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Synergistic chemoprotective mechanisms of dietary phytoestrogens in a select combination against prostate cancer☆

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Abstract

Combination of dietary phytoestrogens with diverse molecular mechanisms may enhance their anticancer efficacy at physiological concentrations, as evidenced in epidemiological studies. A select combination of three dietary phytoestrogens containing 8.33 μM each of genistein (G), quercetin (Q) and biochanin A (B) was found to be more potent in inhibiting the growth of androgen-responsive prostate cancer cells (LNCaP) as well as DU-145 and PC-3 prostate cancer cells in vitro than either 25 μM of G, B or Q or 12.5+12.5 μM of G+Q, Q+B or G+B. Subsequent mechanistic studies in PC-3 cells indicated that the action of phytoestrogens was mediated both through estrogen receptor (ER)-dependent and ER-independent pathways as potent estrogen antagonist ICI-182780 (ICI, 5 μM) could not completely mask the synergistic anticancer effects, which were sustained appreciably in presence of ICI. G+Q+B combination was significantly more effective than individual compounds or their double combinations in increasing ER-β, bax (mRNA expression); phospho-JNK, bax (protein levels); and in decreasing bcl-2, cyclin E, c-*myc* (mRNA expression); phospho-AKT, phospho-ERK, bcl-2, proliferating cell nuclear antigen (protein levels) in PC-3 cells. Phytoestrogens also synergistically stimulated caspase-3 activity. Our findings suggest that selectively combining anticancer phytoestrogens could significantly increase the efficacy of individual components resulting in improved efficacy at physiologically achievable concentrations. The combination mechanism of multiple anticancer phytochemicals may be indicative of the potential of some vegetarian diet components to elicit chemopreventive effects against prostate cancer at their physiologically achievable concentrations, in vivo.

Keywords: Prostate cancer; Genistein; Quercetin; Biochanin A; PC-3; Estrogen receptor

1. Introduction

Prostate cancer (CaP) is the most frequently diagnosed malignancy in men, and though only a small proportion of low-risk CaPs progress to life-threatening disease during the lifetime of the patient, it is still the second most common cause of cancer death [1]. It has been established that the incidence of clinically significant CaP is much lower in parts of world where people eat predominantly a plant based diet [2], and therefore the interest in phytoprevention and phytotherapy of CaP has increased considerably [3]. This is also important from the point of view that many men with low-risk CaP choose comprehensive life-style changes (including plant-based nutrition) over surgery/radiotherapy for disease management [4]. Invasive prostate carcinomas become androgen insensitive due to mutation of androgen receptors [5,6]; nevertheless their estrogen sensitiveness increases as a result of an increased expression of estrogen receptors (ERs) [7]. Phytoestrogens, because of their structural similarity to estradiol, bind to these ERs [8], especially ER-β, which is anti-proliferative and proapoptotic [9] and is expressed in plenty in normal prostate as well as CaP [7,10]. Studies have shown that selective ER modulators (including phytoestrogens) inhibit CaP cell proliferation in vitro [11,12] as well as in vivo [13]. Besides estrogen agonistic/antagonistic activities, phytoestrogens also exert a number of other effects that beneficially modulate several aspects of tumor growth [14]. However, a major constraint in their use as effective chemoprotectants is poor bioavailability and toxicity at efficacious doses. Vegetarian diets contain combinations of different phytochemicals that may act synergistically to modulate the aberrant signaling pathways of cancer cells resulting in chemoprotective effect at physiological concentrations, as reflected in epidemiological studies [15]. Hence, simultaneous administration of dietary phytochemicals with different chemopreventive mechanisms may provide additive or synergistic activity at lower doses of the individual agents, resulting in improved efficacy and reduced toxicity [16]. We selected some phytoestrogens on the basis of their diverse anti-cancer mechanisms (as reported in literature), not necessarily requiring their binding to the ER (which is a common property of all phytoestrogens). This was done to selectively combine phytoestrogens with different anticancer mechanisms together, so as to get a combination effect. Several combinations were tried, and we

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identified a combination of genistein (G), biochanin A (B) and quercetin (Q) that was most effective in inhibiting prostate cancer cell proliferation, in vitro. G is a potent inhibitor of tyrosine kinases [17], B is a powerful agonist of the human aryl-hydrocarbon (ArH) receptor [18] and Q is strongly anti-inflammatory [19,20]. Inhibition of tyrosine kinases by G and activation of ArH receptor by B inhibits prostate carcinogenesis [21,22] while Q mediates suppression of inflammation, which plays a crucial role in pathogenesis of CaP [23]. A rational approach to CaP prevention/treatment using dietary phytoestrogens could include a combination of potent ArH receptor agonism, tyrosine-kinase inhibition and anti-inflammatory activity, and therefore, we studied the combined and individual effects of B, G and Q on prostate cancer cell lines in vitro. To the best of our knowledge, this is the first experimental evidence on a multiple phytoestrogen combination exhibiting significantly enhanced efficacy against prostate cancer cells than three times higher concentration of any individual component.

2. Materials and methods

2.1. Cell culture

Androgen-responsive prostate cancer cells (LNCaP), DU-145 and PC-3 prostate cancer cells were procured from the American Type Culture Collection (ATCC, Manassa, VA, USA). PC-3 and DU-145 cells were grown in Dulbecco's modified eagle medium/Ham's F-12 (1:1; without phenol red; Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (charcoal stripped, Life Technologies), 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate (Sigma-Aldrich, St. Louis, MO, USA) in an atmosphere of 5% CO_2/95% air at 37°C. LNCaP cells were grown in RPMI-1640 medium using gelatin coated plates. Other supplements were same as for PC-3 and DU-145 cells.

2.2. Cell proliferation assay

Prostate cancer cells were seeded in 96-well plates at a density of 2×10^4 cells/well and allowed 24 h for attachment. The cells were then treated separately (in triplicates) with either a single compound (G, Q or B at 25 μM) or double combinations (G+Q; Q+B or G+B at 12.5+12.5 μM) or triple combination (G+Q+B at 8.33+8.33+8.33 μM), for 48 h at 37°C in 5% CO_2 atmosphere. The phytoestrogens (Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO) and diluted with culture medium to different concentrations before adding to the cells. Final concentration of DMSO was not more than 0.05%. Controls were treated with DMSO (0.05% in culture medium). After 48 h of incubation in CO_2 incubator, 10 μl of 5 mg/ml MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)] was added to the cells. After a further incubation of 3 h, the formazan crystals formed in viable cells were dissolved in DMSO and absorbance was measured at 540 nm using a microplate reader (Microquant, Bio-Tek, Winooski, VT, USA).

2.3. Cell proliferation after inhibition of ERs

To examine the role of ERs in the action of phytoestrogens, PC-3 cells were treated (in triplicates) with G, Q or B at 25 μM, or double combinations (G+Q; Q+B or G+B at 12.5+12.5 μM) or triple combination (G+Q+B at 8.33+8.33+8.33 μM), in the presence of a potent estrogen antagonist fulvestrant (ICI-182780, 5.0 μM), for 48 h at 37°C in 5% CO_2 atmosphere. After 48 h incubation in CO_2 incubator, 10 μl of 5 mg/ml MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)] was added to the cells. After a further incubation of 3 h, the formazan crystals formed in viable cells were dissolved in DMSO and absorbance was measured at 540 nm using a microplate reader (Microquant, Bio-Tek).

2.4. Caspase-3 activity assay

PC-3 cells were treated for 12 h with different concentrations and combination of phytoestrogens (as indicated in "cell proliferation assay"), washed with ice-cold phosphate-buffered saline and placed in lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.5% Triton X-100) for 30 min on ice. Cell lysates were mixed with caspase assay buffer (20 mM HEPES, 10% glycerol, 2 mM DTT, pH 7.5) containing 20 μM DEVD-pNA (Sigma-Aldrich) as substrate and incubated for 2 h at 37°C. Enzyme catalyzed release of pNA (p-nitro aniline) was monitored using an ELISA plate reader at 405 nm.

2.5. Effects on gene expression in PC-3 cells using real-time polymerase chain reaction (PCR)

Total RNA of PC-3 cells (treated with phytoestrogens for 48 hrs as indicated above) was extracted using the Tri-reagent (Invitrogen) by following the manufacturer's instructions. The quantity and quality of the purified RNA was evaluated by spectrophotometry. cDNA was prepared from 1.0 to 2.0 μg of total RNA using *Revert Aid H-Minus first strand* cDNA synthesis kit (Fermentas Life Sciences, Glen Burnie, MD, USA). Synthesis was performed for 1 h at 42°C (for reverse transcription) and the thermocycling for each reaction was done in a final volume of 20 μl containing 1 μl of cDNA sample, 0.5 μM of each primer, 2× ready-to-use reaction mix (ABi SYBR green master mix) including Taq DNA polymerase, reaction buffer and deoxyribonucleotide triphosphate mix. After 10 s of initial denaturation at 95°C, the following cycling conditions (45 cycles) were used: denaturation at 95°C for 20 s, annealing at 58°C for 30 s and elongation at 72°C for 30 s. The detection of the PCR reaction based on fluorescence monitoring (Light Cycler 480, Roche) was employed. Quantitative results were obtained by the cycle threshold value where a signal rose above background level. Expression of the investigated genes was compared to the steady expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences used have been detailed in Table 1.

Table 1
Primers sequences

Gene	Forward (5′-3′)	Reverse (5′-3′)
ER-β	AAGAAGATTCCCGGCTTTGT	CTTCTACGCATTTCCCCTCA
bcl-2	AGTACCTGAACCGGCACCT	GGCCGTACAGTTCCACAAA
bax	ATGTTTTCTGACGGCAACTTC	ATCAGTTCCGGCACCTTG
c-*myc*	CACCAGCAGCGACTCTGA	GATCCAGACTCTGACCTTTTGC
Cyclin D-1	GCCATTGATTCATTCATTAGAGTTCCA	ACTGTCCCACTCCAAACCTG
Cyclin E	TGGATCTGAAGAAATACTTGGATTCTA	TCTGGAGATCTGTACCAGAGTGTT
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC

2.6. Western blot analysis

Whole cell lysates of PC-3 prepared in lysis buffer [25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% NP-40, 1% protease inhibitor cocktail (Sigma-Aldrich)] were incubated at room temperature for 10 min and then centrifuged at 10,000×*g* (4°C, 10 min). The protein concentration of the supernatant was determined by Bradford protein assay. Samples were boiled for 10 min in denaturing sample buffer (10% glycerol, 1% SDS, 1% β-mercaptoethanol, 10 mM Tris-HCl, 0.01% bromophenol blue, pH 6.8), and then 50 μg (protein) of each sample was separated on 10% acrylamide gel and transferred to Immobilon-P polyvinyledene fluoride (PVDF) membrane (Millipore). After blocking nonspecific sites with 5% skimmed milk (in 150 mM NaCl, 10 mM Tris-HCl, pH 7.6) the membranes were probed with antibodies for ER-β (1:10,000, Sigma-Aldrich), proliferating cell nuclear antigen (PCNA) (1:10,000, Santa Cruz Biotechnology), bcl-2 and bax (1:10,000, Sigma-Aldrich); phospho-Akt, phospho-JNK, phospho-p38, phospho-ERK, (1:5000, Cell Signaling Technology), separately, and then re-probed with β-actin antibody (1:10,000, Sigma-Aldrich) for loading correction. Subsequently, the blots were washed three times with 0.1% Tween 20 in Tris-buffered saline (TBS) and incubated with 1:20,000 dilution of secondary antibody (anti-immunoglobulin G-horseradish peroxidase conjugate). After extensive washing in 0.1% Tween-20/TBS, substrate solution was added to the membrane, which was incubated for 5 min and exposed at room temperature. The membranes were developed with enhanced chemiluminescence kit (GE Healthcare), by following the manufacturer's protocol.

2.7. Data analysis

Each experiment was repeated three times, and the results were analyzed by Student's *t* test and one-way analysis of variance. Post hoc analyses were made using a more robust Mann Whitney *U* test that compares medians, which are insensitive to outliers compared to means, and therefore less likely than the *t* test to spuriously indicate significance. GraphPad Prism software (Version 4.0) was used for all statistical analyses, and *P* values less than .05 were considered significant.

3. Results

3.1. Dose dependent inhibition of prostate cancer cell proliferation by phytoestrogens at noncytotoxic concentrations

Initially, the dose-dependent growth inhibitory potential of G, Q and B against prostate cancer cells was evaluated by MTT assay. All the three exhibited almost equipotent activity and caused a dose-dependent inhibition of prostate cancer cell proliferation in vitro (data not included). Cytotoxicity of these phytoestrogens was determined against a nonprostate, noncancer Vero (monkey kidney epithelial) cell line by employing the MTT assay. The compounds were found to be safe up to 50 μM as the next higher concentration employed (100 μM) inhibited cell growth (Supplementary Data). Lactate dehydrogenase (LDH) release assay against PC-3 cells was employed to determine the cytotoxicity of G, Q and B at concentrations

and combinations used in the present study. The results indicate that G, Q and B did not cause leakage of cytosolic LDH from PC-3 cells into the culture medium at concentrations and combinations used in the present study (Supplementary Data). Finally, an effective concentration (25 μM) lower than the IC_{50} of G, Q and B against PC-3 cells was selected to conduct all further experiments on synergistic mechanism of various combinations of the three phytoestrogens.

3.2. Synergistic effect of phytoestrogens on prostate cancer cell proliferation through ER-mediated and ER-independent pathways

A synergistic chemoprotective action of phytoestrogens was clearly visible against PC-3 prostate cancer cells after 48 h of treatment with G, Q, B, GQ, QB, GB and GQB (Fig. 1A). Similar effects were also seen against DU-145 and LNCaP prostate cancer cells (Supplementary Data), although the response of DU-145 was better than LNCaP. Using the MTT cell viability assay it was found that at an in vitro concentration of 25 μM, G, Q and B individually reduced cell proliferation of PC-3 by ~40%, with B having a slight edge over G and Q. The double combinations (GQ, QB, GB) at 25 μM (12.5 μM of each phytochemical) were almost as effective as single compound but the triple combination GQB at 25 μM (8.33 μM of each phytochemical) was significantly more effective than 25 μM of G ($P<.001$), Q ($P<.05$) or B ($P<.01$) as well as 12.5+12.5 μM of GQ, QB or GB ($P<.01$), and reduced PC-3 proliferation by ~50% in 48 h. The activity profile was as follows: GQB>B>GQ>QB>Q>GB>G (Fig. 1A). This was the first indication of requirement of more than two compounds for significant synergism. To confirm that the effects were not just additive but actually synergistic, in a subsequent experiment, PC-3 cells were treated independently with 8.33 μM each of G, Q or B; or a GQB combination (8.33+8.33+8.33 μM) and assessed for inhibition of cell proliferation by MTT assay. G, Q and B independently reduced PC-3 cell proliferation by approximately 12.6%, 8.0% and 16.4%,

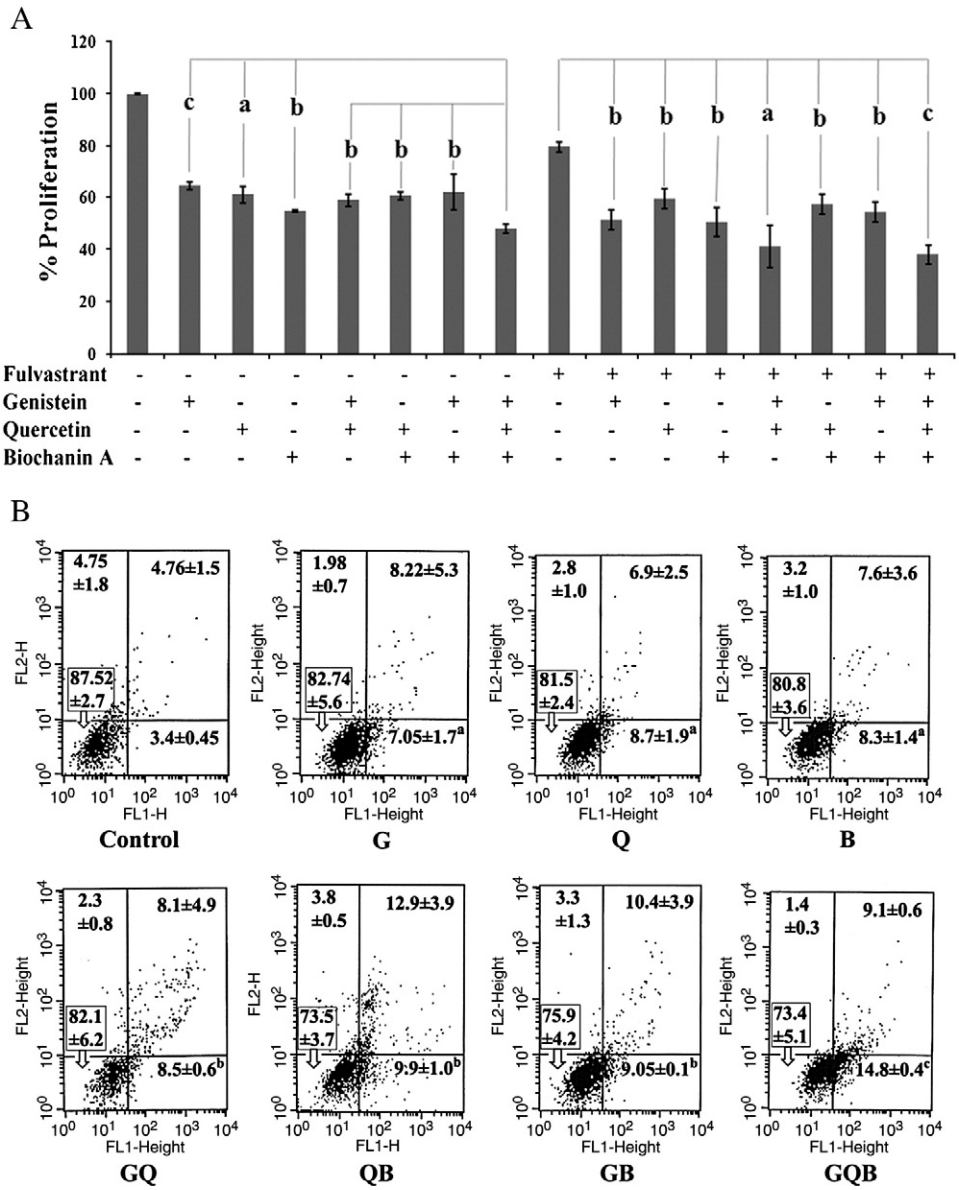

Fig. 1. Growth inhibition and apoptosis of prostate cancer (PC-3) cells induced by 25 μM G, Q and B (B); 12.5+12.5 μM of G+Q, Q+B and G+B and 8.33+8.33+8.33 μM of G+Q+B. MTT cell proliferation assay (Mean±SE, n=9) in presence or absence of Fulvestrant (ICI-182780, 5 μM) (A) and representative cytogram for flow cytometric detection of annexin-V FITC/PI labeling with statistical data (P value vs. control) and percent cell populations in four quadrants identified as: lower left, live; lower right, apoptotic; upper right, late apoptotic; upper left, necrotic (B). $^{a}P<.05$; $^{b}P<.01$; $^{c}P<.001$.

respectively. However, GQB reduced cell proliferation by approximately 55%, which was significantly more than that by either of the compound ($P<.01$), as well as their theoretically calculated additive effect of ~37% (Supplementary Data).

Since phytoestrogens were selected on the basis of their ER-independent anti-cancer properties, we also evaluated their non ER-mediated action against PC-3 cells after completely inhibiting the ERs by using a potent estrogen antagonist fulvestrant (ICI 182780, 5.0 μM). Fulvestrant by itself reduced PC-3 proliferation by ~20% but did not completely mask the effects of phytoestrogens. In presence of fulvestrant, G, Q and B (25 μM) further reduced PC-3 cell proliferation significantly ($P<.01$), but synergism could not be detected markedly in double combinations (GQ, QB and GB). However, a distinct synergism was evident in triple combination GQB, which was significantly more effective ($P<.001$) than single and double compounds (Fig. 1A). The activity profile in presence of fulvestrant was GQB>GQ>B>G>GB>QB>Q. This indicates that the combination effect of multi-phytoestrogens is essentially mediated through both ER-dependent and independent pathways.

3.3. Synergistic induction of apoptosis by phytoestrogens in PC-3 cells

Phytoestrogens synergistically induced apoptosis in PC-3 cells as evidenced by increased annexin-V binding to phosphatidylserine expressed on surface of apoptotic cells. Flow cytometric analysis detected increase in annexin-V FITC binding of PC-3 cells by ~2.0–2.5-fold after single compound treatment ($P<.05$), ~2.5–2.9-fold after double compound treatment ($P<.01$) and ~4.5-fold after triple compound treatment ($P<.001$, Fig. 1B).

3.4. Phytoestrogens synergistically down-regulate c-myc and up-regulate ER-β genes in PC-3

With a view to establish the anti-cancer potential of the phytoestrogen combinations we investigated the expressions of

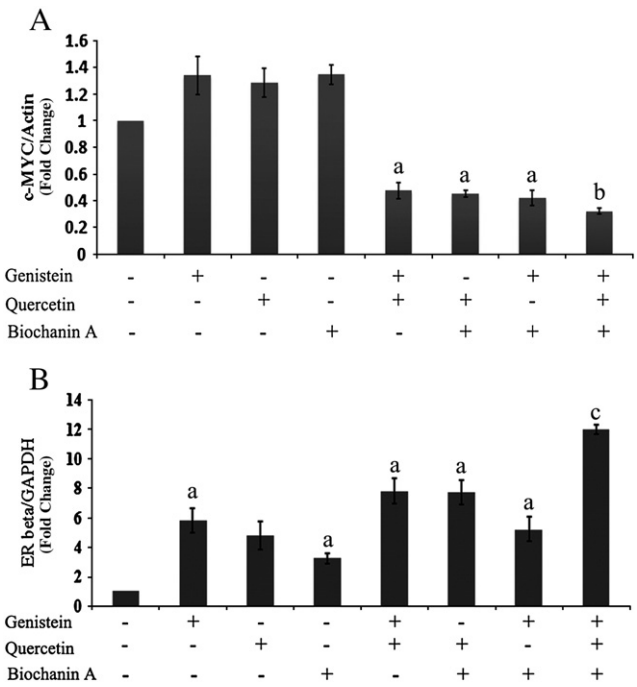

Fig. 2. Changes in expression of c-*myc* (A) and ER-β (B) genes as evidenced by mRNA levels in prostate cancer (PC-3) cells after treatment with 25 μM of G, Q and B (B); 12.5 +12.5 μM of G+Q, Q+B and G+B and 8.33+8.33+8.33 μM of G+Q+B. Significant difference from vehicle treated control cells is indicated as [a]$P<.05$; [b]$P<.01$; [c]$P<.001$.

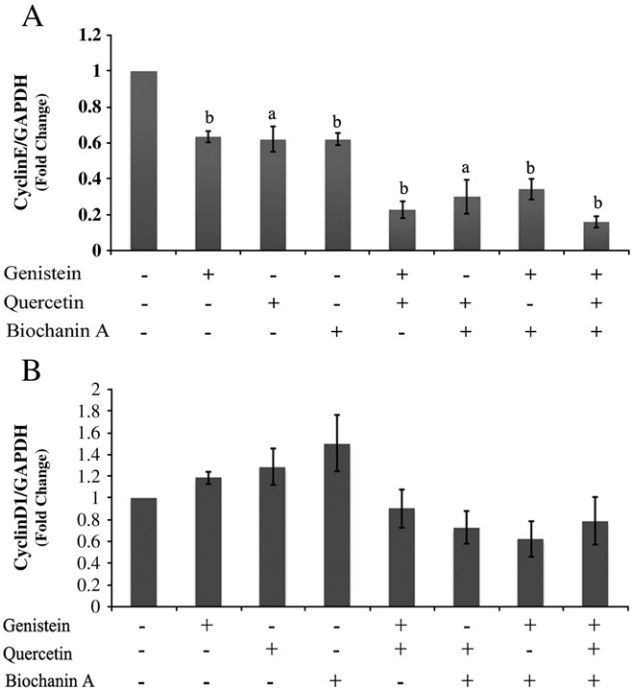

Fig. 3. Changes in expression of cyclin E (A) and cyclin D1 (B) genes as evidenced by mRNA levels in prostate cancer (PC-3) cells after treatment with 25 μM of G, Q and B (B); 12.5+12.5 μM of G+Q, Q+B and G+B and 8.33+8.33+8.33 μM of G+Q+B. Significant difference from vehicle treated control cells is indicated as [a]$P<.05$ and [b]$P<.01$.

oncogene c-*myc* and ER subtype β gene in PC-3 cells treated with either G, Q, B or their double/triple combinations. Individually, G, Q and B did not significantly change the expression of c-*myc*, but GQ, QB and GB reduced c-*myc* expression by >50% ($P<.05$). However, GQB was most effective and reduced c-*myc* expression significantly by >60% ($P<.01$, Fig. 2A). The gene expression of ER-β, which mediates tumor repressor activity in prostate, was increased nonsignificantly by 25 μM of Q and significantly ($P<.05$) by 25 μM of G, B, GQ, QB and GB in PC-3 cells. Here, GQ and QB had an apparent edge over their individual components. However, GQB at a total concentration of 25 μM was once again the most effective and caused ~12-fold increase in ER-β expression ($P<.001$) (Fig. 2B). This may indicate that G, B and Q significantly up-regulate anti-cancer mechanisms in PC-3 when present concurrently in combinations.

3.5. Synergistic inhibition of cell-cycle by down-regulation of cyclins D1 and E gene expression

Inhibition of cell proliferation is usually through an arrest in cell-cycle and/or up-regulation of apoptosis. We investigated effect on cell cycle by evaluating the transcriptional expression of cyclins D1 and E genes in prostate cancer cells. A ~40% reduction in cyclin E gene expression caused by 25 μM of G, Q and B was enhanced further when phytoestrogens were used in either double combinations or triple combination ($P<.01$). GQ, QB and GB reduced cyclin E expression by ~70-80%, whereas GQB reduced it by >80% (Fig. 3A). On the other hand, cyclin D1 expression was down-regulated only in combination treatments but the change was statistically non-significant (Fig. 3B). Nevertheless, a drastic change in cyclin E expression indicated cell-cycle disruption in treated cells. We confirmed cell-cycle arrest by flow cytometric assessment of propidium iodide labeled PC-3 cells. An increased G_1 phase arrest was accompanied by drastic reduction of cells in S-phase after treatment with phytoestrogens. GBQ treated cells had the highest sub-G_0G_1 peak of apoptotitic cells (Supplementary Data).

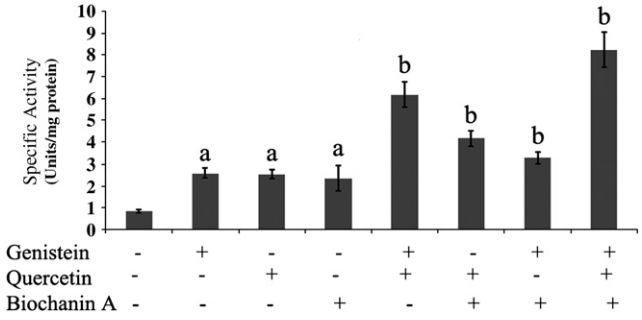

Fig. 4. Changes in activity of caspase-3 enzyme in prostate cancer (PC-3) cells after treatment with 25 μM of G, Q and B (B); 12.5+12.5 μM of G+Q, Q+B and G+B and 8.33 +8.33+8.33 μM of G+Q+B. Significant difference from vehicle treated control cells is indicated as [a]$P<.05$ and [b]$P<.01$.

3.6. Synergistic up-regulation of caspase-3 in PC-3 cells by phytoestrogens

To investigate the induction of apoptosis in PC-3 cells by the synergistic action of G, B and Q, we measured the enzyme activity of caspase-3, the executioner of apoptosis. Caspase-3 activity increased significantly in PC-3 cells treated with G, Q or B (~2.5-fold increase, $P<.05$); GQ, QB or GB (~3.5–6.5-fold increase, $P<.01$) and GQB (~9 fold increase, $P<.01$) at 25 μM in vitro (Fig. 4). This indicates that combined action of phytoestrogens increases apoptosis in prostate cancer cells more efficiently than individual compounds.

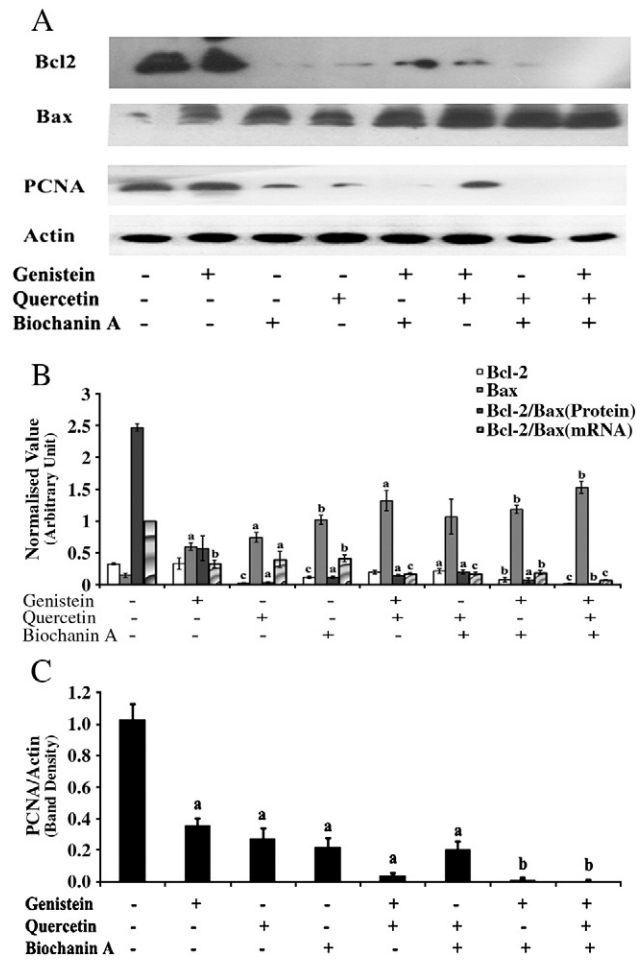

Fig. 5. Changes in expression of bcl-2, bax and PCNA proteins and bcl-2:bax mRNA ratio in prostate cancer (PC-3) cells after treatment with 25 μM of G, Q and B (B); 12.5+12.5 μM of G+Q, Q+B and G+B and 8.33+8.33+8.33 μM of G+Q+B. Representative immunoblot (A); statistical data on bcl-2, bax and bcl-2:bax mRNA and protein expressions in PC-3 cells (B); statistical data on PCNA expression in PC-3 cells (C). Significant difference from vehicle treated control cells is indicated as [a]$P<.05$, [b]$P<.01$ and [c]$P<.001$.

3.7. Down-regulation of bcl-2/bax gene expression ratio in PC-3 cells

A marked reduction in expression of anti-apoptotic bcl-2 and significant increase in expression of pro-apoptotic bax at transcriptional level in PC-3 cells after treatment with G, Q, B, GQ, QB, GB and GQB (data not shown) resulted in a significant reduction in bcl-2/bax gene expression ratio (Fig. 5B). bcl-2:bax mRNA expression ratio was reduced significantly by ~60% in G, Q and B; ~75% in GQ, QB and GB and by >80% ($P<.001$) in GQB treated cells (Fig. 5B).

3.8. Differential regulation of bcl-2 and bax protein expression in PC-3 by phytoestrogens

Q and B markedly reduced bcl-2, while G caused only a marginal change. GQ, QB and GB also very effectively reduced bcl-2 levels in PC-3, with QB having an edge over the other two combinations. However GQB was most effective and reduced bcl-2 to almost undetectable levels. On the other hand, bax protein level was increased markedly by all the three phytoestrogens and their combinations. Once again, combinations were more effective than single compounds (Fig. 5A, B).

3.9. PCNA expression in PC-3

PCNA levels fell sharply in prostate cancer cells after treatment with phytoestrogens. G, Q and B reduced PCNA significantly ($P<.05$) in PC-3 though Q and B had an edge over G. Bi-combinations were more effective than single compounds and QB combination reduced PCNA very significantly ($P<.01$). Nevertheless, GQB was most effective in reducing PCNA in PC-3 ($P<.01$; Fig. 5A and C).

3.10. Down-regulation of PI3K/AKT signaling pathway by phytoestrogens

The involvement of PI3K/AKT pathway in the synergistic anti-cancer effects of phytoestrogen combinations was evaluated by measuring the phospho-AKT (pAkt) protein levels in treated PC-3 cells by immunoblotting. A significant reduction of pAkt by 25 μM of individual phytoestrogens G ($P<.05$), Q ($P<.01$) and B ($P<.01$) in PC-3 cells was diluted in bi-combinations of 12.5+12.5 μM GQ (NS), QB ($P<.05$) and GB ($P<.05$). However, potent activity reappeared in GBQ combination, which was apparently the most effective. Thus, the inhibition of PI3K/AKT signaling pathway is evidently involved in the anti-cancer effects of G, Q and B and also in the synergistic action of GQB (Fig. 6A).

3.11. Down-regulation of mitogen activated protein kinase (MAPK)/ ERK-1/2 signaling in PC-3

The extracellular-signal regulated kinase (ERK-1/2) is an important component of MAP kinase signaling in cells. The involvement of this pathway in anti-CaP effect of phytoestrogens was studied by measuring phospho-ERK (pERK-1/2) levels in treated PC-3 cells using Western blotting. While individual phytoestrogens (G, Q and B) did not change pERK-1/2 levels in PC-3 cells, bi-combinations reduced pERK-1/2 significantly [GQ and QB ($P<.05$), GB ($P<.01$)]. However, once again, GQB was most effective and reduced pERK-1/2 to almost undetectable levels ($P<.001$). Thus, it is quite apparent that the

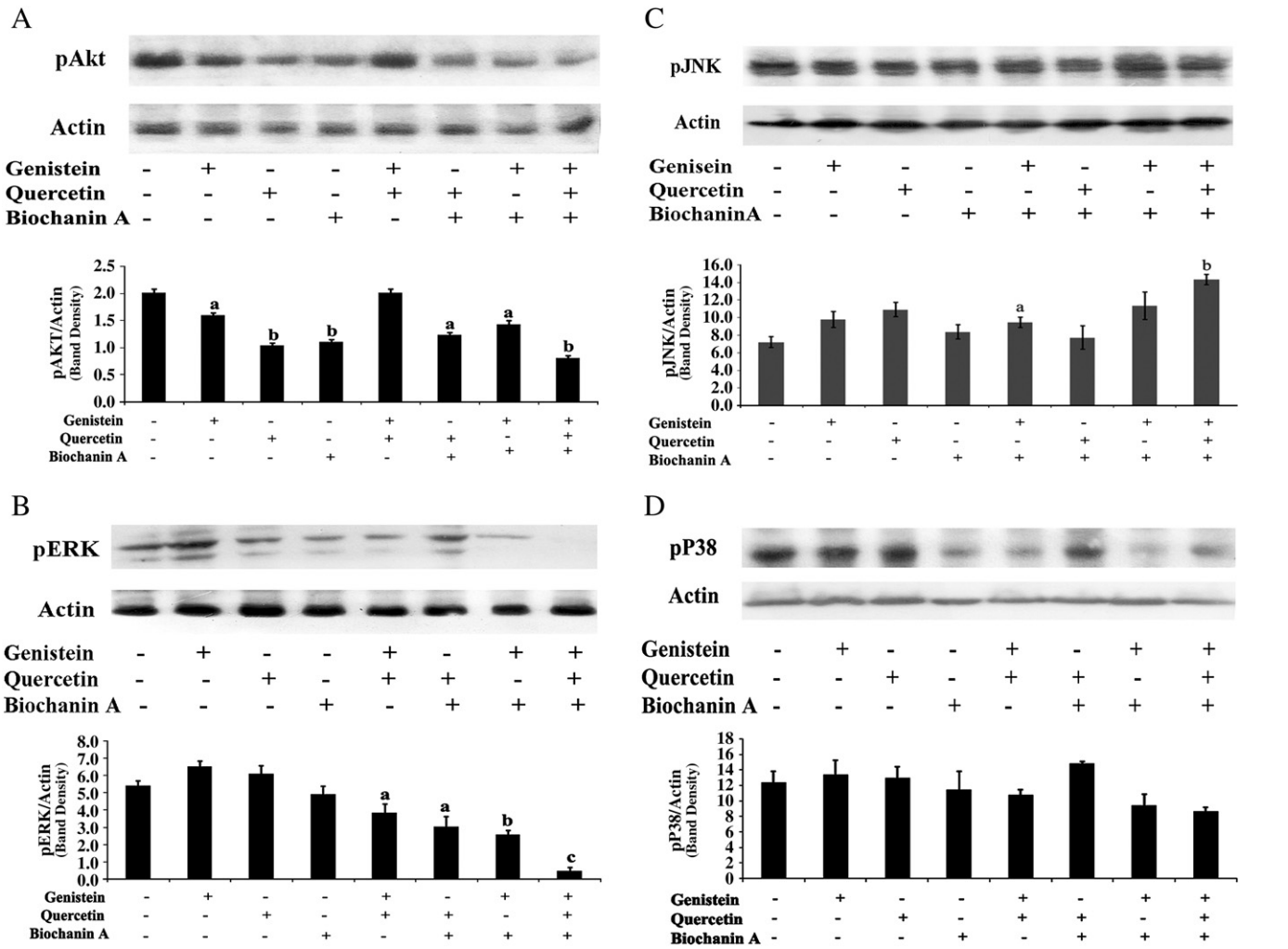

Fig. 6. Changes in phosphorylation of AKT, MAPK/ERK-1/2, MAPK/JNK and MAPK/p38 proteins in prostate cancer (PC-3) cells after treatment with 25 μM of G, Q and B (B); 12.5+12.5 μM of G+Q, Q+B and G+B and 8.33+8.33+8.33 μM of G+Q+B. Representative immunoblot and statistical data on pAkt levels (A); representative immunoblot and statistical data on pERK-1/2 levels (B); representative immunoblot and statistical data on pJNK levels (C), and representative immunoblot and statistical data on p38 levels in PC-3 cells. Significant difference from vehicle treated control cells is indicated as $^{a}P<.05$, $^{b}P<.01$ and $^{c}P<.001$.

synergistic action of G, Q and B is mediated through modulation of MAPK/ERK-1/2 signaling pathway (Fig. 6B).

3.12. Up-regulation of MAPK/c-Jun N-terminal kinase (JNK) signaling in PC-3 by phytoestrogens

The JNK signaling regulates cell survival and apoptosis. In the present study, the role of JNK-signaling pathway in inhibition of PC-3 cell proliferation was studied by estimating the levels of the active enzyme phospho-JNK (pJNK) in treated cells. G, Q and B had no significant effect on JNK signaling in PC-3 cells. Similarly, GQ and QB also failed to affect pJNK levels, although GB increased it significantly ($P<.05$). GQB yet again was most effective in increasing JNK-signaling in PC-3 cells and increased pJNK levels very significantly ($P<.01$; Fig. 6C). Hence, effective activation MAPK/JNK signaling in PC-3 cells required multiple-phytoestrogen treatment.

3.13. Phytoestrogens and MAPK p38 signaling in PC-3 cells

The p38-MAPK is another important component of MAP kinase signaling pathway that controls cell survival and apoptosis. However, G, Q and B did not affect this pathway significantly in PC-3 cells, either per se or in double/triple combinations. Thus, it is apparent that chemoprotective effect of phytoestrogen against prostate cancer may not be mediated through p38 MAPK signaling (Fig. 6D).

4. Discussion

In LNCaP, a direct association between the expression of androgen receptor (AR) and ER-β has been reported [24], and therefore, ER-β agonists like selective ER modulators and phytoestrogens act quite substantially through AR by down-regulating its expression [25] and, consequently, the expression of AR dependent genes [26]. Prostate cancer in its initial stages is androgen responsive and regresses by androgen ablation. However, it generally returns in a more invasive and hostile form that is androgen independent and difficult to manage. Our goal was to study the role of phytoestrogens in management of prostate cancer independent of the AR, exclusively through ER-mediated and non-ER-mediated pathways. Hence, we selected an androgen independent prostate cancer cell line (PC-3) for subsequent mechanistic study. Since PC-3 cell line expresses both ER-α and ER-β, it mimics the in vivo human situation more closely than DU-145, which expresses only ER-β.

Development of dietary compounds as anticancer agents is particularly attractive because of our long-experience of exposure to them, their relatively low toxicity, and encouraging indications from epidemiology [15]. Several preclinical studies have clearly

demonstrated the anti-CaP attribute of dietary phytoestrogens, which support the epidemiological evidences indicating their protective role against CaP [27]. However, the high concentrations of phytoestrogens needed to elicit effects in laboratory assays are difficult to achieve in vivo by dietary consumption. It has been reported that several weak estrogenic compounds can produce an unexpectedly strong synergistic response when mixed together at NOECs (no observed effect concentrations) in vitro [28]. In the present study, a combination of three weak estrogenic compounds at 8.33 μM (each) was significantly more effective than 25 μM of any individual component, in up-regulating anti-cancer mechanisms and inhibiting CaP cell proliferation. Japanese men who consumed cereal bars containing 20 mg soy isoflavones daily for 1 year had 15.8±0.8 μM G in genitourinary tissues [29]. Thus, this study demonstrates that innovative combination of phytoestrogens can elicit significant synergistic effects resulting in improved anticancer efficacy at physiologically achievable concentrations. Though previous studies have shown that G, B and Q individually inhibit proliferation of prostate cancer cells in vitro [30-33], but their combination effect has not been investigated. It has been shown that multiple flavonoid mixture can alter the pharmacokinetics to increase the bioavailability of individual flavonoids [34]. In this first study, we demonstrate synergistic action of three dietary phytoestrogens on human prostate carcinoma cells in vitro. In PCNA Western blot, the double combination (Q+B) (in spite of higher mean band density) appeared as effective as triple combination, statistically. However, it is important to take into consideration that, in the triple GQB combination, the net concentration of Q and B is diluted by 33% with G, which itself was least effective per se and less effective in dual combinations. Hence, higher activity could be achieved with low (physiologically achievable) concentrations of individual components in triple combination due to synergism. The same is true for a few other parameters where, statistically, double combination may appear as effective as triple combination. Increased bioavailability of individual components could be a reason for this combination effect. It has been reported that G enhances the cytosolic bioavailability of green tea catechin by two to five times [35]. G is one of the major isoflavones in human nutrition and is derived mainly from various legumes including soy bean (*Glycine max*), peas, lentils, peanuts and beans. Q is the main representative of the falvonol class and a polyphenolic antioxidant found in a variety of fruits and vegetables including citrus fruits, apples, grapes (red wine), variety of berries, red onions, broccoli, tomato and green leafy vegetables. On the other hand, B is predominantly concentrated in legumes like alfalfa, chick pea and red clover.

The effect of B and Q on ER-β gene expression in PC-3 cells has not been studied before, although a number of studies have provided compelling evidences in favor of the beneficial (anti-proliferative) effects of ER-β activation on prostate cancer [36]. Limited studies conducted with G have shown either inhibition [27] or elevation [12] of ER-β expression in PC-3 cells. However, our studies demonstrate that the ER-β gene is up-regulated in PC-3 cells significantly with G, B, GQ, QB and GB, but very significantly with GQB. Hence, a major ER-β mediated beneficial effect on prostate cancer progression is expected after treatment with GQB combination. Notably, an inverse relationship exists between ER-β expression and prostatic inflammation [37,38], and therefore, ER-β activation may also down-regulate inflammatory responses that promote tumor progression.

Since all phytoestrogens are weak ER agonists/antagonists, the present combination of phytoestrogens was conceived on the basis of their anticancer properties that were apparently independent of their binding to the ER, so as to have a superior synergized action. Besides several overlapping activities, some exclusive properties make the three an apt combination. G is one of the most potent, naturally occurring and specific inhibitor of protein tyrosine kinases with negligible protein serine/threonine kinase inhibitory activity [39]. On the other hand, B and Q have comparatively much lower tyrosine kinase inhibitory activity though Q has been reported to inhibit serine/threonine kinases [40]. Q is also the strongest anti-inflammatory agent amongst the three [20] and is a potent inhibitor of heat shock proteins [41] that play a significant role in survival of prostate cancer cells. In contrast, B is a very potent ArH receptor agonist, an activity not detectable in either G or Q [18]. B is also one of the least investigated flavonoid for anti-cancer, especially anti-CaP activity, possibly because it is a precursor of G and the general belief is that it acts through metabolic conversion to G. However unlike G, B shows distinct beneficial gene expression in breast cancer cells [34]. Also in the present study, GB was more efficacious in inhibiting LNCaP cell proliferation than either G or B, and GBQ was more effective than GQ in all the three cell lines. The present study indicates that the synergistic action of phytoestrogens involve both ER mediated and nonmediated events. For most of the tumor-related molecular parameters studied, single and double compound treatments were either ineffective or less effective than the triple compound treatment in promoting anti-cancer mechanisms, indicating the significance of multi-phytoestrogen action.

The PTEN (phosphatase and tensin homolog deleted on chromosome ten) tumor suppressor gene is lost or mutated in prostate cancer cells [42,43]. However, recombinant PTEN has been shown to dephosphorylate phosphotidylinositol-3,4,5-triphosphate [44], a product of PI3K that recruits AKT to plasma membrane for activation through phosphorylation. pAkt inhibits apoptosis [45], deregulates cell cycle and selectively enhances the production of growth and survival factors [46]. Hyperactivation of PI3K and increased phosphorylation of AKT are almost invariably associated with prostate cancer progression [47]. In the present study, pAkt level was reduced significantly in PC-3 by phytoestrogens, though G was somewhat less effective than Q and B as reported earlier in oral cancer cells [48]. Nevertheless, GQB combination was apparently the most effective, indicating inhibition of PI3K/AKT pathway as one of the modes for synergistic action of phytoestrogens.

The three highly characterized MAPK signal transduction pathways: the extracellular-signal regulated kinase (ERK-1/2), the JNK, and the p38 MAPK, have been implicated in a variety of cancers, including the CaP [49]. The ERK pathway plays an important role in cell survival by inhibiting apoptosis [50] and is activated in several tumors, including the prostate cancer, particularly at advanced stages of the disease [51,52]. JNK and p38 in contrast, are activated by stress signals and promote apoptosis [53]. The GQB combination was most effective in inhibiting MAPK/ERK pathway by reducing pERK-1/2 to undetectable levels in PC-3 cells. The reduction was significantly more than that by any of the double combination while single compounds per se were ineffective. On the other hand, MAPK/JNK pathway was significantly activated only when the three compounds were used in combination. Here, double combination and single compounds were largely ineffective. Thus, modulation of MAPK signaling may play an important role in the combination effect of multiple dietary phytoestrogens to curb prostate cancer cell proliferation. ICI-182780 resistant ER-β located on cell surface can also mediate MAPK/ERK-1/2 cell-signaling [54].

The ratio of B-cell lymphoma protein-2 (bcl-2; apoptosis suppressor) to its associated protein-X (bax; apoptosis inducer) indicates the therapeutic response of cancers (including CaP) to anticancer treatments [55]. A significant up-regulation of bax and down-regulation of bcl-2 proteins in CaP cells by GQB combination caused a considerable decrease in bcl-2/bax ratio, indicating a superior therapeutic potential of multi-phytoestrogen regimen over single compound. It has been shown that over expression of bcl-2 protein resists apoptosis induction by tyrosine kinase inhibitors in prostate cancer cells, which could be countered through a concomitant up-regulation of pro-apoptotic proteins like bax by using

phytoestrogens [56]. In the present study, high levels of bcl-2 were maintained in PC-3 cells after treatment with G (potent tyrosine kinase inhibitor) in spite of increased bax level. On the other hand, Q and B treatments reduced bcl-2 levels very effectively. The combined effect of three compounds reduced bcl-2 to undetectable levels while increasing bax expression quite significantly and, thus, creating an intracellular environment favoring apoptosis [57].

Prostate cancer chemotherapy often requires multiple agents that may contribute to added toxicity requiring dose reduction, which, in turn, may result in poor treatment outcome [58]. However, this study supports a phytotherapeutic/phytopreventive approach to CaP using innovative combinations of phytoestrogens like GQB, where multiple components may exhibit added efficacy at reduced concentrations (and hence toxicity) of individual components. The present study indicates that phytoestrogens exert their maximum beneficial effect when present in combinations of preferably more than two components, and therefore, novel combinations could be designed to improve efficacy and reduce dose, toxicity and adverse effects of single phytoestrogen therapy against CaP [59]. This study also provides a vital clue that selectively adding more components to GQB may further improve its chemoprotective activity without increasing the total concentration, resulting in enhanced efficacy of individual components at concentrations easily achievable in vivo by dietary supplementation. In view of the fact that the phytocompounds used in the present combination may not be available from a single dietary source or be found in appreciable quantities in normal diets of people residing in several countries (including those with high CaP incidence), supplementation may help in chemoprevention, especially in susceptible men over 60. Nevertheless, this study provides some crucial experimental evidence for the possible anti-CaP mechanisms that might be employed by multiple phytochemicals present in vegetarian diets, especially those of Asian people who have a lower incidence of the disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2010.06.003.

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