Iraqi Journal
of Cancer and
Medical Genetics

The Cytotoxic Activity of *Punica granatum* on Growth of Hela and REF Cell Lines

Zaynab S. abdul gany*, Aseel F. Gedhan*, and Rasha A. Hussen*

** Iraqi Center for cancer and medical genetic research.*

Abstract :

The crude aqeouse extracts of punica granatum peel were assessed for their cytotoxic activity assay. This study utilized two different types of cell lines, Hela (Cervical human carcinoma) and REF (Rat Embryo fibroblast).

The results showed that the aqueous extract of punica granatum peel exhibited high cytotoxicity against the REF cell line with an inhibition value of 97.3 µg/ml after 24 h incubation. However, punica granatum peel displayed less toxicity against the Hela cell line with an inhibition value 24.4 µg/ml after 24 h incubation and this effect was dose- and time-dependent.

In conclusion, the results demonstrated that punica granatum peel is potently cytotoxic against REF cell, and lesser extend against Hela cells.

Introduction:

Pomegranate (punica granatum, punicaceae), native to Persia, is an edible fruit cultivated in Mediterreanean countries and some parts of the United States (1).

The pomegranate fruit has long been known in different countries with ancient cultures for its medical purposes (2). Pomegranate fruits are widely consumed fresh and in beverage forms as juice (3). Commercial pomegranate juice shows potent antioxidant (4) and antiatherosclerotic (5) properties attributed to its high content of polyphenols, including ellagic acid in its free and bound forms, gallotannins, and anthocyanins and other flavonoids (3).

The most abundant of these polyphenols is punicalagin, an ellagitannin implicated as the bioactive constituent responsible for >50% of the potent antioxidant activity of the juice (3).

Punicalagin is abundant in the fruit husk and, during processing, is extracted into pomegranate juice in significant quantities reaching levels of > 2g/L juice (3). Pomegranate peel extracts were shown to possess significant antioxidant activity in various models of cell lines (6). An extract of the pomegranate peel was fed to rats, which were then exposed to carbon tetrachloride, a toxic chemical. The pomegranate extracts help to protect the rats' livers from the toxic effects of carbon tetrachloride (6). The extract was evaluated for the antitumor promoting effects, especially involving topical application against skin tumors. The Wisconsin researchers concluded that animals pretreated with pomegranate fruit extract showed 70% less tumor incidence compound to animals that did not receive it (7), the study authors believe that their results provide "clear evidence that pomegranate fruit extract possesses anti skin promoting effects and may possess chemopreventive activity "in a wide range of tumor models" (7).

In view of this, the present study was aimed to evaluate, for the first time, the effects of the aqueous extract of punica granatum peel on the growth of two types of cancer cell lines, REF and Hela cells.

Material and Methods:

Preparation of pomegranate extract:

An aqueous extract of pomegranate was prepared using fresh seeds 100 g 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 filter paper to remove all the residual materials. Then, it was dried at 45˚C using hot air oven, with circulatory fan, and kept at 4˚C until use. For the following experiments, 10g of powdered plant was dissolved into 100ml PBS (as a solvent), filtered and sterilized by using 0.2 µm sterile Millipore filtering system, and the stock solution kept in sterile containers at 4˚C until use.

Cell Growth Assay

Two types of cell lines, Hela and REF, were used in this study. They were obtained kindly from Iraqi Center for cancer and medical genetic research (ICCMGR).

Cells maintained in RPMI-1640 containing 10% bovine calf serum. When the in vitro cells culture forms a monolayer. These cells were treated with trypsin/ versine mixture in order to pursue subculture process.

Cytotoxicity assay

The effect of the aqueous extract of pomegranate on growth of cancer cells was determined by using inhibitory activity assay. Briefly, cell cultures in the micro titration plate were exposed to various concentrations (1562, 781, 390, 195.2, 97.6, 48.8, 24.4, 12.2, 6.1 and 3.05 µg/ml) of plant extract during the log phase. The cytotoxic activity was determined after (24, 48 and 72 h) (8). The inhibition rate was calculated according to the below equation (9).

Inhibition % = [(optical density of control wells -optical density of test wells)/optical density of control wells] x 100

After the end of the exposure period, the medium and the cells decanted off and replaced by 200 µl of 0.01% crystal violet dye. After 20 min. the stain was washed gently with tap water for three times. The plate was left until become dry.

The optical density of each well was read by using a micro-ELISA reader at 492 nm transmitting wave length (8).

Statistical Analysis

Statistical evaluation of the treated and untreated control cells along with the extract and solvent-treated cells was calculated using Student's t-test.

A probability of 0.05 or less was statistically significant.

Results:

The crude aqueous extract derived from the peel of punica granatum was evaluated as a cytotoxic agent using crystal violate assay, against REF cell line and Hela cell line. The inhibition values for the continuous exposure of REF cells after a 72 hr was indicated that the extract is potently cytotoxic to the REF cells with an IC50 value of 6.1µg/ml after 24 h incubation (fig. 1). An additional series of experiments were carried out in order to establish whether the punica granatum extract was cytotoxic in a dose- and time-dependent manner to the REF or not. The results had clearly showed that significant cytotoxic activity was evident after 48 h exposure at a concentration as low as 3.05µg/ml ($P<0.05$) and this effect increased in a dose-dependent manner up to 12.2 µg/ml. Significant cytototoxic activity was also observed after 48 h incubation and this was dose dependent up to 12.2µg/ml ($P<0.05$). However, the cytotoxic effect of punica granatum in these cells was abated after 24 h of incubation.

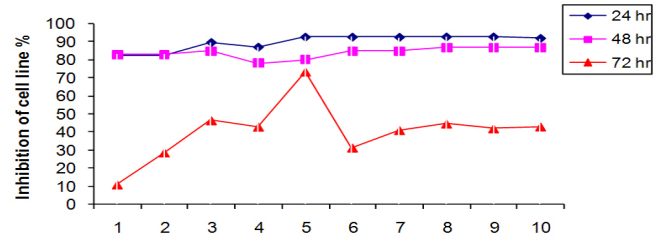

Figure 1: Effect of aqeous extract of pomegranate peel on growth of REF cell line during different exposure periods.

The effects of aqueous extract of punica granatum against Hela cells are shown in Figure 2. The extract exhibited less cytotoxic activity against Hela cells in comparison with REF cells the inhibition rate was 12.2µg/ml after 24 h incubation. On the other hand, Figure 2 showed that there was cytotoxic activity as early as after 24 h incubation ($P<0.005$) and this increased in a dose-dependent manner up to 24.4µg/ml. At 24 h incubation period treatment with 12.2 µg/ml punica granatum extract inhibited cell proliferation of Hela cells by approximately 50% and minimum cytotoxic effect was obtained after 72 h incubation ($P<0.05$).

This study showed that the crude extract of punica

granatum was more effective in inhibition growth of REF cells than the Hela cells.

Discussion:

Ellagic acid and tannins have been shown previously to exhibit in vitro and in vivo anticarcinogenic properties, such as induction of cell cycle arrest and apoptosis, as well as the inhibition of tumor formation and growth in animals (10). Recently, there have been several reports on the antiproliferative, apoptotic, angiogenic and inhibition of nuclear factor-k B (NF-kB) activity and xenograft growth by pomegranate polyphenols (11-15). Pomegranate phytochemicals inhibited the in virto proliferation of three prostate cancer cell lines, LNCaP, PC3 and DU145 and showed in vitro inhibition of xenograft growth in athymic mice (13).

The lower cytotoxic effect of punica granatum against Hela cells was compared with the REF cells might be due to the selectivity of the extract towards the REF cancer cells than Hela cancer cells. The strong cytotoxic effect of punica granatum extract against the REF cells implies that the aquous extract may have a potent anti-tumour effect on proliferation of Hela cancer cell lines.

Further studies are currently underway in our laboratory with the aim of extracting Ellagic acid from punica granatum and determining its mode of molecular action and cytotoxic effect on a variety of other human cancer cell lines.

In addition, the tannins in pomegranate fruit should be taken into consideration for their genotoxic potential which also have been reported to exert a certain genotoxic activity (16).

It is well documented that fruit or plant extracts are a complex mixture of various constituents and in most of the instances it is still not clear whether a single compound or a mixture of compounds is responsible for the reported effects (17). However, evidence is accumulating that often related compounds present in a fruit or herb extract augment each other's biological effect. For example, it has been reported that ellagic acid and quercetin (both are also present in pomegranate) together exert a more pronounced inhibitory effect against cancer cell growth than either compound alone (18).

On the other hand, since the pomegranate fruit is edible; the present work has been prepared by a procedure related to its consumption as an edible fruit.

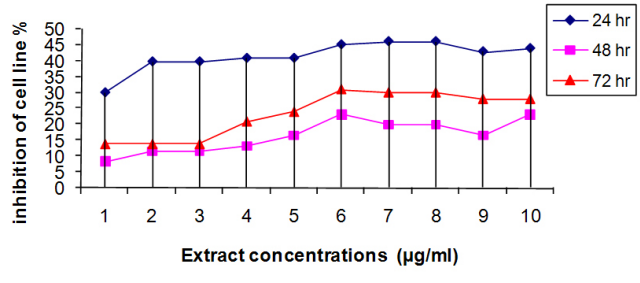

Figure 2: Effect of aqueous extract of pomegranate peel on growth of Hela cell line during different exposure periods.

References:

1. Langley, P. why a pomegranate. (2000) Br. Med. J. 321: 1153-1154.
2. Longtin, R. The pomegranate: nature's power fruit? (2003) J Natl Cancer Inst (95):346-8.
3. Gil, M.I.; Tomas-Barberan, F.A.; Hess-Pierce, B.; Holcroft, D.M. and Kader, A.A. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. (2000) J Agric Food Chem (48): 4581-9.
4. Fuhrman, B. and Aviram, M. Flavonoids protect LDL from oxidation attenuate atherosclerosis. (2001) Curr Opin Lipidol (12): 41-8.
5. Aviram, M.; Rosenbalt, M. and Gaitini, D. Pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduces common carotid intima-media thickness, blood pressure and LDL oxidation. (2004) Clin Nutr (23): 423-33.
6. Chidambara, K.N.; Jayaprakasha, G.K. and Singh, R.P. Studies on antioxidant activity of pomegranate (punica granatum) peel extract using in vivo models. (2002) J Agric Food Chem 50 (17): 4791-5.
7. Afaq, F.; Saleem, M.; Krueger, C.G.; Reed, J.D. and Mukhtar, H. Anthocyanin-and hydrolyzable tannin-rich pomegranate fruit extract modulates

MAPK and NF-kappaB path- ways and inhibits skin tumorigenesis in CD-1 mice. (2004) Int J Cancer. 28.
8. Freshney, RI. Culture of Animal cells. 3rd. ed. Wiley-Liss. USA (1994). Pp267-308.
9. Chiang, W.; Chang, MY. and Lin, CC. In vitro cytotoxic antiviral and immunomodulatory effects of Plantago major and Plantago asiatica. American Journal of Chinese Medicine (2003). 31(2): 225-234.
10. Castomguay A, Gali HU and Perchellet EM. Antitumorigenic and antipromoting activities of ellagic acid, Ellagitannins and oligomeric anthocyanin and procyanidin. Int J Oncol 10:367-73. 1997.
11. Afaq F, Saleem M, Krueger CG, Reed JD and Mukhtar H. Anthocyanin- and hydrolysable tannin-rich pomegranate fruit extract modulates MAPK and NF-κB pathways and inhibits skin tumorgenesis in CD-1 mice. Int J Cancer 113:423-33. 2005.
12. Lansky EP, Jiang W and Mo h. Possible synergistic prostate cancer suppression by anatomically discrete pomegranate fractions. Invest New Drugs 23:11-20. 2005.
13. Albrecht M, Jiang W and Kumi-Diaka J. pomegranate extracts potently suppress proliferation, xenograft growth and invasion of human prostate cancer cells. J Med Food 7:274-83. 2004.
14. Toi M, Bando H and Ramachandran C. Preliminary studies on the anti angiogenic potential of pomegranate fractions in vitro and in vivo. Angiogenesis 6:121-8. 2003.
15. Kim DN, Mehta R and Yu W. Chemopreventive and adjuvant therapeutic potential of pomegranate of human breast cancer. Breast Cancer Res Trear 71:203-17.2002.
16. Ferguson, L.R. Role of plant polyphenols in genomic stability. (2001). Mutation Research (475): 89-111.
17. Schafer A, Chovanova Z, Muchova J, Sumegova K, Liptakova A, Durackova Z and Hogger P. Inhibition of cox-1 and cox-2 activity by plasma of human volunteers after ingestion of fresh maritime pine bark extract (Pycnogenol). Biomed Pharmacother 60:5-9. 2005.
18. Seeram NP, Adams LS, Henning SM, Niu Y, 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 Biomed 16:360-367. 2005.

ζγ ÁȒ *Punica granatum* ȷÅŋÀÖä ğÅGÅó ĞŁŧÊÖä ĞŁÖÅĤÅÖä REF ş Hela ţζφ ĞŁφÅùÀÊÖä ÅΛΨϱå ȓζÛn

*ķÊğ Ķɳɾå ÆƷȝ ÅȚǿ ş Û*ȷåÆŁd ĘΛÅz ÕŁĖèå Û*Ɨ8ÖÄ ÆƷȝ ÁĤĖè ÆΛΛà .ȳ.ȳ

ĞŁƷÛÖää Ğǫäóζõäş ȷÅùÀÊÖää ǫζHƱõ ȕęäÅĤÖää ÂõÄţ*

:ĞȍΨϱå

Hela ÛĞŁφÅùÀÊÖää ÅΛΨϱå ϕδţ ĸķÁΫQƯ ķȝζφ ĜĖäíøÆÖää ĆØŁ ȒĶΫŧŁĤǫĖÙ .ĜŁzΫϱå ĞŁŧÊÖää ϵǫĖÖÅĤÅíõ *Punica granatum*ĝÅÎÕõ ȳÅϱå ȕəÅţå ÒΫÁǫÊţå ŀţę .(fibroblast ÉÀDZ ķADZ) REFs (ŀǵÀÖää ĘΛȝ ȷÅùÀĖ)

čđ ÁĤæ Õɳ/ȳïÀđşÅÒΛÅɳ ĚĒ,Đ ÙĖĞȝĶ ĜŧŁÕæ REF ĜĖΫn ŲϑȞ ÉȒ БÅȝ ĞŁƧ ÅΤşå *Punica granatum* ĝÅÎÕõ ȕəÅţå ÒΫÁǫÊţå ȷÙ ǰəÅǫÁÖää ĶǫĞää čđ ÁĤæ Õɳ/ȳïÀđşÅÒΛÅɳ.čđ,đ ÙĖĞȝĶĜŧŁÕæ Hela ĜĖΫn ŲϑȞ ÉȒ ĞŁƧ Õę̆Ù ĶÛȝå *Punica granatum* ĝÅÎę ȷå ÆDZş ķǵ Ž .ȒΛÅĤǫÖää ɒɳ Ĝȝλè .ĶęşϽΫȝ ĝÆŧǫȞɳş ķȝÀDZ ǰÅõ ĶǫÙǫÖää ăØŁş ȒΛÅĤǫÖää ɒɳ Ĝȝλè

ϵÅΘ Õę̆Ù ĶφÅõ ÅΖǫĖÖÅĤζş REF ĜĖΫn ŲϑȞ ÉȒ òÅĦz ÕÒÎæ ĞŁŧÊÖää ÕΫǫϱ *Punica granatum* ĝÅÎę ȷÙæ ĜĖäíøÆÖää ĆØŁ ɒɳ ǰǫÁǫÊφ ȷå ɒÒζ ş .Hela ŀǵÀÖää ĘΛȝ ǰÅùÀĖ