Effect of alcoholic and aqueous extracts of *Punica granatum* on normal and cancerous cell lines

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Abstract
Pomegranate, or Punica granatum, belongs to the monogeneric family Punicaceae and is primarily found in Iran, which is thought to be its primary place of origin. The pharmacological and toxicological effects of Pg and its chemical constituents include antioxidant, anti-inflammatory (through blocking pro-inflammatory cytokines), anti-cancer, and anti-angiogenesis activity. One of the top five global causes of adult mortality is cancer, and its toll is unreasonably increasing. The Global Cancer Observatory's data show. In this study two Punica grantum extracts-alcoholic and aqueous-were used in this study. The two extracts' capacity to prevent the growth of cancer cells and determine the optimal dose for each cell line. As the concentration of the two extracts increases, the results demonstrate a linear decrease in optical density and an increase in cellular growth inhibition. This study's findings highlight the potential anti-cancer properties of Punica grantum extracts, particularly the Punica grantum extract. When compared to currently available cytotoxic medicines, these extracts demonstrated a significant cytotoxic effect against breast cells while demonstrating non-cytotoxic effects on normal human cells. The findings of this study call for more research, notably on the extracts' cytotoxic mechanisms, which can also be used to create new cancer treatments.

Keywords: alcoholic extracts, aqueous extracts, punica granatum, HdFn and MCF-7 cell line

Introduction

Cancer is one of the top five global causes of adult mortality, and its burden is disproportionately rising. The global cancer observatory (GLOBOCANGCO) estimates that 10.3 million cancer-related deaths and 19.3 million new cases will occur worldwide in 2020. Breast cancer will account for the majority of these cases, with an incidence rate of 11.7%, followed by lung, colorectal, prostate, and stomach cancers (5.6%).

Lungs (18%), colorectal cancer (4%). The deadliest malignancies when first discovered were those of the liver (3%), and stomach (6.9%). (Gopal *et al*. 2021 and Sung *et al*., 2021) [5, 18].

Cancer is the human disease that imposes the highest clinical, social, and financial burden in terms of cause-specific disability-adjusted life years (DALYs).

While acknowledging that addressing preventable risk factors, such as unhealthy eating and smoking, and proper diagnosis/control of non-preventable ones, such as genetic factors and the primordial inflammatory response, is the most effective strategy to lower the risk of developing cancer, the use of natural bioactive chemicals, such as antioxidants, and the repositioning of over-the-counter (OTC) medications not typically used as chemotherapeutic aids, such as metformin, are effective. The remarkable value of fruit-rich diets in lowering the risk of developing certain types of cancer has been demonstrated by a number of cross-sectional and prospective epidemiological studies (Farvid *et al*., 2021; Wigner *et al*., 2022) [4, 21].

Small tree that grows between five and eight meters tall; primarily found in Iran, China, the United States, and the Mediterranean region. Pg is one of the most significant endemic plants in Iran, flourishing in the majority of its arid and semiarid regions due to its capacity to adapt to challenging environmental factors. Punica granatum (Pg) has over 764 varieties, all of which have unique fruit traits, including size, color, flavor, ripening period, and disease resistance. These cultivars have all been grown in the Iranian cities of Saveh and Yazd. The Pg can also be divided into a number of anatomical compartments, such as seed, juice, peel, leaf, flower, bark, and root, all of which have intriguing pharmacological and biological properties.

The aril, which ranges in color from white to deep red or purple and contains water, is the part of the fruit that can be eaten. The edible fruit is a berry that is 5-12 cm in diameter, has a rounded hexagonal form, thick reddish skin, and 600 or more seeds. The seeds are encased in a stringy, white, spongy pulp. Pomegranates are said to grow in the gardens of heaven, and the holy Quran mentions the Pg twice as an illustration of god's excellent creations.

The fruit of the Pg has long been used as a traditional treatment for respiratory disorders, acidosis, dysentery, microbiological infections, diarrhea, and helminth infections. Estrone and estradiol, two estrogenic substances, have also been found in pg seeds. Additionally, the dried pericarp and the fruit's juice are thought to be helpful for treating oral diseases, colic, colitis, menorrhagia, oxyuriasis, headaches, diuretics, acne, piles, and

allergic dermatitis. New scientific studies for the conventional applications of PG have emerged in recent studies.

The pomegranate, also known as Punica granatum L., is referred to as a "Super Fruit" because of its superior nutritional (macro/micronutrients) and functional (bioactive xenobiotics) health-promoting profile (Melgarejo *et al*., 2021) [11].

Although there are many phytochemicals in PMG, polyphenols, which are found in the fruit (aryl; mesocarp), peel (exocarp), seeds, flowers, bark, blossoms, and leaves, have drawn the most attention. In-depth studies have been conducted on the chemical composition, molecular features, and physiological mechanisms underlying PMG polyphenols' oncosuppressive activity for breast, lung, thyroid, colon, and prostate cancer (such as cell cycle arrest at S-G2-M, pro-apoptosis, free-radical inhibition/trafficking, direct DNA damage, antiangiogenesis, and antimigration/invasion).

However, PMG fruits have a variety of genetic varieties. This attribute affects a variety of phenotypical traits, including fruit size, color, and flavor, as well as the phytochemical content and physicochemical properties, all of which have an impact on how bioactive the fruit is. For instance, despite the lack of (disease-specific) cheminformatic (in silico), preclinical (in vitro/in vivo), and clinical (human trials) evidence, strong-to-light colored PMG varietals appear to follow a high-to-low bioactivity trend. However, this pattern may differ between various cancer types and other human diseases.

This study aimed to compare the chemical and phytochemical composition of three PMG fruits with three different flesh (aryl) colors: red (cv. Wonderful), pink (cv. Molar de Elche), and white. The first thorough [in vitro (multiple cell lines, high-throughput chemical characterization) and in silico (cheminformatics)] screening for the genotype-specific anticancer potential of PMG fruits is presented here. This application to the informatics techniques used in food chemical research (foodinformatics) is unique from a computational standpoint.

Materials and Methods

Collection of plant

Plant extracts

Two Punica grantum extracts-one alcoholic and the other aqueous-were employed in this study. The two extracts' capacity to prevent the growth of cancer cells and determine the optimal dose for each cell line assortment of plants The leaves were cleaned and dried for over 4 weeks in a shaded setting with an average temperature of 32°C and a 64% humidity level. The dried ingredients were ground into powder using a blender, stored in airtight plastic containers, and labeled appropriately.

Preparation of alcoholic extracts

The alcoholic extract of Punica grantum and Trigonella foenum was prepared by washing them with distilled water, then with 1% sodium hypochloride solution and washed again with sterile distilled water. 20 g of Punica grantum were weighed and placed in a food perocessor, then 100 ml of 95% ethanol was placed on it and mixed for 2-3 minutes and left in the flask on a shaker for 24 hours to dissolve in ethanol and then filtered using several layers of gauze to get rid of the suspended plant parts and the remaining fibers, then filtered again using a Millipore microfiltration unit with a diameter of 0.45 mm to prevent the passage of germs from the filter. Then the mixture was placed in an electric oven at 40°C until all the alcohol had evaporated Punica grantum and Trigonella foenum's alcoholic extract was made by washing the plants in distilled water, 1% sodium hypochlorite solution, then sterile distilled water once more. Punica grantum was weighed and placed in a food processor. 100 ml of 95% ethanol was added, and the mixture was processed for 2-3 minutes. The flask was shaken for 24 hours to allow the ethanol to dissolve, and it was then filtered. The residual fibers and suspended plant parts were removed using multiple layers of gauze, and the remaining material was then filtered once more using a Millipore microfiltration device with a 0.45 mm diameter to stop bacteria from passing through the filter.

Punica grantum and Trigonella foenum were cleaned with distilled water, 1% sodium hypochlorite solution, and then sterile distilled water again to create the alcoholic extract. Punica grantum was weighed and put in a food processor. 100 ml of 95% ethanol was added, and it was combined for a few minutes before being put in a flask and shaken for 24 hours to dissolve in the ethanol. After that, it was filtered. To remove the remaining fibers and suspended plant components, several layers of gauze were used. The remaining fibers were then filtered once more using a Millipore microfiltration unit with a diameter of 0.45 mm.

Sterlization of alkohol and aqueous extracts and prepration of stock and dilution

After adding 2 g of the powdered alcoholic and aqueous plant extracts to 5 ml of ethanol and distilled water, respectively, to create a stock solution containing 400 mg/ml, the combination was pasteurized before the plant extract dilutions were created from the stock solution.

Cell culture

For this investigation, two cell lines-normal human newborn dermal fibroblast (HDFn) cells and breast carcinoma (MCF-7)-were employed. The cells were kept alive in tissue culture flasks made by Falcon in the USA using Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, USA), 10% fetal bovine serum (FBS), and 1% antibiotic antimycotic (Invitrogen, USA). The cells were incubated at 37°C, 5% CO2, and 95% relative humidity.

Cell densities were calculated to a final level of 1 104 viable cells per ml using cell counts obtained using the trypan blue exclusion method. The 96-well microplates (Falcon, USA) used for the experiments had a flat bottom and were seeded with 1 103 cells per well. The medicine or plant extracts were added after the cells had been cultured for 24 hours. The negative controls were untreated cells and the negative vehicle control was 0.2% DMSO in full DMEM. The cells were treated, and then cultured for 48 hours before being analyzed with the AlamarBlue® assay (Rachelle *et al*., 2017) [12].

Statistical analysis

The information was presented using mean optical density and standard deviation (SD). ANOVA was performed to see whether there were any notable differences between the controls and plant extracts. The resulting dose-response curves were used to calculate the IC50 for extracts and controls.

Results

The following collection of tables and figures illustrates how increasing Punica granatum concentrations and controls affect the cytotoxicity of breast cancer cells. The calculated percentages of cell viability inhibition for methanol and aqueous are displayed in (Table 1 and Table 2) With increasing concentrations of the two extracts, a linear decrease in optical density and an increase in cellular growth inhibition are seen. At concentrations of 30, 60, 125, 250, and 500 g/ml for the two extracts, there was a significant difference (p value 0.001) from the negative control, but there was no significant difference from the positive controls at any of the concentration levels, according to an ANOVA analysis.

Table 1: The cytotoxic effect of *Punica granatum* in methanol extract on HdFn and MCF-7 cell line at 72H

Concentration µg mL-1	Mean viability (%) ± SD	
	HdFn	MCF-7
500	66.39±1.5	29.3±2.31
250	74.76±1.31	38.233±0.94
125	86.42±1.91	51.08±3.08
60	92.5±2.14	64.930±1.36
30	95.216±0.57	73.34±1.27

Table 2: The cytotoxic effect of *Punica granatum* in aqueous extract on HdFn and MCF-7 cell line at 72H

Concentration µg mL-1	Mean viability (%) ± SD	
	HdFn	MCF-7
500	55.8±5.08	30.054±1.3
250	64.12±2.42	39.27±2.2
125	75.8±1.63	51.15±2.53
60	86.9±1.4	65.12±0.9
30	93.8±0.7	73.264±2.04

Figures 1 and 2 of the log-linear regression dose-response curves for methanol and aqueous, along with the slope, measure of linearity (r2), and their respective confidence intervals, were used to calculate the IC50. The Punica granatum extract was the more effective for the two extracts in drastically reducing cell viability in a dose-dependent manner. The IC50 values for the methanol extracts for HdFn and MCF-7 were 155 and 99 g/ml, respectively. These values are lower than the calculated IC50 for mytomycine but higher than the IC50 for colchicine and 5-fluorouracil.

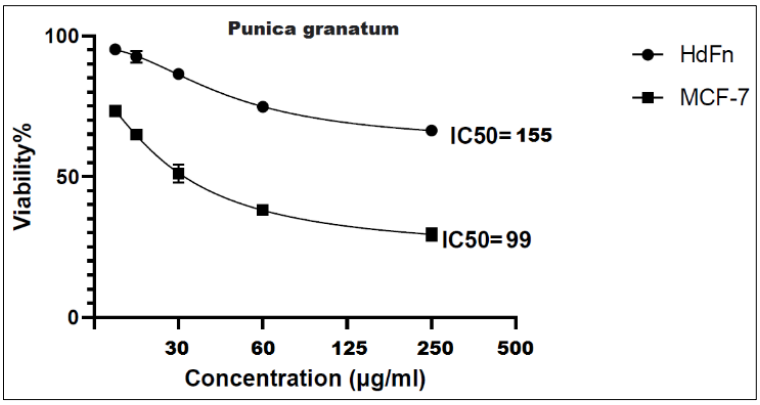

Fig 1: Determine of mtt on HdFn and MCF-7 cell line in methanol *Punica granatum* extract

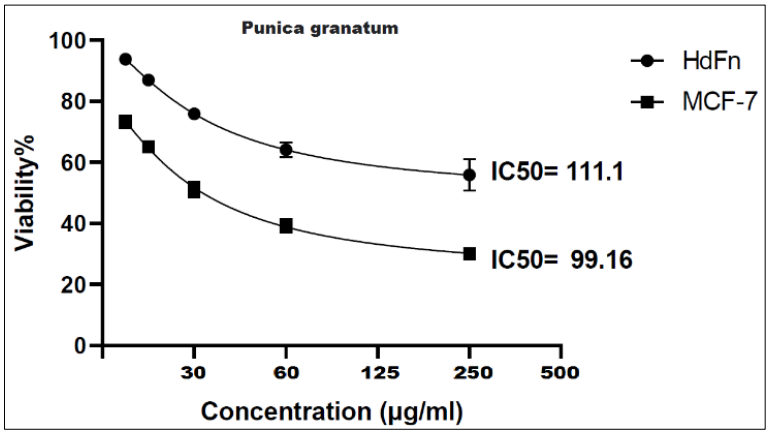

Fig 2: Determine of mtt on HdFn and MCF-7 cell line in aqueous *Punica granatum* extract

Figures 1 and 2 of the log-linear regression dose-response curves for methanol and aqueous were used to calculate the IC50 along with the slope, measure of linearity (r2), and respective confidence intervals. The Punica granatum extract was the more effective of the two extracts at reducing cell viability, and this effect was dose-dependent. The IC50 for the methanol extracts for HdFn and MCF-7, respectively, was 155 and 99 g/ml. These values are significantly lower than the IC50 of mytomycine but higher than the computed IC50 for colchicine and 5-fluorouracil.

By treating HDFn with the identical quantities of Punica granatum extracts and controls used for MCF-7 cell line, the effect on a normal human cell line was evaluated. The cytotoxic effects of the extracts and controls on the normal cells are displayed in Tables 1 and 2. Log-linear regression was used to generate the parameters for the dose-response curve in order to calculate the IC50. The effects of the drug controls on normal cells were significantly more cytotoxic than those of the plant extracts, according to the results. Based on the data, it appears that Punica granatum extracts are not cytotoxic to HDFn normal cells (IC50 > 100 g/ml).

Discussion

Chemotherapy and/or radiation are almost always used in the treatment of cancer, and these cytotoxic procedures can cause serious immune deficiencies, cardiomyopathies, and the emergence of malignancies that are related to the cancer treatment itself. For the scientific community, finding safer therapeutic choices remains a constant problem. Recent studies on plant-based products have uncovered several anti-cancer potentials from various extracts.

The outcomes of this investigation supported the findings of earlier studies by De Moura *et al*. (2012) and Ishikawa *et al*. (2010), showing that the two Punica granatum extracts have a significant potential to be studied further as cytotoxic agents against breast cancer. Further research is necessary due to Punica granatum extract's potent anti-colorectal cytotoxic effect. For HdFn and MCF-7, the IC50 for methanol was 155 and 99 g/ml, respectively. and aqueous extracts against HdFn and MCF-7, respectively, were 111.1 and 99.16 g/ml. Considering the pharmacological restrictions, these IC50 levels against MCF-7 are acceptable. This cancer cell line has low cytotoxic activity, as evidenced by the IC50 values obtained from aqueous and methanol extracts, which are much higher than the pharmacological controls. Additionally, it was found that normal HDFn cells weren't significantly harmed by either of the Punica granatum extracts.

Additional research should be done on the defense mechanisms and effectiveness of Punica grantum extracts against these cancer cells. When compared to drug controls, the Punica grantum extract produced IC50 levels that were noticeably lower for MCF-7 and HdFn.

Pg has inhibitory effects on a variety of malignancies, including lung, breast, colon, and prostate cancers (Rettig *et al*., 2008; Sturgeon *et al*., 2010) [14, 17]. (Kasimsetty *et al*., 2010) [7]. In these investigations, many pathways for pomegranates' anti-cancer properties have been described. In the LAPC4 xenograft model, in vitro, Pg reduces NF-kBand cell survival of prostate cancer cell lines in a dose-dependent manner (Hong *et al*., 2008) [6]. Pg polyphenols, ellagitannin-rich extract, and whole juice extract reduced the gene expression of SRD5A1 (steroid 5alpha reductase type 1), AKR1C3 (aldo-ketoreductase family 1 member C3), and HSD3B2 (3beta-hydroxysteroid dehydrogenase type 2), which are essential androgen-synthesizing enzymes in LNCaP, LNC (Seeram *et al*., 2007) [15].

Pg may have anti-carcinogenesis effects because it inhibits CYP activity/expression, which is required for procarcinogen activation. Some pomegranate metabolites, such as 3,8-dihydroxy-6H-dibenzo[b, d]pyran-6-one (urolithin A, UA), which is made from ellagic anthracens (ETs), may also have anti-cancer properties.

Lung cancer was found to be significantly inhibited after treatment with (50-150 g/mL) pomegranate fruit extract (PFE) for 72 hours. This treatment resulted in dose-dependent arrest of cells in the G0/G1 phase of the cell cycle, induction of WAF1/p21 and KIP1/p27, decrease in the protein expression of cyclins D1, D2, and E, decrease in the expression of cyclin-dependent kinase (PE also changed the levels of Bax and Bcl-2 in the PC-3 cell line. A recent study demonstrated Pg's capacity to prevent angiogenesis.

By measuring VEGF, IL-4, and migration inhibitory factor (MIF) in the conditioned media of estrogen-sensitive (MCF-7) or estrogen-resistant (MDA-MB-231) human breast cancer cells, as well as immortalized normal human breast epithelial cells, Toiet *et al*. evaluated the anti-angiogenic potential of Pg. Human umbilical vein endothelial cells (HUVEC) showed significant potential for inhibitory effects of angiogenesis by Pg fractions as VEGF was significantly decreased in MCF-10A and MCF-7 while MIF was increased in MDA-MB-231 (Rahimi *et al*., 2012) [13]. In TA 100 cells, methanolic extract of Pg (15 mg/plate) exhibits the highest anti-mutagenic activity.

Conclusion

In conclusion, this study's findings highlight the potential anti-cancer properties of Punica grantum extracts, particularly the Punica grantum extract. When compared to currently available cytotoxic medicines, these extracts demonstrated a significant cytotoxic effect against breast cells while demonstrating non-cytotoxic effects on normal human cells. The findings of this study call for further research, particularly on the extracts' cytotoxic mechanisms, which can also be used to create new cancer treatments.

References

1. Al-Najar FM, Abbas HM, Ahmed NM. Effect of alcoholic extracts of Punica grantum and Trigonella foenum on E. coli isolated from UTI. Journal of Advanced Education and Sciences, 2022; 2(3):23-27.
2. Cortez-Trejo MC, Olivas-Aguirre FJ, Dufoo-Hurtado E, Castañeda-Moreno R, Villegas-Quintero H, Medina-Franco JL, *et al*. Potential Anticancer Activity of Pomegranate (*Punica granatum* L.) Fruits of Different

Color: In Vitro and In Silico Evidence. Biomolecules, 2022; 12(11):1649.
3. Faria A, Monteiro R, Azevedo I, Calhau C. Pomegranate juice effects on cytochrome P450S expression: in-vivo studies. J. Med. Food, 2007; 10:643-639.
4. Farvid MS, Barnett JB, Spence ND. Fruit and vegetable consumption and incident breast cancer: A systematic review and meta-analysis of prospective studies. Br. J. Cancer, 2021; 125:284-298.
5. Gopal S, Sharpless NE. Cancer as a global health priority. JAMA, 2021; 326:809-810.
6. Hong MY, Seeram NP, Heber D. Pomegranate polyphenols down-regulate expression of androgen-synthesizing genes in human prostate cancer cells overexpressing the androgen receptor. J. Nutr. Biochem, 2008; 19:848-855.
7. Kasimsetty SG, Bialonska D, Reddy MK, Ma G, Khan SI, Ferreira D. Colon cancer chemopreventive activities of pomegranate ellagitannins and urolithins. J. Agric. Food Chem, 2010; 58:2180-2187.
8. Khan SA. The role of pomegranate (*Punica granatum* L.) in colon cancer. Pak. J. Pharm. Sci, 2009; 22:346-348.
9. Maphetu N, Unuofin JO, Masuku NP, Olisah C, Lebelo SL. Medicinal uses, pharmacological activities, phytochemistry, and the molecular mechanisms of *Punica granatum* L. (pomegranate) plant extracts: A review Biomed Pharmacother, 2022; 153:113256.
10. Mattiuzzi C, Lippi G. Current cancer epidemiology. J. Epidemiol. Glob. Health, 2019; 9:217-222.
11. Melgarejo-Sánchez P, Nunez-Gomez D, Martínez-Nicolás JJ, Hernández F, Legua P, Melgarejo P. Pomegranate variety and pomegranate plant part, relevance from bioactive point of view: A review. Bioresour. Bioprocess, 2021; 8:2.
12. Rachelle PM, Warren SV, Glenn GO. In vitro cytotoxic potential of Yacon (Smallanthus sonchifolius) against HT-29, MCF-7 and HDFn cell lines. Journal of Medicinal Plants Research, 2017; 11(10):207-217.
13. Rahimi HR, Arastoo M, Ostad SN. A comprehensive review of *Punica granatum* (pomegranate) properties in toxicological, pharmacological, cellular and molecular biology researches. Iranian Journal of Pharmaceutical Research: IJPR, 2012; 11(2):385.
14. Rettig MB, Heber D, An J, Seeram NP, Rao JY, Liu H, *et al*. Pomegranate extract inhibits androgen-independent prostate cancer growth through a nuclear factor-kappaB-dependent mechanism. Mol. Cancer Ther, 2008; 7:2662-71.
15. Seeram NP, Aronson WJ, Zhang Y, Henning SM, Moro A, Lee RP, *et al*. Pomegranate ellagitannin-derived metabolites inhibit prostate cancer growth and localize to the mouse prostate gland. J. Agric. Food Chem, 2007; 55:7732-7.
16. Sharma K, Kesharwani P, Prajapati SK, Jain A, Jain D, Mody N, *et al*. An insight into anticancer bioactives from *Punica granatum* (Pomegranate). Anti-Cancer Agents Med. Chem, 2022; 22:694-702.
17. Sturgeon SR, Ronnenberg AG. Pomegranate and breast cancer: possible mechanisms of prevention. Nutr. Rev, 2010; 68:122-128.
18. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, *et al*. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA A Cancer J. Clin, 2021; 71:209-249.
19. Turanli B, Altay O, Borén J, Turkez H, Nielsen J, Uhlen M, *et al*. Systems biology based drug repositioning for development of cancer therapy. In Seminars in Cancer Biology; Academic Press: Waltham, MA, USA, 2021; 68:47-58.
20. Wall-Medrano A, Olivas-Aguirre FJ. Antioxidant phytochemicals in cancer prevention and therapy-An update. In Functional Foods in Cancer Prevention and Therapy; Academic Press: Waltham, MA, USA, 2020, 195-220.
21. Wigner P, Bijak M, Saluk-Bijak J. Clinical potential of fruit in bladder cancer prevention and treatment. Nutrients, 2022; 14:1132.
22. Wong TL, Strandberg KR, Croley CR, Fraser SE, Venkata KCN, Fimognari C, *et al*. Pomegranate bioactive constituents target multiple oncogenic and oncosuppressive signaling for cancer prevention and intervention. In Seminars in Cancer Biology; Academic Press: Waltham, MA, USA, 2021; 73:265-293.
23. Wongwattanasathien O, Kangsadalampai K, Tongyonk L. Antimutagenicity of some flowers grown in Thailand. Food Chem. Toxicol, 2010; 48:1045-1051.