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FULL LENGTH ARTICLE

Health-benefits of date fruits produced in Saudi Arabia based on in vitro antioxidant, anti-inflammatory and human tumor cell proliferation inhibitory assays

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Abstract Date fruits are reported to exhibit health-beneficial effects in addition to its nutritional value. Fruits collected from commercial date palm trees were sequentially extracted with water and methanol. All varieties of date fruits contained sugars, phenolics, triterpenoids, triglycerides, fatty acids and steroids, where sugars were the predominant components. Water and methanolic extracts of date fruits were assayed for antioxidant, antiinflammatory and human tumor cell proliferation inhibitory activities. In MTT antioxidant assay, methanolic extracts at 250 μg/mL exhibited moderate activity with absorbance values between 0.14 and 0.41. Water and methanolic extracts at 100 μg/mL inhibited lipid peroxidation (LPO) by 50–67% and 58–82%, respectively. In anti-inflammatory assay using cyclooxygenase enzymes (COX-1 and -2), water and methanolic extracts at 100 μg/mL showed COX-1 enzyme inhibition by 26–36% and 33–41%, and COX-2 by 45–48% and 48–52%, respectively. At 100 μg/mL concentrations, methanolic extracts of all date fruits showed marginal cell proliferation inhibitory activity against human gastric, prostate, colon, breast and lung tumor cell lines. The bioassay results suggest that varietal difference is not a

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significant factor among the 29 date fruits studied when compared for health-beneficial effects resulting from non-nutritional components present in it.

1. Introduction

Date palm *Phoenix dactylifera* L. (Palmaceae) is grown under arid conditions primarily in the Middle East, North Africa and United States. Its fruit is an important food and dietary component in many countries. Dates are implicated to possess medicinal properties in addition to its nutritional value (Vayalil, 2012). Several studies have reported date fruit with a wide range of bioactivities, such as antioxidant activity due to the presence of phenolics, carotenoids and anthocyanins in it (Mohammed and Al-Okbi, 2004; Saleh et al., 2011; Vayalil, 2002), antimutagenic (Vayalil, 2002), anti-inflammatory (Mohammed and Al-Okbi, 2004), antihyperlipidemic (Tang et al., 2013), antibacterial (Sallal and Ashkenani, 1989) and antifungal (Sallal et al., 1996) activities.

Over 450 date palm varieties or cultivars are grown in the Kingdom of Saudi Arabia and yield more than 1 million metric tons of date fruits accounting for about 14% of the total world production (FAOSTAT, 2014). We had earlier reported the functional food components in Ajwa date fruit, the most expensive date fruit in the market (Zhang et al., 2013). In this study, we determined the health-benefits of 29 significant varieties of date fruits, Barni Al Madinah, Hulwa, Khashram, Khodry, Khalas, Deglet Noor, Dekhaini, Rabeaa, Rushodia, Ruthana, Ruthana Al Sharag, Sabaka, Sukkari Al Qassim, Sullaj, Shalabi, Shaishee, Safawi, Sefri, Segae, Ajwa, Anbara, Luban, Mabroom, Majhool, Mutwah, Meneifi, Nabtat Ali, Naboot Seif and Hilali using in vitro bioassays. The antioxidant activity was evaluated using MTT [3-(4, 5-dimethylthia zole-2-yl)-2,5-diphenyltetrazolium bromide] (Liu and Nair, 2010) and lipid peroxidation (LPO) assays (Bowen-Forbes et al., 2009; Liu and Nair, 2012; Zhang et al., 2013). Using cyclooxygenase enzymes (COX-1 and -2), we determined the anti-inflammatory activity of the date varieties studied (Bowen-Forbes et al., 2009; Liu and Nair, 2012; Zhang et al., 2013). In addition, human tumor cell lines, AGS (gastric), DU-145 and LNCaP (prostate), HCT-116 (colon), MCF-7 (breast) and NCI-H460 (lung) were employed to evaluate the cell proliferation inhibitory activity of water and methanolic extracts of all date fruits (Bowen-Forbes et al., 2009; Liu and Nair, 2012).

2. Material and methods

2.1. General experimental procedures

All solvents used were of ACS reagent grade (Sigma–Aldrich Chemical Company, St. Louis, MO, United States). 250 μm silica gel plates (Analtech, Inc., Newark, DE, United States) were used for thin-layer chromatography (TLC). TLC plates were viewed under UV (ultraviolet) light at 254 and 366 nm in Spectroline CX-20 ultraviolet fluorescence analysis cabinet (Spectroline Corporation, Westbury, NY, United States), and sprayed with 10% sulfuric acid solution. MTT [3-(4, 5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide], *tert*-butylhydroquinone (TBHQ), butylatedhydroxyanisole (BHA), butylatedhydroxytoluene (BHT), Adriamycin, aspirin, naproxen and ibuprofen were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, United States). Similarly, the nonsteroidal anti-inflammatory drug (NSAIDs) Celebrex® was physician's professional sample provided by Dr. Subhash Gupta, Sparrow Pain Center, Sparrow Hospital, Lansing, Michigan. COX-1 and -2 enzymes were prepared in our laboratory from ram seminal vesicles (Oxford Biomedical Research, Inc., Rochester Hills, MI, United States) and insect cells cloned with human PGHS-2 enzyme, respectively. Arachidonic acid was purchased from Oxford Biomedical Research, Inc. (Rochester Hills, MI, United States). 1-Stearoyl-2-lino leoyl-sn-glycero-3-phosphocholine (SLPC) was purchased from Avanti Polar Lipids (Alabaster, AL, United States). The fluorescent probe, 3-(p-(6-phenyl)-1,3,5-hexatrienyl) phenylpropionic acid was purchased from Molecular Probes (Eugene, OR, United States). Fetal bovine serum (FBS) and Roswell Park Memorial Institute 1640 (RPMI-1640) medium were purchased from Gibco BRL (Grand Island, NY, United States). Human tumor cell lines DU-145 and LNCaP (prostate), MCF-7 (breast) and NCI-H460 (lung) were purchased from the National Cancer Institute (NCI, Bethesda, MD, United States). AGS (gastric) and HCT-116 (colon) were purchased from American Type Culture Collection (ATCC, Rockville, MD, United States). All cell lines, enzymes and reagents were stored in the Bioactive Natural Products and Phytoceuticals Laboratory at Michigan State University (East Lansing, MI, United States). Centrifugation was carried out on a Sorvall RC-5C refrigerated high-speed centrifuge (DuPont, Newtown, CT, United States). MTT antioxidant assay plates were read on Bio-Tek Elx800 universal microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, United States). The fluorescent reading in the LPO assay was carried out on a Turner model 450 Fluorometer (Barnstead/Thermolyne Corporation, Dubuque, IA, United States). COX assays were performed in Instech micro oxygen chamber using an oxygen electrode (Instech Laboratories, Plymouth Meeting, PA, United States) attached to a YSI model 5300 biological oxygen monitor (Yellow Springs Instrument, Inc., Yellow Springs, OH, United States).

2.2. Date fruits

The 29 varieties of date fruit samples were collected from commercial farms in Saudi Arabia. The varietal identity of the fruit collected was determined based on the information provided by the farmer and by using the database kept in the Ministry of Agriculture, Saudi Arabia (Table 1) (Anonymous, 2006). Date fruits were packaged and shipped to Michigan State University and kept in −20 °C till analyses.

Table 1 Date fruit variety, date palm identification number, yield of water and methanolic extracts (g) from 100 g date fruits and percent human tumor cell proliferation inhibitory activity of methanolic extracts for DU-145 and LNCaP (prostate), MCF-7 (breast), NCI-H460 (lung) AGS (gastric) and HCT-116 (colon). Water extracts were not active.

Date variety	Plant ID*	Water extract	MeOH extract	AGS	DU-145	LNCaP	HCT-116	MCF-7	NCI-H460
Barni Al Madinah[a]	26	76.4	3.89	11.6	12.4	–	15.7	13.0	15.8
Hulwa[a]	64	72.3	5.30	16.8	–	–	16.6	16.9	17.5
Khashram[b]	80	72.6	1.86	20.5	10.8	–	13.8	16.6	20.1
Khodry[c]	86	77.4	4.23	10.0	15.9	12.3	19.7	22.1	18.4
Khalas[c]	89	80.3	4.60	11.2	–	–	22.8	19.4	14.1
Deglet Noor[c]	98	79.7	5.01	16.1	14.5	–	15.9	11.4	20.1
Dekhaini[a]	100	79.9	3.93	18.5	–	10.0	24.7	15.6	17.1
Rabeaa[a]	111	74.3	3.96	14.7	16.2	–	13.9	17.5	17.2
Rushodia[a]	114	67.2	3.77	19.4	15.2	–	22.7	19.0	18.2
Ruthana[a]	116	73.3	3.60	12.5	–	–	13.6	18.8	17.6
Ruthana Al Sharag[a]	117	80.6	4.16	27.3	–	12.0	18.7	11.2	16.6
Sabaka[a]	124	70.4	5.74	18.6	–	–	14.4	21.6	16.2
Sukkari Al Qassim[a]	138	73.1	4.19	–	13.1	–	18.3	16.7	20.1
Sullaj[c]	142	81.4	2.60	12.2	17.2	–	14.3	13.5	16.6
Shalabi[a]	159	76.4	4.75	22.8	–	10.4	14.6	22.3	18.6
Shaishee[a]	164	73.4	4.12	12.2	16.9	11.1	13.4	14.9	19.7
Safawi[a]	169	80.6	3.95	12.7	14.0	10.1	14.6	16.7	19.2
Sefri[c]	173	72.3	4.97	20.6	–	–	12.5	17.2	18.2
Segae[d]	176	75.2	4.71	21.7	13.5	–	16.4	20.1	18.4
Ajwa[c]	185	75.3	4.64	16.4	–	12.9	22.1	18.6	18.8
Anbara[a]	191	73.3	3.98	13.1	–	–	14.8	10.7	22.2
Luban[a]	226	74.8	4.85	19.0	–	–	11.8	18.9	12.9
Mabroom[a]	231	77.7	4.29	–	–	–	16.5	15.3	18.1
Majhool[c]	234	73.0	5.50	–	–	–	19.9	17.4	20.9
Mutwah[a]	246	74.8	9.43	16.0	–	–	11.4	13.9	11.9
Meneifi[c]	254	76.7	4.98	12.0	13.3	–	18.4	17.7	12.7
Nabtat Ali[c]	273	66.1	6.47	18.5	–	–	12.5	11.4	20.9
Naboot Seif[c]	275	76.9	5.88	15.5	19.0	–	19.7	20.3	20.2
Hilali[a]	285	76.2	5.47	25.5	14.2	–	18.5	19.6	19.4

[a] Procured on November 15, 2011.
[b] Procured on June 20, 2012.
[c] Procured on May 10, 2014.
[d] Procured on April 26, 2014.
* Commercial varieties, Ministry of Agriculture, Saudi Arabia. – : not active.

2.3. Extraction of date fruits for bioassays

The 29 varieties of date fruits, 2 or 3 fruits per variety, were weighed and pitted separately. The pitted dates were cut in small pieces, homogenized and extracted with water (50 mL × 3, 1 h). After centrifugation, the supernatant from each extraction was combined and lyophilized to yield crude water extracts. The residue from water extraction was then extracted with MeOH (100 mL × 3, 2 h), centrifuged and the combined supernatant evaporated under vacuum. The resulting concentrate was lyophilized to yield crude methanolic extract.

2.4. MTT antioxidant assay

MTT assay was evaluated according to our previous report (Liu and Nair, 2010). The stock solution of test extracts (10 mg/mL) and positive controls (Vitamin C and TBHQ at 1 mg/mL) were prepared in DMSO. An aliquot of 10 μL of test samples and 190 μL of MTT water solution (1 mg/mL) were vortexed in a capped glass vial (2 mL) for 1 min, which was then incubated at 37 °C for 24 h. To this, 200 μL of DMSO was added and vortexed again for 1 min. An aliquot (200 μL) of the reaction mixture was pipetted to a 96-well cell culture plate and the absorbance was read at 570 nm on a Bio-Tek Elx800 universal microplate reader. Each sample was tested in duplicate.

2.5. Lipid peroxidation (LPO) inhibitory assay

LPO inhibitory activity was evaluated for the extract (100 μg/mL) and positive controls (BHA, BHT and TBHQ at 1.802, 2.204 and 1.662 μg/mL, respectively) according to our previous report (Bowen-Forbes et al., 2009; Liu and Nair, 2012; Zhang et al., 2013). The liposome, large unilamellar vesicles (LUV), was prepared by re-suspension of the lipid-probe mixture (0.15 M NaCl, 0.1 mM EDTA, and 0.01 M MOPS maintained over chelating resin Chelex 100). The mixture was then subjected to 10 freeze–thaw cycles in a dry ice/EtOH bath and extruded 29 times through a 100 nm pore size membrane (Avestin Inc., Ottawa, ON, Canada). The peroxidation was initiated by the addition of 20 μL of $FeCl_2 \dot{s} 4H_2O$ (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, 3 and every 3 min thereafter up to 21 min on a Turner model 450 Fluorometer. 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.

2.6. Cyclooxygenase enzymes (COX-1 and -2) inhibitory assays

The COX-1 and -2 enzyme inhibitory activities were measured for extracts (100 μg/mL) and the positive controls (commercial Aspirin, Celebrex, Naproxen, and Ibuprofen at 108, 1, 15 and 12 μg/mL, respectively) following the published procedure (Bowen-Forbes et al., 2009; Liu and Nair, 2012; Zhang et al., 2013). The test was carried out by monitoring the initial rate of O_2 uptake using an Instech micro oxygen chamber and electrode attached to an YSI model 5300 Biological Oxygen Monitor at 37 °C. The test samples (6 μL) were initially added to the chamber full of assay buffer (1 mM Tris-phenol buffer, 600 μL, pH = 7, and hemoglobin (17 μg)). COX-1 or -2 enzymes (20 μL) were then added and incubated for 2 min. The substrate arachidonic acid (10 μL of solution at 1 mg/mL 1 mM Tris-phenol buffer, pH = 7) was added to initiate the enzyme reaction or formation of prostaglandin endoperoxide. The data were recorded using QuickLog for windows data acquisition and control software. Each sample was tested in duplicate, and the percent inhibition was calculated with respect to DMSO control. The varying concentration of positive controls used in the assay was to obtain comparable enzyme inhibitions between 50% and 100%.

2.7. Human tumor cell proliferation inhibitory assay

The human tumor cell proliferation inhibitory activities were evaluated for the extracts at 100 μg/mL and positive control adriamycin at 1.5 μg/mL as per published method (Bowen-Forbes et al., 2009; Liu and Nair, 2012). AGS (gastric), DU-145 and LNCaP (prostate), HCT-116 (colon), MCF-7 (breast), and NCI-H460 (lung) human tumor cells were cultured in RPMI-1640 medium containing penicillin–streptomycin and 10% fetal bovine serum (FBS). Aliquots of

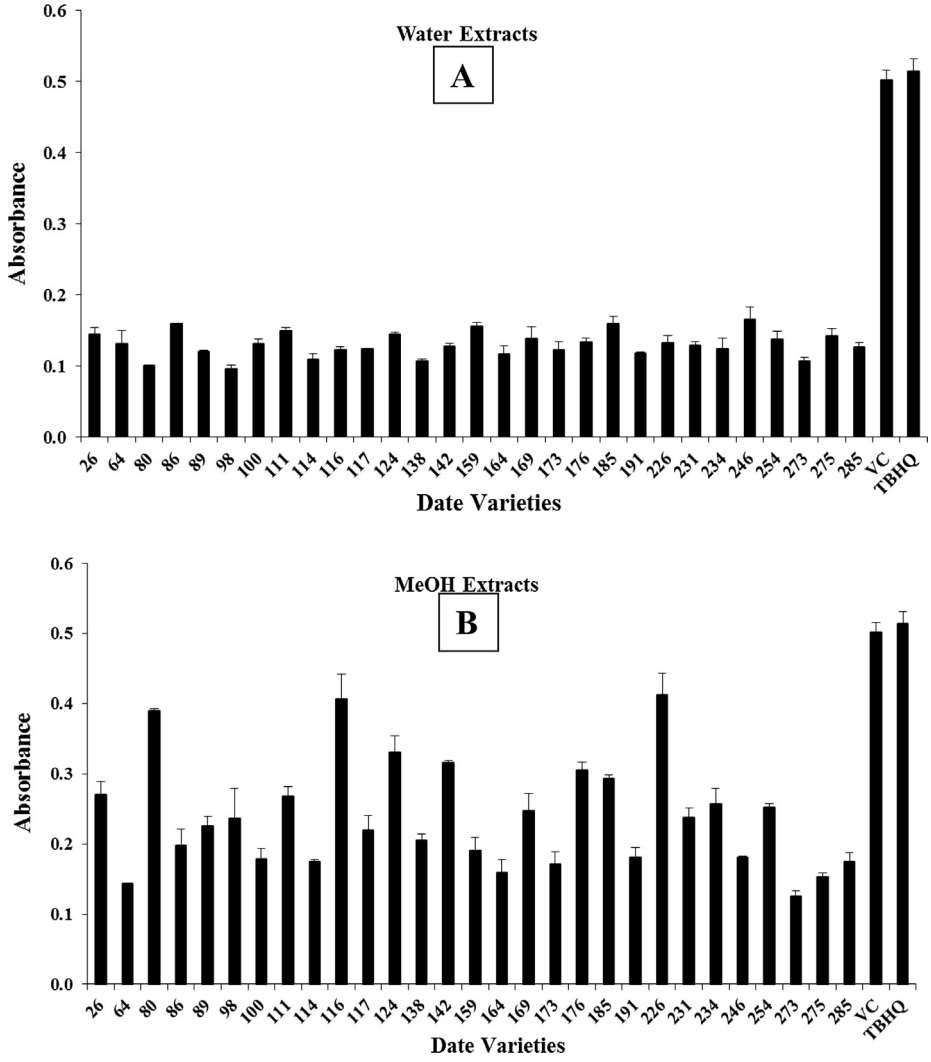

Figure 1 Absorbance values at 570 nm of (A) water and (B) methanolic extracts of 29 varieties of date fruit extracts at 250 μg/mL obtained after reaction with MTT at 37 °C. Vitamin C and TBHQ were used as positive controls at 25 μg/mL. Vertical bars represent the standard deviation of each data point (n = 2).

100 μL of test samples were added to each well of 96-well cell culture plate containing the tumor cells (2000–4000 cells). After incubation for 48 h at 37 °C, an aliquot (25 μL) of MTT in Phosphate Buffer Saline (PBS) solution (5 mg/mL) was added and plates were incubated for another 3 h at 37 °C. The wells were then aspirated followed by the addition of DMSO (200 μL) to dissolve the formazan blue crystals produced by viable cells. The plates were shaken and the absorbance was measured on a Bio-Tek Elx800 universal microplate reader at 570 nm. Each sample was assayed in triplicate.

3. Results and discussion

The date fruits were extracted sequentially with water and methanol to afford the respective crude extracts. The yield of total extracts varied among the varieties of date fruits studied (Table 1) and was between 70.97% and 84.90%. Among them, Khalas, Deglet Noor, Dekhaini, Ruthana Al Sharag, Sullaj, Safawi and Mutwah gave the highest yield of total extracts, about 84%, while Khashram, Rushodia and Nabtat Ali gave the lowest yield, about 72%. Khalas, Deglet Noor, Dekhaini, Ruthana Al Sharag, Sullaj and Safawi showed the highest yield of water extract, about 80%, while Mutwah showed the highest yield of methanolic extract, about 9.43%. The TLC profiles of water extracts showed only sugars and no indication of the presence of secondary metabolites in all date fruits studied. Fructose was predominant along with glucose in all varieties of dates except Nabtat Ali, Sukkari Al Qassim and Deglet Noor showed additional sucrose. In Sukkari Al Qassim and Deglet Noor sucrose was the predominant sugar. Between fructose and glucose, fructose was higher in these varieties containing sucrose. The major components in the methanolic extracts of all date fruits by TLC were still the same sugars detected in water extracts. In order to profile the minor components or secondary metabolites in all varieties of date fruits

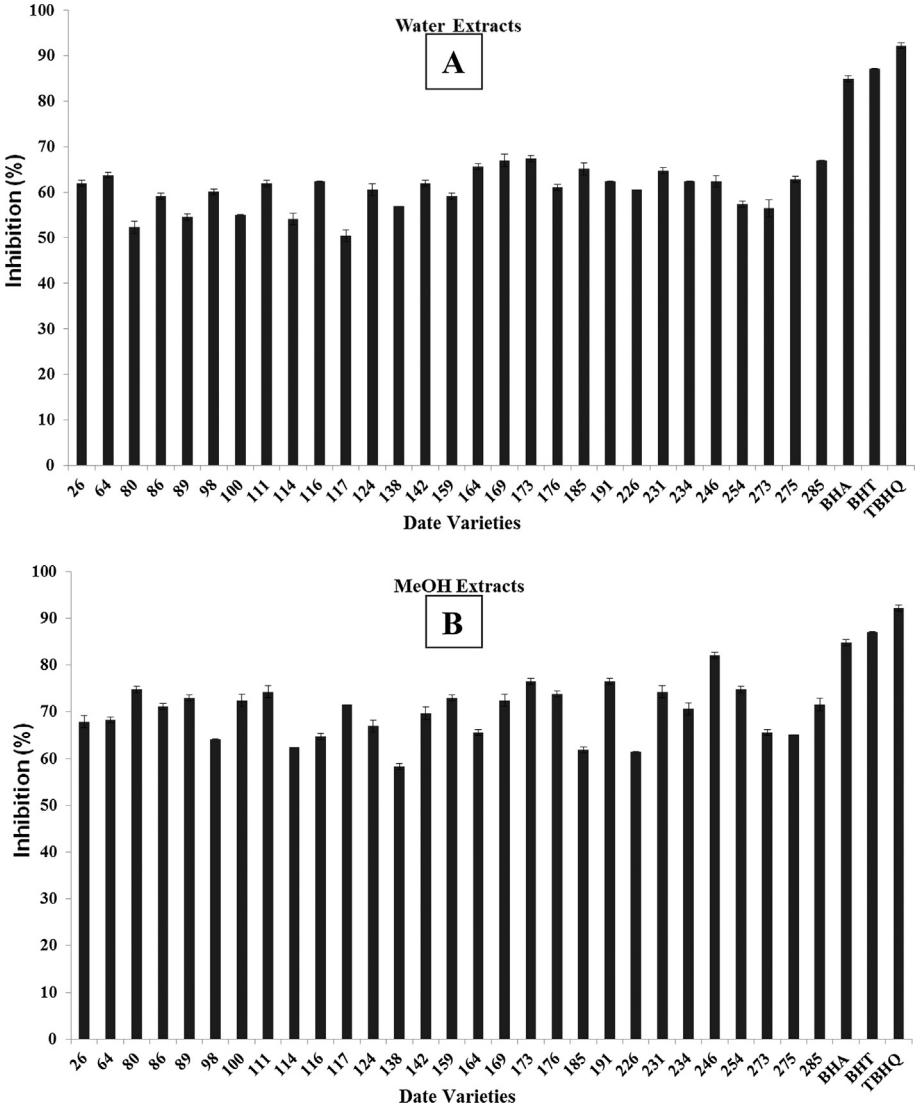

Figure 2 Inhibition of LPO by (A) water and (B) methanolic extracts of date fruits at 100 μg/mL. Commercial antioxidants BHA, BHT and TBHQ were tested at 1.802, 2.204 and 1.662 μg/mL. The oxidation of lipid was initiated by the addition of Fe^{2+} ions. The varying concentrations of positive controls were to yield a comparable activity profiles between 0% and 100% by test extracts and positive controls alike. Vertical bars represent the standard deviation of each data point ($n = 2$).

studied, an aliquot of 400 mg of methanolic extract of each variety was separately stirred with $CHCl_3$–MeOH (4:1, v/v) to yield a sugar free fraction, respectively. Interestingly, the sugar free fractions of all varieties showed identical TLC profiles with variations in concentration of some of its components. The secondary metabolites detected by TLC profiles for all varieties of date fruits were similar to phenolics, triterpenoids, triglycerides, fatty acids and steroids reported for Ajwa date fruit (Zhang et al., 2013). Therefore, characterization of bioactive compounds was not further carried out for the date fruit extracts evaluated in this study for antioxidant, antiinflammatory and tumor cell proliferation inhibitory activities.

The water and methanolic extracts of all varieties of date fruits were evaluated for antioxidant activity using MTT and LPO assays (Bowen-Forbes et al., 2009; Liu and Nair, 2010, 2012; Zhang et al., 2013). MTT assay detects compounds that are capable of reducing or removing oxidative agents while the LPO assay detects components in the extract that are free radical scavengers. In MTT assay, water extracts from all date fruits at 250 μg/mL showed weak activity with absorbance values between 0.10 and 0.16 at 570 nm. However, all methanolic extracts at 250 μg/mL exhibited better activity with absorbance values between 0.14 and 0.41 (Fig. 1). Among these, methanolic extracts of Khashram, Ruthana and Luban showed the highest antioxidant activity on par with the absorbance displayed by the positive controls. In the LPO assay, water and methanolic extracts of all varieties at 100 μg/mL showed inhibition by 50–67% and 58–82%, respectively (Fig. 2). Again, similar to MTT assay results, the methanolic extracts showed better activity than water extracts. For example, among the water extracts, Shaishee, Safawi, Sefri, Ajwa, Mabroom and Hilali showed the highest LPO inhibition by about 65% and the methanolic extracts of Khashram, Rabeaa, Sefri, Anbara,

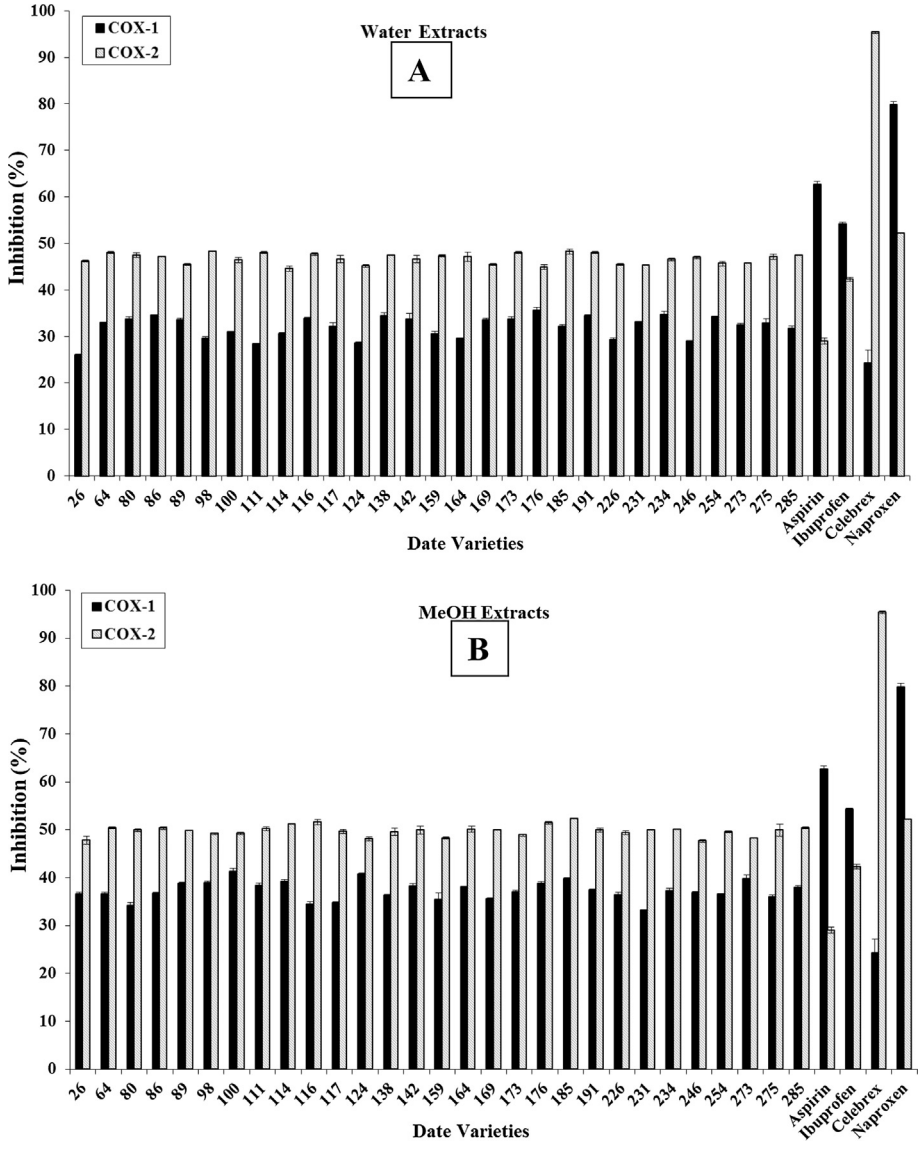

Figure 3 COX-1 and -2 enzyme inhibitory activities of (A) water and (B) methanolic extracts of date fruits at 100 μg/mL and commercial NSAIDs aspirin, Celebrex®, naproxen and ibuprofen used as positive control at 108, 1, 15, and 12 μg/mL, respectively. The varying concentrations of positive controls were to yield a comparable activity profiles between 0% and 100% by test extracts and positive controls alike. Vertical bars represent the standard deviation of each data point ($n = 2$).

Mabroom, Mutwah and Meneifi by around 75%. Methanolic extract of Mutwah showed the highest LPO inhibitory activity, about 82%, and was similar in activity with respect to the positive controls used in the assay. The enhanced activity of methanolic extracts in these assays over water extracts was not surprising since sugars do not function as antioxidants.

The anti-inflammatory activity of all date fruits studied was determined by percent inhibition of COX-1 and -2 enzymes by their water and methanolic extracts (Bowen-Forbes et al., 2009; Liu and Nair, 2012; Zhang et al., 2013). Cyclooxygenase enzymes, especially COX-2 produce inflammatory intermediates and partakes in gene regulations that cause many diseases. Therefore, potential inhibition of COX-2 enzyme by date fruits is highly desirable as a health-beneficial trait. Water and methanolic extracts of all 29 varieties of dates inhibited COX enzymes. At 100 μg/mL, all water extracts showed COX-1 and -2 enzyme inhibition by 26–36% and 45–48%, respectively (Fig. 3). Similarly, all methanolic extracts inhibited COX-1 enzyme by 33–41% and COX-2 by 48–52%. For example, the water extracts of Khodry, Sukkari Al Qassim, Segae, Anbara and Majhool showed almost identical COX-1 enzyme inhibition by about 35%. Among the methanolic extracts, Dekhaini, Sabaka, Ajwa and Nabtat Ali showed the highest COX-1 enzyme inhibition. All extracts showed higher COX-2 enzyme inhibition when compared to COX-1 and methanolic extracts were superior in activity than water extracts. The COX-2 enzyme overexpresses in tumor cells and participates in tumor progression (Achiwa et al., 1999; Murata et al., 1999; Parrett et al., 1997; Tsujii et al., 1997). Based on the COX-2 enzyme inhibitory activity detected for these date fruits, all extracts were tested for cell proliferation inhibition against AGS (gastric), DU-145 and LNCaP (prostate), HCT-116 (colon), MCF-7 (breast) and NCI-H460 (lung) human tumor cell lines (Bowen-Forbes et al., 2009; Liu and Nair, 2012). Water extracts did not inhibit the growth of tumor cell lines studied. The methanolic extracts of all date varieties showed around 20–25% inhibition of breast, colon, lung and gastric human tumor cell lines tested (Table 1). The result suggests that date fruits may not provide significant tumor cell proliferation inhibition when compared to antioxidant and anti-inflammatory activities.

4. Conclusion

Based on in vitro antioxidant and antiinflammatory activities, extracts from all 29 varieties of date fruits showed that it contained components than can act as reducing agents and free radical scavengers in cellular reactions in vivo. Similarly, the inhibition of COX enzymes indicated that the extracts contain compounds that can inhibit the production of inflammation causing hormones such as prostaglandins and thromboxanes by preventing the formation of prostaglandin endoperoxide which then leads to the production of inflammatory intermediates. These results further support the health-benefits of date fruits over and above its nutritional value. The bioassay results also suggest that most varieties of date fruits studied provide similar levels of health benefits. This is the first report on the varietal difference of date fruits with respect to bioactivity and associated health benefits. Results presented in this manuscript are informative to both growers and consumers of date fruits.

Conflict of interest

Authors certify that they have no financial or other conflicts of interest on this research.

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