Punicic and Jacaric acids induce Mitochondrial dysfunction in prostate cancer cell lines

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Abstract: Two fatty acids (punicic and jacaric acids) found in pomegranate and jacaranda seed oils have been used as anticancer agents. Punicic and jacaric acids are long chain polyunsaturated fatty acids. Here we investigated the potential ability of punicic and jacaric acids to affect growth of both androgen dependant prostate cancer cell line (LNCaP) and androgen independent prostate cancer cell line (PC-3). Apoptosis and disrupted cellular mitochondrial membrane potential were induced at 30 and 100μM punicic and jacaric acids in prostate cancer cell lines compared to untreated control cells. We also investigated whether lipid oxidation was required for the function of punicic and jacaric acids by adding 20 μM of the antioxidant tocotrienol to the assays. This resulted in reversal of the effects of punicic acid on apoptosis and disruption of the mitochondrial membrane potential. Finally, we evaluated the role of PKC signaling in the anti-cancer effects of punicic and jacaric acids by performing cell viability (WST-1) assay in the presence of the PKC inhibitor bisindolymaleimide I. Cell death that induced by punicic and jacaric acids was partially blocked in both the LNCap and PC-3 cells. These results suggest that punicic and jacaric acids have prostate cancer inhibitor properties that are dependent on lipid peroxidation and the PKC pathway.
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Key Words: Punicic acid, Jacaric acid, prostate cancer, PKC, cell death, apoptosis and mitochondrial dysfunction.

1. Introduction

Prostate cancer is one of the most important cancers in men worldwide. Its incidence can be influenced by several risk factors including genetic susceptibility, environmental exposure in its largest sense and differences in health care and cancer registration (or a combination of these). It primarily affects males of age 55 and above and is more common in African American males than white males [Crawford, 2003]. Standard treatment of prostate cancer consists of surgery (prostatectomy), hormonal therapy and radiotherapy. While prostatectomy is successful for treating early stage cancer, its use is limited to localized tumors and can cause nerve damage resulting in impotence, incontinence or rectal injury [Bishoff *et al.,* 1998; Talcott *et al.,* 2003; Melman *et al.,* 2004]. Hormonal therapy acts by blocking the action of cancer supporting male hormones and is only effective till the time the cancer can spread independent of male hormones [National Cancer Institute, 2002; Conde and Aronson, 2003]. Similarly, radiotherapy is also effective for early stage prostate cancer and becomes progressively ineffective in later stages (Roomi *et al.,* 2015).

Therefore, there has been a renewed push to identify natural remedies to fight prostate cancer. It has been shown that apoptosis may be induced or inhibited by a variety of nutritional compounds known to have health benefits including fatty acids (Langley, 2000; Gil *et al.,* 2000; Longtin, 2003) .Two fatty acids (jacaric and punicic acids) found in jacaranda and pomegranate seed oil, respectively have the ability to induce apoptosis in human hormone-dependent LNCaP and hormone-independent PC-3 prostate cancer cells, as well as immortalized normal prostate epithelial cells (RWPE-1) (Gasmi and Sanderson, 2013). Jacaric acid activates extrinsic apoptosis via death receptor 5 in LNCaP cells, punicic acids were further found to induce intrinsic apoptosis via a caspase-dependent pathway (Gasmi and Sanderson, 2013).

The mechanisms underlying the cytotoxicity of PA and JA are not very well understood and remain a continuing topic of investigation in prostate cancer cell line. We try to understand the mechanisms of action mediating the anti prostate cancer effects of PA and JA through investigating the disruption of mitochondrial permeability Lipid peroxidation and PKC pathways and confirming if these pathway plays the major role in prostate cancer cell death.

2. Material and Methods

Fatty acids

Jacaric and Punicic acid were purchased from Larodan Fine Chemicals AB (Malmö, Sweden). Each compound was dissolved in DMSO as 1000-fold concentrated stock solutions of 100 mM.

Cell lines and reagents

PC3 and LNCaP cells were obtained from the American Type Culture Collection (Manassas, VA).

PC-3 cells were grown in 1:1 (v/v) Dulbecco's modified Eagle medium/Ham's F-12 nutrient mix (DMEM/F12) supplemented with 10% FBS and 1% penicillin/streptomycin. LNCaP cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 1% HEPES, 1% sodium pyruvate and penicillin/streptomycin in a humidified atmosphere of 95% air and 5% CO2 at 37°C. (DMEM/F12), RPMI-1640 and (penicillin/ streptomycin) were purchased from Gibco by life technologies corporation (Grand Island, NY, USA). HEPES buffer (1 M) and Sodium pyruvate (100 mM) was purchased from Sigma Aldrich (3050 Spruce Street, St. louis, MO, USA). Fetal bovine serum was purchased from Corning (Mediatech, Inc, A corning subsidiary Manassas, VA 20109,USA).

Cell viability

Each cell type was added to 96-well plates at a density of 1×104 cells/200 µl of medium per well. After 24 h, medium was replaced with fresh medium containing various concentrations of Punicic or Jacaric acid (0, 1, 3 10, 30 and 100 µM) in a final DMSO concentration in culture medium of 0.1%. Cell viability was assessed using a WST-1 Cell Proliferation Reagent kit (Roche, Laval, QC) according to the manufacturer's instructions. Absorbance was measured at 440 nm using a SpectraMax M5 multifunctional spectrophotometer (Molecular Devices, Sunnydale, CA).

Fluorescence microscopy (Apoptosis and mitochondrial membrane potential)

PC-3 and LNCaP cells were added to 24-well plates at a density of 1×105 cells/ml of medium containing 2% dextran-coated charcoal-treated FBS. After 24 h, cells were treated with several concentrations of PA and JA (0, 10, 30 and 100 µM) in fresh medium and after another 8 h, Hoechst 33342 (Sigma-Aldrich, St Louis, MO) was added at a concentration of 1 µg/ml per well and tetramethylrhodamine ethyl ester (TMRE) was added to each well at a final concentration of 50 nM. TMRE is a positively charged cell-permeable dye that accumulates in active negatively charged mitochondria. Inactive or depolarized mitochondria fail to sequester TMRE resulting in reduced fluorescence. After a 15 min incubation at 37°C, cells were observed and counted under a Nikon Eclipse (TE-2000U) inverted fluorescent microscope at 20 X magnification. Hoechst- and TMRE positive cells were made visible using filter cubes with excitation wavelengths of 330–380 nm and 532–587 nm, respectively.

SDS–PAGE and immunoblot analysis

Cells were added to 6-well Cell-Bind plates (Fisher Scientific, Ottawa, ON) in complete culture medium for 24 h. Cells were then exposed to Punicic or Jacaric acid (0, 1, 3 10, 30 and 100 µM) for 8h in LNCaP and 24 h in PC-3, dependent on the experiment. Adherent cells were collected using a cell scraper, then rinsed in cold phosphate-buffered saline (PBS) three times followed by centrifugation at 700 × g for 5 min. After removing the PBS, the cell pellets were lysed in RIPA buffer containing 1× protease and phosphatase inhibitor cocktail. Then, cell lysates were centrifuged at 4°C, 15,000 rpm for 15 min and protein concentrations in the supernatant were determined using a BCA protein assay kit (Pierce Biotechnologies, Rockford, IL). Proteins (40 µg) were diluted with loading buffer and boiled for 5 min, then loaded onto 10% sodium dodecyl sulfate-polyacrylamide gels. After electrophoresis, gels were transferred to polyvinylidene diflouride (PVDF) membranes using a Trans-Blot Turbo System (Bio-Rad, Mississauga, ON). Membranes were then blocked using Tris-buffered saline (TBS) containing 5% milk powder (blocking buffer) for 1 h at room temperature, after which the membranes were incubated overnight in blocking buffer with the appropriate primary antibodies (anti CHOP, JNK,p-JNK, PUMA, caspase 12 and β-actin at 1:1000 dilution; Cell Signaling, Beverly, MA) at 4°C. The next day, membranes were washed three times with Tris-buffered saline containing 0.1% Tween (TBS-T) followed by an incubation with the appropriate secondary antibody for 1 h at room temperature. Membranes were washed another three times with TBS-T and then incubated with Immobilon Western Chemiluminescent horseradish peroxidase substrate (EMD Millipore, Billerica, MD) for 5 min to make the bands visible; membranes were sealed in plastic wrap and photographed using a ChemiDoc-It gel documentation system (Bio-Rad). B-actin was used as reference protein and loading control.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). Results are presented as means ± standard deviations of at least three experiments. Statistically significant differences were determined using a two-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc test. A p-value less than 0.05 was considered statistically significant.

3. Results

Punicic and jacaric acids induce apoptosis and mitochondrial dysfunction in PC-3 and LNCaP.

To determine whether the punicic and jacaric acids-induced apoptosis and mitochondrial dysfunction in PC-3 and LNCaP cell lines during apoptosis, PC-3 and LNCaP cells were exposed to different concentration of PA and JA (10, 30 and 100 µM) for 8 h and then stained with Hoechst3342 and

TMRE. A significant decrease in TMRE fluorescence signals that accumulates in active negatively charged Mitochondria at concentrations at 30 and 100 µM PA and JA (Figure 1). Therefore, PA and JA cause inactivate or depolarized the mitochondria and membranes have decreased potential and fail to sequester TMRE.

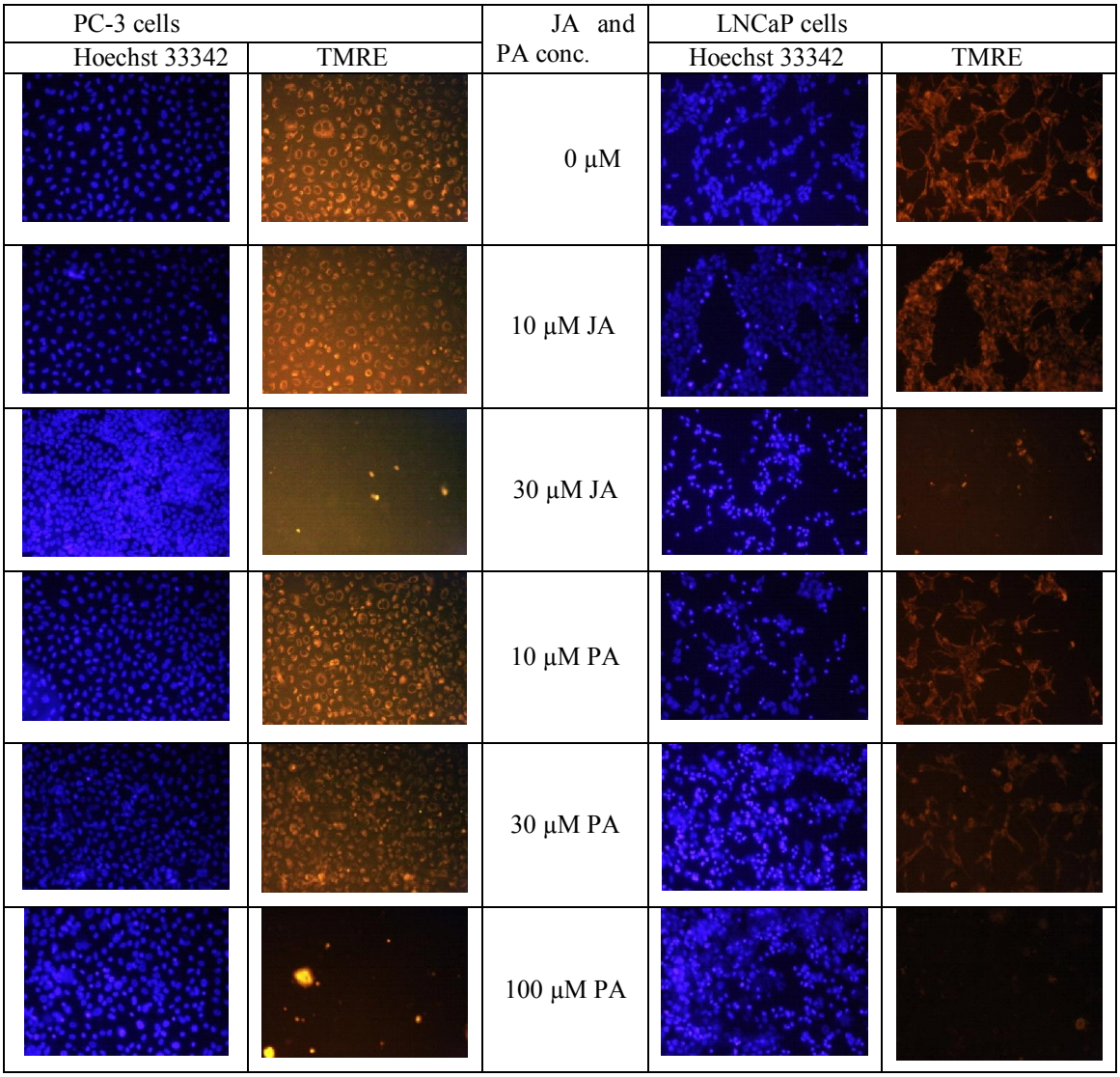

Figure 1. Punicic and jacaric acids induce mitochondrial dysfunction in PC-3 and LNCaP. Cells were exposed to different concentrations of PA and JA for 8 hrs. Mitochondrial membrane permeability of PC-3 and LNCaP cells was measured using TMRE fluorescent dye and nuclei were stained with Hoechest.

Blocking lipid peroxidation by antioxidant alpha tocotrienol, a derivative form of vitamin E

To determine whether the lipid peroxidation play a vital role during apoptosis and mitochondrial dysfunction that induced by PA and JA. PC-3 and LNCaP cells were exposed to 20 µM alpha tocotrienol for 4 hours and then exposed to different concentrations of PA and JA (10, 30 and 100 µM) for 8 h and then stained with Hoechst3342 and TMRE. We found that alpha tocotrienol reduces apoptosis and mitochondrial dysfunction in PC-3 and LNCaP cell lines. (Figures 2 and 3).

To confirm these findings, we investigated the effect of alpha tocotrienol on Puma, P-JNK, caspase 12 and CHOP on both PC-3 and LNCaP (fig.4). We found that alpha tocotrienol reduces Puma expression in PC-3 and LNCaP cell lines when they were exposed to 30 µM of PA for 24 hours. It was also noticed that alpha tocotrienol reduced CHOP and p-JNK in LNCaP cell lines when they were exposed to

30 µM of PA for 24 hours while it didn't have any effect on it in PC3 cells at 30 µM PA.

Response of Prostate cancer cells to PA and JA with PKC inhibitor (BIM I):

PKC activation and PA function. Protein kinase C (PKC) has been shown to be activated by diacylglycerol which can be synthesized using glycerol and PUFA (poly unsaturated fatty acids). We found that the PKC inhibitor bisindolymaleimide I (BIM) partially blocked the antiproliferative effects of PA for PC-3 at (10,30 and 100 µM PA) and also block JA for PC-3 at (30 and 100 µM JA) (Fig 5). The blockage was not significantly different from untreated controls in PC-3 cells treated with 30 and 100 µM JA for the PC-3 cells but the blockage was significantly different from untreated controls in PC-3 cells treated with 10, 30 and 100 µM PA for the PC-3 cells.

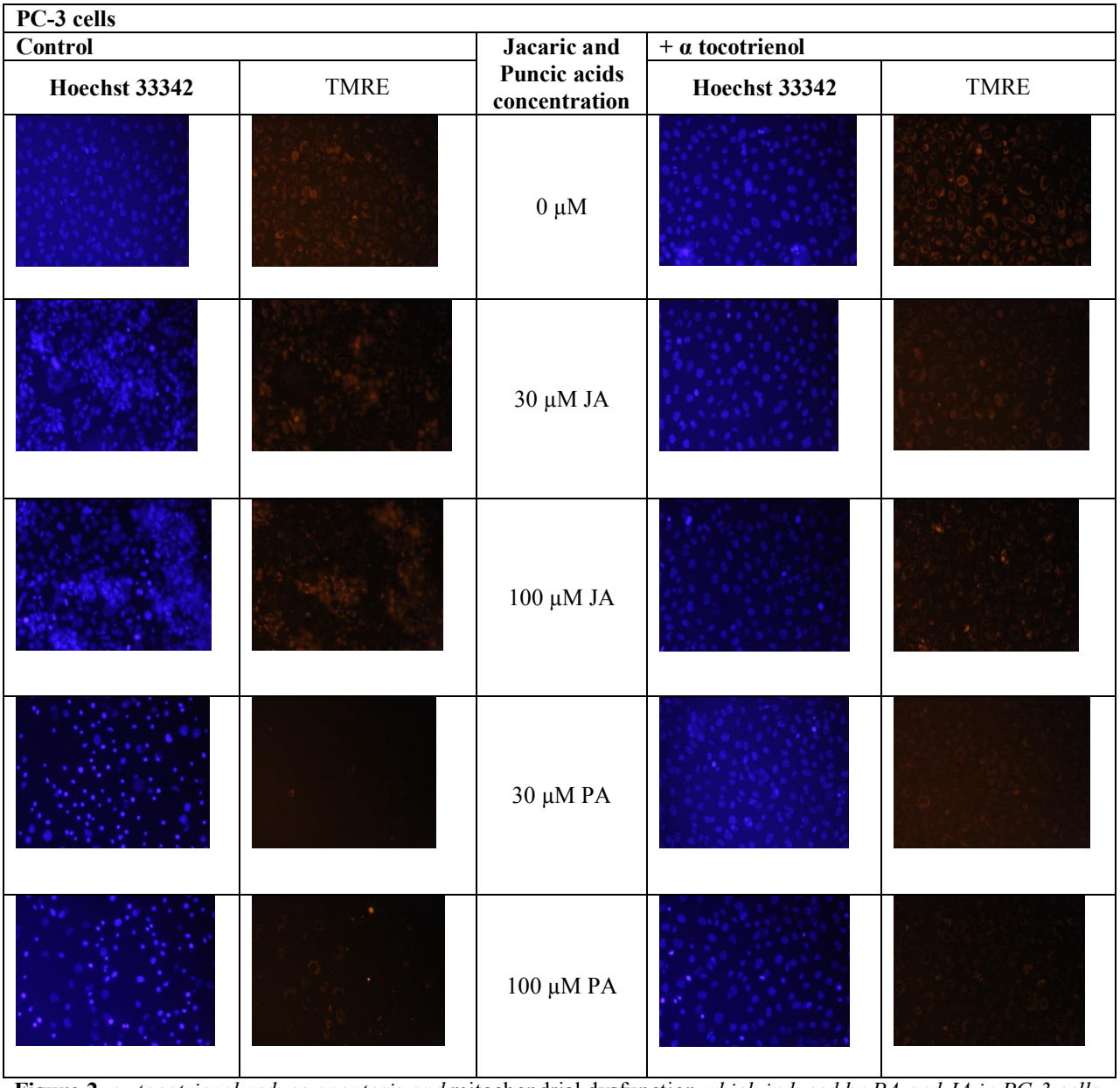

Figure 2. *α -tocotrienol reduce apoptosis and* mitochondrial dysfunction *which induced by PA and JA in PC-3 cells. Apoptotic nuclear morphology (chromatin condensed nuclei and nuclear fragmentation) was observed with Hoechst 33258 staining using fluorescence microscopy.* Mitochondrial membrane permeability of PC-3 cells was measured using TMRE fluorescent dye. *One of three photos per concentration is shown.*

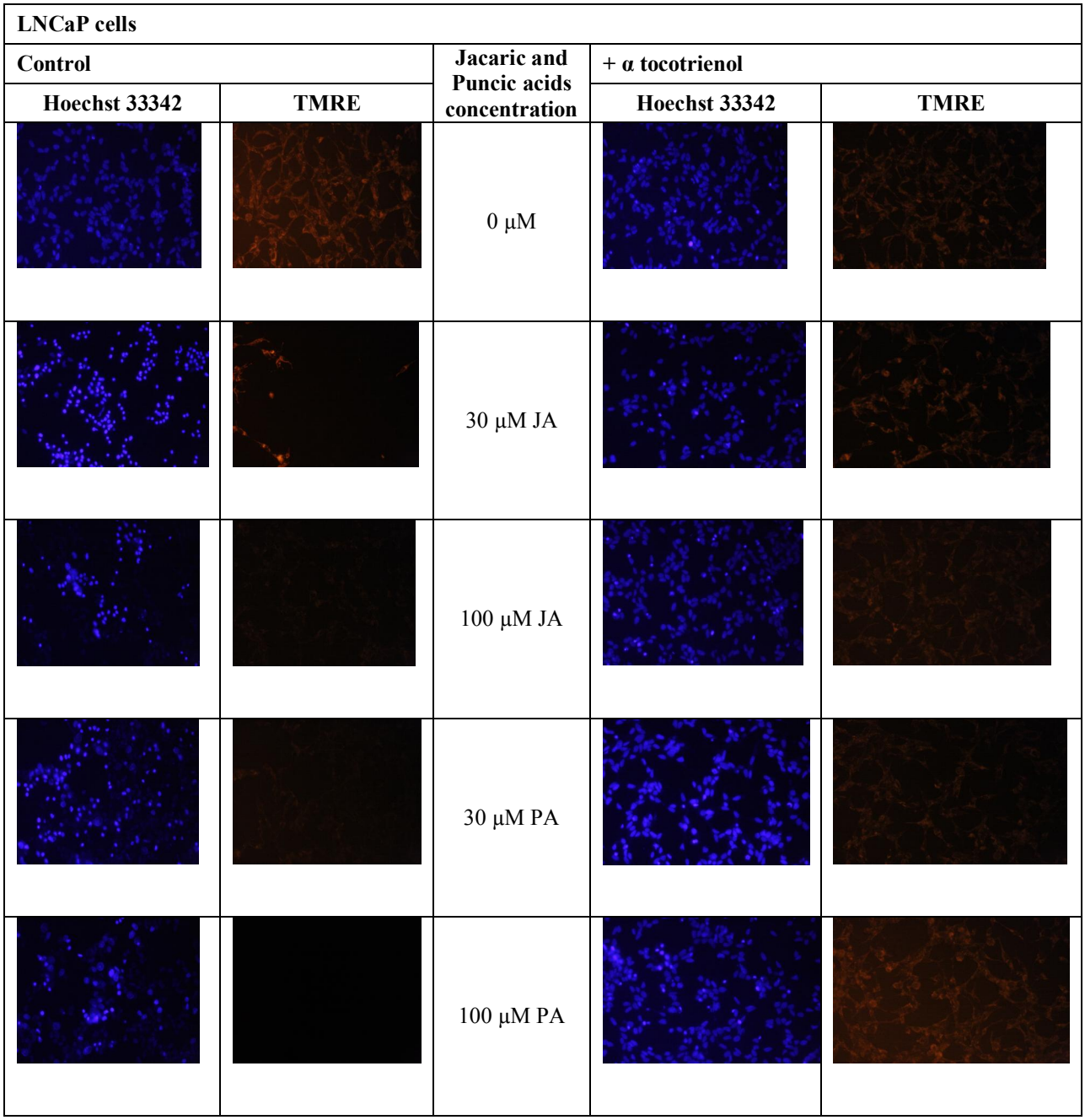

Figure 3. *α -tocotrienol reduce apoptosis and* mitochondrial dysfunction *which induced by PA and JA in LNCaP cells. Apoptotic nuclear morphology (chromatin condensed nuclei and nuclear fragmentation) was observed with Hoechst 33258 staining using fluorescence microscopy.* Mitochondrial membrane permeability of LNCaP cells was measured using TMRE fluorescent dye. *One of three photos per concentration is shown.*

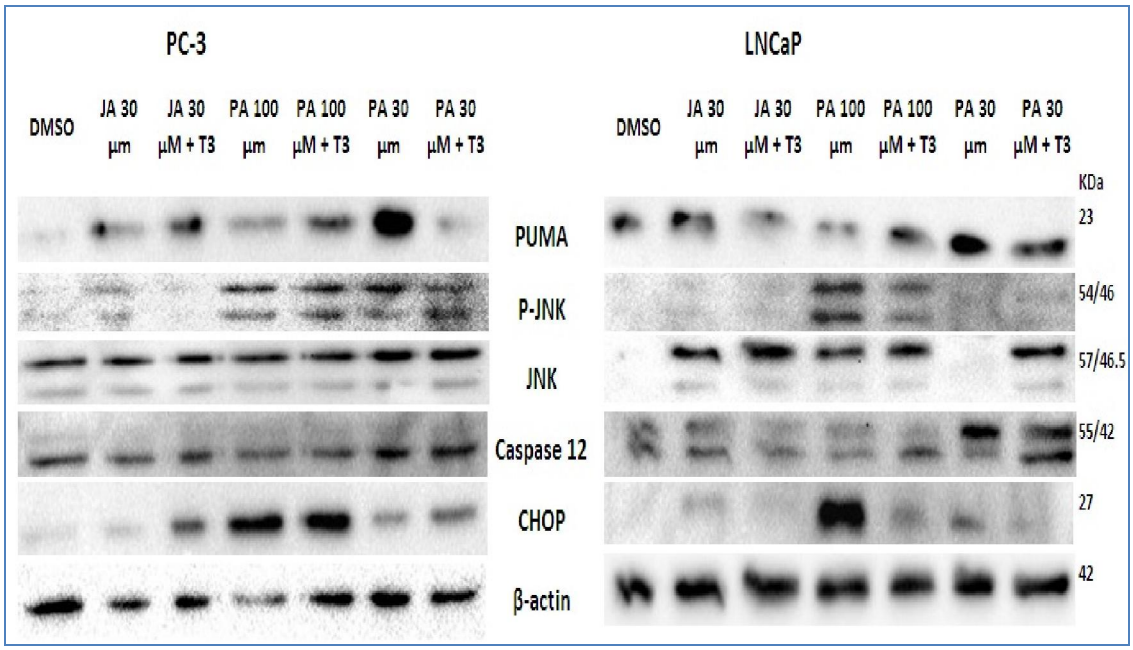

Figure 4. *Effect of α -tocotrienol on CHOP, Caspase 12, p-JNK and PUMA levels in pc-3 and LNCaP cells. α – tocotrienol inhibits PA (100 µM) to induce CHOP and p-JNK in LNCaP cells. Proteins were detected by western blotting. One representative gel of three is shown.*

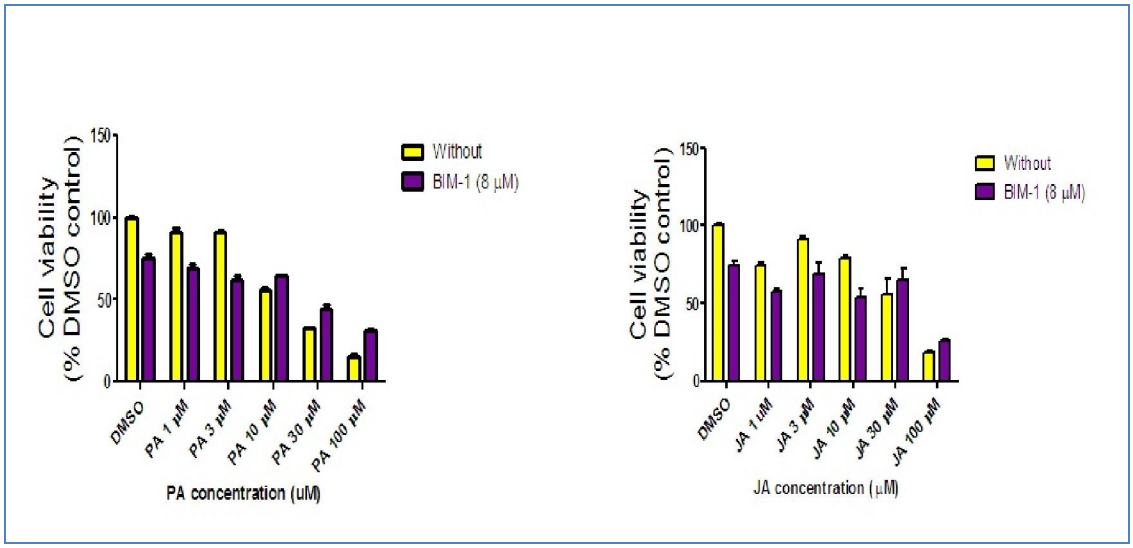

Figure 5. *Response of Prostate cancer cells (PC-3) to PA and JA with PKC inhibitor. Cells were plated, allowed to adhere overnight, pretreated with bisindolylmaleimide I (BIM) for 1 h and then treated with PA in 8 µM BIM for 24 h. The proliferation assays were performed using WST-1 kit. Two-way ANOVA P<0.0001 for differences between the treatments.*

4. Discussion

Punicic and jacaric acids induce apoptosis and mitochondrial dysfunction in PC-3 and LNCaP

Punicic and jacaric acids induced apoptosis and inactivates or impairs the mitochondrial membranes permeability of PC-3 and LNCaP cells. The mitochondrial membranes have potentially decreased and failed to sequester TMRE (Fig.1). A previous study mentioned that Punicic acid disrupted cellular mitochondrial membrane potential in breast cancer (Grossmann *et al.,* 2010). Furthermore, another study noted that compositional and functional alterations in mitochondrial membrane may be an important initiator of apoptosis induced by omega-3PUFAs in human gastric cancer cells (Yin *et al.,* 2007).

Blocking lipid peroxidation by antioxidant alpha tocotrienol, a derivative form of vitamin E decrease cell death that induced by PA and JA on PC-3 and LNCaP cells and improve cellular mitochondrial membrane potential

In the current study, we found that the ability of PA and JA to induce apoptosis and mitochondrial dysfunction were blocked by 20 µM alpha tocotrienol (Figs.2 and 3). Prvious study mentioned that 20 µM alpha tocotrienol completely blocks PA to induce cell death or cause mitochondrial dysfunction in breast cancer (Grossmann *et al.,* 2010.) Another study suggests that the transformation of vitamin E to CEHC is mostly a detoxification mechanism useful to maintain the malignant properties of prostate cancer cells (Conte *et al.,* 2004). Another study reported that TRF, a tocotrienol-rich fraction from grape seeds prevents cellular reactive oxygen species (ROS) and cellular lipid peroxidation induced by tert-butyl hydroperoxide (TBHP) in HepG2 cells (Choi *et al.,* 2010).

In this study, we found that alpha tocotrienol reduces Puma expression in PC-3 and LNCaP cell lines when they were exposed to 30 µM of PA for 24 hours. It was also noticed that alpha tocotrienol reduced CHOP and p-JNK in LNCaP cell lines when they were exposed to 30 µM of PA for 24 hours while it didn't have any effect on it in PC3 cells at 30 µM PA (Fig.4). Previous study mentioned that antioxidant N-Acetylcysteine Attenuates Hepatocarcinogenesis by Inhibiting ER Stress proteins markers (Bip, phospho-eIF2a, eIF2a, CHOP, IRE1a, phospho-JNK1/2 and JNK1/2) in TLR2 Deficient Mouse (Lin *et al.,* 2013). Another study found that the use of antioxidants reduce endoplasmic reticulum stress and improve protein secretion in male C57BL/6 mice (Malhotra *et al.,* 2008).

PKC activation and (PA&JA) function

PKC can be activated by DAG which can be synthesized using glycerol and PUFA (poly unsaturated fatty acids). PKC has been suggested to be involved in a number of aspects of tumor progression (Jude *et al.,* 2006). This may be in part due to the fact that there are several different PKC isozymes that phosphorylate a variety of target proteins which control antiproliferation and apoptosis as well as proliferation and survival (Griner and Kazanietz, 2007). Diacylglycerol is actually an extremely varied molecule that can be composed of various glycerol compounds linked to two fatty acids which can also be highly varied resulting in many different compounds that are all known as DAG. The various DAG molecules can differentially activate the PKC isozymes (Griner and Kazanietz, 2007) and it seems probable that this is in some way linked to the ability of PA and JA to inhibit proliferation (fig. 6). In our study we found that the PKC inhibitor bisindolymaleimide I (BIM 8µM) partially blocked the antiproliferative effects of PA for PC-3 at (10,30 and 100 µM PA) and also block JA for PC-3 at (30 and 100 µM JA) (Fig.5). Prvious study mentioned that bisindolymaleimide I (BIM) partially blocks PA to induce cell death in breast cancer (Grossmann *et al.,* 2010). Another study reported that the cytotoxic effect of phorbol 1 2-myristate 1 3-acetate (PMA) could be blocked by bisindolylmaleimide on human thyroid epithelial cells (Hall-Jackson *et al.,* 1998).

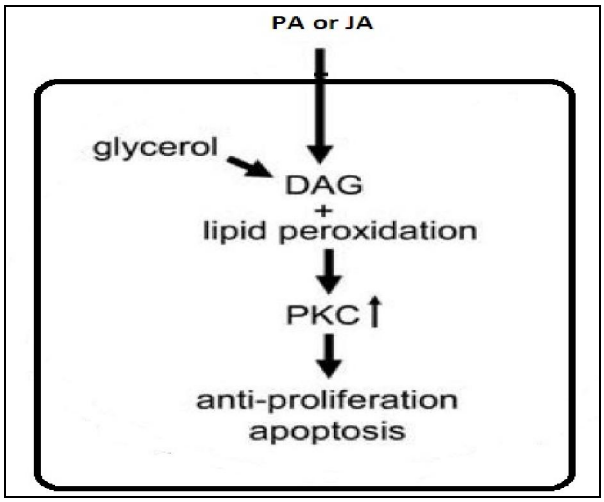

Figure 6. Potential pathway for punicic and Jacaric acids actions on prostate cancer cells.
PA, punicic acid; JA, jacaric acid; DAG, diacylglycerol and PKC, protein kinase C.

Conclusion:

In summary, we have found that punicic and Jacaric acids induced apoptosis, mitochondrial dysfunction, PKC and lipid peroxidation in PC-3 and LNCaP cells during cell death. When PC-3 and LNCap cells were preincubated with 20 µM of the antioxidant tocotrienol, the effects of PA and JA on apoptosis and disruption of the mitochondrial membrane potential were reversed. We've also proved that the lipid peroxidation pathway is an essential requirement for PA and JA to induce cell death while PKC is only a partial requirement. In other words, the cell death here is mainly dependent on the lipid peroxidation mechanism and partially on PKC. It's concluded that lipid peroxidation caused by LCA, PA and JA plays a vital role in apoptosis and necrosis in prostate cancer cells.

Pomegranate extracts (which contain puncic acid) are more readily available than jacaranda seed oils (which contains jacaric aicd), more palatable and more acceptable to individuals around the world. Hopefully the present study will be useful for defining preclinical studies and eventually in making dietary

recommendations to men. Our study provides new information about the use of PA and JA in cancer inhibition.

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