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# Effect of Pomegranate Extract on P53 Expression in Cancer Cell Line (Hep-2)

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## Abstract :

***Background:*** Pomegranate (*Punica granatum*) fruits are widely consumed as juice. The potent antioxidant and anti-atherosclerotic activities of pomegranate are attributed to its polyphenols.
***Aim:*** Pomegranate extract were evaluated for *in vitro* cytotoxic and its effect on the level of p53 in Hep2 (Human larynx epidermal carcinoma).
***Methods:*** Aqueous extraction of pomegranate was prepared and *in vitro* cytotoxicity and amount of p53 was evaluated on cancer cell line Hep2 (Human larynx epidermal carcinoma).
***Results:*** The study showed that the pomegranate extract has greatest antiproliferative activity by inhibition proliferation from 13% to 73.9%, at 10 µg/ml and 24 hr period of exposure. Also, the study showed significant decrease of p53 in treated group in comparison to control.
***Conclusions:*** According to the *in vitro* antiproliferative activity, inhibition of p53 expression pomegramate extracts may be possess a potent anticancer activity.

***Key words:*** *pomegranate extract, cytotoxicity, p53, cancer cell lines.*

## Introduction:

*Punica granatum*, which belongs to the family of Punicaceae, is commonly known as pomegranate"grenade" granats and "punica apple" (1).
*Punica granatum* has been used extensively in a traditional medicine in many countries (2) for the treatment of dysentery, diarrhea, helminthiasis, acidosis, hemorrhage and respiratory disorders (3,4).

In addition, *P. granatum* is reported to have antioxidant (5,6), anti-atherosclerotic (7,8), antibacterial (9,10) and antiviral (11) properties. The constituents of *P. granatum* include gallocatechins, delphinidin, cyanidin, gallic acid, ellagic acid, pelargonidin and sitosterol, which are very well known for their therapeutic properties (12).

*Punica granatum* peel is used to treat infections found in human sexual organs as well as mastitis, acne, folliculitis, pile, allergic dermatitis, tympanitis, scalds (13).

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The potent antioxidant properties of pomegranate juice (PJ) have been attributed to its high content of punicalagin isomers that can reach levels >2 g/L juice (14–16).

Ellagitannins (ETs) have also been identified as active antiatherogenic compounds in PJ (17,18). It has been shown that pomegranate fruit extracts and its purified ETs inhibit the proliferation of human cancer cells and modulate inflammatory subcellular signaling pathways and apoptosis (19-22).

Pomegranate fruit extract also caused significant reduction in prostate tumor growth and prostate-specific antigen (PSA) levels in athymic nude mice implanted with CWR22Rv1 prostate cancer cells (23).

Although apoptosis or programmed cell death is critical for development, tissue homeostasis, and protection against pathogens, it is known that some oncogenic mutations disrupt apoptosis, leading to tumor initiation, progression or metastasis. Furthermore, it has been documented that most cytotoxic anticancer agenst is due to induction of apoptosis, that reduce the sensitivity to therapy.

Thus, apoptosis might be considered as the major mechanism by which most chemopreventive and chemotherapeutic agenst act (24-26).

## Materials and Methods:

### *preparation of the extract:*

An aqueous extraction of pomegranate were prepared by using 100 g dried rind which soaked in 250 ml boiling distilled water for about 6 hours on a hot plate and homogenized. The mixture was then filtered through a piece of soft cloth and filtered through Whatman no. 1 filter paper to remove all the residual materials. Then, it dried at 45˚C by using hot air oven, with circulatory fan, and kept at 4˚C until to be used (27).

For this test, the extract were weighed (0.05 g) and dissolved in phosphate buffer saline (PBS) and dimethylsulphoxide (DMSO) to prepare stock extract solution at constriction of 1000 µg/ml, from which several concentration were prepared (100, 75, 50, 25 and 10) µg/ml and kept at 4˚C until to be used.

### *Preparation of cell line and culture:*

This *in vitro* method was used to evaluate the effect of the water extract of pomegranate on (Hep-2 and Hela tumour cell lines). Solutions were prepared according to Iraqi center of medical and genetic researches standard method. These cells were maintained in RPMI –1640 media with 10% (v/v) bovine serum and incubated at 37˚C in a humidified atmosphere containing 5% CO2.

### *Cytotoxic assay:*

The cytotoxic assay was measured using the crystal violate stain (28). In brief, the extract re dissolved in DMSO then diluted by serum free media (SFM) and (100, 75, 50, 25 and 10) µg/ml concentrations, then tumor cells were seeded in 96- well microplate and incubated for 24 h at 37˚C, then old media was changed with new media SFM containing the serial concentrations of each extract, and then plate incubated for 24 hr of all cell lines and humidified incubator at 37˚C containing 5% CO2.

After finishing the exposure periods, the media was discarded and the wells treated with 200 µl/well of crystal violate dye and incubated the plate for 20 min at 37˚C, the wells were washed with PBS, and the plates left for 15 min at room temperature and then the absorbenc (O.D.) of wells masured by ELISA reader at 492 nm. wave length.

### *P53 detection and quantitative determination:*

The concentration of Pomegranate extract (10 µg/ml) that censed the highest inhibitory effect on Hep-2 cell line after 48 hr period of exposure, was used for p53 kit detection. The detection of p53 protein is carried out by using a P53 ELISA Kit (US Biological Co., p 1001- 25A).

This p53 ELISA Kit is a comxlete kit for the quantitative determination of wild- type and mutant p53 in human, mouse and rat samples. The principle of this kit depends upon the binding of monoclonal antibody (immobilized on a microtiter plate) with p53 in the standards or samples. Purified p53 standards are provided in the kit. A polyclonal antibody to p53 labeled with the enzyme Horseradish peroxidase is added to the standards and samples. This labeled antibody binds to the p53 captured on the plate.

After short incubation, the excess sample and labeled antibody are washed out and substrate is added. After a short incubation, the enzyme reaction is stopped and the color generated is read at 450 nm. by using ELISA reader.

### *Statistical analysis:*

Experiments data were analyzed using statistical Last Significant Design (LSD). The Significance differences between control and samples were determined by using students f-test, at P value $\leq 0.05$.

## Results:

The crude aqueous extract derived from the peel of *Punica granatum* was evaluated as a cytotoxic agent, using the crystal violate assay, and p53 detection and quantitative determination against Hep-2cell line.

The inhibition values for the continuous exposure of Hep2 cells over a 72 hr period to *punica granatum* extract are shown in Figure 1 and in table 1. The results indicated that the extract is potentially cytotoxic to the Hep2 cell line with an inhibition value 73.9% in 10µg/ml after 24 h incubation.

An additional series of experiments were carried out in order to establish if the *punica granatum* extract was cytotoxic in a dose- and time-dependent manner to the Hep2. Results from these studies are presented in Figure 1, and clearly indicated that significant at ($P<0.05$) cytotoxic activity was evident after 48 h and 72 h exposure and this effect increased in a dose-dependent manner up to 10 µg/ml.

Significant cytototoxic activity was also observed after 24 incubation and this was dose and time dependent up to 10 µg/ml, respectively ($P<0.05$). However, the cytotoxic effect of *Punica granatum* in these cells was abated after 24 h of incubation.

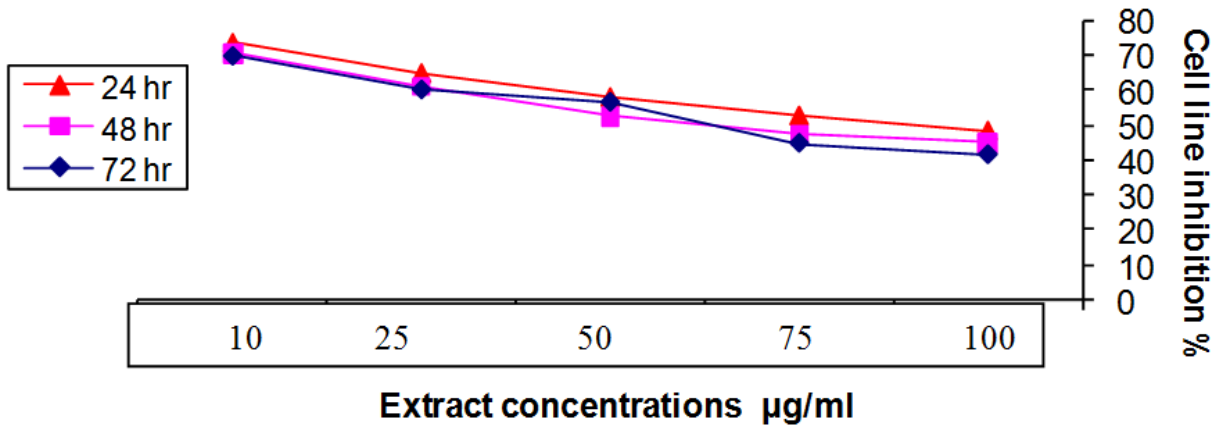


*Figure 1: Effect of pomegranate extract on the growth of Hep-2 cancer cell line during different exposure periods.*

*Table 1: Optical density of growth inhibition of Hep-2 cell line treated with different concentration and periods of exposure.*

| Conc. (µg/ml) of extract | Optical density (O.D) | | |
|---|---|---|---|
| | 24hrs | 48hrs | 72hrs |
| Control | 1.29 | 1.15 | 1.12 |
| 100 | 0.662 | 0.634 | 0.65 |
| 75 | 0.605* | 0.60 | 0.613 |
| 50 | 0.538* | 0.54* | 0.48 |
| 25 | 0.449* | 0.44 | 0.44 |
| 10 | 0.337* | 0.33 | 0.331 |

*(*): siginificant differences at ($p \leq 0.05$) between treated and control groups*

In respect to P53 detection and the result showed that there was a significant reduction of p53 in treated group (58 pg\ml) in comparison with p53 in control group (33.15 pg\ml).

## Discussion:

Over the last decade, remarkable advances in our understanding of cancer biology have led to the realization that apoptosis and the genes that control it have a profound effect on the malignant phenotype (29-31).

In this study we used different ranges of extract concentration until we found the best or ideal range which give high inhibition to the cells used. In present study the rind extract from pomegranate decreased the viable cell number of Hep-2 cell line. In study to investigate the activity of punicalagin, ellagic acid and pomegranate tannin as anti tumor activity they found that these compound from pomegranate have the ability to decrease the viable cell number of human oral, prostate and colon tumor cells; however superior activity was obtained with pure pomegranate juice (32).

Similarly, in the apoptotic studies, pomegranate juice induced apoptosis in HT-29 (human colon cancer cells) when concentrations of punicalagin, ellagic acid and pomegranate tannin equalizes to amounts found in pomegranate juice had no effect. Only when the concentration of these com

pounds was raised to equivalent amount (w/w) with pomegranate juice were they able to induce apoptosis (32).
Other studies reported that pomegranate seed oil act as a safe and effective agent against skin cancer and colon cancer tumors, respectively (33, 34). However, pomegranate seed oil inhibited the proliferation of human breast cancer cells up to 90%, while fermented pomegranate juice polyphenols inhibited 47% of cancerous lesion formation in mammary gland cells from mice (35).

Furthermore, pomegranate peel extract had a potent cytotoxic effect on HL60 cells (8.0 µg/ml), but was non-toxic to normal PBMCs, indicating that as a potential source of antileukemic agents (36).

The p53 tumor suppressor gene encodes a nuclear phosphoprotein with cancer inhibiting properties, and is mutated in more than half of human cancers (37). The p53 protein is induced by many genotoxic stresses which is followed by cell cycle arrest and apoptosis of the injured cells (38). Most p53 mutation in the mutational hot spots of human cancer function in a dominant negative fashion, which interfere with the function of normal protein, culminating in the monoclonal expansion of the cells with p53 mutation and the development of cancer (39).

Differences in the pattern of p53 mutations in human cancers from different tissues are supposed to reflect the effects of specific carcinogens. In the case of liver cancer, G\T transversion is preferentially led by tobacco smoke (40), while in the case of skin carcinoma, C\T transitions at the site of adjacent pyrimidines are detected predominantly, indicating that the mutagen is ultraviolet radiation (41).

Responses to P53 vary in different types of cells and depend on the level of p53 expression. For example, thymocytes undergo p53 dependent apoptosis more readily than do fibroblasts. In some cells, high levels of p53 induce apoptosis; whereas lower levels induce cell cycle arrest (42 and 43). P53 plays an important role in regulating the G2- M transition. Progression from G2 in to mitosis was blocked when p53 was overexpressed in either rat or human fibroblasts. Overexpression of p53 in fibroblasts also causes arrest in G2 and in activation of p53 by human papillomavirus (HPV)- E6 attenuates the G2 delay that normally occurs in response to ionizing radiation (44 and 45).

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# تأثير مستخلص قشور الرمان على التعبير عن جين الـ P53 في خط الخلايا السرطانية Hep-2

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**الخلاصة:**

**السياق:** ثمرة الرمان (punica granatum) شائع استخدامها كشراب. اهميتها تكمن بامتلاكها فعالية مضاد للاكسده و ضد تصلب الشرايين وذلك بسبب احتوائها على فينولات متعددة.

**الهدف:** تقييم سمية مستخلص الرمان (داخل الزجاج) وتأثيره على مستوى الـ p53 الناتج في الخط السرطاني ( خط سرطان الحنجرة ).

**الطرق:** تم عمل مستخلص مائي لقشور الرمان وتم قياس سميته وقياس كمية الـ p53 للخط السرطاني ( خط سرطان الحنجرة ).

**النتائج:** اظهرت الدراسة بان مستخلص الرمان له فعاليته الكبيرة ضد نمو الخلايا السرطانية المستخدمة باحداث تثبيط لنموها من 13%- 73,9%. عند التركيز 10مايكرو غرام/ مل, بعد 24 ساعة الاولى من التعريض اظهرت النتائج انخفاض في مستويات الـ p53 في الخلايا المعاملة بمقدار 73,9 % مقارنة بالخلايا غير المعاملة.

**الاستنتاج:** استنادا الى التأثير المثبط لنمو الخلايا السرطانية الذي أظهره مستخلص الرمان (في الزجاج) وكذلك تثبيط مستوى الـ P53، فأن مستخلص الرمان يمتلك فعاليه قوية مضادة للسرطان.