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Evaluation of the Efficacy of Grape Extracts on Hematological Parameters in Rats with Induced Leukemia

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Annotation: : Leukemia (blood cancer) is a serious disease that affects the bone marrow and leads to the production of large quantities of immature blood cells called blast cells. These cells do not function normally and crowd out healthy cells, leading to weakened immunity, anemia, and easy bleeding, In this study, leukemia was induced in a group of rats (experimental animals) They were then treated with natural substances extracted from grape skins, such as protein precipitate and fatty acid, in addition to methoxate, a chemotherapy drug used in cancer treatment.

After treatment, blood smears were taken from these rats and examined under a microscope to determine the extent of the disease The results showed that some natural extracts had a clear effect in reducing cancer cells and improving blood formation.

Introduction

Leukemia

Liked leukemia is more a malignant disorder which involves blood cells plus bone marrow caused by uncontrolled proliferation of immature white blood cells. Without treatment, this unregulated flourishing suppresses healthy blood cell production which can be fatal. Aggressive forms can be fatal within weeks if not treated immediately..

While the precise etiology remains unclear, leukemia is believed to stem from complex interactions between **genetic predispositions** and **environmental triggers** that induce DNA mutations in hematopoietic cells. Established risk factors include:

- Exposure to ionizing radiation or carcinogenic chemicals
- Prior chemotherapy/radiotherapy treatments
- Familial hematologic malignancies
- Inherited genetic syndromes (e.g., Down syndrome)
- Tobacco use (Aby et al., 2024)

Current research underscores the multifactorial pathogenesis, where **treatment-related factors**, **germline mutations**, **toxic exposures**, and **behavioral influences** collectively contribute to leukemogenesis (Tran et al., 2020; Karakosta et al., 2016; Botsas et al., 2023).

Active compounds and medical usage of plants

Grapes are one of the oldest plants used in traditional medicine and remain an important component of modern medicine due to their rich array of biologically active phenolic compounds. Grapes belong to the Vitaceae family and are widely cultivated worldwide, particularly dark-colored varieties that contain high concentrations of active compounds such as resveratrol, catechin, quercetin, and anthocyanins, as well as gallic and ellagic acids (Al-Sheddi et al., 2023). These compounds, particularly those found in grape seeds and skins, exhibit significant antioxidant activity, inhibiting free radicals and reducing oxidative damage to cells. Research has shown that grape seed extract can protect tissues from oxidative stress resulting from exposure to chemicals or chronic diseases. A 2023 in vivo study published in the journal Antioxidants showed that an extract exhibited ameliorative effects against kidney and liver damage markers as well enhancing native antioxidant status through increased SOD, CAT and GSH (Hussain et al. Moreover, studies have indicated that grape compounds may act as an adjuvant in the treatment of tumors. By using grape extract in animal models of cancer, it was found that the potential reduction in tumor size and improvements in immune markers suggested .(this may be a hopeful complementary therapy (Fong et al., 2023

Grapes are packed with a bunch of bonuses: not only do they prevent constipation, they can also enhance digestion, escape from hemorrhoid trouble, increase energy levels and water supply in the human body; balance blood pressure. They are also consumed for skin health, hair loss, and liver detox. Enhances immunity and also strengthens heart, bone and teeth as well it protects .from cancers and aging diseases. It can also help control blood sugar levels (Farag 2017)

Recent findings also demonstrated that grape skin extract has anti-inflammatory activity via the inhibition of inflammatory cytokines and reduction of the oxidative enzyme activities. Its action is still manifested in the promotion of liver health and regulation of blood lipids, while its intake decreases the concentration of bad cholesterol (LDL) [81] and reduces triglycerides at the same .[82] time increases the content of good cholesterol (HDL)

On an immunological note, grape skin has been shown to improve gut health and boost the immune system, improving the body's ability to combat disease in addition to simply protecting tissues from oxidative damage (Xiang et al. 2023).

Experimental Part

Preparation of Crude Aqueous Extract of Grape Peels

Grape peels (150 g) were crushed in a kitchen blender for 10 min, added distilled water (1 w:1 v), frozen and thawed at room temperature three times. They then mechanically disrupted the cellular system with an ultrasonic device. The mixture was stirred for 2 h with an electrical stirrer in the ice bath as offset. It was then filtered using several layers of gauze, and the extract

was separated using a refrigerated centrifuge to remove undissolved materials for (15) minutes at a speed of (3500 x g) to obtain a clear filtrate (Al-Sumaidi, 2023). The resulting extract was dried using a lyophilizer until it became a powder and stored in a freezer at -20°C in a tightly sealed tube until used in animal experiments. Isolation of Oil from **Grape Peels**

Fatty acids and volatile oils were isolated from grape peels by soaking (30) grams of plant peel powder (paragraph 4.2) in petroleum ether solvent (60-80°C) for 24 hours. The soaked powder was then placed in a Soxhlet extraction device and heated for 16 hours until the solvent became clear. The solvent was evaporated using a rotary evaporator, and the sample was then collected and weighed to calculate the percentage of extracted oil (Al-Samarrai, 2018). The resulting oil was placed in a tightly capped tube and refrigerated until HPLC.

Analysis Animal Experiments

(25) male rats were used in this study, aged (3) months and weighing between (95-150 g). They were placed in cages in a special room with appropriate conditions, including feeding, 12-hour lighting, temperature, and ventilation, in the field of the College of Veterinary Medicine, University of Mosul.

Induced Leukemia in Experimental Animals

Leukemia was induced in fifteen rats by injecting 0.2 ml of a diluted benzene solution (5 ml of benzene in 95 ml of methanol) intravenously through the tail three times a week for three weeks. The development of the disease (leukemia) was observed in 92% of the rats within 2 to 3 weeks after the last benzene injection. El-Moneim, A.E.A., et al. (2017)leukemia burden was assessed using index parameters such as: total white blood cell count, red blood cell count, hematocrit, and hemoglobin concentration. Complete Blood Count (CBC) Analysis Method

The complete blood count (CBC) was measured using a Sysmex XN-1000 (Sysmex Corporation, Kobe, Japan). The CBC was performed to evaluate changes in blood cellular components, including Red blood cells (RBC), hemoglobin (Hb), hematocrit (HCT), white blood cells (WBC), and red blood cell indices (MCV, MCH, MCHC, RDW). Using this device helps reduce human interference and speed up analysis (Rai, A., Kaur, M., & Kaur, M. (2020)). This provides accurate and reliable results that can be used in research to interpret the hematological effects of treatments or diseases (McPherson RA,2017, Pincus MR,2017).)

Blood was drawn from rats into tubes containing the anticoagulant EDTA. Samples were stored at 2–8°C and analyzed within 2 hours of drawing to avoid hemolysis. The cells were collected and the samples were gently mixed to avoid clotting.

Analysis Method

The device was turned on and calibrated using quality control materials according to the company's protocol. The sample was then placed in the device inlet and the appropriate analysis mode (Whole Blood Mode) was selected. The device automatically drew the sample and analyzed it. The results appeared on the device screen, were printed, and documented for use in statistical analysis.

Plant Extraction

Plants have been used for thousands of years to treat various diseases or mitigate their effects. Plants are the primary source of chemical compounds that can be used in medical and pharmaceutical fields. Extraction is the essential step in identifying the desired active bioactive compounds in plants. It involves soaking, drying, or grinding to obtain samples that are easier to use. Solvent extraction is one of the most common methods for extracting active compounds from plants, which are then characterized using more specialized and accurate methods..

Fatty Acids

Fatty acids are important bioactive compounds in nature and are essential for the overall health of living organisms. The fatty acids in the grape table (1-1) peel extract were identified using HPLC chromatography technique, which resulted in five fatty acid bands (Figure 1-1). When compared with the standard fatty acid bands (1-2) in the grape peel fatty extract (1-palmitic acid, 2-stearic acid, 3-oleic acid, 4-linoleic acid, 5-linolenic acid) at a concentration of ((23.3, 8.9, 18.9, 40.6, 1.7) micrograms/kg, respectively.

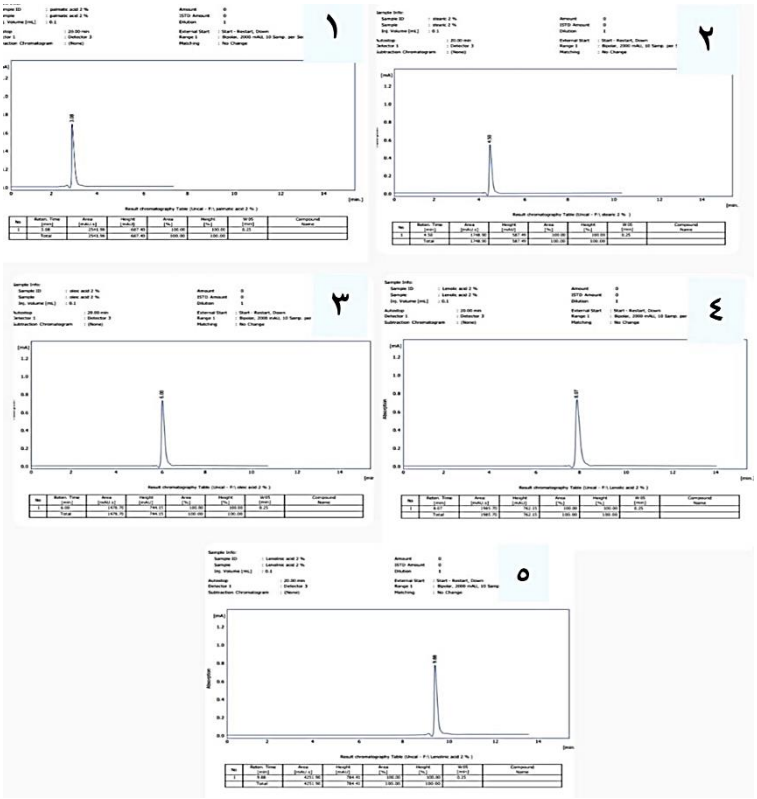
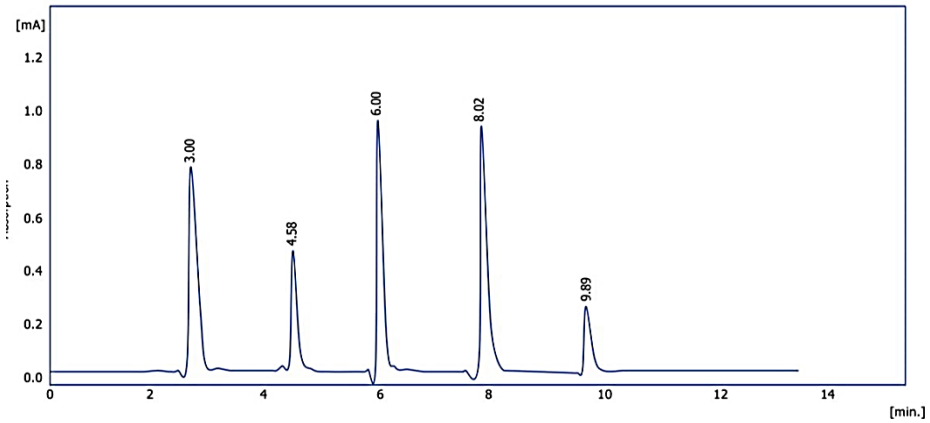


Figure (1-1) HPLC chromatography of standard compounds in fatty acid extract (1-palmitic acid, 2-stearic acid, 3-oleic acid, 4-linoleic acid, 5-linolenic acid).



No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	3.00	7451.08	792.65	20.00	20.00	0.05	
2	4.58	5211.46	404.88	15.00	15.00	0.03	
3	6.00	9856.98	988.59	30.00	30.00	0.08	
4	8.02	9874.48	987.41	30.00	30.00	0.08	
5	9.89	2201.32	266.88	5.00	5.00	0.02	
	Total	34595.08	3440.10	100.00	100.00		

Figure (1-2) HPLC chromatogram of grape peel fatty acid extract

Table (1-1) Analysis of fatty acids in grape peel extract using HPLC

No.	Standard Compounds Retention Time (min)	Extract Retention Time (min)	Fatty Acids Extract	Conc. (µg/gm)
1	3.0	3.0	Palmitic acid	23.3
2	4.58	4.58	Stearic acid	8.9
3	6.0	6.0	Oleic acid	18.9
4	8.02	8.02	Linoleic acid	40.6
5	9.89	9.89	Linolenic acid	1.7

The effect of isolated extracts and drugs from grape peels and the drug on complete blood count (CBC) indicators

A group of healthy rats (healthy control) was compared with a group of rats infected with leukemia (infected control) in terms of several vital blood indicators that reflect the efficiency and function of the circulatory system.

White Blood Cells (WBCs)

The data in Table (1-2) and Figure (1-3) showed a statistically significant increase at a probability level of $p \leq 0.05$ in the number of white blood cells in the infected control group (18.3 ± 1.23) compared to the healthy control group (11.3 ± 0.7). This increase is attributed to the immune response resulting from infection or inflammation, which stimulates the bone marrow to increase the production of white blood cells as a defensive mechanism, as indicated by (Kumar et al. 2019).

By contrast, treating with other extracts (lipid, protein precipitate and methotrexate) reduced the WBCs count significantly compared to the infected control. This might be because the extracts suppress leukemia by changing immunity on one hand and elevating blood cell counts running in .another hand

An example of this is the activity in Active Peptides and Antioxidants of Protein Precipitate which reduce oxidative stress – and hence inflammation. Hyperproduction of white blood cells. These effects are also sustained by a study performed by Zhang et al., 2020, that displayed the fact hydroxyproteins have a role in modifying the immune response in animal models of leukemia The high content of omega-3-w and 6-fatty acids in the oil extract is anti-inflammatory, .El-Bakry, 2023 & immune-modulating, and helps to control WBC (Al-Sayed

Methotrexate is a traditional chemotherapeutic treatment used for leukemia. It has been proved by some studies that it massively decreases the amount of white blood cell and improves haematological parameters, this is due to its ability to inhibit the DHFR enzyme responsible for getting activated in the DNA synthesis process which hampers division of white cells (Khan et al., 2022).

Red Blood Cells (RBCs)

The data display non-significant reduction in the number of red blood cells (RBCs) Table(1-2) and Figure (1-3) infested rats. (5.24±0.22) in leukemia (n = 111; Table 4, Fig 2C) compared to normal controls (6.6±0.70); p This decrease is attributed to leukemia cells displacing normal stem cells in the bone marrow responsible for producing red blood cells, leading to pronounced anemia. This phenomenon is one of the hallmarks of blood tumors, as cancerous cell expansion within the marrow reduces its efficiency in producing normal blood cells. This explanation is supported by a study conducted by researchers Jacek (2002) and Day. The results also indicated a significant increase in the number of red blood cells to the normal level compared to the infected control group when the animals were treated with methotrexate. This indicates a potential role in improving blood production or reducing oxidative damage (Ahmed et al., 2023). When rats were treated with the oil extract in addition to the protein precipitate, there was no significant difference compared to the infected control group.

Hematocrit

Hematocrit (HCT) expresses the percentage of red blood cell volume in the blood relative to the total blood volume. The study (HCT) in Table (3-4) and Figure (3-7) showed that the infected control group (46.6±5.5) did not show a significant decrease at the probability level ($p \leq 0.05$) in the hematocrit (HCT) ratio compared to the healthy group (44.6±4.04), indicating that leukemia-associated anemia may not be primarily related to low hematocrit concentration. In addition, treatment with (lipid precipitate, protein precipitate, methotrexate) contributed to a gradual improvement in this ratio.

Hemoglobin (HB)

The results shown in Table (1-2) and Figure (1-3) showed a significant decrease at the probability level ($p \leq 0.05$). ($p \leq 0.05$) in hemoglobin (Hb) concentration in rats with leukemia (10.43±0.38) compared to the healthy control group (13.6±1.71), reflecting the presence of anemia resulting from inhibited red blood cell production. Hemoglobin is directly related to the number of red blood cells, so anemia resulting from decreased production of these cells automatically leads to a decrease in hemoglobin concentration. This is consistent with a study conducted by (Jasek and Day, 2021).

On the other hand, the results showed a significant increase in Hb levels to the normal level for all treated animals compared to the infected control group, reflecting the ability of these compounds to reduce oxidative stress and improve bone marrow function, which contributes to supporting the hematopoietic process and stimulating normal red blood cell production (Ahmed et al., 2021).

Lymphocytes

The results in Table (1-2) and Figure (1-3) recorded a significant increase at the probability level ($p \leq 0.05$). The percentage of lymphocytes (%) in the infected control group (69.3±0.57) compared to the healthy control group (50.0±1.581) indicates an excessive immune response associated with the cancerous activity of leukemia.Lymphocytes are one of the main types of white blood cells, and their numbers are observed in cases of acute or chronic lymphocytic leukemia (ALL) due to their uncontrolled proliferation in the bone marrow, disrupting the production of normal blood cells. Studies indicate that leukemias, such as chronic lymphocytic leukemia (CLL), are characterized by a marked increase in the number of lymphocytes due to the expansion of these cells, which contributes to disease progression and weakens the natural immune system. For instance, a study by Vicenzetto and colleagues [9] Consistent with this concept, T-LGL expansions were reported to proliferate in the setting of leukemia thus promoting disease progression (2023). These forces include complicated immune interactions . with multiple types of viruses, along with genetic modifications

The percentages of lymphocytes were significantly lower in the treated groups than in the infected group respectively (reflecting inhibitory activities of plant extracts and active component on the hyperimmune reaction and reduce overproliferation normal cells). In addition, methotrexate has been demonstrated to work by decreasing the quantity of lymphocytes by suppressing enzyme dihydrofolate reductase (DHFR) and then going on to cell cycle arrest which prevents division of leukemia cells and any other rapidly dividing cells in the body .(Howard et al. 2016)

Results Table (1-2): represents the effect of isolated extracts from grape peels and the drug on the levels of wbc, Hb, Lympho, hct, Rbc in male rats infected with leukemia.

Complete Blood Count (Mean ± SD)					
Group (n = 8)	WBC (10^3/µL)	RBC (10^6/µL)	Hct (%)	Hb (g/dL)	Lympho (%)
Negative Control	11.3 ± 0.78 a	6.6 ± 0.70 b	44.6 ± 4.04 a	13.6 ± 1.71 b	50.0 ± 1.58 b
Positive Control (Leukemia)	18.3 ± 1.23 d	5.24 ± 0.22 ab	46.6 ± 5.5 ab	10.43 ± 0.38 a	69.3 ± 0.57 d
Positive Control + Protein Precipitate	11.0 ± 0.11 a	5.75 ± 0.43 ab	47.6 ± 7.0 ab	14.2 ± 0.38 a	49.5 ± 1.91 b
Positive Control + Lipid Extract	14.0 ± 0.26 c	5.55 ± 0.66 ab	50.0 ± 1.8 ab	12.9 ± 0.93 b	50.0 ± 2.0 b
Positive Control + Methotrexate	14.4 ± 0.78 c	6.5 ± 0.78 b	54.0 ± 2.6 bc	13.25 ± 0.90 b	57.6 ± 2.5 c

- Different letters vertically indicate a significant difference at the probability level $P \leq 0.05$.

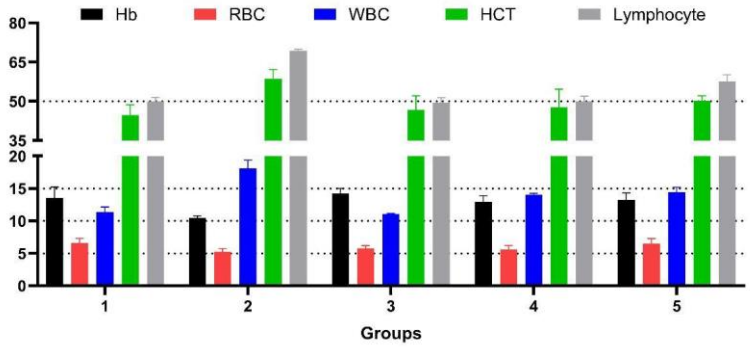


Figure (1-3) Effect of isolated grape peel extracts and the drug on the levels of (wbc), (Hb), Lympho, Hct, and Rbc in male rats with leukemia.

Microscopic analysis of blood smears in leukemic animals

Morphological evaluation of peripheral blood or bone marrow smears is an essential step in the diagnosis and follow-up of acute leukemia. According to the World Health Organization (WHO) classification, the diagnosis of the disease is based on the presence of ≥ 20% of myeloblasts or lymphoblasts in the peripheral blood or bone marrow. In normal cases, these blasts appear in very small proportions in the bone marrow and are not usually present in the peripheral blood. However, in leukemia, these cells proliferate abnormally and invade both tissues. The decrease in the number of blasts and the return of blood cells to their mature, normal forms is an important indicator of the effectiveness of treatment (Bain & Bene, 2019),(Swerdlow et al., 2017).

Figure (1-4) (a) shows microscopic analysis of red blood cells and white blood cells in moderate proportions in the healthy control group. In contrast, image (b) shows a widespread proliferation of blast cells in the untreated infected group, a pattern characteristic of the acute phase of the disease. After treatment with the different doses, as shown in figures (c–h), a gradual decrease in the proportion of blast cells and an increase in mature or differentiated cells was observed, indicating the effectiveness of these treatments in stimulating cellular differentiation. Treatment with the protein precipitate (c) and lipid extract (d) showed a clear cellular improvement, (e) while methotrexate led to a relative restoration of normal hematopoiesis with enlarged red cells.

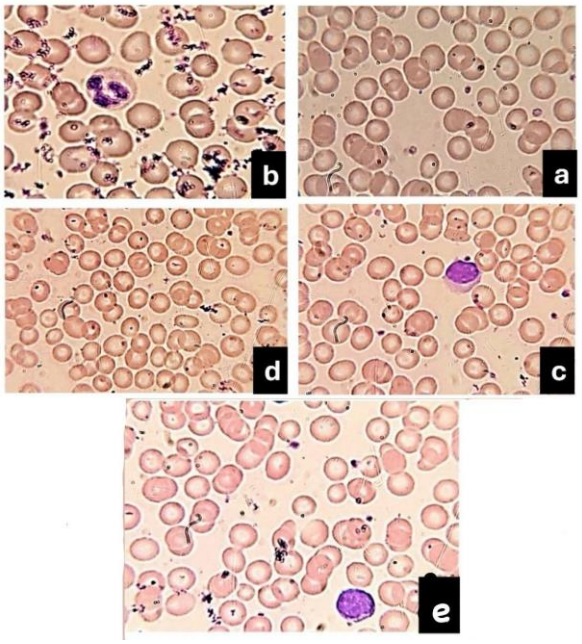


Figure (1-4) shows the microscopic analysis and morphological variables of blood cells in rats. (a): Healthy group (b): Group infected with leukemia ((c): Group infected and treated with protein precipitate (d): Group infected and treated with lipid extract. e): Group infected and treated with methotrexate

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