Proanthocyanidins from the American Cranberry (Vaccinium macrocarpon) Induce Cell Cycle Alterations in DU145 Human Prostate Cancer Cells in Vitro by Affecting the Expression of Cell Cycle-Associated Proteins

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ABSTRACT:

Background: Prostate cancer is one of the most common cancers in the world. There are genetic and environmental factors that can potentially impact the development and progression of many types of cancer, including prostate cancer. As a consequence of environmental factors, such as diet having a potential effect on the development of prostate cancer, considerable interest in the possible health benefits associated with the inclusion and consumption of certain foods in the diet exists.

Context and purpose of this study: This study describes the effects of a proanthocyanidin-enriched fraction (PACs) isolated from the American cranberry (*Vaccinium macrocarpon*) on the behaviour of androgen-refractory (insensitive) DU145 human prostate cancer cells *in vitro*.

Results: Following treatment of DU145 human prostate cancer cells with 25 μg/mL of PACs for six hours, PACs significantly decreased the cellular viability of DU145 cells. PACs treatment (25 μg/mL for 6 hours) of DU145 cells increased the proportion of cells in the G2-M phase of the cell cycle and decreased the proportion of cells in the G1 phase of the cell cycle. These alterations were associated with changes in cell cycle regulatory proteins and other cell cycle associated proteins. PACs increased the expression of cyclin E, cyclin D1, CDK2 and CDK4, and decreased the expression of cyclin A and cyclin B1. The protein expression level of p27 increased, and the protein expression levels of $p16^{INK4a}$, p21, and pRBp107 decreased in response to PACs treatment. The protein expression level of pRBp130 was unchanged in

response to PACs treatment.

Conclusions: These findings demonstrate that proanthocyanidins from the American cranberry can affect the behaviour of human prostate cancer cells *in vitro* and further support the potential health benefits associated with cranberries.

Keywords: Prostate cancer, proanthocyanidin-enriched fraction (PACs), American cranberry (*Vaccinium macrocarpon*)

INTRODUCTION:

Prostate cancer is one of the major health concerns in the Western world [1]. Therefore, research studies focused on determining and describing the etiology and development of prostate cancer, and for the treatment of prostate cancer, including possible chemo-preventative and/or chemo-protective strategies, is warranted. The risk of developing many types of cancer, including prostate cancer, is associated with genetics. Environmental factors may also determine whether cancer will develop [2]. In this regard, the rates of prostate cancer are much lower in East Asian countries as compared to North America [3]. Interestingly, post-migration to North America and the risk for East Asian men developing prostate cancer increases for both themselves and their descendants suggesting an environmental role for the development of prostate cancer [4]. The nature of the diet has also been implicated in affecting the development and/or progression of prostate cancer [5,6].

Considerable interest in examining the effects of whole extracts and their constituent phytochemicals from a wide variety of different foods upon the behaviour of cancer cells both *in vitro* and *in vivo* have arisen. In this regard, many phytochemicals have been shown to have substantial effects on the behaviour of prostate cancer cells [7-14].

Our research focuses on phytochemicals isolated from the American cranberry (*Vaccinium macrocarpon*) fruit. Previously, we have demonstrated that constituents from cranberries can induce and regulate apoptosis and also affect the activity of many key proteins, such as the matrix metalloproteinases, associated with the metastatic potential of cancer in androgen-insensitive (DU145) human prostate cancer cells [11-13]. Due to the cranberry phytochemical extracts' ability to affect apoptosis, it was previously hypothesized that extracts from the American cranberry could cause cell cycle arrest in DU145 human prostate cancer cells. Cell cycle deregulation is a common occurrence in cancer, leading to uncontrolled and unmitigated cell growth and division, and compounds which effect the expression of cell cycle proteins may be a method of controlling or preventing the growth of cancer [15]. The proteins assayed in this study represent an overview of the cell cycle, with cyclins and cyclin dependent kinases working in conjunction to push the cell cycle forward and the cell cycle inhibitory proteins retarding this process [16]. Whole extracts from the cranberries have previously been demonstrated to affect human prostate cancer cell growth via cell cycle arrest by modulating expression of these cell cycle regulatory proteins [16]. In this current study, the effects of a proanthocyanidin–enriched fraction (PACs) from American cranberry (*V. macrocarpon*) on cellular viability, progression

through the cell cycle, and the expression of key cell cycle associated proteins are examined in DU145 human prostate cancer cells *in vitro.*

MATERIALS AND METHODS:

Experimental Materials: All antibodies used in this study were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). They include: anti-actin, anti-CDK2, anti-CDK4, anti-cyclin A, anti-cyclin B1, anti-cyclin D1, anti-cyclin E, anti-p21, anti-p27, anti-p16^{INK4a}, anti-pRBp107, and anti-pRBp130. Reagent-grade acetone, methanol, and ethyl acetate were purchased from Pharmco Products Inc. (Brookfield, CT). Solvents for HPLC analysis were from Fisher Scientific. Diaion® HP-20 was from Supelco, Inc. (Bellefonte, PA), and Sephadex™ LH-20 was from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Procyanidin A2 was from Indofine Chemical Company (Hillsborough, NJ). All other chemicals and materials were purchased as indicated.

Preparation of Cranberry Proanthocyanidin-Enriched Fraction (PACs): Cranberry fruit (Vaccinium macrocarpon) was harvested in November 2009 at the State Bog in Wareham, Massachusetts. The fruit was flash-frozen in liquid nitrogen and stored at -20°C until use. For preparation of whole cranberry polyphenolic extract, 1.04 kg of fruit was macerated in 300 mL solvent in a Waring Blender for five minutes, and then allowed to stand for thirty minutes. The slurry was filtered, and the solids were blended with several additional 100 mL portions of solvent until the solids were nearly colorless. An additional extraction with 200 mL of ethyl acetate was performed; the filtrates were concentrated by rotary evaporation and freeze-dried, then re-dissolved in a minimum volume of distilled water and chromatographed on Diaion HP-20 resin (5.5 x 30 cm) to remove free sugars. Extracts were allowed to adsorb the resin for fifteen minutes. The column was washed with 2 L distilled water to remove sugars and organic acids, and then eluted with 100% methanol. The eluate concentrated in vacuo and freeze-dried to yield 7.11 g de-sugared crude whole cranberry extract. Proanthocyanidins were isolated using a published procedure [17]. Briefly, 0.508 g crude extract was dissolved in a minimum volume of distilled water and applied to a Sephadex LH-20 column packed in 70:30 methanol/water (3 x 22 cm). Elution, first with 600 mL of 70:30 methanol/water, removed phenolic acids, anthocyanins, and flavonol glycosides as colored bands. Then the elution with 100 mL 70:30 acetone/water yielded a fraction that was dried in vacuo to give 132 mg of tan solid. HPLC analysis on a Waters XTerra C8 column, using a published procedure [17], was used to verify the presence of procyanidin A2, other epicatechin oligomers with characteristic absorbance at 279.1 nm, the absence of characteristic anthocyanin (520 nm), and flavonol (355 nm) peaks. The presence of (epi)catechin oligomers of up to 12 degrees of polymerization was verified by MALDI-TOF MS analysis at the University of Massachusetts Amherst Spectroscopic Facility, as described previously [17].

Cell Culture and Treatment with Proanthocyanidin-Enriched Fraction (PACs): Human DU145 prostate adenocarcinoma cells (ATCC, Manassas, VA) were cultured on 100 mm plastic tissue culture dishes (Falcon, Mississauga, ON) in alpha minimal essential media (MEM)

(Gibco, Burlington, ON) and also supplemented with 10% fetal bovine serum (FBS) (Invitrogen Canada, Burlington, ON.) and 1% antibiotic-antimycotic (Invitrogen Canada, Burlington, ON). Cells (4 x 10^5) were cultured on the tissue culture dishes and were initially incubated for twenty-four hours at 37°C in the presence of 5% CO_2. After this 24-hour incubation period, the media was removed and replaced with serum free alpha MEM supplemented with 5 µg/mL of transferrin (Sigma) and 2.5µg/mL of insulin (Sigma). The use of the serum-free medium (defined medium) was to ensure that the effects noted were due to the effects of the PACs and not due to any possible interactions that may have occurred between the PACs and the components of the serum. These plates were again incubated for twenty-four hours at 37°C in the presence of 5% CO_2. After this incubation period, cells were treated with PACs to a final concentration of 25µg/ml for six hours. PACs were dissolved in dimethyl sulfoxide (DMSO) as the vehicle prior to use. The control cells received only the vehicle (DMSO) at a concentration of 0.1%; the concentration of DMSO used in the control cells is the same as the concentration of DMSO introduced to treated cells (as the vehicle for PACs) and DMSO alone has previously been determined to have no effect on these cells at this concentration. After treatment, the media was removed from the cells and stored at -80°C for further analysis. The cells were then washed with PBS and were removed by trypsin (Sigma) diluted in phosphate buffered saline (PBS). The cells were re-suspended with alpha MEM supplemented with 10% FBS and were centrifuged for fourminutes at 500 x g. After centrifugation, the cell pellet was re-suspended with PBS, then was transferred to a micro centrifuge tube, and was centrifuged at 500x g for four minutes. After centrifugation, this cell pellet was then stored at -80°C until subsequent immunoblot analysis as described below.

Cellular Growth Curve: DU145 cells (25,000 cells/plate) were cultured on tissue culture plates in alpha-MEM supplemented with 10% FBS and 1% antibiotic/antimycotic solution. Following twenty-four hours of cell culture, cells were counted using a haemocytometer, and the cell number was determined. This cell count represented the zero-time point. The cells were then cultured in the presence of alpha MEM containing 1% FBS and 1% antibiotic/antimycotic solution supplemented with either DMSO (vehicle) or 25 µg/mL PACs. Cell numbers were evaluated following a further twenty-four, forty-eight, and seventy-two hours of cell culture respectively. Triplicate plates were evaluated for each treatment at each time point.

Alamar Blue Cytotoxicity Assay: The Alamar Blue assay (Invitrogen, Burlington, ON) was used to determine cellular viability after treatment with PACs as per manufacturer's instructions. Briefly, DU145 cells were sub-cultured into a ninety-six well plate at 5,000 cells/well. After twenty-four hours of incubation, the media was replaced with 100µl of serum-free media and was once again incubated for twenty-four hours. After this incubation, the cells were treated with PACs for six hours with a final concentration of DMSO within each well of 0.1%. The control for this experiment was DU145 cells treated with DMSO alone at a total concentration of 0.1% for six hours. Following treatment, 10µl of Alamar Blue was added to each well. The cells were incubated for three hours post exposure to Alamar Blue. After this three-hour incubation period,

the resulting fluorescence was read with an excitation wavelength of 528 nm and an emission wavelength of 590 nm.

Immunoblot Analyses: Protein expression was determined in the whole cytosolic protein fraction. Briefly, cell pellets were reconstituted in 100 µl of 10 mM Tris-HCl buffer (pH 7.4) containing 0.5 mM PMSF (Sigma). Once re-constituted, the cells were then sonicated. The cell lysates were then centrifuged for ten minutes at 9,300 g at 4°C. Following this centrifugation, the supernatant was removed from the pellet, and this "cytosolic lysate" fraction was subsequently evaluated. Equal amounts of protein extracts from lysates were mixed in a 3:1 ratio with standard Laemmli buffer consisting of 50 mM Tris-HCl (pH 6.8), 10% SDS, 0.1% bromophenol blue, 10% glycerol, and 100mM beta-mercaptoethanol. Once mixed, these samples were boiled for three minutes. Once boiled, they were resolved by electrophoresis through 10% SDS-PAGE gels and transferred onto nitrocellulose membranes (Biorad). After transfer, the membranes were incubated in the presence of a 1% BSA (w/v) TBS-Tween (0.05% v/v) solution for one hour at room temperature. The membranes were then incubated with primary antibodies diluted to a 1:200 ratio (v/v). The membranes were incubated in the presence of the primary antibody for twenty-four hours at 4˚C. After incubation, the membranes were washed three times with TBS-Tween (0.05%) for ten minutes and were incubated in alkaline phosphatase-conjugated secondary antibodies (1:2000 dilution) for one hour. After incubation, the membranes were again washed three times with TBS-Tween (0.05%) for ten minutes, washed briefly with distilled water, and then exposed to SigmaFast BCIP-NBT tablets (Sigma) in solution to visualize protein expression levels. Western blots were then analyzed using Infinity Capture software (Lumenera Corp., Ottawa, ON), and densitometry was performed with ImageJ software (National Institute of Health, Bethesda, MD).

Flow cytometry for cell cycle analysis: DU145 cells treated with either 0.1% DMSO (control) or treated with 25 µg/mL for six hours were harvested with trypsin and then washed with alpha MEM containing 10% FBS. The final concentration of DMSO in the PACs treatments was also 0.1%. The cells were then centrifuged for five minutes at 200x g at room temperature. Once centrifuged, the media was removed, and the cells were re-suspended in 25 µl of PBS, which were then placed in 225 µl of 70:30 ethanol:PBS mixture and allowed to fix for two hours on ice. The fixed cells were then centrifuged for five minutes at 200x g at 4˚C and were then re-suspended in 250 µl of ice-cold PBS for five minutes. The cells were then re-centrifuged for five minutes at 200 x g at 4˚C, and the cell pellet was then suspended in a PBS propidium iodide (the propidium iodide was used at a final concentration of 0.02 mg/mL (w/v)) (Sigma) staining solution with Triton-X (Sigma) (which was added at a concentration of 0.09% (v/v)) and 0.4 mg/ml of RNAse (Sigma) and were incubated for thirty minutes at room temperature in the dark. Once incubated, the cells were transferred into a ninety-six round bottom well plate and were analysed using a BD FACSARRAY bio-analyzer equipped with BD FACSARRAY system software version 1.0.3 (BD Biosciences, Mississauga, ON). The results of this analysis were processed using WinMDI software version 2.9 (Scripps Research Institute, La Jolla, CA).

Statistical Analysis: Statistical analysis was done using GraphPad Prism for Windows (GraphPad Software Inc. San Diego, CA) by using an unpaired t-test. Results are expressed with either a statistical significance of $p < 0.05$ (**) or a statistical significance of $p < 0.10$(*).

RESULTS

Characterization of the proanthocyanidin-enriched fraction (PACs): The composition of extractable PACs from whole cranberry fruit had been described previously (12-14). MALDI-TOF MS analysis of the PACs fraction was performed and is presented in Figure 1. As shown in Figure 1, the MALDI-TOF MS analysis of the PACs fraction confirmed the presence of A-linked PACs of between 2-12 degrees of polymerization, as previously reported for cranberry PACs preparations (14-15). Briefly, the masses detected the major constituents of the fraction to be epicatechin dimer (M + Cs = 709 amu confirmed as procyanidin A2 by HPLC comparison to commercial standard), trimer (M + Cs = 997 amu), tetramer (M + Cs = 1285) pentamer (M + Cs = 1573 amu), and hexamer (M + Cs = 1861 amu), each containing one A-type linkage as well as smaller amounts of the larger oligomers (data not shown). The bio-availability of these compounds to the prostate has not been established.
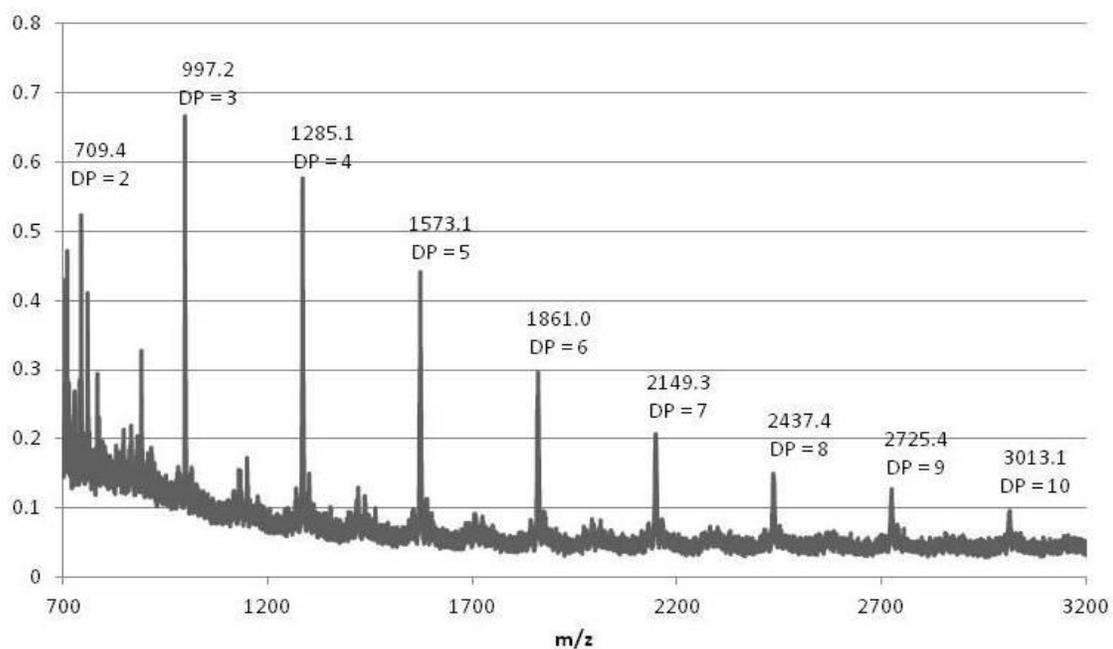

Figure 1: MALDI-TOF mass spectrum of the cranberry proanthocyanidin (PACs) fraction.Major peaks represent [M+Cs] of (epi)catechin oligomers with degree of polymerization (DP) as shown.

PACs are cytotoxic to DU145 human prostate cancer cells *in vitro*: The effects of PACs on DU145 human prostate cancer cells were measured by Alamar Blue assay. As shown in Figure 2, PACs significantly decreased the viability of these cells at a dose of 25 µg/mL. Viability decreased by approximately 30% in response to 25 µg/mL of PACs treatment of DU145 cells for six hours. This concentration of 25 µg/mL PACs was chosen for further study, as it was determined previously to be the effective concentration which was able to exert a substantial effect on the behaviour of DU145 cells *in vitro* [12,13,18]. Furthermore, in time course

experiments, the growth of DU145 cells, in response to PACs, was found to be inhibited (Figure 3).
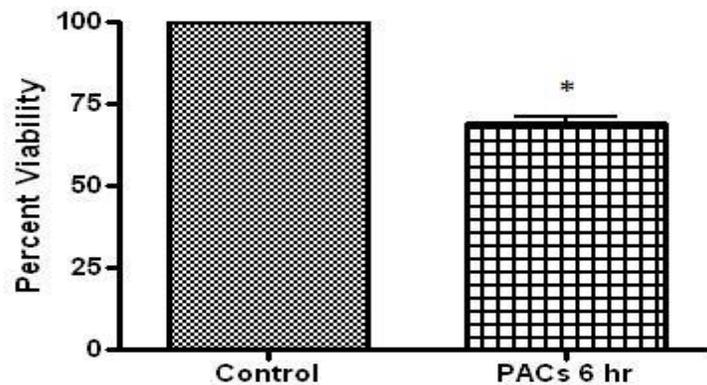

Figure 2: PACs treatment of DU145 cells affects cellular viability. DU145 cell viability was evaluated by Alamar Blue assay following treatment with 25 µg/mL of PACs for six hours. Control cells received 0.1% DMSO (vehicle) for six hours. Fluorescence was read at 528 nm (excitation) and 590 nm (emission) wavelengths. Control cell viability was set to 100% viability. Treatment with PACs (25 µg/mL) for six hours significantly decreased the viability of DU145 cells relative to control cells, which denotes a P-value of <0.10 (*).
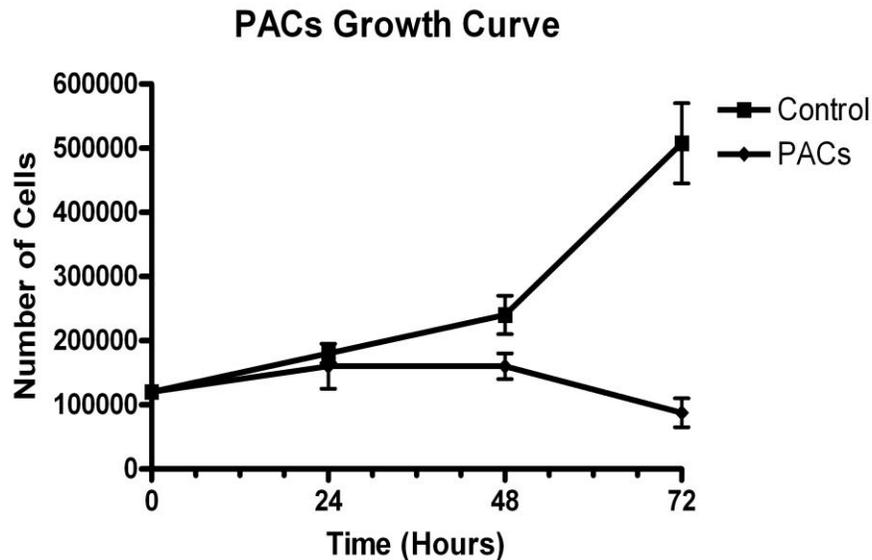
Figure 3: PACs treatment of DU145 cells affects their growth. DU145 cells were cultured in the presence of either 0.1% DMSO (vehicle) (control) or 25 µg/mL PACs for twenty-four, forty eight, and seventy-two hours respectively. The number of cells present under these treatment conditions was determined. Triplicate plates were evaluated for each treatment at each time point.

PACs increases the number of DU145 cells in the G2-M phase and decreases the number of DU145 cells in the G1 phase of the cell cycle: To further investigate the effects of PACs on DU145 cells, flow cytometric analysis was conducted to ascertain if PACs treatment of DU145 cells affected cell cycle progression. As shown in Figures 4A and 4B, after treatment with 25 µg/mL for six hours, PACs increased the number of cells in the G2-M phase of the cell cycle and decreased the number of cells in the G1 phase of the cell cycle. The first image in Figure 4A represents one experiment with vehicle treated cells (0.1% DMSO), and the second image represents one experiment with DU145 cells treated with 25 µg/mL PACs. The summary of flow cytometric replicates is illustrated in Figure 4B. PACs treatment of DU145 cells with 25 µg/mL PACs for six hours results in a block in the G2-M phase of the cell cycle.
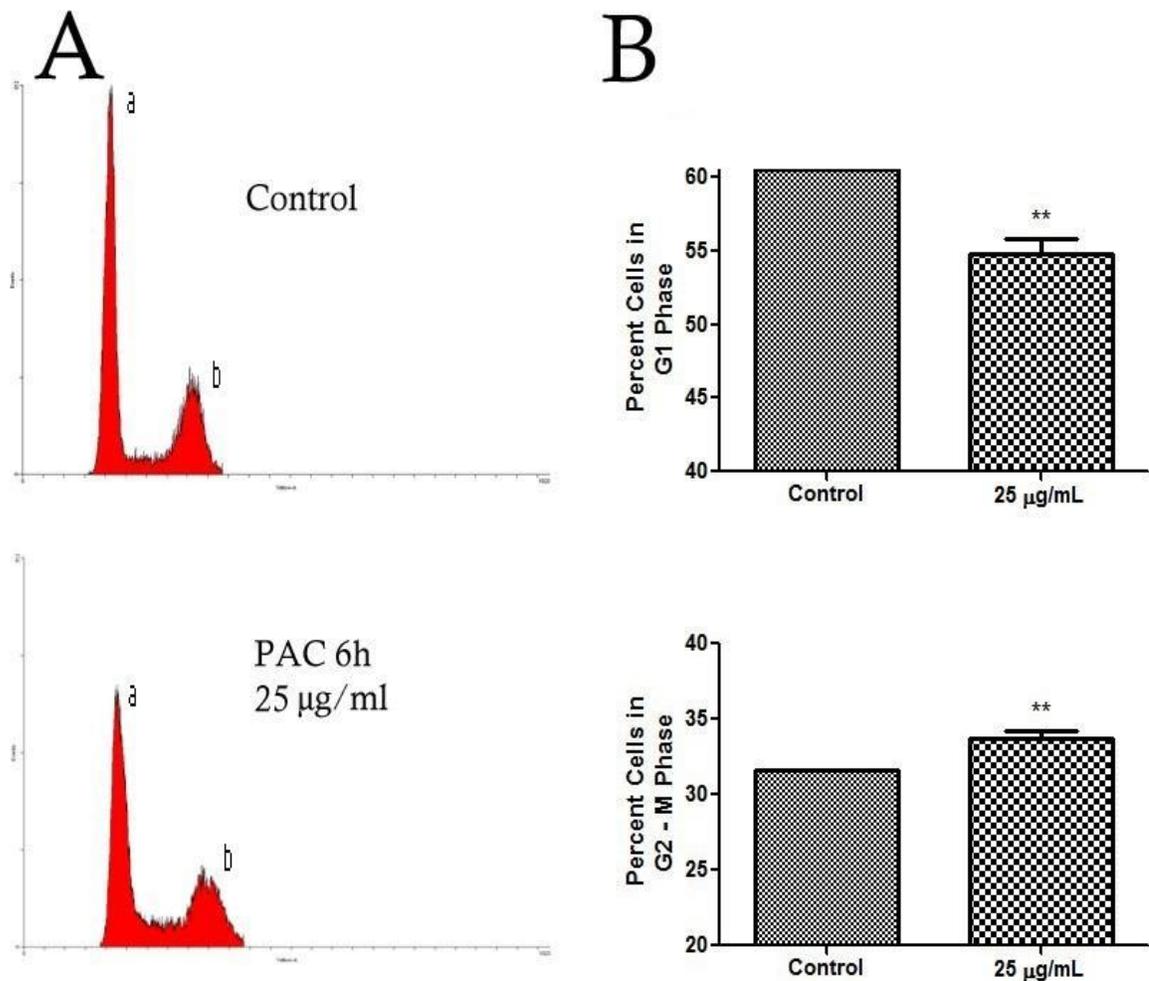

Figure 4: Flow cytometric analysis of DU145 cells treated with vehicle (0.1% DMSO) and 25 µg/mL of PACs for six hours. PACs significantly increases the number of cells in the G2-M phase (b) of the cell cycle and decreases the number of cells in the G1 phase (a) of the cell cycle. Data shown in Figure 4A is from a representative experiment. Figure 4B is histograms which indicate the results of cell cycle analysis of DU145 cells in the presence of PACs (at 25 µg/mL) and shows that PACs significantly effects the distribution of cells which denotes a P value of <0.05 (**).

Effects of PACs treatment on expression of cell cycle related proteins in DU145 prostate cancer cells: As shown in Figure 5, the expression of cyclin A and cyclin B1 protein levels in DU145 prostate cancer cells decreased response to treatment with 25 µg/mL PACs for six hours. The protein expression levels of cyclin E, cyclin D1, CDK2, and CDK4 increased following treatment of DU145 cells with 25 µg/mL PACs for six hours (Figure 5). To ascertain why the expression levels of these proteins were affected by the PACs treatment, the protein expression levels of some key cell cycle inhibitors were also examined.

As shown in Figure 6, after treatment of DU145 cells with 25µg/mL of PACs for six hours, the protein expression level of p27 increased whereas the protein expression levels of p16^{INK4a}, p21, and pRBp107 decreased. However, the protein expression level of pRBp130 was apparently unaffected by 25 µg/mL PACs as it remained unchanged.
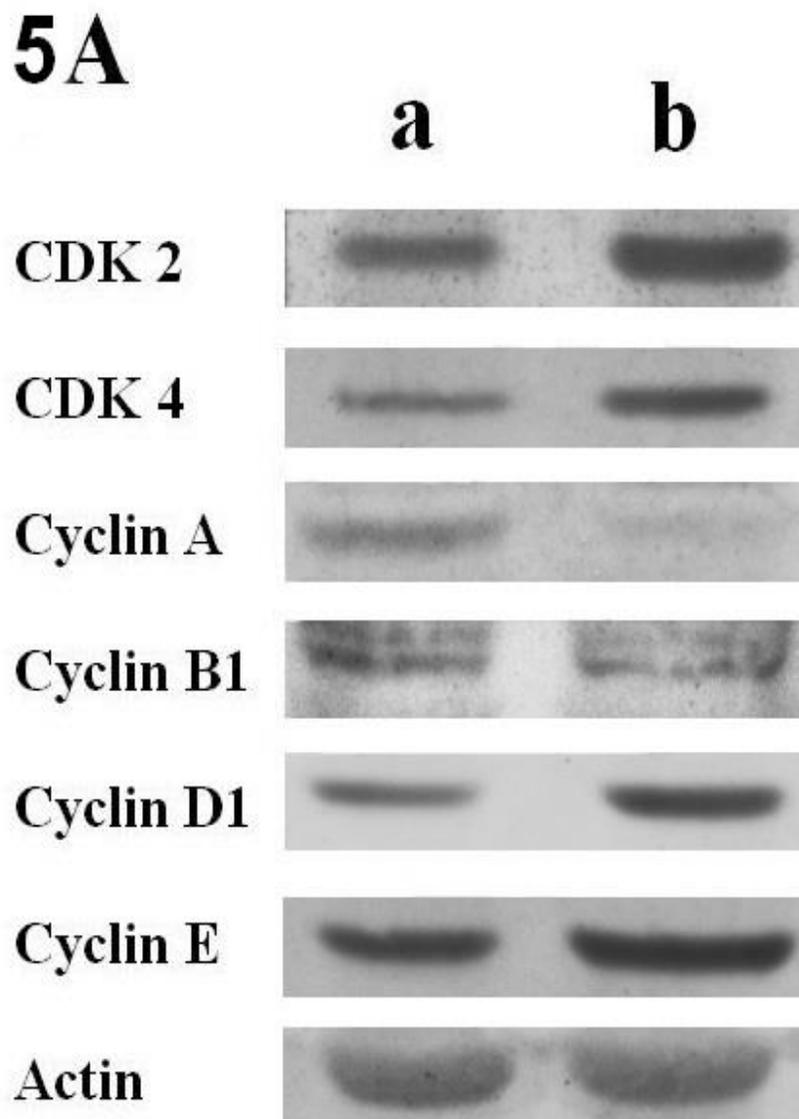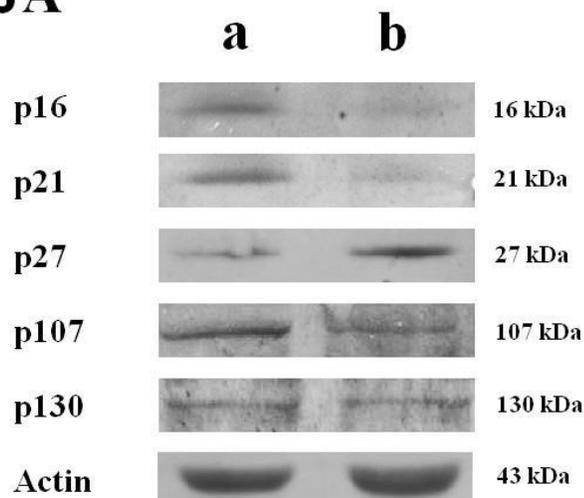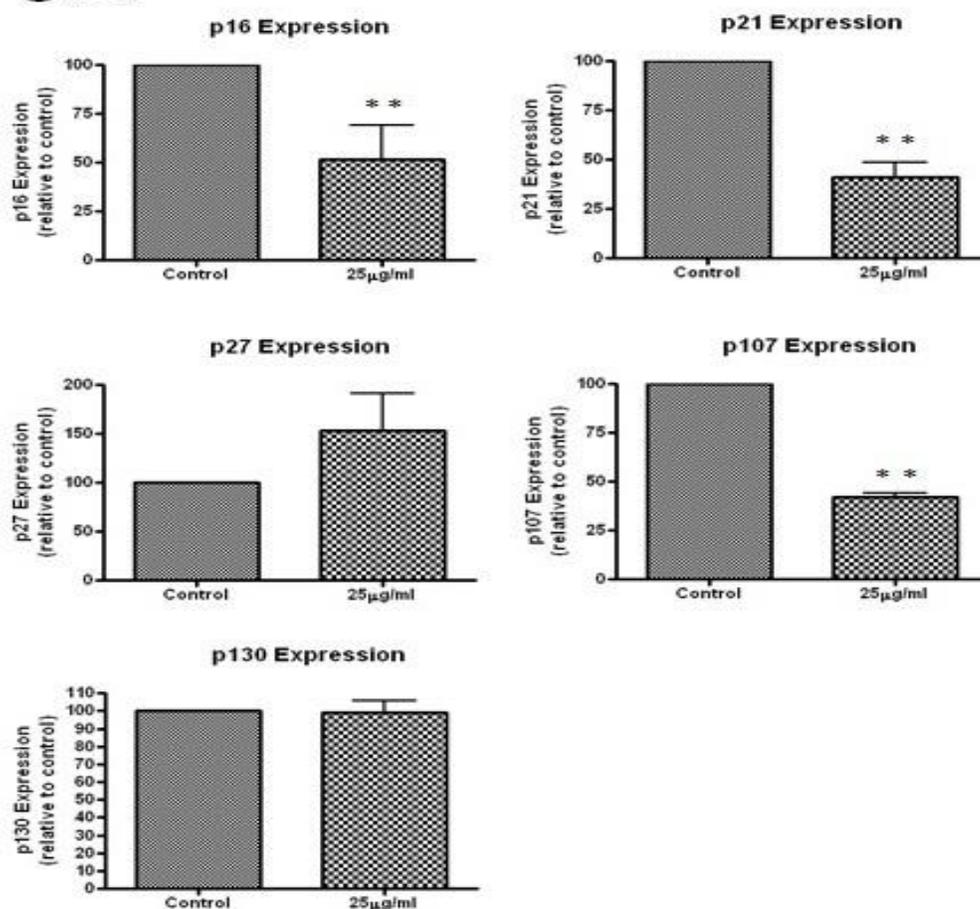
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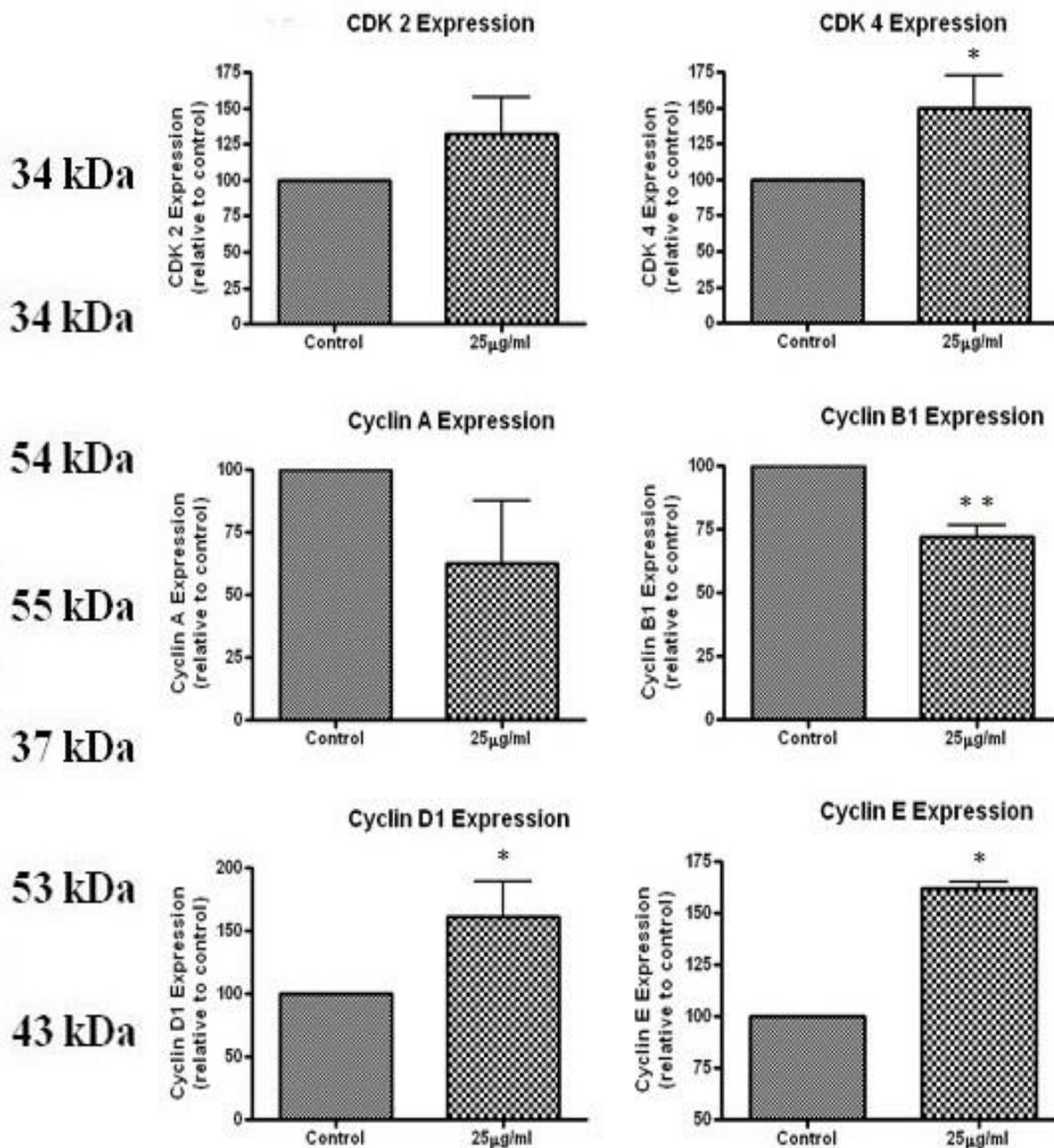
Figure 5A and 5B: PACs effects the expression of cell cycle proteins in DU145 cells. Cells were treated with either vehicle (0.1% DMSO) or with 25 µg/mL PACs for six hours. Figure 5A: CDK2, CDK4, cyclin A, cyclin B1, cyclin D1, and cyclin E protein expression levels are shown. Actin was used as a loading control. Each blot shown is representative of results obtained from three separate experiments with assay triplicates. Figure 5B: Histograms illustrating the protein expression levels in response to PACs treatment.(*) Denotes a P value of < 0.10, and (**) denotes a P-value of <0.05.

Figure 6A and 6B: PACs effects the protein expression levels of cell cycle protein inhibitors. DU145 cells were treated with either vehicle (0.1% DMSO) or with 25 µg/mL PACs for six hours. Figure 6A: p16^{INK4a}, p21, p27, p107, and p130 protein expression levels are shown. Actin

was used as a loading control. Each blot shown is representative of results obtained from three separate experiments with assay triplicates. Figure 6B: Histograms illustrating the protein expression levels in response to PACs treatment. (**) Denotes a P value of <0.05.

DISCUSSION:

In this investigation, the effects of PACs from the American cranberry (*Vaccinium macrocarpon*) on the behaviour of DU145 human prostate cancer cells *in vitro* were determined. PACs were able to induce cytotoxicity at 25 µg/mL after six hours to modulate the expression of several cell cycle associated proteins and to slow the progression of DU145 cells through the cell cycle. The PACs concentration used was previously demonstrated to be able to affect a number of cellular activities in the DU145 cells, including the expression of matrix metalloproteinase and the induction of apoptosis *in vitro* [12,18]. Cranberry PACs are also able to target both ovarian cancer cell viability and angiogenesis *in vitro* [19]. This may suggest that such PACs concentrations may be physiologically relevant. However, whether these concentrations are attainable in the prostate *in vivo* remains to be determined. A further confounding factor is that in an *in vivo* situation, it is most probably the metabolites of the PACs that are impacting upon the behaviour of the prostate. What these metabolites are and how they act on the prostate remains undetermined.

Previously, we have shown that a whole cranberry extract (WCE) is able to affect human prostate cancer cell growth via cell cycle arrest by modulating expression of key cell cycle proteins and their associated cellular regulators [16]. WCE treatment of DU145 cells decreased the proportion of cells in the G2-M phase of the cell cycle and increased the proportion of cells in the G1 phase of the cell cycle following treatment with WCE for six hours [16]. This G1 phase increase is also seen in SEG-1 human esophageal adenocarcinoma cells treated with cranberry proanthocyanidins for twenty-four and forty-eight hours [20] as well as human epidermoid carcinoma A431 cells treated with dietary grape seed proanthocyanidins (containing exclusively B-linked oligomer) [21] and a number of head and neck squamous cell carcinomas treated with grape seed proanthocyanidins [22]. Oligomeric proanthocyanidin complexes (OPC) have been shown to exert anti-proliferative and pro-apoptotic effects on human prostate cancer cells [23]. In this regard, treatment of androgen responsive LNCaP prostate cancer cells with OPC resulted in a decrease in the number of LNCaP cells in the S-phase of the cell cycle and an increase in the number of cells in the sub G1 phase [23].

However, the PACs effect was the opposite with PACs increasing the proportion of cells in the G2-M phase of the cell cycle and decreasing the proportion of cells in the G1 phase of the cell cycle. In keeping with this observation, grape proanthocyanidins have been shown to inhibit pancreatic cancer cell growth *in vitro* and this reduced cellular viability was associated with increased G2/M phase arrest of the cell cycle, which led to the induction of apoptosis [24]. Likewise, purified cranberry proanthocyanidins (PAC-1A) caused pro-apoptotic effects and cytotoxicity in high-risk neuroblastoma cells, and this was in association with PAC-1A's ability to partially block the cell cycle in G2/M phase with a concomitant decrease in the G_0-G1 population [25]. PAC-1 treatment of SKOV-3 ovarian cancer cells also blocked cell cycle progression through the G2/M phase of the cell cycle [19]. The PACs mediated G2-M phase

block seen in DU145 cells is consistent with these findings. It would appear that the PACs mediated cell cycle alterations/responses may be cell–type dependent and perhaps time dependent as well.

Previously, we have reported that several alterations in expression levels of cell cycle activities occurred in response to WCE treatment of DU145 cells [16]. WCE decreased the expression of CDK4, cyclin A, cyclin B1, cyclin D1 and cyclin E [16]. Unlike what occurred in response to WCE, only cyclin A and cyclin B1 protein expression levels decreased in response to PACs treatment of DU145 cells, whereas cyclin D1 and cyclin E protein expression levels increased. An overview of the cell cycle is provided by Schwartz and Shah [26] wherein the functions of the various cyclins and cyclin dependent kinases are explained: cyclins D1 and E in conjunction with CDK2 and 4 drive the cell cycle from G1 to S phase, here cyclin A works with CDK2 to progress to G2 where cyclin B1 causes the cell cycle to enter the M phase. In another study, PAC-1 treatment of neuroblastoma cells resulted in an increase in cyclin D1 expression [25]. On the other hand, grape seed proanthocyanidin treatment of epidermoid carcinoma cells was associated with inhibition of cyclins D1, D2 and cyclin E [21]. In response to treatment of DU145 cells with WCE, p27 protein expression levels increased while CDK2 and p21 protein expression levels were apparently unaffected [16]. This is not what occurred in response to PACs treatment where protein expression of p27, CDK2, and CDK4 in DU145 cells increased and p21 protein expression decreased. These proteins, p21 and p27, act as inhibitors of the cell cycle and specifically target the CDKs [26]. Grape seed proanthocyanidin treatment of head and neck squamous cell carcinoma cells resulted in inhibition of cyclin-dependent kinases (CDK) and increased expression of the CDK inhibitory proteins Cip1/p21 and Kip1/p27 [22]. Grape proanthocyanidin treatment of human non-small cell lung cancer cells is associated with increased expression of Cip1/p21 and Kip1/p27 and a simultaneous decrease in the expression of CDKs and cyclins [27]. The cranberry proanthocyanidin treatment of neuroblastoma cells is associated with up-regulation of cyclin D1 and a down-regulation of CDK6 and p27 [25]. Oligomeric proanthocyanidin complexes (OPC), extracted from either grape seeds or maritime pine bark, were found to inhibit expression of CDKs and cyclins and to stimulate expression of p21 and p27 in LNCaP and PC3 human prostate cancer cells [23].

Previously, we have demonstrated that WCE treatment of DU145 cells resulted in decreased p16^{INK4a} and increased pRBp107 protein expression levels with no apparent change in pRBp130 [16]. The INK4 family of proteins and pRB family of proteins all represent cell cycle inhibitors with the potential to inhibit cell cycle progression. The INK4 family targets cyclin dependent kinases necessary for progression through G1 phase of the cell cycle [28]. The phytochemical apigenin is able to affect the behaviour of DU145 cells by inducing cell cycle arrest and by inducing apoptosis, this apigenin-mediated effect has been suggested to involve an increase in INK^{4a}/p16 in addition to alterations in other cell cycle-related activities [29]. When DU145 cells were treated with cranberry PACs, p16^{INK4a} and pRBp107 protein expression levels decreased and no apparent change occurred in pRBp130 expression. The pRB protein is a phosphorylated protein product required for cell cycle progression and regulation of its expression can be altered in cancer cells [30]. Hypophosphorylation of RBp107 and RB2p130 occurs in response to silibinin in DU145 cells via alterations in cell cycle regulators [31]. The alterations that occurred

in response to PACs treatment may be of biological significance since a molecular link between cellular senescence/aging, and the suppression of tumour growth has been suggested to involve the p16^{INK4a}-RB signalling pathway [32]. In summary, cranberry PACs are cytotoxic to DU145 human prostate cancer cells *in vitro,* and PACs are able to elicit a G2-M phase cell cycle effect associated with this cytotoxicity. This PACs associated cell cycle alteration occurs concomitant with alterations in expression levels of a number of cell cycle associated proteins. The precise reasons why WCE and the PACs have different effects on the cell cycle and its associated activities remains to be elucidated. However, it may be that the non-flavonoid constituents of WCE are responsible for the type of cell cycle alterations seen in response to WCE. Such constituents would be absent in the purified flavonoids /PACs and as such, the effects on cell cycle and its associated activities would be different.

The WCE-mediated cyto-toxicity in DU145 cells was previously shown to involve apoptosis [13]. PACs treatment of DU145 cells also result in an induction of apoptosis [18]. This PACs induced apoptosis is in keeping with other studies that have shown a cell cycle arrest and concomitant induction of apoptosis. In this regard, cranberry PACs have also been shown to induce a G2-M phase arrest in SKOV-3 ovarian cancer cells and to induce apoptosis through activation of both the intrinsic and extrinsic pathways of apoptosis [19]. Purified cranberry proanthocyanidins can also cause pro-apoptotic signalling in high-risk neuroblastoma cells [25]. Oligomeric proanthocyanidins complexes (from grape seeds or maritime pine bark) have been suggested to exert pro-apoptotic effects in androgen sensitive LNCaP prostate cancer cells and in androgen-refractory PC3 prostate cancer cells [23]. Proanthocyanidins have also been suggested to, in addition to modulating cellcycle, induce apoptosis in several cell lines including head and neck squamous cell carcinomas, pancreatic cancer cells, and colon cancer cells [22, 24, 33].

The nature of the event that leads to the cell cycle alterations by PACs may involve an apoptosis-related event, which may impact upon a specific transcription factors that, in turn, would alter the expression of key cellular regulatory activities. In this regard, proanthocyanidins isolated from cranberry have been shown to inhibit the expression of matrix metalloproteinases in DU145 cells, and this inhibition has been shown to involve, at least in part, a decreased translocation of NFkB p65 protein to the nucleus [12]. The exact role of the transcription factors involved, if there is one, in the PACs-mediated alterations in cell cycle of DU145 prostate cancer cells remains to be elucidated.

CONCLUSIONS:

This study demonstrates the cell cycle inhibitory effects of proanthocyanidins on hormone refractory (insensitive) DU145 human prostate cancer cells. The effects of other specific enriched fractions and metabolites of cranberry fruit on cell cycle events need to be examined in order to determine the exact nature of the phytochemicals present in the whole cranberry extract responsible for the observed alterations in DU145 cell behaviour. Such studies are ongoing. Finally, this study revealed many novel effects of proanthocyanidins on DU145 cells, which we suggested may represent a potential source of novel phytochemical anti-cancer agents. As such, more research is warranted to determine the mechanisms by which cranberry and its constituents exhibit their anti-proliferative effects within human prostate cancer cells.

Abbreviations: BCIP: 5-bromo-4-chloro-3-indolyl-phosphate, CDK: cyclin-dependent kinase, Cip:CDK-interacting protein, DMSO:dimethyl sulfoxide, FBS: fetal bovine serum, HPLC: high-performance liquid chromatography, INK: inhibitor of CDK, Kip: kinase inhibitory protein, MALDI-TOF MS: matrix-assisted laser desorption/ionization time of flight mass spectrometry, MEM: minimal essential media, OPC: oligomeric proanthocyanidin complex, PAC: proanthocyanidin, PBS: phosphate buffered saline, PMSF: phenylmethanesulfonylfluoride, RB: retinoblastoma protein, SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis, WCE: whole cranberry extract.

Competing Interests: The authors have no competing interests to declare.

Authors' Contributions: J. Kim who was an undergraduate student in the Hurta lab, B. McKeown who is a current graduate student in the Hurta lab and H. Jahic who was an undergraduate Honours research student in the Hurta lab all contributed to the research presented in this manuscript. J. Kim is listed as the first author as he was responsible for the majority of the research findings described in this manuscript. K. Patel, a previous graduate student in the Neto lab, was responsible for the preparation of the PACs enriched cranberry extract used in the study. A. Catalli, from the Kulka lab, provided key technical support required for the flow cytometry study. M. Kulka, C. Neto and R. Hurta are the primary senior investigators associated with this work. C. Neto and R. Hurta were responsible for the design and the supervision of the research described, read and edited the manuscript, and was responsible for the acquisition of the grant funding required to do the work and to support the students performing the studies.

REFERENCES:
1. P. D. Baade and D. R. Youlden, et al., International epidemiology of prostate cancer: geographical distribution and secular trends, Mol. Nutr. Food Res., 2009, 53(2), 171-184.
2. C. Alberti, Hereditary/familial versus sporadic prostate cancer: few indisputable genetic differences and many similar clinicopathological features, Eur. Rev. Med. Pharmacol. Sci., 2010, 14(1), 31-41.
3. A. W. Hsing, and A. P. Chokkalingam, Prostate cancer epidemiology, Front. Biosci., 2006, 11, 1388-1413.
4. L. S. Cook and M. Goldoft, et al., Incidence of adenocarcinoma of the prostate in Asian immigrants to the United States and their descendants, J. Urol., 1999, 161(1), 152-155.

5. T. Shirai and M. Asamoto, et al., Diet and prostate cancer. Toxicology., 2002, 181-182, 89-94.

6. G. A. Sonn and W. Aronson, et al., Impact of diet on prostate cancer: a review, Prostate Cancer Prostatic Dis., 2005, 8(4), 304-310.

7. B. Neehar and A. Rajesh, Detrimental effect of cancer preventive phytochemicals silymarin, genistein and epigallocatechin 3-gallate on epigenetic events in human prostate carcinoma DU145 cells, Prostate., 2001, 46(2), 98-107.

8. C. Neto and J. W. Amoroso, et al., Anticancer activities of cranberry phytochemicals: An update, Mol. Nutr. Food Res., 2008, 52(S1), S18-S27.

9. D. A. Benitez and M. A. Hermosa, et al., Regulation of cell survival by resveratrol involves inhibition of NFkappaB-regulated gene expression in prostate cancer cells, Prostate., 2009, 69(10), 1045-1054.

10. T. T-Y. Wang and N. W. Schoene, et al., Differential effects of resveratrol and its naturally occurring methylether analogs on cell cycle and apoptosis in human androgen-responsive LNCaP cancer cells, Mol Nutr Food Res., 2010, 54(3), 335-344.

11. M. A. MacLean and M. D. Matchett, et al., Cranberry (Vaccinium macrocarpon) flavonoids inhibit matrix metalloproteinases (MMPs) in human prostate cancer cells, FASEB J., 2007, 21(6), 791.5.

12. B. A. Déziel and K. Patel, et al., Proanthocyanidins from the American cranberry (Vaccinium macrocarpon) inhibit matrix metalloproteinase-2 and matrix metalloproteinase-9 activity in human prostate cancer cells via alterations in multiple cellular signalling pathways, J. Cell Biochem., 2010, 111(3), 742-754.

13. M. A. MacLean and B. Scott, et al., North American cranberry (Vaccinium macrocarpon) stimulates apoptotic pathways in DU145 human prostate cancer cells in vitro, Nutr Cancer., 2011, 63(1), 109-120.

14. C. Neto and C. G. Krueger, et al., MALDI-TOF MS characterization of proanthocyanidins from cranberry fruit Vaccinium macrocarpon that inhibit tumor cell growth and matrix metalloproteinase expression in vitro, J. Sci. Food Agric., 2006, 86(1), 18-25.

15. D. Hanahan and R. A. Weinberg, Hallmarks of Cancer: The Next Generation, Cell, 2011, 144, 646-674.

16. B. Deziel and J. MacPhee, et al., American cranberry (Vaccinium macrocarpon) extract affects human prostate cancer cell growth via cell cycle arrest by modulating expression of cell cycle regulators, Food & Function, 2012, 3, 556-564.

17. K. D. Patel and F. J. Scarano, et al., Proanthocyanidin-rich extracts from cranberry fruit (Vaccinium macrocarpon Ait.) selectively inhibit the growth of human pathogenic fungi Candida spp. and Cryptococcus neoformans, J. Agric.Food Chem, 2011, 59(24), 12864-12873.

18. B. E. Scott, Compounds of Vaccinium macrocarpon inhibit DU145 prostate cancer cells, M.Sc. Thesis, University of Prince Edward Island, 2010.

19. K. K. Kim and A. P. Singh, et al., Anti-angiogenic activity of cranberry proanthocyanidins and cytotoxic properties in ovarian cancer cells, Int. J. Oncol., 2012, 40(1), 227-235.

20. L. A. Kresty and A. B. Howell et al., Cranberry proanthocyanidins induce apoptosis and inhibit acid-induced proliferation of human esophageal adenocarcinoma cells, 2008, J.Agric. Food Chem., 56, 676-680.

21. S. M. Meeram and S. K. Katiyar, Grape seed proanthocyanidins promote apoptosis in human epidermoid carcinoma A431 cells through alterations inCdki-Cdk-cyclin cascade, and caspase-3 activation via loss of mitochondrial membrane potential, Exp.Dermatol., 2007, 16(5), 405-415.

22. R. Prasad and S. K. Katiyar, Bioactive phytochemical proanthocyanidins inhibit growth of head and neck squamous cell carcinoma cells by targeting multiple signalling molecules, PLoS One, 2012, 7(9): e46404.

23. H. Neuwirt and M. C. Arias, et al., Oligomeric proanthocyanidin complexes (OPC) exert anti-proliferative and pro-apoptotic effects on prostate cancer cells, Prostate, 2008, 68(15), 1647-1654.

24. R. Prasad and M. Vaid et al.,Grape proanthocyanidins inhibit pancreatic cancer cell growth in vitro and in vivo through induction of apoptosis and by targeting the PI3K/Akt pathway, PLoS One, 2012, 7(8):e43064.

25. A. P. Singh and T. S. Lange, et al., Purified cranberry proanthocyanidines(PAC-1A) cause pro-apoptotic signalling, ROS generation, cyclophosphamide retention and cytotoxicity in high-risk neuroblastoma cells, Int. J. Oncol., 2012, 40(1), 99-108.

26. G. K. Schwartz and M. A. Shah, Targeting the Cell Cycle: A New Approach to Cancer Therapy, J. Clin. Oncol., 2005, 23, 9408-9421.

27. T. Singh and S. D. Sharma, et al., Grape proanthocyanidins induce apoptosis by loss of mitochondrial membrane potential of human non –small lung cancer cells in vitro and in vivo, PLoS One, 2011, 6(11), e27444.

28. B. B. Aggarwal and S. Banerjee, et al., Curcumin induces the degradation of cyclin E expression through ubiquitin-dependent pathway and up-regulates cyclin-dependent kinase inhibitors p21 and p27 in multiple human tumor cell lines, Bichem.Pharmacol., 2007, 73(7), 1024-1032.

29. S. Shukla and S. Gupta, Molecular mechanisms for apigenin-induced cell-cycle arrest and apoptosis of hormone refractory human prostate carcinoma DU145 cells, Mol. Carcinog., 2004, 114-126.

30. J. Bartek and J. Bartkova, et al., The retinoblastoma protein pathway and the restriction point, Curr. Opin. Cell Biol., 1996, 8(6), 805-814.

31. M. S. Steiner and Y. Wang, et al., p16/MTS1/INK4A suppresses prostate cancer by both pRb dependent and independent pathways, Oncogene, 2000, 19(10), 1297-1307.

32. N. Otani and K. Yamakoshi, et al., The p16 INK4a-RB pathway: molecular link between cellular senescence and tumor suppression, J. Med. Invest., 2004, 51, 146-153.

33. A. M. Liberty and J. W. Amoroso, et al., Cranberry PACs and triterpenoids: anti-cancer activities in colon tumor cell lines, Acta Horticulturae, 2009, 841, 61-66.