

ORIGINAL MANUSCRIPT

Synergistic interactions among flavonoids and acetogenins in Graviola (*Annona muricata*) leaves confer protection against prostate cancer

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Abstract

Phytochemical complexity of plant extracts may offer health-promoting benefits including chemotherapeutic and chemopreventive effects. Isolation of ‘most-active fraction’ or single constituents from whole extracts may not only compromise the therapeutic efficacy but also render toxicity, thus emphasizing the importance of preserving the natural composition of whole extracts. The leaves of *Annona muricata*, commonly known as Graviola, are known to be rich in flavonoids, isoquinoline alkaloids and annonaceous acetogenins. Here, we demonstrate phytochemical synergy among the constituents of Graviola leaf extract (GLE) compared to its flavonoid-enriched (FEF) and acetogenin-enriched (AEF) fractions. Comparative quantitation of flavonoids revealed enrichment of rutin (~7-fold) and quercetin-3-glucoside (Q-3-G, ~3-fold) in FEF compared to GLE. *In vivo* pharmacokinetics and *in vitro* absorption kinetics of flavonoids revealed enhanced bioavailability of rutin in FEF compared to GLE. However, GLE was more effective in inhibiting *in vitro* prostate cancer proliferation, viability and clonogenic capacity compared to FEF. Oral administration of 100 mg/kg bw GLE showed ~1.2-fold higher tumor growth-inhibitory efficacy than FEF in human prostate tumor xenografts although the concentration of rutin and Q-3-G was more in FEF. Contrarily, AEF, despite its superior *in vitro* and *in vivo* efficacy, resulted in death of the mice due to toxicity. Our data indicate that despite lower absorption and bioavailability of rutin, maximum efficacy was achieved in the case of GLE, which also comprises of other phytochemical groups including acetogenins that make up its natural complex environment. Hence, our study emphasizes on evaluating the nature of interactions among Graviola leaf phytochemicals for developing favorable dose regimen for prostate cancer management to achieve optimal therapeutic benefits.

Introduction

Extensive research reports over the past few decades indicate that high intake of fruits and vegetables are associated with reduced cancer risk (1–8). The importance of including fruits and vegetables in our daily diet as a measure of cancer chemoprevention is encouraged by several initiatives by the National Institute of Cancer (NCI) (9). Phytochemicals, constituents of fruits and vegetables, have been shown to target multiple stages of cancer, thus reducing overall cancer burden. These phytochemicals widely present in regular dietary sources include

lutein, zeaxanthin, allyl sulfides (garlic), gingerols, paradols, shogaols (ginger), lycopene (tomato), caffeine (coffee), curcumin (turmeric), gallic acid, punicalagin (pomegranate), sulforaphane, indoles, isothiocyanates (cruciferous vegetables like broccoli, cabbage, brussel sprouts and cauliflower), epigallocatechin gallate (green tea), proanthocyanidins (grape seed extract), etc. (2,5,6,8,9). However, there exist several phytochemicals and plant-based foods whose anticancer therapeutic benefits and mechanisms of action are yet to be explored by mankind.

Received: December 27, 2014; Revised: March 23, 2015; Accepted: March 30, 2015

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Abbreviations

AEF	acetogenin-enriched fraction
CMC	carboxy methyl cellulose
FEF	flavonoid-enriched fraction
GLE	Graviola leaf extract
Q-3-G	quercetin-3-glucoside
PK	pharmacokinetics

Whole foods, comprising an extensive complex array of phytochemicals, have been known to exert complementary and overlapping mechanisms acting via multiple targets to impart optimal health benefits. Isolating a single or a group of active compounds from the whole foods may not be as effective even at high doses but rather have potential toxic side effects. Previous attempts to isolate single pure phytochemicals, as in the case of β -carotene (10,11) have seldom shown an effective pharmacokinetic (PK) behavior of the isolated phytochemicals, in that their optimal physiological concentrations could not be attained even at higher doses. Although pure phytochemicals were shown to be highly active in the *in vitro* setting, their *in vivo* therapeutic failure led to further investigation of various mechanisms contributing to the better efficacy of whole foods. Also, there is a plausibility of superior and crucial roles played by lesser known minor components of complex mixtures (like whole foods) that have been under-studied and ignored in exerting superior bioactivity. For example, the absorption, metabolism and elimination of various phytochemicals may differ and result in their varying bioavailability upon consumption as single agents versus whole foods (5,6,12). Such differences are yet to be unveiled and thus compel further investigation to understand the complexity of whole foods.

Annona muricata, commonly known as Graviola or Soursop, a native to the Amazon basin in South America and Southeast Asia, has been used to cure a wide range of human diseases including inflammation, rheumatism, diabetes, hypertension, insomnia, parasitic infections and cancer (13–20). The fruits, leaves, stems and roots of Graviola are known to be rich in flavonoids, isoquinoline alkaloids and annonaceous acetogenins (13,14,21–27). The active annonaceous acetogenins have been shown to successfully induce death in cancer cells that are resistant even to chemotherapeutic drugs (24,28–31). Despite their remarkable antiproliferative efficacy, these annonaceous acetogenins have been attributed to debilitating side effects such as neurotoxicity suggesting that these components can easily cross the blood–brain barrier and are even known to cause atypical Parkinson's disease, thus restricting their development as new drug entities (28,32,33). However, studies have shown a plausible chemotherapeutic role of whole Graviola extract against cancer (13–15,17,18,34). A recent study reported effects of whole Graviola against cancer cell proliferation, metabolism and tumor growth inhibition in both *in vitro* and *in vivo* pancreatic cancer models (18).

The additive and/or synergistic interplay of constituent phytochemicals in whole foods offer health-promoting, and disease-fighting beneficial effects (5,6). Thus, there is an urgent need to understand the bioactivity-based complexity of whole foods like Graviola. Hence, we undertook a detailed evaluation of the *in vitro* and *in vivo* anticancer efficacy of Graviola Leaf Extract (GLE) in prostate cancer by evaluating the contributions of flavonoid-enriched (FEF) and acetogenin-enriched (AEF) fractions toward GLE's efficacy.

Our data demonstrate the superior anticancer efficacy exerted by GLE in both *in vitro* and *in vivo* prostate cancer

models. We developed a sensitive, specific and robust LC–MS/MS method for quantitation of Graviola flavonoids, rutin, quercetin-3-glucoside (Q-3-G), quercetin and kaempferol to study their collective interactions and pharmacological significance when present in their natural environment. We further monitored the PK parameters of flavonoids upon oral feeding of GLE and FEF to underscore the presence of significant *in vivo* interactions among constituent phytochemicals of Graviola leaves.

Materials and methods

Cell culture, chemicals and reagents

Graviola leaf powder was purchased from Raintree Nutrition (Carson City, NV). Dichloromethane, acetic acid, ethyl acetate, ethanol (EtOH) and methanol (MeOH) were obtained from Fisher Scientific (Pittsburgh, PA). Androgen-independent prostate cancer, PC-3 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and were cultured in RPMI-1640 media supplemented with 10% heat-inactivated fetal bovine serum and 5% penicillin/streptomycin. Luciferase-expressing PC-3 cells (PC3-luc) were purchased from PerkinElmer (Hopkinton, MA) and were maintained in MEM medium with 10% fetal bovine serum, (Hyclone, Pittsburgh, PA). All cells obtained from ATCC or PerkinElmer were immediately expanded and frozen down for future use (every 3 months from a frozen vial) of the same batch of cells. All the cell lines were made sure to be devoid of mycoplasma contamination using Universal Mycoplasma Detection Kit from ATCC (ATCC, Cat#30-1012K, Manassas, VA). Baicalin (internal standard), MTT dye (thiazolyl blue tetrazolium bromide, 98% TLC), rutin, quercetin, kaempferol (>99%), carboxy methyl cellulose (CMC-Na) and dimethyl sulfoxide were from Sigma (St Louis, MO). GLE was prepared by concentrating the leaf powder in ethanol.

Preparation of the GLE and its fractionation

An ethanolic extract of finely milled Graviola leaf powder (100g) with no binders or fillers was prepared. Each extraction was carried out for 8h with 1 l and all the three extractions were pooled and evaporated under vacuum at 50°C to yield a concentrated brown extract. The brown extract was lyophilized to yield a dark powder (11.6g), which was used for further experimentation. The FEF was prepared by dissolving 11.6g of GLE in 500 ml dichloromethane followed by extraction with distilled water (500 ml \times 3). The aqueous fractions were combined, evaporated under vacuum until total volume reduced to 100ml, and then successively loaded on a resin column (8 cm \times 80 cm), eluted with 2000ml of water, 2000ml of 50% water/ethanol and then 1000ml 95% ethanol. The 50% water/ethanol fraction was evaporated under reduced pressure until all of the solvent was removed and lyophilized to FEF, 2.2g.

The AEF was prepared by further extracting the dichloromethane fraction with 1% acetic acid solution (400 ml \times 3). The extraction solvent was pooled and evaporated to yield a dark green extract (3.3g). The extract was dissolved in 5 ml ethyl acetate/hexane (1:1) and loaded on a silica gel column (5 cm \times 50 cm), eluted with 1500 ml (1:1) ethyl acetate/hexanes, 750 ml ethyl acetate, 1000 ml (1:1) ethyl acetate/methanol, then 500 ml methanol. All the fractions were collected based on color and the ethyl acetate fraction was evaporated until all of the solvent was removed and lyophilized to produce AEF, 1.7 g).

Quantitation of most-abundant phytochemicals of GLE

GLE and FEF samples were analysed using liquid chromatography tandem mass spectrometric method (Agilent 6410 series). A positive ionization mode with multiple reaction monitoring (MRM, *m/z* Q1/Q3, retention time) of rutin (*m/z* 611.2/303.0, RT 11.6 min), Q-3-G (*m/z* 465.1/303.0, RT 12.3 min), quercetin (*m/z* 303.1/153.0, RT 17.7 min), kaempferol (*m/z* 287.1/153.0, RT 19.7 min) and Baicalin (internal standard, *m/z* 271.1/123.0, RT 18.6 min) was employed. Quantitation of rutin, quercetin, kaempferol and Q-3-G was performed with external calibration curves. The ion spray voltage was set at 3000V, ionization temperature set as 300°C, drying gas flow rate was 5 l/min and nebulizer gas was set as 40 psi. Data acquisition and quantitation were performed using Mass Hunter software (Agilent

Technologies). Separation was achieved using HP1100 series LC (Agilent Technologies, Wilmington, DE) equipped with a photodiode array detector, using an Agilent Zorbax reversed-phase (SB-C18, 2.1 × 150 mm, 5.0 μm) column. A gradient method was employed to separate the individual GLE components using mobile phase A (0.1% formic acid in water) and mobile phase B (MeOH). The gradient elution method with 20% B at 0 min, 90% B at 30 min, held for 10 min, back to 20% B at 40 min with a flow rate of 0.2 ml/min. An injection volume of 5 μl was used for analysis. Peak area ratio of analyte to internal standard was used for calculating the concentration in all samples.

MTT assay

The effect of GLE, AEF, FEF and rutin on the cellular growth inhibition was assessed by MTT assay. PC-3 cells were seeded in 96-well plates at a density of 3500 cells per well. After 24 h of incubation, the buffer was aspirated and replaced by media containing various concentrations of GLE, AEF, FEF and rutin (1, 10, 25, 50, 75, 100 and 250 μg/ml). A primary stock solution was prepared by dissolving GLE/AEF/FEF/rutin in dimethyl sulfoxide at a concentration of 25 mg/ml from which a secondary concentration of 250 μg/ml was prepared in media. Further dilutions were made using the secondary stock solution. An aliquot of 100 μl was added to each well and incubated for 48 h, in an incubator maintained at 37°C, 5% CO₂ and more than 50% relative humidity. The extract-containing medium was replaced with 100 μl of medium containing MTT (tetrazolium bromide solution in phosphate-buffered saline [5 mg/ml]). The purple tetrazolium crystals were dissolved in 100 μl of dimethyl sulfoxide after 4 h incubation in dark. The absorbance was recorded at 570 nm on a Spectra Max Plus (Molecular Devices, Sunnyvale, CA) multi-well plate reader by employing calibration standards.

Trypan blue and colony survival assay

PC-3 cells (5000 per well) were seeded in a six-well plate and after 24 h of incubation were treated with 65 μg/ml GLE, 60 μg/ml AEF and 90 μg/ml FEF the next day. After 24 h of treatment, cell proliferation was determined using Trypan blue exclusion assay. For the colony assay, PC-3 cells were seeded at appropriate dilutions (~100 cells/well) and were treated with 65 μg/ml GLE, 60 μg/ml AEF and 90 μg/ml FEF for 24 h, washed and replaced with regular RPMI medium. The crystal-violet colonies (each consisting of at least 50 cells) were counted post fixation with 4% formaldehyde solution.

Determination of maximum tolerated dose and acute in vivo toxicity

C57BL6/J mice (3 groups, *n* = 5/group) were fed with a maximum allowed dose of 2 g/kg bw of GLE, AEF and FEF followed by observation of their health conditions. Following one dose, the mice were monitored for toxicity-related signs like loss of appetite, drastic weight loss, decreased activity and motion and signs of hunched back posture (35).

In vivo tumor growth inhibition and bioluminescent imaging

Six-week-old male nude BALB/c mice (Harlan Laboratories, Indianapolis, IN) were subcutaneously injected with PC-3-luc cells (1 × 10⁶) on the right flank. After 15 days, mice with palpable tumors were randomly separated into five groups of six mice each. Control group (*n* = 6) was fed vehicle (0.5% CMC-Na [pH = 7.4]) and the treatment groups (*n* = 6) were fed 100 mg/kg bw GLE, AEF, FEF and rutin (dissolved in 0.5% CMC-Na [pH = 7.4]) by oral gavage daily for 4 weeks. Tumor growth differences between the vehicle and treatment groups were compared by measuring the luciferase activity in live mice via real-time bioluminescent imaging on the IVIS *in vivo* imaging system (PerkinElmer, Hopkinton, MA) equipped with Live Imaging software. Luciferin (30 mg/ml) was injected intraperitoneally in mice anesthetized with isoflurane that were imaged with a CCD camera. Image acquisition was recorded with an integration of 20 s and four binnings of 100 pixels. The relative photon count of the tumors on the mice from vehicle or treatment groups was quantitated twice a week for 4 weeks, whereas tumor volumes were measured using a vernier caliper. Institutional IACUC guidelines were strictly followed while performing the animal experiments (12).

PK study

PK studies were performed in male C57BL6/J mice following a single oral (PO) administration of GLE and FEF at 100 mg/kg. All animals were acclimatized for 3 days before dosing in the experimental area. Mice were fasted for 4 h before dose administration and food was provided 4 h post dose. Water was provided *ad libitum* through the study period. Animals were marked and housed (three per cage) in polypropylene cages and maintained in controlled environmental conditions with 12 h light and dark cycles. The temperature and humidity of the room was maintained between 22 ± 3°C and 30–70%, respectively, and approximately 10–15 fresh air change cycles per hour. A sparse sampling design was used to collect blood samples from animals at 5, 10, 15 and 30 min and at 1, 2, 4, 6, 8, 12 and 24 h into K₂EDTA (200 mM, 20 μl/ml of blood) coated tubes. Plasma was harvested from blood by centrifugation of samples at 8000g for 10 min. All samples were stored below –20°C until analysis.

All samples were processed by protein precipitation method. An aliquot (60 μl) of sample was spiked with 20 μl internal standard (Baicalin), mixed with 220 μl acetonitrile and vortex mixed for 3 min. The tubes were centrifuged at 14000g for 10 min and an aliquot of supernatant was transferred into 1.5 ml auto-sampler vials for analysis. The stock solutions (1 mg/ml) of rutin, quercetin, kaempferol and baicalin (internal standard) were prepared in 30% aqueous acetonitrile. A calibration curve range of 1 ng/ml to 1 μg/ml was employed for the quantification of analytes. The calibration curve consisted of blank, blank with internal standard and 6 non-zero calibration standards. The calibration standards were within ±15% of the nominal concentration and lower limit of quantification was within ± 20% of nominal (12).

All samples were analysed using liquid chromatography tandem mass spectrometric method (Agilent 6410 series). A positive ionization mode with multiple reaction monitoring (measuring the time of retention [RT] and MRM transition [*m/z*] of rutin (*m/z* 611.2/303.0, RT 11.6 min), Q-3-G (*m/z* 465.1/303.0, RT 12.3 min), quercetin (*m/z* 303.1/153.0, RT 17.7 min), kaempferol (*m/z* 287.1/153.0, RT 19.7 min) and IS (*m/z* 271.1/123.0, RT 18.6 min) was employed. The ion spray voltage was set at 3000 V, ionization temperature set as 300°C and drying gas flow rate was 5 l/min and nebulizer gas was set as 40 psi. Data acquisition and quantitation were performed using Mass Hunter software (Agilent Technologies). Separation was achieved using HP1100 series LC (Agilent Technologies, Wilmington, DE) equipped with a photodiode array detector, using an Agilent Zorbax reversed-phase (SB-C18, 2.1 × 150 mm, 5.0 μm) column. A gradient method was employed to separate the individual GLE components using mobile phase A (0.1% formic acid in water) and mobile phase B (MeOH). The gradient elution method with 20% B at 0 min, 90% B at 30 min, held for 10 min, back to 20% B at 40 min with a flow rate of 0.2 ml/min. An injection volume of 5 μl was used for analysis.

PK parameters were calculated from the concentration–time data using the non-compartmental analysis tool of validated Phoenix WinNonlin software (Version 6.3, Pharsight). The area under the concentration–time curve (AUC_{last} and AUC_{inf}) was calculated by the linear trapezoidal rule. Following oral administration, peak concentration (C_{max}) and time for the peak concentration (T_{max}) were the observed values. The elimination rate constant value (*k*) was obtained by linear regression of the log-linear terminal phase of the concentration–time profile using at least three declining concentrations in terminal phase with a correlation coefficient of more than 0.8. The terminal half-life value (T_{1/2}) was calculated using the equation ln2/*k* (12).

In vitro cellular uptake assay

PC-3 cells (1 × 10⁶) were seeded in six-well plates and were allowed to grow overnight. After 24 h, the cells were incubated with or without 65 μg/ml GLE and 90 μg/ml FEF. Cells were collected at 4, 8, 12 and 24 h after treatment. At each time point, the cells were washed 3 times with 1x phosphate-buffered saline, scraped in 1x phosphate-buffered saline, followed by addition of 1 ml lysis buffer. The samples were sonicated and prepared for HPLC-MS/MS analysis for quantitation of the flavonoids using the method described under PK study.

Results

Comparative quantitation of major phytochemicals in GLE and its FEF

Acetogenins and flavonoids comprise the two major phytochemical groups in annonaceous plants. From GLE, we separated

these two groups into a FEF and an AEF (Supplementary Figure 1, available at *Carcinogenesis* Online). The chromatographic fingerprints of the parent extract and the two fractions were obtained using LC-MS/MS analysis in positive-ion mode (Figure 1A). We next wanted to identify the most-abundant flavonoids of GLE and quantitatively compare with that of FEF. HPLC-MS/MS analysis was used to estimate the enrichment of flavonoids in FEF compared to their natural abundance in GLE. A scan for the flavonoids present in GLE revealed rutin as the most-abundant flavonoid followed by Q-3-G, quercetin and kaempferol (Figure 1Bi-ii, Supplementary Figure 1, available at *Carcinogenesis* Online). In order to quantify their abundance, the standard curves of each flavonoid were obtained using HPLC-UV method as described in Materials and methods. Next, the peaks corresponding to the standards in the HPLC-UV profile were identified and integrated after injecting GLE and FEF into the system, followed by calculation of their abundance using the standard curves.

The concentrations of rutin, Q-3-G, quercetin and kaempferol in GLE were found to be 16.7, 1.16, 1.25 and 0.08 $\mu\text{g}/\text{mg}$, respectively, whereas their amounts in FEF were 122.8, 3.53,

1.43 and 0.08 $\mu\text{g}/\text{mg}$, respectively. These data suggest that rutin was ~ 7 -fold enriched in FEF compared with GLE, whereas Q-3-G's enrichment in FEF was about 3-fold (Figure 1Bi). However, there was no significant increase in the content of quercetin and kaempferol between the parent GLE and FEF (Figure 1Bii). The quantitative assessment of acetogenins in GLE and AEF was limited owing to their structural complexity and abundance of acetogenin isomers (14,21,26,28,30,31), which inhibited accurate quantitation of each species. Considering the observed enrichment, the next logical step was to determine the growth inhibitory efficacy of AEF and FEF compared to the parent extract, GLE.

GLE induces cytotoxicity in prostate cancer cells

We performed an MTT assay to determine the growth inhibitory efficacy of GLE, FEF and AEF. PC-3 cells were incubated for 48 h with various concentrations of GLE, AEF and FEF. Since rutin was found to be most-enriched flavonoid in FEF compared with GLE (Figure 1), we also employed pure rutin to assess its contribution in imparting growth inhibitory efficacy to FEF or GLE. Our results indicated a progressive decrease in cell viability with increase in concentration of GLE, AEF, FEF and rutin (Figure 2A).

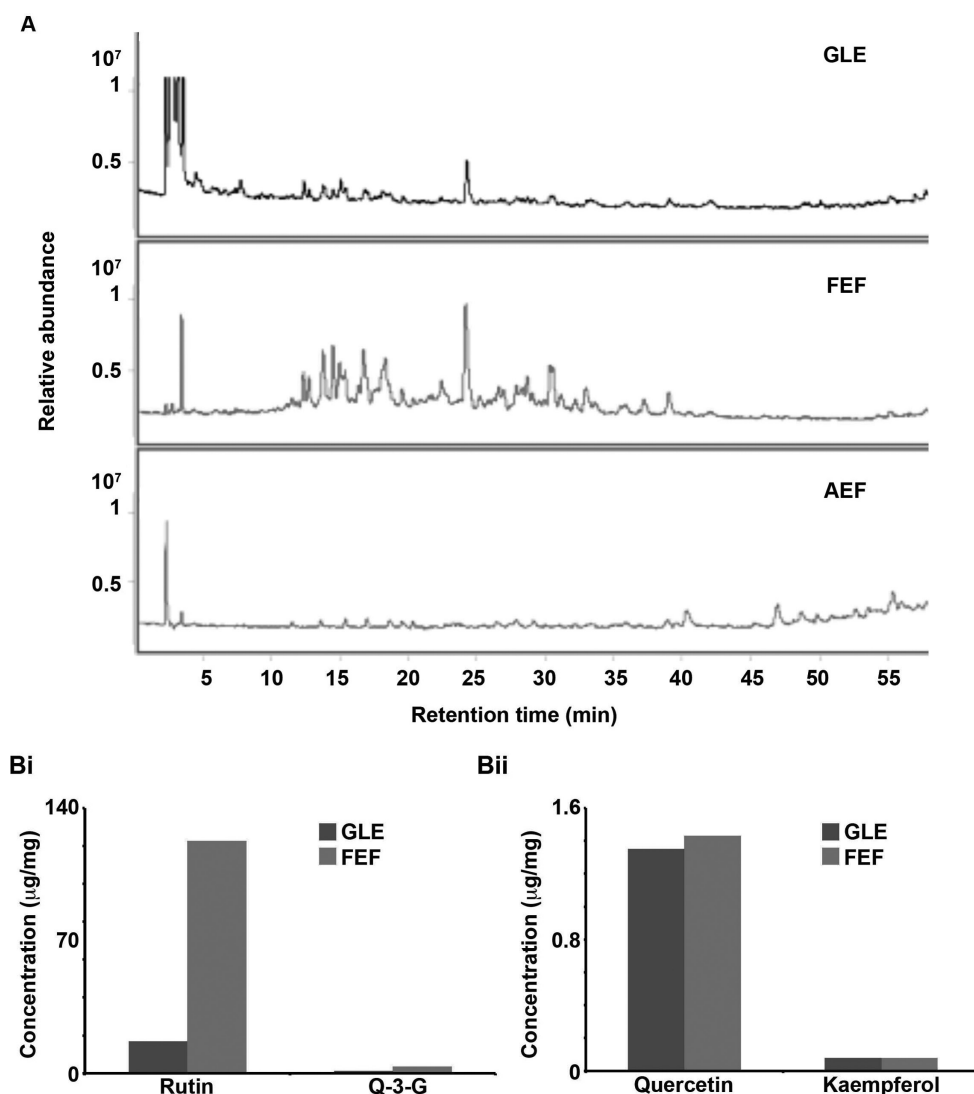


Figure 1. Identification of the most-abundant phytochemicals in GLE. (A) LC-UV/MS (TIC+) comparison of the GLE, FEF and AEF profiles. (B) Quantitation of rutin, quercetin-3-glucoside (Q-3-G), quercetin and kaempferol was performed employing a calibration curve using pure standards.

After 48 h of treatment, the resulting IC_{50} values of GLE, AEF, FEF and rutin for PC-3 cells were 63, 57, 87 and 91 $\mu\text{g/ml}$, respectively (Figure 2B). These results indicate that AEF followed by GLE were most effective in inhibiting the proliferation of human prostate cancer PC-3 cells. However, the enhancement in efficacy of AEF was not significantly different compared to the activity of GLE. Owing to the complexity of isolation and analysis of the acetogenins, their quantitation to correlate their efficacy could not be achieved.

On the other hand, the efficacy of FEF (87 $\mu\text{g/ml}$) and rutin (91 $\mu\text{g/ml}$) was found to be lower than that of GLE. There was no major difference in the activity of FEF and pure rutin (Figure 1A and B), suggesting that rutin contributes maximally toward the growth inhibitory efficacy of FEF. On the contrary, these results clearly indicate that rutin and constituent flavonoids in GLE are not solely responsible for its activity. Hence to further corroborate these observations, we next assessed the trypan blue exclusion viability and colony formation capacities of GLE and its fractions.

The trypan blue assay quantitated PC-3 cell viability upon treatment with GLE, AEF and FEF. We found reduced cell viability of PC-3 cells in the case of AEF (57%) and GLE (78%) (Figure 2C). FEF, however, showed no significant differences in viability compared to controls (Figure 2C). Our observations suggested that while control cells divided profusely and produced several colonies, only a small fraction of GLE- and AEF-treated cells retained their ability to form colonies (Figure 2C). A clonogenic or colony formation assay was performed to determine the ability of a cell to proliferate indefinitely to form a colony upon drug removal

(Figure 2D). FEF-treated cells produced maximum colonies even after FEF removal, suggesting minimal effect of flavonoids on the replicative potential of PC-3 cells. Representative micrographs of colonies in control and treated cells (Figure 2D, top) revealed ~7-fold reduction in the clonogenic capacity of AEF compared to controls, whereas in the case of GLE, there was ~2.5-fold reduction in the number of surviving colonies.

Oral feeding of GLE induces significant inhibition of *in vivo* prostate tumor growth

Having identified *in vitro* anti-proliferative activity of GLE and its fractions, we next aimed at evaluating their *in vivo* efficacy. First, we fed three C47BL/6J mice groups with maximum allowed dose of 2g/kg bw GLE, AEF and FEF. Among these, 100% of the GLE- and FEF-fed groups were alive with no signs of toxicity. However, 100% of AEF-fed mice died concluding that the acetogenins are potentially toxic at such high dose. Upon determining the maximum tolerated dose, we next examined their *in vivo* efficacy to inhibit growth of PC-3 tumor xenografts implanted subcutaneously in athymic nude mice. A PC-3 cell-line stably-expressing luciferase (PC-3-luc), which permits non-invasive real-time visualization of prostate cancer growth was employed in this study. GLE, AEF, FEF, and rutin were all fed orally at a concentration of 100mg/kg bw for 4 weeks to mice in treatment groups, thus achieving a cumulative dose of maximum tolerated dose (i.e. 2g/kg bw) over a period of 28 days. Control mice received the vehicle (0.5% CMC-Na [pH=7.4]) daily by oral-gavage. The relative photon counts obtained via non-invasive bioluminescent

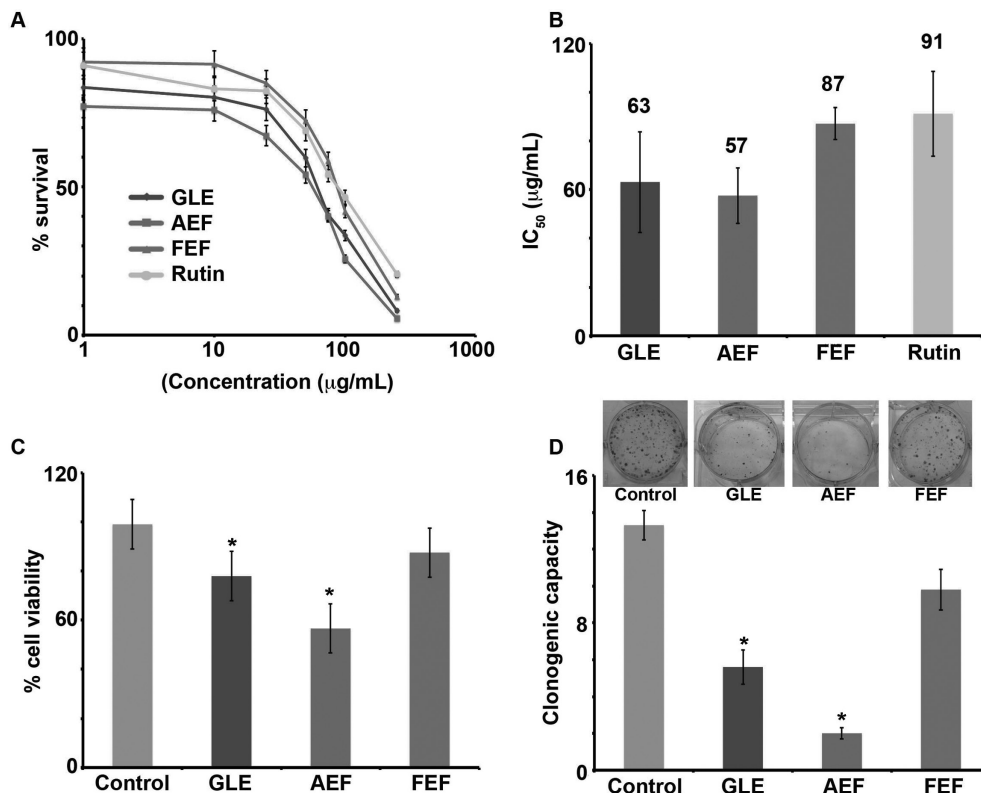


Figure 2. Graviola leaf phytochemicals inhibit proliferation of human prostate cancer cells. (A) Determination of IC_{50} of GLE and its constituents. PC-3 cells were treated with GLE, AEF, FEF and rutin at various concentrations for 48 h. The percentage cell proliferation was measured by MTT assay. (B) Bar-graphical representation of IC_{50} values (mentioned above) of GLE, AEF, FEF and rutin tested in PC-3 cells. (C) GLE and its constituents affect the cell viability of PC-3 cells. Bar graphical representation of percent cell viability of PC-3 cells treated with GLE, AEF and FEF as demonstrated by trypan blue exclusion assay. (D) GLE and its constituents inhibit clonogenic capacity of prostate cancer cells. Bar-graphical representation and photograph of crystal violet-stained surviving colonies from control and GLE-, AEF- and FEF-treated groups. Values and error bars shown in the graphs represent Mean \pm SD. (* P < 0.05, compared with controls).

imaging (Figure 3Ai-ii) and tumor volumes measured using a vernier caliper (Figure 3B) were quantified to evaluate the therapeutic responses of treatment groups.

Among all treatment groups, only GLE displayed significant inhibition of tumor growth over four weeks compared to control vehicle-treated group. Approximately 62% reduction in tumor volume ($P < 0.05$, $n = 6$; Figure 3Aii) was observed at the end of week four in GLE-treated mice, whereas ~51% tumor growth inhibition was found in FEF-treated group. However, rutin-treated group showed only 33% tumor growth inhibition ($P < 0.05$, $n = 6$; Figure 3Aii). Further, tumor volume measurements using vernier calipers showed ~66, ~52 and ~38% decrease in tumor volume for GLE-, FEF- and rutin-treated groups, respectively, compared to control group upon oral feeding for 4 weeks.

The general health and well-being of animals during treatment was assessed by recording body weights twice a week. Mice in GLE-, FEF- and rutin-treated groups exhibited normal weight

gain with no signs of discomfort during the treatment regimen. However, all animals in AEF group could not tolerate the treatment beyond 3 weeks (Figure 3Ai). These mice showed toxicity-related symptoms including loss of appetite, drastic weight loss, decreased activity and motion followed by hunched back posture and were thus euthanized within 3 weeks. However, tumor growth in AEF-treated group was significantly inhibited compared to other treatment groups. In compliance with institutional IACUC guidelines on tumor overburden, mice in control group were euthanized by the end of 4 weeks. In addition, at the end of 4 weeks, tumors from all groups (except AEF) were excised and were weighed upon euthanasia. The tumor weights of GLE-, FEF- and rutin-treated groups were approximately 4.5, 2.3 and 1.2-fold lower compared to tumor weights of control group. These data clearly indicate that GLE exhibits superior tumor-growth inhibitory efficacy compared to FEF, despite maximum flavonoid enrichment in FEF.

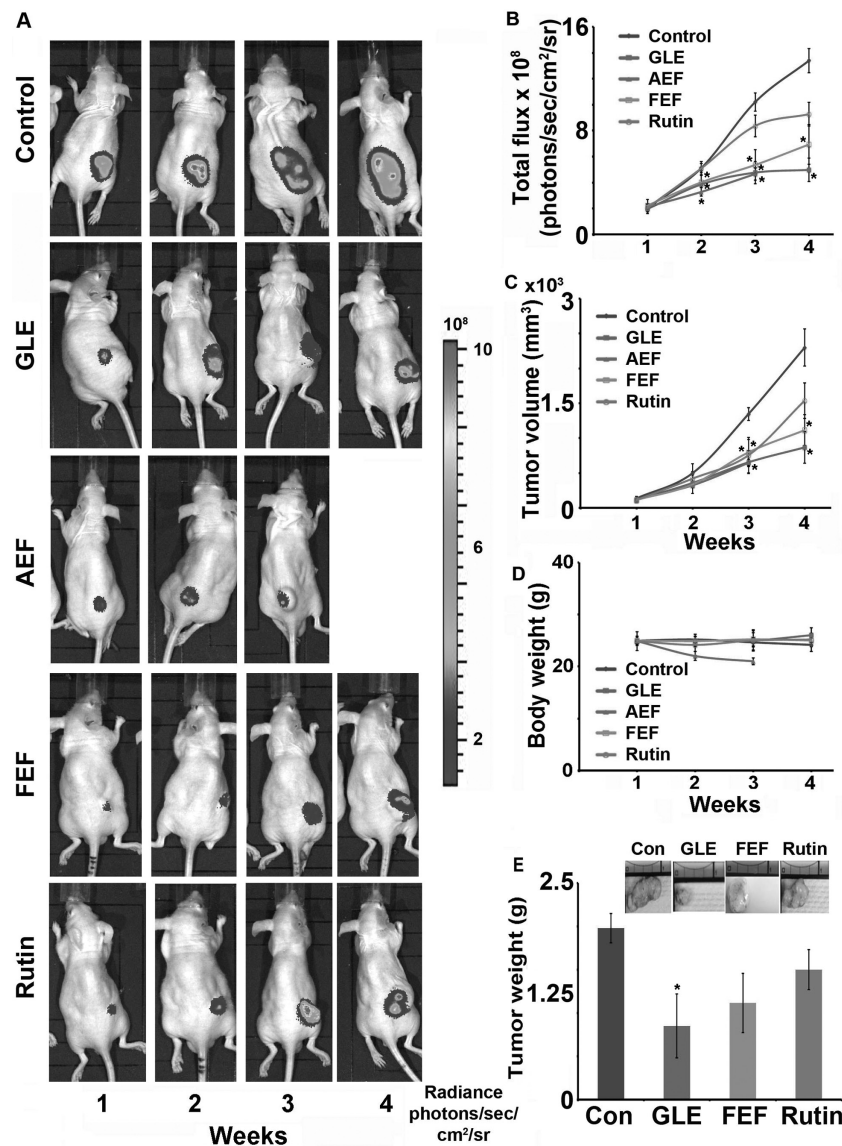


Figure 3. Dietary feeding of GLE showed inhibition of human prostate tumor xenograft growth in nude mice. Male nude mice were subcutaneously injected with 10^6 PC-3-luc cells. (Ai) Bioluminescent images (representative one animal per group) depicting tumor progression over 4 weeks. (Aii) Quantitation of radiance (photons/s/cm²/sr) measured from tumors of vehicle- and GLE-, AEF-, FEF- and rutin-fed mice. (B) Tumor volume (in mm³). (C) Tumor weight comparison along with photographic images of excised tumors. (D) Body weight comparison of vehicle and treatment groups. (* $P < 0.05$; two-way analysis of variance, compared with controls).

To gain deeper insights to reconcile these observations, we wanted to examine physiological concentrations of flavonoids upon oral feeding GLE or FEF administration.

Plasma concentration–time profile of GLE and FEF

To investigate the discrepancy between enrichment and efficacy, we designed a PK study to determine the levels of the most-abundant flavonoids, rutin and Q-3-G, in plasma following oral administration of GLE and FEF (100mg/kg bw) in C57/BL6J mice ($n = 3$ per group) using a sparse sampling design. We excluded AEF from this study owing to its toxicity and complexity of acetogenin quantitation. Plasma samples collected from the two groups were evaluated for the presence of rutin and Q-3-G, followed by their quantitation using LC–MS/MS (Figure 4A and B). A comparison of mean plasma concentration kinetics of GLE and FEF as shown in Figure 4, revealed that higher concentrations of rutin and Q-3-G were achieved upon oral administration of FEF as compared to GLE as expected owing to their enrichment in the former. The C_{\max} values of rutin and Q-3-G were 76ng/ml and 2.4ng/ml, respectively, in the case of FEF dosing (Figure 5A), whereas C_{\max} values of 31.6ng/ml (rutin) and 1ng/ml (Q-3-G) were observed in the case of GLE dosing (Figure 5A).

As determined earlier, FEF showed ~7-fold enrichment of rutin and 3-fold enrichment of Q-3-G compared to GLE (Figure 1Bi). However, *in vivo* plasma concentrations of these flavonoids did not correspond to such high levels of enrichment. Rutin and Q-3-G were found to be ~2.5- and ~2.3-fold higher in the plasma samples, respectively, upon oral administration of FEF compared to GLE (Figure 5A). Similarly, the exposure (AUC_{last}) of rutin and Q-3-G were 2-fold higher when FEF was fed orally compared to GLE. No statistical analysis was performed on AUC_{last} as it was a sparse sampling design with composite profile.

Thus, based on the *in vivo* PK data, which suggested an increase in absorption of flavonoids with their increased enrichment, we next aimed to evaluate the *in vitro* kinetics of GLE and FEF to further understand the discrepancy between the enrichment and efficacy.

In vitro uptake of flavonoids

Next, we designed an *in vitro* cellular uptake study to determine levels of flavonoids in PC-3 cells following incubation with the IC_{50} concentrations of GLE (65 $\mu\text{g/ml}$) and FEF (90 $\mu\text{g/ml}$), obtained from MTT analysis (Figure 2A). Cell samples collected at various time points of treatment (Figure 6A) were processed for LC–MS/MS analysis. The sample analysis allowed the quantitation of

rutin only while other flavonoids were undetectable. This analysis revealed that the cellular absorption of rutin peaks at 8h time point in the case of FEF followed by a sudden decrease, as opposed to a time-dependent increase in the absorption upon treatment with GLE (Figure 6B).

A linear decrease was seen in the difference between rutin levels in GLE and FEF with respect to time (Figure 6). Approximately 6-fold increase in the concentration of rutin was observed after 4h FEF treatment (Figure 6B), followed by ~5.2- and ~2.25-fold increase in the case of 8 and 12h FEF treatments (Figure 6B).

Hence, we envision that despite significant cellular absorption and accumulation of rutin in FEF, the maximum antiproliferative efficacy, as seen for GLE, can perhaps only be achieved with the help of acetogenins (Figures 2 and 3), thus emphasizing the significance of phytocomplexity.

Discussion

Recent advances in understanding mechanistic roles played by constituent phytochemicals of whole foods continue to reveal the importance of synergistic interactions among themselves in their natural complex environment. This has spurred several studies to address a new paradigm, i.e. the imperative need to consume whole foods to attain maximum therapeutic and chemopreventive benefits. Solubility, absorption, pharmacokinetics and toxicity parameters of single agents lend support to the emergence of this paradigm shift (2,5–7). The loss of therapeutic efficacy upon isolation of a single agent or a fraction as compared with the parent underlies the mystifying phyto-complexity of whole foods, which led us to formulate the current study. Our study underscores the importance of different classes of phytochemicals in Graviola leaves, which collaborate and interact with each other to deliver maximum health benefits. Our current study is the first to emphasize that irrespective of the enrichment and improved absorption of flavonoids upon oral feeding of FEF, the presence of acetogenins in GLE contributes to its superior antiproliferative and tumor growth inhibitory efficacy over FEF in both *in vitro* and *in vivo* prostate cancer models.

Our data demonstrate the superior antiproliferative efficacy, viability and clonogenic ability of AEF, followed by GLE compared to FEF (Figure 2). However, upon oral feeding of AEF, the prostate tumor xenograft-bearing mice developed signs of toxicity finally leading to death within 3 weeks of treatment (Figure 3). On the other hand, GLE treatment resulted in remarkable tumor growth

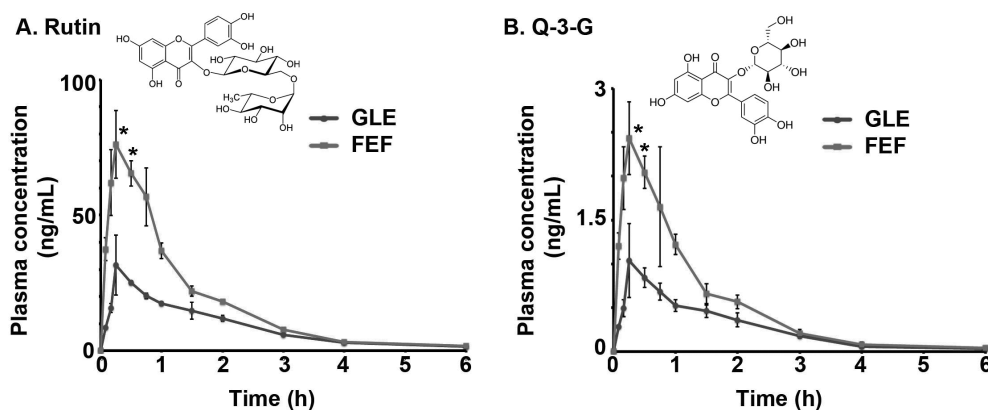


Figure 4. Plasma concentration–time profiles of GLE and FEF (100mg/kg bw). The plasma concentration–time profile of (A) rutin and (B) Q-3-G were quantitated in blood samples collected at various time points (0.08, 0.16, 0.25, 0.5, 1, 2, 4 and 6h) following oral administration of GLE and FEF. Error bars refer to Mean \pm SD (* $P < 0.05$).

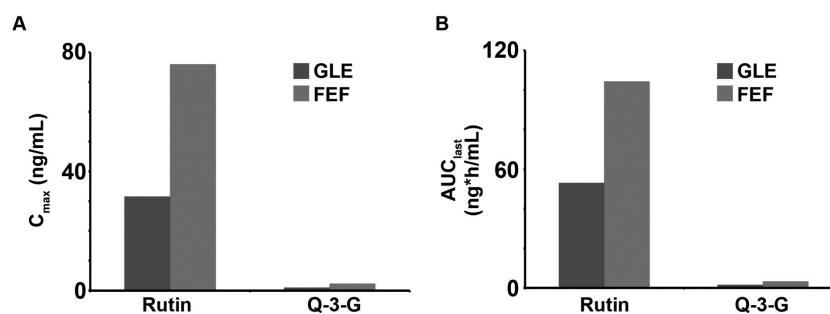


Figure 5. Oral PK parameters of GLE and FEF. (A) Peak plasma concentration of rutin and Q-3-G (C_{max}), (B) area under the concentration-time curve (AUC_{0-6h}) for rutin and Q-3-G determined using plasma samples collected at various time points (0.08, 0.16, 0.25, 0.5, 1, 2, 4 and 6 h) for GLE and FEF.

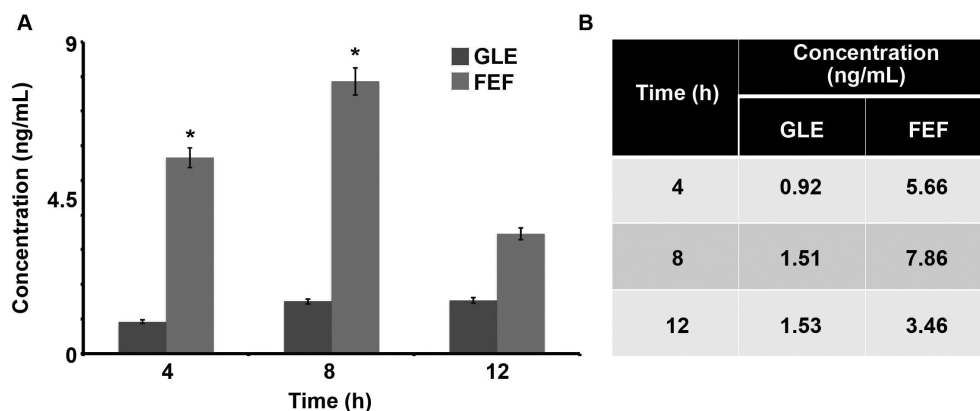


Figure 6. *In vitro* cellular uptake of flavonoids. (A) Comparison of cellular concentrations of rutin in the case of GLE and FEF treatments. (B) Concentration values of rutin in the case of GLE and FEF treatment for 4, 8 and 12 h. Values and error bars refer to Mean \pm SD (* $P < 0.05$).

inhibition with no signs of toxicity at the end of 4 weeks. FEF treatment also resulted in tumor growth inhibition, but was not as effective as GLE (Figure 3). This discrepancy in the *in vivo* efficacy of GLE over FEF, despite the enrichment of flavonoids in FEF, indicates a possible crucial role played by acetogenins in GLE and their synergistic interactions with the flavonoids to contribute toward GLE's improved efficacy.

Although there is an increase in the awareness of whole food consumption, the lack of evaluation of PK and absorption, distribution, metabolism, excretion and toxicity (ADMET) parameters of whole foods is limiting the understanding and acknowledgement of their therapeutic potential. To the best of our knowledge, no studies have been performed to evaluate the PK parameters and bioavailability of *Graviola* phytochemicals. The disparity between enrichment and efficacy of GLE and FEF, led us to evaluate the PK differences in the bioavailability of flavonoids when they are administered as GLE or FEF (Figure 4).

Upon oral administration of GLE or FEF, the plasma concentrations of rutin and Q-3-G were found to be higher in the case of FEF compared to GLE (Figures 4 and 5A, Supplementary Table 1, available at *Carcinogenesis* Online). Also, their bioavailability upon administration of FEF was found to be higher than dosing with GLE (Figures 4 and 5B). To further understand this ambiguity, an *in vitro* cell uptake assay was conducted to assess the absorption of flavonoids by PC-3 cells, which revealed a time-dependent decrease in the difference between rutin quantitation in FEF and GLE. Clearly, these data suggest that though plasma concentration of flavonoids is higher in the case of FEF dosing compared to the parent, the superior efficacy observed in the case of GLE is not dependent only on the flavonoids. The presence of acetogenins in the whole extract seems to be

playing a crucial role in imparting the superior efficacy to GLE compared to FEF, which lack the acetogenins. Furthermore, the absence of rutin in the rat blood stream upon oral administration signifies the possibility of conversion of rutin to quercetin (within 5 min), which further gets metabolized quickly to glucuronide and sulfate conjugates (36). On the contrary, our observations in mice clearly indicate the presence of rutin (70 ng/ml) in the blood plasma (at 5 min). Considering these varying reports, rutin might actually be acting as a secondary player via supportive mechanisms thus helping in prevention of toxic acetogenins crossing the BBB, by activating the Pgp efflux pump in the brain (36,37).

Sufficient evidence indicates that the use of a single acetogenin or a combination of several acetogenins often leads to toxic side effects and even death, despite delivering superior efficacy (21,22,28–33). In the current study, the acetogenins when administered as a part of their natural environment, GLE, appear to have substantially contributed toward GLE's efficacy. However, when administered as AEF, the acetogenins caused toxic symptoms and even led to death. We speculate that this difference in efficacy could be due to the attainment of favorable physiological concentrations of acetogenins in GLE, along with synergistic interactions with the flavonoids. On the contrary, AEF administration may have resulted in toxic build up of acetogenin metabolites due to excessive substrate:enzyme concentrations, hence hindering their effective metabolism. Also, annonaceous acetogenins including bullatacinones, asimicin, rollinone, muricins, etc. have been known to induce severe neurotoxicity, decreased mobility and are attributed to cause atypical Parkinson's disease (24,26,29,32,33). These phytochemicals comprise of 32–34 carbon chains with hydroxyl, keto, epoxide, tetrahydrofurans and

tetrahydropyran groups. It is unlikely for such mega molecules to cross the blood–brain barrier and hence there exists a possibility of their metabolites or degraded products to pass through this barrier causing neurotoxicity (38). Obviously, 100mg/kg of AEF would contain higher concentrations of acetogenins compared to 100mg/kg of GLE, thus prompting the *in vivo* absorption of the right amount of acetogenins to exert maximum efficacy in the latter case. The achievement of the effective physiological concentrations is a key factor in delivering maximum health benefits, whereas undergoing proper metabolism with subsequent elimination to avoid any debilitating side effects.

Based on the above observations, our future attempts include (i) identifying the degradation products or metabolites of various acetogenins, (ii) selectively inhibit the tumor cells while sparing the normal cells, (iii) identifying if they can cross the BBB and (iv) rationally designing novel acetogenin-based analogs that could cause effective tumor growth inhibition while avoiding absorption into brain. Further, the observed toxicity induced by the AEF warrants a detailed analysis of organ distribution with an emphasis on brain partitioning studies along with biochemical analyses of blood plasma to identify the responsible graviola leaf phytochemicals. Further, rutin and quercetin are known to exert antioxidant efficacy and their metabolites have been observed to induce polyglycoprotein expression while activating drug-metabolizing enzymes like CYP3A4 or inhibiting CYP2C8 enzymes (36,37). Also, we aim to determine the chemopreventive efficacy of GLE employing spontaneous prostate tumor models that closely mirrors the human prostate cancer progression like transgenic adenocarcinoma of the mouse prostate (TRAMP) model. Hence, futuristic attempts exploring the possible drug–drug interactions, enterohepatic recirculation of constituent metabolites and antioxidant efficacy exerted by rutin and quercetin metabolites are underway in our laboratory.

In conclusion, the presence of flavonoids along with the active annonaceous acetogenins in GLE appears to be an added benefit to confer maximum therapeutic benefits as well as favorable absorption kinetics and bioavailability of the active Graviola ingredients. Our observations present an opportunity to further investigate and design pioneering combinations/dietary supplements to exploit the beneficial aspects of acetogenins while eliminating their associated toxicities when delivered in their natural matrix.

Supplementary material

Supplementary Table 1 and Figures 1–2 can be found at <http://carcin.oxfordjournals.org/>.

Funding

National Cancer Institute at the National Institutes of Health (R00CA131489, R01 CA169127 to R.A.); American Cancer Society (121728-RSG-12-004-01-CNE).

Conflict of Interest statement: None declared.

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