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Polar biophenolics in sweet potato greens extract synergize to inhibit prostate cancer cell proliferation and *in vivo* tumor growth

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Polyphenolic phytochemicals present in fruits and vegetables indisputably confer anticancer benefits upon regular consumption. Recently, we demonstrated the growth-inhibitory and apoptosis-inducing properties of polyphenol-rich sweet potato greens extract (SPGE) in cell culture and *in vivo* prostate cancer xenograft models. However, the bioactive constituents remain elusive. Here, we report a bioactivity-guided fractionation of SPGE based upon differential solvent polarity using chromatographic techniques that led to the identification of a remarkably active polyphenol-enriched fraction, F5, which was ~100-fold more potent than the parent extract as shown by IC_{50} measurements in human prostate cancer cells. High-performance liquid chromatography–ultraviolet and mass spectrometric analyses of the seven SPGE fractions suggested varying abundance of the major phenols, quinic acid (QA), caffeic acid, its ester chlorogenic acid, and isochlorogenic acids, 4,5-di-CQA, 3,5-di-CQA and 3,4-di-CQA, with a distinct composition of the most active fraction, F5. Subfractionation of F5 resulted in loss of bioactivity, suggesting synergistic interactions among the constituent phytochemicals. Quantitative analyses revealed a ~2.6- and ~3.6-fold enrichment of QA and chlorogenic acid, respectively, in F5 and a definitive ratiometric relationship between the isochlorogenic acids. Daily oral administration of 400 mg/kg body wt of F5 inhibited growth and progression of prostate tumor xenografts by ~75% in nude mice, as evidenced by tumor volume measurements and non-invasive real-time bioluminescence imaging. These data generate compelling grounds to further examine the chemopreventive efficacy of the most active fraction of SPGE and suggest its potential usefulness as a dietary supplement for prostate cancer management.

Introduction

Nutrition research has long favored a reductionist approach emphasizing single phytochemical-based health benefits. However, the idea of synergy among constituent phytochemicals present in whole foods is gaining momentum. Several reports underscore the benefits of a multitargeted approach offered by a synergistic mixture of phytochemicals present in whole foods (1–6). It is becoming recognizable that a whole or partially purified extract of a plant offers significant advantages over a single-isolated ingredient. This can be most appropriately described as the 'herbal shotgun' approach, as opposed to the 'silver bullet' method of conventional medicine (7) and may partially explain the limited success of clinical trials involving individual phytochemicals such as vitamin E and beta-carotene for cancer chemoprevention (8–10). Nonetheless, these data solidify the notion that health benefits from fruits and vegetables may not be solely because of the isolated single compounds but are mainly due to additive and/or synergistic interactions among components that 'partner' together in the concoction at their relative concentrations. For example, studies with skin-bearing apples have demonstrated strong antiproliferative activity in human colon and hepatic cancer cells compared with apples without skin or its most studied constituent, vitamin C (11).

Well known for their abundance in fruits and vegetables, polyphenols are versatile molecules containing several hydroxyl groups with multiple aromatic rings. The amphiphilic phenolic moiety of polyphenols blends the hydrophobic character of its planar aromatic core with the hydrophilic nature of its polar hydroxy substituent (12). The inherent biophysicochemical properties of the phenolic group display a wide repertoire of functional roles, including plant resistance against microbial pathogens and protection against solar radiation. Epidemiological studies suggest an inverse relationship between consumption of polyphenol-rich foods, such as cocoa, red wine, tea, fruits, and vegetables, and the incidence of chronic diseases including cancer (13–15). Although it is easy to evaluate the protective effect of a single phytochemical, for example, a single polyphenolic compound, the health benefits of dietary polyphenols are difficult to discern when numerous phytochemicals including polyphenolics, flavonoids, lignans, and tannins are active and work synergistically. The complexity of polyphenols in foods limits the identification of definitive compositions of partially purified extracts that display superior efficacy compared with single agents or whole foods. Nevertheless, it is likely that a reductionist approach involving fractionation of a whole extract may result in the increased concentration of bioactive constituents in a particular subfraction, thus enhancing efficacy.

Sweet potato greens (SPG), *Ipomoea batatas*, a significant source of dietary polyphenols, are widely consumed as a fresh vegetable in Asia, in particular, Taiwan and China (16). Caffeic, monocaffeoylquinic (chlorogenic acid), dicaffeoylquinic and tricaffeoylquinic acids are reported as the major phenolic constituents of these greens (16,17). Particularly, anthocyanins in SPG have been described to be cyanidin type rather than peonidin type (18). SPG have been shown radical scavenging, antimutagenic, antidiabetic, antibacterial, anti-inflammatory and anticancer activities (19,20). We recently reported the significant anticancer property of sweet potato greens extract (SPGE) in both *in vitro* and *in vivo* prostate cancer models (21). Although several analytical studies have identified major phenolic compounds in SPG, this study is the first report to detail bioactivity-guided fractionation of SPGE, emphasizing the importance of synergistic interactions among various bioactive components to confer remarkable *in vitro* and *in vivo* effects in prostate cancer models.

Abbreviations: CA, caffeic acid; ChA, chlorogenic acid; DMSO, dimethyl sulfoxide; FC, Folin–Ciocalteau; HPLC, high-performance liquid chromatography; MTT, thiazolyl blue tetrazolium bromide; PARP, poly (ADP ribose) polymerase; PC-3-luc, luciferase-expressing PC-3; QA, quinic acid; RT, retention time; SPGE, sweet potato greens extract; TLC, thin-layer chromatography; UV, ultraviolet.

Materials and methods

Cell culture and materials

Human prostate cancer cell lines, PC-3 cells, were cultured in RPMI-1640 media (Mediatech, Manassas, VA) combined with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT) and 1% penicillin/streptomycin solution. Cells were cultured in a humidified atmosphere at 37°C and 5% CO_2. Thiazolyl blue tetrazolium bromide (MTT dye, 98% thin-layer chromatography [TLC]) and dimethyl sulfoxide (DMSO) were from Sigma–Aldrich (St Louis, MO). Quinic acid (QA), chlorogenic acid (ChA), caffeic acid (CA) and Folin–Ciocalteau (FC) reagent, ACS grade methanol, ethyl acetate, hexanes and high-performance liquid chromatography (HPLC) grade solvents were from Sigma–Aldrich. Stably transfected luciferase-expressing PC-3 (PC-3-luc) cells and luciferin were from Caliper Life Sciences (Alameda, CA).

SPGE preparation

SPGE was prepared as described previously (21). Briefly, young Whatley/Loretan (TU-155) variety of sweet potato (*I. batatas*) greens harvested on

day 30 were obtained as part of collaboration with the Nutrition department at Tuskegee University. SPGE was prepared by soaking air-dried leaves in methanol overnight for 3 consecutive days. The supernatant was collected daily and was finally concentrated *in vacuo* (Buchi Rotavap) followed by freeze-drying using a lyophilizer to a solid-powder form, which was stored at −80°C until tested. SPGE stock solution was prepared by dissolving 10 mg in 1 ml of DMSO and various concentrations were obtained by appropriate dilutions. Batch-to-batch variation was evaluated by analysis of polyphenolic content in SPGE by FC method.

Determination of total phenol content

Total phenolic content was determined by FC method using ChA as the standard. ChA (0.5 g) was dissolved in 10 ml ethanol and then diluted to 100 ml with water to make a final concentration of 5 g/l. A total of 50, 100, 250 and 500 mg/l concentrations of standards and 0.5, 1, 2, 3, 4 and 5 mg/ml concentrations of test extracts were prepared in distilled water. A total of 20 µl sample of standard or test extract was dissolved in 1.58 ml water, followed by 100 µl FC reagent. This mixture was mixed thoroughly and incubated no longer than 8 min. Sodium carbonate solution of 300 µl was added to the above mixture and was incubated for 2 h at room temperature. A final volume of 2 ml was measured for absorbance at 765 nm and the results were expressed as milligrams of chlorogenic acid equivalents per gram dry material. The linear range of the calibration curve was 0.02–0.2 mg/ml. All samples were analyzed in triplicates.

Fractionation of SPGE

Classical column chromatographic separation was performed on SPGE (3 g) that was loaded on to a silica gel column, which was run down using hexane: ethyl acetate solvent system starting with 500 ml of 100% hexane. The fraction was collected in a conical flask and stored at 4°C. This was followed by elution using 500 ml of hexane:ethyl acetate solution (90:10). Subsequently, a gradient increase in the percentage of ethyl acetate (10% each time) was incurred in the mobile phase to elute various components of SPGE into different fractions (Supplementary Table 1, available at *Carcinogenesis* Online). After the elution of 50:50 hexane:ethyl acetate fraction, hexane was replaced with 50% methanol to elute the highly polar components. With an increment of 10% methanol each time (starting from 50:50 methanol:ethyl acetate), the column was finally eluted with 100% methanol to ensure complete elution of all components. A total of 17 fractions, thus, obtained were concentrated *in vacuo* (Buchi Rotavap, New Castle, DE) followed by separation on TLC. Based on the observed bands, fractions with similar TLC profiles (Rf values) were pooled to finally obtain seven fractions (F1–F7). All seven fractions were freeze-dried using a lyophilizer and were stored at −80°C until tested.

In vitro proliferation assay

Briefly, 5×10^3 cells/well in a 96-well format were treated with gradient concentrations of test fractions dissolved in DMSO (0.1%). The concentrations used were 1, 10, 25, 50, 75, 100 and 250 µg/ml. F5 was further tested at lower concentrations (0.075, 0.1, 0.5, 1, 5 and 10 µg/ml). After 48 h incubation, cells were washed with phosphate-buffered saline followed by addition of 5 mg/ml MTT solution. Cells were then incubated at 37°C in dark for 4 h. The formazan product was dissolved by adding 100 µl of 100% DMSO after removing the medium from each well. The absorbance was measured at 570 nm using a Spectra Max Plus multiwell plate reader (Molecular Devices, Sunnyvale, CA).

Colony survival assay

A total of 1000 PC-3 cells were seeded in a six-well plate and were treated with 10 µg/ml F5 for 24 and 36 h, washed and then replaced with regular RPMI medium including the controls. After 7 days, each well was washed twice with 1× phosphate-buffered saline, and fixed and stained with the clonogenic reagent for 20 min followed by rinsing with tap water. The stained colonies in the control and treated wells were then counted. A colony was arbitrarily defined to consist of at least 50 cells.

HPLC with ultraviolet and mass spectrometric detection

HPLC–ultraviolet (UV) separation of seven fractions was achieved on a HP1100 series Instrument (Agilent Technologies, Wilmington, DE) equipped with a photodiode array detector, using an Agilent Zorbax reversed-phase (SB-C18, 3.0×250 mm, 5.0 µm) column. The mobile-phase system consisting of solvent A (0.1% formic acid in water) and solvent B (ACN) was employed to achieve the separations. The gradient elution was set as follows: starting at 10% B, achieving 20% B at 20 min followed by 60% B over the next 20 min, which was held for an additional 10 min, reconditioning to 10% B at 51 min and ending the run at 60 min with a flow rate of 0.4 ml/min. A total of 10 µl of each fraction (1.0 mg/ml), dissolved and filtered in 25% ACN, was injected into the system and the resultant HPLC–UV peaks were detected at 326 nm.

The HPLC–MS analyses were performed in tandem with HPLC–UV using the Agilent Zorbax reversed-phase (SB-C18, 3.0×250 mm, 5.0 µm) column interfaced to an Agilent 6400 series Triple quadrupole liquid chromatography–mass spectrometry equipped with an electrospray ionization source, operated in negative-ion mode. The nebulizer and collision gases were nitrogen and helium, respectively, with the former set at 40 psi. A drying gas temperature of 300°C, drying gas flow rate of 9 l/min and capillary voltage of 3000 V were the spray chamber parameters. The presence of QA [m/z = 191, retention time (RT): 2.7 min], ChA (m/z = 353, RT: 11.6 min) and CA (m/z = 179, RT: 15.5 min) in the fractions was confirmed using selected-ion monitoring and the HPLC RT of the same in all the fractions against pure standards.

Subfractionation of F5 using analytical HPLC–UV chromatography

The most active fraction F5 was further fractionated into two subfractions based on the RT, F5-A (0–25 min) and F5-B (25.1–50 min) using analytical HPLC. The subfractions, thus, collected were concentrated and lyophilized to determine the *in vitro* efficacy by MTT assay.

Experimental design for the combination studies and determination of synergy

The pure standards, QA, ChA and CA, were combined as a mixture to mimic their respective concentrations as quantitated in F5. This mixture was used at various increasing gradient concentrations to test its *in vitro* efficacy against PC-3 cells. Specifically, the concentrations used were, 0.075, 0.1, 0.5, 1, 5 and 10 µg/ml. The percentage of cell proliferation was measured by MTT assay.

In vivo tumor growth and bioluminescent imaging

A total of 1×10^6 PC-3-luc cells were subcutaneously injected in the right flank of 6-week old male BALB/c nude mice (Harlan Laboratories, Indianapolis, IN). When mice developed palpable tumors, they were randomly divided into three groups of eight mice each. Control group received vehicle (phosphate-buffered saline with 0.05% Tween-80, pH = 7.4) and the treatment group received 400 mg/kg body wt F5 by oral gavage daily. Real-time bioluminescent imaging of luciferase activity in live mice was employed to monitor tumor growth using the IVIS *in vivo* imaging system (Caliper Life Sciences) using the Live Imaging software. Briefly, mice anesthetized with isoflurane were intraperitoneally injected 25 mg/ml luciferin and imaged with a charge-coupled device camera. An integration of 20 s with four binnings of 100 pixels was used for image acquisition. The relative photon count at the tumor site of the mice from vehicle or F5-treated groups was quantitated as the number of photons leaving a square centimeter of tissue and radiating into a solid angle of 1 steradian (photons/s/cm^2/sr). All animal experiments were performed in compliance with Institutional Animal Care and Use Committee guidelines.

Immunoblot analysis and immunofluorescent microscopy

Tumor lysates treated with vehicle and 400 mg/kg body wt F5 were subjected to western blot analysis. Membranes were probed for cleaved caspase-3 and cleaved poly (ADP ribose) polymerase (PARP) along with β-actin, which was used as a loading control. Paraffin-embedded tumor sections from control and F5-treated groups were processed and immunostained with apoptotic markers, cleaved caspase-3, cleaved PARP and the proliferation marker Ki67. Fluorescent images were captured using confocal microscopy.

Human prostate cancer cell lines PC-3 were treated with 10 µg/ml F5 and cell lysates were collected at 0, 6, 12, 24 and 48 h. Immunoblot analysis was performed on the F5-treated and control samples by probing for cleaved PARP and β-actin to confirm the induction of apoptosis.

Histopathological analysis

Mice were euthanized after 6 weeks of F5 or vehicle feeding by exposing to CO_2 for 2 min. Blood was collected by cardiocentesis in accordance with our standard Institutional Animal Care and Use Committee protocol. The organs were immediately collected, formalin fixed and paraffin embedded. A total of 5 µm sections were stained with hematoxylin and eosin. Microscopic evaluation was performed by a pathologist in a blinded manner.

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 among various treatments, with *P*-values of ≤ 0.05 considered statistically significant. Furthermore, a two-way analysis of variance was performed to evaluate the differences between vehicle- and F5-fed groups *in vivo* and *P*-values were obtained from two-sided tests for statistical significance.

RESULTS

Fractionation of SPGE

SPGE is non-toxic and inhibits prostate cancer growth both *in vitro* and *in vivo* (21). To gain insights into the nature of compounds present in the whole extract, we employed a 'top-down logic' wherein we

fractionated the whole extract using classical column chromatography. This led to the sequential separation of subfractions from the complex whole extract based upon their physicochemical characteristics such as polarity and solubility. To achieve optimal fractionation of SPGE, we employed a mobile-phase system that ranged from the nonpolar hexanes to highly polar methanol (Supplementary Figure 1 and Table 1, available at *Carcinogenesis* Online). The methanolic extract of SPGE was loaded onto the column and binary solvent combinations were used as the mobile phase. Finally, passing 100% methanol through the column ensured complete elution of all compounds. This strategy yielded 17 fractions of varying polarity as shown schematically in Supplementary Figure 1, available at *Carcinogenesis* Online. The 17 fractions, thus, obtained were subjected to TLC and fractions with comparable R_f values were pooled together to finally yield seven fractions (Figure 1A). Our next step was to perform a comparative quantitation of total polyphenolic content of all seven SPGE fractions. Using FC method, different fractions showed varying total polyphenolic content (Figure 1B). The quantitative comparison revealed that F5 contains ~2-fold higher phenolic content compared with SPGE (Figure 1B). Given that polyphenolic content has been correlated with bioactivity, these data prompted us to examine the *in vitro* efficacy of the various SPGE fractions.

A moderately polar fraction, F5, exhibits remarkable antiproliferative activity in prostate cancer cells

Hence, we next determined the half-maximal concentration of growth inhibition (IC_{50}) for the seven SPGE fractions in PC-3 cells using the MTT assay. The IC_{50} values of F1–F7 were in the range of ~1–200 μg/ml (Figure 1C). Indeed the differential total phenolic content and polarity of various components that define a fraction might

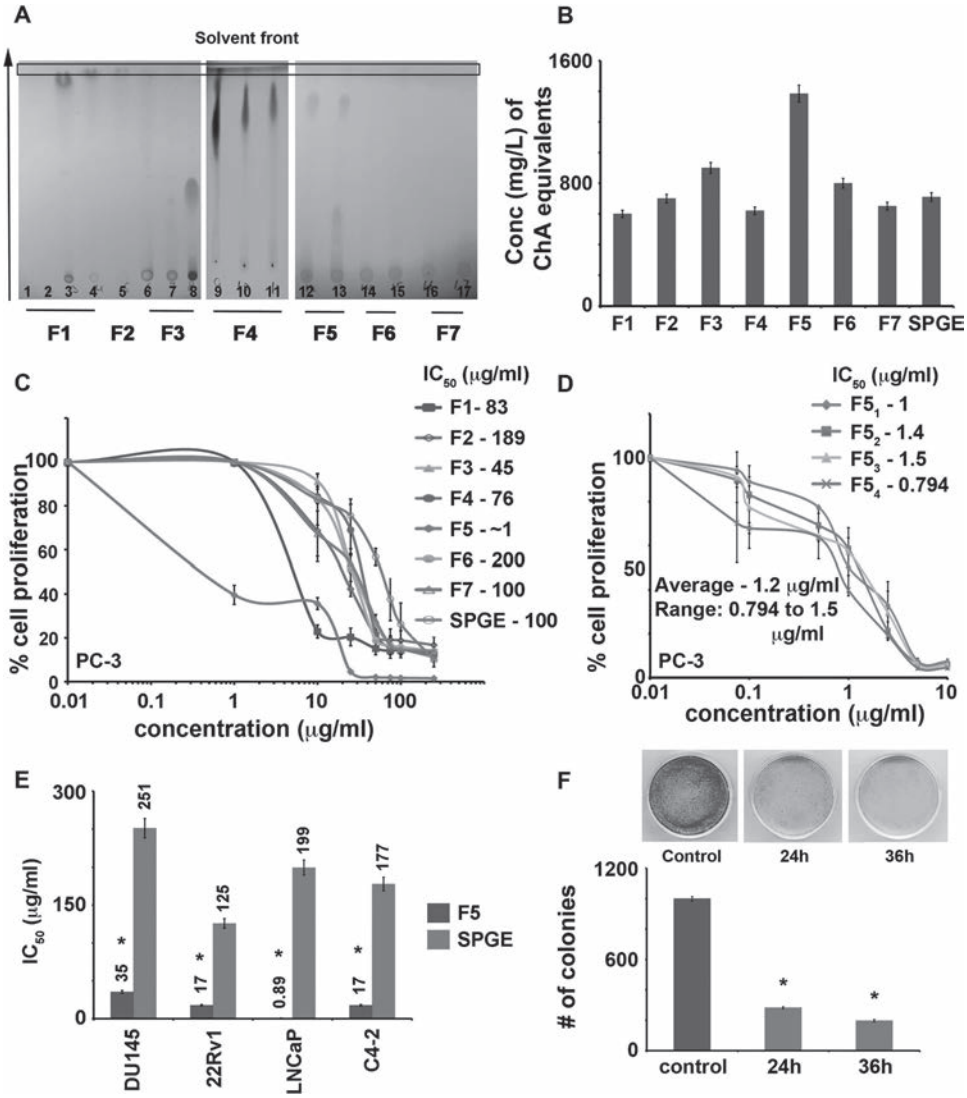

Fig. 1. Fractionation and evaluation of bioactivity of various SPGE fractions. (**A**) TLC of 17 SPGE fractions using an ethyl acetate:hexane (2:1) system for fractions 1–8; dichloromethane:methanol (3:1) for fractions 9–11 and 2:1 dichloromethane:methanol for fractions 12–17. Fractions with similar R_f profiles were pooled together to finally obtain seven fractions. Quantitation of total polyphenol content in all SPGE fractions. (**B**) Quantitation of total phenolic content of all the SPGE fractions quantified via FC method. SPGE and its fractions inhibit proliferation of PC-3 prostate cancer cells. (**C**) Plot of cell survival versus concentrations of fractions tested for PC-3 cells. The IC_{50} values are indicated in the legend of line plot. Cells were treated for 48 h with increasing gradient concentrations of the seven fractions (F1–F7) and the parent SPGE. The percentage of cell proliferation was measured by MTT assay. (**D**) Determination of IC_{50} of F5. PC-3 cells were treated with the most active fraction, F5, from four different batches at increasing concentrations (0.075–10 μg/ml) for 48 h. The percentage cell proliferation was measured by MTT assay. (**E**) F5 shows enhanced inhibition of proliferation in various prostate cancer cells. Bar graphical representation of IC_{50} values (mentioned above) of SPGE and F5 tested in DU145, 22Rv1, LNCaP and C4-2 cells. Cells were treated for 48 h with increasing gradient concentrations of SPGE and F5. The percentage of cell proliferation was measured by MTT assay. (**F**) F5 inhibits the clonogenic capacity of prostate cancer cells. Bar graphical representation and photograph of crystal violet-stained surviving colonies from control and F5-treated groups. Results shown are means from three independent experiments (*, $P < 0.05$ compared with controls).

underlie the range of antiproliferative activity displayed by these fractions. Intriguingly, among the seven fractions, F5 was the most active and its IC_{50} value was initially calculated to be approximately 1 μg/ml (Figure 1C). To precisely determine the IC_{50} value of F5, we then tested still lower concentrations (0.075, 0.1, 0.5, 1, 5 and 10 μg/ml) of F5 subfraction obtained from four different batches ($F5_1$, $F5_2$, $F5_3$ and $F5_4$) in PC-3 cells (Figure 1D). The IC_{50} of F5 was found to be within a range of 0.794–1.5 μg/ml (Figure 1D), which was ~100-fold more potent compared with the whole SPGE extract (IC_{50} = 100 μg/ml). In addition, F5 exhibited better efficacy in other prostate cancer cell lines (LNCaP, 22Rv1, DU145 and C4-2; Figure 1E) compared with SPGE, suggesting the generality of the effect of F5 on a variety of prostate cancer cells.

We next performed a clonogenic or colony formation assay to evaluate the capacity of a cell to proliferate to form a colony upon removal of the drug. Antiproliferative activity of F5 was demonstrated when several PC-3 colonies were observed in case of control, and the F5-treated cells were found to only partially retain their colony forming ability (Figure 1F). The relative clonogenicity of control versus F5-treated PC-3 cells can be visually observed in the representative micrographs shown above the bar graphical quantitation of the colonies in Figure 1F.

F5 shows enrichment of major phenolic components of SPGE

Having identified the differential bioactivity of SPGE fractions, our next step was to perform a comparative quantitation of the phenolics present in all the seven SPGE fractions by LC-UV/MS analysis. Three major phenolics, QA (m/z = 191), CA (m/z = 179) and ChA (m/z = 353), were identified to be present in SPGE (21), along with other isochlorogenic acids such as 4,5-di-caffeoylquinic acid (4,5-di-CQA, m/z = 515), 3.5-di-caffeoylquinic acid (3,5-di-CQA, m/z = 515) and 3,4-di-caffeoylquinic acid (3,4-di-CQA, m/z = 515) (Figure 2Aii, B and Bi). Further analysis of F1 through F7 compared with SPGE demonstrated the differential relative abundance of the major phenols, QA, CA and ChA, and the three isochlorogenic acids (Figure 2A, Ai and Aii and Supplementary Tables 2 and 3, available at *Carcinogenesis* Online). For example, F1 and F2, the fractions of lower polarity, showed an absence of QA, CA and 4,5-di-CQA (Figure 2A, Ai and Aii). The CA content was found to be high in F3 and F4 as opposed to the ChA amounts (Figure 2Ai, Supplementary Table 2, available at *Carcinogenesis* Online). However, the most active fraction F5 exhibited the highest amounts of QA and ChA (Figure 2A, Ai and Aii and Supplementary Table 2, available at *Carcinogenesis* Online). The enrichment of 4,5-di-CQA, 3,4-di-CQA and 3,4-di-CQA was observed from F3 onwards (Figure 2A and Aii). However, there was a decrease in their quantities in F6 and F7 compared with F4 and F5 (Figure 2Aii and Supplementary Table 3, available at *Carcinogenesis* Online). F4 was found to be enriched in all the three isochlorogenic acids with 3,5-di-CQA being the most abundant, whereas the content of 3,4-di-CQA is enhanced in F5 (Figure 2A and Aii and Supplementary Table 3, available at *Carcinogenesis* Online). Fractions 6 and 7 exhibited a decrease in the composition of isochlorogenic acids (Figure 2Aii and Supplementary Table 3, available at *Carcinogenesis* Online).

Differential ratio of constituents in F5 compared with SPGE

The remarkable efficacy of F5 prompted us to examine the composition of this potent fraction. LC-UV/MS analysis of both SPGE and F5 confirmed the differential abundance of several phenolic compounds (Figure 2B and Bi, Supplementary Table 4, available at *Carcinogenesis* Online), which were found to be enriched in F5. The hydroxycinnamic acids, ChA and CA along with QA in SPGE and F5 were quantitated using the respective pure standards. Tandem-mass spectrometric analysis affirmed the presence of QA, ChA and CA in SPGE and F5 (Figure 2B and Bi). The selected-ion monitoring of QA (191), ChA (353) and CA (179) confirmed their elution in both F5 and SPGE exactly at the same RTs (Figure 2A and Bi). Notably, the ratio between QA, ChA and CA differed between F5 and SPGE. The ratio of QA:ChA:CA in F5 has been (as observed in Figure 2Ai) calculated to be 6:1:0.005, whereas these same compounds existed in a 9:1:0.6 ratio in SPGE (Figure 2Ai). On the other hand, the isochlorogenic acids were found to be in a ratio of 1:3:4.6 in F5 compared with the 1:3:1.4 seen in SPGE (Figure 2Aii). Furthermore, the chemical fingerprints of F5 and SPGE as seen in Figure 2B and Bi establish an obvious difference between their compositions. For example, the compound u4 with m/z value of 385 could not be observed in SPGE, whereas it was present at quantifiable levels in F5 (Supplementary Figure 2 and Table 5, available at *Carcinogenesis* Online). QnG including four other unknown compounds, u5–u8, was absent in F5 (Supplementary Figure 2 and Table 5, available at *Carcinogenesis* Online). Among the major phenolics, there was an approximately 2.6- (QA), 3.6- (ChA) and 3-(3,4-di-CQA) fold increase in F5 compared with SPGE. It is, thus, reasonable to speculate that these differences might be responsible for the higher bioactivity of F5 compared with SPGE in prostate cancer cells.

Subfractionation of F5 results in loss of bioactivity

Our next question was to examine if subfractionation of F5 into its constituent components employing analytical liquid chromatography resulted in identification of single agents that were much more active compared with the whole fractions. Repeated injections of 10 μl of F5 were made into the HPLC system and the eluate from 0 to 25 min was collected as subfraction, F5-A (Figure 3Ai) and the remaining part from 25.1 to 50 min as F5-B (Figure 3Aii). As seen in Figure 3Ai–Aiii, F5-A is a combination of QA, ChA and CA, whereas F5-B constituted the three isochlorogenic acids. These subfractions, thus, obtained were concentrated and lyophilized. Next, we again employed a bioactivity-guided approach to determine the efficacy of F5 subfractions. Both the subfractions were reconstituted in DMSO to yield 1 mg/ml stock solutions that were then used to dose PC-3 cells at gradient concentrations for 48 h. An MTT assay that performed postincubation showed that neither of the individual subfractions was as active as F5. Intriguingly, F5-A and F5-B did not show 50% inhibition of cell growth even at the highest test concentration (250 μg/ml) and hence, their IC_{50} values could not be determined. This clearly indicated that F5-A needs F5-B and vice versa to mimic F5's activity, hence suggesting a synergistic interplay among F5 constituents. Additionally, the clear differences among the compositions of each subfraction compelled us to investigate if the loss of activity in the subfractions was related to their respective compositions.

F5 phytochemicals exhibit synergism

To corroborate this observation, we next tested commercially available QA, ChA and CA in combination at varying concentrations against PC-3 cells. Quantitative data point out that 1 mg of F5 contains 115 μg of QA, 16 μg of ChA and 0.1 μg of CA. Given the IC_{50} value of F5 is approximately 1 μg/ml (based on the range of 0.794–1.5 μg/ml, Figure 1D), F5 (1 μg) actually consists of 115 ng QA, 16 ng ChA and 0.1 ng CA. Assuming that these three compounds are the major players that contribute to F5-A's activity, we tested the bioactivity of the mixture of the three pure standards by measuring the percentage of cell proliferation using the MTT assay. PC-3 cells were treated with this mixture in an increasing gradient concentration (0.075, 0.1, 0.5, 1, 5 and 10 μg/ml), ensuring that the relative quantities of the three compounds (QA + ChA + CA, the major constituents of F5-A), at each test concentration bore the same ratiometric relationship as was observed between them in F5. This mixture formulation, thus, mimicked the composition of F5-A (as it exists in F5). Evaluation of the *in vitro* efficacy of this subfraction might also enable exclusion of the possible antagonism of other yet unknown phytochemicals in F5-A. Our data suggested that even at the highest concentration tested (10 μg/ml), the formulated mixture of pure standards did not show 50% inhibition in cell growth. As the pure standard mixture of three compounds could not reproduce equivalent efficacy as of F5, we speculate that the other unknown components in F5-A perhaps did not exert an antagonistic influence. Thus, our results from *in vitro* experiments testing various combinations of pure standards (QA, ChA and CA) suggested that higher efficacy of F5 could not only be ascribed to enhanced total polyphenolic content but also to

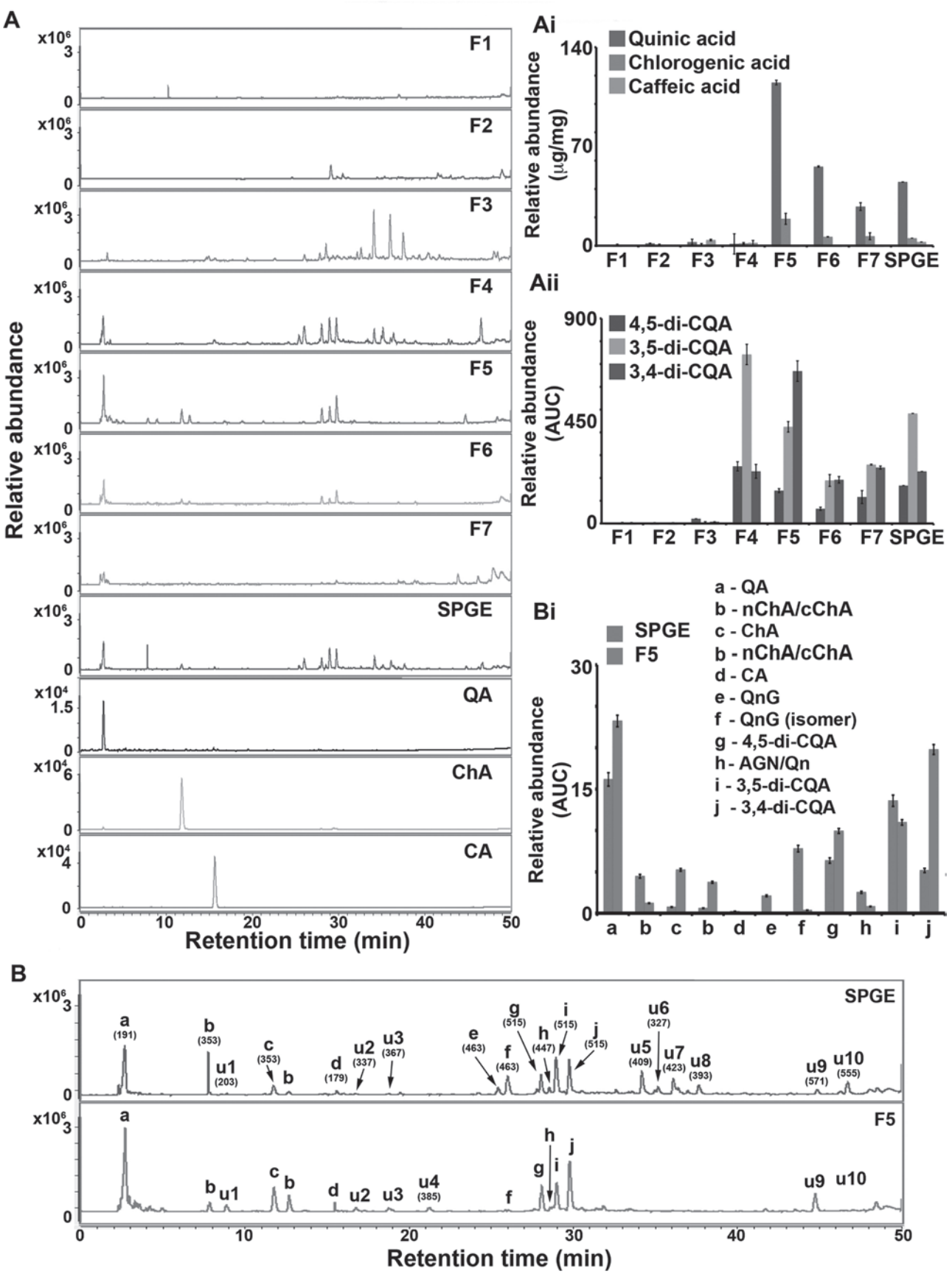

Fig. 2. F5 exhibited the presence of major phenols of SPGE. (**A**) LC-UV/MS comparison of constituents present in F1–F7 with respect to SPGE. Quantitation of the identified phenols, that is, QA, CA and ChA in F1–F7 and SPGE was obtained by performing a standard curve analysis using pure standards. Three other compounds, 4,5-di-CQA, 3,5-di-CQA and 3,4-di-CQA, were quantitated relative to each other using the area under the curves. (**Ai**) Varying concentrations of

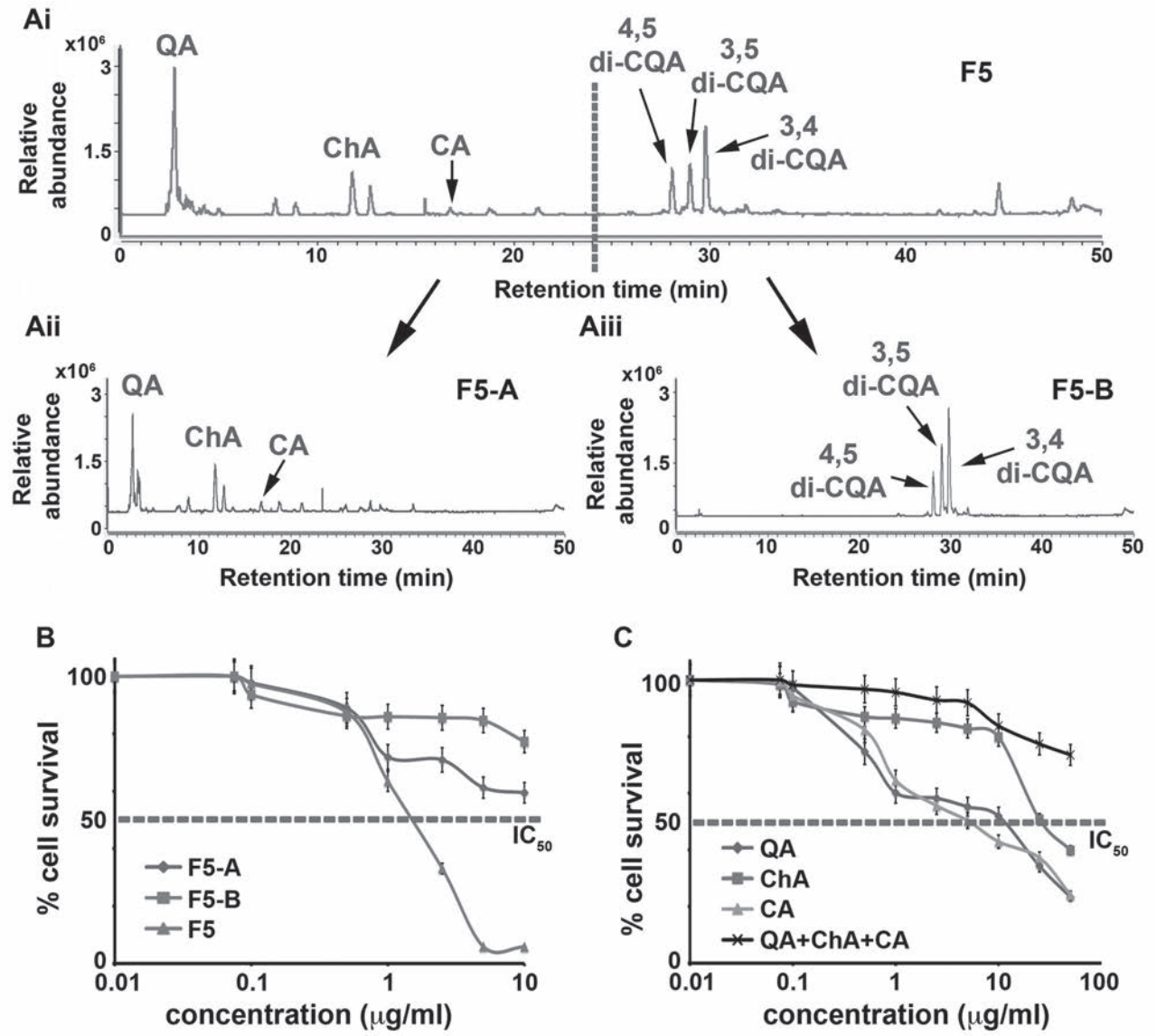

Fig. 3. Sub fractionation of F5. (**Ai**) The chromatographic profile of F5 in MS scan mode. F5 was collected as two parts, F5-A (0–25 min) and F5-B (25.1–50 min) by injecting F5 for 17 times in the analytical HPLC system. MS scan mode chromatograms confirm the presence of (**Aii**) QA, ChA and CA in F5-A, (**Aiii**) 4,5-di-CQA, 3,5-di-CQA and 3,4-di-CQA in F5-B. (**B**) Line plot of percentage cell survival versus concentration of F5 subfractions, F5-A and F5-B, tested against PC-3 cells. Cells were treated for 48 h with increasing gradient concentrations of the two subfractions of F5 and F5. The percentage of cell proliferation was measured by MTT assay. (**C**) Line plot of percentage cell survival versus concentration of a combination of QA, ChA and CA in increasing gradient concentrations treated for 48 h in PC-3 cells. The percentage of cell proliferation was measured by MTT assay. The test mixture of the three pure standards was constituted based upon their individual concentrations as in F5. Results shown are means from three independent experiments.

possible synergistic interactions associated with definitive ratiometric composition of these phenolics.

The other subfraction F5-B also tested to be non-active. Hence, the loss of bioactivity in both subfractions F5-A and F5-B individually suggested existence of synergism among the characterized and the yet unknown F5 components. It is perhaps likely that other identified compounds such as Qn, nChA, cChA, QnG and astragalin contribute to uphold the superior activity of F5. These data also emphasize the importance of the occurrence of QA, ChA, CA, 4,5-di-CQA, 3,5-di-CQA and 3,4-di-CQA in a distinct ratio, as found in F5 to display remarkable activity. To further substantiate our *in vitro* data, we tested the efficacy of F5 in an *in vivo* prostate xenograft model as discussed in the next section.

Oral feeding of F5 inhibits prostate tumor growth in vivo

Given the significant difference in the *in vitro* antiproliferative activity of F5 compared with SPGE, we next evaluated its *in vivo* efficacy 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), which enables real-time

←
QA, CA and ChA in all SPGE fractions. (**Aii**) Varying concentrations of 4,5-di-CQA, 3,5-di-CQA and 3,4-di-CQA in all SPGE fractions. (**Bi**) Different amounts of bioactive constituents such as neochlorogenic acid (nChA, m/z = 353), cryptochlorogenic acid (cChA, m/z = 353), quercetin-glucoside (QnG, m/z = 463) and its isomer (QnG isomer, m/z = 463), quercetin (Qn, m/z = 447) and astragalin (m/z = 447) in SPGE versus F5 were identified using HPLC–UV/MS and tandem-mass spectrometry (LC-MS/MS) analyses. (**B**) Tandem-mass spectrometry identification of 12 constituents (QA, nChA, cChA, ChA, CA, QnG, QnG isomer, 4,5-di-CQA, astragalin, Qn, 3,5-di CQA and 3,4-di-CQA) and 10 other unknown m/z values (u1–u10) in F5 and SPGE. Values and error bars represent average and standard deviations, respectively, of three independent experiments.

visualization and longitudinal monitoring of prostate cancer growth non-invasively in mice. We have shown previously that SPGE inhibits the *in vivo* tumor growth by 69% (21). We found that the treatment group fed with 400 mg/kg body wt F5 daily by oral gavage for 6 weeks (Figure 4Ai and Supplementary Figure 3, available at *Carcinogenesis* Online) showed a time-dependent inhibition of tumor growth (Figure 4Ai, Aii and B) compared with the vehicle-treated control animals. A relative total flux quantitation revealed a ~75% inhibition in tumor volume with a confidence level of $P < 0.05$ ($n = 8$, Figure 4Aii) as measured at week 6 for the F5-fed group, compared with vehicle-treated controls. Body weights were recorded twice a week to evaluate the general health and well-being of animals during treatment. Mice in the F5 treatment group exhibited normal weight gain with no signs of discomfort during the treatment regimen. All animals in the control group were euthanized due to tumor overburden, in compliance with Institutional Animal Care and Use Committee guidelines. At the end of week 6, the excised tumors (Figure 4Di and Dii) were weighed posteuthanasia and a ~74% reduction in tumor weight was observed in F5-treated groups, compared with controls.

F5 mediates apoptosis and reduction of tumor growth in vivo

To evaluate *in vivo* inhibition of tumor growth upon oral feeding of F5, we immunostained for Ki67 (MIB-1), a well-known marker of cell proliferation. Essentially, the Ki67 antigen is a non-histone protein expressed in all phases of the cell cycle except G_0. We found that Ki67-stained tumor sections from F5-fed animals showed decreased immunoreactivity (Figure 5A) compared with vehicle-fed animals. Tumor sections from F5-treated groups also showed an increase in cleaved caspase-3 and PARP staining (Figure 5Ci and Cii) compared with vehicle-fed controls, suggesting induction of robust apoptosis in tumors from SPGE-treated mice.

Furthermore, the tumor tissue lysates were immunoblotted for cyclin D1 and the apoptotic markers, cleaved caspase-3 and cleaved PARP (Figure 5B). Cyclin D1 plays a central role in the regulation of proliferation, linking extracellular signaling environment to cell-cycle progression. There was a decrease in cyclin D1 expression in F5-fed tumor lysates suggesting a cessation of cell-cycle progression. Further, as expected, the cleaved caspase-3 and PARP expression (Figure 5B) were higher in F5-treated tumors compared with controls. Similar trend was observed in PC-3 cell lysates, where F5 treatment

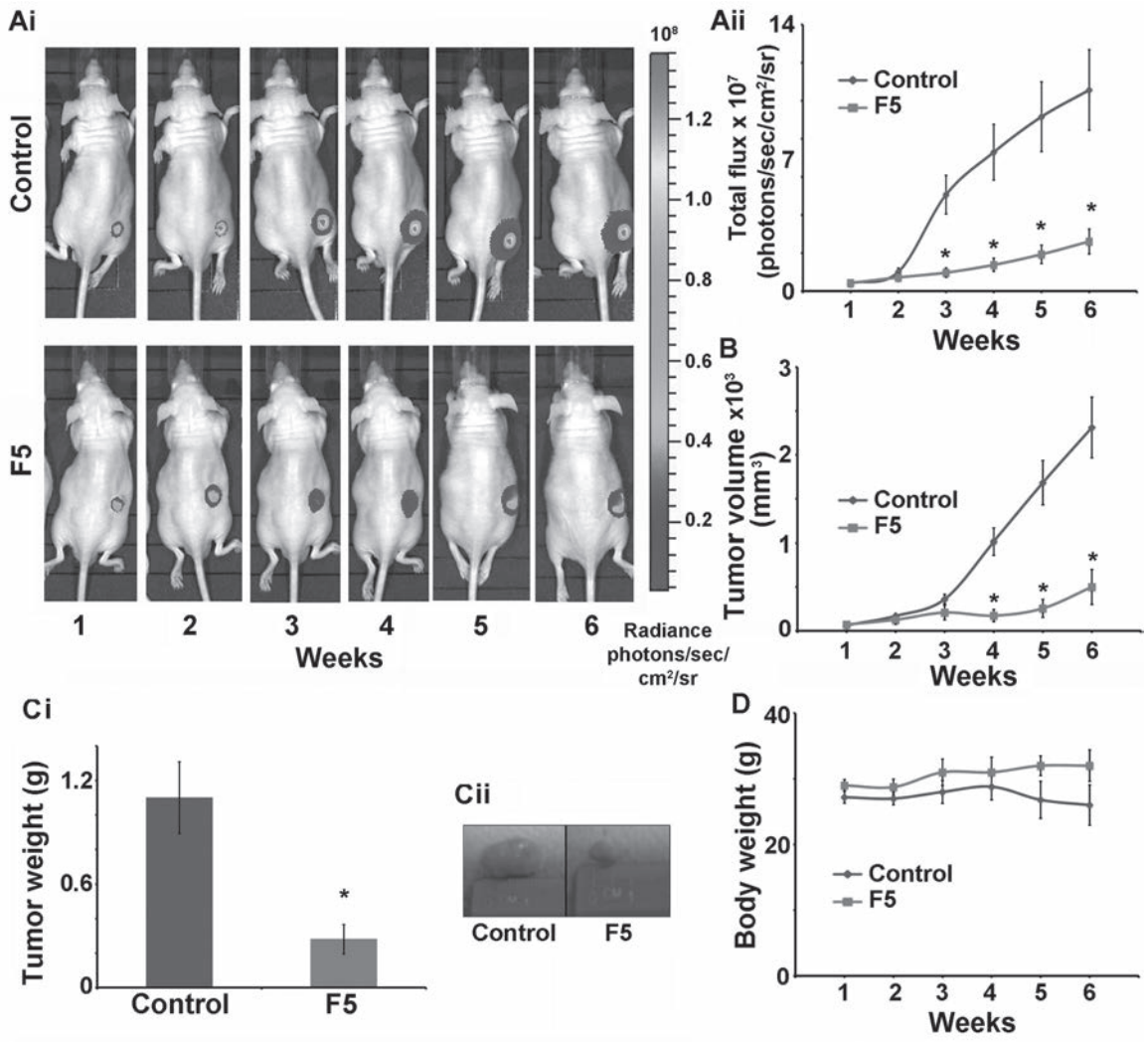

Fig. 4. Dietary feeding of F5 showed enhanced inhibition of human prostate tumor xenograft growth in nude mice compared with SPGE. Male nude mice were subcutaneously injected with 10^6 PC-3-luc cells. (**Ai**) Representative bioluminescent images of one animal per group, indicating progression of tumor growth over 6 weeks. Mice images showing bioluminescent tumors of all animals in vehicle and F5-fed animal groups at week 6 are shown in Supplementary Figure 3, available at *Carcinogenesis* Online. (**Aii**) Graphical representation of quantitative radiance measured as the number of photons leaving a square centimeter of tissue and radiating into a solid angle of 1 steradian (photons/s/cm^2/sr) from vehicle- and F5-treated mice for 6 weeks. (**B**) Tumor growth monitored (by vernier calipers) and presented as tumor volume in cubic millimeter over a period of 6 weeks. (**Ci**) Graphical representation of tumor weight. (**Cii**) Photographic images of excised tumors. (**D**) Graphical representation of body weight of vehicle- and F5-treated mice [*, $P < 0.05$ (two-way analysis of variance), Aii, B and C].

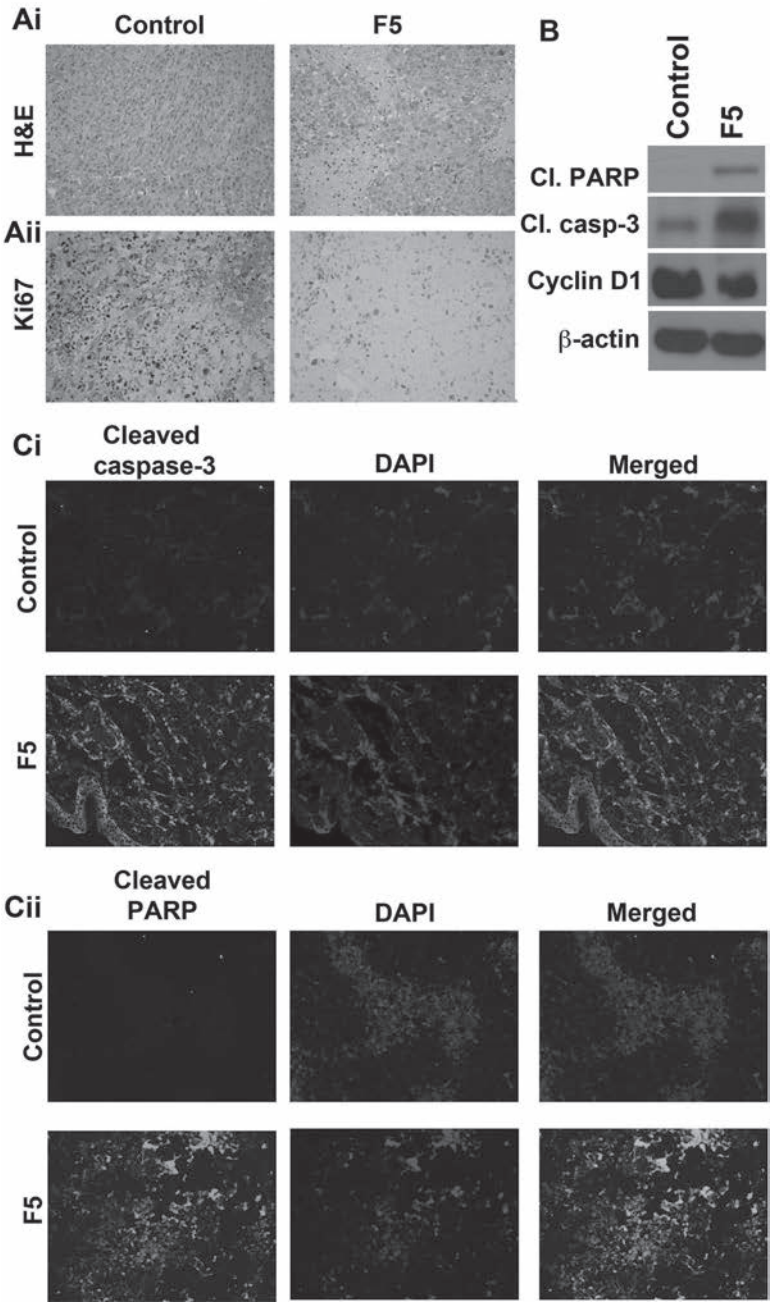

Fig. 5. F5 induces apoptosis. Histochemical micrographs of tumor tissue sections from control and 400 mg/kg F5-fed groups stained for (**Ai**) hematoxylin and eosin and (**Aii**) Ki67. (**B**) Western blot analysis of tumor tissue lysates probed for cleaved caspase-3, cleaved PARP and cyclin D1. β-Actin was used as a loading control. Immunofluorescence micrographs of tumor sections from control and 400 mg/kg body wt F5-treated mice stained for (**Ci**) cleaved caspase-3 and (**Cii**) cleaved PARP. All images and blots shown are representative of three independent experiments.

showed increased cleaved PARP expression compared with controls (Supplementary Figure 4, available at *Carcinogenesis* Online).

Non-toxicity of F5 in vivo

Toxicity is overly concerning and is often observed in prostate cancer patients undergoing either radio or chemotherapy. The histopathological evaluation of the tissues of intestine, spleen, liver, lung, brain, heart, adrenal gland, and testes from both vehicle- and F5-fed mice (Figure 6A) revealed no detectable differences in architecture. Furthermore, analysis of biochemical markers in the sera (alanine transaminase, aspartate transaminase, alkaline phosphate, lactic acid dehydrogenase, creatinine kinase and urea nitrogen) collected from both vehicle- and F5-fed mice was observed to be within the normal range (Figure 6Bi–Biii).

Discussion

Mother Nature is perhaps the best combinatorial chemist providing a broad array of phytochemical constituents naturally occurring in whole foods or whole extracts that may confer optimal health benefits for humans. The use of 'whole extract versus single-isolated constituent' to achieve desirable health benefits has been an issue of ongoing debate the past several years. Based on the hypothesis of reduced disease risk upon consumption of whole grains, fruits and vegetables, attempts to search for the 'magic bullet' in whole foods do not seem unreasonable. However, because carcinogenesis is a multistage process in which several normal cellular pathways go awry, it is unlikely that a single agent could serve to combat this dreaded disease. In addition, accumulating evidence is encouraging the consumption of whole

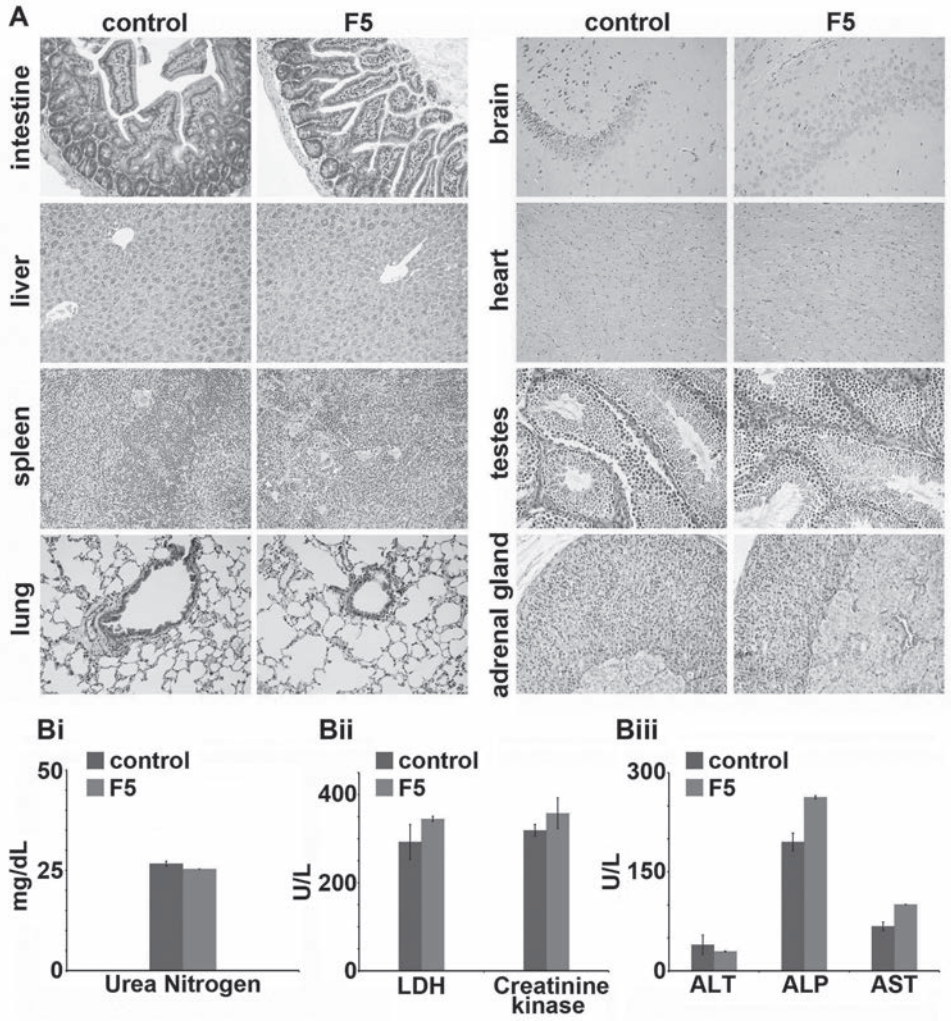

Fig. 6. F5 is non-toxic. (**A**) The panels represented above are paraffin embedded; 5 μm thick tissue sections of intestine, liver, spleen, lung, brain, heart, testes and adrenal glands stained with hematoxylin and eosin and observed using a ×20 objective. No visible differences were observed between the vehicle- and F5-fed groups. (**B**) Organ-associated toxicity was not observed in F5-fed group. (**Bi**) Urea nitrogen levels in F5-fed group were comparable with that of vehicle-fed group. (**Bii**) The lactate dehydrogenase (LDH) and creatinine kinase levels in both the groups did not show any abnormal differences. (**Biii**) The biomarkers of liver, alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were comparable for both the groups.

or partially purified food extracts over single-isolated constituents due to the existence of synergistic interactions among phytochemicals (22,23) in whole foods. Consumption of whole foods, namely fruits and vegetables, although a very healthy option, may necessitate intake of impractical amounts to achieve the optimal physiological concentration of the desired phytochemicals that offers *in vivo* chemopreventive and chemotherapeutic benefits. Thus, a reductionist approach to yield the 'right' fraction that comprises the most efficacious phytochemicals in the most synergistic ratios might serve as an optimal 'middle ground' between a single phytochemical and the intake limitations of whole foods. The fraction that comprises an ideal mix of active ingredients intricately tendering the right hydrophilicity, molecular size and solubility would perhaps target malignant cells via multiple pathways not only to treat but also to prevent disease recurrence. Our study is the first of its kind to provide evidence that phytochemicals, when present in specific compositions, can impart superior efficacy and generates enthusiasm for testing the efficacy of F5 in preventing disease recurrence and imparting chemopreventive benefits. Our study reiterates the importance of the 'natural milieu' created by Mother Nature that exists in whole foods and is perhaps preserved and enhanced in fractionated extracts for excellent anticancer benefits.

Several plant-based extracts have been subjected to rigorous fractionation to identify the active ingredients. However, it has been widely reported that certain fractions of the whole extracts enriched in phytochemicals such as polyphenols, carotenoids, and anthocyanins (24–26) have proven to be more potent than their respective whole extracts. For example, procyanidin-rich fraction obtained from grape seeds (27) and apple extracts (28), polyphenol-rich pomegranate fraction (29) and semipurified proanthocyanidin-rich cranberry fractions (30) have shown efficacy both *in vitro* and *in vivo* compared with their parent counterparts in the induction of apoptosis, inhibition of tumor growth and activation of antiangiogenic pathways in cancer cells. Furthermore, studies investigating the complex interplay between anthocyanins, proanthocyanidins, polyphenols and flavonol glycosides resulting in supra-additive anticancer benefits (31,32) support the concept of synergism among the constituent phytochemicals of a whole extract.

SPG, a commercially available polyphenol-rich vegetable, has been shown to be effective against prostate cancer in both *in vitro* and *in vivo* models (21). Although studies reporting the bioactive components of SPGE (18,33–35) exist, variability in the extraction methods employed preclude reliable interpretation of those bioactivity studies. In the current study, we have employed a relatively simple and holistic approach to make SPGE and have followed a bioactivity-guided fractionation of the whole leaf extract to obtain and identify active fractions/constituents. Our strategy using mobile-phase systems of varying polarity to elute SPGE down the classical silica gel column to fractionate and

elute components of different polarities in different solvent systems (Supplementary Figure 1, available at *Carcinogenesis* Online) has identified a medium-polar fraction, F5, (eluted via 50:50 and 40:60 ethyl acetate and methanol system) that exhibited the highest antiproliferative efficacy in prostate cancer cells (Figure 1C and D). F5 was determined to be ~100-fold more potent compared with the parent, SPGE, which prompted us to further investigate F5's composition. Among the repertoire of bioactive polar phenols enriched in F5, we have identified QA, ChA and CA in a distinct ratio (Figure 2A and Ai). The analytical data revealed higher abundance of QA, and ChA over CA in F5 compared with the whole extract. As it can be observed in Figure 2Ai, the pattern of QA:ChA:CA in F5 is similar to that in SPGE, but these compounds are highly enriched in F5 compared with the parent whole extract. On the contrary, the signature of isochlorogenic acids in SPGE differs from F5 (Figure 2Aii), wherein the most abundant in the whole extract is 3,5-di-CQA and 3,4-di-CQA is maximally present in F5. Furthermore, a 7 day stability study suggested a stable shelf-life of F5 when stored at 4°C, as there were no variations in the concentrations of individual constituents that make up this most active fraction.

The *in vitro* efficacy of F5 was supported by the synergy study performed with the pure standards in combinations (Figure 3C). This strengthened the concept that QA, ChA, CA, 4,5-di-CQA, 3,5-di-CQA and 3,4-di-CQA act synergistically among themselves and with other unknown components to exert maximum efficacy and emphasized the importance of the ratio of phytochemicals for the observed antiproliferative activity. This observation is further supported by the quintessential green tea polyphenol concoction, Polyphenon E, which has been proven to confer optimal anticancer benefits via a specific combination of five different catechins, including epicatechin, gallocatechin gallate, epigallocatechin, epicatechingallate and most abundantly EGCG (36). This specific formulation of green tea is in clinical cancer trials funded through the National Cancer Institute to investigate the benefits of tea catechins in humans.

Furthermore, subfractions of F5 could not outperform F5, suggesting the potential additive or synergistic interactions among F5 phytochemicals (Figure 3B) supporting our speculation. A similar observation made by Liu *et al.*, where the subfractions of black raspberry extract's active fraction, WBR-95, showed diminished antiangiogenic efficacy compared with the refined parent (37), lends support to our observations as seen in Figure 3.

Several studies suggest that individual foods or extracts may offer advantages over their isolated constituents, suggesting that factors within foods may improve the absorption, metabolism or retention of the bioactive food components or that multiple bioactive compounds within the food/extracts can exert additive or synergistic effects. However, in other scenarios, the whole food or its extract itself may not be as effective as its isolated components, suggesting that the food may contain other constituents, which can attenuate the response by negatively modifying the absorption, metabolism or site of action of the bioactive food constituent. Surprisingly, we found that the *in vivo* efficacy of F5 was only slightly higher than the whole parent SPGE extract, which may be due to the inherent complexity of *in vivo* physiological systems. Although bioluminescence imaging measurement showed a ~75% tumor growth inhibition observed in F5-fed group (Figure 4) compared with the vehicle-treated group, this most active fraction of SPGE could only improve the efficacy of the parent by a small ~10% increment (21). We speculate that this could be because of the disorganization of the plant matrix or disruption of 'natural milieu' during the fractionation process, which could have led to the loss of bioactive components that played a crucial role in dictating favorable pharmacokinetic characteristics. We cannot exclude the possibility that F5 may require interreactivity or dependency on other components in the whole food source to augment oral bioavailability, which means that systemic levels of the polyphenols achieved may be several-fold less than their effective concentrations in *in vitro* systems.

Although the rationale of pharmacological efficacy of single-isolated compounds over whole foods exists, there are several factors such as dose, bioavailability, metabolism and toxicity favoring the latter. In particular, specific factors affecting phenolic bioavailability include matrix of food sources, processing condition during food preparations, chemical compositions and molecular physicochemical properties of the phenolic molecules (38). The diverse molecular forms of phenolics due to alterations in sugar moiety such as glycone or aglycone analogs are known to cause variations in bioavailability levels (38). Furthermore, gastrointestinal pH and enzymatic secretion levels, microbiota and age have been established as crucial factors affecting digestion and absorption of phytochemicals. Equally, the role of interactions among food components and their interplay with gastrointestinal secretions contribute significant effects in determining bioavailability of phytochemicals (38).

QA, enriched in F5 compared with the parent extract, is a common constituent of our diet. Reports suggest that although QA is not responsible for any efficacy, it nutritionally supports the *in situ* synthesis of essential metabolites such as tryptophan and nicotinamide in the gastrointestinal tract. Hence, it in turn leads to DNA repair enhancement and NF-kB inhibition via increased nicotinamide and tryptophan production (39). The other phenolic acids in F5, namely CA and ChA, belong to the most abundant class of polyphenols called hydroxycinnamic acids that are widely present in a large variety of fruits and vegetables (40,41). CA is the major representative of this class and extensively exists as a conjugate with QA as seen in ChA (40). Several reports have established that the bioavailability and efficacy of these hydroxycinnamic acids depend on their uptake and metabolism in the gut mucosa (41–43). Literature suggests that although CA is readily absorbed in the small intestine and can be detected in the blood plasma (44–46), ChA is poorly absorbed. However, ChA is detected only in the urine with no structural changes (47,48), indicating its differential metabolism compared with CA. Although the metabolism of ChA is not well studied and is controversial (42), some groups believe that it is usually hydrolyzed into CA and O-methylated metabolites in the lower intestine due to enzymatic reactions by the gut microflora (42,44), suggesting that the bioactivity could be due to CA. Considering this evidence, our quantitation data (Figure 3Ai) leave us pondering if higher levels of ChA in F5 are limiting its *in vivo* bioavailability and thus leveling its efficacy to that observed for the whole extract. Another line of thought that seems plausible is that the remarkable activity of F5 (containing high ChA) as observed *in vitro* is not recapitulated *in vivo* due to limited absorption and metabolic conversions of ChA. Nonetheless, detailed pharmacokinetic evaluation is warranted to delineate the discrepancy between the *in vitro* and *in vivo* data.

Hence, as the natural balance of polyphenol content in SPGE is perturbed during the fractionation process, the differences in the *in vitro* and *in vivo* absorption and metabolism efficiencies due to this perturbation are likely to be responsible for the discrepancy observed between the *in vitro* and *in vivo* efficacy.

Intriguingly, our unpublished data reveal that the composition of whole SPGE and its subfractions thereof is dependent on the variety (Jewel or TU-155), age of leaves at the time of harvest (30, 45, 60 or 75 days) and the mode of processing of the leaves (air-dried, frozen or freeze-dried). Several other confounding variables such as cultivation season, soil, and rainfall are highly likely to play a role in influencing the nature and composition of the plant extract. We, thus, envision that these varietal differences in the total phenolic content, QA/ChA/CA and isochlorogenic acid content among other unknown components may possibly affect the antiproliferative efficacy of the whole extract and its derived fractions.

Nevertheless, the idea of non-toxic dietary supplements of specific phytochemicals in defined ratios as in F5 would be beneficial to bypass the potential limitations in absorption and assimilation of active whole food components, such as variations in human genetic profiles affecting nutritional absorption (49). Also, it is highly likely that the variability of unidentified components in F5 and their standardization to constitute a therapeutic blend might provide valuable insights for a clinical dietary intervention. It is extremely encouraging that F5 was able to retain the anticancer attributes of the whole extract while demonstrating increased solubility, generating impetus to propose its usefulness as a non-toxic dietary supplement, comprised of specific phytochemicals in distinct ratios.

In conclusion, our study strengthens the evidence that plant polyphenols exhibit synergism to confer efficacy to fruits and vegetables. This approach, attempting to deconstruct the possible inherent synergies among the mixture of phytochemicals present in SPGE fractions, unveiled specific enrichment of components in F5 that nestle together to exert superlative activity. Our future efforts are directed toward characterization of the yet unknown constituents in F5 and further investigation of the chemopreventive efficacy of F5 and the mechanisms involved, providing direction for the effective dose regimen and design of a commercialized dietary supplement for prostate cancer management.

Supplementary material

Supplementary Tables 1–5 and Figures 1–4 can be found at http://carcin.oxfordjournals.org/

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