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Phytochemical, antioxidant and cytotoxic potential of *punica granatum* extracts on leukemia cell line

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Abstract--Pomegranate fruit is edible and has been used on leukemia cell lines and protecting DNA damage known antioxidant efficacy the aim of the current work is to explore the potential benefit of pomegranate (*Punica granatum*) extract as antioxidant and cytotoxic to leukemia cell line. Methanol was used to extract pomegranate fruit so that to test its antioxidant and cytotoxicity on two cell lines (Hepatic cell line WRL68 and Leukemia cell line HL60). This assay was classified as determining antioxidant effect using 3-(4,5-dimethylthiazol-2yl) 2,5diphenyl tetrazolium (MTT) assay on two cell lines (Hepatic cell line WRL68 and Leukemia cell line HL60) for different concentrations (25, 50, 100, 200 and 400 $\mu\text{g mL}^{-1}$). These examine the efficacy of the phenol extract on HL60 cell apoptosis, morphology, viability of cells, total nuclear intensity, cell membrane potential, mitochondrial membrane potential, and cytochrome C release were evaluated by High Content Screening (HCS) assay. The current results revealed that the methanol extract of pomegranate fruit had the strongest antioxidant activity, which increased when concentrations were raised (28.27 and 61.57%). The results demonstrated that phenol can inhibit HL60 cell line from growing, with increased concentrations. The inhibition become stronger at 400 $\mu\text{g mL}^{-1}$ of phenol extract. The HL60 cell line were more effective than the WRL68 cell line. High Content Screening (HCS) assay of the following concentrations (12.5, 25, 50, 100, and 200 $\mu\text{g mL}^{-1}$) of methanol extract of pomegranate fruit showed toxic effect on HL60 cell line after 24 hr of treatment at 37 °C, with a decrease in the number of viable cells, a decrease in the mitochondrial membrane potential,

an increase in the membrane potential, an increase in nuclear intensity and increase in cytochrome C release.

Keywords---Pomegranate fruit, DNA damage, leukemia, phenol, MTT assay, HCS method.

Introduction

The effectiveness of these components of pomegranate fruit against tumor activity is considered to be related to communications with the enzymes involved in neoplasia, induced blocking of DNA replication by inhibiting the activity of enzymes such as DNA polymerase II and topoisomerases I and II also take part in inhibition of the cell cycle, which results in blocking production and inducing apoptosis of cancer cells (Kumar and Pandey, 2013). Approximately, 10% of all cancers are blood cancers, with a new finding given every 3 minutes in the United States. In Ireland, blood cancers are the fourth most common cause of cancer-related deaths (Leukemia and Lymphoma Society 2016; Irish Cancer Society 2016). Blood cancer is a wide term used to classify any cancers that affect cells of the blood, or organs where blood cells mature, the bone marrow and lymphatic system. Leukemia, cancer of white blood cells, is one of the most common types of blood cancer (Irish Cancer Society, 2016). Chronic lymphocytic leukemia (CLL) is the most prevalent leukemia in adults in the western world and accounts for 25-30% of all leukemia's. Myelogenous leukemia is a main type of leukemia and characterized by the proliferation of myeloid tissue and increase in the number of granulocytes, myelocytes, and myeloblasts in the circulating blood (Ghia, 2007).

Aim of study

The aims of this study were to evaluate the antitumor and inhibitor activity of pomegranate extracts *in vitro* used WRL68 cell line and HL60 cell line has been evaluated the viability activity by using MTT assay and HCS for detective subsequent cellular parameters: membrane porousness, cell viability, total nuclear intensity, cytochrome C release and mitochondrial membrane potential changes.

Materials and Method

MTT assay: This assay was done according to the instructions of the company, this test done according to (Shing *et al.*, 2015) to determine the viability of cells was from Intron Biotech / Korea.

Multi-parameter cytotoxic assay: The double parameter toxicity experiment was done on WRL68 and HL60 cell lines *in vitro*. The parameters were: Viability cell count, total nuclear intensity, semi-permeable membrane porousness, mitochondrial membrane porousness and cytochrome unharness. Briefly, once twenty-four hours of exposure with totally different concentrations of pomegranate extracts, the treated HL60 cells were stained with cell staining resolution for thirty min at 37 °C. Cells were mounted, permeabilized and blocked before inquiring with primary cytochrome protein and secondary DyLight 649

conjugated goat anti-mouse IgG for sixty min every. Plates were analyzed by Array Scan HCS instrument. Cellomics multiparametric cytotoxicity 3 kit used to determine some parameters changes were obtained from Thermo Scientific USA.

Plant Materials: Pomegranate fruit were purchased from local market in Baghdad city, Iraq.

Methanolic Extraction: A quantity of 50g of pomegranate powder was mixed with 250ml of 96% methanol by soxhlet apparatus for 6 hrs. 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 by (Fawole *et al.*, 2011). Briefly, 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.

MTT Viability assay: The assay was done to determine the viability according to the manufacturer's instructions.

Multi-parameter cytotoxic assay: This assay was done to determine the change in some parameters according to (Al-Saffar *et al.*, 2017).

Experimental designed and statistical analysis: Experiment were conducted using completely randomized design with their replicates as required. 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 ≤ 0.05 were considered statistically significant. Means were also expressed as mean ± standard deviation.

Results

Viability assay

In vitro HL60 cultures were exposed directly to different concentrations (25, 50, 100, 200 and 400 µg mL⁻¹) of pomegranate extracts crude, phenol and flavonoid. A gradual decrease in viability occurred in both cell lines as the concentrations increased. The results for WRL after treatment with crude were 94.83, 94.17, 90.12, 80.83 and 71.95% and for HL60 were 95.06, 83.76, 72.69, 60.96 and 56.25% followed by phenol treatment results for WRL were 94.52, 93.63, 90.35,

80.94 and 71.73% and for HL60 were 83.18, 70.91, 63.17, 44.41 and 38.43% while the treatment of flavonoid showed results for WRL 94.83, 93.79, 93.60, 83.64 and 71.57% and for HL60 were 95.18, 82.72, 71.91, 58.83 and 50.23% respectively. HL60 cell lines decreased after treatment with pomegranate fruit extracts in variable numbers by increasing the concentrations of the extracts, this revealed that the crude extract showed significant decrease in the survival rate of HL60 cell lines in dose dependence and 55.9% cell death rate at 400 $\mu\text{g mL}^{-1}$ with IC_{50} 67.04 $\mu\text{g mL}^{-1}$ (Figure 1). And phenol showed decrease in cell viability which recorded value 61.5% at 400 $\mu\text{g mL}^{-1}$ with IC_{50} 101.1 $\mu\text{g mL}^{-1}$ (Figure 2). While flavonoid extracts showed least activity lower than phenols and crude in cell viability decreased in value 49.7 at 400 $\mu\text{g mL}^{-1}$ with IC_{50} 66.65 $\mu\text{g mL}^{-1}$ (Figure 3).

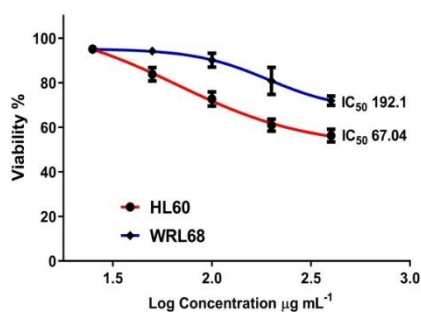


Figure 1. Viability % of crude extract on WRL and HL60 cell lines using MTT assay.

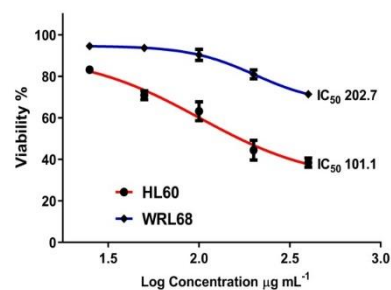


Figure 2. Viability % of phenols extract on WRL and HL60 cell lines using MTT assay.

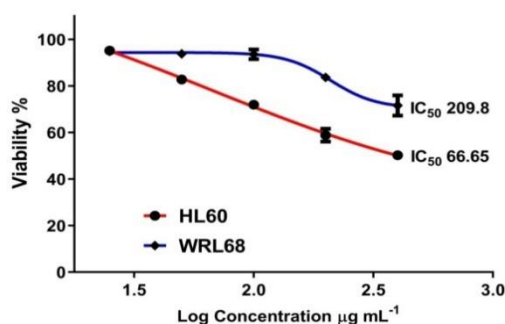


Figure 3. Viability % of flavonoid extract on WRL and HL60 cell lines using MTT assay.

Multi-parameter cytotoxic potential

The results presented in (Figure 4) Indicated that the viable count of HL60 cell treated with phenol compounds decreased at concentration 100 and 200 $\mu\text{g mL}^{-1}$ compared with untreated cells. The 55% reduction in cell counts detected at 200

$\mu\text{g ml}^{-1}$ was significantly different from the control. Other concentrations 12.5, 25 and 50 $\mu\text{g mL}^{-1}$ showed non significantly different when compared with control. Nuclear intensity in HL60 increased significantly when treated with different concentrations 12.5, 25, 50, 100 and 200 $\mu\text{g ml}^{-1}$ of phenol extract (Figure 5) and the increasing percentage of nuclear intensity were 418.5, 422.5, 457.5, 754.5 and 939.5 $\mu\text{g ml}^{-1}$ respectively. While there was no significant difference between the concentrations 12.5, 25, and 50 $\mu\text{g ml}^{-1}$ and un treated cells.

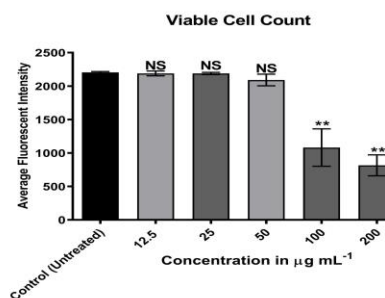


Figure 4. Phenol extract effect on cell viability in HL60 cell line after 24 hr of incubation at 37 °C and evaluated by the array scan HCS reader.

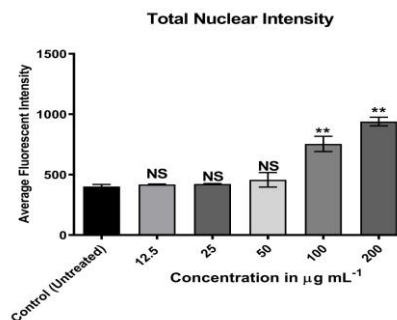


Figure 5. Phenol extract effect on nuclear intensity in HL60 cell line after 24 hr of incubation at 37 °C and evaluated by the array scan HCS reader.

Exposure of HL60 cell line to phenol extracted at different concentrations 12.5, 25, 50, 100 and 200 $\mu\text{g ml}^{-1}$ as a result of enhanced nuclear swelling and cell membrane permeability, the size of the nucleus increased. The increased in nuclear size at the concentration 200 $\mu\text{g ml}^{-1}$ was dependent from control which typically occurs at the high concentrations of phenol extracts. The effects were considerably different from untreated cells, while the other concentrations 12.5, 25, 50 and 100 $\mu\text{g ml}^{-1}$ showed non significantly difference when compared with un treated cells and they were induced at lower concentrations of phenols extract (Figure 6).

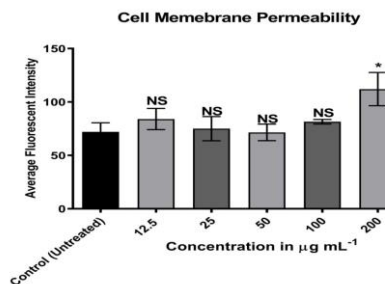


Figure 6. Phenols extract effect on cell membrane permeability in HL60 cell line after 24 hr of incubation at 37 °C and evaluated by the array scan HCS Reader.

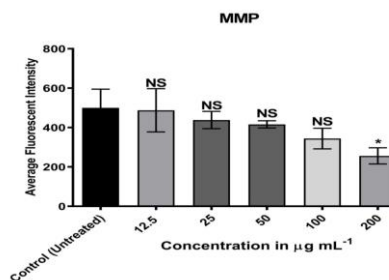


Figure 7. Phenols extract effect on mitochondrial membrane potential in HL60 cell line after 24 hr of incubation at 37 °C and evaluated by the array scan HCS reader.

From (Figure 7) the phenols extract at 200 $\mu\text{g ml}^{-1}$ caused decrease in mitochondrial membrane potential intensity respectively, and the effect of phenols extract was dose dependent. The increased in mitochondrial membrane permeability was detected compared to un treated cells. While the other concentrations 12.5, 25, 50 and 100 $\mu\text{g ml}^{-1}$ showed non significantly difference with un treated cells. Cytochrome C releasing results in (Figure 8) indicated that cytochrome C releasing rise significantly with the increasing of concentration 12.5, 25, 50, 100 and 200 $\mu\text{g ml}^{-1}$ when compared with control and the mean of increasing were 329.0, 386.5, 392.5, 446.0 and 509.0) respectively.

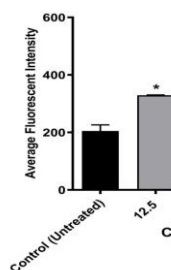


Figure 8. Phenols extract effect on Cytochrome C release in HL60 cell line after 24 hr of incubation at 37 °C and evaluated by the Array Scan HCS Reader.

Discussion

Our study reported that the confirm of some of the essential agents identified from whole Pomegranate fruit and their biological potency. This method of analysis is the simple and is the first line towards check the nature of active principles in this natural extract and this type of study will aid for further detailed in the future study. There is a growing awareness in correlating the phytochemical compounds and their biological activities (Hariprasad and Ramakrishnan, 2011). Many plant species are already being used to treat or prevent growth of cancer. Many researchers have identified species of plants that have demonstrated anticancer properties with a lot of focus on those that have been used in herbal medicine in developing countries (Ochwang *et al.*, 2014). By regulating and evaluating the health of active plant-derived compounds, herbal drugs can help the emergence of a new time of the healthcare system to treat human diseases in the future. Awareness of traditional knowledge and medicinal plants can play a key role in the exploitation and detection of natural plant resources (Jamshidi *et al.*, 2018).

With successful clinical trials drugs being developed from plant origins are popular for clinical development. Their non-toxic effects on normal cells and their cytotoxic effects on cancer cells put them in high demand. A lot of the species investigated are selected from developing countries in Africa and Asia where herbal therapies are practiced and medicinal plants are trusted for primary

treatment (Ochwang *et al.*, 2014). On the other hand, the use of dye stained the membrane permeability and the increased of intensity of this stains, especially at the massive dose received, potentiate the fact that the phenol extract can stimulate apoptosis in HL60 cell lines, the permeability of plasma membrane increases may be because the loss of integrity of membrane that dye penetrate cell easily (Ye *et al.*, 2007; Jasim *et al.*, 2019).

The permeability transition of mitochondrial pores, permitting the transition tiny molecules and ions, like atomic number 20 ions and therefore resulting in the decoupling of the metabolic process chain and unharness of cytochrome of the cytoplasm (Susin *et al.*, 2019). Finally the secrete of cytochrome stimulate a variety of caspases, specifically amino acid proteases, that are essentially support digestion of the cell from within in addition as degradation (Tafani *et al.*, 2003).

Conclusion

Methanolic extract of pomegranate contain many bioactive phytochemicals that have been shown to exhibit cytotoxicity and antioxidant action against the WRL68 and HL60 cell line.

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