

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 pomegranate 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 Puniceae, 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 gallo catechins, 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 anti-atherogenic 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 agent 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 agent 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 concentration 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 violet 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 violet 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 absorbance (O.D.) of wells measured by ELISA reader at 492 nm. wave length.

p53 detection and quantitative determination:

The concentration of Pomegranate extract (10 µg/ml) that caused 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 complete 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 t-test, at P value ≤0.05.

Results:

The crude aqueous extract derived from the peel of *Punica granatum* was evaluated as a cytotoxic agent, using the crystal violet assay, and p53 detection and quantitative determination against Hep-2 cell 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 10 µg/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 cytotoxic 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.

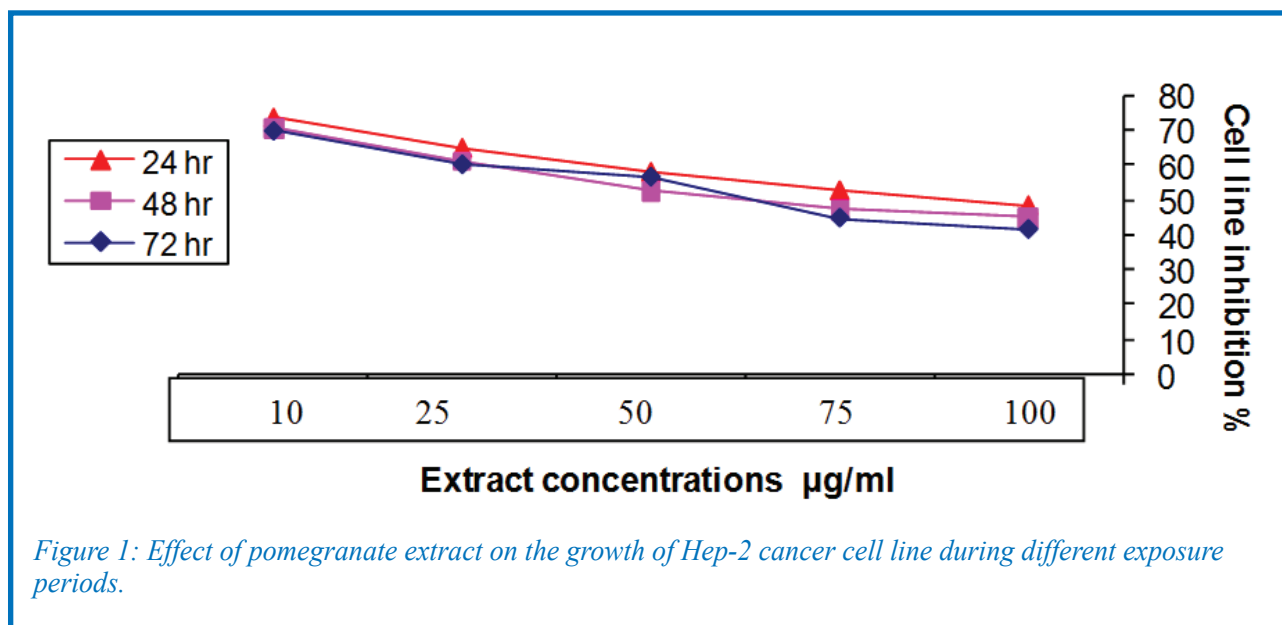


Table 1: Optical density of growth inhibition of Hep-2 cell line treated with different concentration and periods of exposure.

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

(*): significant 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).

References:

- Supayang PV, Treechada S, Surasak L, Thanomjit S, Tetsuya I, Takeshi H. Inhibitory effects of active compounds from *Punica granatum* pericarp on verocytotoxin production by enterohemorrhagic *Escherichia coli* O157:H7. *J Health Sci* 2005; 51: 590–6.
- Chidambara MKN, Jayaprakasha GK and Singh RP. Studies on antioxidant activity of pomegranate (*Punica granatum*) peel extract using *in vivo* models. *J Agric Food Chem* 2002; 50: 4791–5.
- Ricci D, Giamperi L, Bucchini A and Fraternali D. Antioxidant activity of *Punica granatum* fruits. *Fitoterapia* 2006; 77: 310–12.
- Sanchez A, Fonseca G, Fuentes JL, Cozzi R, Cundari E and Fiore M. Assessment of the genotoxic risk of *Punica granatum* L. (Punicaceae) whole fruit extracts. *J Ethnopharmacol* 2008; 115: 416–22.
- Related A, LinksHeber D, Seeram NP, Wyatt H, Henning SM and Zhang Y. Safety and antioxidant activity of a pomegranate ellagitannin-enriched polyphenol dietary supplement in overweight individuals with increased waist size. *J Agric Food Chem* 2007; 55: 10050–4.
- Parmar HS and Kar A. Medicinal values of fruit peels from *Citrus sinensis*, *Punica granatum* and *Musa paradisiaca* with respect to alterations in tissue lipid peroxidation and serum concentration of glucose, insulin and thyroid hormones. *J Medicinal Food* 2008; 11: 376–81.
- Aviram M, Rosenblat M, Gaitini D, Nitecki S, Hoffman A and Dornfeld L. Pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduces common carotid intima-media thickness, blood pressure and LDL oxidation. *Clin Nutr* 2004; 23: 423–33.
- Parmar HS and Kar A. Protective role of *Citrus sinensis*, *Punica granatum* and *Musa paradisiaca* peels against diet induced atherosclerosis. *Nutrition Res* 2007; 27: 710–18.
- Braga LC, Shupp JW, Cummings C, Jett M, Takahashi JA and Carmo LS. Pomegranate extract inhibits *Staphylococcus aureus* growth and subsequent enterotoxin production. *J Ethnopharmacol* 2005; 96: 335–9.
- Naz S, Siddiqi R, Ahmad S, Rasool SA and Sayeed SA. Antibacterial activity directed isolation of compounds from *Punica granatum*. *J Food Sci* 2007; 72: 341–5.
- Zhang J, Zhan B, Yao X, Gao Y and Shong J. Antiviral activity of tannin from the pericarp of *Punica granatum* L. against genital Herpes virus *in vitro*. *Zhongguo Zhong Yao Za Zhi* 1995; 20: 556–8.
- Lansky EP and Newman RA. *Punica granatum* (pomegranate) and its potential for prevention and treatment of inflammation and cancer. *J Ethnopharmacol* 2007; 109: 177–06.
- Singh RP, Chidambara MKN and Jayaprakasha GK. Studies on the antioxidant activity of pomegranate (*Punica granatum*) peel and seed extracts using *in vitro* models. *J Agric Food Chem* 2002; 50: 81–6.
- Clifford MN and Scalbert A. Ellagitannins-nature, occurrence and dietary burden. *J Sci Food Agric* 2000; 80:1118–25.
- Amakura Y, Okada M, Sumiko T and Tonogai Y. High-performance liquid chromatographic determination with photodiode array detection of ellagic acid in fresh and processed fruits. *J Chromatogr A* 2000; 896:87–93.
- Gil MI, Tomas-Barberan FA, Hess-Pierce B, Holcroft DM and Kader AA. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *J Agric Food Chem* 2000; 48:4581–89.
- Cerda B, Ceron JJ, Tomas-Barberan FA, Espin JC. Repeated oral administration of high doses of pomegranate ellagitannin punicalagin to rats for 37 days is not toxic. *J Agric Food Chem*. 2003; 51:3493–501.
- Cerda B, Llorach R, Ceron JJ, Espin JC and Tomas-Barberan FA. Evaluation of the bioavailability and metabolism in the rat of punicalagin, an antioxidant polyphenol from pomegranate juice. *Eur J Nutr*. 2003; 42:18–28.
- Aviram M and Dornfeld L. Pomegranate juice consumption

- inhibits serum angiotensin converting enzyme activity and reduces systolic blood pressure. *Atherosclerosis* 2001; 158:195–8.
20. Aviram M, Dornfield L, Rosenblat M, Volkova N, Kaplan M, Coleman R, Hayek T, Presser D and Fuhrman B. Pomegranate juice consumption reduces oxidative stress, atherogenic modifications to LDL and platelet aggregation: studies in humans and atherosclerotic apolipoprotein E-deficient mice. *Am J Clin Nutr*. 2000; 71:1062–76.
 21. Seeram NP, Adams LS, Henning SM, Niu Yantao, Zhang Y, Nair MG and Heber D. *In vitro* antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. *J Nutr Biochem*. 2005; 16:360–7.
 22. Adams L, Seeram NP, Aggarwal BB, Takada Y, Sand D and Heber D. Pomegranate juice, total pomegranate tannins and punicalagin suppress inflammatory cell signaling in colon cancer cells. *J Agric Food Chem*. 2006; 54:980–85.
 23. Afaq F, Malik A, Syed DN, Maes D, Matsui MS and Mukhtar H. Pomegranate fruit extract modulates UV-B-mediated phosphorylation of mitogen-activated protein kinases and activation of nuclear factor kappa B in normal human epidermal keratinocytes paragraph sign. *Photochem Photobiol*. 2005; 81:38–45.
 24. Johnstone, R.W.; Ruefli, A.A.; Low, S.W. (2002). Apoptosis: a link between cancer genetics and chemotherapy. *Cell* 108: 153–164.
 25. Lowe, S.W. and Lin, A.W. (2000). Apoptosis in cancer. *Carcinogenesis* 21: 485–495.
 26. Gescher, A.J.; Sharma, R.A. and Steward W.P. (2001). Cancer chemoprevention by dietary constituents: a tale of failure and promise. *Lancet Oncol*. 2: 371–379.
 27. Anais, E.Y., Elokda, A.S., AbuGhayun, Y.Y. and Abdulla, F.A. Antidiarrheal activity of the aqueous extract of *Punica granatum* (Pomegranate) peels. *Pharmaceutical Biology* 2007, 45(9):715–720.
 28. Freshney, R.I. Culture of Animal cells. 3rd. ed. Wiley-Liss. USA (1994). Pp267–308.
 29. Johnstone, R.W.; Ruefli, A.A.; Low, S.W. (2002). Apoptosis: a link between cancer genetics and chemotherapy. *Cell* 108: 153–164.
 30. Lowe, S.W. and Lin, A.W. (2000). Apoptosis in cancer. *Carcinogenesis* 21: 485–495.
 31. Gescher, A.J.; Sharma, R.A. and Steward W.P. (2001). Cancer chemoprevention by dietary constituents: a tale of failure and promise. *Lancet Oncol*. 2: 371–379.
 32. Navindra P, Lynn S, Susanne M, Yantao Niu, Yanjun Zhang, Muraleedharan G and David Heber. *In vitro* antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. *Journal of Nutritional Biochemistry* 2005; 16:360–367.
 33. Afaq F, Saleem M, Krueger C, Reed J and Mukhtar H. Anthocyanin- and hydrolyzable tannin-rich pomegranate fruit extract modulates MAPK and NF-kappaB pathways and inhibits skin tumorigenesis in CD-1 mice. *Int J Cancer*. 2005; 113:423–33.
 34. Malik A, Afaq F, Sarfaraz S, Adhami VM, Syed DN and Mukhtar H. Pomegranate fruit juice for chemoprevention and chemotherapy of prostate cancer. *Proc Natl Acad Sci USA*. 2005; 102:14813–8.
 35. Supayang PV, Treechada S, Surasak L, Thanomjit S, Tetsuya I and Takeshi H. Inhibitory effects of active compounds from *Punica granatum* pericarp on verocytotoxin production by enterohemorrhagic *Escherichia coli* O157:H7. *J Health Sci* 2005; 51: 590–6.
 36. Chadarat Ampasavate, Siriporn Okonogi and Songyot Anuchapreeda. Cytotoxicity of extracts from fruit plants against leukemic cell lines. *African Journal of Pharmacy and Pharmacology* 2010; 4(1): 013–021.
 37. Greenblatt, M. S.; Bennet, W. P. ; Hollstein, M. and Harris, C. C. (1994). Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res*. 54: 4855–78 .
 38. Yonish- Rouach, E. (1996). The p53 tumor suppressor gene: a mediator of a G1 growth arrest and of apoptosis. *Experimental*. 52: 1001–7.
 39. Brachmann, R. K.; Vidal. M. and Boek. J. D. (1996). Dominant-negative p53 mutations selected in Yeast hit cancer hot spots. *Proc. Natl. Acad. Sci. USA*. 93: 4091.
 40. Takeshima, Y.; Seyama, T. and Bennett, W. P. (1994). P53 mutations in lung cancers from non- smoking atomic- bomb survivors. *Lancet*. 342: 8886.
 41. Jonason, A. S. ; Kunala, S. and Price, G. J. (1996). Frequent clones of p53 mutated keratinocytes in normal human skin. *Proc. Natl. Acad. Sci. USA*. 93: 14025–9.
 42. Low, S. W.; Schmitt, E. M.; Smith, S. W.; Osborne, B. A. and Jacks, T. (1993). P53 is required for radiation- induced apoptosis in mouse thymocytes. *Nature*. 362, 847–852.
 43. Di Leonardo, A.; Linke, S. P.; Clarkin, K. and Wahl, G. M. (1994). DNA long- term induction of Cip1 in normal human fibroblasts. *Genes. Dev*. 8, 2540–2551.
 44. Agarwal, M. L.; Agarwal, A.; Taylor, W. R. and Stark, G.R. (1995). P53 controls both the G2\ M and the G1 Cell cycle checkpoints and mediates reversible growth arrest in human fibroblast. *Proc. Natl. Acad. Sci. USA*. 92, 8493– 8497.
 45. Stewart, N.; Hicks, G. G.; Paraskevas, F. and Mowat, M. (1995). Evidence for a second cell cycle block at G2\ M by p53. *Oncogene*. 10, 109–115.

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. أهميتها تكمن بامتلاكها فعالية مضاد للاكسدة 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: أظهرت الدراسة بان مستخلص الرمان له فعاليته الكبيرة ضد نمو الخلايا السرطانية المستخدمة باحداث تثبيط لنموها من 13% - 73,9%. عند التركيز 10 مايكرو 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, فإن مستخلص الرمان يمتلك فعالية قوية مضادة للسرطان.