ANTI-CANCER ACTIVITY OF POMEGRANATE (PUNICA GRANATUM) EXTRACTS IN TESTICULAR CANCER

JAYANN MARIE BROWN

ANTI-CANCER ACTIVITY OF POMEGRANATE (*PUNICA GRANATUM*) EXTRACTS IN TESTICULAR CANCER

by

Jayann Marie Brown

A Thesis Submitted to the Faculty of

The Charles E. Schmidt College of Science

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

Florida Atlantic University

Boca Raton, Florida

August 2004

ANTI-CANCER ACTIVITY OF POMEGRANATE (*PUNICA GRANATUM*) EXTRACTS IN TESTICULAR CANCER

by

Jayann Marie Brown

This thesis was prepared under the direction of the candidate's thesis advisor, Dr. James Kumi-Diaka, Department of Biological Sciences, and has been approved by the members of her supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Science and was accepted in partial fulfillment of the requirements for the degree of Master of Science.

SUPERVISORY COMMITTEE:

Thesis Advisor

Chairman, Department of Biological Sciences

Dean, The Charles E. Schmidt College of Science

Vice President for Research and Graduate Studies

July 19, 2004
Date

Acknowledgements

I am extremely grateful to have worked with Dr. Kumi-Diaka, Dr. Frazier and Dr. Baldwin who have assisted and directed me tremendously throughout this research. To my fellow lab partners; Simone, Adeline and Kendra who assisted me tirelessly, my personal thanks to you all.

ABSTRACT

Author:	Jayann Brown
Title:	Anti-Cancer Activity of Pomegranate (*Punica granatum*) Extracts in Testicular Cancer
Institution:	Florida Atlantic University
Thesis Advisor:	Dr. James Kumi-Diaka
Degree:	Master of Science
Year:	2004

Recent advancement in chemotherapy has resulted in higher and longer survival rates of testicular cancer patients. However the use of chemotherapeutic agents are not without serious, sometimes fatal side effects. This study investigated the potential therapeutic efficacy of pomegranate extracts in testis cancer cells, GC1-spg, *in vitro*. A battery of assays was used to determine the chemosensitivity of GC1- spg cells to two pomegranate extracts, S (seed) and P (pericarp), in single and combination treatments: MTS and LDH to determine post-treatment survival rate (growth inhibition) and cytotoxicity respectively; Acridine Orange/Ethidium Bromide fluorescent dye to assess treatment-induced apoptosis/necrosis; Annexin V-FITC and TUNEL assays for early and late apoptosis respectively. Results from the obtained data indicated that both extracts have significant cytotoxic effect on testicular cancer cells (GC1-spg) in single and combination treatments. The data revealed a dose and time dependency of chemosensitivity to both extracts; and that apoptosis was the major mechanism treatment-induced cell death. Synergism was also indicated in growth inhibition by combination treatment. These findings offer strong justification for further studies with pomegranate as potential phytotherapy.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

Dedication

To my mother and father; Ada and Harvey Brown. My sisters; Daphne, Patsy and Shelly and my brothers; Ewart, Danny and Russell. Thank you for your support, this one's for you.

INTRODUCTION

Testicular cancer is a disease in which testicular cells become malignant in one or both testicles. The testicles are a pair of male sex glands that produce and store spermatozoa and are the body's main source of the male hormone testosterone, which controls the development of the reproductive organs and male characteristics.

Testicular cancers may be classified into two general groups: i) seminoma and, ii) non-seminoma (http://cis.nci.nih.gov). About half of all testicular germ cell cancers are seminomas. Seminomas develop from the sperm-producing germ cells of the testicle and make up about 30 percent of all testicular cancers. Typical seminomas are found in men in their 30s through 50 years of age. These tumors usually grow very slowly and usually do not spread to other parts of the body. Nonseminoma tumors are composed of more specialized primitive cells. These cancers tend to develop earlier in life than seminomas and usually occur in men in their 20s. The main types of nonseminoma germ cell cancers are embryonal carcinoma, yolk sac carcinoma, choriocarcinoma and teratoma.

The incidence of testicular cancer has increased three-fold over the past 40 years, with the highest incidence reported in Northern European countries, Scandinavia and Germany and lower rates in Asia and Africa (Kinkade, 1999;

Peerchellet et al., 1992). The American Cancer Society (2003) estimated that 7,600 new cases of testicular cancer occurred in the United States in 2003 and though testicular cancer is very treatable if detected early, approximately 400 men would die from the disease that year. The risk of developing testicular cancer in a man's lifetime is approximately 1 in 500, occurring in approximately 1 in 25,000 men per year. Testicular cancer is four times less common in Afro-American men compared to the incidence in Caucasians (Bosl et al., 2001) with intermediate incidence rates among Native Americans, Hispanics and Asians (Nugteren and Hazelhof 1987). It is the third most commonly diagnosed cancer (14.3% of all male cancers) among males aged 15–19 years (White 1996).

Men at high risk of developing testicular cancer are amongst individuals who have had undescended testicles, abnormal testicular developments, history of testicular cancers, family history of testicular cancers and men with Kleinfelter's syndrome, a sex chromosome disorder characterized by low sperm count, low levels of male hormone, breast enlargement and small testes (White 1996). Although the incidence of testicular cancer has risen somewhat in recent years, advances in treatment imply that most men diagnosed with testicular cancer, especially those diagnosed when the cancer is at an early, treatable stage, can now expect to survive the disease (Beeson, 2000).

Cisplatin-based chemotherapy, which has been the standard treatment for testicular cancers over the past three decades (Liberthal et al., 1996) has had successful survival rates with testicular cancers. Recent data have shown over 85% cure/survival rate in advanced cases with the use of multidisciplinary

therapeutic regimen, involving a combination of surgery and chemotherapy (Dearnaley et al., 2001). Cisplatin, however, can cause intolerable side effects including nausea, vomiting, alopecia, fatigue, and neutropenia. Long-term side effects include peripheral neuropathy and ototoxicity (Gullatte, 2001). In addition, chemotherapy frequently causes azoosperia (which is a complete absence of sperm in the ejaculate), resulting in infertility (Dearnaley et al., 2001). This infertility is frequently temporary; however, in a study of 170 patients who received an orchiectomy and cisplatin-based chemotherapy, the potential for the production of sperm was 48% a year after treatment and 80% after 2 years (Lampe et al., 1997). Further, most of the currently used chemotherapeutic drugs cannot penetrate solid tumor and are only effective against proliferating cancer cells (Tang and Porter, 1997). It is established that the success of chemotherapeutic drugs lie in their ability to initiate apoptosis in cancer cells.

Chemotherapeutic-induced cell death occurs through induction of necrosis and/or apoptosis (Cotter and Martin, 1994). Necrosis is an injurious cell death characterized by swelling and rupturing of the cell membrane with the consequence of invoking inflammatory reaction. On the other hand, apoptosis is a genetically controlled programmed cell death without inflammation; and is characterized by cell shrinkage, membrane blebbing, condensation and fragmentation of chromatin material with consequent formation of apoptotic bodies; the latter of which are immediately cleared by macrophages and other phagocytic cells in the body (Tang and Porter, 1997; White, 1996; Albrecht et al., personal communication; Kerr et al., 1994; Vlietinck and Vanden, 1991). The

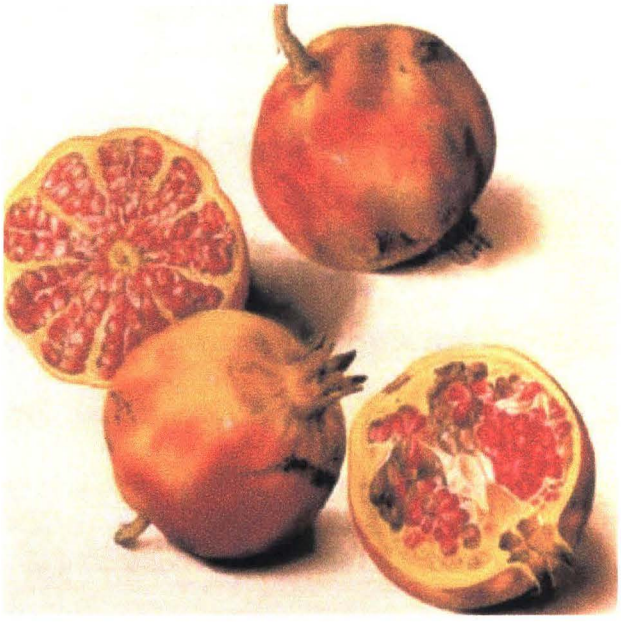
potency of most currently used chemotherapeutic agents is due to their apoptosis induction capabilities; thus suggesting that induction of apoptosis may be a major pathway through which anti-tumor therapies could be aimed.

Apoptosis is essential in many physiological processes including maturation and effector mechanisms of the immune system (Allen et al, Cohen and Duke1992). It has also become evident that alterations in the apoptotic pathways are intimately involved in a variety of disease processes, including cancer (White, 1996; Tang and Porter, 1997; Albrecht et al., Personal correspondent) and all chemotherapeutic agents as well as ionizing radiation, utilize apoptotic mechanisms to induce programmed cell death. (Vlietinck and Vanden, 1991; Pennel et al., 1992; Kerr et al., 1994). Reports indicate that cell death in multi-cellular organisms is subject to genetic control (Ellis and Horvitz, 1986;Vaux et al., 1998) and abnormalities in cell death regulation can cause diseases, including cancer (McDonnel and Korsmeyer, 1991) autoimmunity (Watanabe-Fukunaga et al.,1992.) and possibly degenerative disorders (Bar and Tomei, 1994).

The objective of this study was to investigate anti-cancer potential of *Punica granatum* extracts as chemotherapeutic agents in testis cancer and to study the possible effect of combination treatment. The pomegranate (*Punica granatum*) fruit is among the many natural foods that are currently being analyzed by scientists in search of clinically acceptable chemotherapeutic agents among phytochemicals. Pomegranate is extensively referenced in medical folklore (Kumi-Diaka et al., 2000; Tang and Porter, 1997; deVries et al., 1988; Nam Deuk

et al., 2002). The potential anti-cancer and pharmacological properties of pomegranates have been noted within the past five years. Pomegranate extracts have been shown to potently suppress proliferation of human prostate and breast cancers (Kim et al., 2002; Nugteren and Christ-Hazelhof, 1987).

The pomegranate fruit *(Punica granatum)*

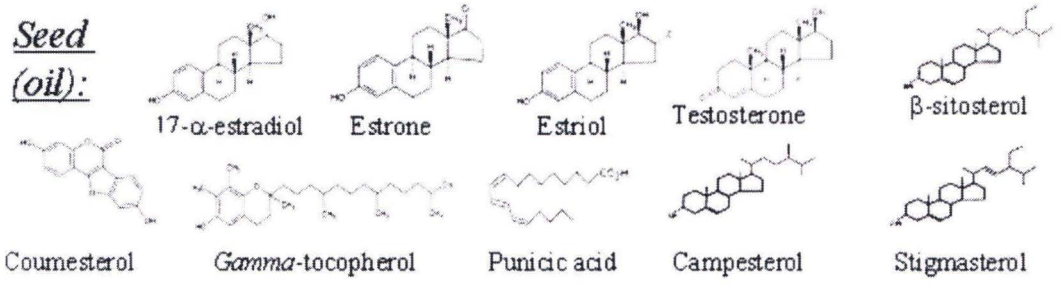


The pomegranate is native of Iran and is grown mainly in India, the near and far Eastern countries and in the USA. The pomegranate belongs to the Punicaceae family (Harde et al., 1970). The Latin name of the tree is *Malus punica* or *Punicum malum*: belonging to the order Granateae. The name granatum was given to it because of it's many seeds. The fruit is the size of an orange, having a thick, reddish-yellow rind, an acid pulp, with large quantities of seeds separated into compartments by membranous walls and a white spongy tissue filled with the tart flavorful juicy pink or red seeds. Its

various extracts contain a rich complement of steroidal and related polyphenolic compounds, (Table I) which in photosynthesizing cells possess an impressive array of pharmacological activities (Hasten, 1983).

Seed oil extract

Select compounds found in pomegranate *(adapted from www.rimonest.com)*

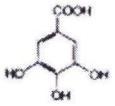


The seed oil is rich in steroids and sterols, including estrone, 17-α-estradiol, campestrol, estriol, testosterone, stigmasterol and sitosterol (Heftmann et al., 1966; Chung et al., 2001). Some of these have cancer chemopreventive properties, including promotion of apoptosis (Nepka et al., 1999). In addition, the oil contains 80% punicic acid, an 18C octadecatrienoic fatty acid and known inhibitor of prostaglandin biosynthesis (Chung et al., 2001). The polyphenol in the oil inhibit cyclooxygenase and lipoxygenase (Albers and Christmas, 2002) which may partially account for it's cancer preventative and therapeutic potential (Raffay and Cohen, 1997).

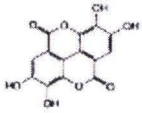
Pericarp extract

Select compounds found in pomegranate *(adapted from www.rimonest.com)*

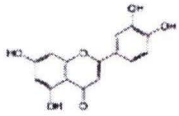
Peels:



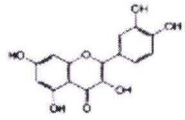
Gallic acid
(A tannic acid)



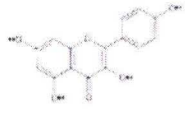
Ellagic acid
(A tannic acid)



Luteolin
(A flavone)



Quercetin
(A flavone)



Kaempferol
(A flavonol)

The pericarp contains tannin flavones, such as gallic acid, quercetin and luteolin with established antioxidant activities. Similarly, flavonol and flavone compounds, such as a kaemopferol, and naringenin, display estrogenic activity and anti-cancer properties (Akagi et al.,1995; Kim et al., 2002; Knowles et al., 2000; Liberthal et al., 1996; Mcdonnel et al .,1995; Iwashita et al., 2000). Commercial pomegranate juice contains anthocyanins and phenolic acids with demonstrated anticancer properties (Kim et al., 2002) and is enhanced with pericarp-derived compounds, which augment the juice's antioxidant activities (Gil et al., 2000).

The hypothesis for this present study is that pomegranate extract will offer interventive action against testicular cancer; further that combination treatment will be more efficacious at killing testicular cancer cells than single treatments.

MATERIALS AND METHODS

2.1 Materials

Commercially prepared pomegranate seed (S) and pericarp (P) extracts were a gift from Ephraim Lansky (Ephraim Lansky, Scientific Director of Rimonest Ltd. Israel). Extracts were dissolved in DMSO to make stock solutions at a final concentration of 0.05% DMSO. Preliminary studies in our lab indicate 0.05%- 0.1% DMSO has no effect on GC1-spg cells. Working solutions were constituted as 4,12,20,40,60,80μg/mL (S_{4-80}; P_{4-80}) and stored at –20°C until used. Combination treatment was carried out using the IC_{50} of one fraction in combination with the varying concentrations of the other fraction. The IC_{50} was determined in preliminary studies. Culture media, antibiotics and trypsin EDTA were purchased from Sigma Scientific (St. Louis, MO). Bioassay kits (LDH, MTS, TUNEL, Acridine orange-Ethidium bromide, Annexin V-FITC, FAM Polycaspase) were purchased from Biovision (Mountain View, CA). Testicular cancer cell line, GC1-spg and normal testicular cell line, Hs-181.Tes, were initially purchased from American Type Culture Collection (Manassas, VA). All cells were cultured and maintained in humidified atmosphere at 37^0C and 5%$C0_2$ in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and 100 μg/mL of penicillin + 100 μg/mL of streptomycin (Sigma, St. Louis, MO. USA)

2.2 Determination of cell viability

Viability was determined using the Trypan Blue exclusion assay, followed by LDH and MTS assays. For this series of experiments $1.0x10^4$ cells/well of the cell line GC1-spg were grown/incubated at 37^0C, 5% CO_2 and exposed to varying concentrations of the agents (S_{4-80}; P_{4-80}) in single and combination treatments and further cultured for 24 and 48 hr. At 24 hr and 48 hr, supernatants were carefully aspirated and stored at -20^0C for LDH determination later; and the adherent cells were processed for MTS determination as described below. For negative controls, cells were grown without exposure to the test agents; and for positive controls, cells were grown and exposed to dexamethazone (DXM), a known apoptosis-inducer in cells. All assays were done in triplicates.

2.2.1 MTS determination: MTS assay depends on the mitochondrial enzyme's reduction of MTS solution to detect and determine cell viability. The MTS cell proliferation assay is a colorimetric method for determining the number of viable cells in proliferation. It is composed of solutions of the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl0-2H-tetrazolium, inner salt; MTS] and an electron-coupling reagent (phenazine ethosulfate; PES). MTS is bioreduced by the cells into formazan product that is soluble in cell culture medium. Following cell culture described above, 100μL of cells were harvested from each treatment group and added to 96 well MTP followed by addition of 20μL of MTS (2.5 μg/mL: Sigma Chemical Co) stock solution to each well. After 2 h incubation under standard conditions of 5% CO_2

and 37°C, the purple formazan product (indicative of reduction of MTS) was visible. The absorbance was read on Multiskan biochromatic automated microplate reader (Multiskan, DC) at 490 nm. Data is collected for each treatment and graphed.

2.2.2 Trypan Blue Assay: Trypan Blue exclusion assay is used to do preliminary estimation of cell death. One of the morphological characteristics associated with cell death is the loss of membrane integrity resulting in the ability to exclude certain chemicals, for example, Trypan Blue dye. Trypan Blue exclusion does not give an indication of the mode of cell death because both necrosis and apoptotic cells permit dye uptake. There is a possibility of underestimating the extent of cell death because cells in the primary stage of apoptosis may retain their membrane integrity for several hours. Briefly equal volumes of Trypan Blue dye and cell suspension were allowed to incubate at room temperature for approximately 2 minutes. 25 μL was then loaded onto unto a hemocytometer, and examined under light microscope at 40X magnification. Cells present in 25 squares of the chamber were counted. The results multiplied by 2 $X10^4$ to account for dilution of the cell sample and the volume of the chamber. Cell viability was calculated as follows:

% viable cells = (# of viable cells/total # of cells) X 100.

2.2.3 LDH determination: LDH determination was done with non-radioactive cytotox kit.

A dying cell will release the cystolic enzyme lactase dehydrogenase into the supernatant therefore, the cytotoxic response of a treatment induced cell death can be quantitatively followed by measuring this enzyme's activity. Briefly, the supernatant of each treated cell suspension was collected at 24 and 48h and frozen at –20°C until use. These were thawed and 100µL of each was transferred into correspondingly labeled 96 well microtiter plate (MTP). 100µL of LDH assay reaction mixture (Kit: dye-catalyst mixture, Cat No 1 644 793:Boehringer-Mannheim, US) was added to each well. The MTP plate was incubated for 1h at 37°C to effect reaction. The absorbance of the reaction-generated color was read at 450nm using an ELISA plate reader. The increase in the amount of dead or plasma membrane damaged cells result in an increase in the LDH dye activity in the supernatant, correlating directly with the amount of tetrazolium salt INT that is reduced to formazan within the hour [Kit contents: catalyst (Diaphorase/NAD+) and dye solution (INT and sodium lactate)]. Data were collected and graphed.

2.3 Determination of apoptosis

It is often advantageous to use several different approaches when studying apoptosis since no single parameter fully defines cell death. Several methods have been developed to distinguish live cells from early and late apoptotic cells and from necrotic cells. To compare the potential apoptosis induction effects of varying concentrations of the agents ($S_{4\text{-}80}$; $P_{4\text{-}80}$) in single and combination treatments 1.0×10^4 cells/well of each cell type was treated as previously described. Apoptosis was detected by fluorescence using Annexin V-FITC

apoptosis detection kit (Fisher Scientific, St. Louis) and Acridine orange/Ethidium bromide nuclear stain. The mechanism of cell death was further confirmed using TUNEL assay. Cells were incubated for 48h at the previously mentioned extract concentrations.

2.3.1 Acridine Orange/Ethidium bromide: This apoptosis detection technique was used according to a modification of Duke and Cohen. It is based on the differential staining of viable/apoptotic cells in a mixture of Acridine orange/ethidium bromide. Briefly, a cocktail of 10μL was added to each cell suspension in the dark for 20 minutes. Aliquots were dropped on glass slides, covered and examined under fluorescence microscope. Detection of apoptosis was based on morphological and fluorescence characteristics as previously described (Duke and Cohen, 1992). Viable cells were indicated by bright green and apoptotic cells by orange. Quantitative assessments were made by determining the percentage of apoptotic cells by counting 60 cells in 5-7 fields of view.

2.3.2 AnnexinV-FITC assay: Apoptosis-associated translocation of phosphatidylserine
from the inner to the outer leaflet of the plasma membrane in GC27 and K833 cells was assessed with the use of FITC-labeled Annexin V, a calcium-dependent phospholipid-binding protein with a high affinity for phosphatidylserine; using AnnexinV-FITC Staining Kit (Boehringer Mannheim). Briefly, 100 μL aliquots of the previously prepared cell suspensions were centrifuged, and the cell pellets

re-suspended in Annexin binding buffer, incubated with AnnexinV-FITC substrate; then cells were smeared onto microscope slides and either evaluated immediately with fluorescence microscope; or smears were fixed with 4% depolymerized paraformaldehyde and stored at -40°C for later examination as previously described. Percentage of apoptosis in the cells was quantified based on morphological and fluorescence characteristics of apoptotic cells as previously described (Kumi-Diaka et al., 1999; Iwashita et al., 2002). Experiments were run in triplicates and repeated three times for each cell line.

2.3.3 DNA fragmentation (TUNEL) assay: The presence of apoptosis was determined by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL), using the ApopTag^R kit (Boehringer Mannheim Co, I-ndianapolis, IN) as previously described (Akagi et al., 1995). The kit reagents detect apoptotic cells *in situ* by specific end labeling and detection of DNA fragments produced by the apoptotic process. In this study, the PBS-suspended cells were pre-stained with supra-vital propidium iodide (PI) stain, and examined under fluoresce microscope to differentiate between apoptosis (PI negative) and necrosis (PI positive), according to previously described procedure (Sinha et al., 1997). To perform the TUNEL assay, slides were prepared with aliquots from the PI-stained cell suspension. The cells (slides) were then permeabilized with Triton X-100 at 4° C for 2 min; then flooded with TdT enzyme and digoxigenin-dUTP reaction buffer (TUNEL) reagent for 60 min at 37°C; followed by further incubation in anti-fluorescein antibody Fab (conjugated with alkaline

phosphatase). After this the cells were stained with chromogenic substrate (Fast Red), and mounted Negative controls were performed by substituting distilled water for TdT enzyme in the preparation of working solutions. The stained mounted cells were examined at 100X, 200X and 400X magnification of the microscope (Olympus BH-2). Cell death was quantified by counting 50 cells in 5-7 separate fields of view per slide, and noting the percentage of apoptotic cells based on morphological appearance, as previously described (Cotter and Martin, 1994; Kumi-Diaka et al., 1999).

2.4 Effect of Pomegranate extracts on normal cells

Potential effects of two pomegranate extracts (S, P) on peripheral blood lymphocytes were assessed. Previously frozen lymphocytes were used. This was venous blood collected from the upper arm and subjected to similar concentration and conditions as cancer cells. In addition, normal testicular cancer cell line Hs181.Tes was subjected to similar concentrations and conditions as described above.

2.5 Mechanism of treatment-induced apoptosis

2.4.1 Detection of caspase3 and caspase 7 Expression: In order to determine the potential role of caspase-3 proteases (CPP32) in the common pathways of pomegranate seed oil and pericarp induced growth inhibition and apoptosis; testicular cancer cell lines were treated as previously described. The treated cells were then subjected to bio-analysis for apoptosis induction, using the FAM

Polycaspase assay protocol according to the manufacturer's instructions (ATCC). The concept of the assay is based on the detection of active caspases by a fluorescent labelled inhibitor FAM-VAD-FMK. These inhibitors are cell permeable and non-cytotoxic and will bind to caspases 1,2,3, 4,5,6,7,8 and/or 9. The green fluorescent signal is a direct measure of the number of active caspase enzymes present in the cell. Cells that contain the bound probe were analyzed by fluorescent microscopy. FAM-VAD-FMK binds primarily to caspase 3 and 7 and can be used to measure the amount of active caspases 3 and 7 (the effector caspases) present in the cell. Briefly, FAM reagent was added to each well of pomegranate treated cell suspension, mixed and allowed to incubate for 1h at 37^0C under 5% CO^2. They were then incubated for 5 min with 1.5 µL Hoechst stain at 37^0C under 5% CO^2. Cells were then washed twice with wash buffer, centrifuged at 10,000 rpm for 10 minutes and the supernatant discarded. Cells were then brought back into suspension with wash buffer. One drop of each cell suspension was placed on a glass slide and observed under fluorescent microscope.

2.6 *Statistical Analysis*.

All the experiments were performed in triplicates and repeated twice, to confirm similar results. Significance of the differences in mean values was determined using Statistical Analytic System (SAS - for both parametric and non-parametric data) (Cody and Smith, 1987), and considering $P < 0.05$ to be statistically significant. Data for LDH and MTS were analyzed using Student t Test while data

for acridine orange/ethidium bromide and Annexin V-FITC were analyzed using Friedman Two-way analysis of variance by ranks test since data did not show a continuous distribution. Wilcoxon-Mann-Whitney test was used for FAM polycaspase assay.

RESULTS

Pomegranate seed oil and pericarp inhibit the growth and proliferation of testicular cancer cells, GC1-spg. Potential pomegranate-induced growth inhibition of GC1-spg testicular cancer cells was investigated using the seed oil (S) and pericarp (P) extracts. MTS and LDH assays are sensitive non-radioactive assays that were used in this study to determine the cytotoxic effects of fractions of the pomegranate fruit on GC1-spg tumor cancer cells. The result revealed by the LDH assay indicates that over time as the concentration of the three preparations increased the number of cells inhibited/killed increased [Figs. 1and 2]. The inverse was also true, as the result of the MTS assays shows, as concentration of the agents increased the number of viable cells decreased [Fig 3]. Although these results indicate that GC1-spg cells were equally affected by the single treatments of P and S and Comb, there was a trend of increasing effect of P at higher concentration of pomegranate ($p>0.05$) [Figs. 1-3]. GC1-spg cells exposed to combination treatment, on the other hand, showed a significant toxicity ($p>0.01$) than either of the single preparations at similar concentrations; concurrent with decreased cell viability with increasing doses of both extracts (S, P)[Figs 1-3]. The reliability of the results was shown by the inverse proportionality of the two bioassays, LDH and MTS [Fig.4]. The results further

revealed that chemosensitivity of GC1-spg cells to both S and P extracts were dose-dependent [Figs 1 -3] showing an increase in cytotoxicity at 48h over 24h.

Pomegranate seed oil and pericarp extracts induce apoptosis in testicular cancer cells. To determine the role of apoptosis and/or necrosis in pomegranate treated GC1-spg cells, cells were treated and assayed using Acridine orange/ethidium bromide nuclear stain. The results revealed that the major mechanism of cell death for these cells was apoptosis [[F_r= 6.91, $p \leq 0.05$; Fig 5-9]. Most noticeable was an increase in cell death via apoptosis with increasing concentration of the agents. Furthermore the combination treatment elicited a higher degree of apoptosis at a lower concentration than either P or S alone [$F_r = 6.91$; $p \leq 0.05$; Fig 7]. Additionally, the same trend was revealed by the Annexin V-FITC and the TUNEL apoptosis assays [[F_r= 6.91, $p \leq 0.05$; Figs. 8,9]. The observed morphological characteristics of apoptosis included; cell shrinkage, membrane blebbing, cytoplasmic contraction and nuclear/DNA condensation [Figs. 12-14].

Pomegranate effect on normal cells. To determine the degree of potential cytotoxic effect of pomegranate in normal cells, normal human testis cell lines, Hs 181.Tes, and peripheral blood lymphocytes were exposed to varying concentrations of the two pomegranate extracts as previously described. The results indicated no significant differences in cytotoxicity between the non-treated controls and the treated normal cells [$F_r = 6.91$; $p \leq 0.05$; Figs. 7,14].

Activity of caspase3 and caspase 7 in pomegranate induced apoptotic cell death. Detection of caspase 3 and caspase 7 involved tagging the active caspases activated by pomegranate seed oil and pomegranate pericarp indicates that the

caspase pathway is one of the major pathway through which apoptosis occurs [W_x =64.5; p=0.0487; Fig.11]. Active caspase enzymes capable of binding the FAM probe were stained green. Cells that were not apoptotic remained unstained. These results indicate that pomegranate induced apoptosis in GC1-spg cell line is dependent on caspase 3 and 7 activation.

Discussion

In this study the cell proliferation and apoptosis induction was investigated *in vitro* using the pomegranate seed oil (S) and pomegranate pericarp (P). The chemosensitivity of testicular cancer cell line, GC1-spg, revealed in the data showed significant inhibition in cell growth and proliferation with increasing dosage of the pomegranate extracts in single and combination treatments, when compared to untreated cells. GC1-spg cells exposed to combination treatment, however, showed a pronounced toxicity than either of the single preparations at similar concentrations. Overall the results indicate that GC1-spg cells were significantly more sensitive to combination rather than to single treatment, indicative of a possible synergism between the two fractions of pomegranate. The results of the combination treatment seem to support the natural synergism of these fractions in plants, designed for their protection and survival (Stermitz et al., 2000).

There existed an inverse relation between the degree of treatment-induced cytotoxicity/cell death and percentage post-treatment live cells, as revealed by the LDH and MTS assays respectively. The LDH assay indicated that the chemosensitivity of GC1-spg to pomegranate was both dose and time dependent with no significant differences in chemosensitivity between the S and P fractions. This result is in conformity with previously reported observations in prostate

(Taylor et al., 1996) and breast cancer (Kim et al., 2002). Anticancer properties of pomegranate have been seen in prostate, breast and skin cancers, with both single and combination treatments, as seen in the present studies.

To determine the potential mechanism of pomegranate-induced apoptosis in GC1-spg cells, both acridine orange/ethidium bromide and TUNEL assays were used. The findings indicate that the major mechanism of pomegranate-induced cell death in GC1-spg cells was apoptosis. The fluorescent characteristics as previously described (Duke and Cohen, 1992), was observed in both single and combination treatments, with distinct orange colored cells indicative of apoptosis. The fluorescence results were further confirmed by the Annexin V-FITC assay. The observed treatment-induced morphological characteristic changes in apoptosis, revealed by the TUNEL assay, was consistent with results in testicular, prostate and breast cancer cells in previous studies (Kim et al., 2002; Kumi-Diaka and Butler, 2000; Albrecht et al., personal communication; Wyllie et al., 1980; Bold et al., 1997).

Further, these data indicate that the tumor cells were considerably more sensitive to pomegranate extracts than were normal non-tumor cells based on cell number and membrane integrity.

This study demonstrated a major pathway for pomegranate-induced apoptosis was through the caspase pathway. The fluorescent detection of caspase inhibitor was an indication that these caspases were active in the treated cells. Caspases are proteolytic enzymes that are activated in response to apoptotic signals resulting in the fragmentation of the cell. (See et al., 1999). FAM caspase

inhibitors mainly bind to caspase 3 and caspase 7 and further the binding of these is indicative that cell death occurs through this death pathway, as these are executioner caspases.

Conclusion:

The overall results from the present study indicate that: i) pomegranate extracts from the seed oil and the pericarp have chemotherapeutic effect on GC1-spg cells, ii) the major mechanism of pomegranate-induced cell death in GC1-spg cells was apoptosis; iii) both the S and P fractions of the pomegranate exerted both time- and dose-dependent effects on the testis, GC1-spg cells cell lines, iv) the differences in sensitivity of GC1-spg cells to the P (pericarp) and S (oil fraction) was not significant, and v) the level of cytotoxicity/chemosensitivity with the combination treatment (S and P) was significantly higher than with single treatment with either S or P alone. The overall results offer strong justifications for further exploration of the anti-cancer properties of pomegranate as a phytotherapy.

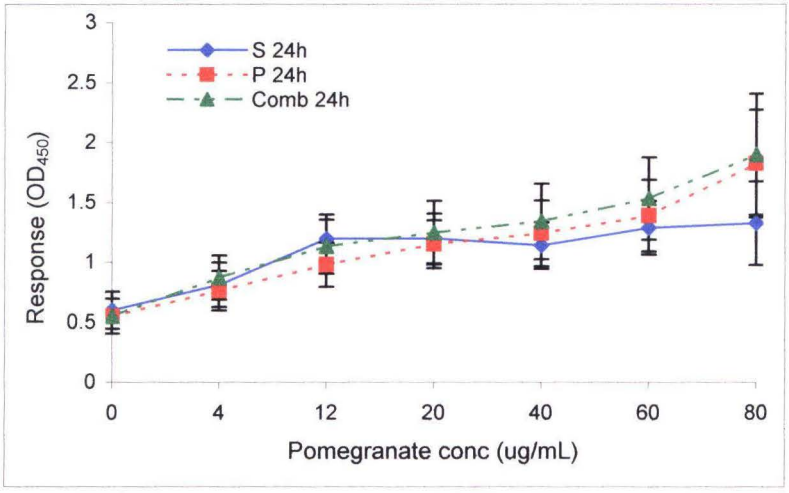


Fig. 1. Comparison of cytotoxicity of GC1-spg cells to (S) and (P) at 24h. GC1-spg cells were co-cultured with varying concentration of (S) and (P) as described above. Treatment induced cytotoxicity was determined using LDH assay. Data points are means $\pm$ SEM of two independently performed experiments done in triplicates. (Student's t test; $p<0.05$).

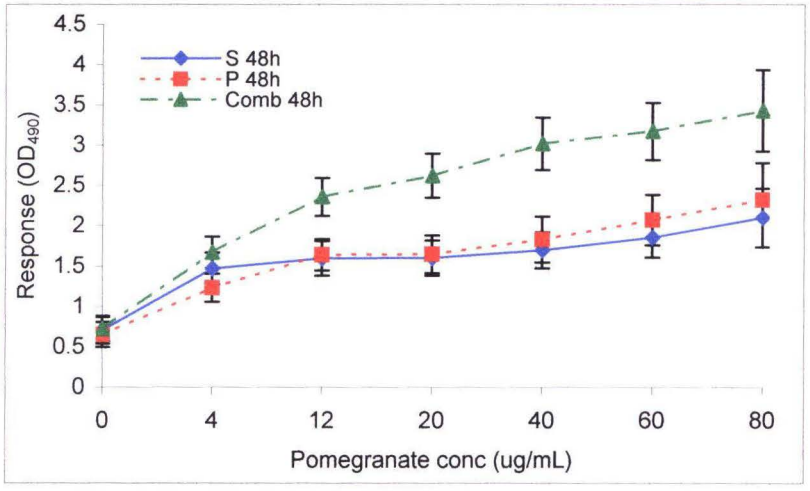


Fig.2. Comparison of cytotoxicity levels in single and combination treatments of GC1-spg cells to (S) and (P) at 48h. GC1-spg cells were co cultured with varying concentration of ($S_{4\text{-}80}$) and ($P_{4\text{-}80}$) as described in the methods. Cells were subjected to LDH assay. Data points are means $\pm$ SEM of two independently performed experiment done in triplicates. (Student's t test; $p<0.05$).

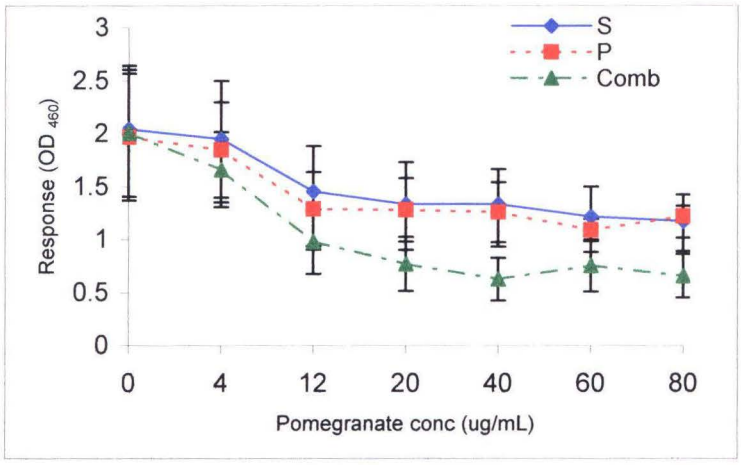


Fig. 3. Comparison of cell survival in GC1-spg cells treated with varying concentration of ($S_{4\text{-}80}$) and ($P_{4\text{-}80}$) as described in the methods. After 48h cells were subjected to MTS assay and viability was determined. Data points are means $\pm$ SEM of two independently performed experiment done in triplicates. (Student's t test; $p<0.05$).

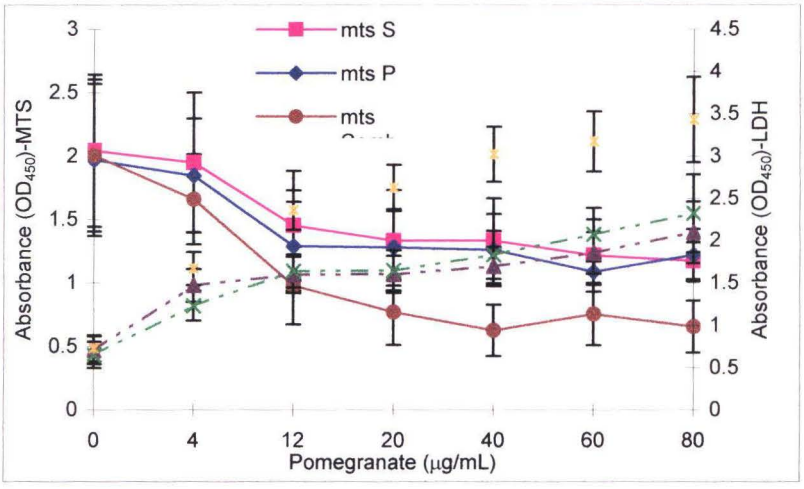


Fig.4. Inverse relation between cell survival and cytotoxicity levels in single and combination treated GC1-spg cells. Cells were cocultured with varying concentration of ($S_{4\text{-}80}$) and ($P_{4\text{-}80}$) as described in the methods. Absorbance of MTS and LDH assays was recorded at 48h. Data points are means $\pm$ SEM of two independently performed experiment done in triplicates. (Student's t test; $p<0.05$).

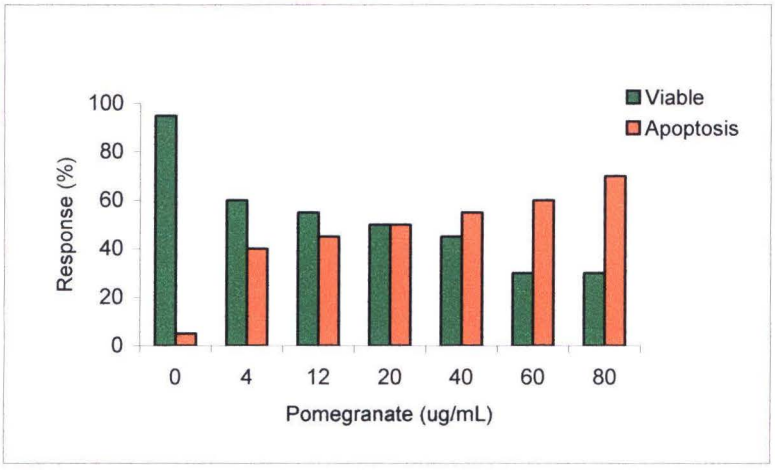


Fig. 5. Apoptosis in GC1-spg cells induced by (S). GC1-spg cells were seeded at a density of 1.0x 10^4 cells/well then cocultured with varying concentration of (S_{4-80}). Treatment induced apoptosis and /or necrosis was determined by Acridine Orange/Ethidium Bromide assay as described in experimental methods. Data points are means of two independently performed experiments. ($F_r = 6.91$; $p \leq 0.05$).

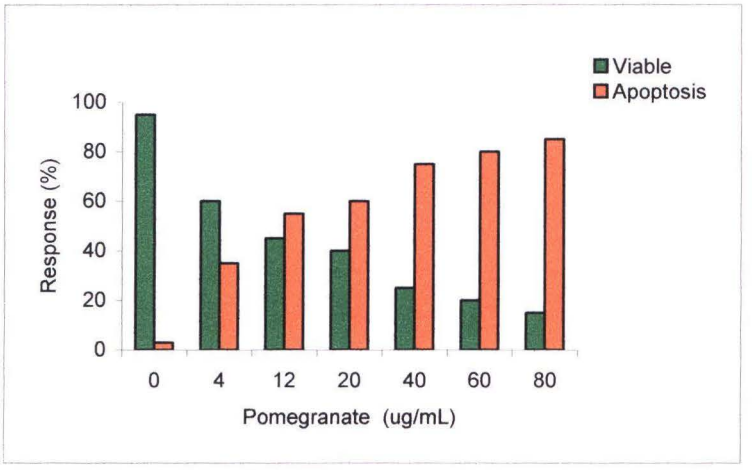


Fig.6. Apoptosis in GC1-spg cells induced by (P). GC1-spg cells were treated as previously with varying concentration of ($P_{4\text{-}80}$) as previously described. The degree of treatment induced-apoptosis and/or necrosis was assessed with Acridine Orange/ Ethidium Bromide dye. Data points are means of two experiments done in triplicates. ($F_r = 6.91$; $p \leq 0.05$).

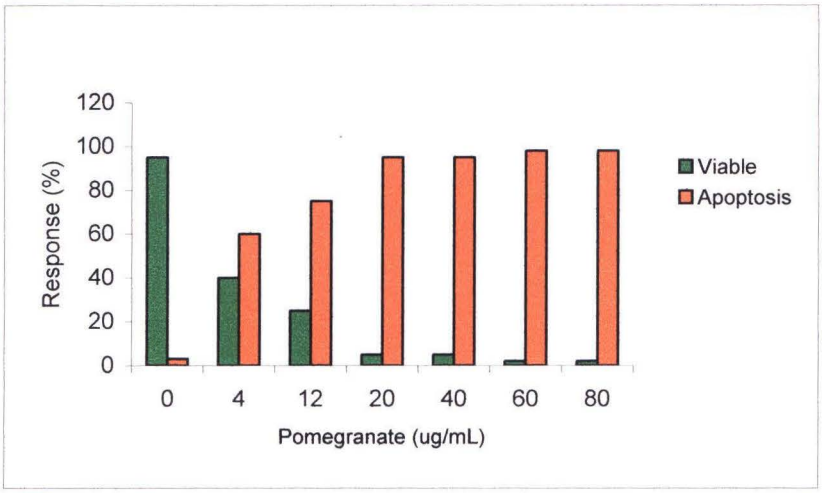


Fig. 7. Apoptosis in GC1-spg cells induced by combination of (P) and (S). GC1-spg cells were cocultured with varying concentration of (P) and (S) as described in the methods. Treatment induced apoptosis was determined by Acridine Orange/Ethidium Bromide assay described in experimental methods. Data points are means of two independently performed experiments. ($F_r = 6.91$; $p \leq 0.05$).

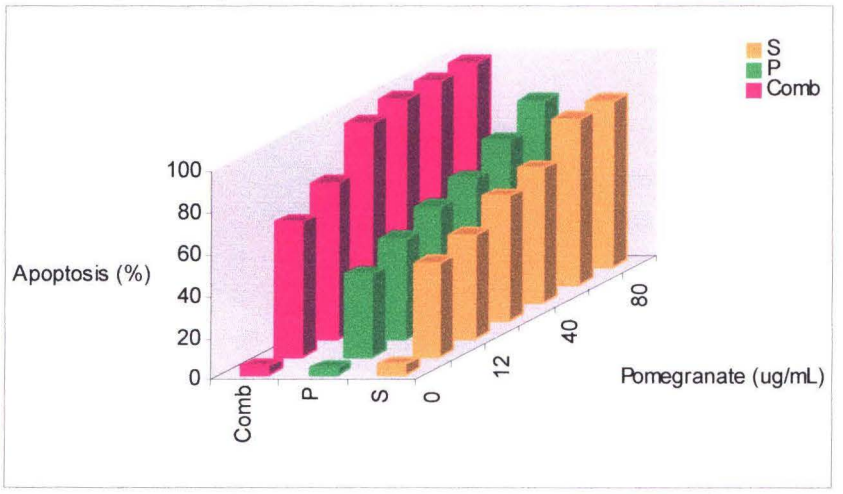


Fig.8. Apoptosis in pomegranate treated GC1-spg cells were subjected to Annexin V-FITC assay. Cells were cocultured with varying concentration of ($S_{4\text{-}80}$) and ($P_{4\text{-}80}$) as described in the methods. After a 24h period under conditions as previously described the results are analyzed as to compare the number of apoptotic cells post-treatment. ($F_r = 6.91$; $p \leq 0.05$).

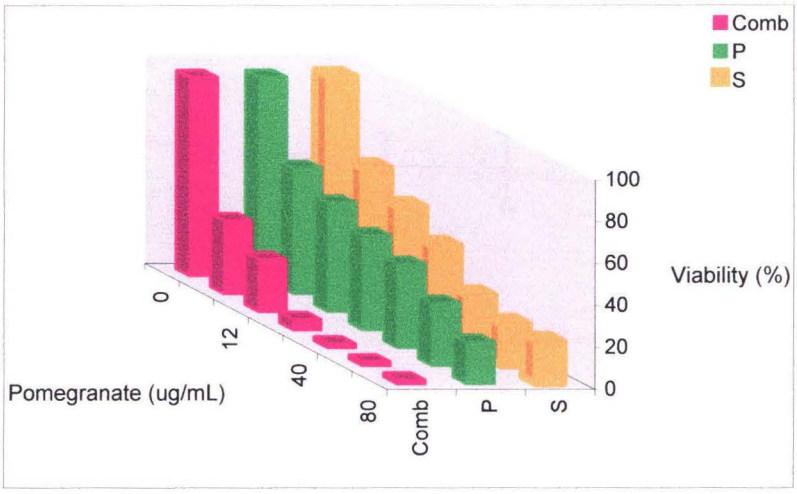


Fig 9. Viability in pomegranate treated GC1-spg cells were subjected to Annexin V-FITC assay. Cells were cocultured with varying concentration of ($S_{4\text{-}80}$) and ($P_{4\text{-}80}$) as described in the methods. After a 24h period under conditions as previously described the results are analyzed to compare the number of viable cells post-treatment. ($F_r = 6.91$; $p \leq 0.05$).

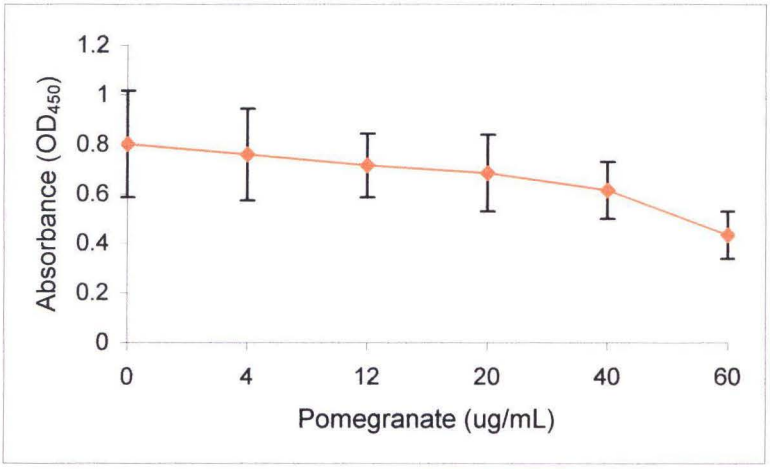


Fig.10. Pomegranate effect on normal testes cells, Hs181.Tes. Cells were seeded at a density of $1x10^6$ cells /well. Concentrations of (P) were added in concentrations of 4, 12, 20, 40 and 60 µg/mL were added as described in the methods. After 48h incubation cells were subjected to MTS assay and cell viability assessed. (Student's t test; $p< 0.05$).

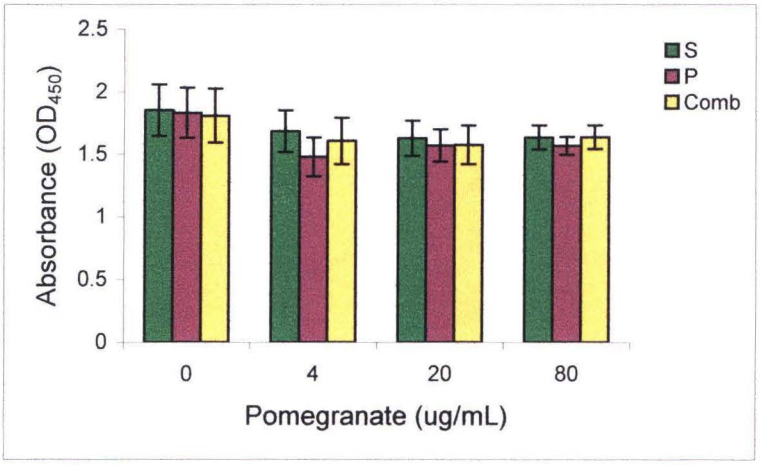


Fig.11. The effect of pomegranate on peripheral lymphocytes in single and combination treatments. Cells were treated as described in the methods at 48h. The results indicate no significant effect in both single and combination treatments on the cells. Data points represent means of two independently performed experiments. (Student's t test; $p < 0.05$).

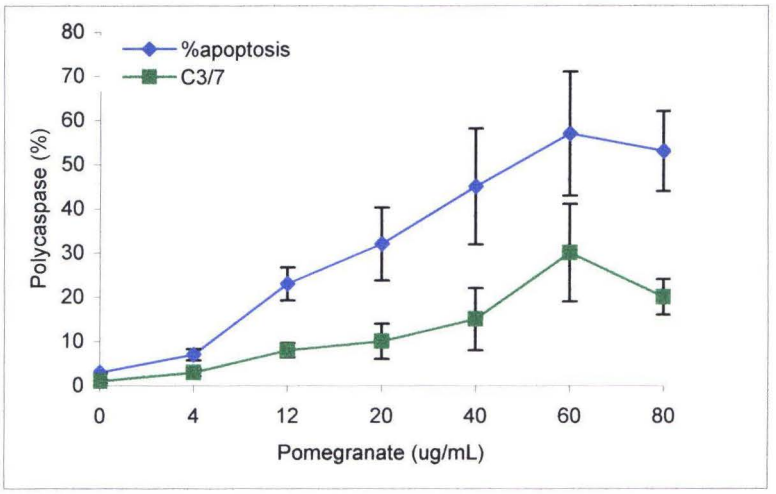


Fig.12. Assessment of the role of caspase in the induction of apoptosis by pomegranate in GC1-spg cells. Cells were cultured and treated as described above with FAM polycaspase probe FAM-VAD-FMK. Data points are means $\pm$ SEM of two independently performed experiment done in triplicates. ($W_x = 64.5$; $p = 0.0487$)

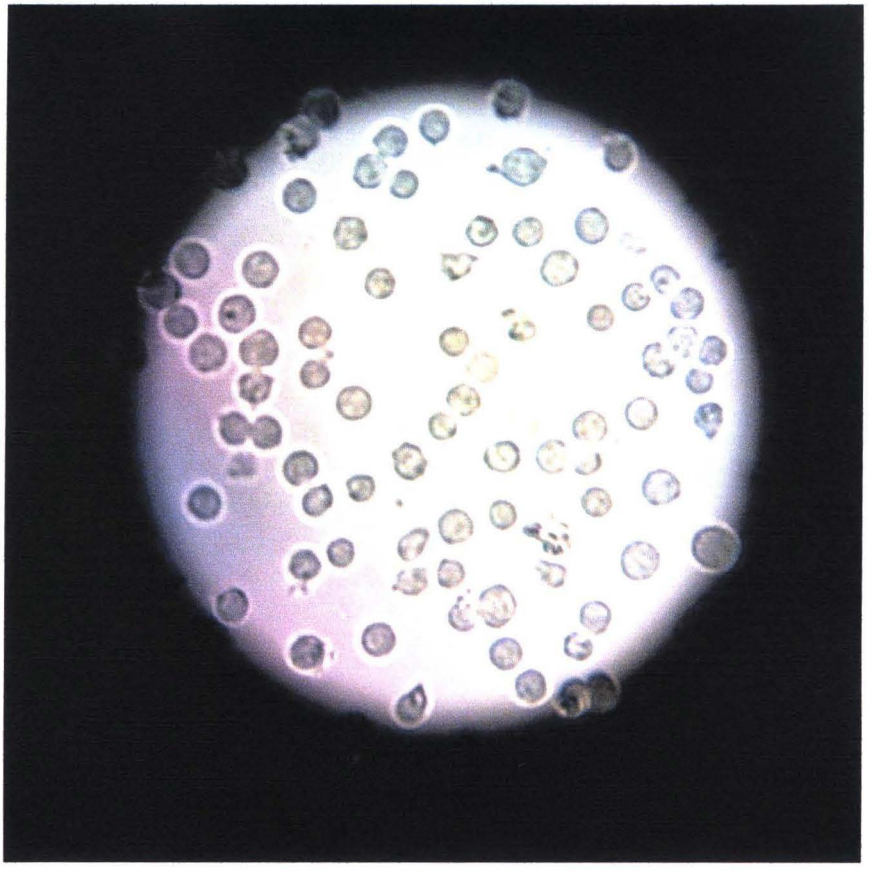


Fig. 13. GC1-spg cells treated with 40 μg/mL (P). Cells are unattached, haloed. Time = 0h.

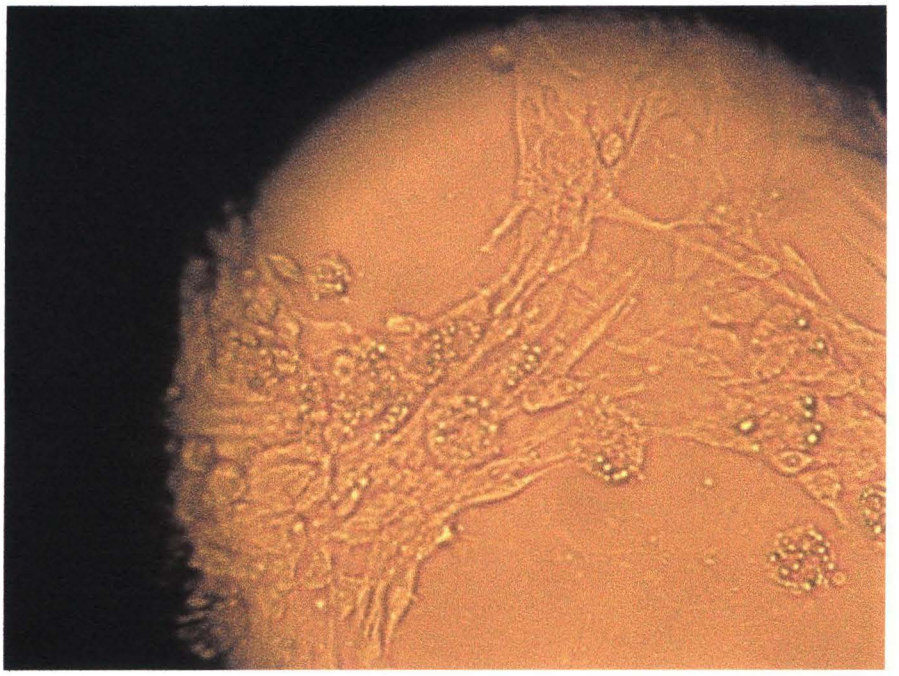


Fig. 14. GC1-spg cells treated with pomegranate combination treatment. At 20 μg/mL widespread apoptosis can be seen. Time = 48h.

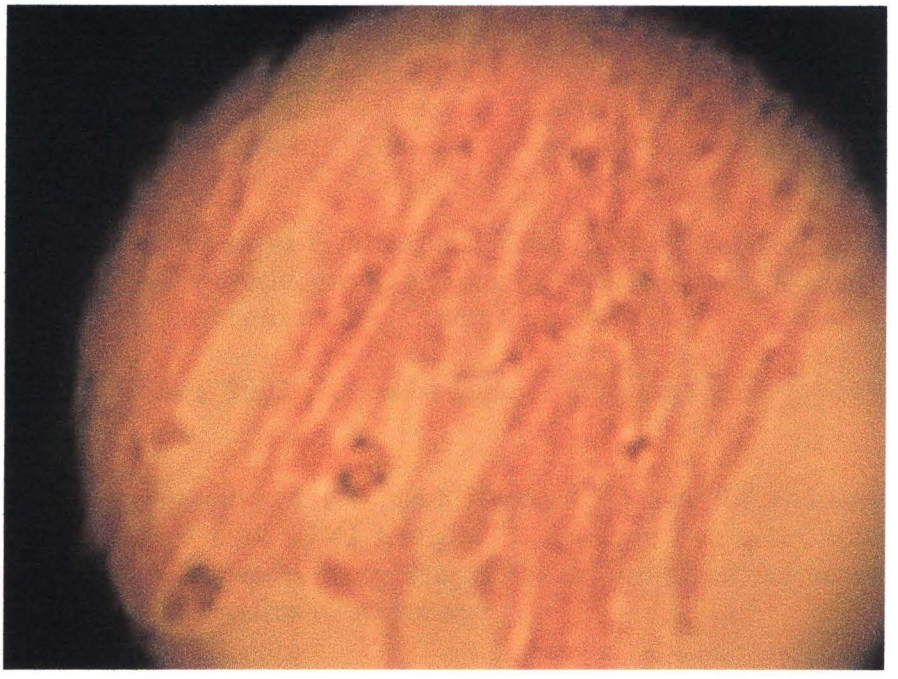


Fig. 15. Hs181.Tes cells treated with pomegranate. Cells are fibroblastic except for few spontaneous apoptosis. Time = 48h.

References

Akagi K, Hirose M, Hoshiya T, Mizoguchi Y, Ito N, Shirai T. 1995. Modulating effects of ellagic acid, vanilla and quercetin in rat medium term multi-organ carcinogenesis model. Cancer Lett; 94:113-121

Albers P, Christmas T. 2002. Testicular Cancer. European Urology; 42 (3) pg I-VIII.

Albrecht M, Gommersall LM, Wenguo J, Mansel RE, Lansky EP, Neeman I, Geldof AA, Kumi-Diaka J, Campbell J. Pomegranate extracts potently suppress proliferation, xenograph growth and invasion of human prostate cancer cells. Personal correspondent.

American Cancer Society. 2003. Report to the nation on the status of cancer. Retrieved from www.cancer.org/eprise/main/docroot/STT/stt_0

Barr PJ, Tomei LD. 1994. Apoptosis and its role in human disease. Biotechnology (N Y). May; 12(5): 487-93.

Beeson D. 2000. Commentary: testicular cancer commonly seen in younger men-(univ.New Mexico). Cancer News. April12.

Bosl GJ, Bajorin, Ssheinfeld DE, Motzer RJ, Chaganti RS. 2001. Cancer of the testis. In V.T. Devita, S. Hellman, & S.A. Rosenberg (Eds.), Cancer: Principles & practices of oncology (pp. 1491-1518). Philadelphia: Lippincott

Cotter TG, Martin SJ. 1996. Techniques in Apoptosis: A User's Guide. Portland Press Ltd. London, pp10-12

Chung LY, Cheung TC, Kong SK, Fung KP, Choy YM, Chan ZY, Kwok TT. 2001. Induction of apoptosis by green tea cathenins in human prostate cancer DUI45 cells. Life Sci; 68:1207-1214

Dearnaley, D., Huddart, R., & Horwich, A. 2001. Regular review: Managing testicular cancer. British Medical Journal, 322 (7302), 1583-1588.

Duke RC, Cohen JJ. Morphological and biochemical assays of apoptosis. In coligan JE, Kruisbeak AM eds, Current protocols in immunology. New York, John Wiley & Sons 1992, 3.17.1

Ellis HM, Horvitz HR. 1986. Genetic control of programmed cell death in the nematode C. elegans. Cell. Mar 28; 44(6): 817-29.

Fotsis T, Pepper MS, Montesano R, Aktas E, Breit S, Schweigerer L, Rasku S, Adlercreutz H, Wahala K. 1998. Phytoestrogens and inhibition of angiogenesis. Baillieres Clin Endocrinol. Metab 12(4): 649-666.

Gil MI, Tomas-Barberan FA, Hess-Pierce B, Holcroft DM, Kader AA. 2000. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. J Agri Food Chem; 48: 4581-4589

Goldberg RB, de Pavia G, Yadegari R. 1994. Plant embryogenesis: zygote to seed. Science 266, 604-614

Gullatte MM. (Ed.). 2001. A chemotherapy handbook: Clinical guide to antineoplastic therapy. Pittsburgh, PA: Oncology Nursing Socety Press.

Harde H., Schumacher W, Firbas F, Denffer D. 1970. In: Strasburg's Textbook of Botany Chaucer, London, p. 2.

Hasten B. 1983. Flavonoids: a class of natural products of high pharmacological potency. Biochemical Pharmacology 32 7, pp. 1141–1148

Heftmann E, Ko ST, Bennet RD. 1966. Response of steroids to sulphuric acid in thin layer chromatography. J Chromatogr; 21:490- 494

Henderson BE, Benton B, Jing J, Yu MC, Pike MC. 1979. Risk factors for cancer of the testis in young men. International Journal of Cancer, 23(5), 598-602.

Iwashita K, Kobori M, Yamaki K, Tsushida T. 2000. Flavonoids inhibit cell growth and induce apoptosis in B16 melanoma 4A5 cells. Biosci Biotechnol Biochem 64:1813-1820

Kerr JF, Winterford CM, Harmon BV. 1994. Apoptosis: its significance in cancer and cancer therapy. Cancer 73: 2013-2226

Kim ND, Mehta R, Yu W, Neeman I, Livney T, Amichay A, Poirier D, Nicholls P, Kirby A, Jiang W, mansel R, Ramachandran C, Rabi T, Kaplan B, Lansky E. 2002. Chemopreventive and adjuvant therapeutic potential of pomegranate (Punica granatum) for human breast cancer. Breast Cancer Res Treat 71: 203-217

Kinkade, S. 1999. Testicular cancer. American Family Physician, 59 (9) 2539-2544, 2549-2550.

Knowles LM, Zigrossi DA, Taubert RA, Hightower C, Milner JA. 2000. Flavonoids suppress androgen –independent human prostate tumor proliferation. Nutr.Cancer : 38 :116-122

Komamine A., Matsumoto M., Tsukahara M., et al (1990) IN PROGRESS In Plant Cellular and Molecular biology (nijkamp, H.J.j.,van der plas,L.H.W. and van Aartrijk, J., eds), pp 307-313,, Kluwer Academic Publishers, Dordrecht

Kumi-Diaka J. 2000. Caspace-3 protease activation during the process of genistein-induced apoptosis in TM4 testis cells. Biology of the Cell, 92, 115-124.

Kumi-Diaka J, Nguyen V, Butler A. 1999. Cytotoxic potential of the phytochemical genistein isoflavone and certain environmental chemical compounds on testicular cells. Biology of the Cell. 91:515-523.

Lampe H, Horwich A, Norman A, Nicholls J, Dearnaley DP. 1997. Fertility after chemotherapy for testicular germ cell cancers. Journal of Clinical Oncology, 15(1), 239-245.

Liberthal W, Triaca V, Levine J. 1996. Mechanism of death induced by cisplatin in proximal tubular epithelial cells: apoptosis vs necrosis. J Am Cell Physiol 270; F700-F708

Mcdonnell TJ, Meyn R E, Robertson LE. 1995. Implications of apoptotic cell death regulation in cancer therapy. Seminars in Cancer and biology: 6, 53-60

Nam Deuk Kim, Rajendra Mehta et al. 2002. Chemopreventive and Ajuvant therapeutic potential of pomegranate (Punica granatum) for human breast cancer. Breast cancer research and treatment 71; 203-217

Nepka C, Asprodini E, Kouretas D. 1999. Tannins, xenobiotic metabolism and cancer chemoprevention in experimental animals. Eur J Drug Metab Pharmacokinet: 24:183-189

Nugteren DH, Christ-Hazelhof E. 1987. Naturally occurring conjugated octadecatrienoic acids are strong inhibitors of prostaglandin biosynthesis. Prostaglandins: 33:403 –417

Peerchellet JP, Gali HU, Perchellet EM, Klish DS, Armbrust AD. 1992. Antitumor promoting activities of tannic acid, ellegic acid, and several gallic acid derivatives in mouse skin. Basic Life Sci: 59:783-801

Pennel RI, Janniche L, Scofield GN, Booij H, deVries SC, Roberts K. 1992. Identification of a transitional cell state in the developmental pathway to carrot somatic embryogenesis. J. Cell Biol 119, 1371-1380 (19,20)

Pilar L, Giorgio P, Olbojn K, Ferran A, Laszlo K, Oscar L, and the EAU Working Group on Oncological Urology. 2001 . EAU Guidelines on Testicular Cancer *European Urology* 40:102-110 15

Raffray M, Cohen GM. 1997. Apoptosis and necrosis in cytoxicology: a continuum or distinct modes of cell death? Pharmacol. Ther. 75: 153-177.

Rowan S. Fisher DE. 1997. Mechanisms of apoptotic cell death. Leukemia: 11,457-465

Schubert SY, Lansky EP, Neeman I. 1999. Antioxidant and eicosanoid enzyme inhibition properties of pomegranate seed oil and fermented juice flavonoids. J Ethnopharmacol; 66: 11-17

Select compounds found in pomegranate.www.rimonest.com

Slee EA, Adrain C, Martin SJ. 1999. Serial killers: ordering caspase activation events in apoptosis. Cell Death Differ. Nov; 6(11): 1067-74. Review

Siegel S, Castellan J. 1988. Nonparametric statistics for the behavioral sciences. 2nd Ed. McGraw Hill Inc.

Sinha Hikim AP, Rajavashish TB, Sinha Hakim I, Lue Y, Bonavera JJ, Leung A, Wang C, Swerdloff RS. 1997. Significance of apoptosis in the temporal and stage-specific loss of germ cells in the adult rat after gonadotropin deprivation. Biol Reprod. Nov; 57 (5): 1193-201.

Stermitz FR, Lorenz P, Tawarajn, Zenewicz LA, Lewis K. 2000. Synergy in a medicinal plant: antimicrobial action of berine potentiated by 5'-methoxyhydrocarpin,a multidrug pump inhibitor. Proc Natl Acad Sci USA 97:1433-1437

Stewart FC, Mapes MO Smith J. 1958. Growth and organized development of cultured cells. Am. J. Bot.5, 693-703

Tang DG, Porter AT. 1997. A hopeful weapon for Prostate cancer. The Prostate 32:284-23.

Thompson C B. 1995. Apoptosis in the pathogenesis and treatment of disease. Science: 267,1456-1462

Vaux DL, Cory S, Adams JM. 1988. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature. Sep 29; 335(6189): 440-2.

de Vries SC, Booij H, Meyerink P et al. 1988. Acquisition of embryogenic potential in carrot cell suspension culture. Planta 176,196-204

Vlietinck AJ, Vanden Bergh DA. 1991. Can ethnopharmacology contribute to the development of anti-viral drugs? *Journal of Ethnopharmacology* (32) pp. 141–153

Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S. 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature. Mar 26; 356 (6367):314-7.

White E. 1996. Life death and the pursuit of apoptosis. Genes and development (10) 1-5,

Wyllie AH, Kerr JFR, Currie AR. 1980. Cell Death: the significance of apoptosis. Internationl review of cytology: 68, 251-305

Xiao-Cheng W M.D., M.P.H., C.T.R, Vivien W. Chen Ph.D., Brooke Steele D.O., Steven Roffers P.A., C.T.R., Judith B. Klotz D.r.P.H., Catherine N. Correa Ph.D. and Susan E. Carozza Ph.D . 2003. Cancer incidence in adolescents and young adults in the United States, 1992–1997Journal of Adolescent Health Volume 32, Issue 6 June 2003, Pages 405-4

Zhang G, Guru V, Kain SR, Yan G. 1997. Early detection of apoptosis using a fluorescent conjugate of Annexin V. Biotechniques 23, 525-531.