Journal of Functional Foods 62 (2019) 103552

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

BetaSweet carrot extracts have antioxidant activity and *in vitro* antiproliferative effects against breast cancer cells

G.K. Jayaprakasha[a,*], K.N. Chidambara Murthy[a,1], Federica Pellati[b], Bhimanagouda S. Patil[a,*]

[a] Vegetable and Fruit Improvement Center, Department of Horticultural Sciences, Texas A&M University, 1500 Research Parkway, Suite A120, College Station, TX 77845, United States

[b] Department of Life Sciences, University of Modena and Reggio Emilia, Via G. Campi 103, 41125 Modena, Italy

ARTICLE INFO

Keywords:
BetaSweet carrots
Anthocyanins
Polyacetylenes
Radical Scavenging activity
Antiproliferative activity
MCF-7 cells

ABSTRACT

Fruits and vegetables have long been studied for their potential roles in reducing certain cancer risks. Herein, lyophilized BetaSweet carrots were extracted with hexane, methanol, and water and their chemical constituents were identified by UPLC-QTOF-HR-MS. Methanol extracts exhibited significantly higher radical scavenging activity compared with hexane and water extracts. Hexane extracts showed significantly higher (88.4%) inhibition of breast cancer cells proliferation after 24 h at 100 μg/mL, followed by water and methanol extracts, likely due to the presence of polyacetylenes. Cells treated with hexane extracts showed the highest cytochrome-*c* release, indicating induction of apoptosis, as supported by DNA fragmentation and *Bax2* and *bcl2* expression and confirmed by staining with propidium iodide and acridine orange. The methanol extract showed the highest antioxidant activity and was purified to obtain cyanidin-3-2″-xylosyl-6″-(ferulosyl-glucosyl)-galactoside. In conclusion, BetaSweet carrots exhibits multiple health benefits to use as functional foods.

1. Introduction

Epidemiological studies have shown that increasing consumption of fruits and vegetables decreases major health risks, such as coronary heart disease (Alissa & Ferns, 2017), cardiovascular disease, obesity (Pearson-Stuttard et al., 2017), diabetes (Wang, Fang, Gao, Zhang, & Xie, 2016), neurological and chronic diseases (Cohen, 2017), and various forms of cancer (Rodriguez-Casado, 2016). The major beneficial phytochemicals reported from fruits and vegetables are ascorbic acid, carotenoids, tocopherols, polyphenolics, and terpenoids. Flavonoids are the major class of polyphenols and include flavones, flavanones, flavonols, isoflavones, catechins, and anthocyanins (Alasalvar, Al-Farsi, Quantick, Shahidi, & Wiktorowicz, 2005). Anthocyanins have special importance, due to their range of food-compatible colors and health benefits (Yousuf, Gul, Wani, & Singh, 2016), such as the reduction of coronary heart disease risk, improved visual acuity, antioxidant activities, and antiproliferative activities (Stintzing & Carle, 2004).

Functional foods contain high levels of these health-promoting compounds. For example, the Vegetable & Fruit Improvement Center at Texas A&M University developed BetaSweet carrots with high levels of anthocyanins for their enhanced antioxidant activity and health benefits. Corn (*Zea mays*) and genetically modified tomato (*Solanum lycopersicum*) are the only other vegetables containing both carotenoids and anthocyanins (Butelli et al., 2008). The major anthocyanin in BetaSweet carrots is cyanidin-3-sinapoylxylosyl-glucosylgalactoside (Lazcano, Yoo, & Pike, 2001). Previous studies showed that BetaSweet carrots contained ~3.0 μg/g volatile principles, 75 mg/100 of total phenolics (5'-caffeoylquinic acid constitutes more than 50% of total phenolics), and 5.38% sugars. BetaSweet carrots are also a good natural source of carotenoids, phenolic acids, polyacetylenes, and anthocyanins, but there are no comprehensive reports on the chemical constituents and biological activities of this variety. The α-carotene content in BetaSweet carrot is twice the amount measured in regular orange carrots and β-carotene was 2.3-fold higher (16 mg/100 g of fresh weight vs 7.0 mg/100 g) (Lee, Yoo, & Patil, 2011). Moreover, the nutrients in BetaSweet carrots appear to be stable during storage. For example, one study showed that the anthocyanin content was stable upon storage at 5 °C for two weeks and carotenoids decreased by 20–25%. These diverse phytochemicals and stabile anthocyanins provide additional health benefits over regular orange carrots (Lee et al., 2011).

Carrots are commonly included in clinical investigations of vegetables and fruits, and have shown the prevention of certain cancers (Akhtar et al., 2017; Kobaek-Larsen et al., 2017; Mazewski & Gonzalez

* Corresponding authors.
E-mail addresses: gkjp@neo.tamu.edu (G.K. Jayaprakasha), b-patil@tamu.edu (B.S. Patil).
[1] Current affiliation: Central Research Laboratory, Ramaiah Medical College, Bengaluru 560054, India.

https://doi.org/10.1016/j.jff.2019.103552
Received 3 May 2019; Received in revised form 1 September 2019; Accepted 2 September 2019

de Mejia, 2018). For example, one clinical study showed that intake of carrot and vitamin A supplements provides significant benefits in reduction of breast cancer associated symptoms (Chen, Shao, Zhang, & Miao, 2018). However, another study showed that consumption of fruit and vegetables in adulthood is not significantly associated with reduction of breast cancer (Smith-Warner et al., 2001). In the United States, one in eight women will develop invasive breast cancer over the course of her lifetime. An estimated 266,120 new cases of invasive breast cancer are expected to be diagnosed, along with 63,960 new cases of non-invasive breast cancer every year (U.S. Breast Cancer Statistics, 2019). The non-steroidal anti-estrogen medicine tamoxifen has been used to treat one-third of the breast cancer patients (Forbes, 1997). Several studies shown that dietary constituents can help in prevention of breast cancer (Heidari, Jalali, Sedaghat, Ehteshami, & Rashidkhani, 2018). Therefore, it is important to understand the mechanisms involved in the preventive role of natural compounds in breast cancer. In the present study, BetaSweet carrots extracts were tested for their antioxidant activity and proliferation inhibition activity on estrogen-dependent cultured human MCF-7 breast cancer cells for the first time. Carrot extracts were analyzed by ultra-high-pressure liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UHPLC-HR-QTOF-MS) for the identification of chemical constituents. The most active fraction was purified to obtain an anthocyanin, which was characterized by NMR and mass spectral data for the first time from BetaSweet carrots.

2. Materials and method

2.1. Plant material, chemicals, reagents, and antibodies

Purple 'BetaSweet' carrots (27 kg) were received from J&D Produce (Edinburgh, TX), chopped into small pieces and lyophilized to obtain dry material (2.717 kg). This was powdered (40–60 mesh size powder) using a Vita-prep blender (Vita-Mix Corporation, Cleveland, OH, USA) and used for extraction. HPLC-grade and ACS grade solvents were obtained from Sigma-Aldrich (St Louis, MO, USA). Water was purified by using a Milli-Q PLUS 185 system from Millipore (Milford, MA, USA). Falcarinol and falcarindiol were kindly gifted by Prof. Christian Zidorn, University of Innsbruck, Austria and Prof. Pia Knuthsen, Technical University of Denmark, Denmark, respectively. Polyacetylenes are not available commercially. Therefore, to confirm the identification of the HPLC peaks, an authentic sample of purple carrot was obtained from Dr. Brandon Metzger (Standard Process Inc., Palmyra, WI, USA). Metzger et al. reported several polyacetylenes from orange and purple carrots (Metzger et al., 2008). This extract was prepared by extraction of the plant material with ethyl acetate under sonication. Dulbecco's Modified Eagle's Medium (DMEM) and chemicals used for cell cultures were purchased from Hyclone (Logan, UT, USA). All primary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescent probes (acridine orange [AO] and propidium iodide [PI]) were obtained from Molecular Probes (Invitrogen Corp., Carlsbad, CA, USA). Unless otherwise mentioned, control cells/treatment refers to MCF-7 cells treated with an equal amount of DMSO under conditions similar to sample treatment.

2.2. Extraction of phytochemicals

BetaSweet carrot powder (2717 g) was sequentially extracted with hexane, methanol, and water at room temperature on an orbital shaker for 24 h each in dark conditions. The filtered extracts were concentrated under vacuum to obtain 30.4 g of oily fraction from hexane. Further concentrated methanol and water extracts were lyophilized to obtain 40.9 and 160 g, respectively, of powder.

2.3. Identification of chemical constituents from the hexane extract

(a) *HPLC-UV/DAD conditions.* The BetaSweet carrot hexane extract was passed through a 0.45 µm PTFE filter and injected into the HPLC-UV/DAD system. Chromatography was performed using an Agilent HPLC (model 1100 system, Waldbronn, Germany), consisting of a vacuum degasser, quaternary pump, autosampler, thermostatted column compartment, and diode array detector. The chromatograms were recorded using an Agilent ChemStation for LC and LC-MS systems (Rev. B.01.03). The analyses were carried out on an Ascentis C_{18} column (250 mm × 4.6 mm I.D., 5 µm, Supelco, Bellefonte, PA, USA). The mobile phase composed of (A) H_2O and (B) acetonitrile (ACN). The gradient elution was modified as follows: 0–5 min 70% B; 5–18 min linear gradient from 70% to 86% B; 18–20 min from 86% to 95% B, 20–40 min 95% B. The post-running time was 5 min. The flow rate was 1.0 mL/min. The column temperature was set at 40 °C. The sample injection volume was 10 µL. The DAD acquisition was performed at 205 and 210 nm.

(b) *HPLC-ESI-MS and MS/MS conditions.* Analyses were performed using two HPLC-ESI-MS systems: (a) an Agilent Technologies modular 1200 system, equipped with a vacuum degasser, a binary pump, an autosampler, a thermostatted column compartment and a 6310 ion trap mass analyzer with an ESI ion source; (b) an Agilent Technologies modular 1200 system, equipped with a vacuum degasser, a binary pump, an autosampler, a thermostatted column compartment and a 6520 Q-TOF mass analyser with an ESI ion source. The HPLC column and the applied chromatographic conditions were the same as those described for the HPLC-UV/DAD system, with the exception of solvent A, which was acidified with 0.1% formic acid. The flow-rate was split 5:1 before the ESI source.

For ESI-ion trap mass analyzer, the parameters were set as follows: the capillary voltage was 3.5 kV, the nebulizer (N_2) pressure was 25 psi, the drying gas (N_2) temperature was 300 °C, the drying gas flow was 8 L/min and the skimmer voltage was 40 V. Data were acquired by Agilent 6300 Series Ion Trap LC/MS system software (version 6.2). The full scan acquisition was performed in the positive ion mode in the *m/z* range 50–1000. MS^2 spectra were automatically performed with helium as the collision gas by using the SmartFrag function.

For ESI-triple quadrupole mass analyzer, the parameters were set as follows: the capillary voltage was 3.5 kV, the nebulizer (N_2) pressure was 25 psi, the drying gas temperature was 300 °C, the drying gas flow was 8 L/min and the fragmentor voltage was 120 V. Data were acquired by Agilent MassHunter Workstation (Rev. B.02.01). The mass spectrometer was operated in the positive ion mode in the *m/z* range 50–1000 and in the product ion scan mode by using nitrogen as the collision gas with collision energy (CE) of 20 V.

2.4. Identification of phenolic compounds from methanol and water extracts

Aliquots of 1 µL of each sample were subjected to chromatography using the 1290 Agilent rapid resolution LC system (Agilent, Santa Clara, CA). The chromatographic separation was carried out on a Zorbax rapid resolution high definition eclipse plus C_{18} column (50 × 2.1 mm, 1.8 µm) at 30 °C with a flow rate of 0.2 mL/min using a binary mobile phase consisting of (A) 0.1% formic acid in water and (B) 0.1% formic acid in water and 0.1% formic acid in acetonitrile (7:3). The separation was achieved in 11 min using the following gradient program, 0–4 min (100–95% A), 4–15 min (95–0% A) and 15–16 min (0–100% A). The column was equilibrated for 2 min before the next injection and peaks were monitored at 280 and 520 nm. Mass spectrometry was performed on a maXis Impact (Bruker Daltonics, Billerica, MA) high resolution Q-TOF operated in the positive ion mode. The mass range was set between *m/z* 50–2000. The nebulizing gas pressure was set at 4 bar, the drying gas flow at 12 L/min, the drying gas temperature at 250 °C. Before each

chromatographic run, 5 mM sodium formate was injected for accurate calibration. The control of the instrument and data processing were done using HyStar v3.2 SR2 software and Data Analysis 4.3 (Bruker Daltonics).

2.5. Radical scavenging activities and total phenolics

(a) *DPPH and ABTS free radical scavenging activities.* The DPPH and ABTS free radical scavenging activity of carrot extracts were measured according to our published protocols (Jayaprakasha, Girennavar, & Patil, 2008b). To understand the free radical scavenging activity, assays were monitored for 15 min using a 96 well microplate reader.
(b) *Total phenolics*. Total phenolics were estimated by the Folin-Ciocalteu method as described by our research group (Chaudhary, Jayaprakasha, Porat, & Patil, 2012) and results were expressed as gallic acid equivalents (mg/g of extract).

2.6. Cell culture and maintenance

Human-derived breast carcinoma MCF-7 cells were obtained from ATCC (VA, USA) and cultured in DMEM using 10% fetal bovine serum with 100 units of penicillin and streptomycin. Cells were cultured and maintained as described in our previous publication (Chidambara Murthy, Jayaprakasha, Kumar, Rathore, & Patil, 2011).

2.7. Cell viability assay

Viability of MCF-7 cells was determined by the MTT (3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide) method (Chidambara Murthy et al., 2011). Approximately 1×10^4 cells/well were seeded and after 24 h, cells were incubated with fresh media containing different concentrations (6.25–100 μg/mL) of samples. After a specific incubation period, each well was treated with 10 μL of MTT reagent (5 mg/mL in sterile phosphate buffered saline, PBS) and incubated at 37 °C for 2 h with gentle agitation. Then, the culture medium was removed and 200 μL of DMSO was added to dissolve the formazan; the optical density of formazan was measured spectrophotometrically at 550 nm in a microplate reader (Bio-Tek, Instruments Inc., Vermont, USA). The percent cell viability was calculated based on the intensity of formazan in samples and control wells.

2.8. Proliferation assay

MCF-7 cells were seeded into 12-well plates at a density of 20×10^3 cells/well and cultured for 24 h. The media was replaced with 1.0 mL of fresh media containing different concentrations (25, 50, 75 and 100 μg/mL) of carrot extracts or camptothecin. Cell count was recorded using Z_1 coulter particle counter after 48 and 96 h by detaching the cells with trypsin-EDTA. Results were expressed as mean ± SD cells/mL of culture. Cell count was recorded in duplicate and each treatment was done in triplicate.

2.9. DNA fragmentation assay

Cells grown up to 80% confluence in 100 mm petri-dish were treated with either 100 μg/mL of carrot extracts or 50 μg/mL of camptothecin. After 24 h of incubation with samples, genomic DNA was extracted using the phenol-chloroform extraction method with slight modifications, as mentioned in our previous publication (Chidambara Murthy et al., 2011).

2.10. Total cellular cytochrome c assay

MCF-7 cells were cultured in 2 mL DMEM at a density of 1×10^6 cells/mL and these cells were incubated for 24 h with or without carrot extract (100 μg/mL of extract). After incubation, cells were washed twice with PBS and suspended in lysis buffer. The lysates were incubated at room temperature for 1 h with gentle mixing. The cytosolic fraction obtained after centrifugation for 15 min at 1000 *g* was diluted with 1 × RD5P (calibration diluents, R&D Systems, Inc. Minneapolis, USA). Following the reaction of cytochrome-*c* with the antibody substrate, cytochrome-*c* concentration was calculated by measuring absorbance at 450 nm (with correction at 540 nm). The amount of cytochrome-*c* was calculated with the help of a standard curve prepared using a human cytochrome-*c* standard.

2.11. RT-PCR analysis

Total RNA was extracted from cultured MCF-7 cells after incubation with carrot extracts for 24 h. RNA was isolated according to the manufacturer's instructions (SV total RNA isolation kit, Promega, USA). The isolated RNA was subjected to DNAse (Invitrogen Corporation, California, USA) treatment. Reverse-transcription reaction was carried out using the Taqman reverse transcription reagents kit (Applied Biosystems, Cat. No. N808-0234) with 400 ng of total RNA and oligo (dT) primers in a 10-μL volume. The resulting cDNA was subjected to PCR amplification using primers 5′CCCTCAAGATTGTCAGCAATGC3′ (forward) and 5′GTCCTCAGTGTAGCCCAGGAT3′ (reverse) for *GAPDH* (*Glyceraldehyde-3-phosphate dehydrogenase*) (control), 5′TAGCAAACTG GTGCTCAAGG3′ (forward) and 5′CGAAGTAGGAGAGGAGGCC3′ (reverse) for *Bax* and 5′TGCACCTGACGCCCTTCAC3′ (forward) and 5′ AGACAGCCAGGAGAAATCAAACAG3′ (reverse) for *Bcl2*. The PCR conditions were as follows: 94 °C for 5 min; 30 cycles of 94 °C for 45 s, annealing for 45 s, extension at 72 °C for 60 s and a final extension at 72 °C for 10 min. Annealing temperatures used were 58, 55 and 52 °C for *GAPDH*, *Bax*, and *Bcl2*, respectively. These PCR products were electrophoresed on 1.6% agarose gel, then visualized following ethidium bromide staining on a transilluminator (FBTIV-816, Fisher Biotech, USA). The image was captured with a CCD camera (Alpha Innotech Corporation, CA, USA) and band intensities were quantified with Alpha Images 5.5 software.

2.12. Protein expression analysis through western blotting

Approximately, 2×10^6 cells were cultured in a 100-mm petri dish overnight prior to incubation with carrot extracts. Plated cells were treated with 100 μg/mL of carrot extract or 50 μg/mL camptothecin for 24 h. Cells were lysed in buffer (containing 130 mM NaCl, 1 mM dithiothreitol, 2 μg/mL leupeptin, 10 mM NaF, 1 mM PMSF and 20 mM Tris, pH 7.4). These lysates were subjected to western blotting to study the expression of apoptosis-associated proteins, such as Bax and Bcl2, as described in our previous publication (Chidambara Murthy et al., 2011)

2.13. Microscopy of cells

In order to understand the induction of apoptosis, MCF-7 cells were cultured in two borosilicate sterile chambered cover glasses (Lab-Tek brand, Nalge Nunc International, NY, USA) for microscopy. Approximately 10×10^5 cells/well SW480 were cultured for 24 h in slides pre-incubated with serum for a few hours and the cells were treated with carrot extracts (100 μg/mL), camptothecin (50 μg/mL), or DMSO (control) in medium for 48 h. The cells were incubated with 5 μM each of acridine orange and propidium iodide at 37 °C for 10 min. The supernatant medium and excess dye were removed after 10 min and cells were washed with DMEM (without phenol red). Fluorescence images were captured using a Zeiss Digital Imaging Workstation.

2.14. Purification of the methanolic extract

The methanolic extract showed the higher antioxidant activity and, therefore, it was subjected to purification to identify the anthocyanins.

The crude extract (39 g) was dissolved in water and loaded onto 500 g SP-70 Sepabeads in a column in water. The column was eluted with water and water:acetonitrile with increasing concentrations of acetonitrile. Fractions containing anthocyanins were pooled, lyophilized, and subjected to flash chromatography on a CombiFlash Rf 4x flash chromatography system with 80 g C_{18} stationary phase for the separation. The column was initially equilibrated with water for five column volumes prior to separations. The compounds were separated using a 45 min pump program with a solvent gradient of water (solvent A) and acetonitrile (solvent B). Initially, 100% A was held for 11 min, linearly increased to 25% B in 4 min, held for 5 min, linearly increased to 30% B in 1 min, held for 5 min, linearly increased to 100% B in 13 min, held for 4 min and returned to 100% A, which was maintained for 2 min. The flow rate was set at 20 mL/min and individual fractions were collected by monitoring the eluting analytes at λ_{max} at 520 nm and 280 nm. A total of 36 fractions of 40 mL each were collected; the eluted fractions corresponding to the major peaks (12–21) were analyzed by HPLC, pooled, and lyophilized to obtain 21 mg.

2.15. Identification by NMR

The structure of the isolated compound was identified by 1D and 2D NMR in methanol-d_4. ^{1}H and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively (JEOL USA, Inc., MA, USA). To assign all the signals, 2D experiments, such as HMBC, HSQC and DQF-COSY, were performed.

2.16. Statistics

The antioxidant activity was expressed as mean ± standard deviation and data was analyzed by Student's *t*-test to compare significant differences at $p < 0.05$. Cell culture results were expressed as mean ± standard error from triplicates with two separate experiments. To evaluate the significant differences, two-way analysis of variance (ANOVA) was performed and data were analyzed by Tukey's post-test analysis using GraphPad prism software version-5.00.288.

3. Results and discussion

3.1. Extraction of phytochemicals from BetaSweet carrots

The current study explores the antioxidant potential and proliferation inhibition of human breast cancer cells by extracts of BetaSweet carrots. The lyophilized BetaSweet carrot powder was successively extracted with different solvents to fractionate non-polar and polar compounds using hexane, methanol, and water. To prevent the degradation of phytochemicals, such as polyacetylenes, phenolic acids, and anthocyanins, a cold extraction was performed for 24 h to obtain 1.1, 1.5, and 5.9% (w/w) yields. All these fractions were stored at −20 °C until further use.

3.2. Characterization of crude extracts by mass spectrometry

(a) *Carrot hexane extract.* The analysis of BetaSweet carrot hexane extract was initially carried out by HPLC-UV/DAD, under reversed-phase conditions. Interestingly, BetaSweet carrots did not possess the typical composition of conventional carrot extracts, which contain falcarindiol, falcarindiol-3-acetate, and falcarinol as the most common polyacetylenic compounds (Metzger & Barnes, 2009; Søltoft et al., 2010). To confirm this observation, a genuine purple carrot crude extract was analysed in parallel by HPLC-UV/DAD under the same chromatographic conditions, showing three main peaks at 9.9, 15.0, and 18.4 min, which correspond to falcarindiol, falcarindiol-3-acetate, and falcarinol (Fig. 1). Falcarinol and falcarindiol standards were also analysed by HPLC-UV/DAD, showing the typical UV spectrum of carrot polyacetylenes (with UV absorption maxima at 230–232, 243–246, and 255–260 nm). The comparison of these chromatographic profiles with those of the samples analysed in this work, together with the analysis of samples spiked with pure falcarinol and falcarindiol, allowed us to identify the presence of a very small amount of falcarinol eluting at 18.8 min (Fig. 1). Falcarindiol and falcarindiol-3-acetate were not detected in the BetaSweet carrot extract analysed in this study.

To confirm the identity of peaks present in the hexane extract, HPLC-ESI-MS analyses were performed with an ion trap mass analyser. The main peak eluting at 23.1 min showed *m/z* at 282.3; it gives also an adduct ion with Na^+ at *m/z* 304.3. MS^2 experiments were then performed to study the fragmentation pattern of this peak. The peak at *m/z* 282.3 produced two product ions at *m/z* 265 and 247. Further HPLC-ESI-MS and MS/MS experiments were then carried out by using a Q-TOF mass analyser. The exact mass of the peak at *m/z* 282.3 was found to be 282.2813, corresponding to the molecular formula $C_{18}H_{35}NO$. On the basis of published data (Schmiech, Alayrac, Witulski, & Hofmann, 2009), this peak could be tentatively assigned to isofalcarinolone, which has a molecular weight of 258.36 and forms an adduct ion with the solvent $[M + H - H_2O + CH_3CN]^+$ at *m/z* 282.

(b) *Methanol and water extracts.* Both polar extracts were subjected to ultra-high-performance liquid chromatography with quadrupole time-of-flight tandem mass spectrometer (UHPLC/HR-QTOFMS) to identify the chemical constituents. Table 1 shows the compounds characterised from methanol and water extracts. A total of 15 compounds were identified by high resolution accurate mass spectra (Table 1, Fig. 2 and Fig. S1). Besides the presence of known antioxidants (vitamins C and E), BetaSweet carrots have attracted the attention of consumers due to their phenolic compounds and anthocyanins, which contribute significantly to the antioxidant capacity. The outer layer of these carrots is indeed an intense purple color, due to the presence of anthocyanins. Along with purple color, anthocyanins have many health benefits, such as the reduction in the risk of coronary heart disease, reduced risk of stroke, anti-proliferative properties, anti-inflammatory effects, and improved cognitive behavior (Mazewski & Gonzalez de Mejia, 2018). BetaSweet carrots have been reported as a very good source of anthocyanins, including acylated forms (Lazcano et al., 2001), but there is no detailed report on their anthocyanin profiles. UPLC chromatograms recorded at 520 nm show the four anthocyanin peaks in BetaSweet carrot extracts. The mass spectra and chemical structures of the identified anthocyanins are shown in Fig. 2. BetaSweet carrot contains mainly acylated anthocyanins. The active fraction was purified to obtain a compounds and structure was elucidated and identified as cyanidin-3, 2″-xylose-6″-feruloyl-glucose-galactoside by 2D NMR spectra (Table 2).

3.3. Radical scavenging activity and total phenolics

All three BetaSweet carrot extracts, purified anthocyanin, and ascorbic acid were evaluated for radical-scavenging ability with ABTS and DPPH free radicals (Fig. 3). All three extracts showed significant differences in both ABTS and DPPH scavenging activities. For instance, the methanol extract exhibited significantly higher free radical scavenging activity, equivalent to ascorbic acid at 200 μg/mL in both methods. Interestingly, significantly lower radical scavenging activity was observed for the water extract 19% (ABTS) and 20% (DPPH) and moderate activity was observed for the hexane extract 41% (ABTS) and 62% (DPPH). The EC_{50} of ABTS and DPPH scavenging activity of cyanidin-3-2″xylose-6″feruloyl-glucose-galactoside was 39.18 ± 0.76 and 40.70 ± 1.59 μM, respectively. The degree of discoloration indicates the scavenging potentials of the samples.

Free radicals cause auto-oxidation of unsaturated lipids in food.

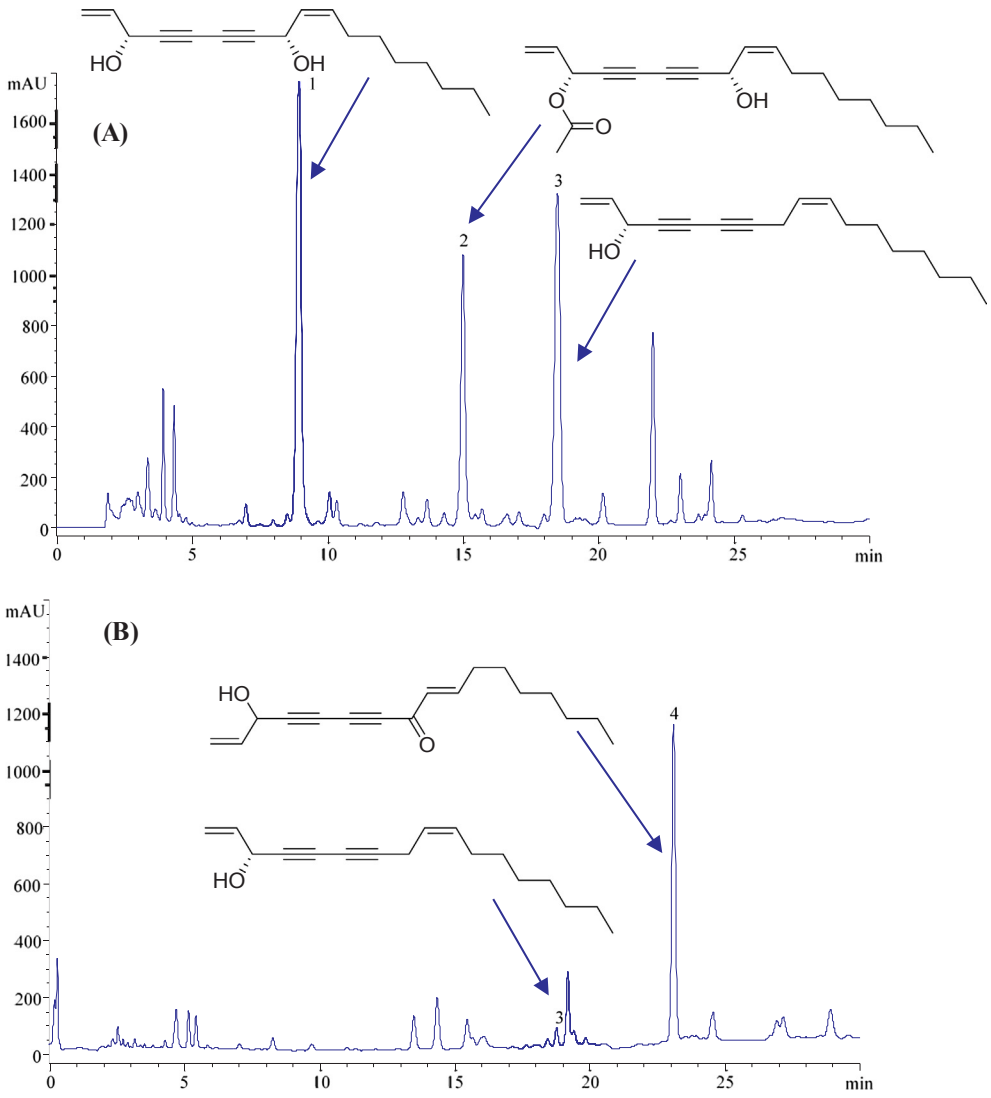

Fig. 1. HPLC-UV/DAD chromatograms at 205 nm of: (A) a reference purple carrot crude extract; (B) BetaSweet carrot crude extract. Peak identification: (1) falcarindiol, (2) falcarindiol-3-acetate, (3) falcarinol and (4) isofalcarinolone.

Table 1
Phenolic compounds and anthocyanins identified in methanol and water extracts from BetaSweet carrots by UPLC-QTOF-HR-MS with electrospray positive ionization.

RT (min)	Identified compound	Molecular Formula	Experimental mass	Adducts	Fragments	Mass error	MeOH	Water
2.51	Phenylalanine	$C_9H_{11}NO_2$	166.0875	$[M+1]^+$	103.0551; 72.9376	−7.3	X	
3.10	Chlorogenic acid	$C_{16}H_{18}O_9$	355.1009	$[M+1]^+$	163.0398	4.1	X	X
3.65	3-Caffeoylquinic acid	$C_{16}H_{18}O_9$	355.1021	$[M+1]^+$	163.0396	0.7	X	X
3.75	5-Caffeoylquinic acid	$C_{16}H_{18}O_9$	355.1015	$[M+1]^+$	163.0396	2.4	X	X
3.82	Feruloyl quinic acid	$C_{17}H_{20}O_9$	369.1177	$[M+1]^+$	287.0545; 163.0385	−0.6	X	X
3.90	5- Feruloyl quinic acid	$C_{17}H_{20}O_9$	369.1216	$[M+1]^+$	323.0581; 163.0407	−11.2	X	X
8.50	Luteoline-O-caffeoyl-pentoside C-hexoside	$C_{35}H_{34}O_{18}$	743.2029	$[M+1]^+$	287.0547	−0.6	X	X
9.05	Cyanidin-3-2″-xylose-6″-sinapoyl-glucose-galctoside	$C_{43}H_{49}O_{24}$	949.2601	$[M^+]$	287.0552	0.3	X	
9.10	Luteoline −7-O-glucoisde	$C_{21}H_{20}O_{11}$	449.1075	$[M+1]^+$	287.0466	−0.6	X	X
9.35	Cyanidin-3-2″-xylose-6″-sinapoyl-glucose-galactoside	$C_{43}H_{49}O_{24}$	949.2610	$[M^+]$	287.0551	1.3	X	
9.55	Cyanidin-3-2″-xylose-6″-feruloyl-glucose-galctoside	$C_{42}H_{47}O_{23}$	919.2522	$[M^+]$	287.0564	−1.5	X	X
9.65	Cyanidin-3-2″-xylose-6″-coumaroyl-glucose-galctoside	$C_{41}H_{45}O_{22}$	889.2407	$[M^+]$	287.0554	−1.2	X	
10.00	Kaempferol-3-O-sorphoroside sinapoyl	$C_{38}H_{40}O_{20}$	817.2191	$[M+1]^+$	287.0548	−1.4	X	X
10.25	Kaempferol-3-feruloydiglucoside	$C_{37}H_{38}O_{19}$	787.2080	$[M+1]^+$	287.0552	−0.2	X	X
10.55	Cyanidin-3-2″-xylose-6″-sinapoyl-glucose-galactoside isomer	$C_{43}H_{49}O_{24}$	949.2610	$[M^+]$	287.0584	−12.3	X	
10.75	Quercetin 3,4″-O-diglucoside	$C_{27}H_{30}O_{17}$	627.1559	$[M+1]^+$	303.0497	−0.5	X	X

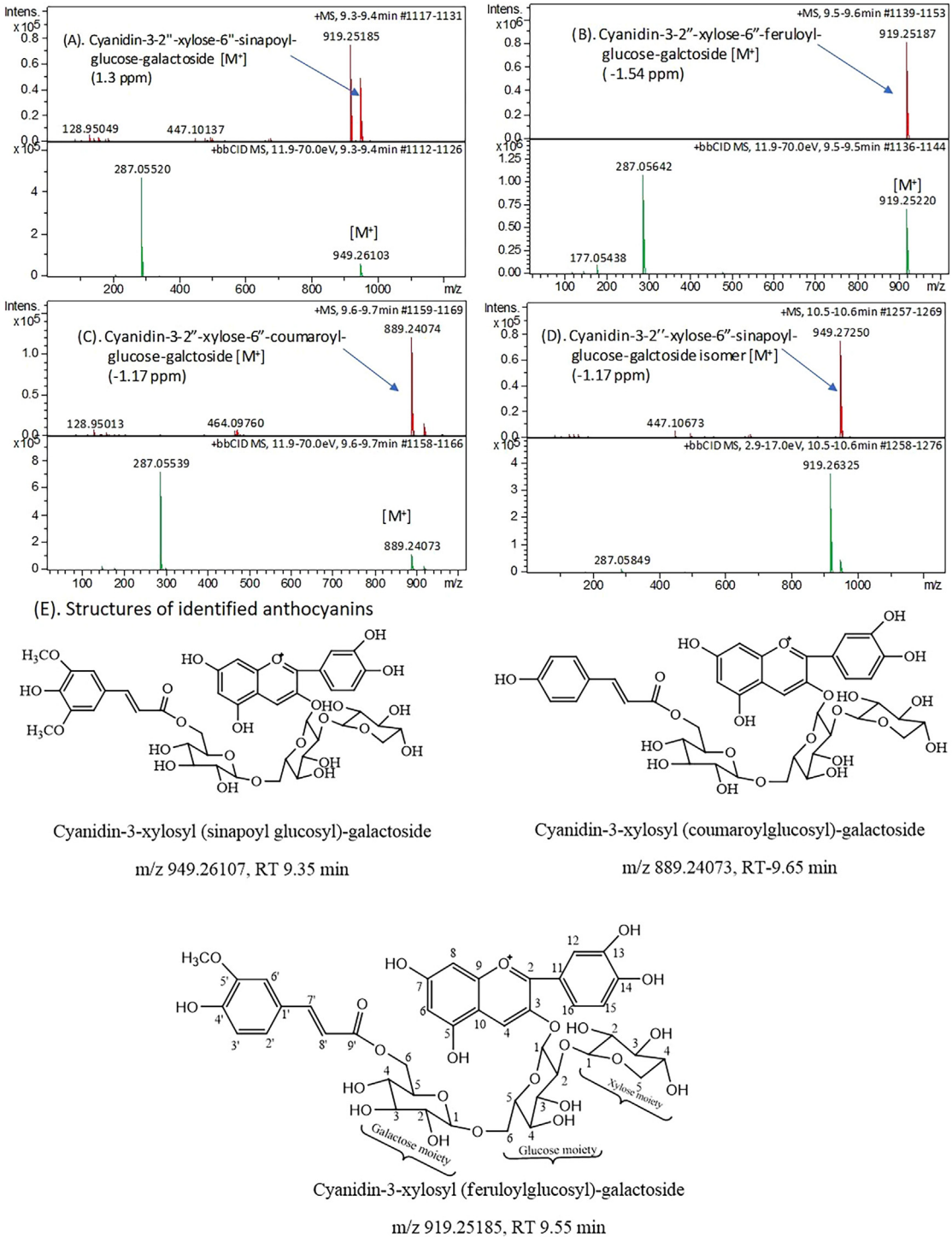

Fig. 2. (A–D). Tandem mass spectra of four major anthocyanins identified from BetaSweet carrots analyzed by UPLC-QTOF-HR-MS. The MS precursor scan (above) and CID product scan (below) obtained by electrospray positive ionization mode; mass errors for precursor ions are shown in brackets. (E). Structures of characterized anthocyanins in the present study with their accurate mass and retention time.

Antioxidants neutralize free radicals by donating hydrogen from the phenolic hydroxyl groups to obtain stable end products. The data obtained from the present study reveal that the carrot extracts are free radical scavengers, which may be attributed to the proton donating ability of their components. Therefore, antioxidant activity of the carrot extracts can be ascribed to their hydrogen donating ability (Jayaprakasha, Girennavar, & Patil, 2008a).

The total phenolic contents of the carrot extracts were determined by the Folin–Ciocalteu method; the results are reported as gallic acid equivalents. Among the three extracts, the methanol extract contains the highest content of total phenolics, (1.09 ± 0.19%), while the water extract has 0.88 ± 0.17% phenolics. The hexane fraction did not show any phenolics.

3.4. Effect of carrot extracts on viability of MCF-7 cells using MTT assay

Extracts of BetaSweet carrots showed inhibition of MCF-7 cancer cells in a dose-dependent manner. All the extracts exhibited a

Table 2
^{1}H- and ^{13}CNMR chemical shifts for the isolated anthocyanin (cyanidin-3-2″-xylose-6″-feruloyl-glucose-galactoside) in CD_3OD.

H/C	Carbon/proton type	^{1}H	^{13}C
2	C		161.46
3	C		144.27
4	CH	8.41 (s)	131.58
5	C		155.28
6	CH	6.58 (Br. s)	101.82
7	C		167.75
8	CH	6.44 (Br.s)	93.66
9	C		157.42
10	C		113.27
11	C		119.69
12	CH	7.82 (Br.s)	117.43
13	C		146.24
14	C		154.46
15	CH	6.98 (d, 8.7 Hz)	116.09
16	CH	8.03, 8.01 (dd, 1.9; 8.7 Hz)	127.39
1′	C		125.26
2′	CH	6.48 (d,d, 1.8, 8.2 Hz)	122.29
3′	CH	6.28 (d, 8.2 Hz)	114.33
4′	C		149.12
5′	C		147.60
6′	CH	6.39 (Br.s)	108.07
7′	CH	7.25 (d, 16 Hz)	146.24
8′	CH	6.08 (d, 15.7 Hz)	114.33
9′	C		168.05
Glucose-1	CH	5.19 (d)	101.82
Glucose-2	CH	4.22 (dd)	79.92
Glucose-3	CH	3.19 (dd)	74.99
Glucose-4	CH	3.39 (dd)	69.05
Glucose-5	CH	4.42 (dd)	75.86
Glucose-6	CH2	4.21 (dd)	72.03
Galactose −1	CH	4.69 (d)	105.02
Galactose −2	CH	4.12 (dd)	73.89
Galactose −3	CH	3.29 (dd)	76.52
Galactose −4	CH	3.92 (dd)	69.62
Galactose −5	CH	3.42 (dd)	73.89
Galactose −6	CH2 (a,b)	5.26 (d), 4.07 (d)	59.51
Xylose-1	CH	4.46 (d)	105.58
Xylose-2	CH	4.10 (dd)	73.26
Xylose-3	CH	3.42 (dd)	76.44
Xylose-4	CH	3.68 (dd)	68.59
Xylose-5	CH2	3.46 (dd)	65.74
5′-OCH_3	CH3	3.78 (s)	54.43

significant ($p < 0.05$) inhibition of cancer cell proliferation at all the tested concentrations (Fig. 4A). Inhibition was independent of time, especially at concentration more than 25 μg/mL. This may be due to immediate accessibility (entering the cells) of compounds for the inhibition activity at higher concentrations (50 μg/mL and higher concentrations). Based on our previous experimental data, concentration of positive control (camptothecin) at more than 25 μg/mL is known to kill the cells completely, and very difficult to compare the outcome of viability and expression of genes/protein. Therefore, in the current study, 25 μg/mL of camptothecin was used for proliferation inhibition assay. Proliferation inhibition was significant ($p < 0.001$) in case of camptothecin compared to all the three extracts at 25 μg/mL and lower concentrations. At 50 μg/mL, inhibition of 51%, 35%, and 62% was observed for hexane, methanol, and water extracts, respectively compared to control at 24 h whereas 86%, 41%, and 90% was observed at 100 μg/mL for the same time point. Unlike antioxidant assay, in which methanol extract showed highest activity and followed by hexane, whereas in case of MTT assay, methanol extract exhibited highest inhibition at lower concentration (6.25, 12.5 and 25 μg/mL). These results indicate that there may be non-antioxidant phytochemicals in the extract that are responsible for the inhibition of proliferation. Along with antioxidant activity, other mechanisms of inhibition of cell proliferation by naturally derived compounds include induction of apoptosis, inhibition of cell cycle/multiplication process, inhibition of key

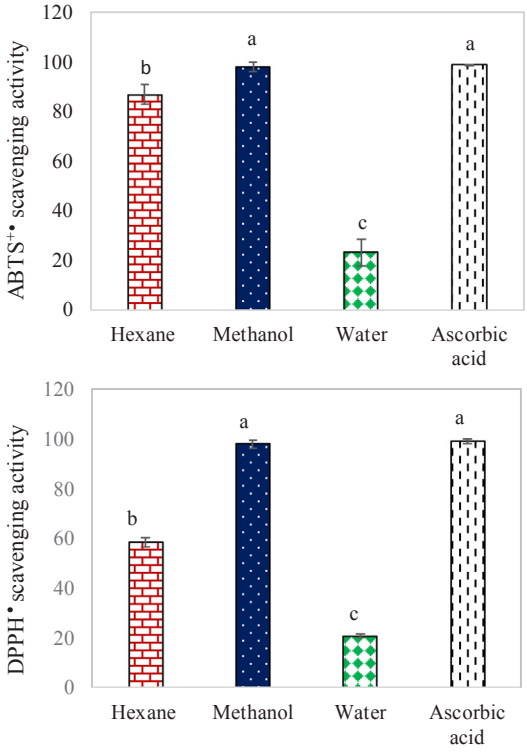

Fig. 3. Free radical scavenging activities (%) of BetaSweet carrot extracts with different solvents and ascorbic acid at 200 μg/mL measured using a 96 well microplate reader for 15 min. [A] ABTS and [B] DPPH. Results were expressed as mean ± SD ($n = 9$), Different letters denotes significant differences ($p < 0.05$) between treatments. Student's *t*-test was done to compare the data and all tests were considered statistically significant at $p < 0.05$.

cell survival signals and activity on hormones (Surh, 2003). Therefore, the relationship between the radical scavenging activity and the inhibition of cancer cell proliferation varies depending on the nature of phytochemicals and its polarity. However, inhibition of cell proliferation and key enzymes, genes associated with apoptosis and cell survival provides further insights on the possible mechanisms. Further, variation in proliferation inhibition activity of all three extracts at lower (< 25 μg/mL) concentration may be attributed to polarity, solubility and permeability of the extracts. Polar extracts, such as those in methanol and water, have water soluble and miscible compounds; they have shown significantly ($p < 0.05$) higher activity compared to hexane extracts at lower concentrations.

3.5. Effect of carrot extracts on viability of MCF-7 cells by cell count

The result of antiproliferative activity of BetaSweet carrot extracts by viable cell count assay was in agreement MTT assay. At 48 h, there was no inhibition of cells at 25 μg/mL compared to control in case of hexane and methanol extracts (Fig. 4B). However, a significant inhibition was observed at 50 μg/mL for all the extracts. Cell counting assay after incubation of cells with extracts for 96 h showed more than 65% inhibition at 25 μg/mL; the inhibition was proportionately increased up to 80–82% at 100 μg/mL. These results were significant ($p < 0.05$) at 100 μg/mL in case of methanol and water extracts along with camptothecin compared to control after 96 h or treatment. Further, analysis of variance indicate that the three extracts showed highly significant ($p < 0.01$) inhibition of proliferation compared to control and inhibition pattern of these extracts at 75 and 100 μg/mL were similar to camptothecin, indicating efficacy of these extracts. Unlike MTT assay, there was not much difference in the inhibition of three extracts at 50, 75, and 100 μg/mL at both the time points. However, at 25 μg/

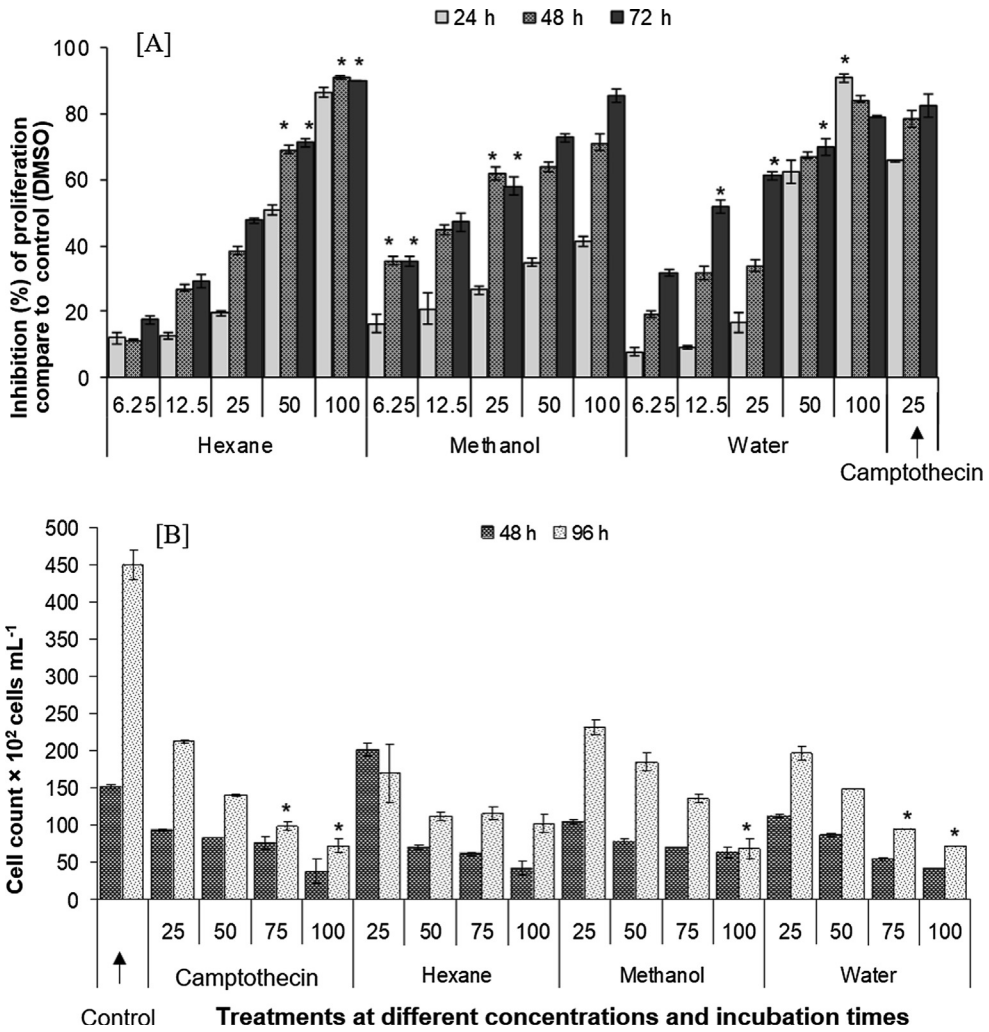

Fig. 4. Proliferation inhibition assay of BetaSweet carrot extracts: x axis represents different concentrations of extracts and positive control and y axis results are expressed in comparison with control (A). MTT assay and (B). Viable cell count assay. Results were expressed as mean ± S.D. from triplicate experiments (n = 9). (*Values were significant ($p < 0.001$) in comparison with control at respective time points; the details of statistics between the concentrations and treatments intergroup is shown in the results section).

mL the inhibition potential of both methanol and water extracts was significantly higher ($p < 0.05$) compared to hexane. At 96 h, the activity of the hexane extract was comparable to the other two extracts.

The MTT and cell count assay results for the three extracts indicate a dose-dependent inhibition of breast cancer (MCF-7) cells. Statistical analysis indicates that proliferation inhibition was comparable to camptothecin by all the three extracts at both 48 and 96 h. Due to multiplication of live cells, the cell number increased in all the treatments after 96 h except in case of hexane extract at 25 μg/mL. Inhibition of cell proliferation showed similar trends with respect to time and concentration in all the three extracts. Multiplication of live cells in the control was 3-fold between 48 and 96 h, but it was less than two-fold for all the extracts and camptothecin treatment.

The comparison of the inhibition potential of the three extracts at different concentrations did not show significant variation except for hexane (25 μg/mL) compared to other treatments at the same concentrations at 48 h. Further, after 96 h, proliferation inhibition by hexane extract was similar to other extracts and standard. There was no significant difference in inhibition potential at different concentrations of hexane extracts.

These extracts were found to be rich mainly in polyacetylenes, phenolic compounds, and anthocyanins, as confirmed by the MS and MS/MS analysis performed in this work. The current study showed that the three extracts have the potential to inhibit breast cancer cells. This can be mainly attributed to falcarinol, isofalcarinolone and minor unidentified polyacetylenes in the hexane extract (Fig. 1), and to phenolic compounds and anthocyanins in the methanol and water extracts (Table 1 and Fig. 2) with different concentrations. These compounds have already shown proliferation inhibition activity in different cancer cells (Mazewski & Gonzalez de Mejia, 2018; Metzger et al., 2008b).

3.6. Effect of carrot extracts on integrity of DNA of MCF-7 cells

Following the results of cell viability assay, it was important to understand the involvement of programmed cell death in inhibiting the proliferation of breast cancer cells. The treatment of MCF-7 cells with BetaSweet carrot extracts at 100 μg/mL for 24 h showed fragmentation of DNA to approximately 200-bp fragments. This is a typical pattern for cells undergoing apoptosis (Fig. 5A). Recent reports indicate that purple carrots also contain non-polar polyacetylenes, such as falcarindiol, falcarindiol 3-acetate and falcarinol (Christensen & Kreutzmann, 2007). These compounds have shown ability to induce cell death in breast cancer cell lines (Lu, Gu, Zhao, Zheng, & Xing, 2017).

3.7. Effect of carrot extracts on cytosolic cytochrome-c content of MCF-7 cells

The release of cytochrome-c into the cytoplasm is another hallmark

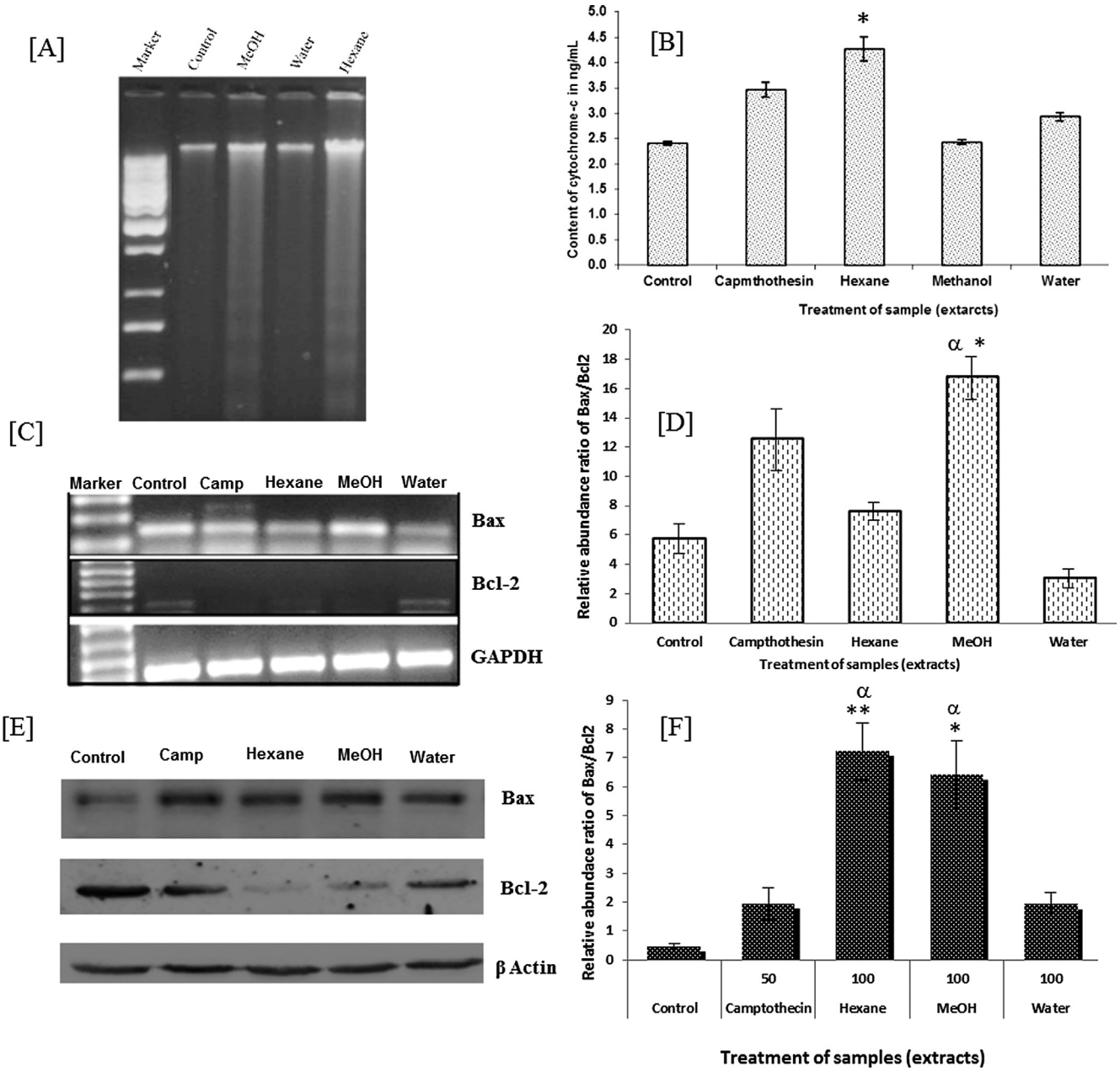

Fig. 5. Influence of BetaSweet cattor extracts with different solvent extracts on (A). DNA fragmentation, (B). Cytosolic cytochrome-*c* (C–D). Expression of apoptosis-related genes and (E–F). Respective proteins in MCF-7 cancer cells, camptothecin (50 μg/mL), BetaSweet carrot hexane, methanol, and water extracts (100 μg/mL). **highly significant compared to control ($p < 0.001$), *significant compared to control ($p < 0.05$) and α indicates significant differences compared to camptothecin, a positive control ($p < 0.05$).

of apoptosis. The measurement of cytochrome-*c* following 24 h treatment of carrot extracts (100 μg/mL) produced a significant increase in the cytochrome-*c* content in cytoplasm compared to control cells (Fig. 5B). The highest content of cytochrome-*c* was observed in the cells incubated with the hexane extract, followed by the water extract. The activity was higher compared to positive control. The difference in activity was significant compared to control ($p < 0.001$) and camptothecin ($p < 0.05$), respectively. Camptothecin is plant-derived compound known to inhibit different tumors mainly through inhibition of DNA topoisomerase I. However, it is known to induce release of cytochrome-*c* as an indirect effect in many cancer cells (Sanchez-Alcazar, Ault, Khodjakov, & Schneider, 2000; Zeng et al., 2012) and, due to its role on tumor inhibition through apoptosis, it is a commonly used as a positive control for studies of proliferation inhibition by naturally derived molecules.

Considering the secondary metabolites identified in the hexane carrot extract, which includes polyacetylenes and carotenoids, it was anticipated that this extract could show significant biological activity. Polyacetylenes have shown radical scavenging activity and proliferation inhibition activity on different cancer cells; therefore, the results of the current study further confirm the activity of these compounds. Cells incubated with the methanol extract did not show any enhancement in the cytochrome-*c* content compared to control. Carotenoids have been reported to maximum in hexane extract, which have antioxidant (Murthy et al., 2005), immunomodulatory, anti-inflammatory, hepatoprotective (Chidambara Murthy, Rajesha, Vanitha, Swamy, & Ravishankar, 2005) and cardioprotective activities (Lidebjer, Leanderson, Ernerudh, & Jonasson, 2007). However, due to their dual roles as antioxidants and pro-oxidants based on the oxygen content, the health benefits of carotenoids have always been questionable (Zhang &

Omaye, 2001). β-carotene has been demonstrated to be a good lipid peroxidation preventing agent under hypoxic conditions; this activity was not affected at 143 torr O_2 tension, but at 722 torr (~14 lb-f in^2) tension, this compound failed to prevent peroxidation of lipids (Zhang & Omaye, 2001). A similar behavior of β-carotene has also been observed in clinical studies on lung cancer prevention in smokers (Omenn et al., 1996). In the current study, the cell proliferation inhibitory activity of the hexane extract may be mainly attributed to polyacetylenes, based on the LC-MS data.

3.8. Effect of carrot extracts on expression of pro-apoptotic Bax and anti-apoptotic Bcl2 genes

The expression of two of the major apoptosis genes, namely *Bax* (*Bcl2 associated protein-X*), a pro-apoptotic gene, and *β-cells of lymphocytes* (*Bcl2*), an anti-apoptotic gene, in cells treated with carrot extracts indicated that apoptosis is the mechanism involved in the inhibition of cell proliferation (Fig. 5C). The expression of *Bax* after normalizing with GAPDH (housekeeping gene) was found to be significantly higher in cells incubated with the methanolic extract ($p < 0.001$ compared to control) compared to all extracts and positive control. Further, there was no difference in *Bax* expression in cells treated with the hexane extract compared to control and this expression was lower compared to camptothecin. Among the extracts, the activity of the methanol extract was highly significant ($p < 0.001$) in comparison with both water and methanol extracts ($p < 0.001$).

Interestingly, the expression of *Bcl2* was completely inhibited in cells incubated with the methanol and hexane extracts. However, *Bcl2* expression in cells treated with the water extract was almost similar to the control. Due to homo- and heterodimer interactions of the encoded proteins, the *Bax/Bcl2* ratio is more important than the individual gene expression for determining apoptosis induction. The *Bax/Bcl2* ratio after normalizing with *GAPDH*, indicated that the potential induction of apoptosis by different extracts was in the order methanol, hexane and water.

Anthocyanins are the polar flavonoids present in BetaSweet carrots and these are relatively stable in the plant matrix. In addition to induction of apoptosis, as a preventive mechanism anthocyanins have also shown inhibition of various cancer cells through different mechanisms, including antioxidant, phase-II metabolic enzyme activation, apoptosis, anti-inflammatory, interfering with cell growth signals, reduce angiogenesis and so on (Mazewski & Gonzalez de Mejia, 2018). To support these findings, the results of the current study demonstrated an inhibition of breast cancer cell by BetaSweet carrot extract by alteration of the *Bax/Bcl2* ratio.

3.9. Effect of carrot extracts on expression of pro-apoptotic Bax and anti-apoptotic Bcl2 proteins

To further confirm the gene expression study results and to rule out the possibility of post transcriptional silencing effects, the levels of the respective proteins were studied using western blotting. Levels of both Bax and Bcl2 were calculated after normalizing with β-Actin. The results of apoptosis related protein expression showed results similar to those obtained by the expression of genes, except for the methanol extract. The overall level of Bax was higher in cells incubated with the carrot extracts compared to untreated cells (Fig. 5D). A clear inhibition of Bcl2 was observed in cells incubated with extracts of BetaSweet carrots which were highest in hexane ($p < 0.001$ compared to control) extract followed by methanol and water extracts. There was no significant difference between control and camptothecin. Unlike gene expression, the activity of hexane extracts was highly significant compared to control ($p < 0.001$) and significant ($p < 0.01$) in comparison with both camptothecin and water extract. Methanol extract showed a relatively higher Bax/Bcl2 ratio compared to the water extract. A complete inhibition of the Bcl2 expression was observed for the hexane and methanol extracts; cells treated with the water extract also exhibited a relatively lower inhibition of Bcl2 protein. The Bax/Bcl2 ratio was higher in cells treated with camptothecin and carrot extract; the increased expression ratio was in the order of hexane > methanol > water.

The results of the current study add further scientific evidence to the health benefits of BetaSweet carrots, as demonstrated by the *in vitro* inhibition of breast cancer cells. Breast cancer cell inhibitory activity was observed for all the three extracts and they were in the order of hexane > methanol > water. The result also indicates that both non-polar and intermediate polarity compounds are imparting proliferation inhibition activity in BetaSweet carrots. Previous research has shown that phenolic compounds, such as luteoline, cyanidin, kaempferol, quercetin and their complexes from plant sources, induce apoptosis through alteration in *Bax* and *Bcl2* genes (Chidambara Murthy, Kim, Vikram, & Patil, 2012; Katsube, Iwashita, Tsushida, Yamaki, & Kobori, 2003). Some compounds identified in these carrot extracts, such as anthocyanins and other phenolics, have shown similar effects on cancer cell lines. Several phytochemicals have shown inhibition of cancer cell proliferation by activation of genes which favor apoptosis and fragmentation of DNA. The pro-apoptotic *Bax* gene and its related protein expression were increased. As a result of this, the mitochondrial membrane potentials were affected, as indicated by the cytosolic cytochrome-*c* content in treated cells. A difference in the pattern of protein expression in relation with the gene expression was observed for the methanolic extract. This may be due to the effect of multiple compounds present in the extract and the modification of translation initiation factors during apoptosis, which led to an overall reduction of protein synthesis (Bushell, Stoneley, Sarnow, & Willis, 2004).

3.10. Fluorescence microscopy of cancer cells treated with the carrot extracts

The microscopic examination of cells treated with the carrot extracts clearly showed cell death along with involvement of apoptosis. The control cells treated with DMSO (highest amount used in sample) had a normal morphology and they did not take up propidium iodide (PI), indicating intact nuclei of the cells (Fig. 6). A higher magnification of the image showed the clear normal morphology with AO-stained organelles inside the nuclei. The cells treated with the hexane extract clearly showed cell death based on number of cells/area at similar magnification. Moreover, cells took mixed stain of AO and PI, indicating un-intact nuclei and disturbed nuclear membranes. Surface morphology of MCF-7 cells treated with hexane and methanol was more irregular and disrupted compared to that of water-soluble extract treatment. Cells treated with water extractable were shrunken but smooth comparatively. This may be due to the presence of non-polar compounds in these to solvent extracts which have potential apoptosis activity. Nuclei were also observed in mixed staining with both AO and PI, indicating the loss in permeability of nuclear membrane. Cells treated with the methanol extract showed cell death at lower magnification, and higher magnification indicated total loss of cellular architecture. Further, membrane blebbing and bleeding were clearly seen as evidence for apoptosis. Similarly, cells pre-treated with the water extract underwent cell death; loss of cellular architecture and higher intensity of PI staining was seen at 400×, suggesting loss of permeability of nuclear membrane and possible loss of nuclei. Further, the surface image of cells provided clear evidence regarding cells membrane blebbing and bleeding, which appeared as rough and irregular masses with all the three extracts and camptothecin-treated cells compared to smooth and intact surface of control cells.

4. Conclusions

In BetaSweet carrots, five phenolic acids, five flavonoids glycosides, four anthocyanins, one amino acid, and two polyacetylenes were

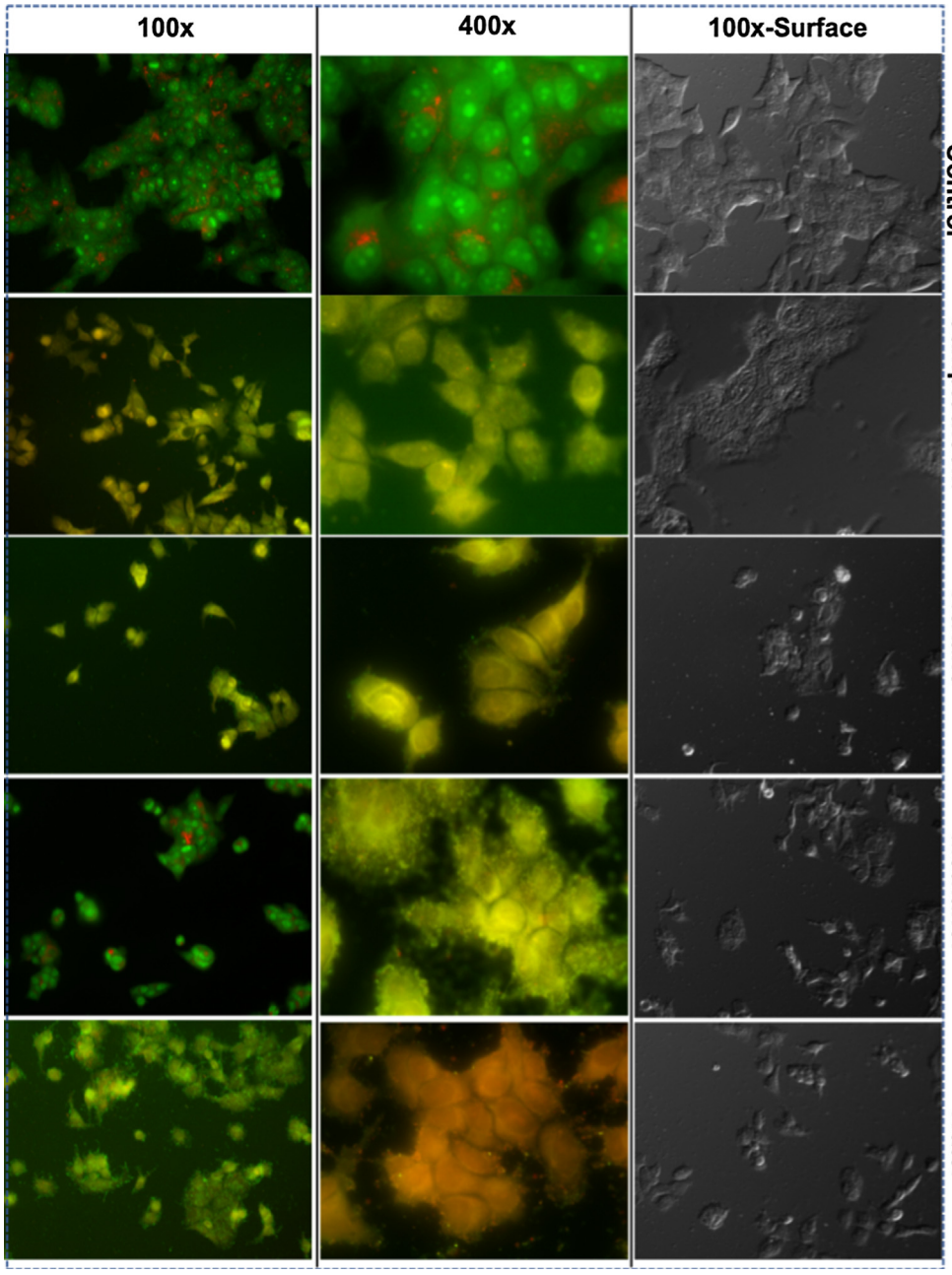

Fig. 6. Micrographs of MCF-7 cells treated with BetaSweet carrot solvent extracts and probed with acridine orange (5 μM) and propidium iodide (5 μM) for detection of apoptosis characteristics. Images were captured at 100 and 400× magnifications, using a fluorescent lamp at 500 nm (excitation) and 530 nm (emission) for acridine orange and 535 nm (excitation) and 617 nm (emission) for propidium iodide. Images were also taken at 100× with all the filters open to examine the effect of compounds on surface morphology.

identified for the first time using mass spectral data. Methanolic extracts showed significantly higher radical scavenging activity as compared to hexane and water extracts. The inhibition of breast cancer cell proliferation was observed in all three extracts. The highest amount of cytochrome-*c* release was observed for the hexane extract, indicating induction of apoptosis, which was also supported by DNA fragmentation and expression of *Bax* and *Bcl2* genes. The activity may be due to the presence of polyacetylenes and other non-polar compounds in the hexane extract, anthocyanins and other phenolic compounds in both the methanol and the water extracts. The results of this study suggest that consumption of whole BetaSweet carrots would be beneficial in the prevention of breast cancer. Considering the health benefits that have been associated with the consumption of anthocyanin-rich foods and diets, BetaSweet carrot seems to be an important food vegetable with a good nutritional value.

Ethics statements

This study does not include human subjects and animal experiments.

Acknowledgements

This study was supported by Texas State Funding 2015-121277 VFIC-TX state appropriation and the United States Department of Agriculture-SCRI-2017-51181-26834 through the National Center of Excellence for Melon at the Vegetable and Fruit Improvement Center of Texas A&M University. Authors thank J&D Produce (Edinburgh, TX) for

the supply of Purple 'BetaSweet' carrots.

Declaration of Competing Interest

All authors declared no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2019.103552.

References

Akhtar, S., Rauf, A., Imran, M., Qamar, M., Riaz, M., & Mubarak, M. S. (2017). Black carrot (*Daucus carota* L.), dietary and health promoting perspectives of its polyphenols: A review. *Trends in Food Science & Technology, 66*, 36–47.

Alasalvar, C., Al-Farsi, M., Quantick, P. C., Shahidi, F., & Wiktorowicz, R. (2005). Effect of chill storage and modified atmosphere packaging (MAP) on antioxidant activity, anthocyanins, carotenoids, phenolics and sensory quality of ready-to-eat shredded orange and purple carrots. *Food Chemistry, 89*(1), 69–76.

Alissa, E. M., & Ferns, G. A. (2017). Dietary fruits and vegetables and cardiovascular diseases risk. *Critical Reviews in Food Science and Nutrition, 57*(9), 1950–1962.

Bushell, M., Stoneley, M., Sarnow, P., & Willis, A. E. (2004). Translation inhibition during the induction of apoptosis: RNA or protein degradation? *Biochemical Society Transactions, 32*(Pt 4), 606–610.

Butelli, E., Titta, L., Giorgio, M., Mock, H.-P., Matros, A., Peterek, S., ... Luo, J. (2008). Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. *Nature Biotechnology, 26*(11), 1301.

Chaudhary, P., Jayaprakasha, G., Porat, R., & Patil, B. S. (2012). Degreening and post-harvest storage influences 'Star Ruby'grapefruit (*Citrus paradisi Macf.*) bioactive compounds. *Food Chemistry, 135*(3), 1667–1675.

Chen, H., Shao, F., Zhang, F., & Miao, Q. (2018). Association between dietary carrot intake and breast cancer: A meta-analysis. *Medicine, 97*(37), e12164.

Chidambara Murthy, K. N., Jayaprakasha, G. K., Kumar, V., Rathore, K. S., & Patil, B. S. (2011). Citrus limonin and its glucoside inhibit colon adenocarcinoma cell proliferation through apoptosis. *Journal of Agricultural and Food Chemistry, 59*(6), 2314–2323. https://doi.org/10.1021/jf104498p.

Chidambara Murthy, K. N., Kim, J., Vikram, A., & Patil, B. S. (2012). Differential inhibition of human colon cancer cells by structurally similar flavonoids of citrus. *Food Chemistry, 132*(1), 27–34.

Chidambara Murthy, K. N., Rajesha, J., Vanitha, A., Swamy, M. M., & Ravishankar, G. A. (2005). Protective effect of Dunaliella salina–A marine micro alga, against carbon tetrachloride-induced hepatotoxicity in rats. *Hepatology Research, 33*(4), 313–319.

Christensen, L. P., & Kreutzmann, S. (2007). Determination of polyacetylenes in carrot roots (*Daucus carota* L.) by high-performance liquid chromatography coupled with diode array detection. *Journal of Separation Science, 30*(4), 483–490. https://doi.org/10.1002/jssc.200600325.

Cohen, M. (2017). Brain Food: Can the foods you eat enhance brain health, or even help manage a neurologic condition? We spoke to the experts about popular diets and reviewed the evidence to find the answers. *Neurology Now, 13*(3), 22–35.

Forbes, J. (1997). The control of breast cancer: The role of tamoxifen. *Seminars in Oncology, 24*(Suppl. 1), 5–19.

Heidari, Z., Jalali, S., Sedaghat, F., Ehteshami, M., & Rashidkhani, B. (2018). Dietary patterns and breast cancer risk among Iranian women: A case-control study. *European Journal of Obstetrics & Gynecology and Reproductive Biology*.

Jayaprakasha, G. K., Girennavar, B., & Patil, B. S. (2008a). Radical scavenging activities of rio red grapefruits and sour orange fruit extracts in different in vitro model systems. *Bioresource Technology, 99*(10), 4484–4494.

Jayaprakasha, G. K., Girennavar, B., & Patil, B. S. (2008b). Antioxidant capacity of pummelo and navel oranges: Extraction efficiency of solvents in sequence. *LWT - Food Science and Technology, 41*(3), 376–384.

Katsube, N., Iwashita, K., Tsushida, T., Yamaki, K., & Kobori, M. (2003). Induction of apoptosis in cancer cells by bilberry (*Vaccinium myrtillus*) and the anthocyanins. *Journal of Agricultural and Food Chemistry, 51*(1), 68–75.

Kobaek-Larsen, M., El-Houri, R. B., Christensen, L. P., Al-Najami, I., Fretté, X., & Baatrup, G. (2017). Dietary polyacetylenes, falcarinol and falcarindiol, isolated from carrots prevents the formation of neoplastic lesions in the colon of azoxymethane-induced rats. *Food & Function, 8*(3), 964–974.

Lazcano, C. A., Yoo, K. S., & Pike, L. M. (2001). A method for measuring anthocyanins after removing carotenes in purple colored carrots. *Scientia Horticulturae, 90*(3–4), 321–324.

Lee, E. J., Yoo, K. S., & Patil, B. S. (2011). Total carotenoid, anthocyanin, and sugar contents in sliced or whole purple (cv. Betasweet) and orange carrots during 4-week cold storage. *Horticulture, Environment, and Biotechnology, 52*(4), 402.

Lidebjer, C., Leanderson, P., Ernerudh, J., & Jonasson, L. (2007). Low plasma levels of oxygenated carotenoids in patients with coronary artery disease. *Nutrition, Metabolism and Cardiovascular Diseases, 17*(6), 448–456.

Lu, T., Gu, M., Zhao, Y., Zheng, X., & Xing, C. (2017). Autophagy contributes to falcarindiol-induced cell death in breast cancer cells with enhanced endoplasmic reticulum stress. *PLoS ONE, 12*(4), e0176348.

Mazewski, C., & Gonzalez de Mejia, E. (2018). Impact of anthocyanins on colorectal cancer. *Advances in plant phenolics: From chemistry to human health: Vol. 1286*, (pp. 339–370). American Chemical Society.

Metzger, B. T., & Barnes, D. M. (2009). Polyacetylene diversity and bioactivity in orange market and locally grown colored carrots *(Daucus carota* L.). *Journal of Agricultural and Food Chemistry, 57*(23), 11134–11139.

Metzger, B. T., Barnes, D. M., & Reed, J. D. (2008). Purple carrot (*Daucus carota* L.) polyacetylenes decrease lipopolysaccharide-induced expression of inflammatory proteins in macrophage and endothelial cells. *Journal of Agricultural and Food Chemistry, 56*(10), 3554–3560.

Murthy, K. N. C., Vanitha, A., Rajesha, J., Swamy, M. M., Sowmya, P. R., & Ravishankar, G. A. (2005). In vivo antioxidant activity of carotenoids from *Dunaliella salina* - A green microalga. *Life Sciences, 76*(12), 1381–1390.

Omenn, G. S., Goodman, G. E., Thornquist, M. D., Balmes, J., Cullen, M. R., Glass, A., ... Williams, J. H. (1996). Risk factors for lung cancer and for intervention effects in CARET, the beta-carotene and retinol efficacy trial. *Journal of the National Cancer Institute, 88*(21), 1550–1559.

Pearson-Stuttard, J., Bandosz, P., Rehm, C. D., Afshin, A., Peñalvo, J. L., Whitsel, L., ... Lloyd-Williams, F. (2017). Comparing effectiveness of mass media campaigns with price reductions targeting fruit and vegetable intake on US cardiovascular disease mortality and race disparities. *The American Journal of Clinical Nutrition,* ajcn143925.

Rodriguez-Casado, A. (2016). The health potential of fruits and vegetables phytochemicals: Notable examples. *Critical Reviews in Food Science and Nutrition, 56*(7), 1097–1107.

Sanchez-Alcazar, J., Ault, J., Khodjakov, A., & Schneider, E. (2000). Increased mitochondrial cytochrome c levels and mitochondrial hyperpolarization precede camptothecin-induced apoptosis in Jurkat cells. *Cell Death and Differentiation, 7*(11), 1090.

Schmiech, L., Alayrac, C., Witulski, B., & Hofmann, T. (2009). Structure determination of bisacetylenic oxylipins in carrots (*Daucus carota* L.) and enantioselective synthesis of falcarindiol. *Journal of Agricultural and Food Chemistry, 57*(22), 11030–11040.

Smith-Warner, S. A., Spiegelman, D., Yaun, S.-S., Adami, H.-O., Beeson, W. L., van den Brandt, P. A., ... Hunter, D. J. (2001). Intake of fruits and vegetables and risk of breast cancer: A pooled analysis of cohort studies. *JAMA, 285*(6), 769–776. https://doi.org/10.1001/jama.285.6.769.

Søltoft, M., Eriksen, M. R., Träger, A. W. B., Nielsen, J., Laursen, K. H., Husted, S., ... Knuthsen, P. (2010). Comparison of polyacetylene content in organically and conventionally grown carrots using a fast ultrasonic liquid extraction method. *Journal of Agricultural and Food Chemistry, 58*(13), 7673–7679.

Stintzing, F. C., & Carle, R. (2004). Functional properties of anthocyanins and betalains in plants, food, and in human nutrition. *Trends in Food Science & Technology, 15*(1), 19–38.

Surh, Y.-J. (2003). Cancer chemoprevention with dietary phytochemicals. *Nature Reviews Cancer, 3*(10), 768.

U.S. Breast Cancer Statistics, 2019. https://www.breastcancer.org/symptoms/understand_bc/statistics.

Wang, P. Y., Fang, J. C., Gao, Z. H., Zhang, C., & Xie, S. Y. (2016). Higher intake of fruits, vegetables or their fiber reduces the risk of type 2 diabetes: A meta-analysis. *Journal of Diabetes Investigation, 7*(1), 56–69.

Yousuf, B., Gul, K., Wani, A. A., & Singh, P. (2016). Health benefits of anthocyanins and their encapsulation for potential use in food systems: A review. *Critical Reviews in Food Science and Nutrition, 56*(13), 2223–2230.

Zeng, C.-W., Zhang, X.-J., Lin, K.-Y., Ye, H., Feng, S.-Y., Zhang, H., & Chen, Y.-Q. (2012). Camptothecin induces apoptosis in cancer cells via microRNA-125b-mediated mitochondrial pathways. *Molecular Pharmacology, 81*(4), 578–586.

Zhang, P., & Omaye, S. T. (2001). Antioxidant and prooxidant roles for [beta]-carotene, [alpha]-tocopherol and ascorbic acid in human lung cells. *Toxicology in Vitro, 15*(1), 13–24.