RESEARCH ARTICLE

Editorial Process: Submission:06/05/2024 Acceptance:11/22/2024

Induction of Apoptosis and Antiproliferative Activity of MCF-7 Human Breast Cancer Cells with Sonicated Aqueous Peel Extract of *Punica granatum* L. (Nimali sp.)

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Abstract

Background: The antioxidant and antiproliferative activities of various parts of the *Punica granatum* L. fruit (Nimali variety) on MCF-7 human breast cancer cells have been investigated. The analysis of the effect on gene regulation and apoptosis induction compared to different extraction methods, was carried out highlighting the fruit's potential anticancer properties attributed to polyphenol-rich composition. **Methods:** This study analyzed alterations in radical scavenging capacity (RSC), phenol (TPC), and flavonoid contents (FC) of pomegranate fruit parts, and antiproliferative activity towards MCF-7 cancer cells using different extraction methods. Most effective peel extract/s were analyzed for total protein content, nitric oxide production, LDH, and Caspase 3 and 8 activities. RT-qPCR was performed with intact RNA to examine the apoptotic pathway and gene expression, and western blot analysis confirmed the presence of tumor suppressor protein/s**. Results:** The sonicated peel extract (SPL) exhibited the highest RSC, TPC, and FC. Fermented juice displayed higher RSC, TPC, and FC compared to fresh juice. Sonicated peel extract showed an IC_{50} value of 130±4.5 μg mL^{-1} against MCF-7 cells, while VERO (healthy) cells had values >1,000 μg mL-1. Sonication was identified as the most effective extraction method for the antiproliferative activity of pomegranate fruit. The study revealed that SPL induced apoptosis via the p21, p53-dependent, caspase 8 pathways, and caspase 3-independent mechanisms in MCF-7 cells. **Conclusion:** Findings emphasize the potential therapeutic effects of SPL in the antiproliferative effect of MCF-7 cells by modulating caspase 8, p53, and p21-dependent pathways, without activating caspase 3.

Keywords: Apoptosis- p53/p21 genes- RT-qPCR- caspase 3/8- MCF-7 breast cancer cells- *Punica granatum* L.

Asian Pac J Cancer Prev, **25 (11)**, 3967-3976

Introduction

Cancer is a major cause of mortality globally, responsible for approximately 10 million deaths in 2020, which equates to nearly one in six deaths. The most prevalent types of cancers include breast, lung, colorectal, and prostate cancers [1]. Sri Lankan conventional medicinal and treatment methods are based on many indigenous herbs, compounds, and many techniques integrated as well. In Sri Lankan home gardens pomegranate has been grown due to its nutritional benefits [2]. Pomegranate (*Punica granatum* L.) is one of the most widely used herbs for the treatment of cancers in Ayurveda with minimum side effects. It is reported that pomegranate, juice, seeds, leaves, peel, pericarp bark have shown several health benefits such as antioxidant, anti-inflammatory, antibacterial activities [3]. There is a paucity of evidence of the Sri Lankan variety (Nimali) of pomegranate and effectiveness as an anticancer medication. Furthermore, the exact mechanism of action as an anticancer drug is not clear.

The *p53* gene is a tumor suppressor gene, mapped in chromosome 17. In the cell, p53 protein binds to DNA and stimulates a gene to produce p21 protein that interacts with a cell division-stimulating protein (cdk2). When p21 is complexed with cdk2 the cell cannot undergo cell division [4]. Thus, it prevents the uncontrollable growth of tumours and undergoes apoptosis, which is induced by the antioxidant activity, polyphenols, flavonoids. The aim of the study was comparing the effect of extraction methods with the radical scavenging capacity, total phenol content, flavonoid content, and cytotoxicity against selected cancer cell line compared to the healthy cells and mechanism of action of its cytotoxicity for the Sri Lankan pomegranate fruit extracts/s.

Materials and Methods

Chemicals and solvents

The major chemicals and kits such as Lactate

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Dehydrogenase Activity Assay Kit, Trizol® reagent Modified Eagle's medium (MEM) (Cat.no. M0769), L-glutathione reduced, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) (Cat. No.M2128) were purchased from Sigma Chemicals Co. (P.O.Box 14508, St. Louis, MO 63178 USA). All other chemicals used in the study were of analytical grade.

Gallic acid, Folin- Ciocalteu reagent, trichloroacetic acid, Sodium bicarbonate, Sodium chloride, EDTA, Modified Eagle's medium (MEM), Glutamin, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], Fetal Bovine Serum (FBS), DPPH, Aluminium chloride and Sulphanilamide, L-ascorbic acid, N-(1-naphthyl-)-ethylene diamine Dihydrochloride, Polyvinyl-polypyrrolidone (PVPP) and Quercetin., Primers were manufactured by Integrated DNA Technologies, Pte. Ltd. & purchased through Avon Pharmo Chem (Pvt.) Ltd. CaspACE™ Assay -3 and -8 (Colourimetric), GoTaq® 1-Step RT– qPCR Systems, Acridine orange/ Ethidium bromide. All the chemicals were in analytical grade or molecular grade. MCF-7, RD and VERO cell lines were used for cytotoxicity assays.

Plant material

Fruits of *Punica granatum* L. (Nimali) a Sri Lankan variety were obtained from Fruit Research Institute, Department of Agriculture, Kalpitiya, Sri Lanka (January 2017). Plant species were taxonomically identified, and a voucher specimen was deposited in the Botany Department, Bandaranayake Memorial Ayurveda Research Institute, Nawinna, Colombo, Sri Lanka (specimen number 2025).

Preparation of Pomegranate peel and pericarp extracts

Pomegranate peel (PL) and pericarp (PC) samples were freeze-dried (Labconco Free Zone Legacy Benchtop Freeze Drier, Model No: 7670530, 220V) at -40ºC until a constant weight. Lyophilized powder was subjected to selected extraction methods with mentioned conditions; Boiling (B) (45 minutes), Sonication (S) (50 kHz, 135 W, 30 minutes), Sonication followed by microwaving (MS) (50 kHz, 135 W, 30 minutes) (2450MHz, 1050W, 3 minutes), Microwaving (M) (2450MHz, 1050W, 3 minutes), Heating in a water bath (W) (50˚C, 20 minutes). Powdered extracts were stored airtight at –20˚C, to prevent deterioration due to moisture and microbial growth.

Cell culture

Human breast cancer cell line (MCF-7) and healthy cell line (VERO - African green monkey) were received by Medical Research Institute, Colombo 08, Sri Lanka, and cultured with Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) (Sigma Chemicals Co. P.O.Box 14508, St. Louis, MO 63178 USA), L – Glutamine (3%), 10,000 U/mL penicillin and10mg/mL streptomycin, 7.7% sodium bicarbonate and HEPES (1M) in 5% CO2 incubator (SHEL LAB/Sheldon Manufacturing Inc., Cornelius, OR 97113, USA) at 37°C in a humidified atmosphere. Cells in exponential growth were used for assays with FBS-free medium and exposed to extractions in different concentrations (100 µL/well) in 96-well plates.

Cell viability assay

Cell viability was determined by MTT cytotoxicity assay at 540nm with 96 microplate readers with extracts of PL and PC on MCF-7 and VERO cells. Cycloheximide (0.1 %, 25 µl) was considered as positive control. Percentage cell cytotoxicity was determined according to the following formula,

$$\% \text{ Cytotoxicity} = (Ab \text{ (control)} - Ab \text{ (sample)}) / Ab \text{ (control)} * 100 \quad (1)$$

Lactate dehydrogenase (LDH) activity

LDH activity of supernatant and lysate of MCF-7 cells, after treatment with sonicated and microwaved pomegranate peel extractions, was measured by the LDH Activity Assay Kit (Sigma-Aldrich, St. Louis, MO; Catalog Number: MAK066) according to the manufacturer's instructions.

Estimation of protein content

The protein content of the cell lysate was determined by Lowry et al. 1951 (Oliver H. Lowry, Nira J. Rosebrough, A. Lewis Farr, 1951), after treatment with pomegranate peel extractions for 24 h. The calibration curve was plotted with Bovine Serum Albumin (BSA). The percentage protein content of treated cells was calculated as follows.

$$\text{Protein content\%} = [\text{Protein content of treated sample} / \text{Protein content of untreated}] * 100 \quad (2)$$

Griess Nitrite Assay for Nitric Oxide production in cells

The cell supernatant obtained from the MTT assay was used to assay nitric oxide production in cells. The standard curve was constructed using Sodium nitrite (0.25- 3 µg/ml) and the nitrite content in the supernatant was determined.

Ethidium bromide and acridine orange (EB/AO) and Giemsa staining

This was performed to determine apoptosis in MCF-7 cells by sonicated peel extract (SPL). Morphological changes were examined using a phase-contrast inverted fluorescence microscope under an ultraviolet (UV) lamp and imaged with a digital imaging system.

DNA fragmentation assay

The isolation of fragmented DNA was carried out according to the procedure of Kasibhatla et al. [5] with slight modifications. Cells (1×10^6) were seeded in 24 well plates and treated with different concentrations of SPL for 24 h respectively. 1000-kbp DNA marker was used for size reference. DNA was photographed by the UVI pro gel documentation system (UVItec UK.)

Caspase 3 activity

Caspase 3 activity of MCF-7 cells was assayed with a Colourimetric Assay Kit (Cat No: G7351, Promega Corporation, 2800 Woods Hollow Road, Madison, USA). The cells were seeded in a 96-well plate and treated with selected concentrations (10, 15, 20 µg/mL) of SPL in

triplicates. The activity of caspase 3 (pmol pNA liberated/hour) was calculated according to the protocol.

Caspase 8 assay

Caspase 8 activity of MCF-7 cancer cells was assayed with colourimetric caspase 8 Assay Kit (Merck KgaA, Darmstadt, CAT NO: APT129). The cells were seeded in a 24-well plate with a density of 1×10^6 cells/well and treated with selected concentrations (10, 15, 30 µg/mL) of sonicated peel extract (SPL) in triplicates. The activity of caspase 8 (nmol pNA liberated/Hr) was calculated according to the protocol.

Reverse Transcription Quantitative PCR (RT-qPCR)

Total RNA was isolated by TRIZOL (Life Technologies) Invitrogen manual extraction. Extracted RNA samples were adequately diluted and quantified by Qubit® RNA HS Assay kit. Extracted RNA was loaded to agarose gel (1%) incorporated with Clorox (3%) stained with ethidium bromide (0.5µg/mL) in 1X TAE to judge the integrity and overall quality. Primer pairs for p53, p21, and β actin were synthesized by Integrated DNA Technologies (IDT) Pte, Ltd. β actin was utilized as an internal reference for p53 and p21 expression. The primers of TP53 were as follows: forward, 5'-ATGAGCCGCCTGAGGTTGG-3' and reverse 5'-CAGCCTGGGCATCCTTGAGT-3'. The primers of TP21 were as follows: forward, 5'-TGGCACCTCACCTGCTCTG-3' and reverse, 5'-GTTTGGAGTGGTAGAAATCTGTCAT-3'. The primers of β actin were as follows: forward: 5'-ATCGTGCGTGTGACATTAAGGAG-3' and reverse, 5'-AGGAAGGAAGGCTGGAAGAGTG-3'. RT-qPCR analysis was executed using GoTaq® 1-step RT-qPCR kit (Cat No: A6020, Promega Corporation, 2800 Woods Hollow Road, Madison, USA. β-actin was used as the housekeeping gene which is inherent to the cell cytoskeleton. Culture samples with selected concentrations of $1/2IC_{50}$, IC_{50}, and $2IC_{50}$ were used with this endogenous control. The relative expression of TP21 and TP53 was figured by the $2^{-\Delta\Delta Ct}$ method applying the Ct values obtained [6].

$$\Delta\Delta Ct = [Ct\ (target, treated)-Ct\ (ref, treated)] - [Ct\ (target, untreated)-Ct\ (ref, untreated)] \quad (3)$$

Statistical analysis

Data were presented as mean ± SD. IC_{50} values (concentration required for 50% growth inhibition) were determined from either linear or logarithmic dose-response curves. The viability of cells with effect from different extraction methods was compared and the statistical significance by unpaired t-test (Microsoft Excel, 2010). P values <0.05 were considered significant.

Results

Determination of Antiproliferative activity of PL and PC extracts

Percentage viability at 50% (IC_{50}) of the PL and PC samples were depicted in Figure 1. PL extracts exhibited

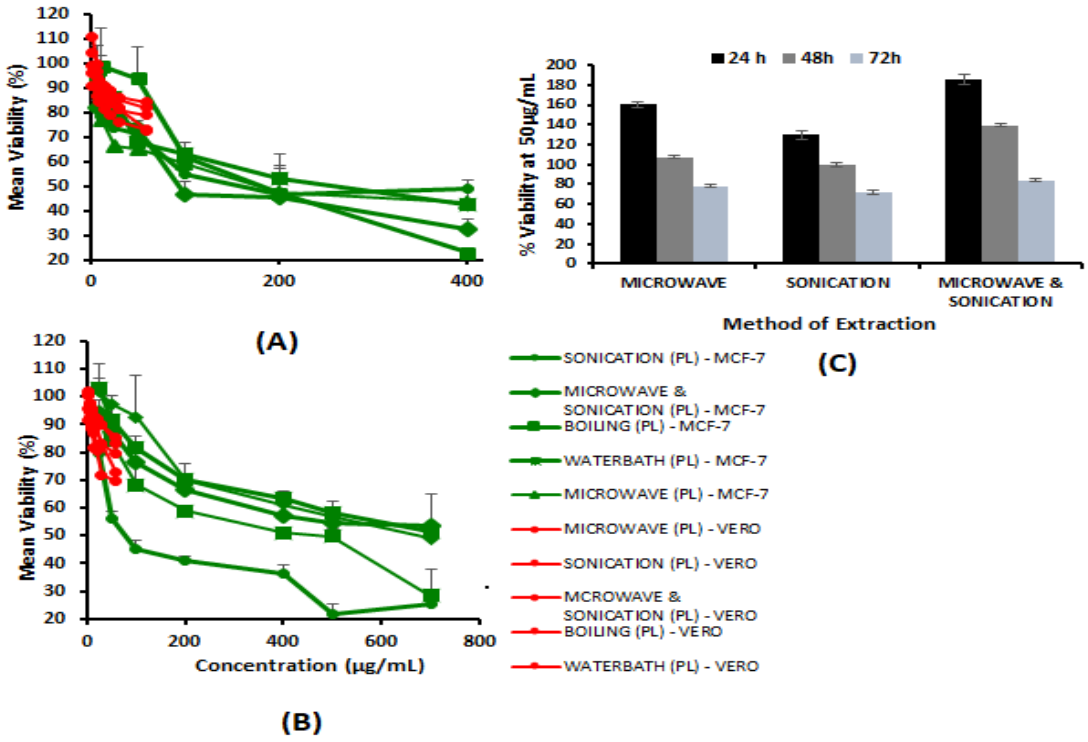

Figure 1. (A) Mean % viability of MCF-7 cancer cells compared to VERO healthy cells with effect of concentration of Punica granatum L. PL extracts with MTT cytotoxicity assay after 24 hrs. (B) Mean % viability of MCF-7 cancer cells compared to VERO healthy cells with effect of concentration of Punica granatum L. PC extracts with MTT cytotoxicity assay after 24 hrs. (C) Concentration of Punica granatum L. PL extracts with at 50% viability (IC50) compared to extraction methods with MTT cytotoxicity assay after 24 hrs, 48 hrs, and 72 hrs incubation with MCF-7 cells. Data represent the mean ± SD (n=3). Microwave (M); Sonication (S); Microwave & Sonication (MS); Boiling (B); Waterbath (W); Peel-(PL); Pericarp-(PC)

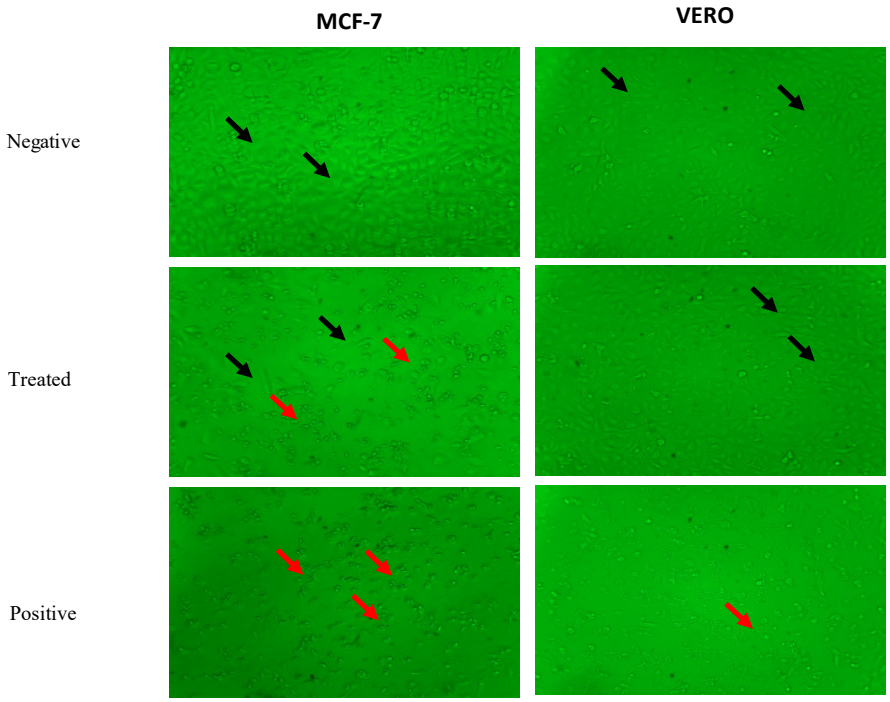

Figure 2. Light Microscopy Images of MCF-7 and VERO Cells Treated with Their Respective IC_{50} Values, with Magnification of 20X. (Black arrow – indicates healthy spindle shape cells; Red arrow – dead and shrinkage cells due to the PL extracts).

higher antiproliferative activity compared to PC extracts. The lowest cell viability was represented with the Sonication method for PL and PC, with (26.0±2.8) and (130.0±4.5) µg/mL on MCF-7 cells respectively after 24h exposure. PL extracts exhibited higher antiproliferative activity compared to PC extracts.

A statistical significance (<0.05) was observed with Microwaving (M), Microwave followed by sonication (MS), Boiling (B), and Heating in a water bath (W) extractions compared to the Sonication (S) method in pomegranate PL extract. Furthermore, MCF-7 cells responded effectively toward decreasing cell viability compared to VERO cells. Distinctly elevated cell viability was denoted with peel extracts on VERO cells (>1000µg/mL IC_{50}) after 24 hr, indicating less toxicity towards healthy cells. Therefore, apoptotic activity was performed with SPL extract. Figure 2 depicted the morphological changes with shrunken appearance with condensed cytoplasm and membrane blebbing of MCF-7, and VERO cells treated with SPL extract.

Ethidium bromide/acridine orange (EB/AO)

Following EB/AO staining, cells treated with SPL caused typical apoptotic morphological changes including chromatin condensation, membrane blebbing, and fragmented nuclei in MCF-7 cells in contrast to the controls. Cycloheximide (50 µg/mL) used as the positive control. Following EB/AO, cells treated with SPL caused typical apoptotic morphological changes including membrane blebbing, chromatin condensation, and fragmented nuclei in MCF-7 cells in contrast to the controls (untreated cells). Cycloheximide (50 µg/mL) treated positive control cells were presented with yellow to red colour reflecting apoptotic features with EB/AO stain (Figure 3).

DNA fragmentation assay

DNA fragmentation with a smear pattern was observed in MCF-7 cells, exposed to SPL for 24 h. Detection of DNA fragments is an integral part of apoptosis. Untreated control cells showed no evidence of DNA fragmentation while the positive control cycloheximide at 50 µg/mL presented DNA fragmentation (Figure 4).

Determination of total protein content, Nitric Oxide production, released LDH concentration, and Caspase 3,

Table 1. The Phenolic Profile of Sonicated *Punica granatum* Peel Powder Extract

No	Active Ingredient	Content %	Unit	Test method
1	Catechin	Not Detected	ppm	In Housed HPLC Method
2	Ellagic Acid	99.8		
3	Punicalin	1.7		
4	Gallic Acid	Not Detected		
5	Punicalagin	24.2		

ppm, parts per million

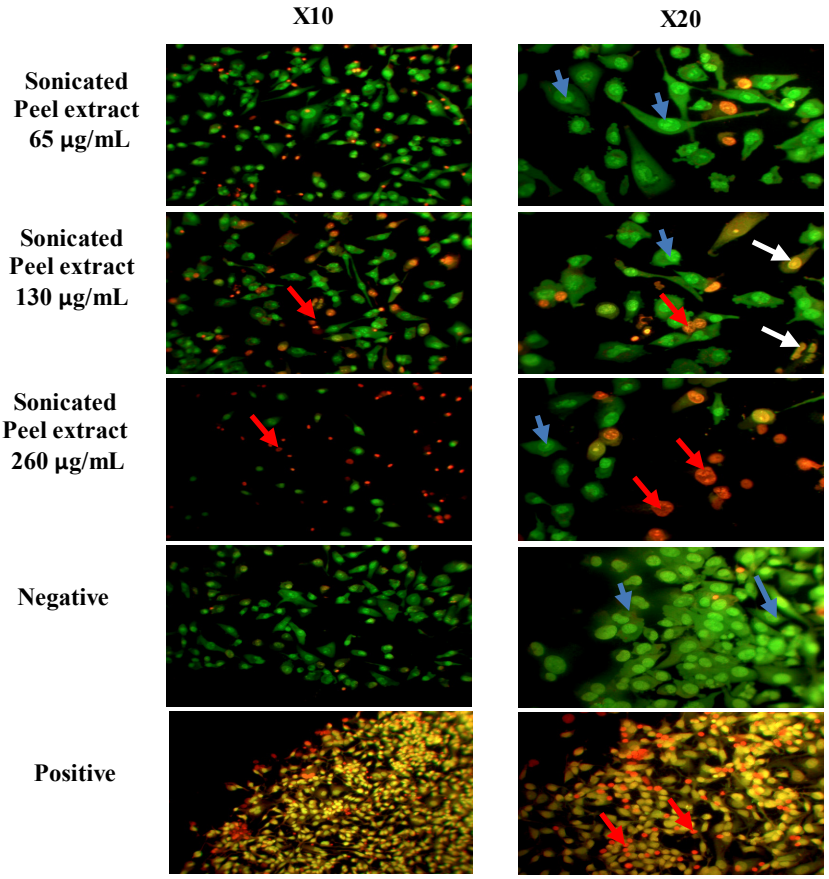

Figure 3. Morphological Changes of Ethidium Bromide / Acridine Orange Stained MCF-7 Cells (representative images from n=6). Blue arrows indicate live cells; White arrows indicate early apoptotic cells with blebbing and Red arrows indicate late apoptotic cells.

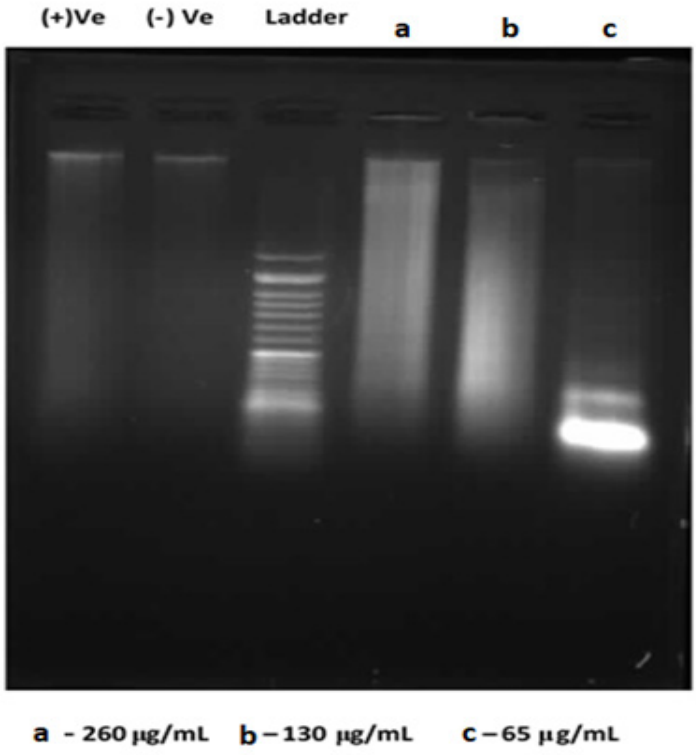

Figure 4. DNA Fragmentation Shown by Agarose Gel Electrophoresis. MCF-7 cells treated with different concentrations of SPL for 24 hours (all concentrations are in µg/mL). The concentration of the Positive control Cycloheximide in all three cell types was 50 µg/mL. (1.2% agarose)

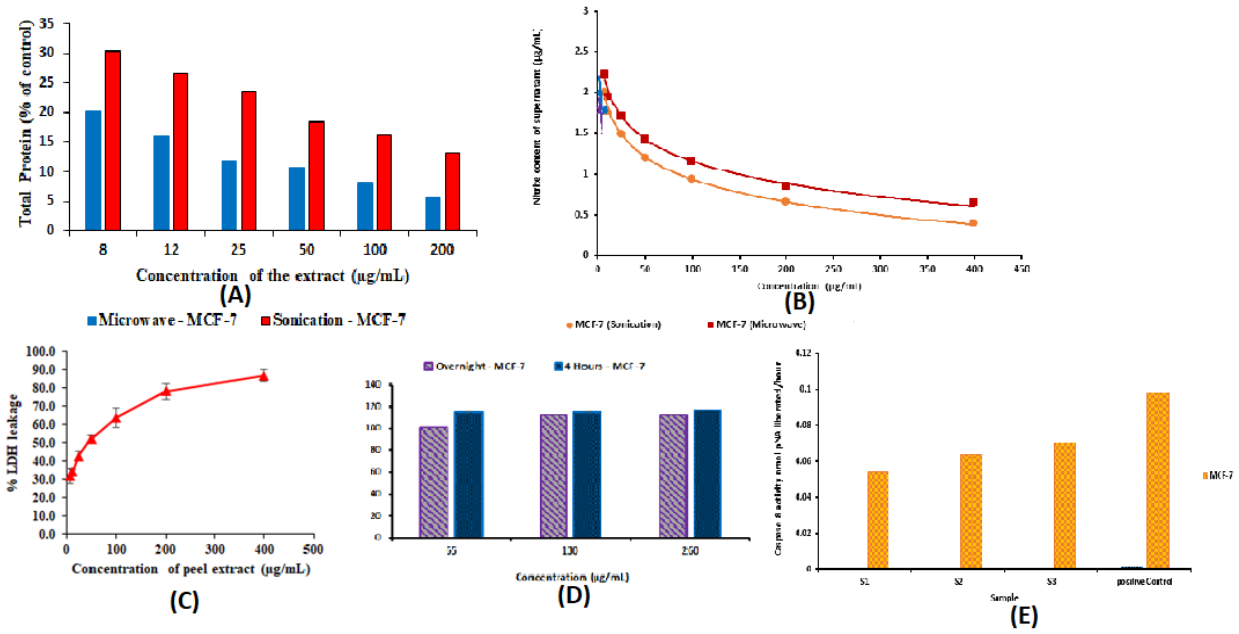

Figure 5. (A) Effect of S and M Extractions of Pomegranate PL at Different Concentrations on Total Protein (B) NO Production in MCF-7 Cell Lysate. (C) Dose dependent LDH concentration after incubation with S and M extractions of pomegranate PL on MCF-7 cells. (D) Caspase-3 activity after treatment with SPL at different concentrations on MCF-7 cells for 4hrs and 24hrs. (E) Caspase-8 activity after treatment with SPL at different concentrations (65, 130, 260µg/mL) on MCF-7 cells for 2hrs. Data represent the mean ± SD (n=3).

8 enzyme activities.

A reduction indicated in total protein content in MCF-7 cells treated with S and M PL extractions in a dose-dependent manner, predicting that the S and M PL extractions induce an inhibitory mechanism of protein synthesis in MCF-7 cells causing cell death. A decrease of nitrite content in the supernatant of S and M, PL extracts is visualized in the graph plotted against concentration.

Leakage of cytoplasmic located enzyme LDH into the extracellular medium is measured in lactate dehydrogenase (LDH) assay. In the present study, a dose-dependent increase in LDH release was observed at increasing concentrations of supernatants and a decrease in lysates on MCF-7 cells.

Caspase 3 enzyme activity has not been induced after 4h and 24h incubation with the concentration of MCF-7 cells indicating that SPL induces Caspase 3 independent apoptotic cell death pathway in MCF-7. Caspase 8 enzyme activity has been induced after 2h incubation with the concentration. Concentration-dependent induced caspase-8 activity was indicated in MCF-7 cells (Figure 5).

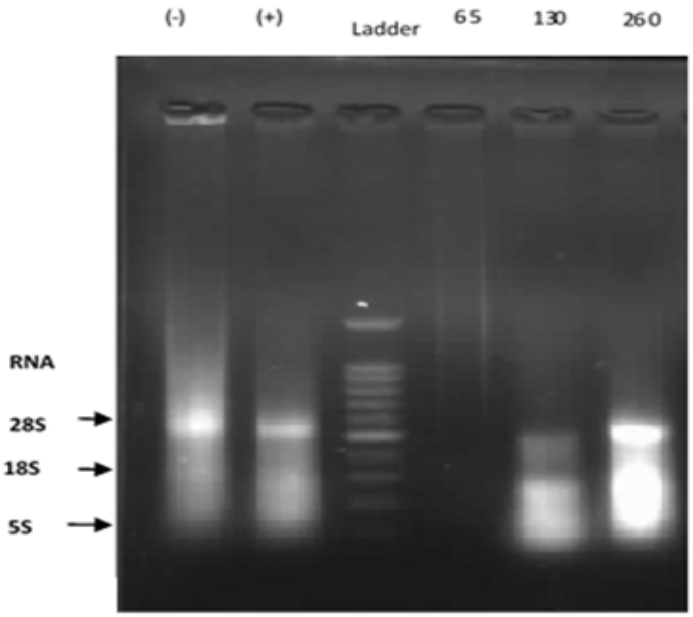

Figure 6. Agarose Gel Photograph of RNA Extraction of MCF-7 Cells Treated with SPL after 24hrs (1.0% agarose gel treated with Clorox). 65µg/mL, 130µg/mL, 260µg/mL SPL concentrations; DNA ladder (100bp)

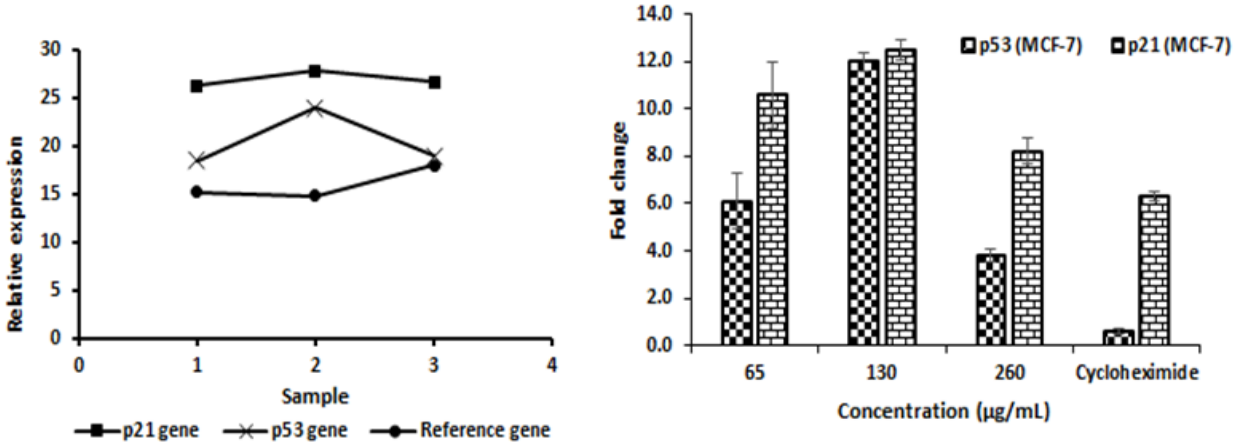

Figure 7. (A) Comparison of expression of p21 and p53 genes reference to β actin, reference gene. (Sample labeled 1=65 µg/mL, 2=130 µg/mL, 3=260 µg/mL). (B) SPL extract effects on p21 and p53 mRNA levels in MCF-7 cells after treatment with 65 µg/mL, 130 µg/mL (IC_{50}), and 260 µg/mL for 24 Hrs. The relative quantification of the target gene was done by the ΔΔCt method using the Qiagen software. Data represent the mean ± SD (n = 3).

Real-Time Quantitative PCR (RT-qPCR)

The extracted RNA was composed of 28S, 18S, and 5S ribosomal components (Figure 6). RT-qPCR was applied to analyze mRNA levels of apoptotic and cell cycle-related genes in MCF-7 cancer cells at concentrations of 65 µg/mL, 130 µg/mL (IC50), and 260 µg/mL exposed to SPL for 24 h. In MCF-7 cells, the expression of the *p21* gene in the SPL-treated cells up-regulated at 65 µg/mL, 130 µg/mL (IC_{50}), and 260 µg/mL, by 10.6, 12.5, and 8.2 fold, while the *p53* gene expression up-regulated by 6.1, 12.0, and 3.8 fold respectively compared to housekeeping gene. Hence, the SPL ensued the apoptosis via *p53* dependent apoptotic pathway in MCF-7 cells (Figure 7). The presence of p53 tumour suppressor protein was further confirmed by visualization of the banding pattern in western blot analysis. The protein bands were separated and tentatively marked them comparative to the BSA standard protein in SDS-PAGE gel. The detected mean protein yield was 1.36 mg/mL as per Lowry's method (Figure 8).

High-Performance Liquid Chromatography (HPLC) evaluation of the phenolic profile of sonicated Punica granatum peel powder extract

Phenolic compounds of sonicated peel extract were identified by comparing their retention times (Rt) with those of pure standards (Table 1). The results were expressed as area % of each identified compound from the total area. Ellagic acid and punicalagin are present as major polyphenolic compounds in the sonicated peel extract (Figure 9).

Discussion

Cancer continues to be a major cause of mortality across the globe. Numerous treatments have been employed, including natural products. Compounds derived from plants offer innovative therapies with fewer adverse effects and, superior effectiveness.

The secondary metabolites in medicinal plants boost their powerful activity in treating various ailments. Their adaptogenic, antioxidant, and anticancer properties are

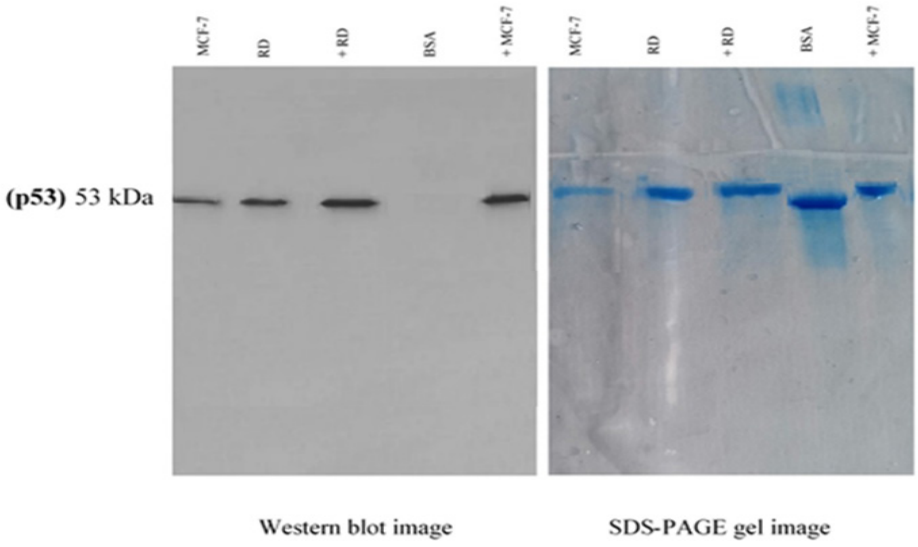

Figure 8. Western Blot Analysis of p53 Protein in Sonicated Peel Aqueous Extracts of *Punica granatum L.* (A) Protein separation by SDS PAGE method (B) MCF-7 treated extracts have run with BSA standard.

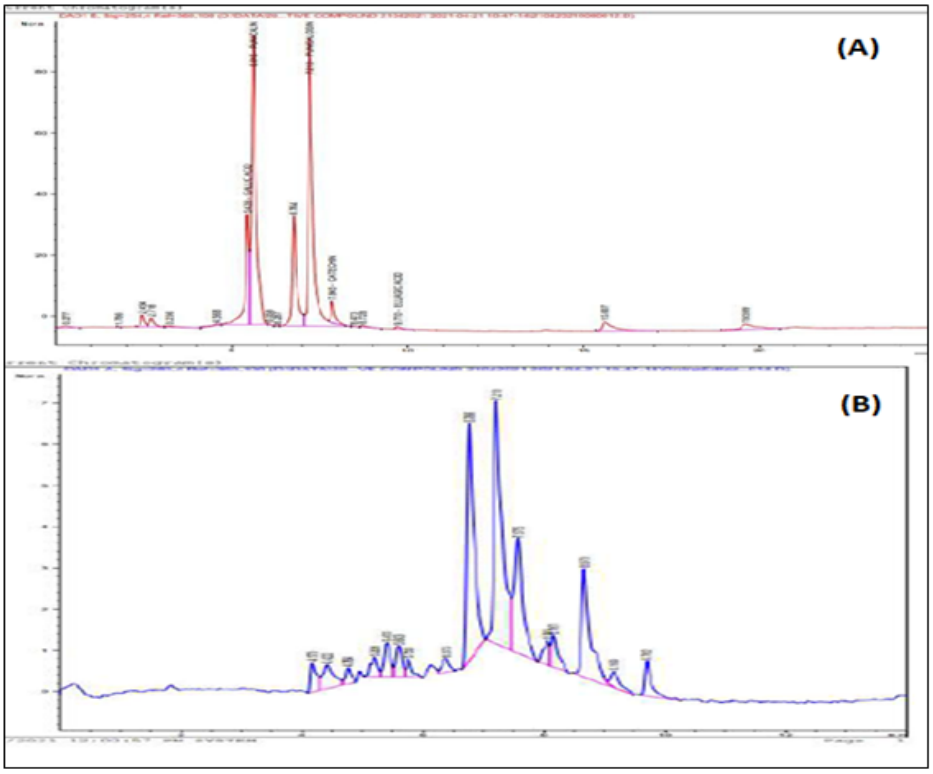

Figure 9. Chromatogram of Sonicated *Punica granatum* Peel Sample (B) compared to standard phenolics (A)

primarily enhanced by phenolic compounds, tannins, and flavonoids. Pomegranate fruit is abundant in strong antioxidants, including tannins and phenolics (ellagic tannins, ellagic acid, and gallic acid). The peel, which is inedible and makes up about 50% of the total fruit weight, contains more polyphenols than the juice and is often discarded as waste. The antiproliferative activity of pomegranate peel against MCF-7 cells was higher than that of the pericarp, regardless of the extraction method used. Additionally, Thelephora ganbajun mushroom, Hibiscus cannabinus oil, and Chinese propolis have shown greater efficacy with ultrasound-assisted extraction compared to other traditional methods (maceration, Soxhlet extraction, supercritical carbon dioxide fluid extraction) for cytotoxicity, yielding higher extraction rates in a shorter time frame [6, 7, 8, 9]. Previously, a positive correlation between antioxidant capacity and total phenolic content of parts of Sri Lankan pomegranate fruit and the highest total phenolic content was determined in the peel. Faster kinetics, high yield in extraction, and preserving thermolabile compounds were recognized benefits of the sonication method [3].

Latest studies revealed that polyphenols are the main phytochemicals involving the antiproliferative activity of plants [10]. Polyphenols, primarily ellagitannins and ellagic acid, which are more prevalent in pomegranate peels, are responsible for the enhanced antiproliferative activity [11]. Pomegranate juice has demonstrated superior antiproliferative, apoptotic, and antioxidant activities compared to isolated polyphenols, indicating synergistic and multifaceted effects from the interaction of multiple compounds. Cellular stress conditions can disrupt signaling pathways involved in protein synthesis [11]. Literature indicates that LDH is a more dependable and precise indicator of cytotoxicity because cells undergo complete fragmentation during extended incubation with substances. We investigated the impact of SPL on the cascade of caspases, which are essential initiators or effectors in cell death pathways. The apoptosis of MCF-7 cells is dependent on caspase 8 and independent of caspase 3. Additional insights were derived from a DNA fragmentation assay, which revealed a distinctive ladder banding pattern or a slight smear. Activated caspases cleave the inhibitor of caspase-activated DNase (ICAD), facilitating the release of active caspase-activated DNase (CAD) [12]. The activated CAD cleaves the nuclear scaffold and cytoskeleton, further oligonucleosomal DNA at the inter-nucleosomal linker sites yielding DNA fragments in multiples of 180 base pairs [13]. One of the key endpoints for understanding the molecular basis of carcinogenesis is the quantitation of gene expression. Due to a very low concentration of RNA, Qubit Fluorometer was performed which is an alternate fluorescence-based detection method.

Apoptosis can be triggered by the p53 protein, a transcription factor that regulates both pro-apoptotic and anti-apoptotic genes. The tumor suppressor gene *p53* is involved in G1 phase arrest and apoptosis. It functions as a transcription factor to stimulate the expression of p21 [14]. The p21 acts as a cell cycle regulator by arresting cells at G1 and G2 phases. The p21 protein, known as cyclin-dependent kinase inhibitor 1A (CDKN1A), tightly binds to cyclin and CDK complexes, inhibiting their activity. The *p21* gene is located on chromosome 6p21.2, containing 3 exons and 2 introns, and encodes the p21 protein. Therefore, the induction of p21 halts the

cell cycle in the G1 phase and mediates the p53 function of preventing the division of DNA-damaged cells. As the most frequently mutated protein in carcinogenesis, p53 induces the expression of *p21* in response to cellular stress, such as DNA damage or oxidative stress [15]. Mutation or deletion of p53 is strongly linked with tumor progression and metastasis. The p21 is important in apoptosis through p53-dependent and p53-independent pathways. Growth and the viability of prostate cancer were suppressed by pomegranate extract through the cki-cyclin-cdk network by upregulating p21 and p27 at G1 phase cell cycle arrest independent of p53 [16].

The pomegranate fruit extract has expressed a significant inhibition of lung cancer in a dose-dependent manner resulting in an induction of WAF1/p21 and KIP1/p27 and reduction of protein expression of cyclins D1, D2, E, cyclin-dependent kinase (cdk) 2, cdk4, and cdk6 presented with a cell cycle arrest in G0 /G1 phase [17]. Pomegranate peel extract is rich in phenolic acids; urosolic acid, α-tocopherol, ellagic acid, quercetin, ellagitannins, luteolin, apigenin, gallic acid, p-hydroxybenzoic acid, chlorogenic acid, caffeic acid which leads to antitumor activity [16]. The cascade pathway is achieved through a decline inactivation of NF-B, a decrease in fatty acid synthase activity and tumor necrosis factor, increased caspase activities, and upregulation of p21 and p53 expression. Scavenging of free radicals, induction of enzymes involved in the metabolism of xenobiotics, regulation of gene expression, and modulation of cellular signaling pathways including DNA damage repair, cell proliferation, and apoptosis were some reported anticancer properties of phenolic acids [18].

Recent research indicates that pomegranate peel is rich in polyphenols such as Delphinidin, Chlorogenic acid, Rutin, Caffeic acid, and Ellagic acid. Notably, Rutin exhibits significant antioxidant, anti-inflammatory, antiangiogenic, proapoptotic, and antiproliferative effects, which can suppress the jab1 oncogene. This gene contributes to cancer progression by deactivating the *p53* and *p27* tumor suppressors, resulting in cell cycle arrest. Furthermore, these polyphenols have been shown to inhibit the Akt/mTOR pathway by regulating its activation proteins. In cervical cancer, the PI3k/Akt/mTOR pathway is frequently dysregulated. While its precise function and regulatory mechanisms are not fully elucidated, it is viewed as a potential biomarker for early detection and a possible therapeutic target for cervical carcinoma [19].

In human ovarian cancer cells (A2780), pomegranate fruit juice and its polyphenols, including ellagic acid and luteolin, were found to inhibit cell proliferation and migration by downregulating matrix metalloproteinases (MMP-2 and 9) in a concentration-dependent manner. Additionally, recent research indicates that urolithin A, a metabolite of ellagitannins, can reduce the viability of prostate cancer cells and promote apoptosis by increasing the expression of p53 and p21. Furthermore, ellagic acid derived from pomegranate can inhibit the proliferation of breast cancer cells (MCF-7) by upregulating cyclin-dependent kinase inhibitors (p21, Cip1, p15, and p19). It suppresses the proliferation of ovarian cancer cells (ES-2 and PA-1) by elevating p53 and p21 levels, leading to cell cycle arrest in the G1 phase [20].

As per current findings, SPL treatment increased the expression of *p21* gene by 10.4-fold and p53 by 7.3-fold in MCF-7 cells, compared to negative controls and the housekeeping gene. These results indicate that the apoptotic effects of SPL cause cell cycle arrest and apoptosis in caspase 3-deficient MCF-7 cells, likely through the upregulation of *p21* and *p53*, along with the activation of caspase 8. Thus, we suggest the polyphenolic substances present in SPL individually or synergistically affect the apoptotic mechanism in MCF-7 cells with the least effect on VERO healthy cells. This knowledge will be helpful for the development of novel chemotherapeutic and chemopreventive agents as a treatment for breast cancer cells in the future.

Author Contribution Statement

All authors contributed equally in this study.

Acknowledgements

Statement of Conflict of Interest

The authors declare no conflict of interest.

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