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CURRENT TOPICS IN NUTRACEUTICAL RESEARCH Vol. 11, No. 4, pp. 137-144, 2013
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CYTOTOXIC EFFECT OF PUMPKIN (*CURCURBITA PEPO)* SEED EXTRACTS IN LNCAP PROSTATE CANCER CELLS IS MEDIATED THROUGH APOPTOSIS

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[Received May 12, 2013; Accepted October 23, 2013]

[Communicated by Prof. Hsin-Ling Yang]

ABSTRACT: *The cytotoxic effects and related mechanisms of pumpkin (Cucurbita pepo) seed ethanolic (PS-ET) and aqueous (PS-AQ) extracts were evaluated in LNCaP prostate cancer cells. LNCaP cells were exposed to extracts for 24hr. The viability of LNCaP cells was significantly decreased following 24 hr treatment with both PS-AQ (IC_{50}= 49 μg/ml) and PS-ET (IC_{50}= 55 μg/ml) extracts. The reactive oxygen species levels were also significantly increased (4.1 ± 0.2 and 3 ± 0.3 fold) and mitochondrial membrane potential was reduced with both PS-AQ and PS-ET extracts respectively. Both extracts induced DNA fragmentation and PolyADP-ribose polymerase (PARP) cleavage. Additionally PS-ET produced a 5* (± 0.2) *and 3.5* (± 0.4) fold *increase in caspase 3 and 9 activities respectively, while PS-AQ produced a 7* (± 0.4) and *3* (± 0.9) fold *increase in caspase 3 and 9 activities respectively. Our results confirm that the cytotoxic activity of both PS extracts is mediated through oxidative stress and mitochondrial depolarization. Further, the cleavage of PARP, caspase activation and DNA fragmentation confirms the induction of apoptosis as the major cytotoxic modality for the extracts. These findings could hold positive implications for potential pumpkin seed extract intervention in prostate cancer therapy.*

KEY WORDS: Cytotoxicity, Pumpkin Seed Extracts, Prostate Cancer Cells

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INTRODUCTION

Prostate cancer is one of the most common cancers in the Jamaican population and the leading cause of cancer related deaths in the Caribbean, despite improvement in screening methods and accessibility to healthcare (Jemal et al., 2011; Gibson et al., 2010). Globally, it is the second most frequently diagnosed cancer and in the United States it is the second leading cause of cancer death in men (Jemal et al., 2011). There are several approaches to prostate cancer treatment depending on the stage of the disease. Chemotherapy is applicable in cases where the cancer has spread outside the prostate gland and when hormone therapy fails. First line chemotherapeutic agents are taxanes such as docetaxel and cabazitaxel. Docetaxel is commonly met with high levels of resistance and adverse events including bleeding, anemia, neuropathy and depressed cardiac function (Hwang, 2012); hence there is significant interest in exploring new chemotherapeutic agents for prostate cancer that might present with safer treatment profiles. One potential source of such anti-cancer agent could be the Pumpkin plant.

Cucurbita pepo Linn that is commonly referred to as Pumpkin, is a plant belonging to the family Cucurbitaceae (Oloyede, 2012). The plant along with its fruits and seeds are a main staple in the diet of many countries including Africa, Europe and Jamaica (Oloyede, 2012; Chevallier, 1996). There is strong evidence of a protective role of Pumpkin seed oil on prostate health, where is has been shown to inhibit the development of testosterone induced prostatic hyperplasia in rats (Gossell-Williams et al., 2006; Tsai et al., 2006) as well as to improve quality of life and prostate specific antigen (PSA) levels in men with benign prostatic hyperplasia (BPH) (Hong et al., 2009). The extracts of pumpkin seeds have also been reported to exhibit anti-androgenic activity (Schmidlin and Kreuter, 2003), improve symptoms of BPH (Friedrich et al., 2000; Carbin et al., 1990) and induce cytotoxic effects in hepatocarcinoma, colon carcinoma (Shokrzadeh et al., 2010) and melanoma cells (Xia et al., 2003). Although there are several studies regarding pumpkin seed extracts and BPH; there is only limited data available regarding the direct cytotoxic effects of these extracts in prostate cancer.

Prostate cancer shares several common serological, anatomical and pathological features with BPH including inhibition of apoptosis, elevated prostate specific antigen levels and structural alterations in DNA (Guess, 2001). Interestingly, diets high in pumpkin seeds have been associated with lower levels of other types of cancers such as gastric, breast, lung, and colorectal cancer (Huang et al., 2004) Based on these lines of evidence, our present study was designed to examine the cytotoxic effects of pumpkin seed extracts on human prostate cancer cells and the associated mechanisms. We evaluated the cytotoxic effects of aqueous and ethanolic extracts from pumpkin seeds on cell viability in the LNCaP prostate cancer cells and our experiments were pivoted specifically on events predicting cell death such as increased oxidative stress, caspase activation, mitochondrial depolarization, DNA fragmentation and poly (ADP-ribose) polymerase (PARP) cleavage. We present evidence to support further studies on potential exploitable benefits of pumpkin seed extracts in prostate cancer.

MATERIALS AND METHODS

Preparation of Pumpkin Seed Extracts

Briefly, Pumpkin seeds obtained from the local fruit (Kingston, Jamaica) were air-dried and milled to a powder. Mr. Patrick Lewis of the University of the West Indies herbarium and a voucher specimen #35661 deposited identified the plant and fruit as Curcurbita pepo. To prepare the ethanol extract (PS-ET), the powder (100 gm) was soaked overnight in 500 ml of 80% ethanol with frequent agitation and filtered. The resulting extract was concentrated to dryness [yield: 9.5% (w/w)] by evaporation and stored at 4ºC for further studies. To prepare the aqueous extract (PS-AQ), the powder (50 gm) was dissolved in 500 ml of distilled water. The mixture was then boiled for 45 minutes, filtered and the resultant extract evaporated to dryness. The solid extract [yield: 6.5% (w/w)] obtained was stored at 4 C°.

Cell Culture

LNCaP cells (ATCC, Washington, D.C, U.S.A) were maintained at 37°C in a humidified atmosphere of 5% CO_2 and propagated as adherent monolayer in 90% RPMI medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were subcultured at approximately 80% confluence and harvested for the various studies after brief trypsinization.

MTT Cytotoxicity Assay

Cells were harvested and counted, then seeded in 96-well microplates (1×10^5 cells/well) and incubated (37°C/ 5% CO_2) for 24 hr prior to the addition of the extracts. For treatment of cells, PS-AQ and PS-ET extracts were diluted using distilled water and ethanol respectively to final concentrations of 20, 40, 80, 120 and 160 μg/ml. Control cells were treated with vehicle only as negative control. The treated cells were incubated for 24 hr (37°C/ 5% CO_2). Following incubation, 10 μl of the MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reagent was added to each well. The plates were further incubated for 4 hrs in the dark, after which 100 μl of DMSO was added to the cells and incubated overnight under the same conditions. Absorbance (OD) was read at 490 nm using a Multiskan microplate reader (Molecular Devices Inc., Sunnyvale, CA, USA). The relative cell viability in percentage was calculated as (*Absorbance 490* of treated samples/*Absorbance 490* of untreated samples) × 100. The IC_{50} for each extract was determined using linear regression from the dose-response curve.

JC-1 Mitochondrial Membrane Potential (Δψm) Analysis

Mitochondrial membrane potential (Δψm) was analyzed using 5,5', 6,6'-tetrachloro-1, 1', 3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1), a fluorescent compound. Mitochondria with intact membrane potentials induce JC-1 aggregation that results in the mitochondria fluorescing a red color. In depolarized mitochondria, there is no JC-1 aggregation and this produces green fluorescence. LNCaP cells were cultured in a 24 well plate until 80% confluency was reached. Cells were treated with PS-AQ and PS-ET (50 μg/ml) respectively, then incubated for 24hr as previously described. The JC-1 dye (25μL) was then added to each well and incubated for a further 15 minutes. The cells were then analyzed with a fluorescent microscope at excitation/emission= 540/570nm for red fluorescence (viable cells) and 485/535nm for green fluorescence (apoptotic cells).

Nitroblue tetrazolium (NBT) Reactive Oxygen Species Assay

The NBT assay is based on the conversion of soluble nitroblue tetrazolium (NBT) to the insoluble NBT-diformazan by reactive oxygen species (ROS) such as the superoxide anion. LNCaP cells (1×10^5 cells/well) were propagated in a six-well plate incubated with PS-AQ and PS-ET as described above for 24hr. Treated cells were then incubated at 37°C with NBT (1 mg/ml) in HBSS medium for 4 hrs in the dark. Following incubation, reduced NBT was solubilized with DMSO. The absorbance was measured for each well at 560 nm using an ELISA plate reader. The ROS production levels were calculated by comparing the absorbance values of the treated samples to that of the control samples.

Assay for Caspase 3 and 9 Activities

The activities of caspase 3 and 9 activity were measured using a colorimetric assay. Following treatment of LNCaP cells with PS-AQ and PS-ET extracts (50μg/ml each) in 6-well plates, cells were collected by centrifugation and lysed with lysis buffer (1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 10 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, 1 mM PMSF, 1 μg/ml Aprotinin, 1 mg/ml Leupeptin). Lysates were transferred to 96-well microplates and incubated with 2 mM substrate specific for caspase 3 and 9 in triplicate respectively at 37°C for 1 hour. Specific substrate was DVED-pNA for caspase 3 and LEHD-pNA for caspase 9. The production of cleaved p-nitroanilide from each substrate was measured at 405 nm using a Molecular Dynamics SpectraMax 250 microplate reader.

Western Blotting: Detection of Cleaved PARP

Protein extracts were prepared for western blotting following treatment of LNCaP cells with PS-AQ and PS-ET (50 μg/ml) and topotecan as previously described for 24 hr. Protein was extracted using the Qiagen- Protein Purification kit, and quantified according to the manufacturer's protocol. In total, 30 *μ*g of sample proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. Membranes were blocked with 5% fat-free dry milk in 1 × Tris-buffered saline (TBS) and incubated with the primary antibody to PARP (Cell Signaling Technology, USA). Protein bands were detected by incubating with horseradish peroxidase-conjugated antibodies (Sigma Aldrich, USA) and visualized with enhanced chemiluminescence reagent.

DNA fragmentation Assay

DNA fragmentation patterns were examined by electrophoresis of extracted genomic DNA from the LNCaP cells following treatment with PS-ET and PS-AQ extracts for 24hr. Topotecan (10μM) was used as positive control. After incubation, the cells were harvested and washed with phosphate-buffered saline (PBS). Cells were resuspended in 200 μl of PBS supplemented with 20 μl of proteinase K. The DNA was extracted using the Qiagen DNeasy Kit by following the manufacturer's protocol. The samples were subjected to electrophoresis at 80 V for 2 hrs in 1.5% agarose gel containing 5 μl of ethidium bromide. DNA fragmentation patterns were visualized using UVP image analyzer.

Statistical Analyses

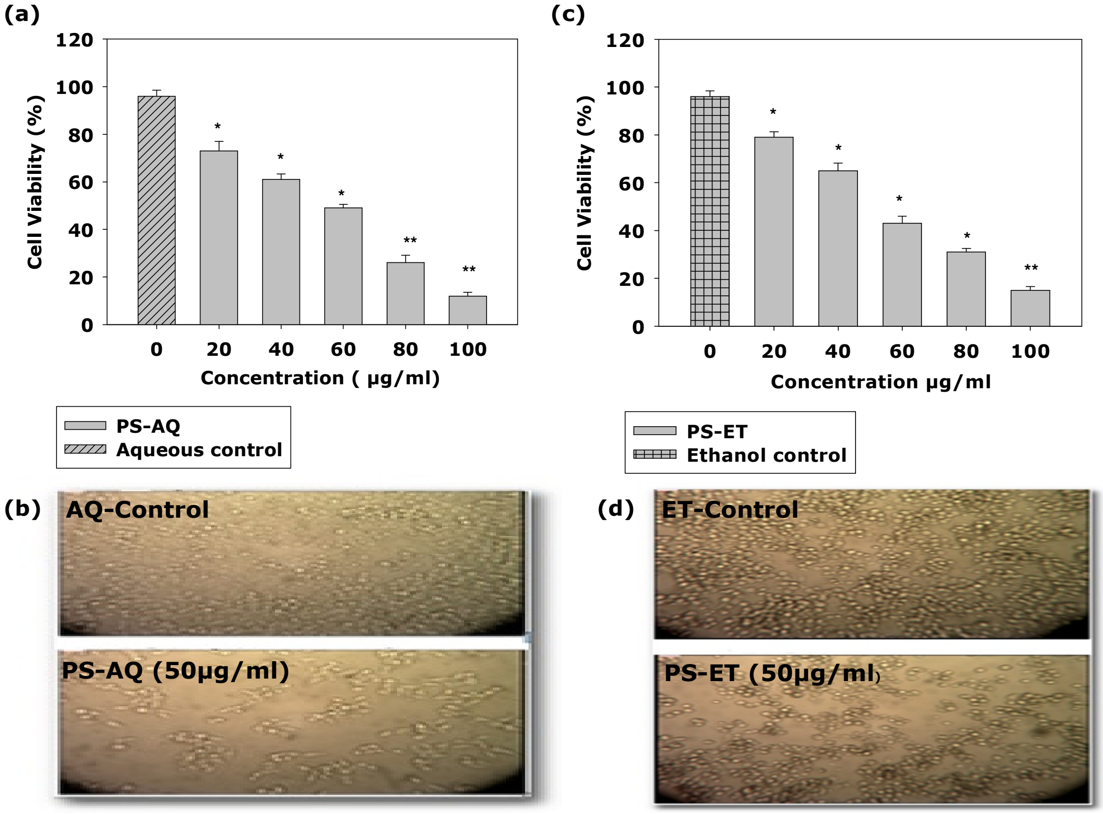
All experiments were performed in triplicate, and data is presented as means ± S.E.M. The Student's t-test (in SigmaPlot 10.0) was used for analysis of the difference between PSO treated and control groups and statistical significance with P value < 0.05 considered significant (*).

RESULTS

Effect of PS Extracts on Cell Viability

The viability of LNCaP cells was significantly ($p \leq 0.05$) reduced in a dose dependent manner by both the PS-AQ (Figures 1a & b) and PS-ET (Figures 1c & d) extracts when compared to vehicle controls. The IC_{50} values were 49 μg/ml and 55μg/ml for PS-AQ and PS-ET extracts respectively. This suggested potent cytotoxic activity of the extracts.

FIGURE 1. Effect of PS-AQ and PS-ET extracts on LNCaP cell viability. Cells were incubated with extracts PS-AQ (a) and PS-ET (b) at concentrations of 20, 40, 60, 80 & 100 μg/ml for 24 hr. Cell viability was determined by the MTT assay. Data is presented as the mean ± S.E.M. from triplicate experiments. Asterisks represent values significantly different from the vehicle control, *($p \leq 0.05$), **($p \leq 0.005$)). Both extracts produced a significant and dose dependent reduction in cell viability with IC_{50} values of 49 μg/ml and 55μg/ml for PS-AQ and PS-ET extracts respectively.



Effect of PS Extracts on Mitochondrial Membrane Potential ($\Delta\psi m$)

Treatment of LNCaP cells with both PS-AQ and PS-ET extracts resulted in decreased $\Delta\psi m$, as is evidenced by the shift from red to green, as shown in figures 2a and b respectively. Control cells showed intact membrane potential. The loss of mitochondrial potential in treated cells therefore implicates mitochondrial depolarization as a mechanism involved in the cytotoxicity induced by the PS extracts.

Effect of PS-Extracts on ROS Generation in LNCaP Cells

ROS levels were significantly increased in LNCaP cells treated with both the PS-AQ ($p \leq 0.0001$) and PS-ET ($p \leq 0.001$) extracts when compared to controls (Figure 3). The PS-AQ induced higher levels of ROS relative to the PS-ET treated cells by increasing the generation of intracellular ROS to 4.1 ± 0.2-fold of the control, compared to the 3 ± 0.3 fold increase produced by PS-ET (Figure 3).

Caspase Activation by PS Extracts

The activities of caspase 3 and caspase 9 were significantly increased by both PS-ET and PS-AQ extracts and this indicated the activation of the apoptotic pathway. PS-ET produced a 5 (± 0.2, $p \leq 0.005$) and 3.5 (± 0.4, $p \leq 0.003$) fold increase in caspase 3 and 9 activities respectively, while PS-AQ produced a 7 (± 0.4, $p \leq 0.003$) and 3 (± 0.9, $p \leq 0.04$) fold increase in caspase 3 and 9 activities respectively when compared to the untreated control cells (Figure 4). In comparison, the standard chemotherapeutic agent topotecan produced a 10 (±0.1, $p \leq 0.0004$) and 5.5 (±0.4, $p \leq 0.001$) fold increase in caspase 3 and caspase 9 activities respectively (Figure 4).

PARP Cleavage in LNCaP Cells treated with PS extracts

PARP cleavage was used as indicator of apoptosis in our study. Both PS-AQ and PS-ET along with topotecan induced PARP cleavage as depicted in figure 5, which is symbolic of induced apoptosis. There was no PARP cleavage evident in cells treated with the ethanol or distilled water. (Figure 5).

FIGURE 2. Effect of PS-AQ and PS-ET extracts on $\Delta\psi m$ in LNCaP cells. LNCaP cells were exposed to the PS-AQ and PS-ET extracts for 24 hr and then stained with JC-1 (25 µl). Red fluorescence indicates mitochondria with intact $\Delta\psi m$. Green fluorescence indicates depolarized mitochondria. Images were taken with a Carl-Zeiss epifluorescence microscope. Panel a, shows cells treated with PS-AQ extracts and aqueous control. Panel b shows cells treated with PS-ET extracts and ethanol control.

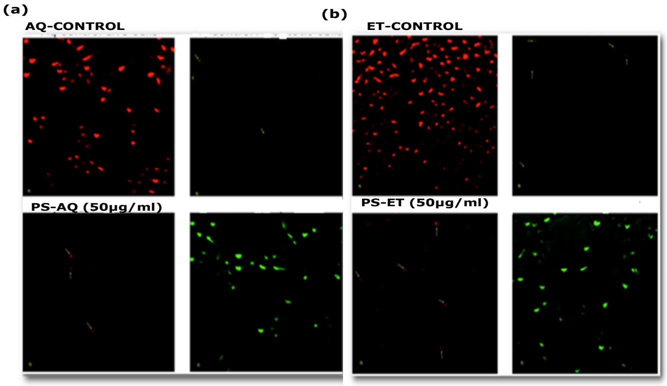


FIGURE 3. ROS generation in LNCaP cells treated with PS extracts. ROS levels (NBT reduction) determined from percent absorbance of formazan relative to untreated controls (1%) following 24hr exposure of LNCaP cells to PS extracts. Data is presented as the mean ± S.E.M. from triplicate experiments. Asterisks represent values significantly different from the vehicle control (*$p \leq 0.001$, **$p \leq 0.0001$). When compared with the control groups, both extracts elevated ROS levels.

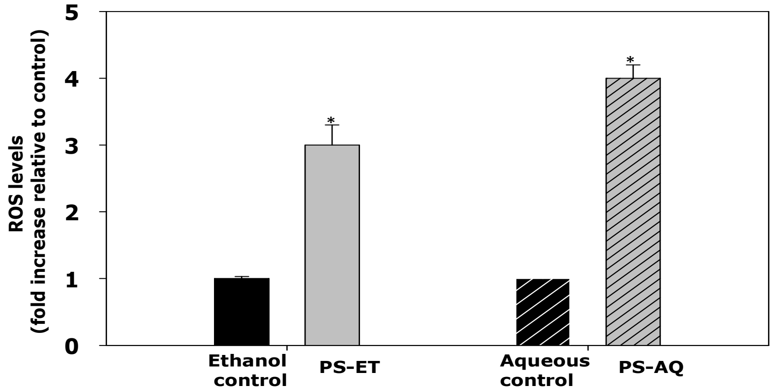


FIGURE 4. PS extracts activate Caspases 3 and 9 in LNCaP cells. Caspase 3 and 9 activities via enzymatic cleavage of Ac-LEHD-pNA and Ac-DEVDpNA in lysates from LNCaP cells following 24 hr exposure to PS-AQ and PS-ET 50µg/ml, respectively. Data is presented as the mean ± S.E.M. from triplicate experiments. Asterisks represent values significantly different from the vehicle control (*$p \leq 0.05$, **$p \leq 0.005$, *** $p \leq 0.005$). PS-ET produced a 5 (± 0.2, $p \leq 0.005$) and 3.5 (± 0.4, $p \leq 0.003$) fold increase in caspase 3 and 9 activities respectively, while PS-AQ produced a 7 (± 0.4, $p \leq 0.003$) and 3 (± 0.9, $p \leq 0.04$) fold increase in caspase 3 and 9 activities respectively when compared to the untreated control cells.

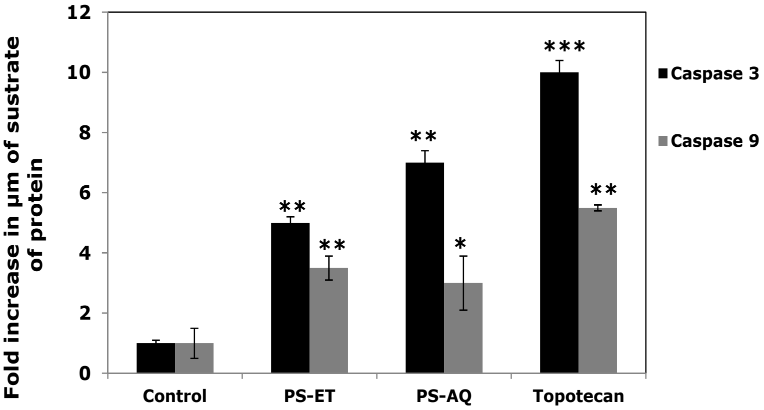
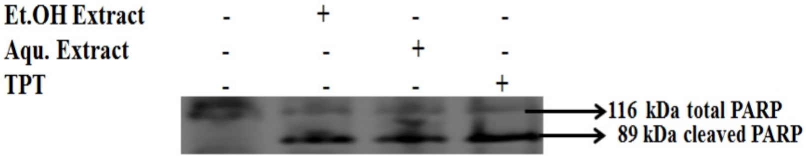


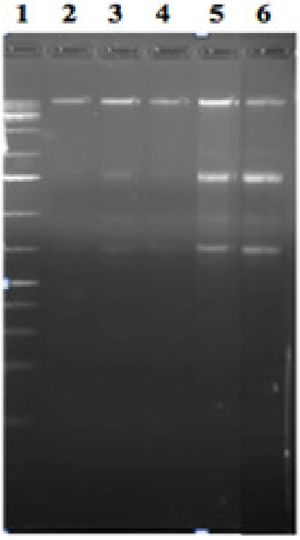
FIGURE 5. Proteolytic cleavage of PARP in LNCaP cells by PS extracts. Cell lysates were analyzed by Western blotting for PARP after exposure to PS-AQ, PS-ET extracts (50μg/ml respectively) and topotecan for 24 hr. β Actin (43kDa) was also detected as the loading control. Cleaved PARP fragments (89kDa) are evident in PS-AQ, PS-ET and topotecan treated cells.



DNA fragmentation in LNCaP Cells Treated with PS Extracts

We assessed further the role of apoptosis in the cytotoxic effects of the PS extracts by examining DNA fragmentation patterns in response to 24 hr exposure, as internucleosomal cleavage of cellular DNA by endonucleases is indicative of apoptotic cell death. PS-AQ and PS-ET as well as topotecan induced distinct DNA fragmentation patterns in contrast to vehicle treated cells that showed no fragmented DNA (Figure 6).

FIGURE 6. PS-extracts induce DNA fragmentation in LNCaP cells. Agarose gel (1.5%) electrophoresis depicting DNA fragmentation patterns in LNCaP cells treated with PS extracts and controls for 24hr. Lane 1, 100 bp DNA ladder; lane 2, control cells exposed to ethanol; lane 3, cells treated with PS-ET (50μg/ml); lane 4, cells treated with distilled water; lane 5, cells treated with PS-AQ (50μg/ml); lane 6, cells treated with topotecan (10 μM) as positive control. DNA fragmentation patterns were visualized using a UVP image analyzer.



DISCUSSION

In developing countries especially, nutrition and traditional medical practices are highly interconnected, and in recent years, the targeted screening of edible plant material for anti-cancer activity has significantly increased. In the case of prostate cancer, this has been driven significantly by the limitations of current conventional therapies that include severe side effects and resistance (Hwang, 2012). Many potent anti-cancer agents have been derived from natural products, and the combination of natural products with conventional agents have also improved efficacy of prostate cancer therapy (Mukherjee et al., 2001). Our study is a continued scientific exploration of natural products such as the pumpkin seed for such properties.

In the current study, we used an ethanol (PS-ET) and an aqueous (PS-AQ) extract obtained from the seeds of the *Cucurbita pepo* fruit and examined their cytotoxic effects in LNCaP prostate cancer cells. Both the PS-ET and PS-AQ extracts induced significant cytotoxicity in these cells as assessed by the MTT cell viability test which is an established assay for determination of cell viability.

To investigate the mechanisms involved in cell death, we evaluated the involvement of apoptosis firstly through DNA fragmentation and poly ADP ribose polymerase (PARP) cleavage analysis following 24hr exposure of LNCaP cells to the PS extracts. Apoptosis is characterized biochemically by the production of 180-200 bp internucleosomal DNA fragments resulting from the activation of endonucleases (Oberhammer et al., 1993). This produces a characteristic pattern of DNA cleavage into a 180 base pair "DNA ladder" that can be visualized by agarose gel electrophoresis with an ethidium bromide stain and ultraviolet illumination (Oberhammer et al., 1993). The PS extracts produced characteristic apoptotic fragmentation patterns in our LNCaP cells. PARP is a nuclear enzyme that is integral in the DNA repair process and its cleavage by caspase 3 during apoptosis disables DNA repair (D'Amours et al., 2001). PARP cleavage was evident in cells treated with both PS extracts and along with the DNA fragmentation observed, this confirmed apoptosis as a modality through which the PS extracts induced cytotoxicity.

We subsequently evaluated the involvement of the caspase cascade in the induced cell death as apoptotic death of mammalian cells is typically mediated by cysteine proteases of which caspases 3, 8 and 9 are key players (Pilchenkov et al., 2004). Apoptosis can be initiated by the extrinsic or intrinsic pathway. The extrinsic apoptotic pathway is initiated by the ligation of a transmembrane death receptor with its ligand, which activates caspase 8, while the intrinsic apoptotic pathway can be triggered by several events including the release of cytochrome c due to loss of Δψm that leads to the activation of caspase 9 (Kim, 2005). Caspase 3 is the executioner caspase common to both extrinsic and intrinsic apoptotic pathways, and its activation is a hallmark sign of apoptosis (Pilchenkov et al., 2004). We found that treatment of LNCaP cells with both PS-ET and PS-AQ extracts increased the activities of caspase

3 and 9, confirming that the intrinsic apoptotic pathway modulates the cell death induced by both extracts.

In addition to the activation of caspases 3 and 9, the PS extracts also induced mitochondrial depolarization in LNCaP cells as evidenced by fluorescent shift from red to green following JC-1 staining. Mitochondrial depolarization is generally due to aberrant mitochondrial function which is presumed to be secondary to opening of the mitochondrial permeability transition pore (MPTP), allowing passage of ions and small molecules (Kroemer et al., 2007). This depolarization often serves as the nodal point where diverse apoptotic stimuli translate from initiation into execution (Ling et al., 2003) through cytochrome c release which leads to the activation of caspase 9 with subsequent activation of the central executioner of apoptosis caspase 3 (Pilchenkov, 2004). Activation of caspase 3 eventually triggers the caspase-activated DNase, which enters the nucleus and thus causes PARP cleavage and DNA fragmentation (Pilchenkov et al., 2004). Taken together these findings outline a pathway of induced cytotoxicity that involves the activation of the intrinsic apoptotic pathway subsequent to mitochondrial depolarization.

We then evaluated ROS release in PS-AQ and PS-ET treated cells as excessively generated ROS have been linked to reduction of mitochondrial membrane potential in apoptotic cancer cells including LNCaP cells (Kroemer et al., 2007; Kim et al., 2007. Both extracts significantly elevated ROS generation when compared to controls, supporting the hypothesis that the induced apoptosis involved increased ROS generation.

The chemical constituents of *Cucurbita pepo* seeds are diverse and include triterpenes (cucurbitacins), carotenoids, fatty acids, minerals, tocopherol and lignins (Abdel-Rahman, 2006). The carotenoids lutein and zeaxanthin have been previously linked to prevention of prostate cancer (Procida et al., 2013; Binns et al., 2004; Jian et al., 2005). These carotenoids are also well established as potent cytotoxic agents in lymphomas, leukemias and breast cancer (Molnar et al., 2004), mainly acting through induction of apoptosis and arrest of cell cycle. Interestingly, pumpkin seeds are also rich is phytoestrogens such as Secoisolariciresinol diglucoside (SDG) (Patel et al., 2012). When ingested, SDG is metabolized to the compounds enterodiol and enterolactone. Enterolactone has been previously reported to exhibit potent cytotoxic activity in LNCaP cells by inducing apoptosis (Chen et al., 2007). Both Enterolactone and enterodiol have also been reported to suppress the growth of prostate cancer cells via hormonally dependent and independent mechanisms (Lin et al., 2001). Therefore, it is tempting to suggest that the cytotoxic activity of the PS extracts in our study could possibly be attributed to carotenoid or phytoestrogenic activity; but in further work we will be conducting phytochemical analysis and activity directed separation of our aqueous and ethanolic extracts in order to identify the bioactive principles.

CONCLUSION

In conclusion the data gathered in our study presents significant evidence to confirm the cytotoxic efficacy of aqueous and ethanolic extracts of pumpkin seeds (*Cucurbita pepo*) in LNCaP cells and suggests a potential use in prostate cancer therapy which will be further explored by our research group.

ACKNOWLEDGEMENTS

The Authors would like to acknowledge the Rumbaugh Goodwin Research Institute and the Health Professions Department at Nova Southeastern University for funding the present research.

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