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Research Article

In vitro and *in vivo* modulation of testosterone mediated alterations in apoptosis related proteins by [6]-gingerol

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Ginger (*Zingiber officinale*, Zingiberaceae) has been widely used as a dietary spice, and as a traditional oriental medicine. The rhizome of ginger contains pungent vanillyl ketones, including [6]-gingerol and [6]-paradol, and have been credited with therapeutic and preventive health benefits, including anti-cancer activity. Prostate cancer is an attractive target for chemoprevention because of its ubiquity, treatment-related morbidity, long latency between premalignant lesions and clinically evident cancer, and defined molecular pathogenesis. Here we are reporting the modulatory effects of [6]-gingerol on testosterone-induced alterations on apoptosis related proteins in both *in vitro*, androgen sensitive LNCaP cells and *in vivo*, ventral prostate of Swiss albino mice. [6]-gingerol treatment resulted apoptosis in LNCaP cells, as indicated by depolarization of mitochondrial membrane potential, increase in sub G1 cell population by flow cytometry and the appearance of DNA laddering pattern in agarose gel electrophoresis. Results of western blot analysis showed that [6]-gingerol upregulated the testosterone depleted levels of p53 in mouse prostate and upregulated its downstream regulator Bax and further activated Caspase-9 and Caspase-3 in both LNCaP cells and in mouse prostate. We also found downregulation of testosterone induced antiapoptotic proteins, Bcl-2 and Survivin expression by [6]-gingerol in both LNCaP cells and in mouse ventral prostate. Thus, [6]-gingerol shows its protective effects in both *in vivo* and *in vitro* prostate cancer models by modulation of proteins involved in apoptosis pathway.

Keywords: Apoptosis / [6]-Gingerol / LNCaP / Mouse prostate / Testosterone

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1 Introduction

Prostate cancer (PCA) is one of the most common cancers in men. Each year ~543 000 new cases are reported worldwide, and the disease kills about 200 000 people in developed countries (http://www.prostatecancerfoundation.org). With the expanding understanding of the pathophysiology of PCA, identification of potential molecular targets and novel therapeutic approaches are required to treat both advanced and localized PCA [1]. Regulation of normal growth, development, and function of the prostate gland is intimately associated with androgen action because many androgen-regulated genes are known to contribute to development/progression of PCA [2]. Molecular biology of PCA and its progression is characterized by aberrant activity of several regulatory pathways both within the prostate cells and in the surrounding tissue. These pathways can be grouped broadly into apoptosis, androgen receptor signaling, signal transduction, cell cycle regulation, cell adhesion and cohesion, and angiogenesis [3]. Variations in the DNA, RNA and/or protein levels of molecules involved in these pathways are all potential candidate markers of prognosis and therapeutic response [4]. Although the underlying basis for rising levels of prostate-specific antigen (PSA) in PCA is not fully understood, but attention has turned to the possi-

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Abbreviations: **FBS,** fetalbovine serum; **GR,** group; **IAP**, inhibitor of apoptosis; **MTT**, 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; **PCA**, Prostate cancer; **PI,** Propidium iodide; **PSA,** Prostate-specific antigen; **TUNEL**, Terminal deoxynucleotide transferase dUTP nick-end labeling

bility that loss of normal p53 function might be directly involved in the pathogenesis [5]. Few studies have shown p53 protein accumulation and gene mutation in the progression of human PCA [6, 7]. Earlier reports have shown the concept of the targeted suppression of Bcl-2 anti-apoptotic family members using multitarget inhibition strategies for PCA, through the effective induction of apoptosis [8]. Besides this, accumulating evidence shows that Survivin, a new member of the inhibitor of apoptosis (IAP) family is associated with both PCA progression and drug resistance. Survivin plays a potentially important role in hormone therapy resistance and targeting of Survivin may enhance sensitivity to antiandrogen therapy in PCA [9]. A number of naturally occurring products have been shown to possess inhibitory effects on proliferation of prostate tumor cells associated with cell cycle blockage and apoptosis [10, 11].

Plant of ginger (*Zingiber officinale*) family is one of the most highly consumed dietary substances in the world. The oleoresin from rhizhome of ginger contains pungent ingredients including gingerol, shoagol, and zingerone [12]. Recently, these phenolic substances have been found to possess many interesting pharmacological and physiological activities [13]. Of these, [6]-gingerol (1-(4′-hydroxy-3′-methoxyphenyl)-5-hydroxy-3-decanone), the major pungent principle of ginger, has antioxidant, anti-inflammatory and anti-tumor promoting activities [12]. For instance, [6]-gingerol inhibited pulmonary metastasis in mice bearing B16F10 melanoma cells through the activation of CD8+ T cells [14]. It also inhibited the tumor promotion in ICR mouse induced skin tumor by tumor promoter, and blocked the azoxymethane-induced intestinal carcinogenesis in rodents [13, 15]. [6]-gingerol interfered with EGF-induced transformation of mouse epidermal JB6 cell line, and reduced the activation of activator protein-1, which plays a critical role in tumor promotion [16]. Moreover, [6]-gingerol exerted inhibitory effects on cell viability, DNA synthesis and also induced apoptosis in promyelocytic leukemia HL-60 cells [17] and in mutant p53-expressing pancreatic cancer cells [18]. Recently it has been shown that upregulation of MAP kinase phosphatase-5 by [6]-gingerol may contribute to its chemopreventive actions by decreasing prostatic inflammation [19]. It suggests that [6]-gingerol has some chemopreventive effects against PCA.

Our working hypothesis is that testosterone-mediated induction of antiapoptotic proteins is an important contributor of PCA development, and [6]-gingerol by inhibiting their expression will result in prevention of PCA. Validation of this hypothesis could lead to novel strategies for developing preventive approaches for human PCA. This study is designed to investigate both, under *in vitro* and *in vivo* situations, (i) the effect of testosterone on cell proliferation and apoptosis and (ii) whether [6]-gingerol can ameliorate these responses.

2 Materials and methods

2.1 Materials

Testosterone, Rhodamine 123, Propidium iodide and anti β-actin (clone AC-74) antibody were purchased from Sigma (St Louis, USA). [6]- gingerol was purchased from Calbiochem (Darmstadt, Germany). The p53, Bcl-2, Bax, Survivin and activated Caspase-3 and Caspase-9 antibodies were procured from Oncogene Cell Signaling Technogoly (Beverly, MA, USA). The anti-mouse and anti-rabbit secondary antibodies conjugated with horseradish peroxidase were obtained from Bangalore Genei (Bangalore, India). The nitrocellulose membrane and ECL detection kits were obtained from Millipore (Bedford, MA, USA). Rests of the chemicals were of analytical grade of purity and was procured locally.

2.2 Cell culture conditions and treatment

Human PCA cells LNCaP were purchased from National Centre for Cell Science, Pune, India and cultured as monolayers in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 μg/mL penicillin streptomycin (Gibco Lifetech, Karlsruche, Germany) and maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. To study the effect of testosterone alone, heat-inactivated FBS was replaced with charcoal stripped FBS and cells (80% confluence) were treated either with vehicle (ethanol; final concentration, 0.1%) or specified dose of testosterone (50 nM) in the same volume of ethanol. At 12 h post-treatment, the medium was removed; cells were washed twice with ice-cold PBS, and harvested cells were processed for further study.

Further, to study the modulatory effects of [6]-gingerol on testosterone-induced alterations, the cells were treated with ethanol alone (vehicle control) or testosterone (50 nM in 0.1% ethanol). After 12 h, the cells (80% confluent) were treated with varying doses of [6]-gingerol (5 – 75 μM) in ethanol (0.1%) for different time periods. Cells were harvested, washed with PBS, and processed for analysis.

2.3 Cell proliferation assay

The effect of [6]-gingerol on the proliferation of LNCaP cells was determined by MTT, (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), assay. The cells were plated at 1×10^4 cells per well in 200 μL of complete culture medium and exposed with testosterone (50 nM) and various concentrations of [6]-gingerol (5 – 75 μM) as mentioned in Section 2.2 in 96-well microtiter plates. After incubation for 24, 48 and 72 h at 37°C in a humidified incubator, cell proliferation was determined. MTT (5 mg/mL in phosphate buffered saline) was added to each well and incubated for 5 h

after which the plate was centrifuged at 1800 rpm for 5 min at 4°C. The supernatant was removed from the wells by aspiration. After careful removal of the medium, 0.1 mL of buffered DMSO was added to each well, and plates were shaken. The absorbance was recorded on a microplate reader at the wavelength of 530 nm. The assay was done three times independently, and the values are shown as mean ± SD. The effect of [6]-gingerol on growth inhibition was assessed as percent cell proliferation where cells treated with the vehicle were taken as 100% viable.

2.4 Flow cytometric analysis of apoptotic cell population in LNCaP Cells

Sub-confluent LNCaP cells were treated with or without testosterone (50 nM) for 12 h and with post-treatment of [6]-gingerol for 24 h (50 and 75 μM). Thereafter, the cells were harvested, washed with cold PBS, and processed for cell cycle analysis as described earlier [20]. Briefly, 1×10^5 cells were suspended in 500 μL of cold PBS, to which 500 μLcold methanol was added, and the cells were then incubated for 1 h at 4°C. The cells were centrifuged at 1100 rpm for 5 min. The pellet was washed with cold PBS, resuspended in 500 μL PBS, and incubated with 5 μL RNase (20 μg/mL final concentration) for 30 min. The cells were incubated with propidium iodide (PI) (50 μg/mLfinal concentration) for 30 min in the dark. The acquisition and analysis of samples were done three times, independently, and the values are shown as mean ± SD. The percent cells in sub-G1 population of the total cells were then determined using flow cytometry (BD-LSR-II, San Jose, CA, USA) equipped with Cell Quest 3.3 software.

2.5 ELISA for PSA in LNCaP cells

An ELISA kit from United Biotech (Mountain View, CA, USA) was used for measurement of PSA in both conditioned media (secreted) and cell lysate (cellular) [21]. LNCaP cells were gently washed three times with PBS to reduce background PSA. Fresh medium containing testosterone was then added for 12 h. Cells were then treated with [6]-gingerol (50 and 75 μM) for 24 h. Conditioned media samples were collected and centrifuged to remove detached cells. Cell lysate was prepared in PBS by freezing on dry ice and thawing for three cycles and sonicated for 15 s. The supernatant obtained after centrifugation (16 000 × g, 20 min, 4°C) was collected and stored at −20°C. Protein content of the lysate was determined by Lowry [22] method for normalization of results. The assay was carried out three times, independently, and the values are shown as mean ± SD.

2.6 Total cell lysate preparation of LNCaP cells

For dose dependent studies, the cells (50% confluent) were treated with testosterone and [6]-gingerol as mentioned in Section 2.2. Cells that served as controls were incubated with the vehicle (ethanol) alone. Following treatment of the cells, the medium was aspirated and the cells were washed twice with cold PBS (10 mM, pH 7.4). Ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP-40, 1% Triton X-100, 1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, pH 7.4) was added to the plates, which were then placed over ice for 30 min [23]. The cells were scraped, and the lysate was collected in a microfuge tube and passed through a 21G needle to break up the cell aggregates. The lysates was cleared by centrifugation at 14 000 × g for 15 min at 4°C and the supernatant (total cell lysate) was either used immediately or stored at −80°C.

2.7 Mitochondrial membrane potential analysis

The change in the mitochondrial membrane potential after treatment with [6]-gingerol was determined by flow cytometry using rhodamine 123 dye. Briefly, LNCaP cells were treated with testosterone and [6]-gingerol (50 and 75 μM) as mentioned in Section 2.2, harvested, washed with PBS. One times 10^6 cells were incubated in 1 mL PBS (pH 7.4) containing rhodamine 123 (5 μg/mL) for 60 min in the dark at 37°C. Stained cells were washed, resuspended in 500 μL PBS. The mitochondrial membrane potential was measured using flow cytometry and was defined in terms of mean fluorescence intensity (FL-1, 530 nm) of 10 000 cells [24]. The assay was done three times, independently, and the values of MFI are shown as mean ± SD.

2.8 DNA fragmentation assay

The DNA fragmentation assay was carried out by agarose gel electrophoresis as described earlier [20]. An aliquot of 2 mL (1.5×10^6 cells/mL) was incubated with testosterone and [6]-gingerol (50 and 75 μM) as described. At the end of incubation, cells were pelleted by centrifugation at 200 × g for 10 min and the pellet lyzed with 0.5 mL lysis buffer (10 mM Tris-HCl, pH 7.5, 20 mM EDTA, 0.5% Triton X-100) on ice for 30 min. The DNA in lyzed solution was extracted with phenol/chloroform and precipitated with 3 M sodium acetate (pH 5.2) and cold ethanol. The purity of DNA was determined by taking absorbance at 260 and 280 nm. Ratio obtained between 1.7 – 1.9, indicated the purity of sample. DNA (2 μg) was then loaded on 1.5% agarose gel and electrophoresis was carried out. The bands were visualized by ethidium bromide staining under ultra-violet light and recorded on Versa Doc Imaging System (BioRad Model 4000).

2.9 Animals and treatment

Adult male Swiss albino mice (~25 g/body weight.) were taken for the study from the Industrial Toxicology Research Centre animal-breeding colony. Animals were quarantined

for one week on a 12/12 h. light-dark cycle and were fed synthetic solid pellet diet (Ashirwad, Chandigarh, India) and water *ad libitum*. The animals were divided into five groups (Gr.) comprising ten animals each. The animals of Gr. I were kept untreated while animals of Gr. II, III and IV were given testosterone (5 mg/kg body weight dissolved in minimal amount of ethanol and suitably diluted in corn oil) subcutaneously for 15 consecutive days. [6]-gingerol (10 mg/kg body weight dissolved in minimal amount of ethanol and suitably diluted in corn oil) was given orally 1 h prior and post to testosterone administration in Gr. III and IV respectively. Gr. V served as vehicle control and were given [6]-gingerol orally for 15 days. Animals from all the Grs. were examined every day for gross morphological changes including body weight changes during the entire study period. Animals were sacrificed, 24 h after the last treatment, blood and ventral prostate tissue was taken out and stored at -80°C until further use. Weight of prostate of each animal from all Grs. was recorded.

2.10 Preparation of prostate tissue samples

For protein source 100 mg of prostate tissue was homogenized in ice-cold lysis buffer (containing 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS supplemented with protease inhibitor mixture) followed by a 30 min incubation at 4°C. Supernatant were collected after centrifugation at $10\,000 \times$ g for 10 min at 4°C and were either used immediately or stored at -80°C.

2.11 Western blotting

Western blotting was carried out as described by Arora *et al.,* [25] in prostate tissue samples and total cell lysates prepared. Protein concentration was estimated [22], using BSA as standard. Protein samples (100 μg) were resolved on 10% SDS-polyacrylamide gels followed by electro-transfer onto an immobile PVDF membrane (Millipore). The blots were blocked overnight with 5% nonfat dry milk and probed with primary antibody at dilutions (1:1000) recommended by the suppliers. Immunoblots were detected by horseradish peroxidase conjugated anti mouse or anti rabbit IgG using chemiluminescence kit of Millipore and visualized by Versa Doc Imaging System (BioRad Model 4000). To quantify equal loading, membranes were reprobed with β-actin antibody. The intensity was given in terms of relative pixel density for each band normalized to band of β-actin. The intensity of the bands were measured using software UNSCAN- IT automated digital system version 5.1. The blotting was done three times, independently, and the pixel densities are shown as mean ± SD.

2.12 TUNEL assay

We used Apo-BrdU terminal deoxynucleotide transferase dUTP nick-end labeling (TUNEL) kit (Molecular Probes, Eugene, OR) to measure the extent of apoptosis in prostate by exploiting the fact that the DNA breaks expose a large number of 3′-hydroxyl ends. These hydroxyl groups can then serve as starting points for terminal deoxynucleotidyl transferase (TdT) to add deoxyribonucleotides in a template-independent fashion. Addition of BrdUTP to the TdT reaction serves to label these break sites. Once incorporated into the DNA, BrdU can be detected by FITC (fluorescein isothiocyanate) or alexa flour 488 dye conjugated anti-BrdU antibody and PI to simultaneously give cell cycle and apoptotic data using flow cytometry.

For this assay, the single cell suspension of treated and untreated mouse prostate was prepared using Madimachine (BD LSR II). The cell suspension was washed with PBS and the cells (1×10^6) were fixed overnight in ethanol (90%). The cells were washed and labeled with UTP-BrdU overnight, washed again with PBS and incubated with alexa flour 488 Anti-BrdU antibody followed by counterstaining with PI. Cells were analyzed using a flow cytometer (BD LSR II). The analysis were performed using Cell Quest software (BD LSR II) for apoptosis.

2.13 Statistical analysis

All data are expressed as ± SD of three independent experiments. Statistical analyses between Grs. were performed by Student's *t*-test.

3 Results

3.1 Effect of testosterone and [6]-gingerol on cell proliferation of LNCaP cells

Testosterone treatment to LNCaP cells resulted in increased cell proliferation in a time dependent manner in comparison to vehicle treated cells (data not shown). However, [6]-gingerol treatment to cells resulted in a dose-dependent inhibition of cell proliferation, assessed at 24, 48 and 72 h post treatment. IC_{50} was found to be 50 μM at 24 h time period ($p < 0.05$). Based on this observation we further planned our study with 50 μM and 75 μM of [6]-gingerol treatment to cells.

3.2 Effect of [6]-gingerol on PSA levels in LNCaP cells

Treatment of LNCaP cells with [6]-gingerol resulted in a concentration-dependent decrease in cellular PSA level induced by testosterone (Fig. 1). The value for cellular PSA was 100 ng/mg protein based on ELISA in vehicle treated cells, which was significantly increased up to 150 ng/mg protein by testosterone treatment (Fig. 1(A)). However, [6]-gingerol (50 – 75 μM) decreased the amount of cellular PSA level by 40 and 60% ($p < 0.05$), respectively in comparison to testosterone treated cells. Cells which were incu-

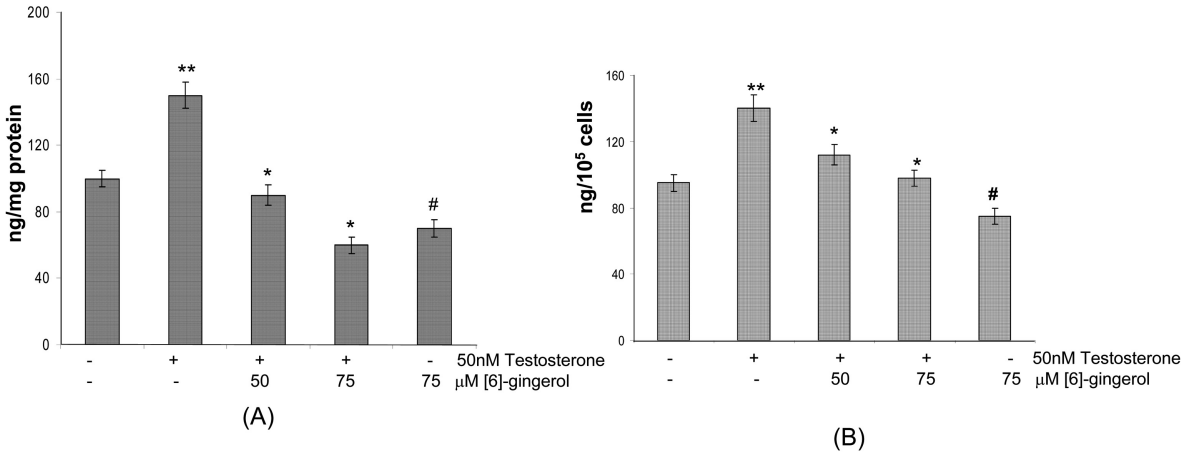

Figure 1. Effect of [6]-gingerol on testosterone induced PSA levels in LNCaP cells (A) cellular (B) secretory PSA level. Cells were treated with specified concentration of both testosterone and [6]-gingerol. PSA level was estimated using ELISA kit. Data represents mean value of percent viable cells ± SD of three independent experiments with similar results. **shows significant increase over vehicle treated cells, *shows significant decrease over testosterone treated cells, #shows significant decrease over vehicle treated cells. $p < 0.05$.

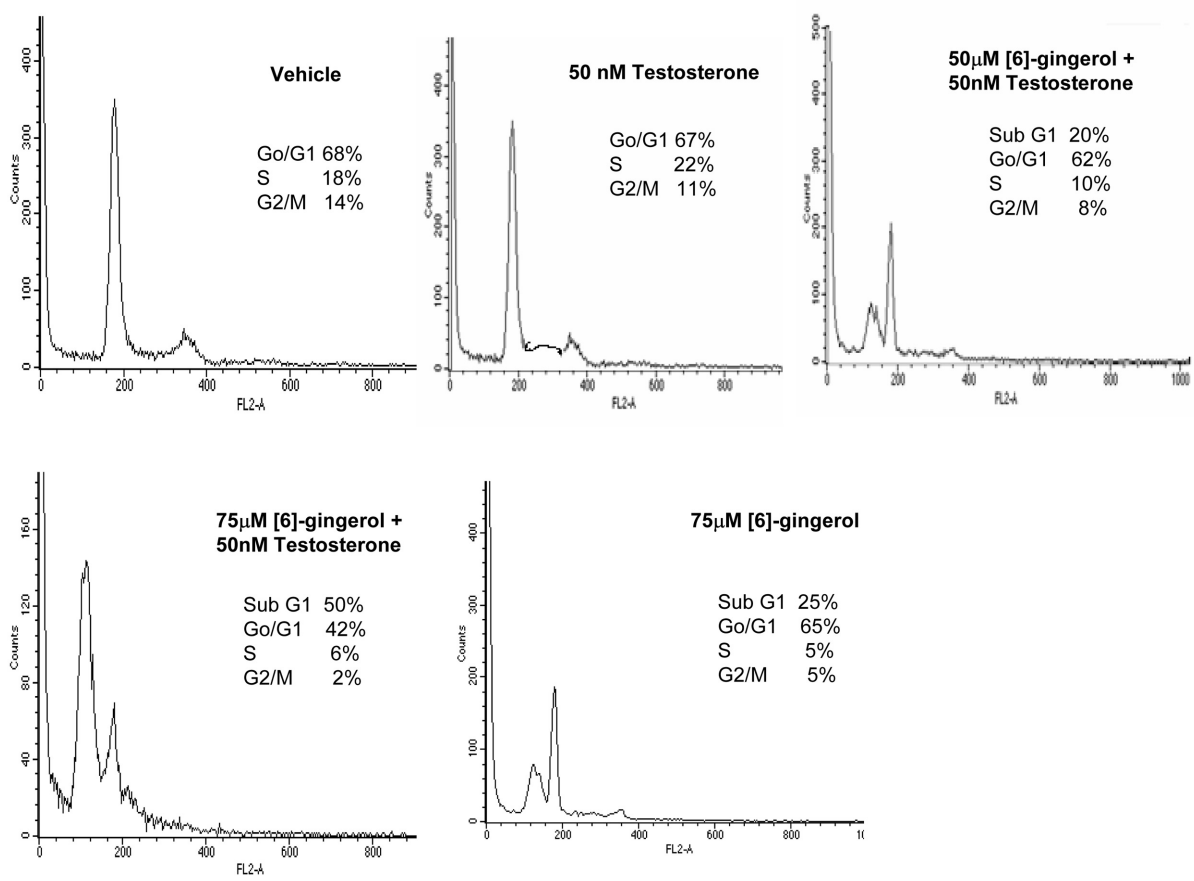

Figure 2. [6]-gingerol-induced apoptosis in LNCaP cells as shown by flow cytometry analysis: The cells were treated with a specified concentration of testosterone and [6]-gingerol. Sub G1 peak, indicative of apoptosis was obtained by [6]-gingerol treatment in a dose dependent manner. Data represents mean value of ± SD of three independent experiments. The details are described in Section 2.

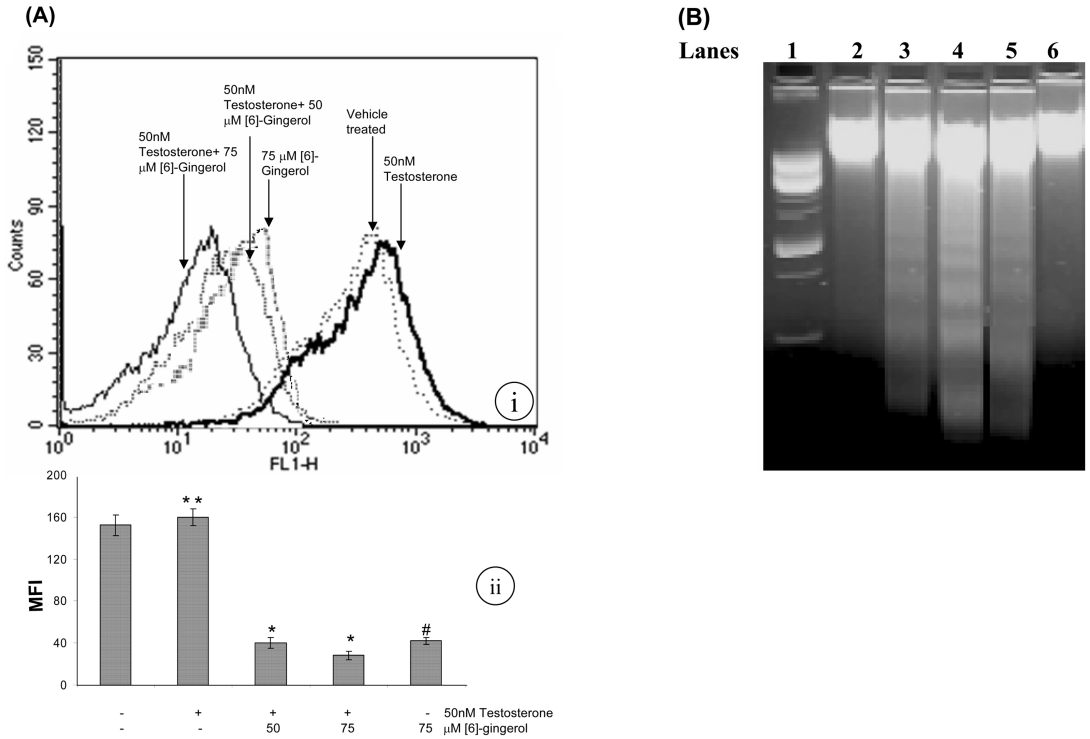

Figure 3. Effect of [6]-gingerol on (A) mitochondrial membrane potential and (B) DNA fragmentation. Freshly isolated LNCaP (1.5×10^6) treated with testosterone and [6]-gingerol (50 – 75 μM) for 24 h. (A) For determination of mitochondrial membrane potential rhodamine 123 was added, incubated for 60 min and its fluorescence measured using a flow cytometer with FL-1 filter. Results expressed as representative histogram (i) and mean fluorescence obtained from the histogram statistics (ii). ** shows significant increase over vehicle treated cells, * shows significant decrease over testosterone treated cells, # shows significant decrease over vehicle treated cells. $p < 0.05$. (B) Apoptotic effect was assessed by DNA fragmentation by (1.5%) agarose gel electrophoresis. Vehicle treated cells showing no ladder formation or single band of DNA (lane 2) while ladders were observed in both 50 and 75 μM [6]-gingerol treated cells (lane 3 and 4) as well as 75 μM [6]-gingerol alone treated cells (lane 5). Vehicle treated cells (lane 6). Marker was run in lane 1. The result shown here is representative of three independent experiments with similar results.

bated with [6]-gingerol alone also showed a decrease in the cellular PSA levels up to 30% ($p < 0.05$) in comparison to vehicle treated cells. Vehicle treated cells secreted about 95 ng PSA per T25 flask (95 ng/10^5 cells) during this 12 h period and this was increased to 140 ng/10^5 cells by testosterone treatment (Fig. 1(B)). [6]-gingerol (50 – 75 μM) at a given concentration reduced this amount to about 30 ($p < 0.05$) and 20% ($p < 0.05$), respectively in comparison to testosterone treated cells. Cells incubated with [6]-gingerol alone also showed a decrease in the secretary PSA levels up to 21% ($p < 0.05$) in comparison to untreated cells. Thus, it appeared that cellular PSA induced by testosterone was inhibited to a greater magnitude by [6]-gingerol than was the secretary PSA.

3.3 [6]-gingerol induces apoptosis in LNCaP cells

Testosterone treatment to LNCaP cells resulted in increased S phase in comparison to vehicle treated control. However, significant increase in sub-G1 population of LNCaP cells was observed after 24 h of post-treatment with [6]-gingerol, *i.e.* 50 μM (20% of total cell population, $p < 0.05$) and 75 μM (50% of total cell population, $p < 0.05$) with concomitant decrease in the S phase population (Fig. 2). [6]-gingerol treatment alone also induces a significant increase in sub-G1 cell population.

3.4 [6]-gingerol depolarizes the mitochondrial membrane potential and induces DNA fragmentation in LNCaP cells

Rhodamine 123, a lipophillic cationic fluorescent dye, is selectively taken up by mitochondria and its uptake is directly proportional to mitochondrial membrane potential of cells [24]. An increased mitochondrial membrane potential was recorded by testosterone treatment in LNCaP cells in comparison to vehicle treated LNCaP cells. However, [6]-gingerol treatment along with testosterone resulted in a decrease in mitochondrial membrane potential in a dose depended manner, as an early event of apoptosis (Fig. 3(A)). Cells treated with [6]-gingerol alone also showed a decrease in the mitochondrial membrane potential in com-

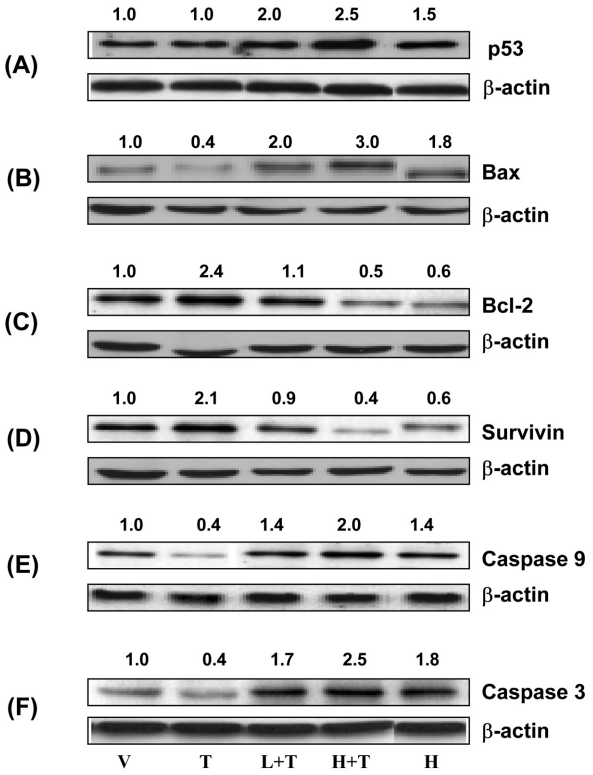

Figure 4. Effect of [6]-gingerol on p53 (A), Bax (B), Bcl-2 (C), Survivin (D), Caspase-9 (E) and Caspase-3 (F) expression in LNCaP cells. Western blotting was done in LNCaP cells treated with testosterone and [6]-gingerol (50–75 μM) for 24 h. (Vehicle treated control (V), Testosterone (T), 50 μM [6]-gingerol (L) + testosterone, 75 μM [6]-gingerol (H) + testosterone, 75 μM [6]-gingerol (H)). Samples were incubated with primary antibody specific for each protein and secondary antibody. Equal loading was confirmed by stripping immunoblots and reprobing them for β-actin. Values given above each lane on the immunoblot represents the relative density of the bands normalized to β-actin. The immunoblots shown here are representative of three independent experiments with similar results. The details are described in Section 2.

parison to vehicle treated control. DNA laddering pattern was also recorded by [6]-gingerol treatment along with testosterone in LNCaP cells in a dose dependent manner (Fig. 3(B)). However, no fragments of DNA could be observed either with testosterone or with vehicle treatments to LNCaP cells. Cells treated with [6]-gingerol alone also showed DNA laddering pattern in LNCaP cells (Fig. 3(B)).

3.5 Effect of [6]-gingerol on cell apoptosis related protein expression in LNCaP cells

p53 is known to play a central role in sensing and signaling for growth arrest and apoptosis in cells with DNA damage. It is one of the most commonly mutated genes found in human tumors [26]. p53 is a sequence specific transcription factor that binds to DNA and thereby activates Bax to induce apoptosis and represses Bcl-2 [27, 28]. To study the effect of [6]-gingerol on apoptosis related proteins, LNCaP cells were treated with testosterone and 50 and 75 μM of [6]-gingerol for 24 h. Results of western blotting showed that testosterone treatment had no effect on p53 expression however, down regulated Bax expression in comparison to vehicle treated control ($p < 0.05$) (Fig. 4(A) and (B)). [6]-gingerol treatment along with testosterone leads to over-expression of p53 and its downstream regulator Bax ($p < 0.05$) (Fig. 4(A) and (B)) in a dose dependent manner. Further, testosterone application significantly enhanced the level of antiapoptotic protein Bcl-2 ($p < 0.05$) which was restored to the normal level by [6]-gingerol in a dose dependent manner (Fig. 4(C)). Cells treated with [6]-gingerol alone showed upregulation of p53 and Bax with concomitant decrease in the Bcl-2 protein expression in comparison to vehicle treated control (Fig. 4) ($p < 0.05$). We further investigated the effect of testosterone on another antiapoptotic protein, Survivin. We found that testosterone significantly upregulated the protein expression of Survivin ($p < 0.05$) over vehicle treated control. However, [6]-gingerol treatment to LNCaP cells, along with testosterone, resulted in downregulation of protein up to the normal levels (Fig. 4(D)) ($p < 0.05$). Cells treated with [6]-gingerol alone also showed down regulation of Survivin protein in comparison to vehicle treated control ($p < 0.05$). Caspases are a family of highly specific cysteine proteases, which plays a central role in apoptosis. They are expressed as inactive proenzymes and participate in a cascade triggered in response to pro-apoptotic signals [29]. Caspase-9 is an initiator protease whereas Caspase-3 is considered to be a major executioner protease [29]. Next we showed that testosterone treatment resulted in downregulation of activated Caspase-9 and Caspase-3 proteins expression in LNCaP cells (Fig. 4(E) and (F)) ($p < 0.05$). However, [6]-gingerol treatment along with testosterone upregulated the expression of both activated Caspase-9 and Caspase-3 in LNCaP cells in a dose dependent manner (Fig. 4(E) and (F)) ($p < 0.05$). Cells treated with [6]-gingerol alone also showed upregulation of activated Caspase-3 and Caspase-9 in comparison to vehicle treated control (Fig. 4(E) and (F)) ($p < 0.05$).

3.6 Effect of [6]-gingerol on prostate weight of mice

During the course of the study, no marked change in the food and water consumption, any sign of overt toxicity, change in body weight and mortality was recorded in the animals of treated groups in comparison to controls. Results showed that testosterone treatment consecutively for 15 days resulted in significant enlargement of mouse prostate ($p < 0.05$). However, there was significant decrease in the prostate weight with supplementation of [6]-gingerol given both prior and post to testosterone. [6]-gingerol alone did not cause any significant change in the weight of prostate (Fig. 5(A)).

3.7 [6]-gingerol causes apoptosis in prostate

Result of the TUNEL assay showed a marked induction of apoptosis in [6]-gingerol treated mouse prostate. Treatment of [6]-gingerol, prior and post to testosterone, resulted in a significant increase in apoptosis (~42 and ~38%, respectively) as FL3 positive population compared to untreated control Gr. I (Fig. 5(B)). Comparatively, no apoptosis was observed either in testosterone or [6]-gingerol alone treated mouse prostate (Fig. 5(B)).

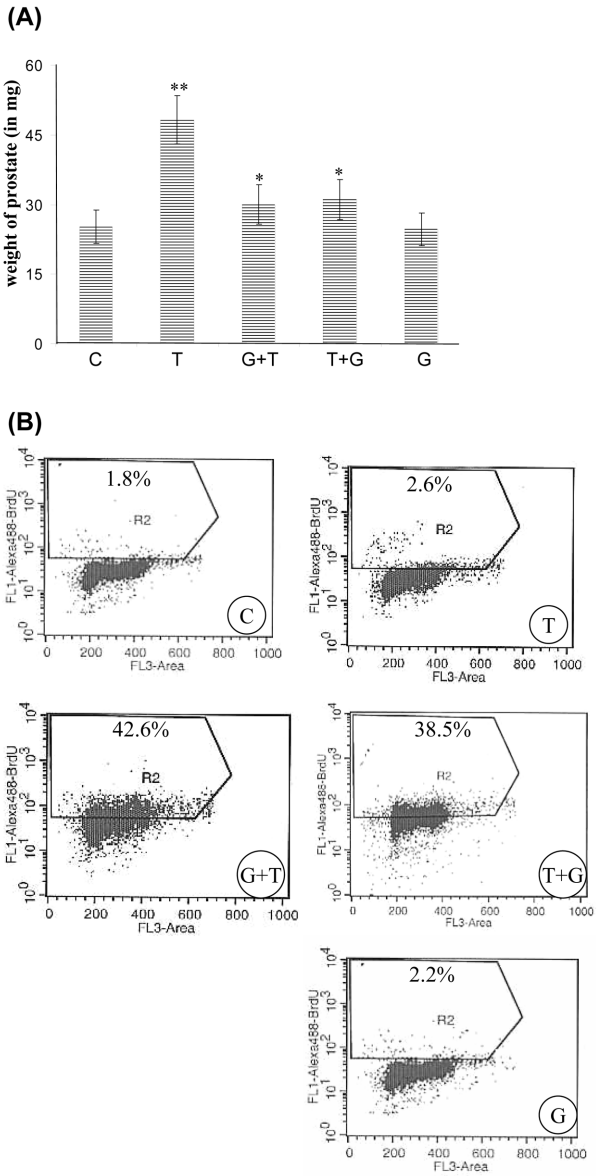

Figure 5. Effect of [6] gingerol on prostate weight (A) and induction of apoptosis (B). The animals were followed for the development of benign hyperplasia and experiment was terminated after 15 days. (A) Weight of each mouse prostate was taken. Results are expressed in average weight of prostate in each Gr. $*p < 0.01$ was considered to be statistically significant compared with controls. (B) The extent of apoptosis was assessed with the APO-BrdU TUNEL Assay kit. The fragmentation of DNA in apoptotic cells is measured by BrdU incorporation which is visualized by conjugation to an Alexa Fluor 488 dye-labeled anti-BrdU antibody. BrdU incorporation was analyzed with flow cytometer. The data is expressed as mean ± SD of three experiments ($*p < 0.01$). Untreated control (C), Testosterone alone (T), [6]-gingerol + Testosterone (G+T), Testosterone + [6]-gingerol (T+G), [6]-gingerol alone (G).

3.8 Effect of [6]-gingerol on apoptosis related protein expression in prostate

Western blot analysis followed by densitometry of the bands revealed that testosterone treatment resulted in a decrease in the level of p53 (Fig. 6(A)). [6]-gingerol supplementation resulted in upregulation of the p53 levels in both Gr. III and Gr. IV, in comparison to testosterone treated Gr. II (Fig. 6(A)). Further, Western blot analysis of Bcl-2 family proteins revealed that testosterone (Gr. II) upregulated Bcl-2

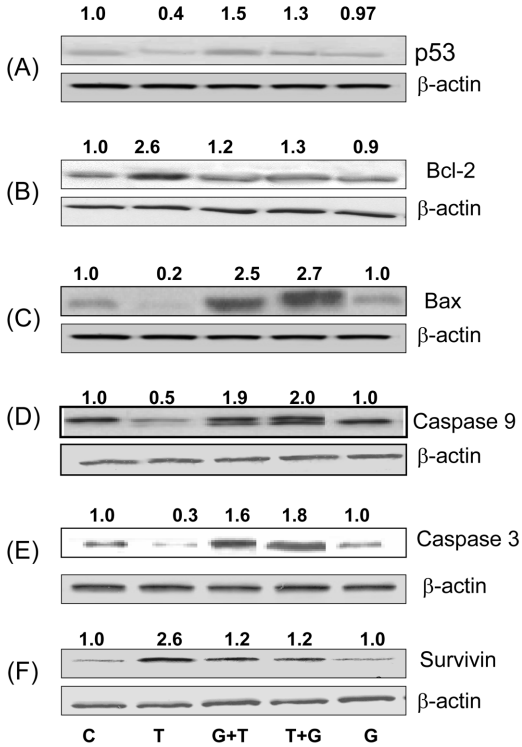

Figure 6. Effect of [6]-gingerol on wild type p53 (A), Bcl-2 (B), Bax (C), Caspase-9 (D), Caspase-3 (E) and Survivin (F) expression in mouse prostate. Western blotting was done in prostate tissue samples. Samples were incubated with primary antibody specific for each protein and secondary antibody. Equal loading was confirmed by stripping immunoblots and reprobing them for β-actin. Values given above each lane on the immunoblot represents the relative density of the bands normalized to β-actin. The immunoblots shown here are representative of three independent experiments with similar results. The details are described in Section 2 (Untreated control (C), Testosterone alone (T), [6]-gingerol + Testosterone (G+T), Testosterone + [6]-gingerol (T+G), [6]-gingerol alone (G).

(Fig. 6(B)) with concomitant decrease in Bax protein (Fig. 6(C)) expression. Densitometry of the immunoblots showed that the ratio of Bax/Bcl-2 was significantly higher in [6]-gingerol-treated Gr. III and IV. [6]-gingerol supplementation significantly enhanced the Bax protein level and decreased testosterone-mediated induction in the level of Bcl-2 protein. However, [6]-gingerol alone (Gr. V) had no effect on the level of Bcl-2 and Bax protein (Fig. 6(B) and (C)) in mouse prostate.

Further extending this work we also showed the effect of [6]-gingerol on testosterone induced altered levels of Caspases expressions. Western blot analysis revealed that testosterone (Gr. II) induced downregulation of both activated Caspase-9 (Fig. 6(D)) and Caspase-3 proteins (Fig. 6(E)). Densitometry of the immunoblot showed that there was significant increase in the expression level of both activated Caspase-9 (Fig. 6(D)) and Caspase-3 (Fig. 6(E)) in [6]-gingerol treated Gr. III and Gr. IV in comparison to testosterone-treated Gr. II. However, [6]-gingerol alone (Gr. V) had no effect on the level of Caspase-9 and Caspase-3 protein expression in mouse prostate. Testosterone treatment (Gr. II) also resulted in the upregulation of antiapoptotic protein Survivin (Fig. 6(F)) in comparison to untreated control. Densitometry of the immunoblots revealed a significant decrease in the level of Survivin in [6]-gingerol supplemented Gr. III and Gr. IV when compared to Gr. II. [6]-gingerol alone (Gr. V) had no effect on the level of Survivin.

4 Discussion

The long latency observed in PCA is unique among malignancies and provides a lengthy window of opportunity for intervention by chemopreventive agents. The hope of PCA prevention by "natural" dietary products, also known as phytochemicals, has become increasingly popular and concordantly the mechanism(s) of action of phytochemicals has become an active area of research. The anti-tumorigenic potential of [6]-gingerol have been studied in *in vivo* as well as in *in vitro* [15, 17, 30] but so far there is no study which correlates the chemopreventive effect of [6]-gingerol to PCA prevention. Here, for the first time we showed a probable mechanism of growth regulatory effects of [6]-gingerol in both *in vitro* and *in vivo* experimental models of PCA.

Androgens are essential for normal prostate physiology and have been shown to play a key role in the development and pathogenesis of PCA [28]. Studies have also shown that androgen deprivation may prevent the development of PCA [31]. Therefore, we used androgen-sensitive LNCaP cells for our *in vitro* studies. In our study, [6]-gingerol inhibited testosterone induced cell growth and PSA level. In the clinic, serum PSA measurements are being widely used to screen for PCA [32, 33]. Several studies have reported the usefulness of serum PSA as a follow-up marker for local recurrence and/or distant disease in the patients after radical prostatectomy, radiation and hormonal therapy [34, 35].

Further, we showed that [6]-gingerol treatment induced apoptosis in LNCaP cells as shown by increase in Sub G1 peak, decrease in membrane potential and DNA fragmentation (Figs. 2 and 3). It is reported that accumulation of p53, activation of Caspases and reductions in IAP abundance promote the apoptosis of PCA cells [36]. In the present study, [6]-gingerol treatment in LNCaP cells resulted in upregulation of p53 expression (Fig. 4(A)). It was shown before that testosterone produced robust stimulation of Survivin mRNA and also had a modest effect on antiapoptotic *bcl-2* mRNA in hormone responsive ALVA-101 human prostate cancer cell line and pyrrolidinedithiocarbamate, a potent inducer of apoptosis, exerts antiandrogen-like action by reversing the expression of both Survivin and Bcl-2 [37]. We also showed that testosterone treatment resulted in downregulation of Bax (Fig. 4(B)) with concomitant upregulation of both Bcl-2 (Fig. 4(C)) and Survivin (Fig. 4(D)). However, [6]-gingerol treatment reversed the expression of proteins in favor of apoptosis (Fig. 4). Survivin inhibits apoptosis by binding to microtubules of the mitotic spindles [38] and ultimately inactivating Caspase-3 activity [39]. Further, transfection in prostate cancer cell lines with oligonucleotide of Bcl-2 is associated with increased resistance to apoptosis, with concomitant decrease in mitochondrial membrane disruption and cytochrome-*c* release. Cytosolic cytochrome-*c* forms a complex with Apaf-1 and Caspase-9, resulting in the activation of Caspase-9 and Caspase-3 [40]. Our results showed that testosterone repressed levels of activated Caspases were found to be increased by [6]-gingerol treatment in LNCaP cells (Fig. 4(E) and (F)). Our results were supported by an earlier report showing that androgen interferes with induction of PCA cell death induced by a variety of stimuli. The effect of androgen on cell death occurs predominantly by interference with Caspases activation and attenuation of Bax expression [41].

Our next objective was to investigate whether these *in vitro* findings could also be translated to an *in vivo* situation. Our results clearly demonstrated that [6]-gingerol significantly inhibited the enlargement of ventral prostate (Fig. 5(A)) induced by testosterone. This study also demonstrated that [6]-gingerol induced apoptosis in mouse prostate, characterized by nicked DNA (Fig. 5(B)), may also contribute to inhibition of prostate enlargement. Next, we showed that [6]-gingerol treatment upregulated the testosterone repressed p53 expression (Fig. 6(A)) in mouse ventral prostate. Previous studies have shown that hormone-regulated apoptosis of the ventral prostate is associated with increased expression of p53 message and protein [42]. Concerning the mitochondrial pathway, further studies have suggested that the Bcl-2 family of proteins could be involved in the prostate cell death apoptotic process. First, a raise in the Bax/Bcl-2 ratio has been observed in ventral prostate during apoptotic cell death process induced by

androgen withdrawal [42]. The downregulation of Bcl-2 and Survivin and upregulation of Bax Capase-9 and Caspase-3 (Fig. 6), in the present study, may be associated with inhibition of testosterone induced prostate growth by inducing apoptosis. It has also been reported that testosterone inhibits the cell death process in the ventral prostate of rat by regulating Caspase-3 and -6 mRNA levels as well as procaspase and active Caspase-3 and -6 proteins levels [43].

Caspase family of cysteine proteases are involved in the execution of cells in response to many apoptotic stimuli including death-receptor (extrinsic) and mitochondria-dependent (intrinsic) pathways [44]. Caspases cleave a variety of substrates including components of cellular DNA repair machinery and activate DNase [45]. The [6]-gingerol induced Caspase -3 and -9 may result in DNA fragmentation as observed by the TUNEL assay in mouse prostate.

We showed that [6]-gingerol treatment alone induces cell death in cancer cells, however its treatment to the ventral prostate tissue does not have any effect on the proteins involved in apoptosis. It shows that [6]-gingerol has dual efficacy in that it kills cancer cells without having any effect on the normal cells. Treatment of testosterone on either cancer cells or to ventral prostate resulted in induction of anti-apoptotic signals. However, [6]-gingerol treatment to cancer cells and to ventral prostate reversed the alterations caused by testosterone in favor of apoptosis. Since, some anti-androgenic drugs like finsteride and dutasteride are shown to reduce PSA levels in human [46] and LNCaP cells [47], results of the present study also showed that [6]-gingerol may regulate the androgen levels by reducing the PSA in both *in vivo* and *in vitro* conditions. Therefore, [6]-gingerol is a probable botanical compound showing potential to be used in the management of PCA.

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