

Assessment of Anti-cancer and Anti-viral potential of Pomegranate peel extract against human Prostate, and Larynx cancer cell lines: *In-Vitro* Study

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Abstract: The present work aimed to evaluate the anticancer and antiviral potentials of Pomegranate (*Punica granatum*) peel extract (PPE). Data recorded revealed that PPE was Cytotoxic to cancer cells in a concentration and cell type dependent manner, where the HEp-2 cells were more sensitive to the tested extract than PC-3 recording a significantly reduced IC₅₀ value (P<0.05) in HEp-2 than PC-3 with records 115 and 206 µg / ml respectively. Also, the anticancer activity was assured via the up/down regulation of pro-apoptotic gene (Caspase-3) and metastasis contributing gene (MMP-1), both genes expression was cell type dependent as well. Also, the apoptotic potential of PPE was confirmed via arresting of cells in the G2/M phase and elevated apoptotic cell % in the pre-G1 phase. Also, the initiation of PPE antiviral potential was confirmed by monitoring the MxA gene post Vero cell treatment for 24 hours as the expression of MxA gene was significantly elevated (P<0.05) than non treated cells used as control.

[Fadya Mohamed El-Garhy, Doaa abd el aziz badr and Aly Fahmy Mohamed. **Assessment of Anti-cancer and Anti-viral potential of Pomegranate peel extract against human Prostate, and Larynx cancer cell lines: *In-Vitro* Study.** *Cancer Biology* 2017;7(2):65-73]. ISSN: 2150-1041 (print); ISSN: 2150-105X (online). <http://www.cancerbio.net>. 9. doi:[10.7537/marscbj070217.09](https://doi.org/10.7537/marscbj070217.09).

Keywords: PPE, PC-3, HEp-2, Caspase-3, MMP-1, MxA gene, and cell cycle.

1. Introduction

Cancer is a disease of unrestricted cell proliferation, occasionally it is considered as genetically originated disease, also considered to be a multifactorial heterogeneous disease, and the major causes of morbidity and mortality worldwide (Jemal *et al.*, 2011). Carcinogenesis, is the process of tumor development which is a multistep process involving three distinct stages are initiation, promotion, and progression (Oliveira *et al.*, 2007). The dysregulated cellular evolution during carcinogenesis drives cells to acquire six phenotypic hallmarks of cancer which are Sustaining proliferative signaling, evading growth suppressor, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, and resisting apoptotic signals; thus initiating the transformation of a normal cell to a malignant one (Hanahan & Weinberg, 2011). Thereby targeting multiple molecular pathways that are incapable of deregulation during carcinogenesis; is the major focus in cancer prevention and treatment. Over the last decades research has been established beyond doubt that diverse of epigenetics and environmental factors play an important role in the development and progression of cancer (Minamoto *et al.*, 1999; Pogribny & Rusyn, 2013). Despite the significant advancements in treatment options, the incidence and mortality rates from cancer continue to increase (Siegel *et al.*, 2015; Jemal *et al.*, 2016). It is predictable that by the year of 2025, there will be virtually 20 million cancer patients (Jemal *et al.*,

2016). Consequently, attention is being focused on cancer prevention as an ultimate strategy for management of the cancer epidemic (Key *et al.*, 2004; DiMarco-Crook *et al.*, 2015). In addition to that two-thirds of cancer related deaths might be prevented through lifestyle variation, mostly through dietary means (Key *et al.*, 2004; Barnard, 2004).

Cancer chemoprevention refers to the usage of synthetic or natural chemical agents that are able to prevent, or suppress the processes of carcinogenesis or tumor progression (Priyadarsini & Nagini, 2012). Dietary plants such as fruits, vegetables, spices, and nuts are important source of bioactive natural compounds with anti-carcinogenic activity (Huang *et al.*, 2010). So far more than 10000 different dietary phytochemicals have been described; they are widely present in plant derived foods and beverages (Russo *et al.*, 2010; Tasinov *et al.*, 2012; Ivanova, 2013). Cancer prevention by dietary phytochemicals is an attractive approach due to their natural biological activity, affordability, easy availability, lack of toxicity and ability to modulate a variety of signaling pathways and cell processes (Priyadarsini & Nagini, 2012; Ivanova, 2013). Phytochemicals belong to various structural and functional chemical classes such as phenolic compounds, vitamins, alkaloids, and carotenoids (Fresco *et al.*, 2010; Ivanova, 2013). Phytochemicals are able to act through multiple mechanisms such as cell receptors targeting, structural proteins, reactive oxygen species (ROS), membrane lipids, the oxidative damaged biomolecule products,

signaling pathway molecules involved in the cell cycle, apoptosis, differentiation and migration (Fresco *et al.*, 2010; Priyadarsini & Nagini, 2012; Ivanova 2013).

Pomegranate (*Punica granatum L.*) seeds are edible; holding a strong antioxidant and anti-inflammatory properties due to their high content of hydrolysable tannins and anthocyanins (Viuda-Martos *et al.*, 2010). As compared to the antioxidant activity of vitamin E, β -carotene, and ascorbic acid; the pomegranate antioxidants appear unique due to combinations of a wide array of polyphenols, having a broader range of action against several types of free radicals (Aviram *et al.*, 2005). As compared to the recognized antioxidants in red wine and green tea, anthocyanins from pomegranate fruit possess significantly higher antioxidant activity (Gil *et al.*, 2000). Pomegranate has been used in various medicinal systems for the treatment of a multitude of diseases and ailments. In the ancient Indian medicinal system, i.e., in Ayurvedic medicine, the pomegranate was considered to be a whole pharmacy into itself. It was recommended to be used as an anti-parasitic agent also to treat diarrhea and ulcers (Naqvi *et al.*, 1976; Caceres *et al.*, 1987). The medicinal properties of pomegranate have sparked significant interest in today's scientific community as evidenced by the scientific research relating to health benefits of pomegranate that have been published in the last few decades (Khan *et al.*, 2008; Faria & Calhau, 2011). Remarkably, it is not just the pomegranate fruit itself, but other parts of the plant as well, including the peels, and leaves of the pomegranate fruit, which are rich in molecular constituents with therapeutic properties (Naqvi *et al.*, 2017; Lansky *et al.*, 2007).

Studies have shown that pomegranate constituents can efficiently affect multiple signaling pathways involved in inflammation, cellular transformation, hyper-proliferation, angiogenesis, initiation of tumorigenesis, and eventually suppressing the final steps of tumorigenesis and metastasis (Khan *et al.*, 2008; Faria & Calhau, 2011). Moreover, the pomegranate constituents are shown to modulate transcription factors, pro-apoptotic proteins, cell cycle regulator molecules, anti-apoptotic proteins, protein kinases, cell adhesion molecules, pro-inflammatory mediators, and growth factors in various types of cancers.

The present work aims to evaluate the anticancer potential of pomegranate peel extract (PPE) against HEP-2, and PC3 cancer cell lines, related cell cycle and gene profile. As well as investigating the anti-viral activity of PPE on Vero cells against Vesicular Stomatitis virus (VSV), and its related MxA gene expression.

2. Materials and Methods

Vesicular Stomatitis virus (VSV)

Vesicular Stomatitis virus Indiana strain-156 (VSV), was supplied from Dr Aly Fahmy, Head of R & D sector VACSERA-Egypt, it was at infectivity titer of $7.5 \log_{(10)} / \text{ml}$, seed stock of VSV was prepared on Vero cells, VSV was liquated and cryopreserved at -80°C (Revco-USA) till use.

Determinations of VSV infectivity titer using cell culture assay

Infectivity titer of VSV stock was determined according to (Bussereau F *et al.*, 1982) TC harvested virus seed stock was 10 fold serially diluted in MEM-E supplemented with 2% FCS. Virus dilutions were dispensed to a precultured Vero cells as 0.1 ml /well. Plates were examined using inverted microscope (Hund-Germany) for 7 days post inoculation for detection of cytopathic effect (CPE) of VSV. 50% end point induced CPE was determined according to (Reed LT & Muench HA, 1938).

Preparation of pomegranate peel extract

Fresh pomegranate fruits were collected from Local market, Giza governorate, Methanol and Ethanol were purchased from [Sigma, Aldrich-USA]. Husks were peeled manually; the husks were dried using oven and then blended with 70% ethanol. The mixture was sterile filtrated through a $0.22 \mu\text{m}$ (Millipore-USA) and kept in aliquots at 4°C according to (Abdel Motaal & Shaker, 2011).

Cell cultures

Prostate (PC-3) and Larynx (HEp-2) cancer cell lines were provided by tissue culture department (VACSERA-Egypt). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (GIBCO, USA) and 1% penicillin-streptomycin (Invitrogen, USA) and then incubated (Jouan-France) at 37°C in a humidified atmosphere containing 5% CO_2 .

Cell Viability (MTT Assay)

Human prostate and larynx cancer cells were propagated in 75 cm^2 cell culture as previously mentioned according to (Bussereau F *et al.*, 1982), where confluent sheet were detached using 0.25% (w/v) trypsin solution and 0.05% (v/v) ethylene diaminetetra acetic acid (GIBCO-USA) for 5 min. Cells were plated at a concentration of 2×10^5 cell/ml in 96-well cell culture plates and incubated at a temperature of 37°C for 24 hours to achieve confluency. The growth medium was decanted and fresh medium containing various concentrations of PPE was dispensed to cells (4 wells). Dead cells were washed out using phosphate-buffered-saline (PBS) and $50 \mu\text{l}$ of MTT stock solution (5 mg/ml) were added to each well. After 4 hours incubation period, the supernatants were discarded and the formazan precipitates were solubilized by addition of $50 \mu\text{l}$ per

well of dimethyl sulfoxide (DMSO). Plates were incubated in the dark for 30 min at a temperature of 37°C and absorbance was determined at a wavelength of 570 nm using micro plate reader (ELX -800, Biotek- USA). The cell viability percentage was calculated using the following formula:

$$\text{Cell Viability \%} = \frac{\text{Mean OD of test dilution} \times 100}{\text{OD of control wells}}$$

Determination of PPE IC₅₀

Cytotoxicity was conducted according to (Van den Berghe *et al.*, 1978), where growth medium was decanted from tissue culture Prostate and larynx cancer cell lines precultured 96 micro titer plates. Pomegranate peel extract (PPE) sample was applied as starting concentration of 10 mg / ml in a series of 2 fold serially diluted pattern in serum free RPMI-1640 medium as 0.1 ml of each dilution/well. Negative non-treated cell control was considered. Plate was incubated at 37 °C (Jouan- France) and examined frequently for up to 3 days. Cells were checked for any toxic as partial or complete loss of monolayer, shrinkage, rounding, or cell granulation. The IC₅₀ value was determined using Master Plex 2010 Software.

Cell Cycle Analysis

PC-3 and HEP-2 were pre-cultured in 25 cm² cell culture flasks were treated with the IC₅₀ of tested product (PPE) in RPMI-1640 medium, for 24h. For cell cycle analysis, the cells were harvested and fixed gently with 70% (v/v) ethanol in PBS, maintained at a temperature of 4°C overnight, and re-suspended in PBS containing 40µg/ml PI, 0.1 mg/ml RNase and 0.1% (v/v) Triton X-100 in a dark room. After 30 min at 37°C, the cells were analyzed using a flow-cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an argon ion laser at a wavelength of 488 nm. The cell cycle and sub-G1 group were determined and analyzed, as described previously.

Real time PCR: mRNA Expression of Cell Apoptosis-related genes

Total RNA was extracted from control and treated HEP-2 and PC-3 cells using the Gene JET RNA Purification kit (Fermantus-UK) according to the manufacturer's protocol. The concentration and the integrity of RNA were assessed spectrophotometrically at 260/280 nm ratio and by gel electrophoresis, respectively. First-strand cDNA was synthesized with 1 µg of total RNA using a Quantitect Reverse Transcription kit (Qiagen, Germany) in accordance with the manufacturer's instructions. These samples were subsequently frozen at a temperature of -80°C until use for determination of the expression levels of Caspase-3 and MMP-1 genes using real-time PCR. Quantitative real-time PCR was

performed on a Rotor-Gene Q cycler (Qiagen, Germany) using QuantiTect SYBR Green PCR kits (Qiagen, Germany) and forward and reverse primers for each gene. The nucleic acid sequences of the primers were as follows CASP3-F 5'-TTC ATT ATT CAG GCC TGC CGA GG-3' and R 5'-TTC TGA CAG GCC ATG TCA TCC TCA-3' and the MMP1-F 5'-CTG GCC ACA ACT GCC AAA TG-3 and R 5'-CTG TCC CTG AAC AGC CCA GTA CTTA-3'. Real-time PCR mixture consisted of 12.5 µl 2x SYBR Green PCR Master Mix, 1 µL of each primer (10 pmol/µl), 2 µl cDNA and 8.5 µl Rnase-free water in a total volume of 25 µl. Amplification conditions and cycle counts were a temperature of 95°C for 15 min for the initial activation, followed by 40 cycles of denaturation at 94°C for 15s, annealing at 60°C for 30s and elongation at 72°C for 30s. Melting curves were performed after real-time PCR to demonstrate the specific amplification of single product of interest. A standard curve was performed to determine the amplification efficiency of the primers used, as well as relative fold changes in both of them.

Determination of Antiviral Activity

The antiviral potential of PPE was monitored using (indirect way), where precultured Vero cells were treated with the safe concentrations of PPE for 24 hrs. Treatment media was decanted and test VSV was prepared according to (Fenard D *et al.*, 1999) where virus was 10 fold serially diluted. Virus dilutions were dispensed to the treated and non-treated cells. Virus infectivity titer was evaluated according to (Fernandez S *et al.*, 2014) and the antiviral activity was determined by subtracting the virus infectivity titer of virus infectivity titer in treated cells from that in non treated cells.

Real time PCR: MxA Gene Detection

Total RNA was extracted from control non-treated and treated Vero cells using the same previously mentioned Gene JET RNA Purification kit (Fermantus-UK) according to the manufacturer's protocol. Samples were frozen at -80°C until use for determination of the expression levels of MxA gene using the following primers F 5'-AAA TGG CTC AAG AGG TGGA-3' R 5'-TAT CGC TGA CAG TTG GGTG-3'. Real-time PCR mixture, amplification conditions, and the cycle counts were the same as previously mentioned in Caspas-3 and MMP-1 expression measuring. Melting curves, as well as standard curve were performed.

Statistical Analysis

All experiments were carried out in three independent tests. Data were expressed as the mean standard deviation (SD) and analyzed using one-way analysis of variance (ANOVA). The results were considered statistically significant at probability <0.05.

3. Results

Cytotoxicity of Pomegranate peel extracts PPE

The Cytotoxic effect of PPE was assessed by recording the various morphological changes of cells. The viability % of treated cells was concentration dependant, it was found that HEp-2 was more sensitive to PPE than PC3 in a non significant way ($P>0.05$). In the same time the related IC₅₀ value of PPE against HEp-2 cells was significantly reduced than its value in PC-3 treated cells (Figure 1- A & B).

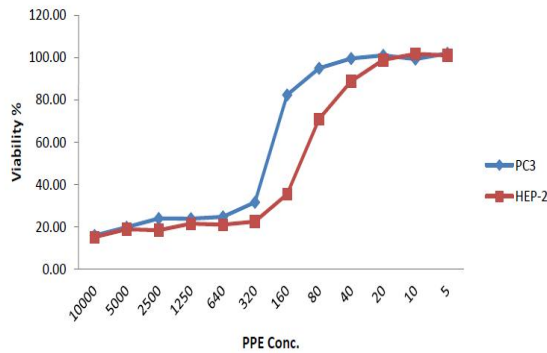


Figure 1: (A) Illustrate the correlation between the PPE concentration and the cell viability

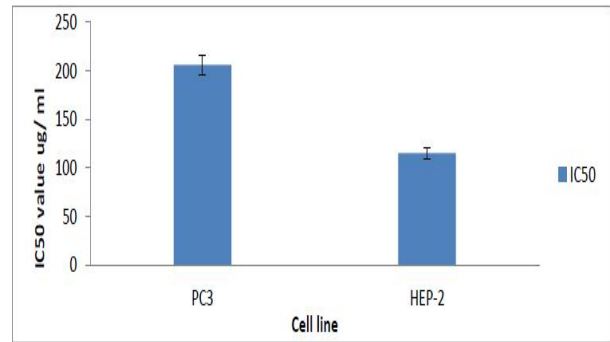


Figure 1: (B) Assessment of IC₅₀ values of PPE post HEp-2 and PC-3 cancer cell lines using MTT assay

Cell Cycle and apoptosis

Regarding the effect of tested extract on cell cycle profile it was noted that cells were accumulated in the G₂-M phase in a significant way; 14.38 % ($P<0.05$) compared to control of PC-3 cell line. Also, cell arrest was detected in HEp-2 cell line but in a non-significant way ($P>0.05$) recording 7.58 and 6.64 in treated and control cells respectively. It was noticed that cell arrest was accompanied with apoptotic profile in the pre-G₁ phase in a significant way in case of both cell lines ($P<0.05$) (Figure 2-A & B).

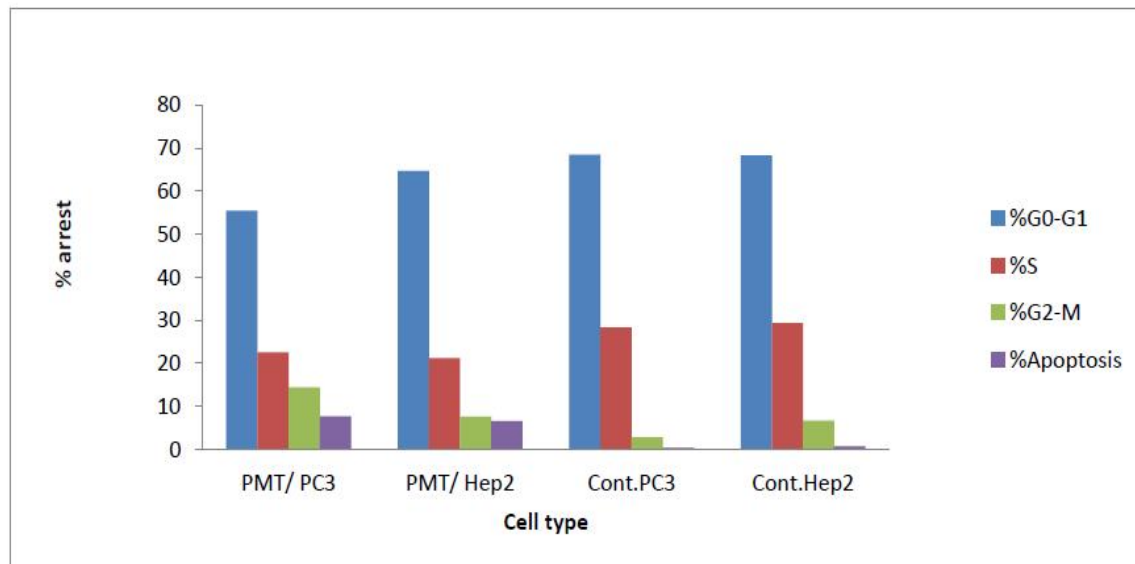


Figure 2: (A) Illustrate the cell cycle analysis and related % of arrested cells in different cell cycle phases post treatment with PPE

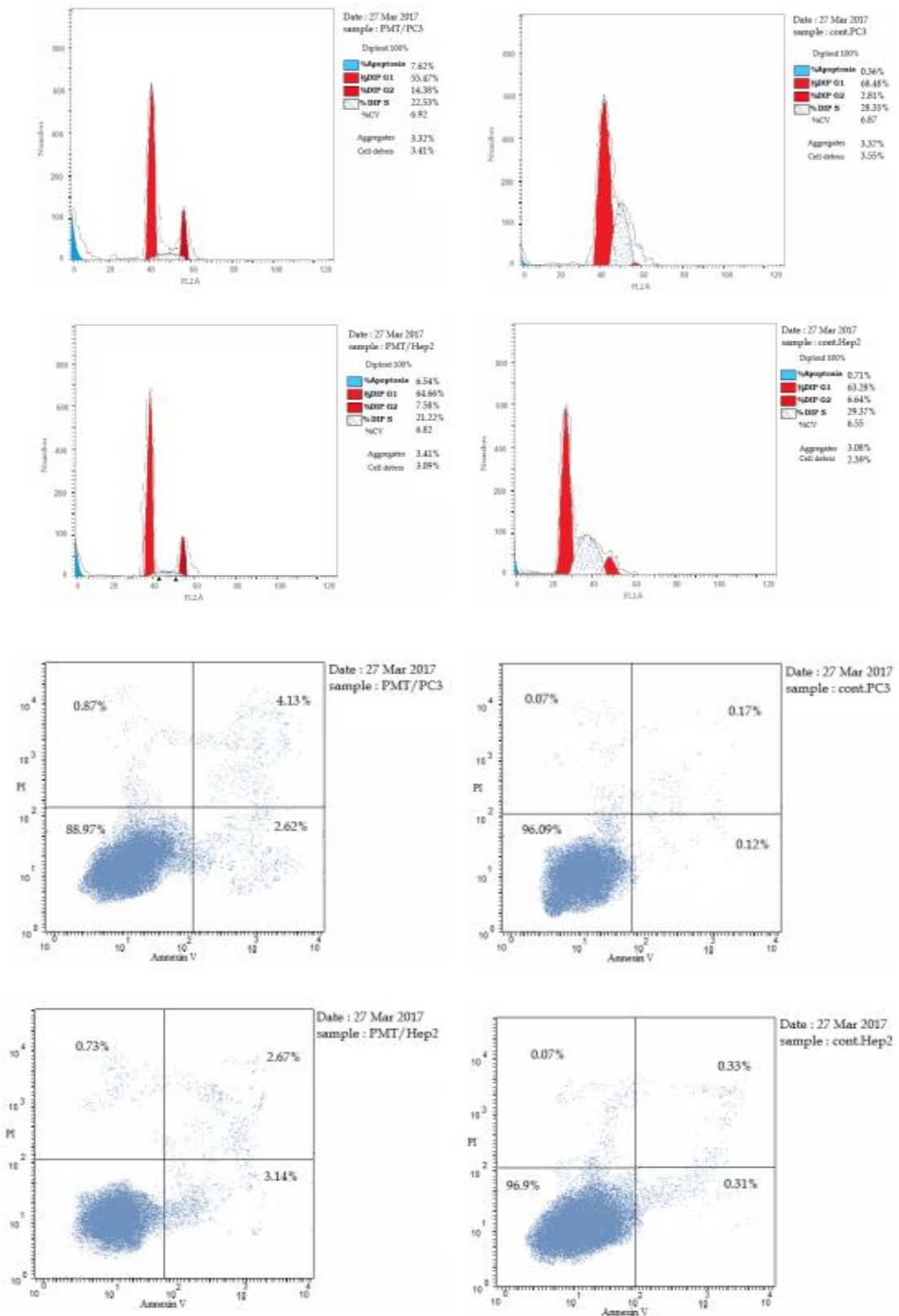


Figure 2: (B) Flow cytometric cell cycle analysis and related % of arrested cells in different cell cycle phases, as well as the apoptotic % post treatment with PPE

Polymerase Chain Reaction (PCR): Apoptosis related genes

Evaluating the expression of pro-apoptotic gene (Caspase-3), and the metalloproteinase protein (MMP-1) metastasis contributing gene; were carried out using the newly synthesized cDNA as template for PCR. According to the measured expression of Caspase-3 and MMP-1 genes, also as illustrated; the Prostate cancer cell line showed higher response to the claimed anticancerous extract [PPE] than in larynx cancer cell lines. Whereas the pro-apoptotic Caspase-3 gene expression was highly elevated in the PC-3 than in the HEp-2 cell lines, the expression rate was significantly elevated ($P < 0.05$) in treated cells more than 8 folds in non-treated cells as control. While the MMP-1 gene was significantly reduced in both cell lines PC-3, & HEp-2 which indicates the anticancerous effect of the tested PPE through the down regulation of the genes contribute in the metastasis process (Figure 3).

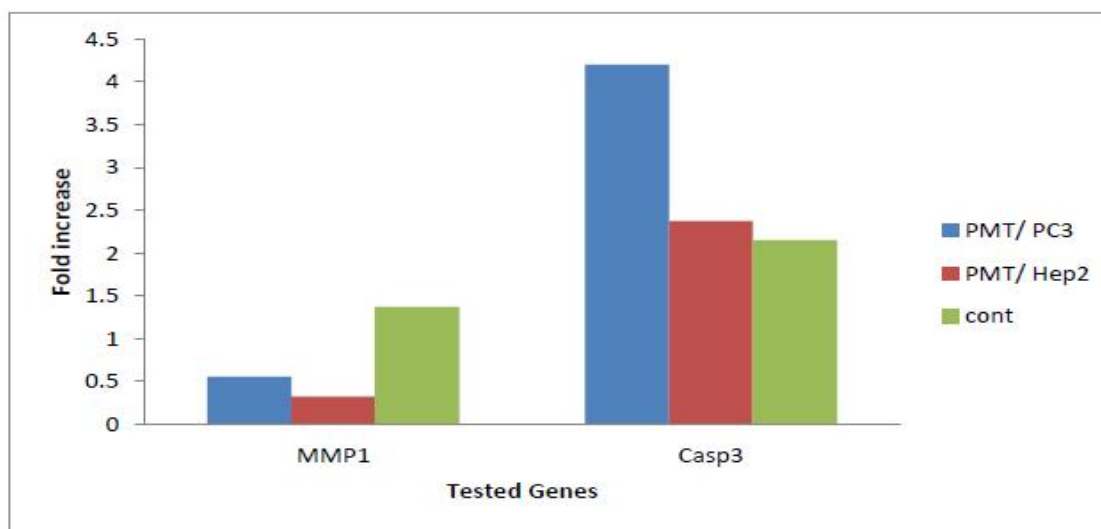


Figure 3: Illustrate both Caspase-3 and MMP-1 expression in PC-3 and HEp-2 post treatment with PPE compared to the non-treated cells negative control

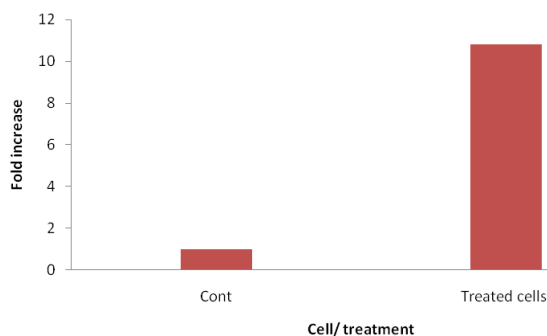


Figure 4: Assessment of MxA gene expression in Vero cells post treatment with PPE compared to the non-treated cell control

Polymerase Chain Reaction (PCR): Antiviral activity determination (MxA gene expression)

Evaluating the expression of anti-viral activity using indicator gene (MxA), was carried out by using the newly synthesized cDNA as template for PCR. Regarding the antiviral potential of PPE against VSV, it was noticed that there was a mild antiviral activity of $0.67 \log_{10} / 0.1 \text{ ml}$ post cell treatment with PPE for 24 hrs pre-infection. Since the moderate antiviral potential of MxA gene was significantly elevated,

therefore MxA gene expression was used as a post cell treatment biomarker with tested material compared to its value with non-treated cell control ($P < 0.05$) (Figure 4).

4. Discussion

The present study was designed to investigate the anticancer potential of PPE in human HEp-2, and PC-3 cancer cells. The growth inhibition was concentration and cell type dependent. The results regarding the antiproliferative and cytotoxic potential matched the study recorded by (El-Awady *et al.*, 2015), whereas the applied MTT assay of different *Rhza strecta* and pomegranate extracts on HepG2 and Caco-2 cell lines. In supportive way with the present work, the extracts showed different anti-proliferative profiles which were extract concentration and cell type dependent. The *Rhza H* extract was significantly cytotoxic against HepG2 and Caco-2 cells recording IC_{50} values in the order of $25 \mu\text{g/ml}$, and $35 \mu\text{g/ml}$ respectively. Several reports described that the anticancer activity of the medicinal plants may be due to the presence of antioxidants such as polyphenols, flavonoids, vitamins, carotene, enzymes, minerals, polysaccharides, lignins, and xanthonnes.

The present study results were in agreement with different studies that were conducted to evaluate the cytotoxic effect of different pomegranate extracts against different cancerous cell lines (**Abdel Motaal and Shaker 2011; Aqil et al., 2012; Yazici et al., 2012; Banerjee et al., 2012**). Also, (**Jeune et al., 2005**) recorded that both pomegranate extracts and genistein have significant *in vitro* inhibitory effect on the growth rate of MCF-7 breast cancer cells. Also, demonstrated that both pomegranate and genistein, in single and combination treatments, induced apoptosis in the cancer cells in a time and dose dependent manner. In consistency with the previous reports (**Kumi-Diaka et al., 2000**) study showed that the treatment induced morphological changes in the cells as indicative of cell differentiation, inhibition, and cell death were consistent with previously observed results. In supportive with the present work, both cell lines HEP-2, and PC-3 were arrested in the pre-G1 phase due to the induced apoptosis.

In the present study, gene profile was performed to determine the expression value of Caspase-3, and MMP-1 genes pre and post-treatment. Recently and as stated by (**Sudhakar et al., 2015**) the potent antioxidant and anti-cancer activities of pomegranate were attributed due to its polyphenols constituents. Also, Ellagitannins (ETs) have also been identified as active anti-atherogenic compounds in Pomegranate juice (PJ) as it has been shown that pomegranate fruit extracts and its purified ETs inhibit the proliferation of human cancer cells and modulate inflammatory sub-cellular signaling pathways and apoptosis. The crude aqueous extract derived from the peel of *Punica granatum* was evaluated as a cytotoxic agent, using the crystal violet assay. P⁵³ detection and quantitative determination against HEP-2 cell line by the ease of p⁵³ ELISA Kit for the determination of wild-type and mutant p⁵³ in human, mouse and rat samples.

Larrosa et al., (2006), studied the induction of apoptosis in Caco-2 cells by punicalagin and ellagic acid from PJ. The study revealed that treatment of Caco-2 cells with these agents resulted in the release of mitochondrial cytochrome C into the cytosol, activation of Caspase-3, Caspase-9, and down-regulation of anti-apoptotic Bcl-xL. Both punicalagin and ellagic acid treated Caco-2 cells resulted in decreased protein expression of cyclin Kinases, as well as arrest of cells in S phase of the cell-cycle. The authors suggested that the anti-carcinogenic effect of pomegranate ellagitannins could largely be due to their hydrolysis product Ellagic acid, which induced apoptosis in colon cancer cells.

As well as (**Malik et al., 2005**) study which demonstrated that Pomegranate fruit extract (PFE) treatment against PC-3 cells resulted in cell apoptosis. The effect of PFE on cell cycle regulatory molecules

operative in the G₁ phase of the cell cycle were examined. As well as the effect of PFE on the induction of WAF1/p21 and KIP/p27 were assessed, which are known to regulate the entry of cells at the G₁-S phase transition checkpoint and induce apoptosis. Immunoblot analysis revealed that PFE treatment of the cells resulted in a marked induction of WAF1/p21 and KIP1/p27 in a dose-dependent manner compared with the basal levels. Using immunoblot analysis, the effect of PFE treatment on the protein expressions of the cyclins and cdk was also assessed, which regulated by WAF1/p21. Revealing that apoptosis was induced in PC-3 cells through the induction of WAF1/p21/KIP1/p27 with the inhibition of Cyclins and cdk.

According to (**Dell'Agli et al., 2010**) study on pomegranate fraction enriched in tannins against infected human THP-1 monocytic cells; PPE extract at 50µg/ml inhibited the secretion of MMP-9 by 61%. When the extract was deprived of tannins, the inhibitory effect was dramatically reduced. This result indicated that tannins were likely to be the active principles.

Regarding the antiviral activity in the present study, MxA-gene expression assured the antiviral potential of the PPE. Limited studies have been conducted on the antiviral activities associated with pomegranate and its extracts. The fruit's antiviral effects have been reported against influenza virus, herpes virus, poxviruses, and human immunodeficiency virus (HIV-1) (**Haidari et al., 2009; Kotwal et al., 2008**). The hydrolyzable tannins and anthocyanins are the main compounds associated with the beneficial effects of pomegranate consumption on other health effects including the antibacterial activities, and also may be responsible for the antiviral activity (**Kasimsetty et al., 2009**). It was reported that among four flavonoid compounds associated with pomegranates (ellagic acid, caffeic acid, luteolin, and punicalagin); only punicalagin was shown to have inhibitory effects on influenza virus (**Houri-Haddad et al., 2005**).

5. Conclusion

It was concluded that pomegranate has an antiproliferative potential against PC-3, and HEP-2 cancer cells through induction of apoptosis proved via up regulation of pro-apoptotic genes such as Caspase-3. As well as down regulation of metastasis contributing genes such as MMP-1. However, apoptosis induction is both dose and time dependent. For further studies on the anticancer activities of the PPE; it is recommended to extend the range of cancer cell lines. Furthermore, *in-vivo* studies must be arranged in parallel with *In-vitro* studies.

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