Polyphenol-rich sweet potato greens extract inhibits proliferation and induces apoptosis in prostate cancer cells in vitro and in vivo

Prasanthi Karna, Sushma R. Gundala, Meenakshi V. Gupta, Shahab A. Shamsi, Ralphenia D. Pace, Clayton Yates, Satya Narayan, Ritu Aneja*

Department of 1Biology and 3Chemistry, Georgia State University, Atlanta, GA-30303; 2West Georgia Hospital, LaGrange, GA-30240; Departments of 4Nutrition and 5Biology, Tuskegee University, Tuskegee, AL-36088; 6UF Shands Cancer Center, University of Florida, Gainesville, FL-32610

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Both authors contributed equally

*Correspondence be addressed to:
Ritu Aneja, Department of Biology, Georgia State University, Atlanta, GA-30303
Email: raneja@gsu.edu; Phone: 404-413-5417; Fax: 404-413-5301

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Sweet potato (*Ipomoea batatas*) leaves, or greens, extensively consumed as a vegetable in Africa and Asia, are an excellent source of dietary polyphenols such as anthocyanins and phenolic acids. Here we show that sweet potato greens extract (SPGE) has the maximum polyphenol content compared to several commercial vegetables including spinach. The polyphenol-rich SPGE exerts significant antiproliferative activity in a panel of prostate cancer cell lines while sparing normal prostate epithelial cells. Mechanistically, SPGE perturbed cell-cycle progression, reduced clonogenic survival, modulated cell-cycle and apoptosis regulatory molecules, and induced apoptosis in human prostate cancer PC-3 cells both *in vitro* and *in vivo*. SPGE-induced apoptosis has a mitochondrially-mediated component, which was attenuated by pretreatment with cyclosporin A. We also observed alterations of apoptosis regulatory molecules such as inactivation of Bcl2, upregulation of BAX, cytochrome c release, and activation of downstream apoptotic signaling. SPGE caused DNA degradation as evident by terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling (TUNEL) staining of increased concentration of 3’-DNA ends. Furthermore, apoptotic induction was caspase-dependent as shown by cleavage of caspase substrate, poly(ADP)ribose polymerase. Oral administration of 400 mg/kg SPGE remarkably inhibited growth and progression of prostate tumor xenografts by ~69% in nude mice, as shown by tumor volume measurements and non-invasive real-time bioluminescent imaging. Most importantly, SPGE did not cause any detectable toxicity to rapidly dividing normal tissues such as gut and bone-marrow. This is the first report to demonstrate the *in vitro* and *in vivo* anticancer activity of sweet potato greens in prostate cancer.
INTRODUCTION

Nearly one-third of all cancer deaths in the US can be prevented through appropriate dietary modification [1-3]. Regular consumption of fruits and vegetables (“five servings per day”) [4] is highly recommended today in the American and European diet, mainly because the constituent phytochemicals, in particular, polyphenols, they contain are known to play important roles in long-term health protection, notably by reducing the risk of chronic and degenerative diseases including cancer [5,6]. Prostate cancer is particularly amenable to dietary chemopreventive strategies since it presents a significantly large-window of latency (about 20-30 years) and its mean age of diagnosis is about 68 years [7-10]. About 35 plant-based foods identified by the NCI display effective anticancer properties including garlic, turmeric, cruciferous vegetables (e.g., broccoli, brussels sprouts, cabbage), and grape-seed extracts [8,11-14]. Many fruit and vegetable whole extracts have also been tested for their efficacy in inhibiting prostate cancer growth [7,8,10,13,15].

Plant polyphenols, a class of naturally-occurring water soluble phenolic compounds, are crucial for optimal human health benefits and are being increasingly recognized owing to their abundance in fruits, vegetables, and derived foodstuffs [16]. The conformational flexibility of polyphenols facilitates complex oligo/polymeric assemblies that enable plants to take advantage of the remarkably diverse range of bio-physicochemical properties exhibited by the phenol functional group thus making plant polyphenolics as unique and intriguing natural products [16]. No wonder polyphenols have sparked a new appraisal of diverse plant-derived foods and beverages such as tea, red-wine, coffee, cider, chocolate, as well as many other food commodities derived from fruits, including berries. The ability of phenolics to homolytically release a hydrogen atom is one of the fundamental processes that underlie the acclaimed health-benefiting antioxidative property of polyphenolics to act as scavengers of free radicals and reactive oxidative species (ROS) that may drive malignant transformation and carcinogenesis [16].

Sweet potato (*Ipomoea batatas*) leaves, or greens, are commonly consumed as a fresh vegetable in West Africa and Asia, in particular, Taiwan and China [17]. Rich in vitamin B, β-carotene, iron, calcium, and zinc, sweet potato greens (SPG) are highly nutritive and contain as many
vitamins, minerals and other nutrients as spinach [18]. SPG are an excellent source of antioxidative polyphenolics, namely anthocyanins and phenolic acids such as caffeic, monoaacaffeoylquinic (chlorogenic), dicaffeoylquinic and tricaacaffeoylquinic acids [19,20]. The major anthocyanins in SPG are cyanidin-type rather than peonidin-type [21]. The constituent polyphenolics of SPG display antimutagenic, antidiabetic, antibacterial, anti-inflammatory, and anticancer activity [18,22]. The chemopreventive action of SPG is suggested by a case-control study in Taiwan reporting that higher SPG consumption is associated with reduced lung cancer risk [23].

Although sporadic studies have reported identification of bioactive polyphenolics and anthocyanin constituents of SPG [24], there has heretofore not been a study that offers a detailed evaluation of the anticancer potential of sweet potato greens extract (SPGE). To the best of our knowledge, we are the first to investigate the anticancer attributes of SPGE in vitro and in vivo and to develop it as a mechanism-based anticancer agent for prostate cancer. In this study, we examine the anticancer effects of SPGE in a panel of prostate cancer cells by evaluating its effects on cellular proliferation, cell-cycle progression, and apoptosis. Our results demonstrate that SPGE causes growth inhibition by inducing a G1-phase arrest followed by a mitochondrially-mediated caspase-dependent intrinsic apoptosis in prostate cancer, PC-3 cells. In vivo studies show that SPGE remarkably inhibits tumor growth of subcutaneously implanted PC-3 human tumor xenografts in nude mice models without any detectable toxicity.

MATERIALS AND METHODS

Cell-culture, antibodies and reagents: Human prostate cancer cell lines (LNCaP, DU145, PC-3, C4-2, C4-2B) were cultured in RPMI medium (Mediatech, Inc., Manassas, VA) with 10% FBS. Luciferase-expressing PC-3 cells (PC3-luc) were from Calipers (Hopkinton, MA) and were maintained in MEM medium with 10% FBS. Antibodies to cyclin D1, cyclin A, cytochrome c, Bcl2, phosho-Bcl2, cleaved caspase-3, and cleaved poly (ADP-ribose) polymerase (PARP) were from Cell Signaling (Beverly, MA). BAX, p21, cyclin E, p53, and β-actin were from Santa Cruz (Santa Cruz, CA). Chlorogenic acid (ChA) and caffeic acid (CA) were from Sigma (St. Louis, MO).
Preparation of SPGE and estimation of polyphenolics: 45-day-old sweet potato (*Ipomoea batatas*) greens (Whatley/Loretan, (TU-155) variety) were obtained from Tuskegee University Agriculture Department. Extracts were prepared by soaking shade-dried leaves in methanol overnight for three consecutive days. The supernatant was collected daily and finally concentrated in vacuo (Buchi-Rotavap) followed by freeze-drying to powder using a lyophilizer. SPGE stock solution was prepared by dissolving 200 mg/ml DMSO and various concentrations were obtained by appropriate dilutions. Batch-to-batch variation was evaluated by analysis of total polyphenolic (~6.5g/100g) [25,26] and anthocyanin (~10.8 CV/g powder) [21], which was observed to be consistent across batches of similar age.

*In vitro* cell-proliferation and colony survival assay: Cells plated in 96-well format were treated with gradient concentrations (1-1000 μg/ml) of SPGE the next day. After 72h SPGE treatment, cell-proliferation was determined using the Alamar Blue assay. For the colony assay, PC-3 cells were seeded at appropriate dilutions (~100 cells/well) and were treated with 250 μg/ml SPGE for 48h, washed, and replaced with regular RPMI-medium. A colony was arbitrarily defined to consist of at least 50 cells. After 10 days, colonies were fixed with 4% formaldehyde, stained with crystal-violet and counted.

Cell-cycle analysis, immunofluorescence microscopy and immunoblot analysis: Cell-cycle studies were performed as previously described [27]. For immunofluorescence microscopy, cells were grown on coverslips, treated with SPGE, and processed as described earlier [28]. Immunoblotting was performed as formerly described [28].

Determination of mitochondrial transmembrane potential and caspase-3 activity: Mitochondrial transmembrane potential was measured flow-cytometrically using JC-1 staining and caspase-3 activity was measured using a fluorescent substrate as described previously [28].

Annexin-V and terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay: SPGE-treated cells were stained with Alexa-Fluor 488-conjugated Annexin-V using the Vybrant-Apoptosis Assay Kit from Invitrogen as per the manufacturer’s protocol. Annexin-positive cells were visualized using confocal microscopy and quantitated flow-cytometrically. DNA strand-breaks were quantified flow-cytometrically using the TUNEL-assay as described [27].
In vivo tumor growth, SPGE treatment and bioluminescent imaging: Six-week old male nude mice were obtained from NCI (Frederick, MD, USA) and 1x10⁶ PC-3-luc cells in 100 µl PBS were injected subcutaneously in the right flank. When tumors were palpable, mice were randomly divided into two groups of eight mice each. Control group received vehicle (PBS with 0.05% Tween-80, pH=7.4) and the treatment group received 400 mg/kg bw SPGE daily by oral gavage. Tumor growth was monitored in real time by bioluminescent imaging of luciferase activity in live mice using the cryogenically cooled IVIS-imaging system (Calipers Inc.) with the live imaging software. Briefly, mice were anesthesized with isoflurane, intraperitoneally injected 25 mg/ml luciferin and imaged with a CCD-camera. Integration of 20s with 4 binnings of 100 pixels was used for image acquisition and signal intensity was quantitated as sum of all detected photon counts within the lesion. Mice from vehicle or SPGE-treated groups were imaged twice a week allowing temporal assessment of in vivo tumor growth. All animal experiments were performed in compliance with institutional IACUC guidelines.

Histopathologic and immunohistochemical analyses: After 6 weeks of SPGE or vehicle feeding, mice were euthanized. Organs and tumors were either formalin-fixed or frozen immediately. Tumor or organ sections were stained with hematoxylin and eosin (H&E). Cleaved caspase-3, cleaved PARP, Ki67 and TUNEL-staining of tumor sections was performed as described previously [29,30]. Microscopic evaluation was performed by a pathologist in a blinded-manner.

High performance liquid chromatography (HPLC) with UV and mass spectrometric detection: The HPLC-UV separations were achieved on a HP1100 series Instrument (Agilent Technologies, Wilmington, DE) equipped with a UV-photodiode array detector using an Eclipse plus reversed phase C-18 column (3.5 µm, 4.6 x 150 mm), as per conditions described in the Supplementary Data section.

Statistical analysis: The mean and standard deviations were calculated for all quantitative experiments using Microsoft-Excel software. The Student’s t-test was used to determine the differences between groups with p-values of <0.05 considered as statistically significant.

RESULTS

SPGE has the highest polyphenol content
Nature has selectively enriched plants with the phenolic functional group as a special means to equip and elaborate complex secondary metabolites useful for their development and survival. It comes as no surprise that plant extracts, herbs and spices rich in polyphenolics have been used for thousands of years in traditional oriental medicine. Thus, we first attempted to determine the total polyphenolic content of SPGE compared to several commercially-available vegetables like spinach, mustard greens, kale, okra, green onions and collard greens (Fig. 1). Estimating polyphenolic content in terms of chlorogenic acid equivalents expressed as mg/L, our data showed that sweet potato greens had the highest polyphenolic concentrations which were ~43% higher than spinach (Fig. 1A). We also quantified the anthocyanin content and found that SPG had ~2.5 fold higher anthocyanin pigments compared to spinach (Fig. 1B). These data encouraged us to evaluate the antiproliferative potential of SPGE that was investigated next.

**SPGE inhibits proliferation of human prostate cancer cells**

Given that prostate cancer has a long latency time and is ideal for chemopreventive intervention by non-toxic dietary extracts, we asked if SPGE inhibited growth of prostate cancer cells (LNCaP, DU145, PC-3, C4-2 and C4-2B) in a concentration gradient-dependent manner. SPGE significantly inhibited cellular proliferation of all prostate cancer cells with IC$_{50}$ values in the range of 145-315 µg/ml (Fig. 2A). The order of sensitivity was C4-2>LNCaP>DU145>C4-2B>PC-3, with C4-2 being the most sensitive and PC-3 the least. Importantly, the IC$_{50}$ of SPGE in normal prostate epithelial cells (PrEC and RWPE-1) was between 1000-1250 µg/ml (Fig. 2B), which was ~5 fold higher than for cancer cells suggesting that SPGE specifically targets cancer cells while sparing normal cells.

Next, we performed a clonogenic or colony formation assay that evaluates the capacity of a cell to proliferate indefinitely upon drug removal to form a colony or clone (Fig. 2C). The most resistant cell line (i.e., highest IC$_{50}$), PC-3, was selected for clonogenic assay and subsequent studies to delineate mechanisms of SPGE action. While controls produced several colonies, only a fraction of SPGE-treated cells retained the ability to form colonies. Fig. 2C shows the effect of 250 µg/ml SPGE on the relative clonogenicity of control and SPGE-treated PC-3 cells. Representative micrographs of colonies in control and SPGE-treated cells shown at the apex of bar-graphs quantitated to a ~80% reduction in number and size of surviving colonies upon
SPGE-treatment (Fig. 2C). We also examined nuclear Ki67 expression, which correlates well with growth fraction, and found that Ki67 immunostaining (green) was significantly more intense in control cells compared to 250 μg/ml SPGE-treated cells over 24h (Fig. 2Di). Bar-graph quantitation of Ki67-positive cells scored in both control and SPGE-treated cells showed a ~82% decrease in treated cells (Fig. 2Dii). Furthermore, DAPI staining (Fig. 2Di) indicated a ~5 fold increase in cells with nuclear fragmentation (Fig. 2Dii) compared to controls, suggesting SPGE-induced apoptotic cell death. In addition, trypan blue data showed that SPGE-induced cell death over time (0, 12, 24, 48, 72h) at 250 μg/ml in PC-3 cells (Suppl. Fig. 1).

**SPGE perturbs cell-cycle progression and modulates cell-cycle regulatory molecules**

We next asked if SPGE-mediated growth suppression was due to its cell-cycle intervention. To this end, we evaluated the effect of varying dose and time of SPGE exposure on the cell-cycle progression of PC-3 cells. Fig. 3Ai and 3Aii show dose- and time- courses of SPGE-treatment in a three-dimensional format. SPGE caused cells to accumulate in the G1-phase at doses less than or equal to 100 μg/ml over 24h, and at a dose of 250 μg/ml up to 12h (Suppl. Fig. 2). This was followed by a dose- and time- dependent increase in sub-G1 population, representing cells with hypodiploid (<2N) fragmented DNA, a hallmark of apoptosis. The quantitation of sub-G1 population over varying dose-levels and times is shown in Fig. 3Aiii and 3Aiv, respectively. Since SPGE arrested cell-cycle in the G1-phase at low doses and shorter time periods, we examined this acute effect of SPGE on G1-phase regulators. Immunoblot analysis revealed a decrease in protein levels of cyclin D1, cyclin A and cyclin E after 24h of 250 μg/ml SPGE treatment (Fig. 3B). An increase in Cip1/p21 levels was evident at 24h, in agreement with the G1-arrest (Fig. 3B).

**SPGE induces robust apoptosis**

Although an increase of sub-G1 population upon SPGE treatment indicated fragmented DNA suggesting apoptosis, we validated apoptosis both qualitatively and quantitatively by Annexin-V staining using confocal microscopy and flow cytometry methods. Immunofluorescence confocal micrographs showed that SPGE-treatment for 48h at 250 μg/ml externalized phosphatidylserine to the outer leaflet of the plasma membrane (observed as green rim) in PC-3 cells, a hallmark of early apoptosis (Fig. 3Ci). Flow-cytometric quantitation suggested a steady increase in Annexin-
positive cells to ~37% at 72h (Fig. 3Cii). It is well appreciated that altered cellular morphology, including membrane blebbing, formation of apoptotic bodies, disruption of cytoskeleton, hypercondensation, and fragmentation of chromatin characterize termination of apoptosis. Thus, we next quantified the increase in concentration of 3′-DNA ends due to DNA fragmentation using a flow cytometry-based TUNEL-assay. We found that SPGE-treated cells showed ~42% TUNEL-positive cells (Fig. 3Cii) at 72h compared to controls, suggesting extensive DNA cleavage.

**SPGE induces caspase-dependent apoptosis**

Both extrinsic and intrinsic apoptotic pathways are well recognized as major mechanisms of cell death in most cellular systems [31]. Having identified that SPGE induced robust apoptosis, we next evaluated whether the apoptosis was caspase-driven. Our data showed that treatment of PC-3 cells with SPGE did not result in caspase-8 activation and cleavage (data not shown) indicating non-recruitment of the extrinsic apoptotic pathway. However, SPGE demonstrated a strong time-dependent cleavage of caspase-3 and PARP, as observed by immunofluorescence and immunoblotting methods (Fig. 4Ai-Aii and Supl. Fig. 3Ai-ii). Caspase involvement was further confirmed by measuring caspase-3/7 activity using a fluorescent substrate (Fig. 4Aiii). Activation of caspase-3/7 without an effect on caspase-8 suggested involvement of the intrinsic pathway. To establish that this was the major mechanism of SPGE-induced apoptotic death in PC-3 cells, we pretreated cells for 3h with pan-caspase inhibitor z-vad-fmk followed by a 48h treatment with 250 μg/ml SPGE treatment. The extent of apoptosis was then determined by estimating the sub-G1 population flow-cytometrically. We observed that z-vad.fmk pretreatment significantly inhibited SPGE-induced apoptosis by ~65% (p<0.01), suggesting that cell death was primarily caspase-mediated (Suppl. Fig. 3Bi-iii).

**SPGE induces mitochondrially-mediated intrinsic apoptosis**

We further confirmed intrinsic apoptosis by measuring the collapse of mitochondrial transmembrane potential (Ψm) and examining release of mitochondrial cytochrome c into the cytosol [32]. The effect of 24h SPGE-treatment on Ψm was observed by staining with JC-1, a cationic-dye which exhibits potential-dependent mitochondrial accumulation [33]. An increase in JC-1 (green profile) monomeric form indicative of Ψm collapse was quantitatively determined
using flow-cytometry. As seen in Fig. 4Bi, 250 µg/ml SPGE-treated cells at 24h showed a right-shift in the mean-fluorescence intensity of green JC-1 monomers (blue-profile) compared to controls. There was a ~90% increase in the mean-fluorescence intensity of SPGE-treated JC-1-stained cells compared to controls (Fig. 4Bii). Most often, disruption of Ψm accompanies alterations in expression level of Bcl2 members, in particular, the ratio of antiapoptotic Bcl2 to proapoptotic BAX. We found that a 24h 250 µg/ml SPGE-treatment increased the levels of phosphorylated Bcl2 indicating its inactivation, while total Bcl2 levels remained unchanged (Fig. 4Biii). A significant increase in BAX levels was observed at 24h of SPGE-treatment (Fig. 4Biii). In addition, cytosolic cytochrome c was elevated upon a 24h SPGE-exposure (Fig. 4Biii). Thus, these data strongly indicated a mitochondrially-driven apoptosis upon SPGE-treatment. We further confirmed the extent of contribution of the mitochondrial pathway toward SPGE-induced apoptosis using cyclosporin A, a mitochondrial permeability transition pore inhibitor. Our results show that pretreatment of cells with cyclosporin A for 3h before SPGE treatment for 24h resulted in ~38% sub-G1 population compared with ~62% upon SPGE treatment alone (Suppl. Fig. 4A). Our experiments to study the drop in Ψm correlated with our flow cytometry data, in that we observed a diminution of the number of cells with depolarized mitochondria when cyclosporin A was added 3h before SPGE treatment compared to when SPGE was administered alone (Suppl. Fig. 4B). This is clearly indicative of the protective effect of cyclosporin A. These results suggest that there is a significant mitochondrial component to the total apoptotic response of SPGE.

**Oral SPGE feeding significantly inhibits PC-3 tumor-growth**

Having identified significant *in vitro* anti-proliferative and pro-apoptotic activity of SPGE, we were curious to examine the *in vivo* efficacy of SPGE to inhibit human prostate tumor xenografts subcutaneously implanted in athymic nude mice. We employed a PC-3 cell-line stably-expressing luciferase (PC-3-luc) that allowed real-time visualization and monitoring of prostate cancer growth non-invasively [34]. Animals in the treatment group were fed daily with 400 mg/kg bw SPGE by oral-gavage for six weeks and treatment responses were followed by bioluminescent imaging in longitudinal studies using the same cohorts of mice (Fig. 5Ai). In vehicle-treated control animals, tumors showed unrestricted progression (Fig. 5Ai,ii). In contrast, SPGE-feeding showed a time-dependent inhibition of tumor growth over six weeks (Fig. 5Ai,ii), though significant retardation was evident as early as 2-3 weeks post-treatment (Fig. 5Aii).
Quantification of relative photon counts revealed a ~69% reduction in tumor-volume with a confidence level of p<0.05 (n=8, Fig. 5Aii) at week six compared to vehicle-treated controls. To assess overall general-health and well-being of animals during treatment, body weights were recorded twice a week. SPGE-treatment was well-tolerated and mice maintained normal weight gain (data not shown) with no signs of discomfort during the treatment regimen. To corroborate our bioluminescent imaging data, we also measured tumor volumes using a vernier caliper. As shown in Fig. 5Bi, tumor-volume measurements demonstrated that oral SPGE-treatment for six weeks (42 days) reduced tumor-volume by ~75%. All animals in the control group were euthanized by day 42 post-inoculation due to tumor overburden, in compliance with IACUC-guidelines. At the end point of animal experiments (week six), the excised tumors (Fig. 5Bii) were weighed and a ~65% reduction in tumor weight was observed in SPGE-treated group compared to controls. We next determined the longevity of surviving mice by monitoring them for general health and well-being for ten weeks. Kaplan-Meier analysis revealed a significantly increased survival time with 87.5% animals treated with SPGE surviving until 10 weeks (p<0.05; Fig. 5C). This was a significant prolongation of survival compared to controls where median survival time was only six weeks.

**In vivo mechanisms of SPGE-mediated reduction of tumor growth**

To evaluate the *in vivo* effect of SPGE feeding on the antiproliferative response associated with tumor growth inhibition, tumor tissue-lysates were analyzed for cyclins (including cyclins D1, A, E) and cyclin-dependent kinase inhibitor, p21, using immunoblotting methods (Fig. 6A). SPGE-treatment caused a decrease in cyclin D1, A and E, which correlated with our *in vitro* findings in PC-3 cells (Fig. 6A). In addition, p21 upregulation was evident as a potential mechanism of cell-cycle inhibition of tumor cells (Fig. 6A), which was in accordance with the G1-phase cell-cycle arrest observed *in vitro*. *In vivo* apoptotic response of SPGE feeding in PC-3-luc tumor xenografts was evaluated by caspase 3/7-activity assay and immunoblotting of tumor-lysates for cleaved caspase-3 expression. As expected, cleaved caspase-3 expression (Fig. 6A) as well as caspase-3/7 activity (Fig. 6B) was higher in SPGE-treated tumors compared to controls.

We further asked if SPGE caused regression of xenografted tumors by inhibiting proliferation and triggering apoptosis. H&E-stained tumor sections from SPGE-treated animals revealed large areas of tumor cell death seen as tumor necrosis adjacent to normal looking healthy cells.
Significant loss of tumorigenic cells in SPGE-treated animals (Fig. 6Ci, right, arrow) was consistent with the therapeutic effect of SPGE. However, some viable tumor cells were observed at the periphery of cell death zones. In contrast, microsections from control tumor tissues revealed sheets of tumor cells with high-grade pleomorphic nuclei and angiolymphatic invasion (Fig. 6Ci, left). Furthermore, Ki67-stained tumor sections from SPGE-fed animals showed weak immunoreactivity (Fig. 6Cii) compared to vehicle-fed animals. Tumor sections from SPGE-treated groups also showed a marked increase in cleaved caspase-3 and PARP staining (Fig. 6D) compared with vehicle-fed controls, suggesting induction of robust apoptosis in tumors from SPGE-treated mice.

**Non-toxic effects of SPGE**
Toxicity, particularly in tissues with actively proliferating cells, remains a major concern in prostate cancer patients treated either radiotherapeutically or by chemotherapeutic drug regimes. We found that there were no detectable differences in the histological appearance of tissues including the gut, liver, spleen, lung, brain, heart, testes and bone-marrow from vehicle and SPGE-treated tumor-bearing mice (Suppl. Fig. 5). In addition, colonic crypts from both mice groups showed comparable nuclear Ki67 staining (Suppl. Fig. 6), suggesting that SPGE did not affect normal tissues with rapidly-proliferating cells. Furthermore, complete blood count (e.g. RBC, WBC, Lymphocytes, HGB), serum biochemical-profile markers (ALT, AST, ALP for hepatic function and creatinine, blood urea’s nitrogen (BUN), and electrolytes including potassium, magnesium, sodium, calcium and chlorides for renal function) were within the normal range and similar between the control and SPGE-treated groups (Suppl. Fig. 7).

**Identification of bioactive phytochemicals in SPGE**
Given the significant activity of SPGE, we next attempted to examine and identify its bioactive constituents. To this end, we first performed a simultaneous on-line HPLC-UV and HPLC-MS comparative detection in both positive and negative ion modes for SPGE using acetonitrile (ACN): water (H₂O) solvent system (gradient conditions detailed in Supplementary Data section). The HPLC-UV chromatograms (Suppl. Fig. 8Ai and Bi) show the appearance of 11 peaks. However, when SPGE passed through the MS detector after eluting from UV detector, new peaks (those numbered in red (Suppl. Fig. 8Aii-Aiii) and blue (Suppl. Fig. 8Bii-Biii)
appeared in both positive and negative ion modes, which were lacking UV chromophores. Two
bioconstituents, chlorogenic acid (ChA) and caffeic acid (CA) with m/z values of 353.0 and
179.0 respectively, have been successfully identified in SPGE (Suppl. Fig. 8C) using tandem-
mass spectrometry (MS-MS) technique. The MRM comparison for the respective product ions,
191 for ChA and 135 for CA, between SPGE (Suppl. Fig. 8Di) and a mixture of pure standards
(Suppl. Fig. 8Dii) confirmed the presence of both caffeic and chlorogenic acids in SPGE.
However, 2 additional peaks (in red boxes, Suppl. Fig. 8Di) were observed to be having the same
m/z values as the product ion of ChA (which was not seen in case of pure standards), thus raising
a possibility of the presence of ChA derivatives, which follow similar fragmentation pattern
(353 → 191). Work in our laboratory is underway to unravel the identity of the active ingredients
present in SPGE using state-of-art HPLC-MS techniques.

**DISCUSSION**

The management of advanced prostate cancer or prostate cancer after androgen-therapy failure
poses a critical challenge because options such as radiotherapy and chemotherapy are associated
with serious side effects. Several studies in recent years have convincingly shown that
chemopreventive agents affect the process of carcinogenesis by targeting pathways such as
carcinogen activation, detoxification, DNA-repair, cell-cycle progression, differentiation, and
induction of apoptosis in transformed cells. Besides displaying potent anticancer activity, the
‘golden-rule’ for an agent to qualify as a chemopreventive is that it should be well-tolerated, non-
toxic, easily-available and inexpensive.

Fruits and vegetables are excellent sources of chemotherapeutic and chemopreventive agents
[35] and there is a uniformity of opinion emphasizing consumption of five or more servings of
fruits and vegetables daily to minimize the risk of cancer [4]. Several plant-based food extracts
have been shown to be effective in cancer therapy and prevention such as ripe berry-extracts and
grape-seed extracts [8,36-38]. Essentially, the beneficial effects of fruits and vegetables are due
to their constituent phytochemicals that include polyphenolics, anthocyanins, carotenoids,
alcaloids, and nitrogen- and sulfur- compounds. These phytochemicals have been shown to target
multiple events of neoplastic stages to confer therapeutic benefits and reduce overall cancer risk [39,40]. In addition, several reports indicate that a variety of naturally-occurring compounds such as grape seed-extract, silibinin, green-tea catechins and apples also play an important role in the prevention and treatment of prostate cancer [41-44].

Although widely consumed as a vegetable in several parts of the world such as West Africa and Asia [17], sweet potato greens represent an untapped food resource in the US. According to a USDA report, the greens can be consumed in several forms including raw, cooked, steamed and processed. In addition, the polyphenolic content in leaves is much higher than in other parts of sweet potato such as the petioles, outer-skin and storage-root [18]. Several other reasons exist that merit the encouragement of SPG as a more common vegetable in the US. First, oxalic acid content which is a concern in vegetables because of its predisposition to form crystals within the kidneys is roughly one-fifth in SPG compared to spinach. Second, as a crop, SPG is more tolerant to diseases, pests and moisture than any other leafy-vegetable grown tropically. SPG may be grown even during monsoon season of the tropics thus making it the only vegetable that can be grown right after floods or typhoons. Finally, this vegetable can be harvested several times during the year [24]. Because of these attributes, sweet potato is one of the crops selected by US National Aeronautics and Space Administration (NASA) to be grown in a controlled ecological life support system as a primary food source [45].

Several groups, mostly from Japan, have characterized various polyphenolics and anthocyanins present in sweet potato greens [21]. A recent study reported the growth-suppressive activity of sweet potato leaves in colon cancer cells [19]. Given the several health-promoting attributes of SPG, the principle objective of the present study was to evaluate and establish the anticancer efficacy and associated mechanisms of SPGE-treatment in human prostate cancer cells in vitro and to translate these findings to an in vivo preclinical cancer model. Our study reveals that SPGE causes cell growth inhibition, induces G1-phase arrest accompanied by upregulation of p21 and induction of apoptosis in PC-3 cells. In these studies, down-regulation of cell-cycle effectors, in particular the G1-cyclins, including cyclins D1, A and E is revealed as a plausible antiproliferative mechanism of SPGE in PC-3 cells.
Selective induction of apoptosis is a highly desirable trait of ideal chemopreventive and chemotherapeutic regimens. Our data showed that SPGE efficiently induces apoptosis in PC-3 cells as determined by Annexin-V and TUNEL staining assays. Insights into molecular mechanisms reveal that SPGE-induced apoptosis is largely mitochondrially-mediated and associated with the collapse of the transmembrane potential which results in the expulsion of key apoptogenic molecules such as cytochrome c from the mitochondria. Oral-feeding of SPGE remarkably inhibits tumor growth, which is accompanied by antiproliferative and pro-apoptotic effects together with a decline in cyclin levels, increased expression of p21 and activated caspase-3.

Although dismaying, it is true that present day chemotherapeutic approaches for cancer patients can be as deadly as the disease itself. Toxicity normally includes myelosuppression, immunosuppression, cardiotoxicity and peripheral neuropathy. To assess safety of SPGE, we evaluated hematologic and histopathological toxicity and found no deviations in hematologic variables and organ-associated toxicities in treated mice compared to controls. In addition, the acid-base and electrolyte balances in SPGE-treated animals were also normal compared to controls. Finally, evidence for the potential usefulness of SPGE as a chemopreventive agent in humans was postulated using a body surface area (BSA) normalization method [46]. Using calculations involving the effective \textit{in vivo} dose (400 mg/kg) data, the human-equivalent dose (HED) was determined to be 30 mg/kg SPGE. For an average, 70 kg adult, this translates to an equivalent dosage of \textasciitilde 2.1g SPGE. Considering these facts and the USDA’s Food Guide Pyramid, the HED can be obtained from \textasciitilde 85g, or about a half-cup of raw greens, which can be easily incorporated in a normal daily-diet.

The presence of polar acids eluting early (ChA and CA) with retention times less than 10 min (Suppl. Fig. 8Ai, 8Bi), and relatively non-polar compounds eluting later (peaks with retention times greater than 30 min, Suppl. Fig. 8Ai, 8Bi), might facilitate fractionation of SPGE into two fractions via HPLC-UV, using semi-preparative higher-diameter HPLC columns (allowing higher sample loading) to further identify and characterize the bioactive constituent(s) present in SPGE. In the light of a recent paradigm shift which recognizes that the anticancer attributes of fruits and vegetables are due to an additive or synergistic interplay of the complex phytochemical
mixtures in whole foods [47], it is perhaps likely that the whole SPG extract works through complementary and overlapping mechanisms to offer the most optimal benefits [47,48]. In this case, single bioactive constituents may show anticancer activity at much higher doses that may be toxic, whereas a mixture of multiple compounds may show enhanced activity at lower, non-toxic doses.

In conclusion, our current study is the first to identify the remarkable anticancer activity of sweet potato greens extract in prostate cancer. Our data generate compelling evidence for further evaluation of sweet potato greens as a chemopreventive regimen for prostate cancer. Currently, work in our laboratory is underway to identify and characterize the bioactive constituent(s) of SPGE that either work alone or in an additive or synergistic manner to offer the significant anticancer benefits.

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FIGURE LEGENDS

Figure 1. SPGE is highest in (A) polyphenolic content and (B) anthocyanin content compared to other commercial vegetables like spinach, kale, okra, mustard greens, collard greens and green onions. Values and error-bars shown in the graph represent average and standard deviations, respectively, of three independent experiments (P<0.05).

Figure 2. SPGE inhibits the growth and reproductive capacity of prostate cancer cells. (A) Bar-graphical representation of IC_{50} values of SPGE for various prostate cancer cells and (B) normal prostate epithelial cells. (C) Bar-graph representation and photograph of crystal violet-stained surviving colonies from control and SPGE-treated groups. (Di) Fluorescence micrographs of PC-3 cells stained for Ki67 (green) or DAPI (blue). (Dii) Bar-graph quantitation of Ki67-positive or DAPI-stained cells treated with vehicle or 250 µg/ml SPGE. Values and error-bars represent average and standard deviations, respectively, of three independent experiments (P<0.05).

Figure 3. (A). SPGE perturbs cell-cycle progression by causing a G1-arrest and increases sub-G1 cell population, indicative of apoptosis. (Ai) Cell-cycle progression over dose (0-600 µg/ml) and (Aii) time (0-72h) is shown in a three-dimensional format. (Aiii) Bar-graphs depicting sub-G1 population of PC-3 cells treated with SPGE over varying doses and (Aiv) time. (*,P < 0.05 compared to controls). (B) Immunoblots of cell-lysates treated in absence or presence of 250 µg/ml SPGE for cyclins D1, A, E and p21. The protein levels were determined by quantifying the pixel values of the protein bands using ImageJ on the immunoblots and normalized to the measured value at 0h treatment. Uniform loading was confirmed by β-actin. (Ci) Confocal micrographs of Annexin-V positive cells (green-staining at cellular rim) upon SPGE-treatment for 24h and (Cii) quantitation of Annexin-V or TUNEL positive cells for vehicle and SPGE-treatment over time determined flow-cytometrically. (*,P < 0.05 compared to controls).

Figure 4. SPGE activates the intrinsic apoptotic pathway. (Ai) Immunofluorescence micrographs of vehicle and 250 µg/ml SPGE-treated cells stained for cleaved caspase-3 and PARP. (Aii) Immunoblot analysis of cleaved caspase-3 and cleaved PARP levels in cell-lysates from vehicle and SPGE-treated PC-3 cells. (Aiii) Caspase-3/7 activity assay over time. SPGE alters
mitochondrial transmembrane potential. (*, P < 0.05 compared to controls). (Bi) Histogram-profiles and (Bii) quantitation of cytosolic monomeric JC-1 in unstained (red), control (green) and SPGE-treated (blue) cells that were read flow-cytometrically (*, P < 0.05 compared to controls). SPGE-induced collapse of transmembrane potential was measured by increased green fluorescence. (Biii) Immunoblot analyses for p-Bcl2, total Bcl2, BAX and cytosolic cytochrome c. The protein levels were determined by quantifying the pixel values of the protein bands using ImageJ on the immunoblots and normalized to the measured value at 0h treatment or controls.

**Figure 5.** Dietary feeding of SPGE inhibits human prostate tumor xenograft growth in nude mice. Male nude mice were subcutaneously injected with 10⁶ PC-3-luc cells. (Ai) Bioluminescent images indicating inhibition of tumor-growth over a period of time. (Aii) Graphical representation of the quantitative photon count from control and SPGE-treated mice for six weeks. (Bi) Tumor-growth monitored (by vernier calipers) and presented as tumor-volume in mm³, over a period of 42 days. (Bii) Photographic images of excised tumors and graphical representation of tumor weight. (C) Kaplan-Meier survival graphs of SPGE-treatment over 10 weeks. (*, P<0.05, Aii, Bi).

**Figure 6.** Tumor tissue-lysates express high apoptotic and low proliferation markers. (A) Western blot analysis, (B) Caspase-3/7 activity (*, P<0.05), (Ci) H&E, (Cii) Ki67, (D) cleaved caspase-3 and PARP from control and 400 mg/kg bw SPGE-treated mice tumors.
REFERENCES


Figure 1 (Karna et al.)

A

conc. of CHA equivalents (mg/L)

SPG  spinach  mustard  green  kale  okra  green onion  collard green

Polyphenols

B

monomeric anthocyanin (mg/L)

SPG  spinach  mustard  green  kale  okra  green onion  collard green

Anthocyanins
Figure 4 (Karna et al.)

Ai  control  SPGE

cleaved casp-3

20 μm  20 μm

Ai  0  24  48  72 hr

cleaved casp-3

1  2.2  6.3  12.5

cleaved PARP

1  2.2  1.8  4.0

β-actin

Aiii

casp-3/7 activity x 10^3

0  24  48  72

time (h)

* * *

Bi

Bii

p-Bcl2

Bcl2

BAX

cytochrome c

β-actin

Bi

Bi

Bi
Figure 6 (Karna et al.)

(A) Western blot analysis of cyclin D1, cyclin A, cyclin E, p21, cleaved caspase-3, and β-actin in control (con) and SPGE-treated samples.

(B) Bar graph showing Caspase 3/7 activity in control and SPGE-treated samples. * indicates statistical significance.

(Ci) Hematoxylin and eosin (H&E) staining of control and SPGE-treated samples.

(Cii) Immunohistochemical staining for cleaved caspase-3 in control and SPGE-treated samples.

Ki67 staining in control and SPGE-treated samples.
Suppl. Fig. 1: SPGE suppresses proliferation of human prostate cancer PC-3 cells. (A) Phase-contrast images of PC-3 cells treated with 250 µg/ml SPGE for 0, 24, 48 and 72h. Concentration- and time- dependent cell death of PC-3 cells following SPGE treatment. (B) Cells were plated at a density of 5,000/cm² overnight and treated with either DMSO alone or SPGE at concentrations of 150 to 2000 µg/ml in DMSO for 48h. After 48h, cells were collected upon a brief trypsinization and counted with hemocytometer after trypan blue staining. (C) PC-3 cells were treated with 250µg/ml SPGE for 0, 12, 24, 48 and 72h. Columns, mean total number of blue (dead) cells from three independent samples for each treatment; bars, SD. P<0.05 versus control.
Suppl. Fig. 2: SPGE perturbs cell-cycle kinetics of PC-3 prostate cancer cells in a concentration-time dependent manner. Upon treatment with noted doses and times, cells were harvested for analysis, fixed and stained with propidium iodide, and analyzed by flow cytometry [fluorescence-activated cell sorting (FACS)] using the FloJo software. (A) Quantitative bar-graphical representation of relative percentage of cell population in various phases of the cell-cycle over increasing concentrations of SPGE. This visual depiction shows that there is an increase of G1 phase arrest until 100 µg/ml; thereafter, an arrest in the G2 phase is observable at 200 µg/ml followed by massive cell death (sub-G1) at still higher concentrations. (B) Quantitative representation of relative percentage of cell population in various cell-cycle over time of SPGE treatment. There is an increase in G1 phase population indicating a G1-arrest up to 12h followed by a decline in G1 population and emergence of a significant sub-G1 cell population indicating apoptosis. Representative results of three independent experiments.
Suppl. Fig. 3: SPGE activates the intrinsic apoptotic pathway. (Ai) Quantitation of activated caspase-3 and (Aii) cleaved PARP positive cells. (Bi) Cell-cycle profiles of PC-3 cells that were pretreated with the caspase inhibitor z-vad-fmk before SPGE-treatment. (Bii) Quantitation of sub-G1 from groups as shown in Bi. (Biii) Western-blot analysis of cleaved caspase-3, PARP and β-actin from cell-lysates collected from the group shown in Bi. The protein levels were determined by quantifying the pixel values of the protein bands using ImageJ on the immunoblots and normalized to the measured value at 0h treatment and controls.
Suppl. Fig. 4: (A) Cyclosporin A pretreatment attenuates SPGE-induced apoptosis. Cells were pretreated with cyclosporin A for 3h before treatment with SPGE (250 µg/ml) for 48h, and the apoptotic sub-G1 population was quantified flow cytometrically. (*, P < 0.05). (B) Cyclosporin A pretreatment before SPGE exposure causes attenuation in the drop of mitochondrial transmembrane potential. Bar graph shows quantitation of number of depolarized cells upon various treatments. The decrease in the number of depolarized cells upon SPGE/cyclosporin A treatment was significant at both 24 and 48h compared with the number of depolarized cells upon SPGE treatment alone (*, P < 0.05).
**Suppl. Fig. 5:** SPGE (400 mg/kg bw) does not cause any detectable pathological abnormalities in normal tissues such as gut, liver, spleen, lung, brain, heart, testes and bone marrow. Panels represent hematoxylin and eosin staining of paraffin-embedded 5 µm thick sections of these tissues from vehicle-treated and SPGE treated groups of mice using a 20X objective. These tissues were indistinguishable between the SPGE and vehicle- treated control groups.
Suppl. Fig. 6: Immunohistochemical staining of paraffin-embedded colonic sections from vehicle-treated control and SPGE-treated groups, for Ki67, an indicator of proliferation index. Images were captured by a light microscope (Olympus BX40) using a 10X and 40X objective. The staining pattern was indistinguishable among the control and treated groups indicating absence of SPGE toxicity in tissue with rapidly proliferating tissues.
Suppl. Fig. 7: SPGE feeding did not cause any observable hematological toxicity. (A) Complete blood count analysis of tumor-bearing SPGE-treated mice compared with vehicle-treated control mice. No significant difference could be detected between the two groups in the WBC count (WBC), RBC count (RBC), lymphocytes, hemoglobin concentration (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocytes, and Absolute reticulocyte count (Abs Retic). (B) Organ-associated toxicity was also assessed for both groups and the levels of alkaline phosphatase (ALP), alanineaminotransferase (ALT), aspartate aminotransferase (AST), glucose, and blood urea nitrogen were comparable for the treated and the control groups. (C) Standard electrolyte panel (sodium, potassium, chloride, bicarbonate) also showed no abnormalities in electrolytes. In addition, anion gap, a useful indicator of abnormalities in the acid-base balance, was also indistinguishable among the two groups.
The mobile phase for profiling SPGE by HPLC consisted of solvent A (2.5% formic acid in water) and solvent B (2.5% formic acid in acetonitrile (ACN)). The mobile phase gradient elution was set as follows: initial 10% B for 25 min, achieving 40% B at 30 min followed by 70% B over the next 5 min which was held for an additional 5 min; reconditioning to 40% B at 45 min and finally decreasing to 10% B in next ten minutes ending the run at 60 min with a flow rate of 0.9 ml/min. 0.5 mg/ml of 10 µl SPGE, dissolved and filtered in pure methanol, was injected into the system and the resultant HPLC-UV peaks were detected at 380 nm.

The HPLC-MS analyses were performed in tandem with HPLC-UV using the same column and mobile phase as described above on HP1100 series Instrument (Agilent Technologies, Wilmington, DE) interfaced to an Agilent 6400 Series Triple Quadrupole LC/MS equipped with an electrospray ionization source, which can be operated in both the positive and negative-ion modes. The nebulizer and collision gases were nitrogen and helium, respectively, with the former set at 40 psi and the instrument was scanned over the mass range 100-900 Da in MS2 Scan mode followed by selective ion monitoring (SIM) of the most abundant ions in MS2 SIM mode (both positive and negative ion modes). A drying gas temperature of 300°C, drying gas flow rate of 9 l/min and capillary voltage of ±3000V were the spray chamber parameters for both scan and SIM modes. Tandem mass spectrometry analysis acquired separately by direct infusion of SPGE revealed the presence of chlorogenic acid (ChA, m/z = 353) and caffeic acid (CA, m/z = 179). This was further confirmed by multiple reaction monitoring (MRM) of ChA and CA in SPGE against pure standards using MRM mode. ChA followed the fragmentation pattern of 353→191, where 191 is the m/z of its product ion and CA fragmented from 179 to a product ion with m/z of 135. Collision energies of 15 eV and 10 eV were used to obtain the above mentioned product ions for ChA and CA respectively, at a fragmentor voltage of 135 V.

Suppl. Fig. 8. Identification of bioactive constituents in SPGE using HPLC-UV/MS and tandem mass spectrometry (LC-MS/MS) analyses. (Ai and Bi) HPLC-UV profile of SPGE (Aii-Aiii) HPLC-MS group selective ion monitoring (SIM) of most abundant ions in SPGE in positive ion mode with m/z values ranging from 100-450 (Aii) and 450-900 (Aiii). The m/z values in positive ion mode of peaks marked as 1=432, 460; 2=118, 144; 6a=460, 520; 6=212, 401, 432, 460, 599; 7=330, 401; 8=144, 149, 330, 401, 432; 9=342, 401, 429, 432, 460, 473; 11a=149, 401. The peaks in red lack UV chromophore and were only identified in LC-MS (Bi-Biii) HPLC-MS SIM
of most abundant ions in SPGE in **negative ion mode** with m/z values ranging from 100-450 (Bii) and 450-900 (Biii). The m/z values in negative ion mode of peaks marked as \(1=397, 441; \ 2=191, 201, 341, 353; \ 3=191; \ 4=191, 353; \ 5=179; \ 6=289, 327, 341, 441; \ 7=201, 327; \ 8=289, 341; \ 9=191, 193, 201, 353, 419, 529, 533; \ 9a=191, 397, 519; \ 10a=191, 311, 327, 341; \ 10=191, 311, 353, 397, 441, 519; \ 10b=397, 529; \ 11=311; \ 12=489.\) The very low abundant peaks in blue were only identified in LC-MS and lack UV chromophore. (C) Tandem mass spectrometry identification of known standards, Caffeic acid (CA, m/z=179.0) and Chlorogenic acid (ChA, m/z=353.0) in SPGE. The numbers associated with the ions represent the respective peak numbers in Fig. 7Bii. For example, the negative ion, 191, as observed in Fig. 7C, was found to be abundant in peaks 2, 3, 9, 9a, 10 and 10a of Fig. 7Bii. However, the ions, 136, 161 and 173 were not observed to be abundant enough in the scan mode, and thus were not chosen for group SIM. (Di-Dii) Identification and comparison of CA and ChA in SPGE (Di) with respect to pure standards (Dii) in MRM mode of LC-MS. The product ions of CA and ChA are 135 and 191 respectively. The peaks in red boxes (Di) are unknown compounds with m/zs similar to the product ions as ChA.