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Assessment the cytotoxicity of pomegranate extracts on leukemia cell line

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***Abstract*---**The most abundant phytochemicals in pomegranate fruit are high antioxidant properties. Free radicals in excess cause cell and tissue damage, which can lead to asthma, Alzheimer's disease and cancer. This study investigates the antioxidant capacity of phenol extract of pomegranate fruit by scavenging activity against 1,1-diphenyl-2-picrylhydrazine (DPPH). The scavenging activity of the pomegranate fruit extracts was determined at the concentrations 12.5, 25, 50, 100, and 200 µg mL^{-1}. The results for phenol extracts recorded 16.74, 25.12, 40.63, 68.87 and 81.98% respectively compared with ascorbic acid. The immunomodulatory effect of phenol extract on HL60 cell line exhibited a decrease in the levels of tumor necrosis factor alpha (TNF-α). Anti-inflammatory was stimulated when Leukemia cell line treated with phenol extract at concentrations 200 and 400 µg ml^{-1}, TNF-α were significantly reduced and the greater reduction at the concentration 400 µg ml^{-1}. The reduction ranged from 299.30 to 129.6 Pg mL^{-1} when compared with control (untreated) cells. Additionally, interleukin produced by immune system was estimated after treatment of leukemia cells with the extracts which led to a decreased in the amount of interleukin produced to (15.40 and 7.79 pg mL^{-1}) at (200 and 400 µg mL^{-1}) respectively when compared to untreated cells.

***Keywords*---**pomegranate fruit, antioxidant, DPPH, TNFα, interleukin-6, leukemia cell line.

Introduction

Blood malignancies account for about 10% of all cancers, with a new diagnosis being made every 3 minutes in the United States. Blood malignancies are the fourth leading cause of cancer-related death in Ireland (Leukemia and Lymphoma Society 2016; Irish Cancer Society 2016). Blood cancer refers to any malignancy that affects blood cells or tissues where blood cells form, as such as the bone marrow and lymphatic system. White blood cell cancer, or leukemia, is one of the most frequent types of blood cancer (Irish Cancer Society, 2016). For many years, medicinal plants have been utilized to cure a variety of diseases. Plants' with medicinal properties have also been studied for neurological and fungal disorders, diabetes, respiratory diseases, children's diseases, hyperlipidemia and obesity, malignancies, liver diseases, cardiovascular diseases, and other diseases (Rouhi *et al.*, 2016). The potential of using pomegranate extract on inhibiting cell proliferation and induction of apoptosis was investigated on different leukemia cells. Pomegranate (*Punica granatum* L.) is a major source for phytochemicals. Fruit is one of the oldest edible fruits. It is widely grown in parts of Asia, North Africa, around the Mediterranean areas and in the Middle East. Pomegranate is used in Indian Unani medicine for treatment of diabetes mellitus (Rita *et al* ., 2020). These fruits have achieved great attention for its health benefits in the last years (Ronald *et al.*, 2013). Phenols and other antioxidant compounds are very significant for human health. They are widely present in fruits, vegetables, and beverages: coffee, tea, fruit juices, wines and other products. Pomegranate has become more popular because of the attribution of important physiological properties such as anticancer (Das *et al.*, 2012).

Materials and Methods

DPPH from SIGMA-ALDRICH / USA solutions was tested to evaluate the scavenging activity. These solutions were prepared according to previous study conducted by Patel *et al.* (2011). Aliquots (0.5ml) of two-fold serial dilution from pomegranate extracts and ascorbic acid (12.5, 25, 50, 100 and 200µg mL^{-1}) were added into reaction test tubes. Simultaneously, 3ml of methanol-DMSO mixture and 0.3ml of DPPH solution were added to each concentration. Samples were shaken few seconds and allowed to stand at room temperature for 60 minutes. Radical scavenging activity of samples against the stable DPPH radical was determined spectrophotometrically. The colorimetric changes (from deep violet to light yellow) appeared when DPPH was.

Tumor Necrosis Factor alpha kit from Elabscience / USA was used, and the standard working solution was added to the first two columns, each concentration of the solution was added to the well, (100 uL for each well). The plate was covered with a sealer provided in the kit, then incubated for 90 min at 37 °C. The solutions were added to the bottom of the micro ELISA plate well to avoid touching the inside wall and foaming. The liquid was removed out of each well, 100 µL of biotinylated detection Ab working solution was added immediately to each well, then covered with a plate sealer, mixed up gently then incubated for 1 hr at 37 °C. The solution was decanted from each well then 350 uL of wash buffer was added to each well, soaked for 1-2 min and the solution was aspirated from each well and dried using clean absorbent paper. Wash step was repeated 3

times. Aliquot of 100 µL from HRP conjugated working solution was added to each well, covered with the plate sealer then incubated for 30 min at 37 °C. The solution was aspirated from each well and the wash process was repeated for five times. Aliquot of 90 µL from substrate reagent was added to each well, covered with a new plate sealer then incubated for 15 min at 37 °C, and the plates were protected from light. The reaction time was shortened and extended according to the actual color change. Aliquot of 50 µL from stop solution was added to each well. The optical density (OD value) was determined for each well at once with a micro-plate reader set on 450 nm.

Interleukin-6 Human IL-6 ELISA Kit from Elabscience / USA was used. The standard working solution was added to the first two columns. Each concentration of the solution was added in duplicate, to one well each, side by side (100 uL for each well). The samples were added to the other wells (100 uL for each well). The plate was covered with the sealer provided in the kit. The standard was incubated for 90 min at 37 °C. Solutions were added to the bottom of the micro ELISA plate well avoiding touch inside tube wall as much as possible which may cause foaming. The liquid was decanted from each well and immediately 100 µL of Biotinylated Detection Ab working solution was added to each well, then covered with the plate sealer and gently mixed up then incubated for 1 hr at 37 °C. The solution was decanted from each well, 350 uL of wash buffer was added to each well. They soaked for 1-2 min and the solution was decanted from each well and dried with the aid of absorbent paper. Wash step was repeated 3 times. Aliquot of 100 µL from HRP conjugate working solution was added to each well, covered with the plate sealer, then incubated for 30 min at 37 °C. The solution was decanted from each well. Aliquot of 90 µL from substrate reagent was added to each well then covered with a new plate sealer, incubated for about 15 min at 37 °C and the plate was protected from light. The reaction time was shortened and extended according to the actual color change. Aliquot of 50 µL from stop solution was added to each well. Optical density (OD value) of each well was read at once with a micro-plate reader set on 450 nm.

Methanolic Extraction: A quantity of 50g of pomegranate powder was mixed with 250ml of 96% methanol and placed in Soxhlet apparatus for 6 hr at 70 °C, then the solvent was removed under reduced pressure by a rotary evaporator at 40 °C. The crude solid extracts were kept in a deep freezer until use (Satheesh *et al.*, 2012).

Total phenolic concentration

Total phenolic content (TPC) was determined by the Folin-Ciocalteu colorimetric method described by Fawole *et al.* (2011). Briefly, aliquot of extract (50 µL) was mixed with 450 µL of 50% methanol followed by addition of 500 µL Folin–C and then sodium carbonate 2% solution after 2 min. The mixture was vortexed and absorbance read at 725 nm using UV–visible spectrophotometer. Results were expressed as Gallic acid equivalents (GAE) per 100 mL extracts.

Cell Culture

Human hepatic cell line (WRL 68) and Leukemia cell line (HL60) obtain from Centre of Biotechnology Research, Al-Nahrain University, Baghdad, Iraq. Both cell lines were cultured in RPMI 1640 medium containing 10% (v/v) fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in a humidified 5% CO2 incubator. Freshney's, (2010) protocols were followed while preparing the solutions and media for cell culture.

Experimental designed and statistical analysis

Experiments were conducted using completely randomized design with three replicates. One-way analysis of variance (ANOVA) was performed. The significance of the results and correlation was evaluated using Graph Pad Prism version 9. P values at ≤ 0.05 were considered statistically significant. Means were also expressed as mean ± standard deviation.

Results

Free radical scavenging activity using DPPH

The scavenging activities of the pomegranate fruit extract at the concentrations 12.5, 25, 50, 100 and 200µg mL^{-1} were measured (Figure 1). The results display an effective free radical scavenging activity of phenols were 16.74, 25.12, 40.63, 68.87 and 81.98 µg mL^{-1} at concentrations above respectively. The pomegranate fruit extract reduced the pattern of free radical DPPH significantly at 25 and 50 µg mL^{-1}. While no significance at 12.5, 100 and 200 µg mL^{-1} when compared with ascorbic acid. This indicates that the phenol compounds are strong antioxidant on leukemia cell line.

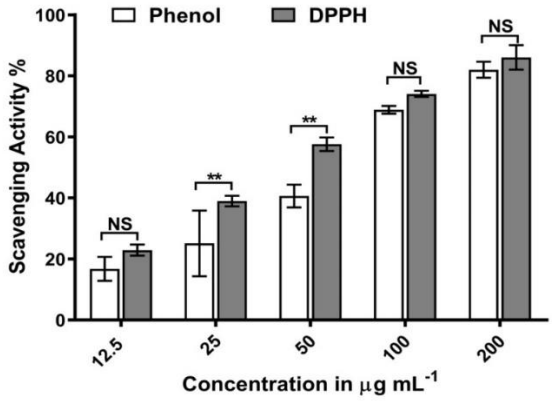

Figure 1. Scavenging activity of phenol compounds vis scavenging activity of ascorbic acid

Tumor necrosis factor alpha (TNF-α)

Anti-inflammatory was stimulated when Leukemia cell lines were treated with phenols at the concentrations 200 and 400 µg ml^{-1} (Figure 2). TNF-α were significantly reduced and the reduction occurred at the concentrations of 200 and 400 µg ml^{-1} was 299.30 and 129.6 pg ml^{-1} when compared with control (untreated) cells.

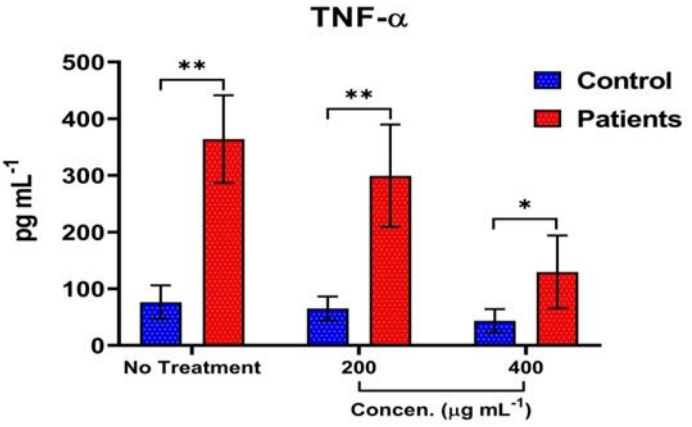

Figure 2. Effect of pomegranate phenol extract on production of tumor necrosis factor alpha in the HL60 cancer cell line.

Interleukin

The following concentrations 200 and 400 µg ml^{-1} of pomegranate fruit extracts were tested on HL60 cell line to detect the decreased production of interleukin-6. The production of IL-6 in the HL60 cells reduced when treated with 400 µg ml^{-1} when compared with control and there is significance between the two concentrations.

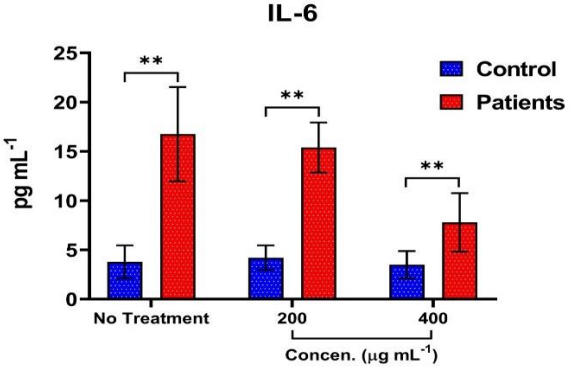

Figure 3. Effect of pomegranate phenol extract on the production of interleukin- 6 in the HL60 cancer cell line

Discussions

The results were compared with free radicals scavenging activity of ascorbic acid as a positive antioxidant, the methanolic extract of the pomegranate fruit showed almost the same pattern of ascorbic acid with free radical scavenging activity particularly at concentrations 12.5, 100 and 200 µg ml^{-1} of pomegranate extract. Pomegranate extract is rich in phenolics and flavonoids thus presented an excellent DPPH radical scavenger activity (Suleman *et al.*, 2019). The DPPH is characterized as a stable radical owing to the delocalization of the spare electron with maximum absorption at about 517 nm, and it has been frequently used to determine the antioxidant activity of different substances including plant extracts (Jean *et al.,* 2012). Also, the results above showed a strong reduction in the production of TNF-α and IL-6 in HL60 cell line, interleukin 6 is a pro-inflammatory and anti-inflammatory cytokine that is one of the most prominent mediators of the acute phase response. This interleukin regulates TNF-α activity and stimulate the secretion of (VEGF) vascular endothelial growth factor, which is a well-known potent angiogenesis factor with pro-inflammatory effects in cancer cells these results indicating strong anti-inflammatory effects. Such results make extracts of pomegranate promising source of anti-inflammatory candidates to be used in the pharmacological industry (Kasprzyk et al., 2012).

Conclusions

Methanolic extract of pomegranate contain many bioactive phytochemicals that have been shown to exhibit antioxidant action against HL60 cell line.

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