″), 28.9 (C-4′, 4″), 23.9 (C-7′, 7″), 22.9 (C-5′, 5″), 14.0 (C-6′, 6″), 11.0 (C-8′, 8″).[28]

Bis(2-ethylheptyl) phthalate (PH2): colorless oil; APCI-MS, m/z 419 [M + H]+; ^{1}H NMR (500 MHz, CDCl$_3$) δ 7.71 (2H, br dd, J = 8.5, 3.5 Hz, H-3, 5), 7.54 (2H, br dd, J = 8.5, 3.5 Hz, H-4, 6), 4.22 (4H, m, H$_2$-1′, 1″), 1.69 (2H, m, H-2′, 2″), 1.25–1.46 (20H, m, H$_2$-3′,

3″, 4′, 4″, 5′, 5″, 6′, 6″, 8′, 8″), 0.93 (6H, m, H$_3$-9′, 9″), 0.89 (6H, m, H$_3$-7′, 7″).[29]

β-D-Fructopyranose and β-D-fructofuranose mixture: white powder; ^{1}H NMR (500 MHz, D$_2$O) δ 4.16 (m, fur H-3, 4), 4.09 (m, pyr H-5, 6a), 3.95 (m, pyr H-4), 3.88 (m, fur H-5, 6a), 3.84 (m, pyr H-3), 3.76 (m, pyr H-1a, 6b), 3.72 (m, fur H-6b), 3.64 (m, fur H-1a), 3.61 (m, pyr H-1b), 3.60 (m, fur H-1b); ^{13}C NMR (125 MHz, D$_2$O) δ 103.0 (fur C-2), 99.6 (pyr C-2), 82.2 (fur C-5), 76.9 (fur C-3), 76.0 (fur C-4), 71.2 (pyr C-4), 70.7 (pyr C-5), 69.1 (pyr C-3), 65.4 (pyr C-1), 64.9 (pyr C-6), 64.2 (fur C-1), 63.8 (fur C-6).[31]

β-D-Glucopyranose and α-D-glucopyranose mixture: white powder; ^{1}H NMR (500 MHz, D$_2$O) δ 5.28 (m, α H-1), 4.69 (m, β H-1), 3.86–3.94 (m, β H-4, 6a, α H-4, 6a), 3.74–3.84 (m, β H-6b, α H-3, 6b), 3.42–3.60 (m, β H-3, 5, α H-2, 5), 3.29 (m, β H-2); ^{13}C NMR (125 MHz, D$_2$O) δ 97.4 (β C-1), 93.6 (α C-1), 77.4 (β C-5), 77.3 (β C-3), 75.6 (β C-2), 74.3 (α C-3), 73.0 (α C-5), 72.9 (α C-2), 71.2 (α C-4), 71.1 (β C-4), 62.3 (β C-6), 62.1 (α C-6).[31]

Proteinaceous fraction K: brown powder; ^{1}H NMR (500 MHz, D$_2$O) δ 5.70 (m), 5.11 (m), 3.00–4.60 (m), 2.61 (m), 2.46 (m), 2.33 (m), 2.06 (m); ^{13}C NMR (125 MHz, D$_2$O) δ 170–184, 94–110, 63–82, 55.5, 46.8, 44.5. The overlapping signals in ^{1}H and ^{13}C NMR spectra and the chemical shifts suggested that fraction K was protein-aceous in nature. The exact nature and structure determination of it requires additional purification and detailed high-resolution NMR and mass spectral analyses of the resulting fractions or isolates. Because characterization of proteins was not within the objectives of this research, it was kept aside for future investigations.

MTT Antioxidant Assay. The MTT assay was performed according to our previous paper.[13−15] Stock solutions of test extracts, selected fraction K and compounds, and positive controls (vitamin C and TBHQ) were prepared in DMSO (10 mg/mL for extracts, 2 mg/mL for fraction, 4 mg/mL for compounds 1−3, and 1 mg/mL for controls, compound 4, phthalates, and mixture of fatty acids). An aliquot of 10 μL of test samples, 190 μL of MTT water solution (1 mg/mL), and 200 μL of DMSO was vortexed in a capped glass vial (2 mL) for 1 min, which was then incubated at 37 °C for 24 h. An aliquot (200 μL) of the reaction mixture was pipetted to a 96-well cell culture plate, and the absorbance was tested at 570 nm in duplicate on a Bio-Tek Elx800 universal microplate reader (Bio-Tek Instruments, Inc.). The assay was conducted in duplicate and repeated twice.

Lipid Peroxidation Inhibitory Assay. The extract (250 μg/mL), fraction K (50 μg/mL), compounds 1−3 (100 μg/mL), compound 4, phthalates and mixture of fatty acids (25 μg/mL), and positive controls (BHA, BHT, and TBHQ at 10 μM) were tested for lipid peroxidation (LPO) inhibitory activities by using fluorescence spectroscopy on a Turner model 450 fluorometer (Barnstead/Thermolyne Corp.) according to the reported procedure.[14−18] The liposome, unilamellar vesicles (ULV), was prepared according to the published procedure. The peroxidation was initiated by the addition of 20 μL of FeCl$_2$·4H$_2$O (0.5 mM) to the assay mixture [HEPES (100 μL), 1 M NaCl (200 μL), N$_2$-sparged Millipore water (1.64 mL), DMSO or test sample (20 μL)] and 20 μL of liposome suspension. The fluorescence was monitored at 0, 1, and 3 min and every 3 min thereafter up to 21 min. The decrease in fluorescence intensity over time (21 min) indicated the rate of peroxidation. Each sample was assayed in duplicate, and the percent inhibition was calculated with respect to DMSO control.

COX-1 and -2 Enzyme Inhibitory Assay. The COX-1 and -2 enzyme (prepared from ram seminal vesicles and insect cells cloned with human COX-2 enzyme, respectively, in our laboratory) inhibitory effects of test samples were measured by monitoring the initial rate of O$_2$ uptake using an Instech micro oxygen chamber and electrode (Instech Laboratories) attached to a YSI model 5300 biological oxygen monitor (Yellow Springs Instrument, Inc.) at 37 °C following the published procedure.[14−18] The test samples (6 μL) were initially added to the chamber full of assay buffer (Tris 1 mM phenol buffer, 600 μL, pH 7) and hemoglobin (17 μg). COX-1 or COX-2 enzyme (20 μL) was then added and incubated for 2 min. Arachidonic acid (10 μL of solution at 1 mg/mL) was added to initiate the reaction. The data were recorded using QuickLog for Windows data acquisition
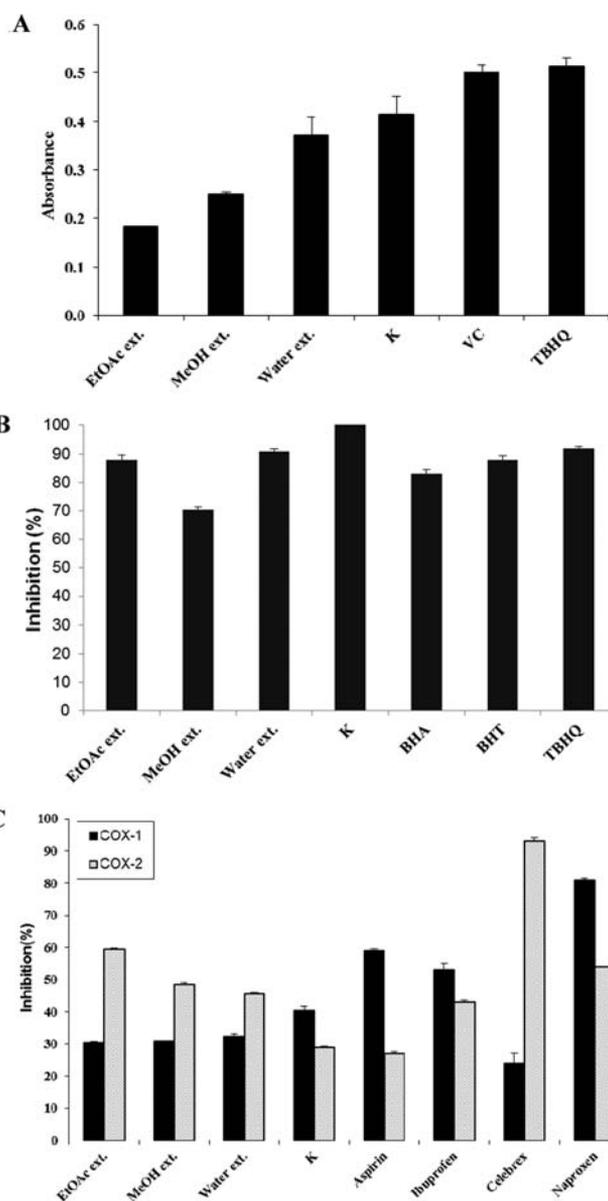

Figure 1. (A) Absorbance values at 570 nm of extracts at 250 μg/mL and proteinaceous fraction K at 50 μg/mL obtained after reaction with MTT at 37 °C. Vitamin C and TBHQ were used as positive controls at 25 μg/mL. (B) Inhibition of LPO by extracts at 250 μg/mL and K at 50 μg/mL. Commercial antioxidants BHA, BHT, and TBHQ were tested at 10 μM. The oxidation of lipid was initiated by the addition of Fe^{2+} ions. (C) COX-1 and COX-2 enzyme inhibitory activities of extracts at 100 μg/mL, K at 50 μg/mL, and commercial NSAIDs aspirin, Celebrex, naproxen, and ibuprofen used as positive control at 108, 1, 15, and 12 μg/mL, respectively. The standard error of the mean was represented for n = 4 (P < 0.05, t test, paired, two tailed). For the COX assay, vertical bars represent the standard deviation of each data point (n = 2). The varying concentrations of positive controls used in these assays were to yield comparable activity profiles between 0 and 100% by test extracts, fraction, and positive controls alike.

and control software (Strawberry Tree Inc., Sunnyvale, CA, USA). The extract and compounds were tested at 100 and 50 μg/mL concentrations, respectively. The positive controls, commercial aspirin, Celebrex, naproxen, and ibuprofen were tested at 108, 1, 15, and 12 μg/mL, respectively. Each sample was tested in duplicate, and the percent inhibition calculated with respect to DMSO control.
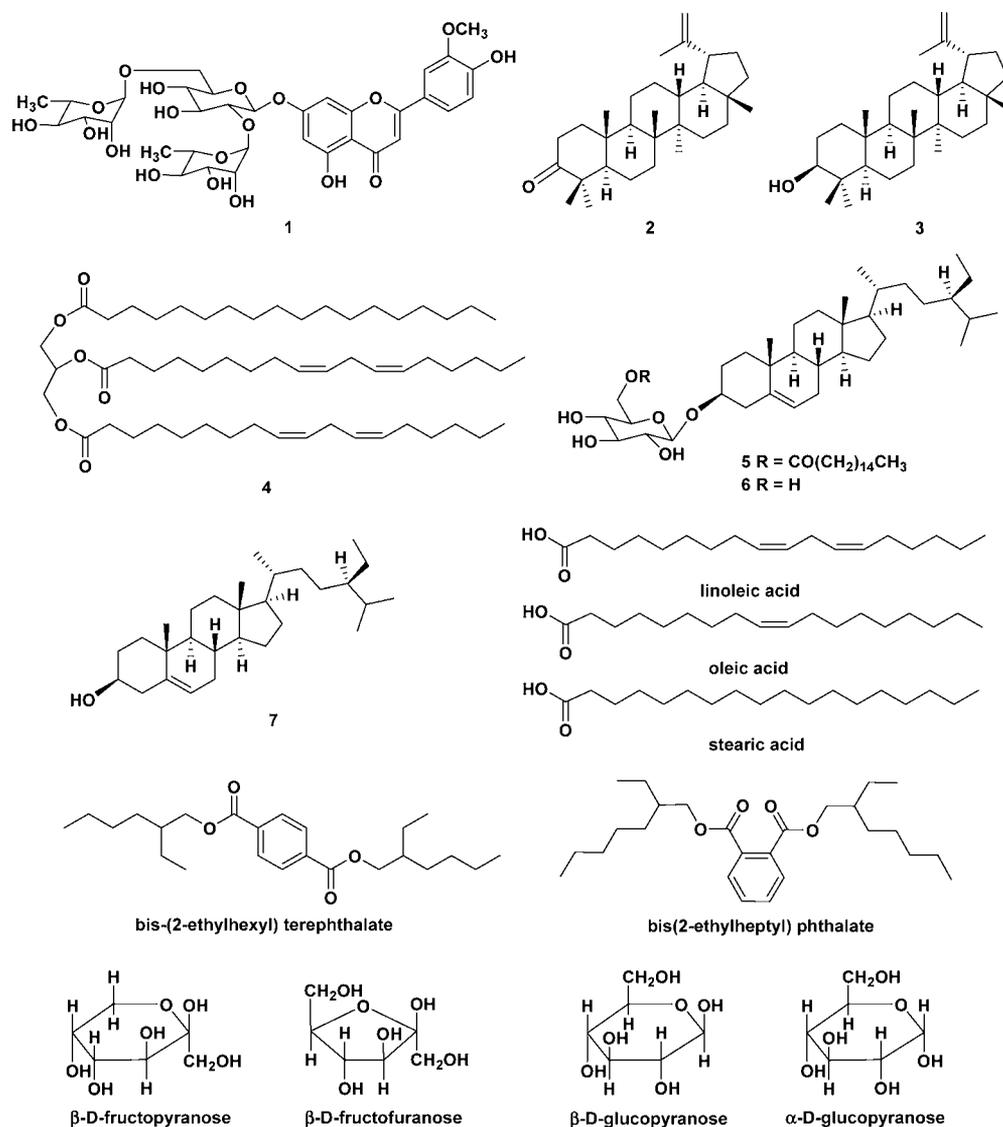
Figure 2. Structures of pure isolates from Ajwa dates: flavonoid glycoside (**1**), triterpenoids (**2** and **3**), triglyceride (**4**), steroids (**5**–**7**), mixture of fatty acids (**FA**), phthalates (bis(2-ethylhexyl) terephthalate (**PH1**) and bis(2-ethylheptyl) phthalate) (**PH2**), and isomers of fructose and glucose.

■ RESULTS AND DISCUSSION

The Ajwa dates used in this study was deep-brown in color and nonsticky compared to many varieties of dates available to consumers. To determine the accurate dry weight of the dates used for extraction, the pit-free fruits were blended with reverse osmosis (RO) water and lyophilized. The lyophilized date powder was then sequentially extracted with hexane, ethyl acetate, methanol, and water to afford lipid-soluble compounds (e.g., pigments, triglycerides, fatty alcohols, and acids), lipid-insoluble compounds (e.g., phenolics, its glycosides, and steroidal glycosides), and water-soluble compounds (e.g., sugars, glycosides, polysaccharides, and proteins). The yield of hexane extract was minute in quantity and combined on the basis of the TLC profiles prior to purification. The bulk of soluble compounds in Ajwa date fruit was sugars, the main constituent in both methanolic and water extracts. The methanolic extract contained trace amounts of components in the ethyl acetate extract, as observed by TLC. The lyophilized water extract was processed with methanol to separate fructose and glucose and thus yielded the proteinaceous fraction **K**, soluble only in water.

Before purification, extracts and proteinaceous fraction **K** were evaluated for their antioxidant and anti-inflammatory activities (Figure 1). We routinely use MTT and LPO assays to determine the antioxidant activity of natural extracts and pure isolates. The MTT assay is based on redox reaction and hence detects most antioxidant compounds that are reducing agents. On the other hand, inhibition of LPO detects free radical scavenging capacity of extracts and test compounds. Biochemical reactions in vivo generate free radicals. The reaction of free radicals with lipids, proteins, and nucleic acids result in oxidative damage and leads to a number of diseases including cancer, cardiovascular disease, and arthritis.[15,18] Antioxidants scavenge these free radicals generated in vivo and prevent such unwanted biochemical reactions. Similarly, inflammation signaling pathways produce intermediates or inflammation-causing hormones. Cyclooxygenase enzymes, COX-1 and -2, play a significant role in producing such intermediates as prostaglandins and thromboxanes. Compounds in food have the ability to inhibit COX enzymes and hence prevent or modulate the inflammation signaling pathways. Therefore, the anti-inflammatory activity of extracts and isolates
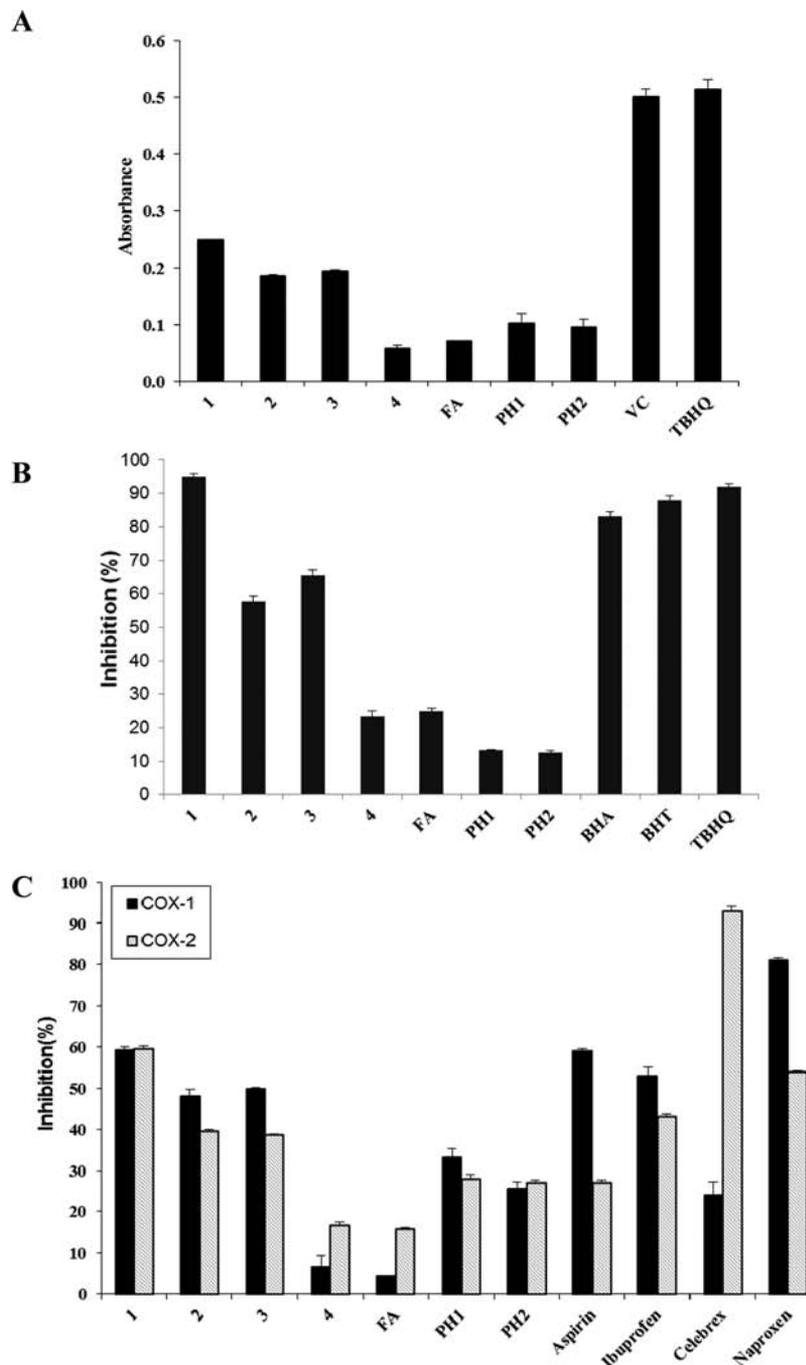
Figure 3. (A) Absorbance values at 570 nm of compounds **1−3** at 100 μg/mL and compound **4**, mixture of fatty acids (**FA**), bis(2-ethylhexyl) terephthalate (**PH1**), and bis(2-ethylheptyl) phthalate (**PH2**) at 25 μg/mL obtained after reaction with MTT at 37 °C. Vitamin C and TBHQ were used as positive controls at 25 μg/mL. (B) Inhibition of LPO by compounds **1−3** at 100 μg/mL and compound **4**, mixture of fatty acids (**FA**), and bis(2-ethylhexyl) terephthalate (**PH1**), and bis(2-ethylheptyl) phthalate (**PH2**) at 25 μg/mL. Commercial antioxidants BHA, BHT, and TBHQ were tested at 10 μM. The oxidation of lipid was initiated by the addition of Fe^{2+} ions. (C) COX-1 and COX-2 enzyme inhibitory activities of compounds **1−4**, mixture of fatty acids (**FA**), bis(2-ethylhexyl) terephthalate (**PH1**), and bis(2-ethylheptyl) phthalate (**PH2**) at 50 μg/mL concentration and commercial NSAIDs aspirin, Celebrex, naproxen, and ibuprofen used as positive control at 108, 1, 15, and 12 μg/mL, respectively. The various concentrations of positive controls used in these assays were to yield comparable activity profiles between 0 and 100% by test extracts, fraction, and positive controls alike. The standard error of the mean was represented for $n = 4$ ($P < 0.05$, t test, paired, two tailed). For the COX assay, vertical bars represent the standard deviation of each data point ($n = 2$).

of Ajwa dates were tested by measuring the inhibition of COX-1 and -2 enzymes. This assay determines the ability of COX enzymes to convert arachidonic acid to prostaglandins, which initialize the inflammatory process in the body.[15,18] At 250 μg/mL, the ethyl acetate, methanolic, and water extracts gave absorbance values of 0.18, 0.25, and 0.37 at 570 nm, respectively (Figure 1A).

Fraction **K** was the most active and showed an absorbance value of 0.41 at 50 μg/mL, similar to the activity of vitamin C and TBHQ at 25 μg/mL (Figure 1A). The ethyl acetate, methanolic, and water extracts of Ajwa fruits inhibited LPO by 88, 70, and 91% at 250 μg/mL, respectively (Figure 1B). As in the case of MTT assay, fraction **K** was the most active among

the extracts tested and showed LPO inhibitory activity by about 100% at 50 μg/mL. In the COX inhibitory assays at 100 μg/mL, these extracts showed a higher COX-2 enzyme inhibition, by 59, 48, and 45%, respectively, when compared to the COX-1 enzyme (30, 31, and 32%, respectively) (Figure 1C). However, the trend was opposite in the case of fraction **K**.

Purification of extracts, as described under Materials and Methods, afforded pure isolates chrysoeriol-7-O-(2,6-dirhamnosyl)-glucoside (**1**),[20] lup-20(29)-en-3-one (**2**),[21,22] lupeol (**3**),[23] 1,2-dilinoleoyl-3-stearin (**4**),[24] β-sitosteryl-3β-glucopyranoside-6′-O-palmitate (**5**),[25] β-sitosteryl-3-O-β-glucoside (**6**),[26,27] β-sitosterol (**7**)[26,27] bis(2-ethylhexyl) terephthalate,[28] and bis(2-ethylheptyl) phthalate,[29] which were elucidated by NMR spectroscopic analyses (see Figure 2 for structures). The fatty acid mixture isolated, contained linoleic, oleic, and stearic acids.[17,30] The sugars purified from methanolic and water extracts were monosaccharides and their structures confirmed as mixtures of β-D- and α-D-glucopyranose,[31] and mixtures of β-D-fructopyranose and β-D-fructofuranose[31] by proton and carbon NMR spectral experiments. Proton and carbon NMR spectral data revealed that fraction **K** was proteinaceous in nature and hence was not studied further to elucidate its structure(s). It is important to note that several flavonoid glycosides were detected in the methanolic extract by analytical TLC. However, the paucity of the extract containing these phenolics allowed only the isolation and characterization of compound **1**.

The isolates from Ajwa dates were tested for antioxidant and anti-inflammatory activities, as in the case of extracts, using MTT, LPO, and COX-1 and -2 enzyme inhibitory assays. Compounds **5**−**7** were not assayed because their activities have been reported from our laboratory earlier.[26,32] At 100 μg/mL concentration, compounds **1**−**3** gave absorbance values of 0.28, 0.19, and 0.19, respectively (Figure 3A). Compound **4**, the mixture of fatty acids, and phthalates **PH1** and **PH2** showed little or no activity as indicated by the poor absorbance values of 0.06, 0.07, 0.10, and 0.10 at 25 μg/mL concentration. These compounds were not tested at higher concentrations due to poor solubility. At 100 μg/mL concentration, flavonoid glycoside **1** showed the highest LPO inhibitory activity at 95%. Triterpenoids **2** and **3** also showed moderate LPO inhibition at 58 and 66%, respectively, at 100 μg/mL concentration. Again, due to poor solubility compound **4**, the mixture of fatty acids, and phthalates were tested at the highest concentration of 25 μg/mL and showed very weak LPO inhibition as indicated by 23, 25, 13, and 13%, respectively (Figure 3B).

The anti-inflammatory activity of the pure isolates from Ajwa fruits was revealed by their COX-1 and -2 enzyme inhibitions. At 50 μg/mL concentration, compounds **1**−**4**, the mixture of fatty acids, and phthalates inhibited COX-1 enzyme by 59, 48, 50, 6, 4, 33, and 26% and COX-2 enzyme by 60, 40, 39, 17, 16, 28, and 27%, respectively (Figure 3C). Among these, flavonoid glycoside **1** showed the highest COX-1 enzyme inhibitory profile, similar to that of aspirin, and COX-2 enzyme inhibition similar to that of naproxen. Triterpenoids **2** and **3** also showed moderate COX-1 and -2 enzyme inhibitions, similar to ibuprofen.

This is the first report of the isolation of compounds **1**−**6** from date fruits as well as the active proteinaceous fraction. In addition, the detailed chemical evaluation and biological activities of Ajwa fruit and biological activities described for its pure isolates **1**−**4** and the phthalates **PH1** and **PH2** are reported for the first time. Ajwa dates contain 39.06% of fructose, a major portion of the total sugar in the fruit. It is

important to note that fructose has the lowest glycemic index among natural sugars and has been proven to be very effective in controlling glycemia in type-2 diabetic patients.[33−35] The overall composition of Ajwa dates in this study showed that it contained 13.24% seeds, 6.21% moisture, and 11.01% fibrous material. The major metabolites in Ajwa fruit were primary metabolites, sugars and proteins. Interestingly, only monosaccharides were present in Ajwa fruit, composed of isomeric mixtures of fructose and glucose, totaling about 65% of the total weight of the fruit. Because fructose was considerably higher than glucose in its total sugar content, consumption of date fruits are less harmful to persons having issues with sugar modulation, as in the case of type-2 diabetics. The presence of phthalates in date fruits could very well be an artifact from plastics involved in pre- and postharvest handling of dates from farms to market. The triglyceride and free fatty acids in date fruits are probably leachates from its seed because seeds are the main storage location of fatty acids and their glycerides. Although we did not fully characterize the proteinaceous fraction, it accounted for 1.33% of the total weight of the fruit. Also, it showed strong antioxidant and anti-inflammatory activities. This is interesting because secondary metabolites are generally reported or implied to possess such biological activities. On the basis of the results presented herein, it is clear that Ajwa date fruit may have added health benefits beyond nutrition and fit in the category of functional foods.

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Notes
The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; LPO, lipid peroxidation; COX, cyclooxygenase; TLC, thin-layer chromatography; UV, ultraviolet; NMR, nuclear magnetic resonance; TBHQ, tert-butylhydroquinone; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; NSAIDs, nonsteroidal anti-inflammatory drugs; SLPC, 1-stearoyl-2-linoleoyl-sn-glycerol-3-phosphocholine; APCI-MS, atmospheric pressure chemical ionization mass spectrometry; DMSO, dimethyl sulfoxide

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