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Comparison of the Tumor-Suppressing Effects of Aqueous and Hydroalcoholic Extracts of Pomegranate Peel on Gastric Cancer

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Authors:
Nooshin Asadmasjedi[1,2]
Marzieh Anam[2]
Fateme Kiani[3]
Zahra Behvandi[3]
Haniye Shahoon[3]
Marzieh Zeinvand-lorestani[4]

1. Department of Medical Laboratory Sciences, School of Paramedical Sciences, Dezful University of Medical Sciences, Dezful, Iran.
2. Infectious and Tropical Disease Research Center, Dezful University of Medical Sciences, Dezful, Iran.
3. Student Research Committee, Dezful University of Medical Sciences, Dezful, Iran.
4. Department of Chemical Technologies, Iranian Research Organization for Science and Technology (IROST), Tehran, Iran.

**** Corresponding author:***
M. Zeinvand-lorestani

Tel/ Fax: +982156276494
Email: *m.zeinvand@irost.ir*
ORCID: 0000-0002-6515-4372

ABSTRACT

Background: The active compounds of pomegranate peel (PP) have different effects and their solubility varies based on their polarity. There are limited studies that thoroughly examine and compare the effects of hydroalcoholic and aqueous extracts of its components so this study will investigate which extract (aqueous or hydroalcoholic) has a greater effect on gastric cancer cells (AGS cell line).

Methods: Gastric cancer cell line (AGS) used and subjected to different concentrations of aqueous or hydroalcoholic extract of PPE, from 0.25 to 8 mg/ml then viability, and IC50 assays were performed.

Results: In our study, both aqueous and hydroalcoholic extracts of pomegranate peel exhibited anti-tumor properties against AGS cells ($p \leq 0.01$). The IC50 value of the aqueous extract was determined to be 2.784 mg/mL, indicating a higher potency in inhibiting gastric cancer cell proliferation. In comparison, the hydroalcoholic extract exhibited a higher IC50 value of 3.58856 mg/mL, suggesting it is less effective at the same concentration .

Conclusion: Therefore, the aqueous extract demonstrates a stronger cytotoxic effect against gastric cancer cells compared to the hydroalcoholic extract.

Keywords: AGS; Pomegranate; Gastric cancer; MTT.

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1. Introduction

Given the high prevalence of gastric cancer worldwide and in Iran, current treatments, despite their high cost and complications, have limited effectiveness (1-3). There is substantial evidence regarding the preventive properties of various plants – consumed as food, fruits, spices, and vegetables – against cancer (1, 4). For this reason, dietary habits, especially diets containing fruits and vegetables, are considered variables that, by possessing effective plant compounds, reduce the risk of cancer(5, 6). Considering that 60% of the anticancer agents used today are derived from natural substances such as plants, one can rely on herbal medicine and focus on the richness of pomegranate peel in polyphenols, which, in addition to their antioxidant properties, also possess apoptotic and anti-metastatic properties(6, 7).

Pomegranate, scientifically known as Punica granatum, is a large tree or shrub belonging to the

class of dicotyledonous angiosperms and the punicaceae family. The pomegranate tree is native to Iran and was cultivated in ancient times in the Mediterranean region of Asia, Africa, and Europe. Due to the presence of secondary metabolites in the fruit peel, fruit, flower, and bark, pomegranate is of great importance in the pharmacology sector. According to numerous studies, polyphenolic compounds such as punicalagin in pomegranate peel give it antioxidant properties, and this compound slows down or inhibits cancer growth(7, 8).

Pomegranate peel is characterized by significant amounts of phenolic compounds, including flavonoids (anthocyanins, catechins, and other complex flavonoids) and hydrolysable tannins (punicalin, pedunculagin, punicalagin, gallic acid, ellagic acid). These compounds are concentrated in the peel and juice of pomegranate, accounting for 92% of the antioxidant activities associated with the fruit and possessing potential anticancer properties.

Aqueous extract primarily utilizes water as the solvent, which is a highly polar medium. This enables efficient solubilization of water-soluble phytochemicals such as phenolic acids, flavonoids, tannins, saponins, alkaloids (if they are hydrophilic), and hydrophilic vitamins (9). In contrast, hydroalcoholic extracts employ a mixture of water and organic solvents, typically ethanol, at varying ratios like 70% ethanol and 30% water. The combined solvent exhibits intermediate polarity, capable of dissolving a broader spectrum of phytochemicals, including polar, medium-polar, and some non-polar compounds such as lipophilic flavonoids, phenolic compounds, terpenoids, essential oils, alkaloids with varying polarity, and glycosides(10-13).

The choice between aqueous and hydroalcoholic extraction is thus critically dependent on the target compounds' polarity, stability, and the specific application requirements(14).

Given the antioxidant and pro-apoptotic effects of pomegranate peel extract and its application in traditional medicine and pharmacology(15), this study will investigate its inhibitory effects on the AGS cell line belonging to gastric cancer. In addition, in this article, we will determine which extract (aqueous or hydroalcoholic extracts) has a greater effect on gastric cancer cells.

2.MATERIALS AND METHODS

2.1. Cell Culture

The human gastric adenocarcinoma cell line (AGS, ATCC® CRL-1739™)) was obtained from National Cell Bank of Iran, Pasteur Institute of Iran. These cells were maintained in RPMI 1640 medium supplemented with 10% FBS, and 1% pen/strep, in a humidified incubator (37 °C & 5% CO_2).

This study was conducted in accordance with ethical standards and was approved by the relevant ethics committee (Approval Code: IR.DUMS.REC.1401.066).

2.2. Experimental design

The AGS cells were incubated with PP at different concentrations and duration times.

The cells were grouped into 13 categories:

1. Control: treated with plain culture medium
2. PP Aqueous extract (0.25): exposed to the culture medium supplemented with 250 µg/ml PP for 24, 48 hr
3. PP Aqueous extract (0.5): exposed to the culture medium supplemented with 500 µg/ml PP for 24, 48 hr
4. PP Aqueous extract (1): exposed to the culture medium supplemented with 1000 µg/ml PP for 24, 48 hr
5. PP Aqueous extract (2): exposed to the culture medium supplemented with 2000 µg/ml PP for 24, 48 hr
6. PP Aqueous extract (4): exposed to the culture medium supplemented with 4000 µg/ml PP for 24, 48 hr
7. PP Aqueous extract (8): exposed to the culture medium supplemented with 8000 µg/ml PP for 24, 48 hr
8. PP Hydroalcoholic extract (0.25): exposed to the culture medium supplemented with 250 µg/ml PP for 24, 48 hr
9. PP Hydroalcoholic extract (0.5): exposed to the culture medium supplemented with 500 µg/ml PP for 24, 48 hr
10. PP Hydroalcoholic extract (1): exposed to the culture medium supplemented with 1000 µg/ml PP for 24, 48 hr
11. PP Hydroalcoholic extract (2): exposed to the culture medium supplemented with 2000 µg/ml PP for 24, 48 hr
12. PP Hydroalcoholic extract (4): exposed to the culture medium supplemented with 4000

μg/ml PP for 24, 48 hr

13. PP Hydroalcoholic extract (8): exposed to the culture medium supplemented with 8000 μg/ml PP for 24, 48 hr

2.3. Extraction

The pomegranate peel is washed, dried in the shade, and then completely powdered.

Hydroalcoholic Extraction: This method involves grinding the plant into a powder and mixing it with 80% ethanol at a ratio of 1:10. The mixture is kept at room temperature, away from light, for 48 hours. After this time, the extract is filtered and centrifuged using sterile gauze. The clear supernatant liquid is concentrated using a rotary evaporator and dried at 45 °C. The dried extract is stored in a dark container in the refrigerator.

Aqueous Extraction: The aqueous extraction method is similar to hydroalcoholic extraction, but hot distilled water (80 °C) is used instead of ethanol. The extraction time is 2 hours. The remaining steps are the same, including filtration, centrifugation, and drying.

2.4. MTT Assay for Cell Viability Assessment

Cell viability is assessed using the MTT assay. The yellow MTT salt, upon entering the cell culture medium, is converted into a formazan compound with a stable purple color by the activity of dehydrogenase enzymes in living cells. Since the dehydrogenase content of cells of a given type is relatively constant, the amount of formazan produced is proportional to the number of cells. In this method, cells are seeded at a density of 5000 per well in a 96-well plate. After 24 hours, allowing the cells to attach to the bottom of the wells, they are individually exposed to serial dilutions of 8, 4, 2, 1, 0.5 and 0.25 mg/mL of extract. Cell viability is assessed at 24- and 48-hours post-exposure. After the exposure period is complete, the culture medium from each well is removed and replaced with 100 μL of fresh culture medium containing 20 μL of a 5 mg/mL MTT salt solution. The plate is incubated for 3-4 hours at 37°C, after which the contents of the wells are gently removed and 100 μL of DMSO is added to each well. At this stage, the formazan compound is formed. Absorbance is measured in each well using an ELISA reader at a wavelength of 570 nm. All experiments are performed in triplicate. The percentage of cell survival is calculated using the following formula (2).

$$\text{Survival Fraction} = \frac{OD\ of\ the\ sample}{OD\ of\ the\ control} \times 100$$

2.5. IC50 Calculation

To calculate the IC50 (the concentration of an extract that inhibits cell growth by 50% compared to the control), the results related to 24 hours after exposure are used. Cell growth curves are plotted against different dilutions of each extract (dose-response curve), and then the IC50 is calculated through a nonlinear regression equation obtained from these curves (4).

2.6. Statistical analyses

Statistical calculations were performed by GraphPad Prism version 10.4.2. In the current study, all tests were repeated 3 times and the results were reported as the mean of these repetitions ± standard deviation (SD). Data were analyzed, using ANOVA test. $P < 0.05$ was considered statistically significant. Graph pad prism software was used to show cell inhibition and half-maximal inhibitory concentration (IC_{50}).

3. RESULTS

3.1. Cellular changes examination with microscope

Microscopic studies revealed that AGS cells exposed to PP at various concentrations showed very distinct and concentration-dependent differences, such that cellular deformation became progressively clearer with increasing time and at higher concentrations nevertheless, at doses of 8 mg/mL we observed a decrease in the effect of the extract on the cancer cells in both water and hydroalcoholic extract.

Inhibition of cell growth, was characterized by stellate morphology, atrophy and vacuolation, reduced cytoplasm, and nuclear pigmentation. These two changes, namely atrophy and nuclear pigmentation, are characteristics of the final stages of programmed cell death (apoptosis). Morphological changes including levels of vacuolation and a reduction in nuclear condensation may reflect metabolic alterations that can be investigated at the molecular level in cells treated with PP. The results indicated that the intracytoplasmic and nuclear changes induced by PP may be related to the toxic effects of PP on AGS cells.

3.2. MTT assay

Based on the results of this research, the PP,

has cytotoxic effects on the AGS cell line. The assessment of cell viability using the MTT assay on the AGS cell line showed that the viability of these cells decreased in a concentration- and time-dependent manner.

A comparative analysis of the survival percentage graphs for the two samples—hydroalcoholic extract and aqueous extract—revealed noticeable variations. These differences are documented as follows (Fig. 1 and 2): In the hydroalcoholic extract, over a 24-hour period, we observed a gradual decrease in the viability of cancer cells from concentrations of 0.25 mg/mL to 2 mg/mL, indicated by a gentle slope. At a concentration of 4, the lowest cell viability was recorded, followed by an increase in viability at a concentration of 8 mg/mL ($p < 0.01$).

Regarding the aqueous extract, the reduction in cell viability occurred with a steeper slope, and notably, the viability dropped below 50% at a concentration of 1 mg/mL ($p < 0.01$). However, similar to the hydroalcoholic extract, an increase in cell viability was observed at a concentration of 8 mg/mL ($p < 0.01$).

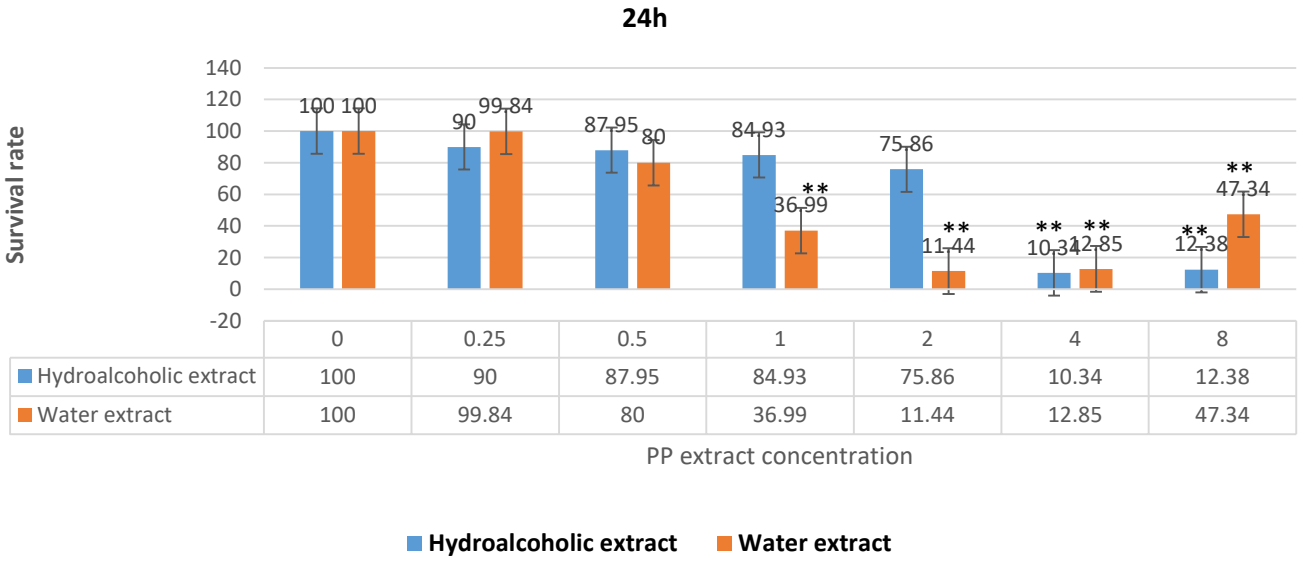

Figure 1: Cell survival rate of AGS cells exposed to different concentrations of aqueous and hydroalcoholic pomegranate peel extract for 24 hours. Cytotoxicity was measured using MTT dye. Values are shown as mean ± SD of three separate experiments (n=3), ($p < 0.01$):.**

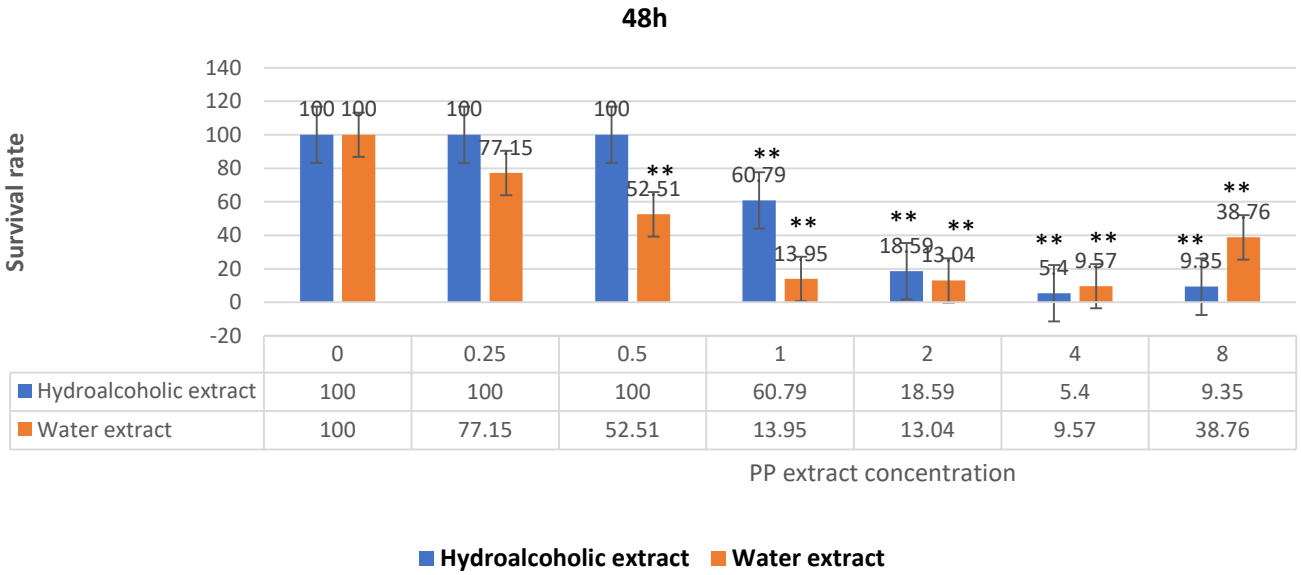

Figure2: Cell survival rate of AGS cells exposed to different concentrations of aqueous and hydroalcoholic pomegranate peel extract for 48 hours. Cytotoxicity was measured using MTT dye. Values are shown as mean ± SD of three separate experiments (n=3), ($p < 0.01$):.**

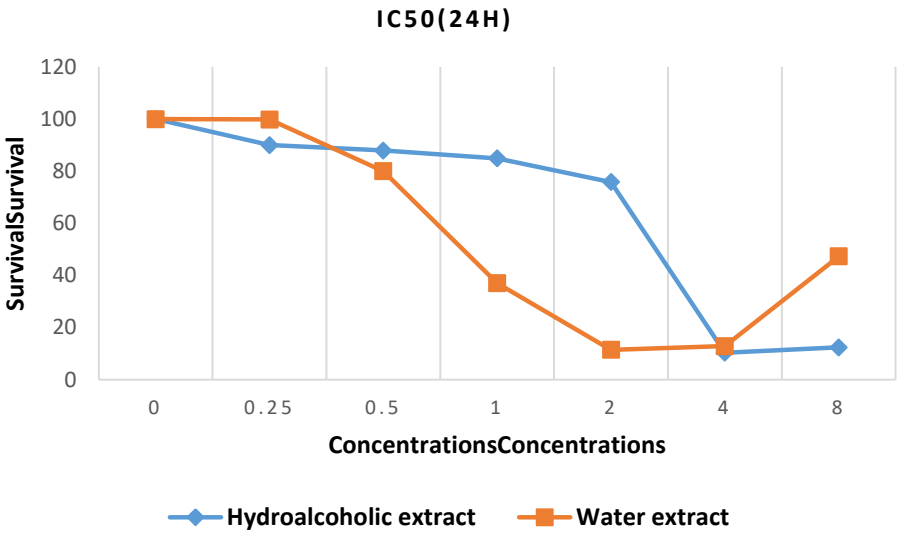

Figure 3. Comparison chart of IC50 values of AGS cells exposed to different concentrations of aqueous and hydroalcoholic extracts of pomegranate peel. The IC50 value of the aqueous extract was determined to be 2.784 mg/mL, indicating a higher potency in inhibiting gastric cancer cell proliferation. In comparison, the hydroalcoholic extract exhibited a higher IC50 value of 3.58856 mg/mL, suggesting it is less effective at the same concentration.

Over the 48-hour period, the changes in cell viability in response to the hydroalcoholic extract were more pronounced, with nearly half of the cells undergoing cell death at a concentration of 1 mg/mL ($p < 0.01$). However, similar to the 24-hour pattern, this reduction was more rapid with the aqueous extract, and an increase in viability was observed at a concentration of 8 mg/mL ($p < 0.01$).

3.3. IC50 Calculation

To calculate the IC50 (the concentration of an extract that inhibits cell growth by 50% compared to the control), the results related to 24 hours after exposure are used. The IC50 value of the aqueous extract was determined to be 2.8 mg/mL, indicating a higher potency in inhibiting gastric cancer cell proliferation. In comparison, the hydroalcoholic extract exhibited a higher IC50 value of 3.6 mg/mL, suggesting it is less effective at the same concentration. Therefore, the aqueous extract demonstrates a stronger cytotoxic effect against gastric cancer cells compared to the hydroalcoholic extract (Fig. 3).

4. DISCUSSION

Gastric cancers account for a significant burden of mortality worldwide. The rate of diagnosis of early gastric cancers is low due to the lack of specific symptoms of early cancer, and therefore, most patients present with advanced stage disease. Some patients even miss the opportunity for surgery, and the overall prognosis for this type of cancer is poor due to the potential for advanced metastatic disease. Accordingly, given the intractable nature of this disease, attention has been focused on the underlying mechanisms of cancer cell survival and migration(1, 16).

Consumption of beneficial polyphenol-rich foods has been widely investigated as a health-promoting measure for longevity and disease treatment. Pomegranate is a well-known highly beneficial food with multifaceted health benefits (17, 18).

Several studies have been performed to evaluate the efficacy of pomegranate products obtained from arils, peel and oils as anti-proliferative, anti-invasive, and pro-apoptotic agents against various cancer cell lines(19-21). Adams et al. revealed that pomegranate juice suppresses cancer activity through the combined antioxidant and antinflammatory effects by modulating the inflammatory cell signaling in colon cancer cells(19). Malik et al. suggested that pomegranate juice may have cancer chemopreventive as well as cancer-chemotherapeutic effects against prostate cancer in humans(20). Pomegranate fruit extracts, also including seed oil, possess proven antitumor-promoting effects in mouse skin(21). Although the active compounds of this plant have different effects and their solubility varies based on their polarity, there are limited studies that thoroughly

examine and compare the effects of hydroalcoholic and aqueous extracts of its components. In our study, both aqueous and hydroalcoholic extracts of pomegranate peel exhibited anti-tumor properties against AGS cells. However, the aqueous extract clearly showed more pronounced effects, indicating the presence of more potent tumor-inhibitory compounds.

Pomegranate peel is a rich source of polyphenols, especially anthocyanins, tannins, and other flavonoids(22, 23). These active compounds may have antioxidant, anti-inflammatory, and anti-cancer properties and play an important role in the medicinal and nutritional value of pomegranate peel. Therefore, consuming pomegranate peel or extracting polyphenols from it may have significant health benefits(21, 23). Many polyphenols are primarily hydrophilic (water-loving). These compounds often contain hydroxyl groups (−OH) that strongly interact with water, making them soluble in aqueous solutions. For example, flavonoids, tannins, and anthocyanins— which are all groups of polyphenols—are typically water-soluble and have significant hydrophilic properties. These characteristics enable polyphenols to be soluble in water-based extraction systems and play important roles in biological environments(10-13, 21, 23).

5. Conclusion

Based on this, we can conclude that the stronger effects of the aqueous pomegranate peel extract may be due to the accumulation of polyphenols in this type of extract. Therefore, the impact of these compounds on gastric cancer is likely more significant compared to less polar compounds.

Ethical considerations

This study was conducted in accordance with ethical standards and was approved by the relevant ethics committee (Approval Code: IR.DUMS.REC.1401.066).

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Authors' contributions

Nooshin Asadmasjedi developed the concept, conducted the experiments and data analysis. Marzieh Anam, Fateme Kiani, Zahra Behvandi, Haniye Shahoon vand, developed the concept, conducted the experiments. Marzieh Zeinvand-lorestani analyzed the data, wrote, and revised the manuscript.

Conflict of interest

The authors declared no conflict of interest.

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