

## Functional Variation of Soluble Polyphenols in Oak Apple Gall and Pomegranate Peels and their Inhibition Activity in Leukemia K562 Cells

Chrow Khurshid<sup>1\*</sup> and Osamah Ahmed<sup>2</sup>

<sup>1</sup>University of Salford, School of Environment & Life Sciences, Peel Building, Salford, M5 4WT, UK

<sup>2</sup>School of Agriculture, University of Kirkuk, Kirkuk, Iraq

\*Correspondence author: Chrow Khurshid, University of Salford, School of Environment & Life Sciences, Peel Building, Salford, M5 4WT, UK, Tel: +44-161-295-4656; Fax: +82-2-921-5357; E-mail: C.A.Khurshid@edu.salford.ac.uk

Received date: September 2, 2015; Accepted date: October 24, 2015; Published date: October 29, 2015

Copyright: © 2015 Khurshid C, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

### Abstract

The possibility of finding anti-cancer drugs has generated interest in natural products. Several studies have in vitro observed potent anti-cancer properties of pomegranate juice against various cancers including leukemia. Although a few studies have described the bioactivities of hydrolysable tannins extracted from pomegranate juices, limited attention has been paid to other tannins extracted from different parts of a plant. Recently, polyphenols, which are found in plants, have become the most studied phytochemicals owing to their significant chemical properties and biological activities. Tannin is an astringent plant polyphenolic compound and has been observed to have anti-oxidant and anti-cancer properties. This study uses a novel approach to study variation in the structure of hydrolysable tannins from two different types of plants and their inhibition activity in leukemia K562 cells. We examined data showing anti-proliferation activity of hydrolysable tannins extracted from pomegranate peels within two concentrations of 1 mg/ml and 100 µg/ml. Hydrolysable tannin extracted from oak apple gall induces different effects than that extracted from pomegranate tannin. At the highest concentration of 100 µg/ml, the tannin from oak apple gall did not have any noticeable effects on cells, whereas at the highest concentration of 1 mg/ml, cell cycle arrest seemed to have occurred after 72 h of treatment. This most likely led to senescence caused by over-stimulation of the cells by specific polyphenols compounds in oak apple gall, which affected the cells either directly or indirectly by changing the culture's environment.

**Keywords:** Apoptosis; Leukemia; Polyphenols; Pomegranate; Oak apple gall; Tannin

### Introduction

For many decades, traditional Kurdish communities in Northern Iraq have therapeutically used pomegranate and oak apple gall extracts owing to their antimicrobial properties. Several researchers have recently attempted to identify polyphenols found in these plants. They have concluded that the quantitative and qualitative properties of polyphenols depend on the extraction method, and analytical techniques have been developed.

Polyphenol compounds in oak wood have various biological activities owing to their structural complexity. For example, they play an essential role in wine maturation because of their potent anti-oxidant effects as well as in making ink. It has also been suggested that many distinctive properties of oak could be attributed to the ellagitannins in their structure [1].

In a previous study that used non-tumor hematopoietic stem cells as control cells, anti-proliferation and apoptosis properties of pomegranate juice extracts have been observed in eight leukemia cell lines (four lymphoid and four myeloid) [2]. Polyphenol compounds are the major and the highest proportion of phytochemicals in pomegranates. Hydrolysable tannins make up one of two major types of polyphenol compounds in pomegranate [3,4].

Among other commonly studied fruits such as red grapes, cherry, blueberry, lemon and apple, pomegranate has been found to have the

highest antioxidant activity owing to the high amount of tannin [5]. The anti-proliferation and apoptosis properties of pomegranate against breast, colon, lung, skin and prostate cancers have been demonstrated in vitro by numerous studies [2,6-9]. Many researchers also reported other bioactivities of polyphenol compounds and tannin acid in addition to their significant influence on the nutritive value of many foods. They have been known to reduce blood pressure and accelerate blood clotting [10-12].

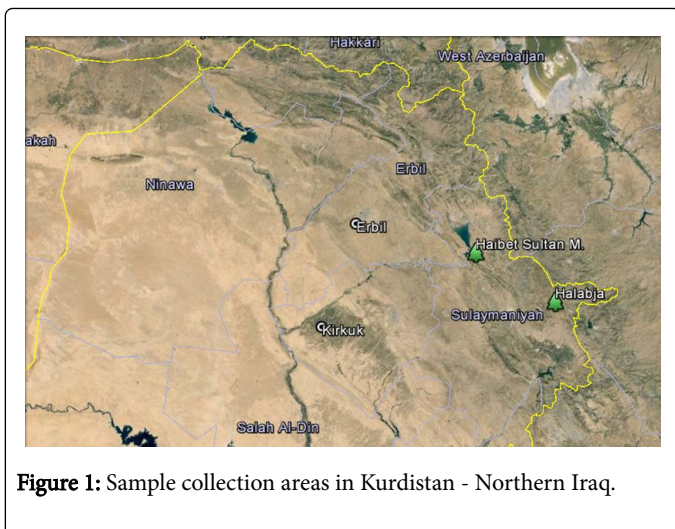
These compounds have been found to have tumor-promoting properties at certain concentrations. An experimental animal developed tumors during treatment with tannin. Therefore, tannin has been considered to have both carcinogenic and mutagenic properties [13,14].

However, the tannin dose required to induce cancer is believed to be far greater than is normally consumable through food [15].

### Material and Methods

#### Sample preparation

The oak apple sample was collected from the *Quercus aegilops* trees at the Haibet Sultan Mountains in the Sulaymaniyah-Kurdistan region of Northern Iraq. Pomegranate peels were obtained from the fresh fruits of *Punica granatum L.* trees at Halapja town in the same region (Figure 1). Each sample was washed several times with cold water to remove dust, left to air dry in the laboratory and then grinded in preparation for extraction.



**Figure 1:** Sample collection areas in Kurdistan - Northern Iraq.

### Sample extraction

Crude powder of each plant was extracted at room temperature for 24 h with 600 ml of water/methanol (1:1). Each plant extract was filtered on a Buchner funnel, and a rotary evaporator was used at 40°C for sample concentration. Liquid-liquid extraction was used to fractionate a solution containing ethyl acetate and diethyl ether. The organic fraction was dried and re-dissolved in methanol while the aqueous sample was freeze-dried.

### Cell culture

Human leukemia cells were obtained from the Public Health England cell collections. K562, a human erythroleukemic cell line, is used in this study. The cells were grown in RPMI 1640 media supplemented with 10% fetal calf serum and 1% w/v of L-glutamine.

### Cell proliferation assay

The K562 cells were proliferated by a standard colorimetric MTT reduction assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) and seeded at a concentration of approximately 2×10<sup>4</sup> cells/200 µl/well into 96 well plates. The extracted powders of the pomegranate peels and oak apple gall were separately dissolved in complete media to prepare stock solutions. The K562 cells were treated with two different concentrations, 1 mg/ml and 100 µg/ml, of both solutions. The plates were incubated in 5% CO<sub>2</sub> at 37°C for 92 h and then 50 µl of MTT (5 mg/ml in phosphate buffer saline) was added to each well and then incubated for another 3 h at 37°C. Eventually, the supernatants were aspirated and 200 µl of dimethyl sulfoxide was added to each well; the amount of MTT which converted to formazan is indicative of the number of viable cells. The absorbance was measured at 570 nm using a multi-well plate reader. 1-25 µM cisplatin was used as the positive detector and the control cell was seeded in the same plate without treatment. The test has triplicated for each treatment in one plate and the assay been repeated at least three times for each treatment.

### Trypan blue exclusion assay

The trypan blue exclusion assay was used to quantify the number of live cells in the suspension culture before treatment with oak apple gall

and pomegranate peel stock solutions. The main aim of this assay was to determine the total number of live cells used in the MTT assay.

### Protein extraction from K562

The K562 cells were treated in 25 cm flasks with 1 mg/ml of media for each 10<sup>6</sup> cells at different time points (24 h, 72 h and 92 h). The protein samples were extracted using a cell disruption strategy which determines the accessibility of intracellular proteins. The Ready Prep Protein Extraction Kit for total protein (BioRad, No. 163-2086-MSDS) was used for this purpose. The protein concentration was determined by the bicinchoninic acid protein assay kit purchased from Fisher Scientific through comparison with the standard BSA curve.

### SDS-PAGE gel electrophoresis

Protein electrophoresis involves many steps. First, the method which could provide better resolutions and data must be selected. Second, the optimum sample preparation method must be chosen on the basis of the sample type. Third, the gels and buffers for finally performing the electrophoresis must be prepared, and finally a visualization technique for detecting and analyzing the protein must be selected. In this study, we used the SDS-PAGE gel for protein samples extracted from K562 cells at different time points. A 12% resolving gel (deionised H<sub>2</sub>O, 1.5 M Tris-HCl, pH 8.8, 30% acrylamide solution, sodium dodecyl sulphate (10% w/v), ammonium peroxydisulphate (10% w/v), TEMED (N,N,N,N-tetramethyl-ethylenediamine) and a 4% stacking gel (deionised H<sub>2</sub>O, 1.5 M Tris-HCl, pH 8.8, 30% acrylamide solution, sodium dodecyl sulphate (10% w/v), ammonium peroxydisulphate (10% w/v), TEMED (N,N,N,N-tetramethyl-ethylenediamine) were used in addition to 20 µL of a 2X sample loading buffer (0.125M Tris-HCL, 20% glycerol, 4% SDS, 0.02% BPB, 0.2 M DTT) which was added in equal amounts to the total cell protein sample. The gel was run for 2 h at 120 V using a 1× running buffer (30.3 g Tris-base, 144.0 g glycine and 10.0 g SDS). The gel was fixed with a fixing solution (50% methanol, 40% H<sub>2</sub>O and 10% acetic acid) and then visualized with the Coomassie blue stain. Then, a de-staining step was performed for 4-24 h until a clear background has obtained.

### Statistical analysis

Each experiment was performed a minimum of three times, with three replicates performed within each experiment. Student's t test and one-way analysis of variance were used to compare the data, and all tests were considered statistically significant at p<0.05. Sigmaplot 2000 software was used for analysis of data obtained from the cell proliferation assay.

## Results and Discussion

### Contributions of tannin in pomegranate peel and oak apple gall extractions

The study results illustrated that oak apple gall contains 27.05% of the polyphenol tannin, whereas pomegranate peels contain 1.06%. The oak apple gall sample obtained from *Quercus aegilops* has a percentage of tannin compounds similar to the sample obtained from *Quercus robur L.* in a previous study, and it was nearly 47% [16]. Moreover, its tannin content is similar to that of an oak apple gall species grown in the Zagros Mountains in Iran [17].

The observation by Saada et al. [18] reported that pomegranate juice extract contained polyphenol and tannin in ration (14.3%). However, the observation by Farag et al. [19] indicated that peel juice contained higher amounts of total polyphenols and flavonoids, approximately 1.22 and 1.43 times that in juice of leaves.

### Effects of pomegranate juice and oak apple gall on cell proliferation and cell arrest

After 72 h, a significant increase was observed in the number of apoptotic and dead cells. The sensitivity of the K562 cells was different between the two different extractions. Leukemia cell lines were more affected by pomegranate polyphenol extractions than the oak apple gall extraction. The percentage of viable cells decreased to 27.6% after 92 h of treatment with 1 mg/ml of pomegranate peel extraction; whereas the percentage decreased to 4.40% after treatment with 100 µg/ml of the extraction for the same duration (Tables 1-4 and Figures 2-5).

% Growth	% Growth
<50	≥ 50
Growth below 50	Growth above 50
38.53318	56.54831
Dose below 50	Dose above 50
0.25	0.5
IC50	0.4091µM

**Table 1:** Oak apple gall's percentage of growth and IC50 at a dose of 1 mg/ml.

% Growth	% Growth
<50	≥ 50
Growth below 50	Growth above 50
8.692308	92.4359
Dose below 50	Dose above 50
100	1.5625
IC50	51.4443µM

**Table 2:** Oak apple gall's percentage of growth and IC50 at a dose of 100 µg/ml.

The IC50s for the K562 cells treated with 1 mg/ml and 100 µg/ml of pomegranate peel extraction are about 0.1026 µM and 7.9167 µM, respectively. This was compared with cisplatin as a positive DNA damaging agent at a concentration of 1-25 µM/ml (Table 5). The cells were treated with the tannin extracted from oak apple gall sample and their growth was inhibited to 22.5% at a concentration of 0.03 mg/ml, thus indicating that the polyphenol tannin caused cell cycle arrest at this concentration. The number of cell deaths is reduced by increasing the concentration of the oak apple gall tannin to a final concentration of 1 mg/ml. The percentage of cell growth starts increasing significantly to 84.9%. Dahlawi [2] has suggested the tannin in pomegranate juice to be a potent therapeutic agent for leukemia cells when non-tumor hematopoietic stem cells are used as control cells.

Many studies have suggested anti-proliferation and apoptosis properties of pomegranate fruit extract against lung cancer [8].

% Growth	% Growth
<50	≥ 50
Growth below 50	Growth above 50
31.59164	83.03859
Dose below 50	Dose above 50
0.125	0.0625
IC50	0.1026µM

**Table 3:** Pomegranate's percentage of growth and IC50 at a dose of 1 mg/ml.

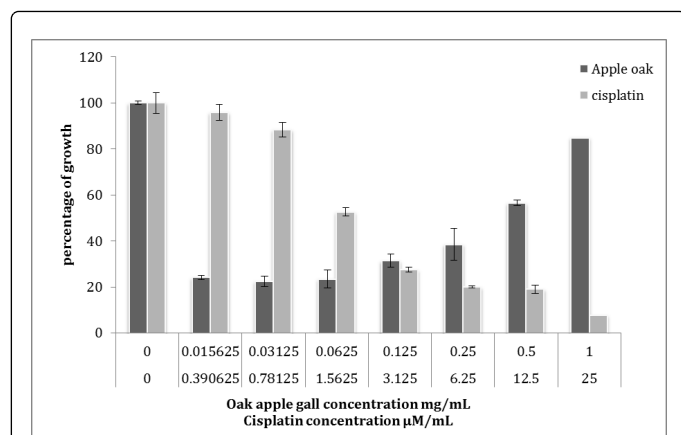
% Growth	% Growth
<50	≥ 50
Growth below 50	Growth above 50
20.30501	81.04575
Dose below 50	Dose above 50
12.5	3.125
IC50	7.9167µM

**Table 4:** Pomegranate's percentage of growth and IC50 at a dose of 100 µg/ml.

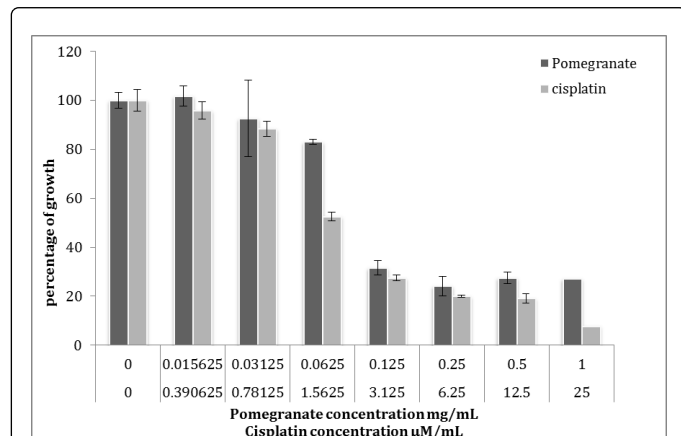
% Growth	% Growth
<50	≥ 50
Growth below 50	Growth above 50
27.46921	52.57602
Dose below 50	Dose above 50
3.125	1.5625
IC50	1.7228µM

**Table 5:** Cisplatin percentage of growth and IC50 at a dose of 25 µM.

The observation by Khan et al. [20] also suggested that pomegranate fruit extract selectively induced apoptosis following 72 h of treatment within the two breast cancer cell lines and did not affect the non-tumor MCF-7 cells. In addition, Dahlawi [2] reported that most leukemia cell lines treated with pomegranate juice display a significant S-phase arrest at high doses, whereas the cells showed G0/G1 arrest at low doses. This induced senescence within treated cells as demonstrated by Schmitt [21]. Many studies have observed S-phase arrest in HT-29 colon cells and Caco-2 cells during treatment with pomegranate juice [22,23].



**Figure 2:** Anti-proliferation activity assay of oak apple gall within the highest concentration of 1 mg/ml against leukemia cells K562 compared with cisplatin as the positive DNA damage agent.



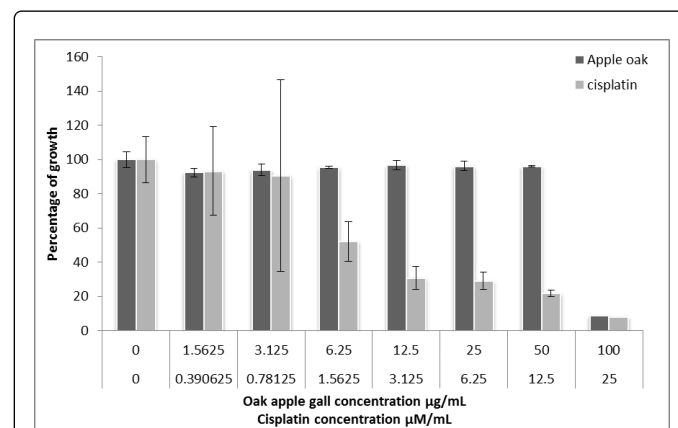
**Figure 3:** Anti-proliferation activity assay of pomegranate extraction within highest concentration of 1 mg/ml against leukemia cells K562 compared with cisplatin as the positive DNA damage agent.

This study aimed to investigate the tannin extractions from pomegranate peels with any potential bioactive agents as crude samples instead of pure fractions. Thus, the response from the K562 cells was as a result of complex mechanisms and pathways through a combination of materials in the crude peel extraction. We observed unexpected activity for the tannin extracted from oak apple gall. In the proliferation assay and apoptosis studies, the oak apple gall extraction induced apoptosis and cell cycle arrest in the K562 cells during treatment for 72 h at low doses. When the oak apple gall extraction was increased to a certain concentration (0.5-1.0 mg/ml), the number of viable cells increased significantly with the percentage of growth exceeding 80% between 72 and 92 h of incubation. These results indicate that a polyphenol compound in the oak apple gall is acting as a cancer promoting agent. This might support the hypothesis presented by Chung et al. [15] that tannin might promote cancer when the concentration of tannin exceeds a specific range and seems to be a sword with two edges.

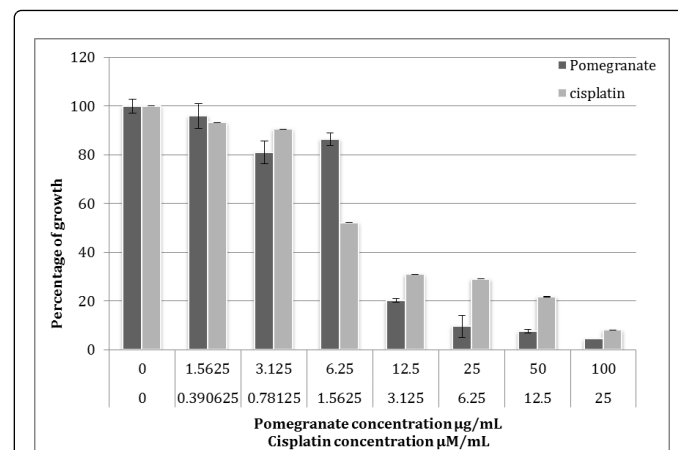
### SDS-Gel analysis of collected cell lysates

Equal amounts of protein were loaded in all lanes in the SDS-Gel along with a protein marker as a reference lane. The concentrations of tannin acid in both sample extractions could induce elevated levels of proteins in the treated cells compared with the control (non-treated) cells.

The protein expression level changed at different time points of the treatment. Fewer changes could be noticed after 24 h (Figure 6) of incubation of cells with both extractions compared with those in the control cells, whereas obvious signs of apoptosis could be observed after 48 h and 72 h (Figures 7 and Figure 8).

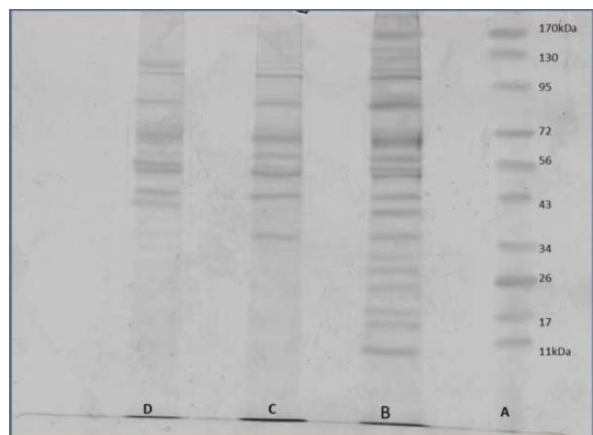


**Figure 4:** Non-inhibition activity of oak apple gall in K562 cells within the highest concentration used 100 μg/ml; the activity is compared with cisplatin.

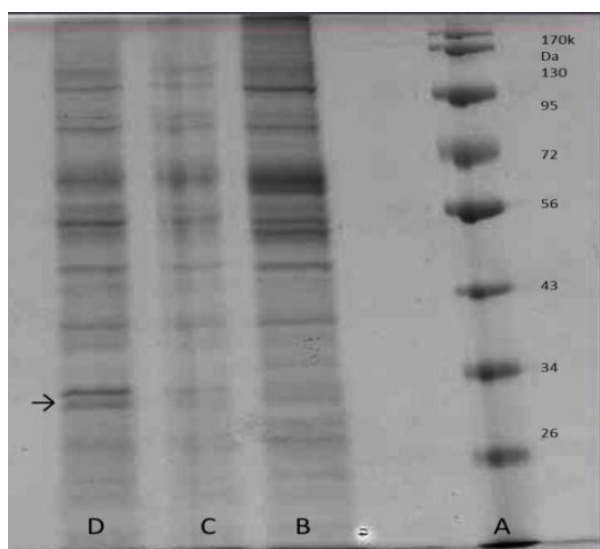


**Figure 5:** Inhibition activity of pomegranate extraction within the highest concentration of 100 μg/ml.

The presence of a band with molecular mass 34 kDa was detected after 72 h of treatment with the pomegranate extract, and regulation in proteins is observed within the 34 kDa MW range. On the other hand, under similar treatment conditions, a weaker expression was detected for the cells treated with the oak apple gall extract. These results indicate that under these conditions, only tannic acid from pomegranate might bind to DNA in a way that leads to cleavage of endonucleases or up-regulation of cell surface receptors.



**Figure 6:** SDS-Gel for total protein extraction from untreated and treated K562 cells after 24 h incubation. Lane A: a reference protein with range 170 kDa to 11 kDa MW. Lane B: an untreated cell (control cell). Lane C: cells treated with 1 mg/ml of oak apple gall extraction for each 106 cells. Lane D: cells were treated with 1 mg/ml of pomegranate peel extraction for each 106 cells. All lanes showed similar patterns after treatment for 24 h.

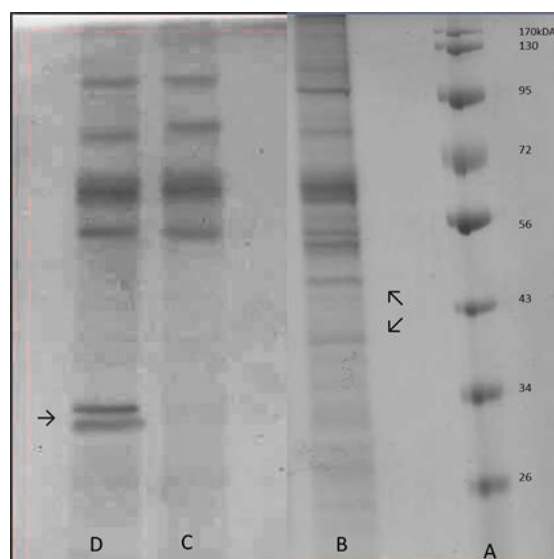


**Figure 7:** SDS-Gel for total protein extraction from untreated and treated K562 cells after incubation for 48 h. Lane A: a reference protein with range 170 kDa to 26 kDa MW. Lane B: an untreated cell (control cell). Lane C: cells treated with 1 mg/ml of oak apple gall extraction for each 106 cells. Lane D: cells treated with 1 mg/ml of pomegranate extraction for each 106 cells.

SDS-Gel analysis of the treated K562 cell lines indicated that some proteins are activated, whereas on exposure to tannin compounds from oak apple gall and pomegranate peels caused a cleavage of other proteins. Level of expression and cleavage of proteins, or in other words, up-regulated and down-regulated proteins vary between the two treatments. Studying these proteins quantitatively and

differentially in a large-scale approach will provide a better understanding of the mechanisms of action of these natural products on cancer cells. The LC-MS/MS technique must be employed in future work.

The mechanism of apoptosis induction by these natural substances is still unknown. Numerous mechanisms could initiate apoptosis in treated K562 cells, including activation of cell surface receptors or death receptors or either mitochondrial or DNA damage caused by extracellular stress. The structure and distribution of tannin is not homogenous among plant tissues. Moreover, it has various impacts and bioactivities depending on its doses and duration of treatment.



**Figure 8:** SDS-Gel for total protein extraction from untreated and treated K562 cells after incubation for 72 h. Lane A: a reference protein with range 170 kDa to 26 kDa MW. Lane B: untreated cell (control cell). Lane C: cells treated with 1 mg/ml of oak apple gall extraction for each 106 cells. Lane D: cells treated with 1 mg/ml of pomegranate extraction for each 106 cells.

## Conclusion

Polyphenol compounds, especially tannin, are found in varying amounts and structures among different parts or tissues of the same plant. These compounds have been used by people since many decades owing to their therapeutic value. Many studies have suggested the therapeutic capacity of these natural products as anti-oxidation, anti-bacterial and anti-cancer drugs. Other researchers have reported the possibility of tannin inducing or promoting cancer at some crucial concentration or when consumed in high amounts through food.

To further understand these polyphenol structures, additional studies of tannin's structure–function relationship are needed. This could be done by using many analytical techniques such as HPLC and NMR. Determined the fine structure of different polyphenol tannin considers as the key aspect for their biological functions and the mechanisms of their “double edges” as carcinogenic or non-carcinogenic can also determine by LC/MS-MS technique. Our results indicate significant inhibition of leukemia cell growth during treatment at specific doses of tannin extracted from pomegranate

peels. Moreover, we observed unclear activity of tannin extracted from the oak apple gall sample towards leukemia cells proliferation, supporting the idea that some polyphenols might promote cancer by over-activation of these cells. This difference in mechanism of activities must be further analyzed and is an interesting topic in a dynamic field of research.

## Acknowledgements

We are grateful to the Iraqi cultural attaché-London, University of Salford Manchester/ Kidsan laboratory and University of Kirkuk for financial support and lab collaboration.

## References

1. Zhang B, Cai J, Duan CQ, et al. (2015) A review of polyphenolics in oak woods. *Int J Mol Sci* 16: 6978-7014.
2. Dahlawi H, Jordan-Mahy N, Clench MR, Le Maitre CL (2012) Bioactive actions of pomegranate fruit extracts on leukemia cell lines in vitro hold promise for new therapeutic agents for leukemia. *Nutr Cancer* 64: 100-110.
3. Lansky EP, Newman RA (2007) *Punica granatum* (pomegranate) and its potential for prevention and treatment of inflammation and cancer. *J Ethnopharmacol* 109: 177-206.
4. Gil MI, Tomás-Barberán FA, Hess-Pierce B, Holcroft DM, Kader AA (2000) Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *J Agric Food Chem* 48: 4581-4589.
5. Wolfe KL, Kang X, He X, Dong M, Zhang Q, et al. (2008) Cellular antioxidant activity of common fruits. *J Agric Food Chem* 56: 8418-8426.
6. Adams LS, Seeram NP, Aggarwal BB, Takada Y, Sand D, et al. (2006) Pomegranate juice, total pomegranate ellagitannins, and punicalagin suppress inflammatory cell signaling in colon cancer cells. *J Agric Food Chem* 54: 980-985.
7. Kim ND, Mehta R, Yu W, Neeman I, Livney T, et al. (2002) Chemopreventive and adjuvant therapeutic potential of pomegranate (*Punica granatum*) for human breast cancer. *Breast Cancer Res Treat* 71: 203-217.
8. Khan, N, Mukhtar, H (2007) Pomegranate fruit as a lung cancer chemopreventive agent. *Drugs of the Future*, 32: 549-554.
9. Lansky EP, Harrison G, Froom P, Jiang WG (2005) Pomegranate (*Punica granatum*) pure chemicals show possible synergistic inhibition of human PC-3 prostate cancer cell invasion across Matrigel™. *Invest New Drugs* 23: 121-122.
10. Ratnoff od, Crum jd (1964) Activation of hageman factor by solutions of ellagic acid. *J Lab Clin Med* 63: 359-377.
11. Clifton EE (1967) The effects of ellagic acid on the coagulation mechanisms and on traumatic bleeding. *Am J Med Sci* 254: 483-490.
12. Bhargava UC, Westfall BA (1969) The mechanism of blood pressure depression by ellagic acid. *Proc Soc Exp Biol Med* 132: 754-756.
13. KORPASSY B (1961) Tannins as hepatic carcinogens. *Prog Exp Tumor Res* 2: 245-290.
14. Bichel J, Bach A (1968) Investigation on the toxicity of small chronic doses of tannic acid with special reference to possible carcinogenicity. *Acta Pharmacol Toxicol (Copenh)* 26: 41-45.
15. Chung KT, Wong TY, Wei CI, Huang YW, Lin Y (1998) Tannins and human health: a review. *Crit Rev Food Sci Nutr* 38: 421-464.
16. Paaver U, Matto V, Raal A (2010) Total tannin content in distinct *Quercus robur* L. galls. *Journal of Medicinal Plants Research* Vol. 4:, pp. 702-705.
17. Tabaraki R, Safari A, Faal AY (2013) Ultrasonic-assisted extraction of condensed tannin from acron, gland, leaf and gall of oak using response surface methodology. *Journal of Applied Chemical Research*, 7, 3, 67-77.
18. Saada H, El Bouhtoury C, Pizzi A, Rode K, Charrier B, et al. (2012) Characterization of pomegranate peels tannin extractives. *Industrial Crops and Products* 40: 239-24.
19. Farag RS, Abdel-Latif MS, Sekina SE, Layla S (2014) Phytochemical screening and polyphenol constituents of pomegranate peels and leave juices.
20. Khan GN, Gorin MA, Rosenthal D, Pan Q, Bao LW, et al. (2009) Pomegranate fruit extract impairs invasion and motility in human breast cancer. *Integr Cancer Ther* 8: 242-253.
21. Schmitt CA (2007) Cellular senescence and cancer treatment. *Biochim Biophys Acta* 1775: 5-20.
22. Kasimsetty SG, Bialonska D, Reddy MK, Ma G, Khan SI, et al. (2010) Colon cancer chemopreventive activities of pomegranate ellagitannins and urolithins. *J Agric Food Chem* 58: 2180-2187.
23. Herst PM, Davis JE, Neeson P, Berridge MV, and Ritchie DS (2009) The anti-cancer drug, phenoxodiol, kills primary myeloid and lymphoid leukemic blasts and rapidly proliferating T cells. *Haematologica* 94: 928-934.