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Study the effect of the toxic alcoholic extract of *Nerium Oleander* on the liver cancer cell line in vivo and the effects on the liver histology in Mus Musculus

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Abstract

The current study included two axes, The aim of the first axis was to investigate the cytotoxic effect of the N.oleander of alcohol extract in the liver cancer cell line by using 10 concentrations with half-fearing measures, The study found a toxic effect of the crude alcohol extract of the N.oleander plant in the cancer cells studied. The second axis was intended to know the effects of the alcohol extract of the plant in the inside of the body. In this study, 16 mice with Mus musculus were used with a mean weight of (2 ± 25 g). They were divided into four groups: Daily for two weeks at a concentration of (1 ml / kg), The third group was administered for two weeks at a concentration of 1.5 ml / kg. The third group was administered for (2 weeks) at a concentration of (2 ml / kg), The oral dosage was given by the gavage. In the histopathological examination, many tissue lesions were observed as a result of treatment in the liver, If the second group showed a slight tissue effect compared with the high concentration doses in the third and fourth groups, it is characterized by focal distention in the liver cells either in which the daily dose was observed in the liver. The blood vessels, as well as the programmed death of cells, start with increased cell damage and blood hemorrhage containing yellow hematodin granules, which is significant in the liver of the fourth group and the expansion of the central vein with liver congestion at high doses.

Keywords: *Nerium Oleander*,

INTRODUCTION

Nerium Oleander this plant belongs to the Apocynaceae family and is classified as a family of Apocyuaceae. Most of the plants of this family are poisonous belong to the class of Gentianales Nangaliposida of the Magnoliophiyta Division.The common name of the plant is called: Nerium oleander this plant is called several names in Arabic, including: donkey poison [1].It is an evergreen ornamental plant, which is often used in landscaping, parks and outdoor roads. The Mediterranean, South and East Asia are endemic, rarely found in central and western Europe because they are sensitive to low temperatures.The plant spreads in most parts of Iraq and grows wildly in the northern regions, as found in the streets of Baghdad [2].The shape of N. oleander considered from an evergreen ornamental plant. It is about four meters long. Its leaves are between (10 and 20 cm) long. A layer of green color is formed from the top and light green surface of the lower surface. It has a prominent middle perspiration, Pink, red, purple, purple, orange, copper, and five petals. The cup was hung around the violets in the form of a tentacle and the talisman in the belly of the petal is long and narrow [3].The therapeutic uses of N.oleander , in the first decades of this century the plant is still used as an effective herbicide against many agricultural pests and rodents. Recent studies have revealed the use of the plant for the treatment of heart disease, which extracts the glycosin-like corticosteroids of the heart muscles[4].It is also used in the treatment of hyperglycemia [5].

A recent study found that the flower extract protects nerve and nerve cells from damage and is an anti-carcinogen because it has the potential to stimulate and stimulate the production of natural killer cells in humans to kill cancer cells[6].

Studies have confirmed that it works to stop the growth of cancerous tumors in animals [7].as prostate cancer[8].Mutations in sperm-producing cells cause infertility in male animals [9].Another study confirmed the importance of the extract in the treatment of malaria [10]. and viral diseases such as Hepatitis (A, B,C) recent study has shown that N.oleander enhances the immune response by stimulating cells to produce gamma-interferon (IFN-γ) , [11].The active compounds in this plant has important medical active compounds such as Cardiac glycosides [12].Cardiac cholecosides are the most common active substances, especially in leaves and have the most impact on the heart [13].Oleandrin is the active and effective type of these calcosides in the N.oleander plant, which is a colorless, tasteless and tasteless substance [14].Other components include Rosagenin, Astrychenin, multiple flavones, volatile oils, fats, sugars, rubbers, hydrocyanic acid, Saponin and Tannins [15].The toxicity of N.oleander One of the plants with high toxicity is the fact that it contains the cardiac calcosides, which are a high source of toxicity and are found in all parts of the plant. The case of poisoning comes from the absorption of the nectar, the chewing of plant parts, especially leaves, the use of plant branches such as roasting, Which is produced by bees feeding on the flowers of the state or inhalation of smoke rising from the burning parts of the plant or use of the plant for medical purposes without knowledge and scrutiny, and one leaf of the plant is sufficient to kill a child or sheep in the case of eating this paper and the most important symptoms of poisoning with the baby : Weakness, dizziness, poor vision, loss of appetite, Vomiting , nausea, diarrhea, irregular heart attack strikes, low blood pressure, inability to coordinate movements, headache, fainting and in most cases of death [16].

MATERIALS AND METHODS:-

1-GET the plant:-

The plant was obtained from the national herbicide / Baghdad.

2-Cytotoxic effects:-

The multi-drill tissue cultivars (96) and the flat bottom were used to conduct this test. The experiment included three stages:

a-Planting or sowing cells:-

After the process of development and growth of cancer cells took the vessels with full growth. Cells were harvested using trypsin-farsin solution. Add (20 ml) of the plant medium with the serum to each container and mix well, then count the blood cells using the dye Tryban blue [17].Take 0.1 mL by micro-pipette from the cell suspension and distributed on the dish. The surface of the drill was then covered with a special sterile adhesive tape and gently

stirred the dish, then incubated at (37 ° C) until the next day to allow cell adhesion .

b-Treatment (exposure) of cancer cells with plant extract

Sequential hemorrhage was done in sterile test tubes for each type of plant extract using the serum-free plant medium the next day from the seeding chamber. The cavity started gradually from (1/1/1/10/1) which gave concentrations of (1000 - 1.95 kg / ml), respectively, taking into account the preparation of the anesthesia at work. In the center of the plant, cultivate the tissue culture dishes after removing the adhesive and in column 1 as a negative control and add (0.2 mL) of serum free medium. Columns from (2-12) were added to the extracts of the (0.2 ml / hole), and then put a new layer of adhesive paper on top of the dish. The dishes were incubated at (37 ° C), while the exposure periods were (24, 48, 72) hours .

c-Detection of toxic effect:-

Crystal violet dye was used to detect the cytotoxic effect of extracts in cancer cells and according to the following :

After the incubation periods, the dishes were taken, the contents were washed and then washed with the PBS solution. Then add (0.1 ml) of the crystallized violet dye prepared in (16) to each hole of the dish and left for (20) minutes. The cells were then washed with PBS solution several times After the dish was completely dried, the results were read using a special optical spectrometer device for fine calibration dishes at a wavelength of (492nm).

3-Extraction of *N. oleander*:-

The extract was prepared with (20 g) of dried leaves powder and was dried by spreading green leaves on a floor covered with clean leaves, while stirring them daily in a dark place with good ventilation and then dissolved in (200 ml) of (95%) ethyl alcohol shake well and leave it for (24) hours after the mixture was sprayed through several layers of gauze and take the leachate produced in Soxhlet extractor (which works on the basis of vaporization under pressure and temperature not more than (45) m, After evaporation of all the alcohol in the mix was dried to obtain dry matter With dark green color and softened with distilled water Harm the required dose [18].

4-Laboratory animals Laboratory Animals:-

In this study 16 mice from Swiss white mice were used, ranging from (25 to 28 g). Obtained from Pharmacology Department / General Pharmaceutical Company for General Industries. The animals were in good health and were placed in laboratory cages dedicated to the breeding of plastic mice with metal caps. They were sprinkled with sawdust and cleaned with cages and sterilized twice a week .

During the experiment, the animals were placed in uniform laboratory conditions in terms of ventilation and temperature. The rats' rattles were continuously fed throughout the experiment in special containers for not being contaminated with sawdust.

5-Group design:-

The animals were randomly divided into four groups in plastic cages. Each cage contained four mice, and treated as follows :-

1-Group A The First Group:-

Control group: consists of (4) mice injected with normal solution Saline (0. 1 mg / kg) .

2-Group II The Second Group:-

Was composed of (4) mice injected with (1 mg / kg) for (2)weeks. The dose was determined according to the (weight / ml / kg body weight) equation [19].

3-Group C The Third Group:-

:This group consists of 4 mice. This group was vaccinated for two weeks at a concentration of (1,5 mg / kg), and explained after the end of the period of direct dosage .

4-Group Four The Fourth Group:-

Therapeutic group B: This group consists of 4 mice. This group was dosage for (2) weeks at a concentration of (2 mg / kg) .

6-Kill the animals:-

At the end of the experiment and after (24) hours of the last dose given to the animals numb by putting it in a glass container containing a piece of cotton wet with Chloroform and be closed and left for a short period until the mouse stopped moving and then explained the mice opening the abdominal cavity by creating a longitudinal incision from the bottom of the abdomen Even the shear bone, and the liver was extracted for study.

7-Preparation of tissue sections:-

After dissecting the animals and placing liver in the Formalin (10%) solution, the samples to be studied were converted to ethyl alcohol at 70% concentration, the following steps were taken:

a- Dehydration, b- Clearing, c- Infiltration, d- Embedding, e- Sectioning, f- Staining, g- Mounting .

RESULTS

The cytotoxicity effect of purified N. oleander extract extracted from t N. oleander umor cell line was studied by evaluating its effect on (Hepatic cell) HEPG2 cell line (passage 18) on exposure time of 48 hours at various concentrations of N. oleander extract (150.0, 75.0, 37.5, 18.75, 9.37, 4.68, 2.34 and 1.17µg/ml) using Neutral Red assay. The optical density measured at transmitting wave length (450 nm) and (492 nm).

The Neutral Red assay is a cell survival/viability assay based on the ability of viable cells to incorporate and bind Neutral Red dye. The Neutral Red uptake assay provides one of the most used cytotoxicity tests with many biomedical and environmental applications. Therefore it was selected for determination of N. oleander cytotoxicity.

Following exposure to extract, cells are incubated in the presence of Neutral Red dye. The dye readily penetrates cell membranes and accumulates intracellularly in lysosomes. As Neutral Red is a vital stain, it was used for staining living cells. Changes of the cells brought about by the action of the purified N. oleander extract causes a decreased in the uptake and binding of Neutral Red. After washing cells with PBS and treating with distaining solution to release any excess of dye taken up, the level cells damage was evaluated by measuring the optical density of treated cell solution and comparing it to untreated negative control samples. Microliter plate reader equipped with (450 nm) and (492 nm) filter.

Results indicated in figure (1) showed that N. oleander extract showed gradual decrease in the viability of the cells in comparison with negative controls.

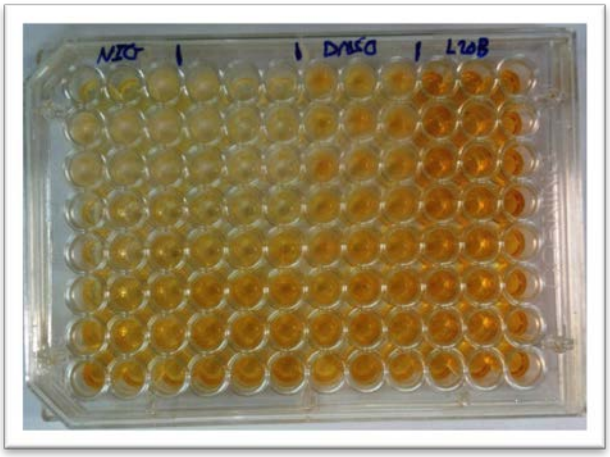

Figure 1: Cytotoxicity of purified N. oleander extract from N. oleander, against HEPG2 cell line using Neutral Red assay.

A: Test sample: the HEPG2 cell line after treatment with N. oleander extract (six times duplicate at each concentration)

B: Blank: the HEPG2 cell line after treatment with only phosphate buffer saline C: Negative control : the HEPG2 cell line without any treatment .

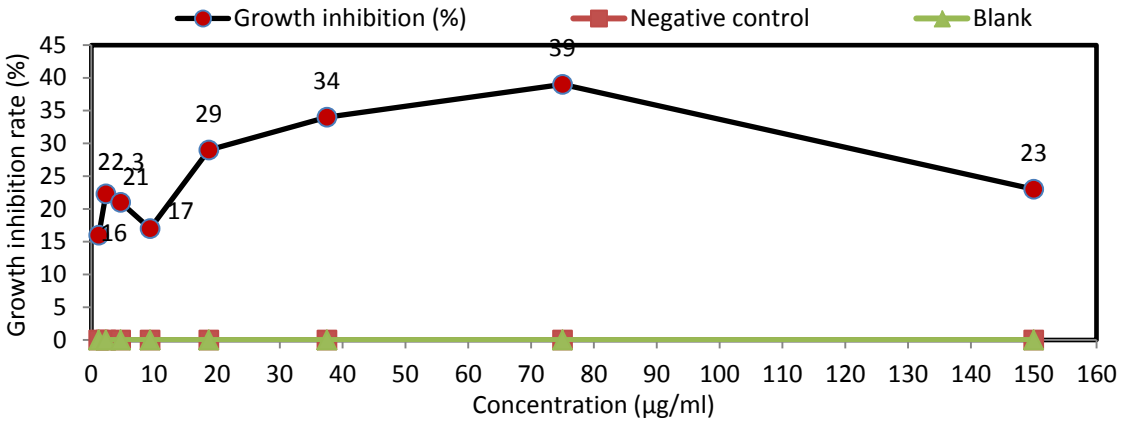

Figure 2: Cytotoxicity effect of purified N. oleander extract m on HEPG2 cell line after incubation period for 48 hours at 450nm.

The (96)-microliter plate was used as a standard tool for cytotoxicity analysis; the cytotoxicity effect depends on the percentage of inhibition growth rate (I.R) which represents the cytotoxicity of N. oleander extract. Remaining activity percentage and cell survival percentage were estimated.

Results indicated in figure (1) showed that N. oleander extract has significant cytotoxicity effect on (HEP G2) cell line in concentration range between (150 µg/ml to 1.17 µg/ml) in comparison with the negative control (the same cell line without any treatment) and blank (the same cell line treated only with phosphate buffered saline).

Results indicated in figure (2) showed that growth inhibition of tumor cell line was increased gradually with the increase of N. oleander extract concentration treated with. Maximum inhibitory effect of N. oleander extract was reached (39%) growth when the cell culture of HEPG2 was treated with (75µg/ml) of N. oleander extract, then the inhibitory effect was decreased to (23%) after treatment with (150 µg/ml) of N. oleander extract.

The inhibitory effect was increased with the increase in extract concentration and reaches the maximum (34.3%) after the treatment with purified N. oleander extract at a concentration of (75 µg/ml), then the inhibitory effect was decreased to (24%) after the treatment with (150 µg/ml) of N. oleander extract.

Cell survivals (%) and remaining activity (%) of HEPG2 tumor cell line after treatment with purified e N. oleander extract (for 48 hours) was depends on the concentration of N. oleander extract at both wave lengths (450 and 492nm).

Results indicated in table (2) also showed that cell survivals of HEPG2 cell line at(450nm) was highly reduced after incubation with N. oleander extract at concentrations (4.68 and 37.5 µg/ml), at these concentrations, the survivals of HEPG2 cell line were (70.8%) and (72.7%) respectively, while the cell line remaining activity percentage was reduced to (60.7%) and (65.9%) after incubation with N. oleander extract concentration of (75 and 37.5 µg/ml) respectively.

Table 1: Cytotoxicity effect of different concentrations of purified N. oleander, on HEPG2 tumor cell line after. Incubation for 48 hours measured at (450 nm) .

N.oleander extract concentration (µg/ml)	Growth inhibition (%)	Cell survival (%)
150	76.9	74.8
75	60.7	73.2
37.5	65.9	72.7
18.75	55	73.0
9.37	40	74.5
4.68	35	70.8
2.34	20	75.9
1.17	12	75.2

Results of histological sections

First group / the histological sections of the liver were studied with light microscopy. It was noted that the normal liver tissue consists of a number of lobules. Each section contains a central vein surrounded by cords of hexagonal or polygonal cells, in which the blood vessels are separated. These are known as the genes that line up two types of cells: the endothelial cells and the pharyngeal cells (Kupffer cells) showed in (figure 3).

Second group / the results of this group showed that most of the liver cells were similar to those in the control group and although some liver cells appeared normal, other hepatocytes were observed to have necrosis and liver cells on cells with a natural nucleus, Of cells containing photosynthesis showed in (figure 4)

Group III and IV / The results of the optical microscopy examination of the current study showed that this group undergoes stages of degeneration of the liver cells, and that some of the nuclei of the liver cells are characterized by their stages of programmed death, hemorrhage, cell membrane thickness, fat accumulation, enlargement of the germs, inflammation of the inflammatory cells, the emergence of hemosiderin and necrosis of the cells showed in (figures5,6 and 7).

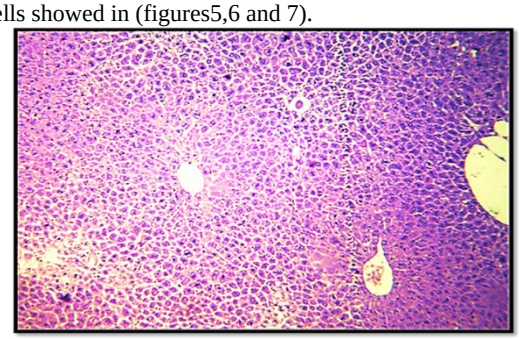

Figure 3: Liver of control group shows central vein CV. , Hepatocyte plate (H & E 400 X)

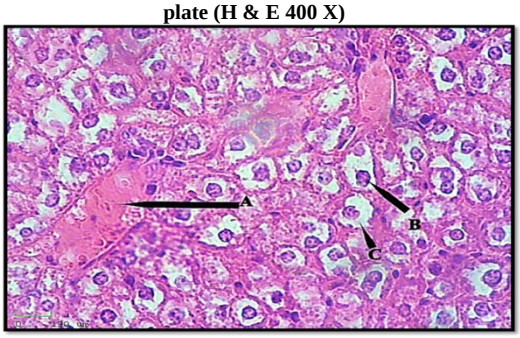

Figure 4: The liver of the rats at the concentration of (1) ml / kg, showing: 1 - blood analysis in the central vein CV. 2 - the nucleus of the nucleus, 3 - Cytoplasmic necrosis, (H & E ,400 X)

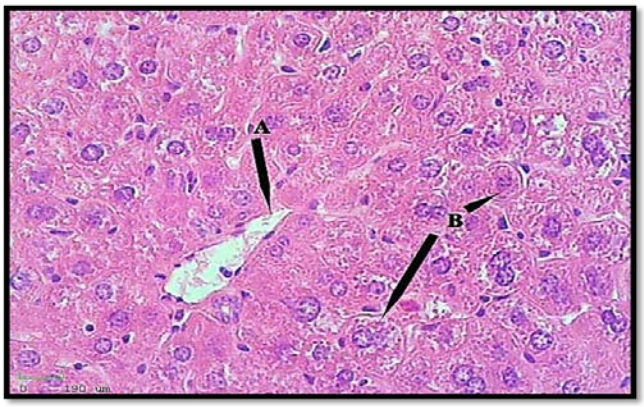

Figure 5: The liver of the rats at the concentration of (1.5) ml / kg, showing: 1 - rupture of the central vein CV., 2-nucleicysis, (H & E, 400 X).

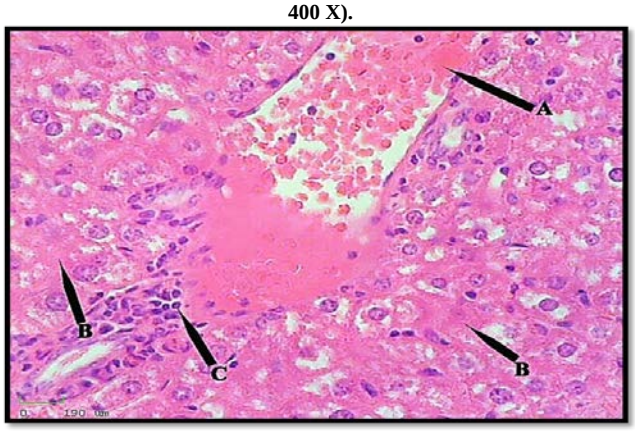

Figure 6: The liver of the mice at the concentration of (2) ml / kg, showing: 1-decomposition of hema-todine 2-nucleicysis, 3-lymphocytes IL, and the section shows the damage of all liver cells (H &X,400 X) .

Figure 7: The liver of the mice with a concentration of (2) ml / kg, showing: 1- Bleeding in the C.V .)2- apoptosis , 3 - nuclear decomposition (H & E ,400 X

DISCUSSION

Due to the importance of finding effective compounds against cancer and finding more types of plants that possess these compounds, N.oleander, one of the locally available medicinal plants with different therapeutic properties, was selected to identify the effects of raw water extracts in cells Human cervical cancer and the extent to which these extracts can be used as medical treatments against cancer in the future. The test of detecting the toxic effects of a substance in cancer cells in glass is one of the important technologies that are initially adopted in the investigation of the possession of these substances fatal to these malignant cells, which may hold hopes as a future treatment.

The results of the present study found that the raw extracts played a role in killing cancer cells, inhibiting their growth and their dissociation outside the organism. The results indicated that the toxic effect of HEPG2 cell line was based primarily on the concentration used, the duration of exposure.

A study found a comparison between the two types of alcohol and water extracts, and found that the alcoholic extracts are more effective than their water-type Hep-2 , This may be due to the fact that the ratio of the active ingredient extracted with ethyl alcohol (70%) is greater than it is when using the water extract, as indicated [20].

Alkaloids are the most important and most active substances found in these extracts, but the mechanism of action is by inhibiting the process of division of the thread, to keep the cells in the tropical phase Metaphase This is mediated by blocking the polymerization of the tubulin protein Tubulin responsible for the formation of spindle yarns [21,22], as well as acting alkaloids to inhibit the building of nucleic acids outside the vivo [23]. Several previous studies by researchers have also indicated that alkaloids have been effective against cancer cells, including human cervical cancer cells (Hela cells). Low concentrations cause spindle action to be discouraged [24]. Confirmed [25] that these alkaloids affect the cancerous lymphocytic lines of the rat and in the lines of human ovarian cancer cells that are resistant to commonly used chemical treatments such as Cisplatin, as well as more effective than Taxol and Adriamycin. The toxicity of these compounds to Leukemia (L1210) leukemia cells is associated with their effect on Tubulin T protein and its spinular arrangement [26, 27].

The effectiveness of phenolic compounds, including flavonoids, is characterized by its antioxidant activity, which works to remove the free radicals that are generated. The cell is directed to enter the programmed cell death [28].

Many active compounds act in opposite directions depending on the concentration used. As noted in the above results, high concentrations inhibited the growth HEPG2 cell, while the low concentrations of these cells stimulated growth. However, the increase in rates ranged between 101-115% Relative to 100% control. This indicates that the extract under study has the effect of (Biphasic Effect) Or so-called Hormetic Effect [29], there are a lot of chemical compounds , antibiotics , and toxins in the work of the phenomenon of Hormetic (a pheno-menon of biological common in toxicology as it works at low concentrations of stimulation, which may be useful to the organism, especially when activated immune cells, while causing High doses of partial or total inhibition of cells [30]. It is worth mentioning that the extract used in this study is a crude extract, that is, it contains many types of active compounds that have been addressed to effectiveness or that are not mentioned, which supports the results of the emergence of antagonism in the impact on cancer cells depending on the concentration used. It is likely that its effect on the genetic material in two directions, the first cause the inhibition of certain genes, while the other stimulates growth and multiplication. It should be noted that the effect of anticancer agents is not just different from the sense of cell type, but passing Passage cells because of mutations in the genetic material after several transitions, the cellular antigens of cancer cells as well as their other characteristics will differ. The mother cell, which is isolated from the patient, is genetically different and antigenic to those that have grown for long periods. The chromosome body does not resemble what is present in the cells of origin from which it originated, resulting in a different response to the substance Therapeutic Treatment [31].Therefore, these results are evidence of the presence of inhibition outside the vivo, and then test therapeutic efficiency within the body .

Discussion of the reasons for obtaining the results of studied histological sections

This is consistent with [32], who explained the cause of this effect to the toxic effect of the extract of leaves of the plant by the active compounds found in the studied extract. Histopa-thological lesions were generally lymphatic cell aggregation and infiltration, liver plate necrosis, cell degeneration with fecal necrosis in cells and in the walls of blood vessels, apoptotic cells, hyper-eutrophic kupffer cell, damage of some bile ducts, The results of these lesions are consistent with those observed by[33],necrosis surrounding the lobules expanding towards the ocean, the presence of decomposing acidic cells in the dye, And early analysis Cellular areas surrounding the canal with the presence of H. pylori cells to lymphocytes. It is consistent with the findings of [34].when the rabbits were extracted with the crude alcohol extract of the seeds of the syrup at a concentration of 25 and 50 mg / ml for 15 days, noting the hepatic cell proliferation.

The toxicity of the extract leads to an imbalance in the effectiveness of liver enzymes. If toxin is removed, toxic substances are associated with liver cells and cause necrosis, thus causing cell death and liver damage [35].

[36] explained the bleeding in the tissue that may occur due to increased pressure inside the vessels due to the effects of toxic substances, thus causing a break in the wall of the vascularity and the release of red blood cells And increased bleeding causes the increase of fibrinogen (fibrin generator).The cause of the decomposition of red blood cells is the interactions between toxic compounds and clotathione forming compounds that lead to the breakdown of pellets. This is in line with what[37] explained that DMAB caused red blood cell decomposition in the treated rat liver, where it was reported that the discrete rings of the substance had the ability to interact with clotathione, forming compounds that would lead to hemolysis[38].Red blood cells are analyzed by thrombocytopenia due to local hemorrhage. This decomposition leads to the formation of the chlorophyll and the hem, as the chlorine is reduced by its liquefaction in the body fluids and the hem breaks down to give the hematodine and hemosiderin. Hematodine is deposited in the site of bleeding in golden yellow Either hemosiderin Deposition in the large macrophages and this is consistent with the results of this study as it was observed the presence of yellow granules in the site of bleeding, which is a hematodine giving the color of oil or yellowish red .

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