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Antileukemic Effect of Polyphenol of Local Grape Seeds Extract from Duhok/Kurdistan of Iraq: *in vitro* Study

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Abstract The present study was conducted to evaluate the cytotoxic effect of secondary metabolites extract (polyphenol) from local grape seeds *of vitis viniferra* (rash meu) from Duhok Governorate / Kurdistan of Iraq using MTT assay. Different concentrations 39.1 µg/ml, 78.125 µg/ml, 156.25 µg/ml, 312.5 µg/ml µg/ml 625 µg/ml, µg/ml 1250 µg/ml, and 2500µg/ml of polyphenol extract were tested against the proliferation of acute leukemic lymphocytes of Acute Myeloid Leukemia (AML) and acute Lymphoid Leukemia (ALL) patients in culture within 24 and 48 hours exposure. This study showed that a polyphenolic extract that isolated from seeds of local grape is substantially rich in phenolic content and flavonoid. The result of cytotoxicity assay revealed that all concentrations of polyphenol extract were effective against the proliferation of both types of acute leukemic lymphocytes of AML and ALL patients. Statistical analysis for all patients with AML and all patients with ALL was revealed that the higher concentrations 1250µg/ml and 2500µg/ml were effected after both time of exposure 24 and 48hours. The result of cytotoxicity assay showed that the highest concentration 2500µg/ml was more effected after 24 and 48 hours of all acute leukemic cases. The CC50 value was 859µg/ml after 24hours of treatment. The result of present study showed no significant different between two type of acute leukemia AML and ALL in to response to cytotoxic effect of polyphenol extract.

Keywords Leukemia, Polyphenol, Cytotoxicity, CC50.grape seed extract

1. Introduction

Leukemia is a type of cancer that related to blood or bone marrow and characterized by an abnormal increase of white blood cells which called blasts (Tenen, 2003). Leukemia was classified according to pathological and clinical criteria into: Acute and chronic leukemia.

Acute lymphoblastic leukemia is malignant disease of lymphocyte cell precursors and characterized by the overproduction and accumulation of cancerous, immature white blood cells, known as lymphoblasts (Ersvær, 2007). Acute myelogenous leukemia is an aggressive malignancy characterized by accumulation of immature myeloid cells in the bone marrow and interferes with the production of normal blood cells (Ersvær, 2007).

The incidence of leukemia in the world is about 1 per 100,000 per year, estimated new cases in 2015 over world was about 54,270 cases which was about 3.3% of all new cancer cases, while estimated deaths in 2015 was about 24,450 which was 4.1% of all cancer death (NCI, 2015). Study of CAGS, (2015) has shown that leukemia is one of the 10 most common malignancies in Jordan, Lebanon, Bahrain, Egypt, Libya, Kuwait, Oman, Qatar, Saudi Arabia, Syria, the United Arab Emirates, and Iraq. In most of these countries, leukemia is also the major form of pediatric cancer. The most common leukemic form is ALL, followed by AML. Abdulbari, (2007) found a high rate of ALL32% among males in the United Arab Emirates. The increased rate of leukemia in several Iraqi cities has been linked to exposure to depleted uranium, which was used during the consequent wars in Iraq from 1990 to 2010 (Abdullatif, 2006). During 1980 to 2010, there were 1222 cases of leukemia of whom 250 female and 401 male cases diagnosed among age groups (1–19), and 265 female and 306 male cases diagnosed among age groups (20 - ≥70) in Ninawa\ Iraq (Muzahem, 2013).

In Kurdistan region\ Sulaimaniya governorate, Leukemias is the second malignancy after lung cancer in males and in females after breast cancer, and in both sexes account for the third most common cancer, the annual incidence rates in both sexes were 4.1/ 100,000 (Khoshnaw, 2015).

Plant extracts are one of the most commonly used complementary and alternative therapies by people with cancer. Some studies have shown that as many as 6 out of every 10 people with cancer use medical plant extracts alongside conventional cancer treatments (Mukherjee,

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2001). There are mainly three major types of plant derived extract that include inhibitor of carcinogen formation, blockers of carcinogen interaction and suppressor of tumor progression (Mukherjee, 2001). Polyphenols are a group of chemical compounds found naturally in plants such as apples, grapes, wines, strawberries, this natural extracts attracted much attention in the last decades because they act as antioxidants which provide numerous health benefits by protecting cells against damage caused by free radicals (Ramos, 2007). The antibacterial, anti-inflammatory and anti-allergenic properties of these products have also contributed to the prevention of many diseases, such as different types of leukemia (Ramos, 2007). The most commonly treatment used for leukemia are chemotherapies. These chemotherapies drugs are expensive, mutagenic and considerable side effects. In addition, patients often fail to get complete disease remission, due to increased occurance of drug resistance. Therefore current have lead to the search for new compounds for the treatment of leukemia (Dahlawi *et al*., 2012).

Phenolic compound are the most abundant secondary metabolites present in the plant kingdom, grape seed extract causes commit cell suicide *in vitro*. Wang, (2012) found that 76 percent of leukemic cell had died after being exposed to the polyphenol grape seeds extract within 24hours. It activated c-Jun NH2-terminal kinase (JNK), a protein that regulates the apoptotic pathway, and it lead to cell death or apoptosis.

Because no study was found in our region about the evaluation the cytogenetic abnormality in AML and ALL patients and no study was found also about the cytotoxic effect of local grape seeds extract (polyphenol) on acute leukemic lymphocytes, Therefore the present study was conducted to: determine the cytotoxic effect of polyphenol extract from seeds of local grape on the growth of acute leukemic lymphocytes of both AML & ALL patients.

2. Material and Methods

Grape seed collection and polyphenol extraction

Vitis vinifera (rash meu) black grape 20Kg were collected from Dohuk governorate (Amedi). Approximately 900gm of the seeds was isolated from these grape fruit. All seeds were isolated from grape fruits dried at room temperature seeds were ground into powder by electrical grinder (mesh No.0.5mm) the powdered was kept in plastic tubes in deep freezer -20 until the time of use. Fifty gram of powdered seeds was suspended with 200ml of aqueous acetone 70% in a conical flask one liter volume and stirred on a magnetic stirrer, the mixture was left in magnetic stirrer about 24hours at room temperature, then filter through gauze and then by filter paper, the resultant solution put in Petridis that was pre weighted, and placed in laboratory until dried Harborne, (1984). Acetone was evaporated, and the Petridis was weighted, again after drying the yield of extraction was calculated by using the following equation: (Yield of extraction)%=Weight of Petridis with extract-weight of empty Petridis\50x100. The powder then put in refrigerator prior biological test (Yu and Dahlgren, 2000).

Blood sample collection

Venous blood 4ml was taken by disposable syringe from 20 untreated of leukemic patients that attendance to Hivi hospital, Azadi hospital, and central laboratory in Duhok city from 1.11.2015-1.7.2016. All blood samples were transformed into heparinized tubes for cytotoxicity assay.

Isolation of lymphocytes from blood

Lymphoflot density gradient centrifugation method according to Blasiak *et al.*, (2001) was used to isolation of lymphocytes. The viable cell appear normal don't stain but dead cell appear blue because take up the stain (Darling and Morgan, 1990). More than 95% of the lymphocyte, viability was ensured to perform the next assay study (Boyum *et al.,* 2002).

Lymphocyte culturing:

After counting, cell were suspended in 50 ml culture RPMI 1640 medium in culture flask supplemented with 5% fetal bovine serum (FBS), 10ml/20 m M/L hepes buffer and 1% antibiotics (100 MI\ ml penicillin and 100 unites\ ml streptomycin) PH7. Culture flask was incubated at 37°C in humidified incubator containing 5% CO2 and 95% air about 24 hours (Dogan *et al.,* 2004).

MTT assay for detect the cytotoxic effect of polyphenol extract on lymphocyte cell:

This assay display a correlation between cell number and the spectrophotometric absorbance (Juan *et al*., 2003). After 24 hours of incubation, cells suspension for each patient was transformed into two groups (A and B) of Eppendorf tubes each group contains 35 tubes (100µl) each, then adding 100µL of different concentration (39.1, 78.125, 156.25, 312.5, 625, 1250, 2500 µg/ml) of polyphenol that extracts from grape seeds (5 replicate for each concentration), 100µL of RPMI -1640 medium was added to other 10 epindoff tubes that contains 100 µL of cell suspension as a control. All tubes transfer into CO2 incubator. Tubes of group A were incubate for 24hours, after this time all tubes subsequently centrifuge at 1500rpm for 10min., then supernatant was discarded, and 120µL of growth RPMI 1640 was added to each epindoff, after that 20µL of MTT added to each tube. Put back all tubes to CO2 incubator for 3hours, after that centrifuge in 1500rpm for 10min. discard the supernatant and remaining about 20µL transfered to each well 96-micro titration plate of ELISA, at the end adding 180µL of DMSO to each well and shaking about 15 minutes by shaker. Tubes of group B incubate for 48hours after this time all tubes subsequently centrifuge at 1500rpm for 10min., then supernatant was discarded and 120µL of growth RPMI -1640 medium was added to each epindoff, after that 20µL of MTT added to each tube, put back all tubes to CO2 incubator for 3hours, after that centrifuge in 1500rpm for 10min. discard the supernatant and remaining about 20µL transfered to each well 96micro titration plate of ELISA, at the end adding

180µL of DMSO to each well and shaking about 15 minutes by shaker. The optical density of each well was read using Enzyme Linked Immunosorbent Assay (ELISA) Reader at transmitting wavelength on 492nm (Betancur-Galvis *et al*., 2002). To know the percentage of cytotoxicity this can be done by using the following equation= (A-B)\AX100.

A= the mean of O.D. of untreated cells
B=the O.D. of cells, treated with extract

The cytotoxic concentration 50% (CC50%) was defined as the concentration (µg/ml) of extract that required to the reduction of cell viability by 50%was calculated from concentration effect-curves after linear regression analysis (Hayslett and Patrick, 1981).

Statistical analysis:

Lymphocytic cell MTT assay experiments results were subjected to statistical analysis by using SSPS program to find the effect of-different factors(treatment, time of exposure, and there interaction) for cytotoxicity of different concentrations of polyphenol that was extracted from *vitis viniferra*. The least significant difference (LSD) was used to determine the significant differences between levels of each factor (Steel and Torrie, 1980).

3. Results

The result of secondary metabolites (polyphenol) extract from grape seeds of *vitis vinifera* (rash meu) revealed that the polyphenol as a Semi-solid brown matter, the yield of extraction was 12.%.

Cytotoxic effect of polyphenol extracted from *vitis vinifera* grape seeds on lymphocytes of AML and ALL patients using MTT assay.

Blood samples were collected from11AML patients and 9ALL patients. Lymphocytes were isolated using (Lymphoflot density gradient centrifugation) Viability of lymphocyte were detected by using trypan blue exclusive assay, by which cell viability was 95%-98%.

Cytotoxic effect of polyphenol extract *vitis vinifera* grape seeds on the growth of leukemic cells of AML type:

The result of statistical analysis for data (O.D) of leukemic cells of all eleven patients with AML type in the present study showed highly significant differences ($p \leq 0.001$) between two times of exposure (24,48 hours) and among all concentrations on the proliferation of leukemic cells. The differences were non-significant in interaction between the time of exposure and the concentrations. The statistical analysis by L.S.D revealed that both times of treatment 24 and 48hours has the same effect against the proliferation of leukemic cell of AML patients (L.S.D=0.046) (Table 1). The highly effective concentrations were 1250 µg/ml and 2500 µg/ml after 24 and 48 hours of treatment the O.D were 0.1602±0.0058, and 0.1425 ± 0.0049, (L.S.D=0.054) (Table 1).

The result of this study showed that the effect of polyphenol extract of *vitis viniffera* grape seeds on AML type after 24hours has a value of 1015µg/ml CC50.

Table (1). Mean ± SE for the effect of different concentrations of polyphenol extract of *vitis vinifera* grape seeds on the proliferation of leukemic cells of AML type after 24 and 48 hours in culture (observation O.D)

Concentration µg/ml	24 Hours Mean ± SE	48 Hours Mean ± SE
0	0.3321±0.0134	0.3252±0.0088
39 µm	0.2938±0.0121	0.2906±0.0085
78 µm	0.2630±0.0108	0.2575±0.0081
156 µm	0.2347±0.0969	0.2282±0.0076
312 µm	0.2061±0.0850	0.1979±0.0068
625 µm	0.1836±0.0070	0.1683±0.0066
1250 µm	0.1602±0.0058*	0.1388±0.0053
2500 µm	0.1425±0.0049*	0.1156±0.0046

Cytotoxic effect of polyphenol extract *vitis viniffera* grape seeds on the growth of leukemic cells of ALL type:

Statistical analysis of leukemic cells of all nine patients with ALL type in this study showed highly significant differences ($p \leq 0.001$) between two times of exposure (24,48 hours) and among all concentrations on the proliferation of leukemic cells in ALL type, whereas the differences were non-significant in interaction between the time of exposure and the concentrations. The result of present study revealed that both times of treatment 24 and 48hours has the same effect against the proliferation of leukemic cell of ALL patients (LSD = 0.0417) (Table 2). The highly effective concentrations were 1250 µg/ml and 2500 µg/ml after 24 and 48 hours of treatment the O.D were 0.1591± 0.005, 0.1341±0.004 (.LSD…0.0421) (Table 2). The polyphenol extract on lymphocytic cells of AML type presented CC50 value of 1091µg/ml after 24hours.

Table (2). Mean±SE for the effect of polyphenol extract of *vitis vinifera* grape seeds on the proliferation of leukemic cells of ALL

Concentration µg/ml	24 Hours Mean ± SE	48 Hours Mean ± SE
0	0.3397±0.0127	0.3156±0.007
39 µm	0.3025±0.01184	0.2833±0.006
78 µm	0.2718±0.0106	0.2560±0.006
156 µm	0.2377±0.009	0.2281±0.0057
312 µm	0.2088±0.008	0.1973±0.0050
625 µm	0.1872±0.006	0.1700±0.0048
1250 µm	0.1592±0.005*	0.1410±0.0043
2500 µm	0.1341±0.004*	0.1170±0.0035

Effect of polyphenol extract of *vitis vinifera* grape seeds on types of leukemia

Although polyphenol extract of grape seeds showed significant cytotoxic effect on both types of acute leukemia (AML and ALL). However statistical analysis showed no significant different ($p \leq 0.05$) between two type of acute leukemia AML and ALL 0.2211±0.0030 and

0.2218±0.0031 respectively in response to effect of polyphenol extract against the proliferation of leukemic cells, as shown in figure (1).

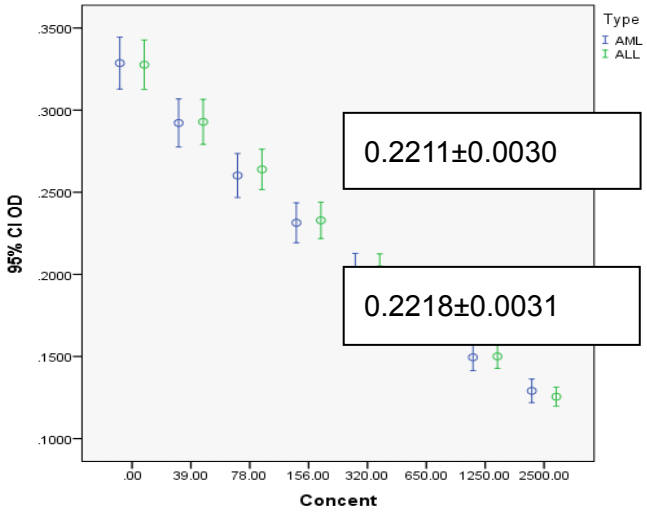

Figure 1. The effect of polyphenol extract of *vitis vinifera* grape seeds on two types of leukemic cells AML, ALL

Cytotoxic effects of polyphenol extract of vitis vinifera grape seeds on the growth of acute Leukemic cells in leukemia patients

Statistical analysis in this study shows highly significant differences ($p \leq 0.001$) between two times of exposure (24,48 hours) and among all concentrations on the proliferation of leukemic cells in both types, whereas the differences were non-significant in interaction between the time of exposure and the concentrations.

Table (3). Mean±SE for the effect of polyphenol extract of *vitis vinifera* grape seeds on the growth of acute Leukemia patients

Concentration (µg/ml)	24 Hours Mean ± SE	48 Hours Mean ± SE
0	0.3354±01314	0.3209±00882
39	0.2976±01211*	0.2873±.00855*
78	0.2669±01088*	0.2568±00811*
156	0.2360±.00969*	0.2282±00763*
312	0.2070±00850*	0.1976±00687*
625	0.1852±00703*	0.1691±00668*
1250	0.1592±00588*	0.1397±.00539*
2500	0.1387±00497*	0.1163±.00464*

All concentrations of polyphenol extract (39.1µg/ml, 78.125µg/ml, 156.25µg/ml, 312.5µg/ml, 625µg/ml, 1250µg/ml, and 2500µg/ml) were effective against the proliferation of leukocytic cells of acute leukemia and in both times of exposure (24,48 hours). The result of present study shows no difference between two times of exposure 24and 48 hours of leukemic cells into polyphenol extract (LSD=0.0436) (Table 3). The highly effective concentration was the highest concentration there O.D=0.01387±0.007 µg/ml.

The result of this study showed that the polyphenol extract of *vitis vinifera* grape seeds on both type of acute leukemic cell has a value of 859 µg/ml CC50 after 24 hours.

4. Discussion

Polyphenol extract of grape seeds of *vitis vinifera*

The result of extraction in this study reveals that the yield of extraction of polyphenol was 12.4%. In the current study the acetone was used to polyphenol extraction from grape seed of *vitis vinifera*, because this solvent is the best solvent that used in extraction method (Dumon., 1990). Dumon, (1990) found among the liquid / liquid extraction systems, acetone/water produces a better desired result of extraction of proanthocyanidins than the other liquid/ liquid systems. This section will therefore not try to establish a standard of extraction of proanthocyanidins. Rather it is just for overview of what else may be used to extract proanthocyanidins from various plant materials, not limited to grape seeds. The result that was obtained by (Alassady *et al*., 2011) revealed that the yield of extraction of polyphenol from Mature fruits of *Capparis spinosa* when used acetone as a solvent in extraction was 15.3%. The acetone had significant effect on polyphenol content and antioxidant activity. Polyphenol extract from black tea by acetone had the greatest antioxidant activity (Nihal *et al*., 2005). The results of (Nihal *et al*., 2005) shows that the solvent with different polarity had significant effect on polyphenol content and antioxidant activity, a high correlation between polyphenol content and antioxidant activity of tea extracts was observed. According to phytochemical test the result of this study showed a positive result for the presence of flavonoids and polyphenol.

Cytotoxic effect of polyphenol extract of vitis vinifera grape seeds on the lymphocytes of AML and ALL types:

Statistical analysis revealed that both times of treatment 24 and 48hours has the same effect against the proliferation of lymphocytes of AML patients. The highly effective concentrations were 1250 µg/ml and 2500 µg/ml after 24 and 48 hours of treatment. The result of this study showed that the effect of polyphenol extract on AML type after 24hours has a value of 1015µg/ml CC50. Grape seed extract was represented a hopeful treatment in AML type (Ning, 2009). An additional study was reported that grape seed extract promotes the death of human AML cells *in vitro* (Hu and Qin, 2006). Another study by Hong and Yi-Min, (2006) was confirmed also that the grape seed polyphenol extract induced apoptosis at 50Mg/ml on AML human cell 14.3D10 cells. The activity of grape seeds extract against the prpliferation of AML cells in this study maybe according to the presence of Resveratrol in this extract which is found abundantly in grapes (Ghorbani, 2015). Goa X *et al*., (2002) found that the Resveratrol (trans -3,4; 5-trihydroxystilbene) that extracted from grape seeds

has antileukemic activity *in vitro* and *in vivo* using a mouse myeloid leukemia cell line (32Dp210). Study of GaoX *et al*., (2002) confirmed that the treatment of 32Dp210 leukemia cells with 25-50micromol/L resveratrol was significantly and irreversibility inhibited their clonal growth *in vitro*. The clonal growth inhibition by resveratrol was associated with extensive cell death and an increase in hypodiploid (sub-G1) cells. Resveratrol caused internucleusomal DNA fragmentation, suggesting apoptosis as the mode of cell death in 32Dp210 cells.

Statistically the same result was revealed in case of ALL patients. The highly effective concentrations were 1250 µg/ml and 2500 µg/ml after 24 and 48 hours of treatment the CC50 value was 1091 µg/ml after 24 hours of treatment. The inhibition activity of polyphenol that extracted from the seeds of local grape against ALL cells in current study can be traced into Resveratrol, which is found abundantly in grapes (Ghorbani, 2015). Resveratrol is a DNA damage agent and has ability to inhibition the growth of ALL cells (Arman Ghorbani, 2015). Study of Dorrie *et al., (*2001) showed that the resveratrol can activating the cell death via mitochondria depolarization and activation of caspase-9 in resveratrol treated cells.

Differential Effect of polyphenol extract of *vitis vinifera* grape seeds in human Acute leukemia (AML andALL):

Statistical analysis showed no significant different ($p \leq 0.05$) between two types of acute leukemia AML and ALL in regards to cytotoxic effect of polyphenol extract against the proliferation of leukemic cells. The similarity in sensitivity of two types of Acute leukemia AML and ALL into polyphenol extract maybe due to the presence of some compounds in this polyphenol extract that extracted from the seeds of local grape such as; rhein, *cis* –stilbene, and *trans*-stilbene. Because both types of leukemic cells AML and ALL cell lines were revealed the same sensitivity to rhein, *cis* –stilbene, and *trans*-stilbene compounds (Mahbob *et al*., 2013).

Cytotoxic effect of polyphenol extract of *vitis vinifera* grape seeds on lymphocytes of acute leukemia (both types) patients:

The result of present study showed that all concentrations of polyphenol extract (39.1µg/ml, 78.125µg/ml, 156.25µg/ml, 312.5µg/ml, 625 µg/ml, 1250µg/ml, and 2500µg/ml) were effective against the proliferation of leukocytic cells of acute leukemia and there are no differences between two times of exposure 24 and 48 hours of leukemic cells into polyphenol extract. The highly effective concentration was the highest concentration 2500µg/ml. The polyphenol that extracted from the seeds of local grape may have ability to induces the programmed cell death of human leukemia cells. Because the grape seeds extract has ability to activating the enzymes of the apoptosis pathways and induces the programmed cell death of human leukemia cells in the laboratory Hu H, Qin YM., (2009). Study of Ning *et al*., (2009) shows that the grape seed extract has a functional role of c-JunNH(2)-terminal kinase (JNK) in treated leukemic cell (Jurkat cell), their result showed that the leukemic cell that treated with 50mg/ml of grape seeds extract for 12 and 24hours were revealed increase in apoptosis and caspase activation also increase in levels of phospho-JNK.

In cell culture, study of Clement *et al*., (1998) shows that the Resveratrol induced apoptosis via activation of CD95/CD95 system in HL60 and U937 leukemic cell lines. Resveratrol has antiproliferative roles in many types of cancer such as breast, melanoma, and gastric cancer and leukemias (Athar *et al*., 2007).

5. Conclusions

1. Polyphenol extracted from seeds of local grape, exhibited different biological and chemical characteristics.
2. Polyphenol extract from seeds of local grape caused great inhibition activity on the acute leukemic lymphocytes in both types of ALL and AML patients in highest concentration (2500 µg/ml) in both time (24,48 hours) of exposure.

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