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The Protective role of aloe vera gel on bone marrow and liver functions changes caused by azathioprine in male rats

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Submitted to the Council of the College of Veterinary Medicine, University of Kerbala in Partial Fulfillment of the Requirements for the Master of Degree of Science in Veterinary Medicine / Physiology

By

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بسم الله الرحمن الرحيم

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I certify this thesis entitled **(Effect of Aloe Vera Extract on Bone Marrow and Liver Functions Changes Induced by Azathioprine in Male Rats)** has been prepared by ***Ahmed Razzaq Kareem*** under my supervision at the college of Veterinary Medicine, University of Kerbala in partial fulfillment of the requirements for the Degree of Master in the Sciences of Veterinary Medicine in Physiology, Biochemistry and Pharmacology.

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Dedication

If sincerity is a part of loyalty, then I dedicate this research

To the one who paved the path of knowledge for me, to the one whose name I carry with pride and who is absent from me due to God's decree and destiny... ***my dear father***, may God Almighty have mercy on him. To whom her prayers were the secret of my success and the most precious thing I had in life. A symbol of love and an ocean of tenderness... ***my dear mother***

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Abstract

The goal of the current study was to evaluate the protective activity of Aloe Vera extract in hematological , biochemical parameters , Liver oxidant & antioxidant enzymes side effects brought on by azathioprine with Histopathological examination for Liver and bone marrow. The experiment employed 40 male rats. The animals was split into four groups:- the control group (10 male rats) which received sterile water single daily dose administered orally for four weeks and the Aloe vera group (10 male rats) which received aloe vera (500mg/kg b.w) single daily dose administered orally for four weeks and the Azathioprine group (10 male rats) which received azathioprine (50mg/kg b.w) single daily dose administered orally for four weeks and the combination aloe vera with azathioprine group (10 male rats) which received aloe vera (500mg/kg b.w) combination with azathioprine(50mg/kg b.w) single daily dose administered orally for four weeks. Rats were given a chloroform anesthetic to make them unconscious, and then blood samples were taken from the heart to examine blood tests, liver enzymes oxidants and antioxidants, and dissected animals to get the liver and bone marrow for a histological investigation.The present study showed WBC (4.19±0.10c), RBC (4.74±0.15b), PCV (30.76±0.45c) a significant decreased in azathioprine group comparatively to control group and aloe vera groups while there were elevation for WBC (9.68±0.37b), RBC (7.14±0.24a) and PCV (37.10±1.40b) in combination aloe vera and azathioprine group in comparison with azathioprine group.Addition that showed significant decrement in MCV (50.09±0.45b) , MCH (14.42±0.52a) and MCHC (30.78±0.42b) in azathioprine group in comparison with control group and aloe vera group. Also a significant increment for MCV (58.53±0.78a) , MCH (22.65±0.47c) and MCHC (37.48±0.95c) in the group treated aloe vera plus azathioprine as compared to the azathioprine group. While showed in examination of Reticulocyte Count (18.00±0.19b) in azathioprine group showed a significant increase when compared with control group and aloe vera groups while the combination azathioprine plus aloe vera group showed (11.00±0.08c) a

significant decrease as compare to azathioprine group. While showed in examination of cell bone marrow smears in azathioprine group showed a significant increase in mylocyte (29.00±0.75a) and promylocytes (12.00±1.50a) while showed a significant decrease in neutrophil (14.00±0.50c) , eosinophil (7.00±0.50b) and lymphocytes (17.00 ±1.50a) when compared with the control and aloe vera group , Also there were a significant decrease in mylocyte (22.00±0.75b) and promylocytes (10.11±1.25a) in addition there were a significant increase in neutrophil (16.00±1.15ab) , eosinophil (9.00±0.50a) and lymphocyte (28.00±1.00c) in the group treated aloe vera plus azathioprine group as compared to azathioprine group. In this study showed a significant increase in AST (128.00±3.9c) , ALT (40.61±2.3c) and Arginase I level (9.33±0.33b) in azathioprine group as compare to control and aloe vera group on the other hand there were observed a significant decrease in AST (100.00±2.3d) , ALT (34.81±0.4d) and Arginase I level (6.65±0.30c) in combination aloe Vera and azathioprine in comparison with azathioprine groups. While in liver oxidant and antioxidant enzyme in azathioprine group showed a significant increase in MDA (16.52±1.4b) and a significant decrease in GSH (22.52±1.7b) and GST (9.16±0.83c) when compared with control and aloe vera group , Also there were a significant decrease MDA (11.21±1.1c) and a significant increase in GSH (35.81±2.1c) and GST (15.98±0.76d) in the group treated aloe vera plus azathioprine as compare to azathioprine group. Histological examination of liver section of azathioprine group as showed a sever central vein dilatation , significant hepatic degenerative changes and marked perivascular polymorph nuclear inflammatory cells infiltration , hepatocytes with significant rounded large nuclei and central vein dilation. Also the histological examination of bone marrow section of azathioprine group were staind with haematocylin and eosin , as showed a significant decrease in hemopoitic tissue formation with marked less cellularity bone marrow and remarkable reduction of main cellular components of bone marrow , some enlargments of hollow spaces. The aloe vera extract as showed effectiveness as hepatoprotective and protection against side effects of azathioprine with normal hepatocyte appearance There are no changes

in the liver cell's normal central vein and no changes to the hepatic cells. Histological examination of bone marrow section of aloe vera group as showed a normal hemopoitic tissue formation , normal bone marrow cellularity with normal megakaryocytes and normal erythrocytes (reticulocytes) with normal osteoblast, osteoid, and osteocyte are visible in the bone marrow tissue with thick periosteum. All of these histological changes were reversed in the combination aloe vera and azathioprine group as showed mild hepatocytes degeneration and normal central vein with mild hepatic swelling and hepatic inflammatory cells infiltration. Addation that in histological examination of bone marrow section of combination azathioprine and aloe vera group as showed a significant and sever hyperplasia in hemopoitic tissue , noticeable increase of bone marrow cellularity with noticeable hyperplasia in bone marrow cellularity, significant large Megakaryocytes and presence of erythroid islands.

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List of abbreviations

Abbreviations	Full word
A.V	Aloe vera

ALT	Alanine aminotransferase
ARG	Arginase I
AST	Aspartate aminotransferase
AZA	Azathioprine
BM-MSCs	Bone marrow - mesenchymal stem cells
CBC	complete blood count
CCC	countercurrent chromatography
DILI	Drug-induced liver injury
eNOS	endothelium nitric oxide synthase
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
GI	gastrointestinal tract
GSH	glutathione concentration
GST	Glutathione-S-transferase
HPLC	high performance liquid chromatography
LC-MS	liquid chromatography-mass spectrometry
M6P	Mannose 6 Phosphate
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MDA	Malondialdehyde
MMP-3	matrix metallopeptidase-3
MSCs	mesenchymal stem cells

NRH	Nodular Regenerative Hyperplasia
NSP	non-starch polysaccharides
PCOS	polycystic ovarian syndrome
PCV	Packed cell volume
RBCs	Total number of erythrocytes
RNS	reactive nitrogen species
ROS	reactive oxygen species
SEC	size exclusion chromatography
TG	triglycerides
TLC	thin layer chromatography
TPMT	thiopurine S-methyltransferase
VLDL	very-low density lipoprotein
WBCs	total number of leukocytes

Chapter One: Introduction

1.Introduction

Bone marrow is a semi-solid tissue found in the spongy portion of bones and is the main site of blood cell production , bone marrow produces about 500 billion blood cells per day, which enter the systemic circulation via permeable vasculature sinusoids within the bone marrow cavity (**Lindberg & Lamps, 2018).** Bone marrow consists of hematopoietic cells, and supporting stromal cells **(Monga *et al.*, 2022).**

White blood cells which fight infection, platelets, and red blood cells, which carry oxygen, are all made in the bone marrow , Since bone marrow is a vital component of your body, its absence can be fatal , The red bone marrow is where the majority of platelets, white blood cells, and red blood cells are created , Fat, cartilage, and bone are produced by the yellow bone marrow **(Alana *et al.*, 2021).**

Red blood cells last roughly 120 days, platelets for about 10 days, and white blood cells for a few hours to a few days , Since each blood cell only has a certain amount of time left in its life, bone marrow must constantly replace these cells , More blood cells may be produced under certain circumstances , This could occur if there is a low oxygen concentration in the human tissues, if there is blood loss, anemia, or a drop in the level of red blood cells , Erythropoietin, a hormone that prompts the bone marrow to create more red blood cells, is produced and released by the kidneys if these things take place **(Shin *et al.*, 2022).**

Additionally, in reaction to infections, the bone marrow makes and releases more white blood cells, as well as more platelets in response to bleeding , Yellow bone marrow can activate and change into red bone marrow in the event of significant blood loss , A variety of processes and activities depend on a healthy bone marrow **(Alana *et al.*, 2021).**

The liver plays a key role in multiple physiological processes, including Intra hepatic detoxification of xenobiotics and hormones e.g., Insulin-like growth factors,

Angiotensinogen **(Almutairi *et al.,* 2022).** Also its a metabolic organ that held fundamental3 pathways to produce energy and metabolism of carbohydrates, lipids, and proteins **(Judge & Dodd, 2020).** Rather than the liver regulating blood clot factors, produces essential proteins e.g. albumin as well as production of bile acid, very-low density lipoprotein (VLDL), cholesterol, store glucose as glycogen and lipid as triglycerides (TG) as well as other vitamins and mineral **(Bizzaro *et al.,* 2019; Huang *et al*., 2022; Ishikawa *et al.,* 2021).**

Besides these roles, the liver is considered an immune organ since there are immune cells that are ready to eliminate the pathogen from the gastrointestinal tract (GI) **(Hastings *et al.,* 2020).** The impact of medications on the body's organs like Drug-induced liver injury (DILI) is a condition of hepatic disorder that occurs due to drug abuse or other natural medicine such as herbs or other supplements, Inflammation, immune response as well as a high level of oxidative stress trigger Hepatic cell death by necrosis, apoptosis, and other types of cell death **(Almutairi *et al.,* 2022).**

Oxidative stress is a major mechanism in drug abused liver injury normally during liver metabolism and detoxification liver produce free radicals ROS and reactive nitrogen species (RNS) these free radicals establish an important normal physiological function but when their cellular concentration exceeds the normal level it is depleted cellular antioxidant and attacked cell membrane, form proteins adduct, DNA modification and trigger lipid peroxidation **(Almutairi *et al.,* 2022)**. And Several tissues contain mesenchymal stem cells (MSCs) such as liver, kidney, skin, adipose tissue, and bone marrow , One of the primary sources of stem cells is Bone marrow constitute a specific population of adult stem cells with multiple characteristics that can activate the mechanisms of repair after injuring tissue **(Rafiei & Sobhani, 2022).**

Azathioprine (AZA) is an immunosuppressive drug that is used to treat leukemia, acute lymphoblastic leukemia, inflammatory bowel disease, and rheumatoid arthritis , Azathioprine in combination with corticosteroids is the best treatment for preventing organ rejection , Despite its extensive use, AZA has been linked to a number of adverse effects, including the suppression of the patient's lymphocytes and toxicity in the bone marrow, gastrointestinal system, and liver **(Adam *et al.*, 2018).**

The drug's toxicity causes free radical generation in organs and tissues, as well as oxidative damage , It works by selectively suppressing purine of nucleotide (adenine) synthesis and decreasing DNA synthesis in a range of immune and other specialized cells, including hepatocytes , Its influence on the creation of reactive oxygen radicals Tissue alterations, necrosis, increased mitochondria, and the rough endoplasmic reticulum are all caused by this drug **(Hashim *et al.*, 2022).** Since ancient times, medicinal powers have been utilized , Plants and the things they produce have been used as a significant source of medicine for thousands of years , Almost 80% of people in the under developed globe still predominantly rely on traditional medications for primary healthcare , Aleo Vera, one of these herbs, has a more than 2000-year history of use in traditional medicine , It has been utilized all throughout the world because of its medicinal qualities **(Golmohammadi, 2022).**

To the family Liliaceae belongs **aloe Vera** (Aloes) , Aloe vera extract (Aloe Gel) has been demonstrated to have antioxidant effects in both humans and animals **(Rozani & Kusbaryanto, 2019).** It has been used as a herbal medicine for thousands of years in different cultures for a number of purposes , Aloe Vera gel contains antioxidants, vitamins that are both fat-soluble and water-soluble, enzymes, minerals, polysaccharides, phenolic compounds, and organic acids , It also exhibits radical scavenging activity that is more effective than capacity **(Singh *et al.*, 2021).**

It has a wide range of biological effects, including anti-inflammatory, arthritis, dermatitis, gout reduction, anti-cancer, antioxidant, UV protection, anti-diabetes (hypoglycemic agent), decrease of macrophage activation, antiprotozoal, antifungal, gastro-protective (peptic ulcer), burn treatment, and anti-cancer , By encouraging the formation of epithelial and fibrous tissue **(Hamza, A. M *et al.*, 2022).** It aids in wound healing By enhancing the activities of a liver enzyme linked to carcinogen metabolism in rats, aloes gel also helps to prevent damage to the liver, which serves as the body's main organ of detoxification, Aloe Vera has shown other therapeutic properties including anticancer, antioxidant, antidiabetic, and anti-hyperlipidemia. Aloe Vera contains more than 75 different compounds, including vitamins (vitamin A, C, E, and B12), enzymes (i.e., amylase, catalase, and peroxidase), minerals (i.e., zinc, copper, selenium, and calcium), sugars (monosaccharides such as mannose-6-phosphate and polysaccharides such as glucomannans), anthraquinones (aloin and emodin), fatty acids (i.e., lupeol and campesterol), hormones (auxins and gibberellins), and others (i.e., salicylic acid, lignin, and saponins) **(Sánchez *et al.*, 2020).**

Aims of Study

The current study attempted to assess the protective activity of Aloe Vera extract in alleviating hematological , Biochemical and histopathological changes caused by Azathioprine by measure the following parameters:-

1) Hematological tests (RBCs , WBCs , PCV% , MCV , MCH , MCHC, reticulocyte count and Examination of bone marrow smears).

2) Biochemical parameters (Arginase 1, ALT , AST).

3) Liver oxidant & antioxidant enzymes (GSH, G-S transferase , MDA).

4) Histopathological examination for Liver and bone marrow.

Chapter two : literature review

2. 1 : Aloe Vera

About 70% of people from around the world prefer to use medicinal plants as alternative solution for the chemical and drugs to treatment disease as assessed by the world health organization where these medicinal plants contain many active substances that has healing capabilities those used by human in particular to protect them from diseases **(Maduna & Patnaik, 2023).** A. Vera has been used for various purposes in dermatology and applied in the production of a variety of pharmaceuticals **(Haghani *et al*., 2022).**

2.1.1. Taxonomy of Aloe Vera:-

Kingdom : Plantae

Division : Tracheophyta

Subdivision : Spermatophytina

Class : Magnoliopsida

Order : Asparagales

Family : Xanthorrhoeaceae

Genus : Aloe (L.)

Species : Aloe vera (L.).

Common Names: Aloe vera; Aloe vera Linné; True aloe; Aloe barbadensis; Barbados aloe; Curaçao aloe; Mediterranean aloe; Ghritakumari; Lu Hui; Luhui, **(Buiza & Narbona, 2017).**

Latin names: Aloe arborescens, Aloe vera, Aloe africana, and Aloe barbadensis.

2.1.2. Distribution of Aloe Vera

Aloe Vera is recognized as having no clear origin, making it challenging to identify its origin , Aloe Vera may have originated in North Africa, the Canary Islands, and the Madeira Islands, though , Aloe Vera has also been found in the Mediterranean, the United States, South Australia, and East and Southern Africa. **(Aida *et al.*, 2022).**

2.1.3. Description of Aloe Vera

Xerophytes are plants that have a great capacity to survive in areas with little to no water because of their tissue's capacity to retain significant amounts of water, such as Aloe Vera, which is a perennial succulent plant of the Liliaceae family , The ability of xerophytes to metabolize crassulacean acid, which promotes acclimation during the photosynthetic pathway to create malic acid, is another characteristic of xerophytes **(Zeljkovic *et al.*, 2020).** The thick, fleshy, succulent green leaves of the Aloe Vera plant are distinguished by their thick covering of cuticle , A layer of vascular tissue that surrounds parenchymal cells and has an interior filled with clear gel lies underneath this one. In a rosette-like arrangement, all of the leaves are present and connected at the base **(Hamza, A. M *et al*., 2022).** The vascular tissue carries nutrients produced in the leaves down to the roots and distributes (the latex) throughout the entire leaf's edge to act as a storage mechanism, among other activities **(Zeljkovic *et al.*, 2020).**

Aloe Vera generally contains two different kinds of fluids (the latex and the gel) The latex is distinguished by its yellow color and is found below the firm cuticle epidermis of the leaf through the vascular system. It contains a high concentration of anthraquinone, which has long been used as a cathartic in addition to its use as a medicine for purges **(Singh & Singh, 2021).** The gel is a clear, mucilaginous substance made by the parenchymal cells (central layer), Aloe vera gel contains a wide range of therapeutic characteristics, making it useful for treating a wide range of illnesses, particularly for wound healing , The color of the leaf is predominantly green, with white spots appearing on its surface while the leaf is young **(Singh & Singh, 2021).** The Aloe vera plant's components, such as the gel that is extracted from the plant, are typically processed in a variety of ways, including heating, drying, and grinding. In fact, the derived gel has been used in a variety of industries, including the food, pharmaceutical, and medical sectors , One of these applications is on the medical level, where the gel contains numerous polysaccharides that are crucial in the treatment of

many diseases, even though these processing methods and many others exist and has the same curing effects **(Chaithanya, 2018).**

2.1.4. Phytochemicals composition of Aloe vera

The Aloe Vera plant contains lectins, flavonoids, terpenoids, unsaturated fats, cholesterol, anthraquinones, chromones mono and polysaccharides, tannins, sterols, salicylic corrosive, destructive regular acids, proteins, saponins, vitamins, minerals, complex mucopolysaccharides like hyaluronic corrosive, sapogenins, and chemical enzymes like catalase, celluase and alliinase **(Jalinda *et al.*, 2022)**. According to studies, the peel of aloe vera leaves contains 20 % to 30 % of its total weight in lipid, 6% of protein, 11 % of carbohydrates like glucose, and 13.5 % of ash like calcium , (NSP) non-starch polysaccharides and the chemical compound lignin form 62,3 % of the weight of the Aloe vera leaf, whereas the inner central part (the pulp) is composed of 70 to 80 percent of the leaf's total weight and contains 4,2 % of lipid, 7,3 % of protein, 16,5 % of sugars like glucose, 15,4 % of ash as calcium, and 57,6 % of NSP (non-starch polys) , it has been demonstrated that the polysaccharides (acetylated mannan) are also present in the aloe Vera plant's gel **(Hamza, A. M *et al*., 2022).**

The chemical and physical components vary depending on the plant's part, the species, and the environment , Researchers have been studying and analyzing the chemical makeup of aloe Vera for a long time. A variety of chromatographic techniques, such as thin layer chromatography (TLC), size exclusion chromatography (SEC), countercurrent chromatography (CCC), gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and high performance liquid chromatography (HPLC), have been used **(Arsene *et al.*, 2022).**

Aloe Vera chemical investigations demonstrated that the plant contains a variety of chemically active substances, including carbohydrates and organic and inorganic gradients , When an Aloe Vera leaf is broken, a large amount of a substance that

resembles a secretion (exudate) is released , This exudate is analyzed and found to contain phenols, which are identified by chromatography, as well as (Anthraquinones, chromone, and anthrone) or their derivatives, which have also been identified in the same rejoin **(Maiuolo *et al.*, 2022).**

The Aloe Vera gel, which is present in the inner cells that contains about 75 nutrients that are very healthy , also contains sugar in the mucilaginous layer as monosaccharides like (Mannose), (aldopentose, glucose), (L-rhamnose), and polysaccharides like (acemannan), (glucomannans), (galactan), (cellulose), and (pectic), and as mentioned in several studies, the ability of wound healing is best when consumed fresh including vitamins such as (vitamin B1, vitamin B2, vitamin B6, choline, -carotene, and folic acid), proteins such as (lectin), anthraquinones such as (Aloesaponarin I, Aloesaponarin I I, Chrysophanol, Aloe-emodin, aloetic-acid, emodin, chromones, Helminth **(Hamza, A. M *et al*., 2022).**

2.1.5. Pharmacological effects of Aloe Vera

2.1.5.1. Medical use

The Aloe Vera plant is regarded as one of the most significant plants used in the medical field. This plant is still used as a medicine plant in Europe, Asia as India, China, and Japan as well as the Mediterranean, in addition to many other countries, and in the 1930s in the United States of America, it was mentioned that the Aloe vera is effective to curing burns caused by X-rays **(Mondal *et al.*, 2021).** Anthraquinone glycosides, a phytochemical with laxative effects, are used as a guide to support the ability of Aloe vera in traditional medicine as a treatment and as evidence for its important cuing properties. The World Health Organization confirmed that the whole leaf of Aloe vera, including the gel and other parts, have treatment abilities by providing clinical data **(Egbuna *et al.*, 2020).**

The gel in the leaves of Aloe vera contains an efficient component called polysaccharides, which have numerous therapeutic properties. Recently, extract of

Aloe vera has been used orally to cure and protect from several diseases **(Mondal *et al.*, 2021).**

2.1.5.1.1. Wound Healing and Tissue Burn

Aloe Vera is referred to as a plant that heals. Many cultures have employed A.Vera for traditional medical uses **(Saikat *et al.*, 2021).** A. Vera extracts used in vitro promote the division of various cell types , Numerous studies have demonstrated that using entire A. Vera gel extracts resulted in quicker wound healing , which has proven the effect of A. Vera on accelerating wound contraction and collagen production, A. Vera may have a direct impact on the wound healing process as a whole, This is demonstrated by an increase in the rate of wound area contraction, The mannose-6-phosphate that is known to be found in A. Vera gel is responsible for this characteristic **(Saikat *et al.*, 2021).**

The polysaccharides in Aloe Vera increases the formation of hydroxyl proline and hyaluronic acid as well as fibroblast proliferation in Fibroblasts, which are crucial to the remodeling of the extracellular matrix during wound healing **(Majchrzak *et al.*, 2022).** In primary human periodontal ligament cells, acemannan significantly stimulates periodontal ligament cell proliferation, upregulates growth/differentiation factor 5, and activates type I collagen and alkaline phosphatase **(Majchrzak *et al.*, 2022).** In a clinical trial to compare the effectiveness of A. Vera gel and 1% silver sulfadiazine cream as a burn dressing for the treatment of superficial and partial thickness burns, A. vera treatment patients healed their burn wounds remarkably sooner than patients receiving 1% silver sulfadiazine treatment **(Irani *et al.*, 2022).**

Rat skin wound healing is aided by polysaccharides extracted from A. vera via inducing the expression of` matrix metallopeptidase (MMP)-3 and metallopeptidase inhibitor-2 genes. This directly influences how well A. vera gel heals wounds **(Saikat *et al.*, 2021).**

2.1.5.1.2. Anti-inflammatory effects

One of the body's reactions is inflammatory activity, which manifests as swelling, discomfort, dysfunction, and redness **(Gakhar, 2021).** While this is a typical reaction, it can slow down healing and prevent inflammation from spreading , Mannose 6 Phosphate (M6P) is a molecule that plays a role in the immune system by binding to lectin , This role is similar to that of a phytochemical (acetylated mannan) found in the gel of Aloe vera, where the gel of Aloe Vera has the capacity to limit and minimize inflammation by building up the synthesize of prostaglandin in addition to elevation of leucocyte infiltration **(Yang *et al.*, 2022).**

2.1.5.1.3. Antidiabetic Effects

Clinical investigations indicate that A. Vera gel may be a safe anti-hyperglycemic and anti-hypercholesterolemic therapy for type 2 diabetic patients, with no notable impact on other normal blood lipid levels or liver/kidney function **(Haghani *et al.*, 2022).** In vivo and in vitro studies show that the water soluble fraction of Aloe species , has glucose-lowering properties, and that several of its components influence glucose transporter-4 mRNA expression **(Maida & Banik, 2020).** A. Vera gel complex lowered body weight, body fat mass, and insulin resistance in obese prediabetic and early nontreated diabetic patients in a randomized controlled experiment , Furthermore, in an 8-week pilot study, two Aloe products tended to reverse the impaired glucose tolerance in patients with prFasting glucose and reduced glucose tolerance have been seen in prediabetes/metabolic syndrome patients **(Saikat *et al.*, 2021).** ediabete demonstrated that dietary Aloe formula decreased obesity-induced glucose tolerance not only by decreasing inflammatory responses but also by promoting glucose tolerance **(Hamza, A. M *et al*., 2022)**.

2.1.5.1.4. Anti-oxidant Effects

A. Vera contains significant levels of antioxidants such as -tocopherol (vitamin E), carotenoids, ascorbic acid (vitamin C), flavonoids, and tannins and antioxidant action

may be an important characteristic of plant medicines used in the treatment of numerous disorders **(Saikat *et al.*, 2021).** Topical A. saponaria treatment demonstrated anti-nociceptive and anti-inflammatory benefits in a UVB-induced sunburn model due to antioxidant components found in gel , An in vitro investigation of the radioprotective activity of Aloe vera gel found that it can scavenge the free radicals 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and nitric oxide in a concentration-dependent manner **(Maida & Banik, 2020).** The administration of an ethanolic extract of A. Vera gel to tissue antioxidants resulted in a decrease in Blood glucose levels in diabetic rats are reduced, which helps to prevent excessive free radical production via multiple metabolic pathways and also minimizes the potential glycation of enzymes **(Zadeh Gharaboghaz *et al.*, 2020).**

The antioxidant potentials of a polysaccharide derived from A. vera gel were studied in vitro and in vivo Enzymatic extracts of A. vera gel were made utilizing ten digestive enzymes, including five carbohydrases and five proteases. Aloe polysaccharides were found to protect against 2,2′-azobis (2-amidinopropane) dihydrochloride-induced oxidative stress and cell death in kidney epithelial cells (Vero cells) and an in vivo zebrafish model **(Svitina *et al.*, 2019).** In one study, the total phenolic content of A. vera leaf skin extracts was measured, and a significant association was found between the total phenolic content and the antioxidant power **(Foda *et al.*, 2023).** Methanol extracts of A. vera leaf skins and flowers were also tested for antioxidant and antimycoplasmic activity, and both extracts displayed antioxidant activity in vitro, with the leaf skin extract being the most active **(Brankiewicz *et al.*, 2023).**

2.1.5.1.5. Antimicrobial Activity

Antibacterial properties have been described for A. Vera **(Chaudhary *et al.*, 2019).** The 14 kDa (kilo Dalton) Aloe protein extracted from the A. vera leaf gel displayed significant antifungal action against Candida paraprilosis, Candida krusei,

and Candida albicans **(Adetunji *et al.*, 2022).** Anthraquinones are an active component in A. Vera Tetracycline's structural counterpart, Anthraquinones work similarly to tetracycline in that they limit bacterial protein synthesis by inhibiting the ribosomal A site where the aminoacylated tRNA enters), As a result, bacteria cannot grow in media containing A. Vera extract. Pandey and Mishra demonstrated the susceptibility of Gram-positive and Gram-negative bacteria to an extract of A. Vera inner gel **(Maida & Banik, 2020).**

Polysaccharides from A. Vera gel have been linked to direct bacterial activity by stimulating phagocytic leucocytes to kill bacteria , A. Vera contains pyrocatechol, a hydroxylated phenol that has been shown to be harmful to bacteria **(Sánchez *et al.*, 2020).** A recent study found that the A. Vera inner gel has antibacterial properties against both susceptible and resistant Helicobacter pylori strains and has an impact on the antimicrobial resistance phenomenon of H. pylori, suggesting that the A. vera inner gel could be used in combination with antibiotics to treat H. pylori gastric infection **(Yahya *et al.*, 2022).**

2.1.5.1.6. Anti lipidemia activity

A. Vera is well-known for its antihyperlipidemic properties, which serve to prevent the formation of fatty streaks and may help to limit the development of atherosclerosis by modifying risk factors **(Nafiu *et al.*, 2018).** The efficacy of A. vera leaf gel has been tested in hyperlipidemic type 2 diabetes patients: a randomized double-blind placebo-controlled clinical trial found that it dramatically lowered total cholesterol and LDL levels **(Deora & Venkatraman, 2022).** A recent study also found that administering phytosterols derived from A. vera gel reduces visceral fat mass while improving hyperglycemia in Zucker diabetic fatty rats **(Prasad *et al.*, 2022).**

In high-fat diet- and fructose-induced hyperlipidemic rats, dried pulp of Aloe succotrina leaves dramatically lowered serum levels of total cholesterol, total triglycerides, low-density lipoprotein-cholesterol, very low-density lipoprotein, and

high-density lipoprotein-cholesterol **(Saikat *et al.*, 2021).** Previous research found that A. vera extract-treated polycystic ovarian syndrome (PCOS) rats had a significant drop in plasma triglyceride and LDL cholesterol levels, with an increase in high-density lipoprotein-cholesterol PCOS, with hyperlipidemia being one of the main outcomes **(Maida & Banik, 2020).** The gel treatment also resulted in the reversion of aberrant estrous cyclicity, glucose intolerance, and lipid dysregulation. enzyme activity are metabolized and returned to normal , It contains phytocomponents that have antihyperlipidemic properties and has demonstrated success in the treatment of PCOS as well as the related metabolic problems **(Saikat *et al.*, 2021).**

2.1.5.1.7. Anticancer effects

The efficiency of the gel of Aloe vera to treat cancer was indicated in numerous scientific studies, where it was discovered that the gel can reduce the tumor's size. Aloe vera is a plant that contains two significant active substances (polysaccharides and glycoproteins) that are effective against cancer and tumors, Acemannan, a specific type of polysaccharide that is important anticancer derived from Aloe vera, has been demonstrated in several lab experiments on many Increased immune system response caused by the polysaccharides in aloe vera's polysaccharides is one potential method through which aloe vera gel's capacity to combat cancer can be explained **(Yahya *et al.*, 2022).**

2.1.5.1.8. Immune effects of Aloe Vera

Numerous studies have demonstrated that the aloe vera gel's active ingredient can boost immunity by stimulating macrophage, which release cytokines like TNF- (tumor necrosis factor-alpha) and produce nitric oxide , Interleukins with immune-boosting properties include IL-1 (interleukin-1), IL-6 (interleukin-6), and INF- (interferon) **(Ma *et al.*, 2019).** The active ingredient acemannan found in aloe Vera gel has been shown to have immunostimulant properties, including the ability to stimulate the production of macrophages and other cytokines as well as immune cells (lymphocytes) , In studies conducted on mice in the lab, acemannan caused the production of lymphocytes of all

types in the spleen and bone marrow , Aloe vera gel contains other substances with the ability to stimulate the immune system, such as lectins, which are glycoproteins **(Gutierrez *et al*., 2022).**

2.1.5.1.9. Hepatoprotective effects

One of the most significant medicinal benefits of aloe Vera is its ability to protect hepatic tissue from damage brought on by a variety of factors, including chemicals, drugs, and other factors **(Sehitoglu *et al*., 2019).** Aloe Vera significantly reduces the defect in the liver tissue from damage, as well as the level of elevated biochemical tests that rise as a result of damage , In a similar manner, aloe Vera treatment also reduces the ratio of defect brought on by the treatment , The bile solids (organic and inorganic substance composition of bile) may be owing to Aloe vera's activities in catalyzing the secretory cells of the liver **(Atta *et al*., 2022).** Aloe vera's antioxidant effects also have a role as hepatoprotective by maintaining the hepatic metabolism enzymes , Inflammations brought on by obesity are reduced by a decrease in inflammatory cytokines, and the gel of Aloe vera has the potential to be hepatoprotective by regulating the causes of damage **(Bjørklund *et al*., 2018).**

2.2. Azathioprine (AZA)

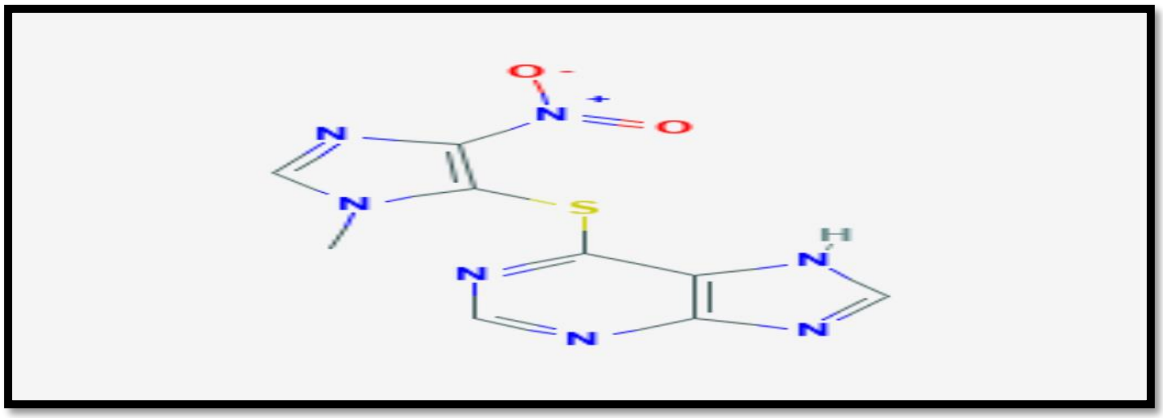
Azathioprine a 6-mercaptopurine thiopurine in which the mercapto hydrogen is replaced by a 1-methyl-4-nitroimidazol-5-yl group , The drug AZA is widely used in medical conditions, particularly to prevent organ rejection during transplantation. It is also used as therapy for many autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease, and systemic lupus erythemato **(Giglhuber & Berthele, 2022).**

Despite all of these therapeutic benefits, Azathioprine's detrimental side effects on the liver and bone might severely limit its use as a treatment , AZA has extremely serious side effects in 15 to 30% of people **(Grzechocińska *et al*., 2023).** The immunosuppressive substance (Azathioprine) is a common medicine used to treat

numerous autoimmune disease , Typically AZA is associated with a variety of symptoms as a side effect of the drug, ranging from nausea to bone marrow suppression to liver damage due to toxic effects in hepatic tissue , Azathioprine has a half-life of about 5 hours **(Patwa & Parab, 2022).**

2.2.1. Basic Chemistry of Azathioprine

Chemical Formula:- C9 H7 N7 O2 S



Figure(2-1) Azathioprine chemical structure (Davarani *et al.*, 2017).

Properties:- Physical Properties Azathioprine appears as pale yellow cryatals from 50% aq acetone.

Molecular Weight:- Solubility while azathioprine alone is insoluble in water , an aqueous solution may be prepared with the addition of one molar equivlent of alkali , the sodium salt of azathioprine is soluble in water up to 10 mg/ml. **(Samohvalov *et al.*, *2021)*.**

2.2.2. Azathioprine hepatotoxicity

The hepatotoxicity of Azathioprine (AZA) is considered one of the most significant challenges limiting its use as therapy, particularly as an immunosuppressant to treat inflammation disease **(Johnson *et al.*, 2019).** The toxic effects of AZA that lead to liver damage typically involve acute drug induce liver damage in addition to NRH (Nodular Regenerative Hyperplasia), peliosis hepatis, and hepatic veno-occlusive disease , The increased activation of thiopurine methyltransferase causes hypermethylation, which leads to hepatotoxicity by oxidative stress caused by ROS

generated primarily by the oxidation of AZA by xanthine oxidase, and therefore the increase in oxidation causes liver damage and hepatotoxicity **(Bayoumy *et al.*, 2020).**

2.2.3. mechanism of Azathioprine Indueced hepatotoxicity

Azathioprine is categorized as an immunosuppressive and antiproliferative medication , Azathioprine inhibits purine metabolism primarily through its metabolites and may prevent the synthesis of DNA, RNA, and proteins , It might also prevent mitosis and impede cellular metabolism **(Panopoulos *et al.*, 2020).** Azathioprine is nonenzymatically converted to 6-MP, a nucleophile counterpart of hypoxanthine, after being exposed to nucleophiles such as glutathione or cysteine Many, but not all, of the pharmacological and toxicological effects of azathioprine are thought to be caused by this conversion **(Grzechocińska *et al.*, 2023 Fig (2-3).**

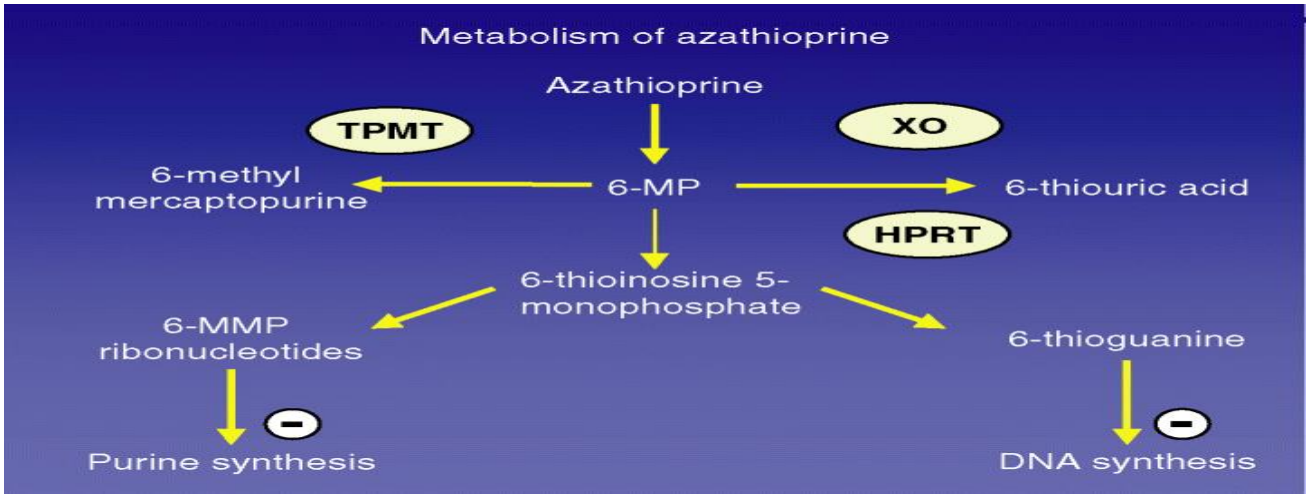


Figure (2-2):- Metabolism pathway of Azathioprine (Grzechocińska et al., 2023).

At least three potential processes, including the following, have been identified as the cause of azathioprine/6-MP toxicity **(Eltantawy *et al.*, 2023):-**

1) Thioguanine nucleotides, which are present in azathioprine/6-MP, combine with DNA to produce aberrant DNA, which then impairs the activity of DNA polymerases, ligases, and endonucleases.

2) Inhibitors of enzymes necessary for de novo purine synthesis are produced by the catalysis of aathioprine and 6-MP.

3) Azathioprine and 6-MP encourage apoptosis, which is a quick kind of cell death, and they also cause further alterations in B lymphocytes that encourage apoptotic processes in those cells. The delayed commencement of azathioprine's pharmacologic activity, which may take 8 to 12 weeks to manifest, is one of its distinguishing characteristics , This could be because 6-TGN slowly builds up inside cells **(Schwartz *et al.*, 2022).** The hazardous effects of azathioprine, some of which may develop at any point during treatment, are not always the same (e.g., bone marrow suppression) , Azathioprine and 6-MP appear to have different pharmacodynamics and pharmacokinetics, as well as differences in the relative abundance of various metabolites that are produced after their administration, which may explain why azathioprine appears to be a more potent immunosuppressive agent than 6-MP itself **(Grzechocińska *et al.*, 2023).** According to research done on hepatocytes, azathioprine toxicity results in reduced glutathione depletion, mitochondrial damage, significant ATP depletion, and necrotic cell death , Strong antioxidants, glycine, and inhibiting the mitochondrial permeability transition pore stopped cell death **(Raghu *et al.*, 2021).**

2.3. Bone Marrow

Bone marrow is a semi-solid tissue found within the spongy (also known as cancellous) portions of bones, In birds and mammals, bone marrow is the primary site of new blood cell production (or haematopoiesis) is composed of hematopoietic cells, marrow adipose tissue, and supportive stromal cells **(Kim & Ko, 2023).** In adult humans, bone marrow is primarily located in the ribs, vertebrae, sternum, and bones of the pelvis , Bone marrow comprises approximately 5% of total body mass in healthy adult humans, such that a man weighing 73 kg (161 lbs) will have around 3.7 kg (8 lbs) of bone marrow **(Suchacki *et al* ., 2020).**

Structuralliy the composition of bone marrow is dynamic, as the mixture of cellular and non-cellular components (connective tissue) shifts with age and in response to systemic factors , In humans, marrow is colloquially characterized as "red" or "yellow" marrow depending on the prevalence of hematopoietic cells vs fat cells ,

While the precise mechanisms underlying marrow regulation are not understood, compositional changes occur according to stereotypical patterns **(Everts *et al* ., 2019).**

2.3.1 Bone marrow suppression

Bone marrow suppression can be defined as low blood cells are produced inside the bone, and it is includes neutropenia , anemia , and thrombocytopenia. The bone marrow supression can be resulted from the side effects of chemotherapy for cancer treatment, the use of cytokines in clinical settings can help alleviate bone marrow suppression brought on by cancer chemotherapy, also known as granulocyte colony-stimulating factor, is effective in treating neutropenia , erythropoietin is effective in treating anemia **(Bhokare, 2017).**

Additionally it is found AZA frequently causes bone marrow inhibition, and this severe inhibition is associated with reduced thiopurine S-methyltransferase (TPMT) activity , The patient's activity of thiopurine S-methyltransferase (TPMT) and the pathogenic mutation of TPMT are important to severe bone marrow suppression **(Zhou *et al* ., 2021).** In an other diffrerent study confirmed the myelosuppression and pancytopenia are one of the main side effects of the immunosuppressive medicine azathioprine, a purine antimetabolite, particularly in patients with some degree of TPMT (Thiopurine Methyltransferase) **(Ghalamkari *et al* ., 2019).**

Also use of the Concurrent therapy with azathioprine like allopurinol should be avoided as it enhances the hematological toxicity because allopurinol stops xanthine oxidase from metabolizing mercaptopurine , 60 Combining ribavirin and azathioprine can also cause myelosuppression because ribavirin alters the metabolism of 6-mercaptopurine and inhibits the enzyme IMPDH, Myelosuppression is common and might begin unexpectedly , Leukopenia and neutropenia are dose-related in terms of severity, despite large interindividual variability in sensitivity , Although some people remain mildly leukopenic and it can take many months for the white blood cell count to recover to normal, myelosuppression is often reversible **(Ponticelli & Glassock,**

2019). Also it found several antibiotics have the side effect of suppressing bone marrow, especially when used for lengthy treatment regimens **(Li *et al* ., 2020).**

2.3.2.Mechanism of azathioprine induced bone marrow suppression

Myelosuppression is an important and potentially lethal complication of azathioprine treatment , Myelotoxicity of azathioprine developed at anytime during drug treatment (range 2 weeks-liyears after starting the drug) and occurredeither suddenly or over several months **(Feinman *et al* ., 2020).** share similar clinical and side effects , Cytotoxicity isbelieved to result from the incorporation of thiopurine metabolites into cellular nucleic acids,while the immunosuppressive effects aresecondary to inhibition of de novo purine ribonucleotide synthesis and interconversion , the bone marrow toxicity varied and The reasons for individual variation in bone marrow susceptibility to azathioprine deserveattention , Bone marrow toxicity seems to berelated to the excessive intracellular concentration of the cytotoxic active metabolities 6- thioguanine nucleotides , These substances may accumulate if the activity of the catalytic enzyme thiopurine methyltransferase is deficient **(Dean, 2020).**

Bone marrow toxicity Most of the time, dose-dependent and The immunosuppressive activity of AZA is due to its ability to inhibit the delayed hypersensitivity reaction and its cytotoxicity is due in part to the incorporation of 6-thioguanine nucleotide for DNA that occurs during renal allotransplantation Mostly, immunosuppressive drugs cause anaemia and increase the production of lipid peroxides leading to an imbalance between oxidant-antioxidant which causes the liver and other organs to have oxidative damage **(Turbayne *et al* ., 2022).** This drug converts into 6-MP through reactions with glutathione rapidly and is absorbed as a prodrug for mercaptopurine , Mercaptopurine blocks the DNA producing enzymes thereby affecting cells with high dividing capacity like T and B lymphocytes , Metabolic intercellular of 6-MP is noted for its inactive metabolites - 6-thioguanine nucleotide (6-TGN) and 6-methyl mercaptopurine (6-MMP) that elicit azathioprine

immunosuppressive features. Azathioprine causes stimulation of the stressactivated protein kinase pathway and mitochondria dysfunction causing necrotic cell death in intact isolated rat mitochondria **(Alrekabi *et al* ., 2020).**

Chapter Three : Materials and Methods

3. Materials and Methods

3.1. Materials

3.1.1. Instruments and Equipment for the Laboratory

Laboratory equipment's and apparatuses used in this study are listed in table (3-1) the table below lists the instruments and tools utilized in the lab for this study:-

NO	Equipment & Instrument	Company	Country
1.	sensitive analytical balance	Sartorius	Germany
2.	Balance	Shimadu company	Japan
3.	Centrifuge	Hettich Roto fix11	Japan
4.	Digital camera	Toup Cam	China
5.	Eppendorf tube	Biolabse	England
6.	Freezer	Newal	Turkish
7.	Incubator	BINDER	Germany
8.	Jell tube	AFMA-Dispo	Japan
9.	Latex gloves	Great glove	Malaysia
10.	Light microscope	Leica	China
11.	Micropipette 100-1000 µl	CYAN	Germany
12.	Plan tube	A F M A- Dispo	Japan
13.	Spectrophotometer	Labomed	UK
14.	Sterile syringes	PROTON	Malaysia
15.	Slides and Cover Slides	Marienfeld	Germany
16.	Serum tube	Lab	China
17.	Glassware different sizes and	Duran	Germany

	shapes		
18.	Digital camera	Sonyo	Japan
19.	Binocular Light Microscope	Olympus	Japan
20.	Anatomical set(Scissors, Forceps, Scalpel)	Chemo lab	China
21.	Beakers	Chemo lab	India
22.	Filter paper	Chemo lab	India

3.1.2. Chemicals

Chemicals used in this study and their suppliers are listed in table (3-2) the chemical were used in this study and their sources :-

NO.	Chemicals	Company	Country
1.	Arginase I Kit	Bioassay assay brand	China
2.	ALT Kit	Bioassay Technology Laboratory	Shanghai Korain
3.	AST Kit	Bioassay Technology Laboratory	Shanghai Korain
4.	Azathioprine	Aspen	**Ireland**
5.	Chloroform	Noorbrok	England
6.	Leishman stain	-	-
7.	Aloe Vera Gel	Local	Iraq
8.	Ethanol 70%	Merck	Germany
9.	Formalin 10 %	TEDIA Company	USA
10.	Glutathione (GSH)	laboratory ct	Italy
11.	Malondialdehyde (MDA)	Ela science	USA
12.	Glutathione s-transferase (**G-S)**	Ela science	USA
13.	Reticulocytes stain	-	-

3.2. Methods

3.2.1. Preparation of Azathioprine (AZA)

Drug: Azathioprine (AZA) (Imuran®, aspen, Ireland). A commercially available formulation of AZA 50 mg/ tablet were purchased from a local private pharmacy. AZA was administered through orally administrated , AZA was prepared as the following procedure , AZA was prepared by dissolving 50 mg\kg of AZA powder in 2 mL of distilled water , with stirring for 30 , AZA solution was administered orally for each rat in AZA groups.

3.2.2. Preparation of Aloe Vera gel

Mature, healthy and fresh Aloe Vera leaves about 75 to 90 cm long were washed with fresh water. The leaves were cut transversely into pieces. Thickened epidermis has been selectively removed , Natural Aloe Vera gel was seen, the outer part of the Aloe Vera leaf was peeled off to obtain Aloe Vera gel directly, using a small spoon, the entire gel was extracted , Then the gel was transferred to a blender to obtain a mixture and foam ready to be dosed to the animals under study **(Alkaabi *et al*., 2022 Fig 3-1).**

Figure (3-1): Preparation Aloe Vera gel 500mg/kg

3.2.3. Animals Experimental :-

Forty (40) male white rats weighing (200-220 g) were used in the current study. Taken from the College of Veterinary Medicine , Tikrit University – Iraq , and their ages are between (12-15) weeks , animals were placed in good condition in special plastic cages. They are provided with appropriate conditions and ventilation. the light system was 12 hours. per day with a relative humidity of 50 ± 5%. They were kept for 2 weeks to adjust to standard experimental conditions. The experiment begins November 30th and ends December 30th. The temperature was maintained At (21-25)°C using a room thermostat, the room air was changed continuously Using vacuum ventilation, feed the animals a pellet of fresh food ration.

3.3. The Experimental Design

In the current investigation, 40 male white rats were employed They were split into 4 groups (n = 10) as listed in the points below (Fig 3-2):

1) Group (G1): 10 rats administrating sterile water and introduce as single daily dose administered orally for four weeks**.**

2) Group (G2): 10 rats administrating azathioprine (50mg/kg. b. w) was dissolved in sterile water and introduce as single daily dose administered orally for four weeks **(Alrekabi *et al*., 2020).**

3) Group (G3): 10 rats administrating Aloe vera gel extract (500mg/kg b.w) was dissolved in sterile water and introduce as single daily dose administered orally for four weeks **(Rashid *et al*., 2022).**

4) Group (G4): 10 rats administrating azathioprine (50mg/kg. b. w) and Aloe vera gel extract (500mg/kg b.w) was dissolved in sterile water and introduce as single daily dose administered orally for four weeks.

3.4. Blood Sample and Hematological study

Blood sample were collected via cardiac puncture from each male rat, placed in serum tube and left for 30 minutes, then to be centrifuged (3000 rpm for 10 minutes) and kept frozen at -20 °C to obtain the serum which then was transferred to the Eppendorf tubes. All these tubes were stored at (-4c) until analyze for biochemical Evaluation, also blood was put in EDTA tube and immediately transfer of samples for examination with Hematological test **(Nasir, 2018).**

3.5. Histopathological examination

Liver and Bone marrow was to be removed and the organs were being fixed in to 10% of formalin for histological examination.

3.6. Hematological study

3.6.1. Complete blood count

was obtained automatically by using a device **Swelab Alfa ,** as shown in Appendices I.

3.6.2. Reticulocyte Count

The test for determining bone marrow function and assessing erythropoietic activity is the reticulocyte count , It is used to categorize and track anemia treatment , Erythropoietic activity grows along with the amount of reticulocytes **(Tanaka *et al*., 2023)** , as shown in Appendices II.

3.6.3. Examination of bone marrow smears

The reason of severe anemia or thrombocytopenia (low platelet count) should be ascertained. Find aberrant chromosomes to assess your risk and make treatment plans **(Melo Bisneto *et al*., 2021)** , as shown in Appendices III.

3.7. Biochemical Analysis

3.7.1. Arginase I (ARG1)

Rat arginase I (ARG1) concentration was determined by using a special ELISA kit (bioassay assay brand , china) **(Zhang *et al*., 2022)** , as shown in Appendices IV

3.7.2. Alanine aminotransferase (ALT/GPT)

Alanine aminotransferase concentration was determined by using a special ALT Kit (Bio systems, Spain) **(Hussein *et al*., 2022)**, as shown in Appendices V.

3.7.3. Aspartate aminotransferase (AST/GOT)

Aspartate aminotransferase (AST) concentration was determined by using a special AST Kit (Bio systems, Spain) **(Hussein *et al*., 2022)**, as shown in Appendices VI.

3.8. Liver antioxidant

3.8.1. Determination of Serum Malondialdehyde Level (MDA) Concentration (µ mol/L)

Malondialdehyhe was estimated by Thiobarbituric acid (TBA) assay method **(Firdausa *et al*., 2022)** on spectrophotometer , as shown in Appendices VII.

3.8.2. Estimation of serum Glutathione-S-transferase (GST) concentration

The procedure was done according to the instructions of the manufacture of ELIZA Kit -Elabscience biotechnology/ china **(Onuoha *et al*., 2023)** , as shown in Appendices VIII.

3.8.3. Serum reduced glutathione concentration (GSH)

The assay involves carefully optimized enzymatic recycling method using glutathione reductase and Ellman's reagent according to **(Abdelrazek *et al*., 2022)** as described in Appendices IX.

3.9. Histological study

The liver and bone marrow of each animal were quickly removed and rapidly weighed then prepared for histological study according to **(Mostafa *et al*., 2022)** , as shown in Appendices X.

3.10. Statistical analysis

Statistical analysis of data was performed using SAS (Statistical Analysis System - version 9.1). One-way ANOVA and Least significant differences (LSD) post hoc test were performed to assess significant differences among means. $P < 0.05$ is considered statistically significant **(SAS, 2010).**

Chapter Four : Results And Discussion

4. Results And Discussion

4.1. Hematological profile

4.1.1. Effect of Azathioprine , Aloe Vera extract on total number of leukocytes (WBCs) , Total number of erythrocytes (RBCs) and Packed cell volume (PCV)%

The main value of RBC , WBC and PCV were decreased in significant value (P<0.05) in azathioprine group comparatively to control group and aloe vera groups while there were elevation in value (P<0.05) for RBC , WBC and PCV in combination aloe vera and azathioprine group in comparison with azathioprine group.

Table 4-1: Effect of Aloe Vera extract 500 (mg/kg/BW) and Azathioprine 50 (mg/kg/BW) on WBC , RBC and PCV in male rats. `

Groups	WBC	RBC	PCV
Control group	12.52±0.36a	7.19±0.17a	39.29±0.53a
Aloe Vera group	13.04±0.29a	7.38±0.12a	41.39±0.33a
Azathioprine group	4.19±0.10c	4.74±0.15b	30.76±0.45c
Aloe Vera & Azathioprine group	9.68±0.37b	7.14±0.24a	37.10±1.40b
LSD	0.91	0.53	2.41

Data represented as mean ± SD different letters significant differences at P-value (P<0.05).

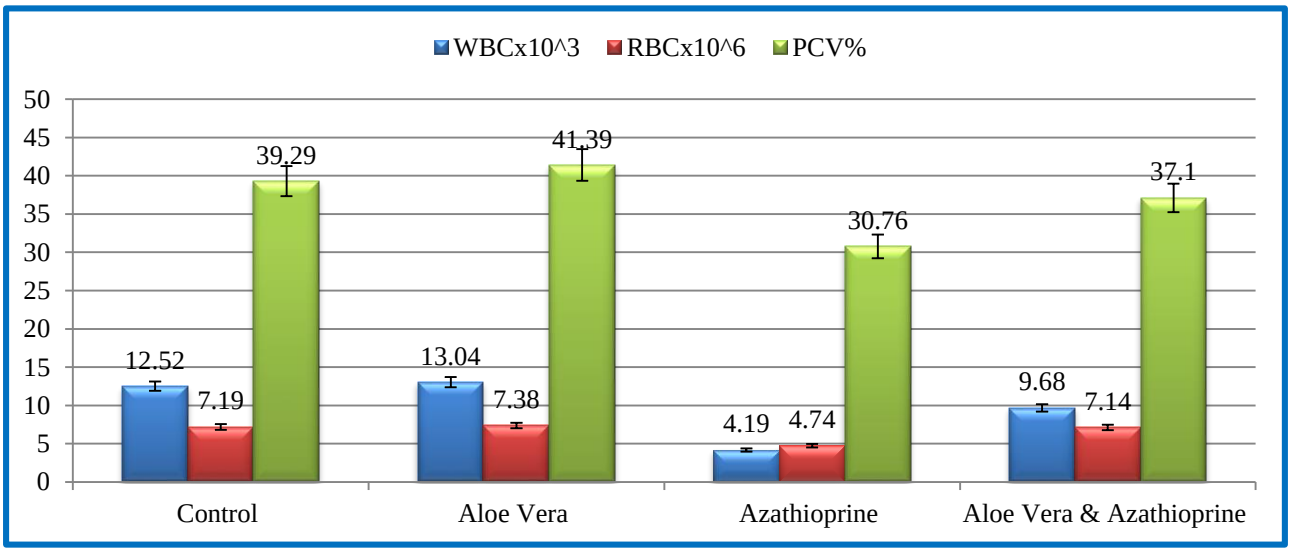


Figure (4-1) Effect of azathioprine and aloe vera extract on WBC , RBC , PCV

Azathioprine has the ability to Myelosuppression and a reduction in thymus size were anticipated side effects of azathioprine therapy and can cause the decrease in WBC , RBC and PCV this result by a process that decreases 6-mercaptopurine (6-MP), a thiopurine, which results in an inhibition of RBC , PCV and WBC count as a result of the bone marrow's inability to produce blood cells **(Lee *et al.*, 2022).** cytotoxicity is thought to be caused by the incorporation of thiopurine metabolites into cellular nucleic acids, which damages RBC and WBC and lowers their count **(Zakerska-Banaszak *et al.*, 2022; El-Sherbiny, 2018).**

Furthermore, that Azathioprine has harmful effects on the liver, including drug-induced hepatotoxicity that can cause release reactive oxygen species (ROS) and is consequently ascribed to hepatotoxicity. Oxidation may play a role in these effects that lead to decrease in RBC , PCV and WBC count **(Donato *et al.*, 2022).** In this study aloe vera extract has been showed improvement in hematological parameters include WBC , RBC and PCV this may be due to purported ability of aloe vera extract to stimulate bone marrow and promote erythropoiesis , It may be related to the stimulation of factors that help stimulate erythrocyte proliferation and differentiation, such as interleukins **(Tanaka *et al.*, 2023).**

Additionally, aloe vera is known to contain minerals such as iron, copper, and folic acid, which are the building blocks for red blood cell production, as well as vitamins such as A, C, E, B1, B2, B5, B6, and B12 **(Nku *et al.*, 2015).**

4.1.2. Effect of Azathioprine , *Aloe Vera* extract on mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) in male rats

Depending on the result clarified in table (4-2) there were a significant (P>0.05) decrement for MCV , MCH and MCHC in azathioprine group in comparison with control group and aloe vera group. Also the same table revealed a significant (P<0.05) increment for MCV , MCH and MCHC in the group treated aloe vera plus azathioprine as compared to the azathioprine group.

Table 4-2: Effect of Aloe Vera extract 500 (mg/kg/BW) and Azathioprine 50 (mg/kg/BW) on MCV , MCH and MCHC in male rats.

Groups	MCV	MCH	MCHC
Control group	59.30±0.27a	18.84±0.27b	35.30±0.20a
Aloe Vera group	60.38±0.90a	19.71±0.64b	36.80±0.36a
Azathioprine group	50.09±0.45b	14.42±0.52a	30.78±0.42b
Aloe Vera & Azathioprine group	58.53±0.78a	22.65±0.47c	37.48±0.95c
LSD	1.95	1.49	1.68

Data represented as mean ± SD different letters significant differences at P-value (P<0.05)

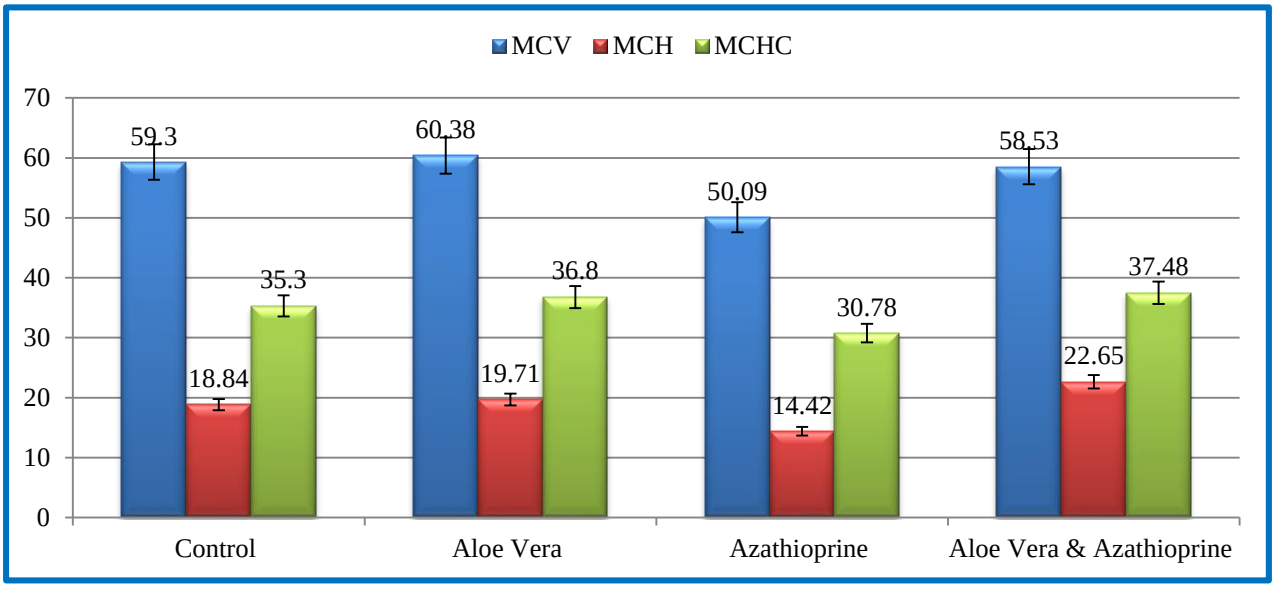


Figure (4-2) Effect of azathioprine and aloe vera extract on MCV , MCH , MCHC

The azathioprine causes MCV, MCH, and MCHC levels Reduced that imply that erythropoiesis was suppressed, which led to the development of microcytic hypochromic anemia , add on ROS (reactive oxygen species) promotes hemoglobin glycation, erythrocyte fragility, and direct oxidation can harm bone marrow **(Bosing *et al.*, 2012; Gao *et al.*, 2013; Kausar *et al.*, 2019).** Reactive oxygen species (ROS) lead to enhance glycemia, erythrocyte fragility, and the risk of direct oxidative damage to bone marrow , More precisely, azathioprine may be etiological by increasing oxidative stress **(Niforou *et al.*, 2014).**

While the result of many studies showed the aloe vera leaf extract significant increases in hematological parameters such as MCV , MCH and MCHC This increase may be due to variations in erythrocyte size, shape, and hemoglobin content **(Egong *et al.*, 2018; Channa *et al.*, 2014).** This effect and increased level have been attributed to the efficacy of essential vitamins such as riboflavin, thiamine, and folic acid as well as essential and non-essential amino acids in A. vera that are required for hemoglobin synthesis **(Iji *et al.*, 2010).**

4.1.3. Effect of Azathioprine , *Aloe Vera* extract on Reticulocyte Count in male rats

The main value of Reticulocyte Count in azathioprine group showed a significant ($P<0.05$) increase when compared with control group and aloe vera groups while the combination azathioprine plus aloe vera group showed a significant ($P<0.05$) decrease as compare to azathioprine group as show in table (4-3).

Table 4-3: Effect of Aloe Vera extract 500 (mg/kg/BW) and Azathioprine 50 (mg/kg/BW) on Reticulocyte Count in male rats.

Groups	Number of Reticulocyte Count	Reticulocyte Count %
Control group	6.00±1.00a	0.6
Aloe Vera group	5.50±0.50a	0.55
Azathioprine group	18.00±0.19b	1.8
Aloe Vera & Azathioprine group	11.00±0.08c	1.1
LSD	2.50	

Data represented as mean ± SD different letters significant differences at P-value (P<0.05)

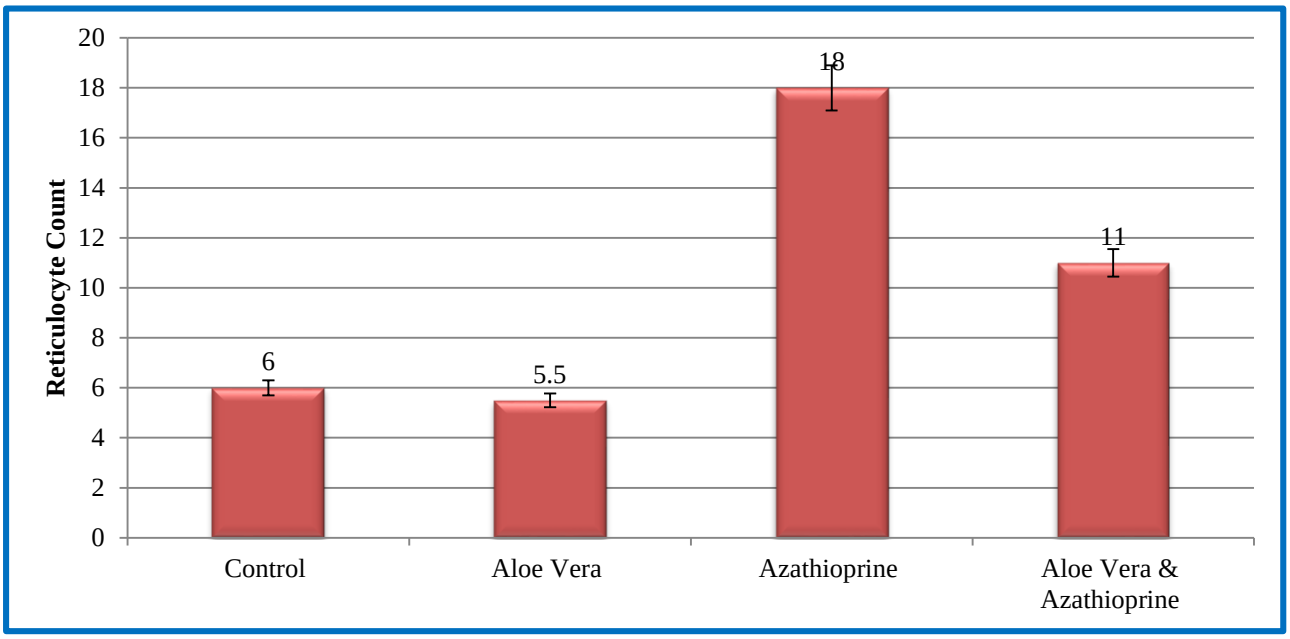


Figure (4-3) Effect of azathioprine and aloe vera extract on Reticulocyte count

Azathioprine is an immunomodulatory medication that is frequently used to treat autoimmune illnesses and prevent the rejection of donated organs and can cause had significantly upper average reticulocyte counts when compared to the control group **(Agarwal *et al.*, 2017).** This may be due to the anemia that may have been caused by the suppression of hematopoiesis , Additionally, there was a considerable decline in phagocytic activity and lymphocyte proportion, which toxicity in the bone marrow

(Alrekabi *et al.*, 2020). Furthermore After in vivo erythropoietin treatment, reticulocyte counts in peripheral whole blood were considerably upper by azathioprine at the effective dose in rats, indicating a minimal risk for anemia **(Nakamura *et al.*, 2017).**

AZA-treated rats' bone marrow showed increased reticulocyte counts due to granulocytic addition-induced bone marrow toxicity, which was defined as the production of peripheral blood pancytopenia , In addition to leucopenia, neutropaenia, anemia, pancytopenia, thrombocytopenia, and thrombocytosis in the peripheral blood, the marrow was hypocellular with decreases in the myeloid, erythroid, and megakaryocyte series **(Molyneux *et al.*, 2008; Ahmed *et al.*, 2014).** While oral aloe Vera therapy (500 mg/kg B.W.) proved effective in reducing azathioprine toxicity, it also changed the anemia, leucopenia brought on by the drug , Furthermore, by lowering the increased activity of blood hepatic enzymes, aloe vera exerted considerable protection against liver damage brought on by azathioprine , Aloe vera also has the ability to reduce the toxicity caused by azathioprine **(Alrekabi *et al.*, 2020).**

Rats given the extract of A. vera demonstrated the presence of young red blood cells in the circulation, indicating stimulation of the development of immature erythrocytes, commonly known as reticulocytes , When active blood regeneration occurs, reticulocytes typically appear as the initial response to stimulation of the haematopoietic system , They are characterized morphologically by increases in the size of red cells in circulation, and they are traditionally used to treat anemia **(Ekanade *et al.*, 2015).**

4.1.4. Effect of Azathioprine , Aloe Vera extract on Bone Marrow Smears in male rats

The main value of bone marrow smears in azathioprine group showed a significant (P<0.05) increase in mylocyte and promylocytes while showed a significant (P<0.05) decrease in neutrophil , eosinophil and lymphocytes when compared with the control and aloe vera group , Also there were a significant (P<0.05) decrease in mylocyte and promylocytes in addition there were a significant (P<0.05) increase in neutrophil , eosinophil and lymphocyte in the group treated aloe vera plus azathioprine group as compared to azathioprine group.

Table 4-4: Effect of Aloe Vera extract 500 (mg/kg/BW) and Azathioprine 50 (mg/kg/BW) on Bone Marrow Smears in adult male rats.

Groups	Mylocyte	Promylocytes	Neutrophil	Eosinophil	Lymphocyte
Control group	25.00±1.00b	7.00±1.10ab	20.00±1.00a	10.00±0.44a	22.00±0.50b
Aloe Vera group	24.00±1.00b	5.00±1.00b	17.00±1.00ab	9.00±1.00a	21.00±1.00b
Azathioprine group	29.00±0.75a	12.00±1.50a	14.00±0.50c	7.00±0.50b	17.00±1.50a
Aloe Vera & Azathioprine group	22.00±0.75b	10.11±1.25a	16.00±1.15ab	9.00±0.50a	28.00±1.00c
LSD	3.92	4.01	4.20	1.50	3.90

Data represented as mean ± SD different letters significant differences at P-value (P<0.05)

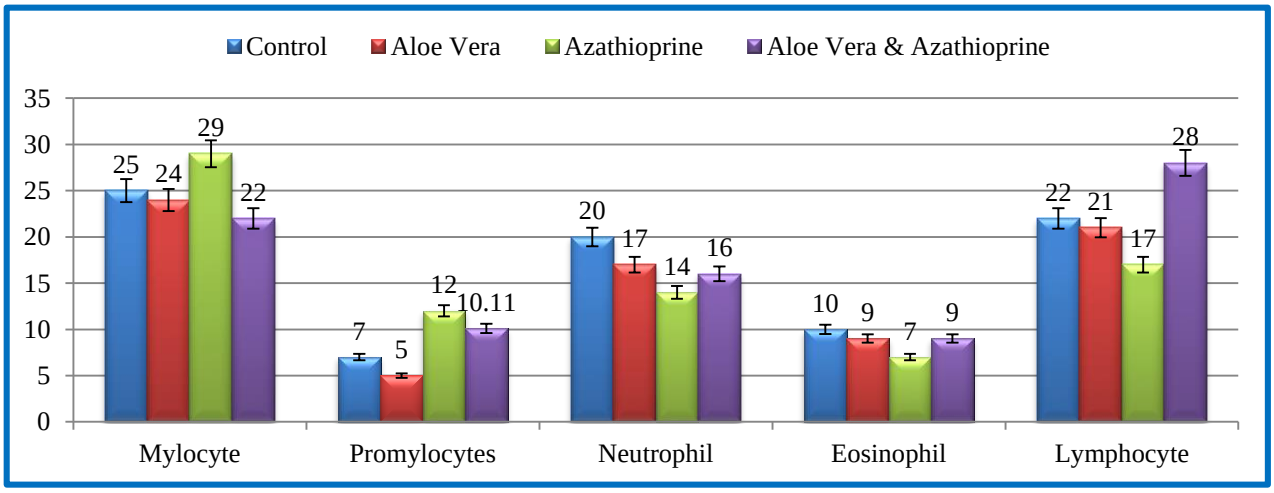


Figure (4-4) Effect of azathioprine and aloe vera extract on mylocyte , promylocytes , neutrophil , eosinophil and lymphocyte

The vast variety of cells that make up bone marrow react to different toxicological insults in different ways , Additionally, the presence or absence of hematologic alterations in laboratory animals exposed to new pharmacological compounds or environmental contaminants may offer useful tools for assessing the toxicity of various agents **(Othman *et al.,* 2009).** The results of the current study, which looked at how azathioprine administration affected bone marrow activity through a differential analysis of different cell types, revealed that Mylocyte and promylocyte of bone marrow cells, with the exception of eosinophils, neutrophils, and lymphocytes, significantly increased in response to these toxins **(Kanderi *et al.,* 2020).**

This result could be a sign that the poisons stimulated the bone marrow's activity , Endotoxins interact with cell membranes because of their makeup, which has a significant impact on how cells develop and function Lipopolysaccharide (LPS) entry into the cell membrane and binding to cellular receptors or soluble proteins can result in these consequences **(Al-Sagair *et al.,* 2009).** Rats have experienced the same bone marrow activity-stimulating effects of endotoxins , An serious and potentially fatal side effect of azathioprine therapy is myelosuppression , Male rats administered with 50 mg/kg/day of azathioprine showed bone marrow toxicity and myelotoxicity **(Ghalamkari *et al.,* 2019).** prompting a reassessment of the bone marrow smear in all patients receiving the medication , When azathioprine is administered in a moderate dose, bone marrow suppression is also frequently reported, but it can be severe , Adenosine, a purine that occurs naturally, is analogous to the synthetic compound azathioprine **(Nku *et al.,* 2015).**

These medications have both cytotoxic and immunosuppressive qualities since they are converted to 6-mercaptopurine, and because their following metabolism is identical, the dose of 6-mercaptopurine needed to induce equivalent therapeutic and harmful effects as azathioprine is around 55% that of azathioprine **(Eltantawy *et al.,* 2023).**

The incorporation of thiopurine metabolites into cellular nucleic acids is thought to cause cytotoxicity, while suppression of de novo purine ribonucleotide synthesis and interconversion is thought to cause immunosuppressive effects , This impact, which is assumed to be dose-dependent, is connected to a change in the ratio of myeloid to erythroid cells and a shift toward less mature forms , Before the peripheral blood count, accompanying bone marrow modifications in granulopoiesis are not visible **(Broen *et al.*, 2020).**

A more severe form of bone marrow suppression happens when using greater doses of azathioprine, an uncommon response may appear without warning , In these circumstances, altered marrow cellularity and poor cell line development lead to agranulocytosis or pancytopenia , It is important to consider the causes of individual differences in azathioprine susceptibility in bone marrow , The high intracellular concentration of the cytotoxic active metabolites 6-thioguanine nucleotides appears to be a contributing factor in bone marrow toxicity **(Carey, 2014).** If the catalytic enzyme thiopurine methyltransferase is not active enough, these compounds may build up , Our experience using azathioprine in bone marrow demonstrates that there is a low risk of myelotoxicity, but there could be substantial clinical consequences , The most prevalent and harmful side effect of azathioprine on the bone marrow is leucopenia , All male rats that experienced bone marrow suppression signs had significant leucopenia , The onset of symptoms as early warning signals of myelosuppression was delayed **(Chen *et al.*, 2014).** Our findings suggested that azathioprine may be to blame for the changes in the bone marrow's histology, including a progressive decline in the RBC and WBC counts in circulation, which indicates active bone marrow suppression brought on by azathioprine-induced toxicity, a reduction in the cellularity of hematopoietic cells, and a corresponding rise in adipocytes , Following exposure to cytostatic drugs that cause the loss of bone marrow cells, the lymphocytes and lymphomas decrease in the bone marrow and peripheral blood **(Kailo *et al.*, 2008).**

Pancytopenia and bone marrow failure are features of aplastic anemia , It might result from autoimmune conditions, radiation, medications, or toxins , Aplastic anemia is primarily brought on by azathioprine, non-steroidal anti-inflammatory drugs, antiepileptic medications, gold salts, and antithyroid medications Pancytopenia frequently causes clinical signs and symptoms, such as anemia and bleeding problems , Neutropenia can result in fever and sepsis in some people **(Ghalamkari *et al.*, 2019; Hassankhani *et al.*, 2017).** When treating a disease with azathioprine, a condition known as pancytopenia or bone marrow suppression with complete blood count test because lower WBC and platelet counts, accumulate polyglutamate in the liver and bone marrow precursors over time, and decrease natural folic acid levels in the tissue, all of which can have toxic effects , The pancytopenia is a moderate side effect of azathioprine caused by the transformation of azathioprine to 6-mercaptopurine, which increases the risk for pancytopenia and hepatotoxicity connected to azathioprine suppression in the bone marrow **(Cotoraci *et al.*, 2021).**

Inhibition of immune system cells and decreased generation of blood cells due to hematopoiesis inhibition In addition to the fact that rats treated with AZA had decreased white blood cell counts, this may be related to the fact that bone marrow lymphocyte and monocyte numbers were declining **(Jensen *et al.*, 2018).** Numerous studies with the azathioprine drug's chemical structure as a result of complications from AZA consider significant issues, one of which is bone marrow suppression and toxicity, support the fact that a decrease in complete blood counts from which platelets and white blood cells occurred due to the treatment with azathioprine and causing myelosuppression and pancytopenia, also can cause toxicity of bone marrow **(Kivity *et al.*, 2014; Ahmadzadeh *et al.*, 2019).** There are numerous medicinal plants that are used in utilized to treat leukopenia and thrombocytopenia as well as cases of anemia of all kinds by boosting the hematopoietic process through its bone marrow precursors, the cause is related to AZA's toxic effects, which cause myelosuppression by suppressing bone marrow **(Mhatre & Marar, 2016).**

Aloe vera has been linked to numerous medical health benefits and good public health , Mesenchymal stem cells (BMSCs) generated from bone marrow have numerous potential applications in tissue regeneration **(Guo & Mei, 2016; Haroun *et al.*, 2020).** Aloe vera is a medical plant that also has antioxidant, anti-diabetic, anti-cancer, and anti-hyperlipidemic properties , Aloe vera gel extract contains a wide range of compounds, including minerals, enzymes, hormones, and carbohydrates , Mesenchymal stem cells (MSCs), biologically active progenitor cells that can self-renew and give rise to a variety of cell types , Plant extracts have been used to encourage the proliferation and differentiation of MSCs **(Farid *et al.*, 2022).** Male rats can be treated with aloe vera gel and bone marrow (BM)-MSCs By creating the oxidative stress, the antioxidant enzymes in aloe vera gel were investigated , The results showed that utilizingand aloe vera gel MSC transplantation has been linked to improvements in bone marrow cells, decreased levels of oxidative stress and pro-inflammatory cytokines, and decreased NF-B activation , Aloe vera gel assisted transplanted MSCs in differentiating , When coupled with MSCs, aloe vera gel has a considerable effect on MSC differentiation **(Farid *et al.*, 2022).**

4.2. Biochemical parameters

4.2.1. Effect of Azathioprine , *Aloe Vera* extract on Arginase I, Alanine aminotransferase enzyme (ALT) and aspartate aminotransferase enzyme (AST) levels in serum male rats

The data in this study table (4-5) showed a significant ($P<0.05$) increase in AST , ALT and Arginase I level in azathioprine group as compare to control and aloe vera group on the other hand there were observed a significant decrease in AST , ALT and Arginase I level in combination aloe vera and azathioprine in comparison with azathioprine group.

Table 4-5: Effect of Aloe Vera extract 500 (mg/kg/BW) and Azathioprine 50 (mg/kg/BW) on serum AST , ALT and Arginase I level in male rats.

Groups	AST	ALT	Arginase I
Control group	50.00±1.8a	28.36±0.6a	3.37±0.10a
Aloe Vera group	48.00±1.3b	30.00±0.2b	4.67±0.10a
Azathioprine group	128.00±3.9c	40.61±2.3c	9.33±0.33b
Aloe Vera & Azathioprine group	100.00±2.3d	34.81±0.4d	6.65±0.30c
LSD	4.85	3.71	1.07

Data represented as mean ± SD different letters significant differences at P-value (P<0.05)

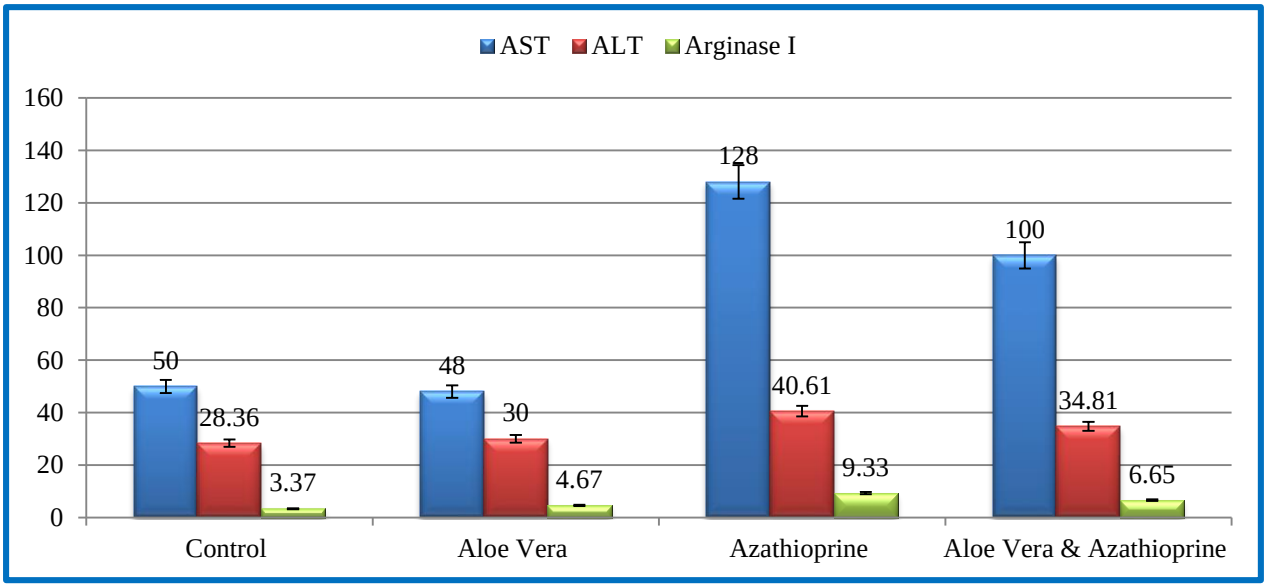


Figure (4-5) Effect of azathioprine and aloe vera extract on AST , ALT , Arginase I

These result about AST and ALT agreement with **(El Joumaa & Borjac, 2022).** That suggested that the elevation in liver function tests such as AST and ALT have close relationship with induction of liver dysfunction by azathioprine and can be sign for hepatotoxicity , Azathioprine is one of many medications used to treat cancer and immunological disorders, and it is the most popular , Numerous reports suggest that azathioprine has hepatotoxic side effects, which manifest as an increase in serum AST and ALT **(Belizna *et al.*, 2020).** The harmful effects of azathioprine on liver

cells are part of its mechanical toxicity, which reduces levels of free radicals that disrupt hepatocyte mitochondria, lowers ATP levels, and eventually causes necrosis, which leads to liver damage **(Choudhary *et al*., 2012; Al-Abbassi *et al*., 2023).**

While the result revealed a substantial reduction in Arginase I , The opposite of its major function is regulation, which is linked to the role of the promoter azathioprine, due to the variable regulation of arginase-specific oxidative stress in different degenerative illnesses via modifying NO **(Al-Shahari *et al*., 2022).** Rats with liver toxicity had lower arginase activity, which may have been caused by a decline in antioxidant activity , Arginase activation could also have resulted in the uncoupling of eNOS due to an increase in ROS , As a result, the uncoupled eNOS produces superoxide using molecular oxygen, further reducing the NO level **(Xiong *et al*., 2017; Akomolafe *et al*., 2018).** By decreasing the availability of arginine to endothelium nitric oxide synthase (eNOS), increased arginase activity in the body has been shown to reduce endothelium dependent vasorelaxation, which ultimately results in a decrease in NO production and subsequently clogs the blood arteries , Arginase activity is found to increase when toxicants such azathioprine medications are administered by phenolic-rich dietary plants to inhibit **(Shatanawi *et al*., 2015; Oyeleye *et al*., 2020; Kashyap *et al*., 2022).**

Arginase has been shown to be superior to ALT and AST in the acute and chronic rat liver damage caused by toxicants , The mitochondria are important targets for drug toxicity, either directly or indirectly through the creation of reactive metabolites, according to an analysis of the mechanisms causing drug-induced liver damage and the release of soluble products like AST, ALT, and arginase , These abnormalities typically result in mitochondrial oxidative stress and the production of peroxynitrite **(Bailey *et al*., 2019).** which affects the structural integrity of proteins and mitochondrial DNA , Apoptosis-inducing factor and other intermembrane proteins are also released , Nuclear DNA fragmentation results from the nuclear translocation of and endonuclease G , These things happen together to cause necrotic

cell death. On the other hand, mitochondrial release of cytochrome C and other proapoptotic elements can encourage caspase activation and apoptotic cell death **(Abdel-Azeem *et al.*, 2013; Ramachandran & Jaeschke, 2017).**

The results of several articles indicates the hepatoprotective effect of aloe vera against hepatotoxicity there are many medical plants available to mend the hepatotoxicity of liver damage ,The treatment with herbals like Aloe Vera might be utilized instead of some drugs to get rid of their toxic activity as well as employed to treat their adverse effects **(Jangra *et al.*, 2022).** The treatment with herbals like Aloe vera could be used instead of some drug to rid of their toxic activity as well as used to treat their side effects , Aloe vera gel has been shown to play a protective effect in preventing liver damage, which is indicated by elevated AST and ALT levels and a considerable decline in those values **(Madhav and Bairy, 2011).** The results of the current study shown that the biological system's numerous organs depend on NO production, which is increased when arginase activity is inhibited by aloe vera and azathioprine treatment **(Dada *et al.*, 2020).** Arginase is a crucial mediator in the etiology of vascular disease, injury, and inflammation, according to mounting evidence , Hepatotoxicity illness problems associated with inflammation, hypertension, and bodily function failure have all been linked to increased arginase activity **(Rojas *et al.*, 2017).**

4.3. Liver Oxidant & Antioxidant Enzymes

4.3.1. Effect of Azathioprine , Aloe Vera extract on reduced glutathione(GSH), glutathione s-transferase (G-S transferase) and malondialdehyde (MDA)

The main value of liver oxidant and antioxidant enzyme in azathioprine group showed a significant ($P<0.05$) increase in MDA and a significant decrease in GSH and GST when compared with control and aloe vera group , Also there were a significant ($P<0.05$) decrease MDA and a significant increase in GSH and GST in the

group treated aloe vera plus azathioprine as compare to azathioprine group as show in table (4-6).

Table (4-6) :- Effect of Azathioprine , Aloe Vera extract on serum oxidant MDA and antioxidant GSH and GST in male rats

Groups	MDA	GSH	GST
Control group	8.62±0.8a	40.78±2.6a	13.30±0.43a
Aloe Vera group	9.41±1.0ba	38.62±2.0a	12.98±0.47b
Azathioprine group	16.52±1.4b	22.52±1.7b	9.16±0.83c
Aloe Vera & Azathioprine group	11.21±1.1c	35.81±2.1c	15.98±0.76d
LSD	2.03	3.21	1.91

Means with a different letter in the same column are significantly different (P<0.05)

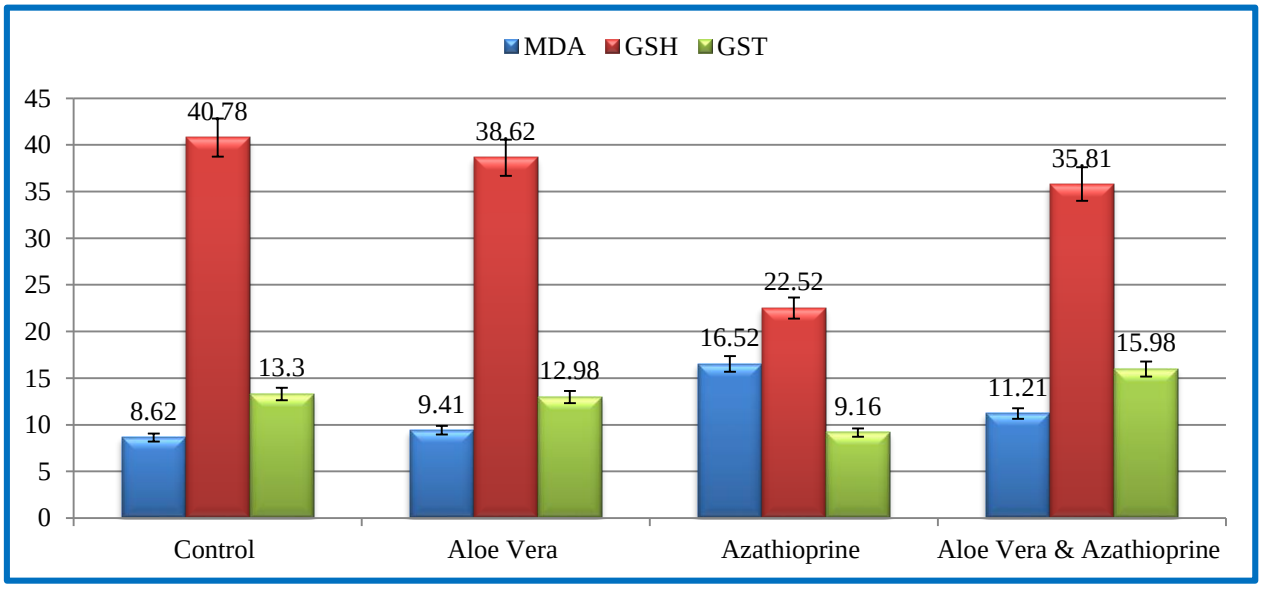


Figure (4-6) Effect of azathioprine and aloe vera extract on MDA , GSH , GST

MDA has been utilized as a marker for lipid oxidative damage because it is one of the aldehydes end products of lipid peroxidation , In the sequence of oxidative stress, increasing levels of MDA are associated with several illnesses, including liver disorders , DNA and protein changes are brought on by MDA. In this work, oral administration of AZA to rats increased MDA while significantly lowering GSH and

G-S transferase levels was seen as a result, confirming the involvement of oxidative stress and lipid peroxidation in AZA-induced liver damage , Depletion of GSH contributes to the toxicity of AZA on hepatocytes, which causes mitochondrial damage, significant ATP depletion, and cell death via necrosis **(Ahmed *et al*., 2014).** The elevated MDA levels in liver tissue indicates increased lipid peroxidation that damages tissue and compromises the antioxidant defense mechanism , Based on the observed GSH depletion seen after azathioprine poisoning, the elevated liver MDA in the azathioprine-treated rats can be explained , According to reports, the generation of lipid peroxides and GSH depletion are closely related , Azathioprine caused hepatotoxicity, which was evident in the livers of rats treated with the drug by significant pathological alterations , Similar histological lesions have been seen in the rats treated with AZA in other instances **(Ayala *et al.*, 2014).** Aldehyde and other by products of lipid oxidation have a significant negative impact on the liver, leading to the formation of high molecular mass protein aggregate within the membrane **(Rosenzweig *et al.*, 2019).**

Therefore, an elevated level of MDA and its related products, such as conjugated dienes, are a real sign of lipid peroxidation that highlights the harmful effects of MDA on the liver , Thiols are believed to be essential for protecting cells from lipid oxidation and The cellular protein surveillance network includes important components, such as ATP-dependent chaperones, that are engaged in a wide range of protein functions **(Xu *et al.*, 2021).** Additionally the Aloe vera reduced the levels of MDA in the liver and increased the amount of GSH and GST , Aloe vera treatment dramatically increased metallothionein induction in male rats treated across the body, reduced lipid peroxidation, and activated antioxidant enzymes crucial for managing oxidative stress and boosting immunity **(Farid *et al.*, 2022).** It appears that Aloe vera extract has a protective function against oxidative stress-induced cell damage and induced protein synthesis because administration of Aloe vera extract increased

hepatic antioxidant enzymes in mice, which in turn decreased hepatic MDA level and increased GSH and GST level **(Hashim *et al.*, 2022).**

A study on the benefits of AV extract against liver damage brought on by oxidative stress found that AV decreased the production of lipid peroxidation , When rats administered with AV were compared to rats administered with AV, the effects on enzyme levels were comparable to antioxidants in terms of glutathione and glutathione s-transferase **(Iftkhar *et al.*, 2022; Nahar *et al.*, 2022).**

4.4. Histopathological study

4.4.1. Effect of Azathioprine , *Aloe Vera* extract on Liver

following the animals had undergone anesthesia, the liver was removed, and following fixation, sections of the liver tissue were obtained and fixed with formalin (10%) , Following processing in an alcohol and paraffin, the blocking of the samples was divided into sections, and the samples were then stained with (H&E) histological examination of liver section of control group were staind with haematocylin and eosin , as showed a normal hepatocyte appearance, normal liver lobular structure, and a strikingly normal central vein, as seen in the figure (4-7) **(Younes *et al.*, 2018).**

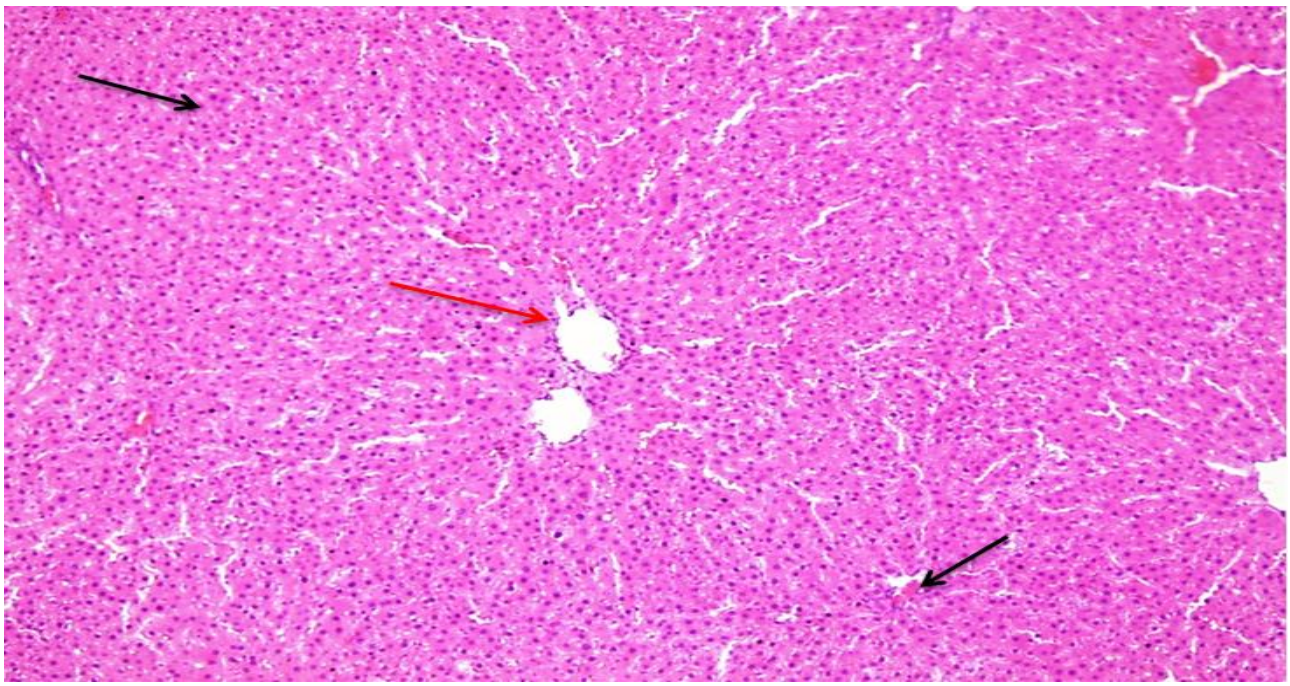


Figure (4-7) Photomicrograph of rats liver tissue section from control group , showed hepatic tissue with normal liver lobules (black arrow) and remarkable normal central vein (red arrow). (H and E ,10X).

Histological examination of liver section of aloe vera group were staind with haematocylin and eosin , as showed a normal hepatocyte appearance There are no changes in the liver cell's normal central vein and no changes to the hepatic cells as showed the liver sections have a normal pattern in his investigation of employing Aloe vera extract as a hepatoprotective **(Alabbood *et al.*, 2019).** Aloe vera extract treatment has been shown to the ability of antioxidant enzymes in the liver of mice addtion to plays a crucial function in protecting hepatocytes from oxidation-induced membrane and cellular damage by increasing the activity of antioxidant enzymes by reducing the amount of malondialdehyde (MDA) that occurs in the liver as a result of oxidative stress. Aloe vera extract regulates and has the capacity to increase Glutathione (GSH), the primary factor in enhancing liver damage and improving hepatocyte alterations **(Guo & Mei, 2016).**

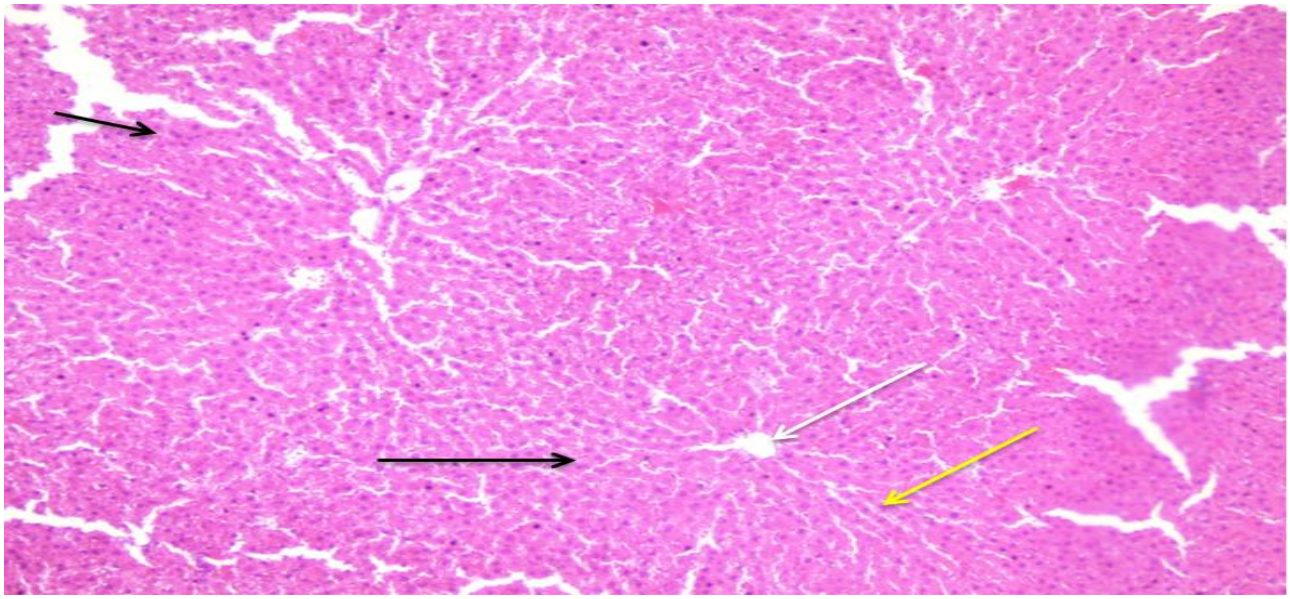


Figure (4-8) Photomicrograph of rats liver tissue section from Aloe group , showed hepatic tissue with normal liver lobules (black arrow),normal hepatocytes arrangements radiating (yellow arrow), around central vein(white arrow) .(H and E ,10X).

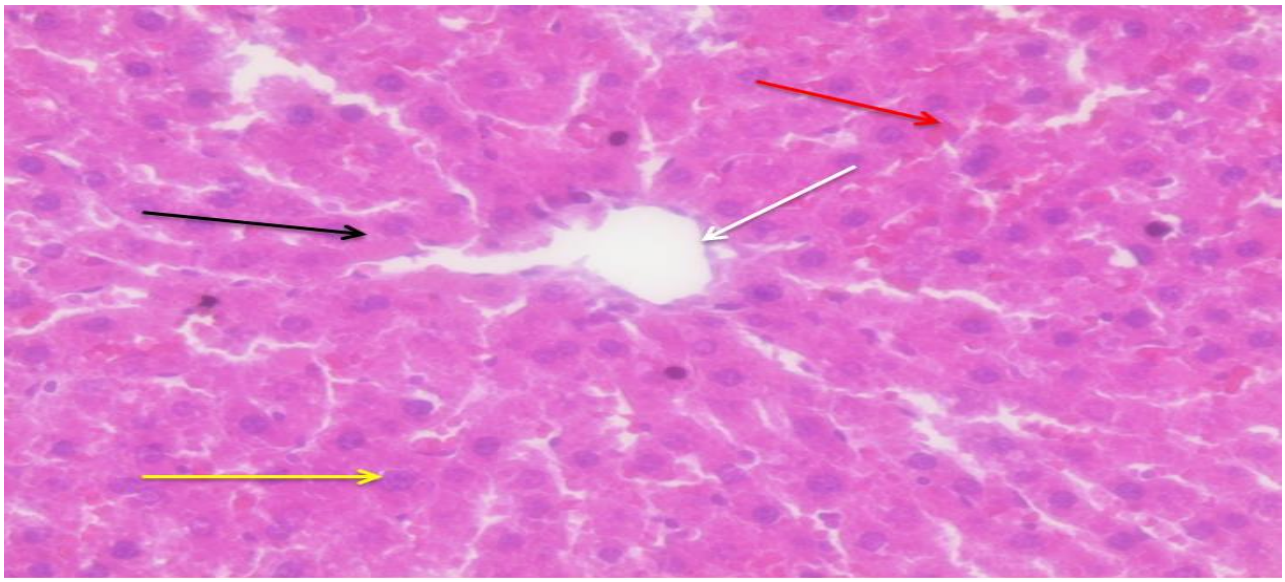


Figure (4-9) Photomicrograph of rats liver tissue section from Aloe group , showed hepatic tissue with normal liver lobules (black arrow), slight sinusoidal congestion (red arrow), around central vein(white arrow), hepatocytes with significant rounded large nuclei (yellow arrow). (H and E ,40X).

Histological examination of liver section of azathioprine group were staind with haematocylin and eosin , as showed a sever central vein dilatation , significant hepatic degenerative changes and marked perivascular polymorph nuclear inflammatory cells infiltration , hepatocytes with significant rounded large nuclei and central vein dilation , Azathioprine is a popular immunosuppressant used to treat a variety of diseases , However, its negative side effects are extremely severe, and the most prevalent cause of liver damage is likely due to a variety of factors, including liver oxidation that occurs during drug metabolism and the detoxification process , The most hazardous stages of the liver damage, on the other hand, are fibrosis, cirrhosis, and even hepatic cancer **(Mannaa *et al.*, 2015; Roselli *et al.*, 2020).**

Azathioprine treatment for abnormal organization and non-organized architecture of hepatic tissue resulted in minor hepatocyte destruction, as seen in the liver portion suffered from apoptosis, or programmed cell death, as well as an evident harm to the liver cell **(Meijer *et al.*, 2017).** Azathioprine can damage the liver and cause hepatotoxicity due to defects in the function of cellular organelles like the mitochondria and rough endoplasmic reticulum, which affect the pathways of the

stress activated protein kinase and also result in a reduction in glutathione levels within hepatocytes , These defects can be seen in the histological section **(Matsuo *et al.*, 2014).**

Additionally, about azathioprine reported an increase in the amount of lipid peroxidation, which stimulated both the apoptosis and necrotic processes in the liver cells and the metabolism of azathioprine in the cells of hepatic tissue can result in a number of problems, including the reduction of the antioxidant Glutathione (GSH), mitochondrial damage, a drop in ATP levels, and cell death by apoptosis **(Cui *et al.*, 2014).** The rise in reactive oxygen species (ROS), which are free radicals, is one of the main factors contributing to the harmful effects of azathioprine on liver cells , The methylation aberrations that create metabolic abnormalities and indicate through methylated metabolites are typically linked to the hepatotoxicity that occurs in hepatocytes affected by azathioprine, where the buildup of methylated toxic metabolites of azathioprine can induce liver damage **(Ardeshiri *et al.*, 2012).**

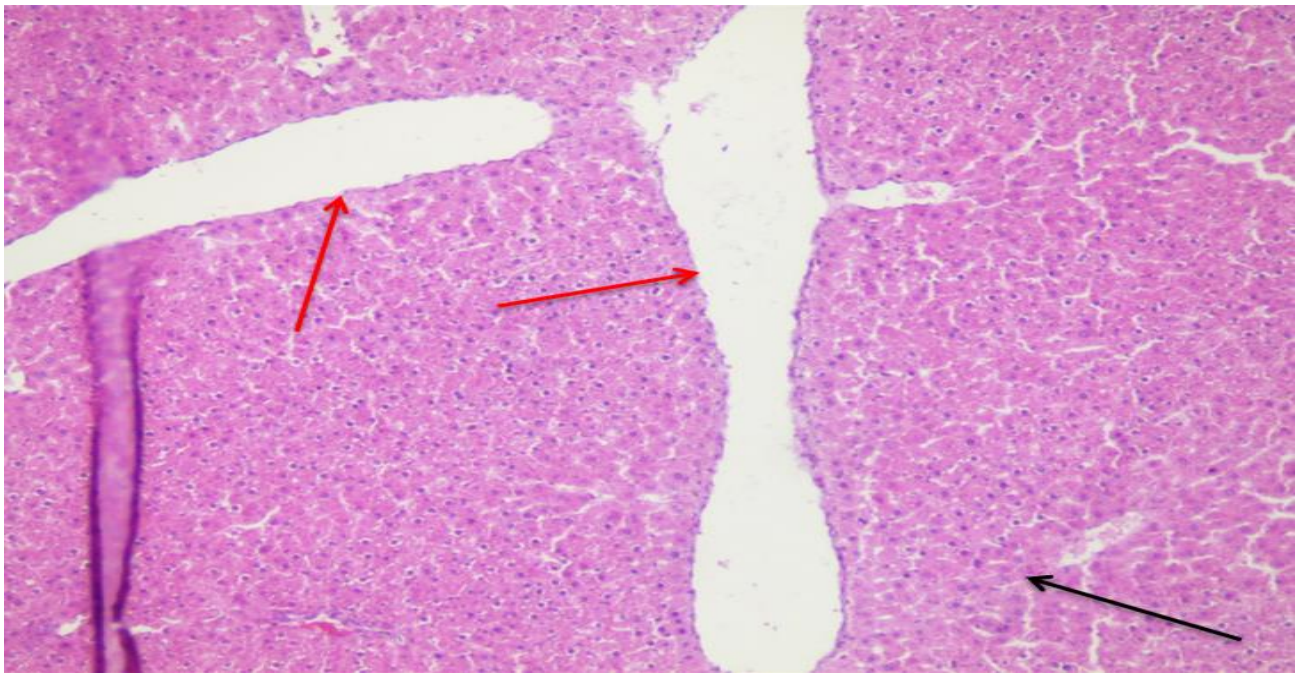


Figure (4-10) Photomicrograph of rats liver tissue section of AZA group , showed sever central vein dilatation (red arrow) , significant hepatic degenerative changes (black arrow). (H and E ,20X).

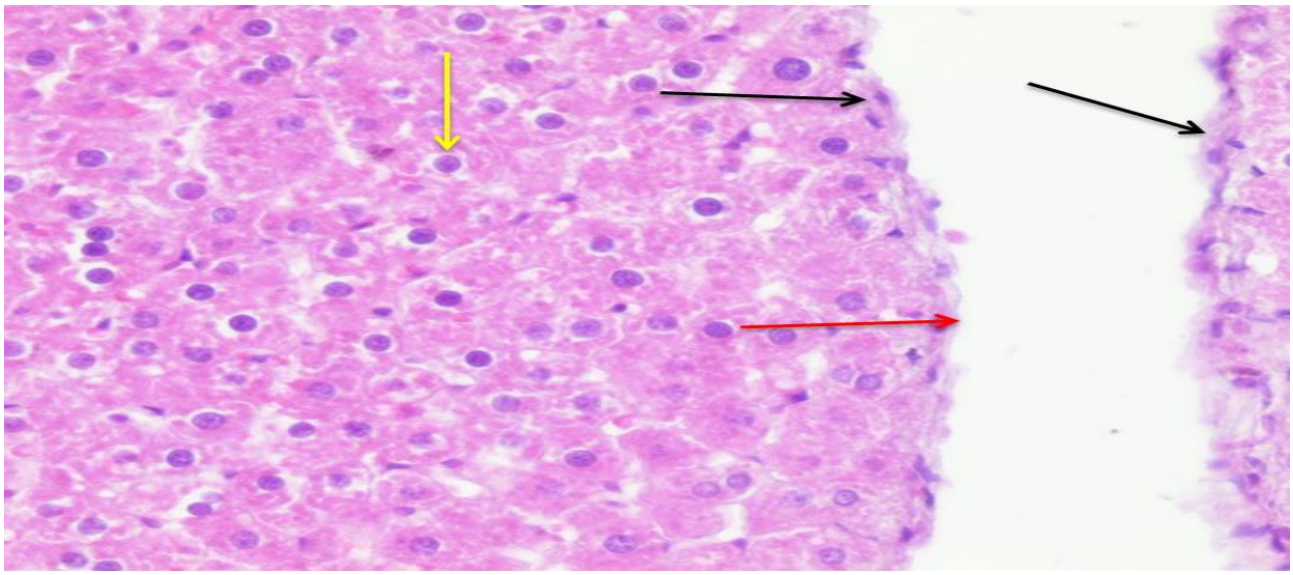


Figure (4-11) Photomicrograph of rats liver tissue section of AZA group , showed marked perivascular polymorph nuclear inflammatory cells infiltration (black arrow), hepatocytes with significant rounded large nuclei (yellow arrow) and central vein dilation (red arrow). (H and E ,40X).

Histological examination of liver section of azathioprine and aloe vera group were staind with haematocylin and eosin , as showed mild hepatocytes degeneration and normal central vein with mild hepatic swelling and hepatic inflammatory cells infiltration that Use medicinal herbs and plants as a natural alternative treatment that is not harmful and has curative effects because the toxic effects of many drugs that cause numerous disorders from numerous causes in many organs, with the liver being the most effective and considered to be too sensitive to be treated directly with drugs and chemicals **(Al-Abdaly *et al.*, 2021; Mannaa *et al.*, 2015).**

Aloe Vera is a popular medicinal plant whose gel plays a part in both protecting and healing individuals , The ability of Aloe vera gel to protect the liver from injury and toxic effects of many drugs and chemicals is known as hepatoprotection , The active ingredients, such as the high concentrations of polysaccharides that have inflammatory effects, as well as the gel's ability to act as an antioxidant by reducing the oxidation that occurs in hepatocytes by the final metabolite, malondialdehyde (MDA), is decreased by Aloe vera extracts ,Cell damage is lessened during lipid peroxidation events occurring inside of cells **(Al-Abbassi *et al.*, 2023).**

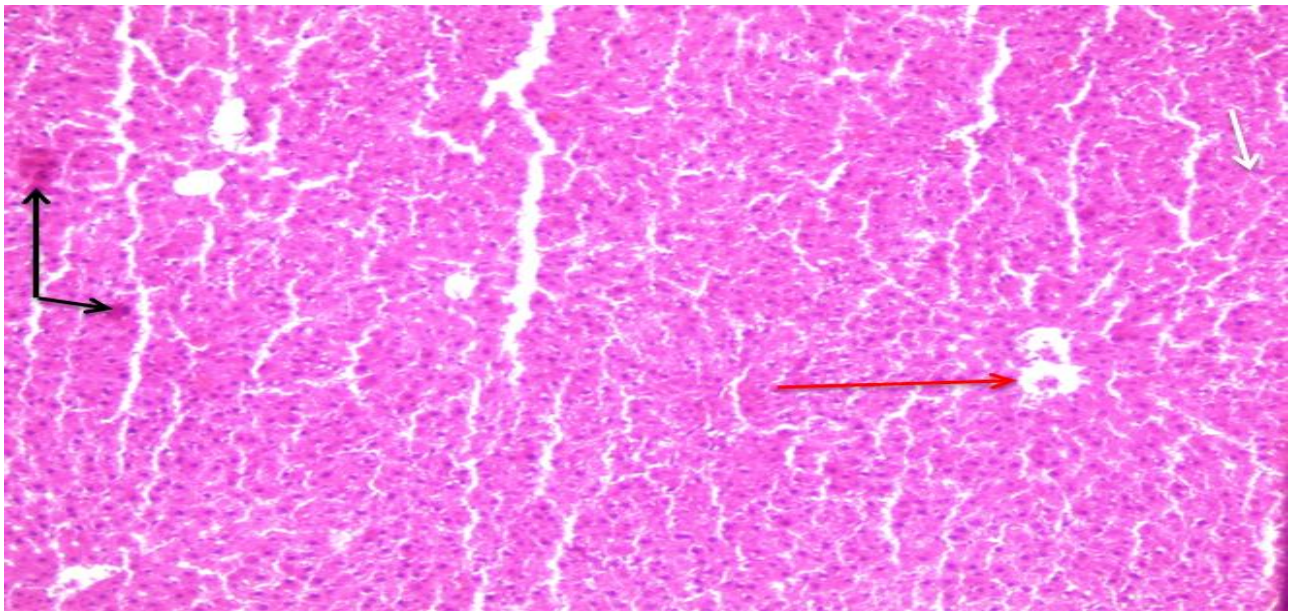


Figure (4-12) Photomicrograph of rats liver tissue section of AZA + Aloe group , showed mild hepatocytes degeneration (black arrow), and normal central vein (red arrow). (H and E ,20X).

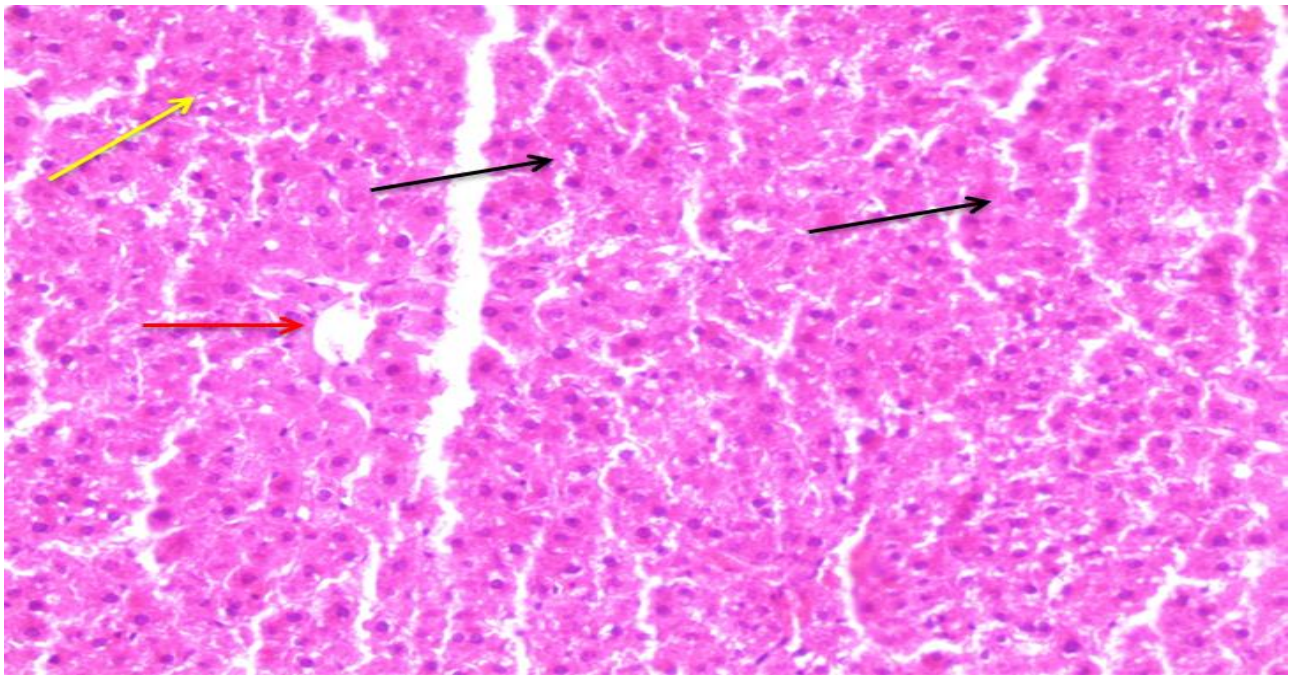


Figure (4-13) Photomicrograph of rats liver tissue section of AZA + Aloe group , showed the mild hepatic swelling (black arrow), hepatic inflammatory cells infiltration (yellow arrow) and normal central vein (red arrow). (H and E ,40X).

4.4.2. Effect of Azathioprine , Aloe Vera extract on Bone Marrow

The bone marrow was extracted from the animals after they had undergone anesthesia, and then after fixation, portions of the bone tissue were obtained and fixed with formalin (10%) , The blocking of the samples was split into sections after processing in an alcohol and paraffin, and the samples were then stained with (H&E)

, histological examination of bone marrow section of control group were staind with haematocylin and eosin , as showed a bone marrow tissue without significant pathological alterations and normal appearance of bone marrow with osteocyte addition to as showed normal osteocyte, notable pathological changes, and normal bone marrow represented by normal reticular fibers , Male rats given standard saline treatment had bone marrow with normal reticulocyte morphology in addition to hematopoiesis and adipose tissue within normal ranges **(Parker *et al.*, 2019).**

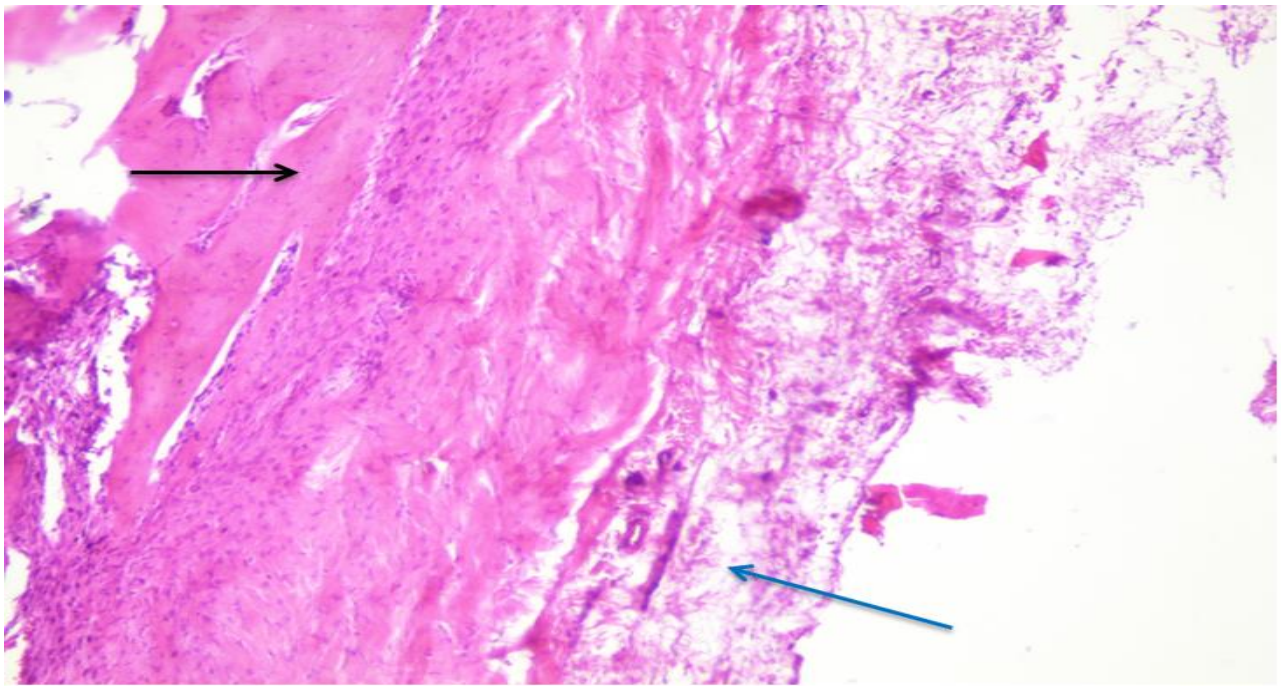


Figure (4-14) Photomicrograph of rats bone marrow section of control group , showed the bone tissue without significant pathological alterations (black arrow), normal appearance of bone marrow (blue arrow), osteocyte (yellow arrow) . (H and E ,10X).

Histological examination of bone marrow section of aloe vera group were staind with haematocylin and eosin , as showed a normal hemopoitic tissue formation , normal bone marrow cellularity with normal megakaryocytes and normal erythrocytes (reticulocytes) with normal osteoblast, osteoid, and osteocyte are visible in the bone marrow tissue with thick periosteum in the Aloe Vera group as showed in figure, There are many different types of medicinal plants that are used to cure anemia of all kinds by boosting the hematopoietic process through its bone marrow

precursors, which also boosts leukopenia and thrombocytopenia **(Al-Hijazi *et al*., 2015).**

Aloe Vera is a medicinal plant , It has anti-cancer, anti-diabetic, antioxidant, and anti-hyperlipidemic properties , Aloe vera gel extract contains a wide range of compounds, including minerals, enzymes, hormones, and carbohydrates. Since mesenchymal stem cells (MSCs) and aloe vera are physiologically active precursor cells with the ability to self-renew and give rise to a variety of cell types, they are also known as progenitor cells **(Farid *et al*., 2022).** Plant extracts have been used to encourage MSC differentiation and proliferation , As a result, bone marrow (BM)-MSCs and aloe vera gel showed therapeutic effects on male rats and study antioxidant enzymes, oxidative stress, and the antioxidant and anti-inflammatory properties of Aloe vera gel , The results showed that aloe vera gel and MSC transplantation decreased oxidative stress and pro-inflammatory cytokines, and that aloe vera gel also functioned as By promoting the differentiation of transplanted MSCs and minimizing liver damage, it also promotes other critical tasks **(Haridyy *et al*., 2022).**

The anti-inflammatory and antioxidant properties of aloe vera as well as its capacity to promote MSC proliferation in the liver When used in combination with lyophilized aloe Vera gel, MSC differentiation is greatly altered , Addition to The bone marrow cells produced binucleate cells and micronuclei, which indicated that these cells had a low level of cytotoxicity and mutagenicity **(Werawatganon *et al*., 2014).** According to the histopathology test, which demonstrated normal bone marrow cellularity and hemopoitic tissue development, aloe vera had no oxidative effects at all Megakaryocytes and reticulocytes in normal range **(Thadani, 2018).**

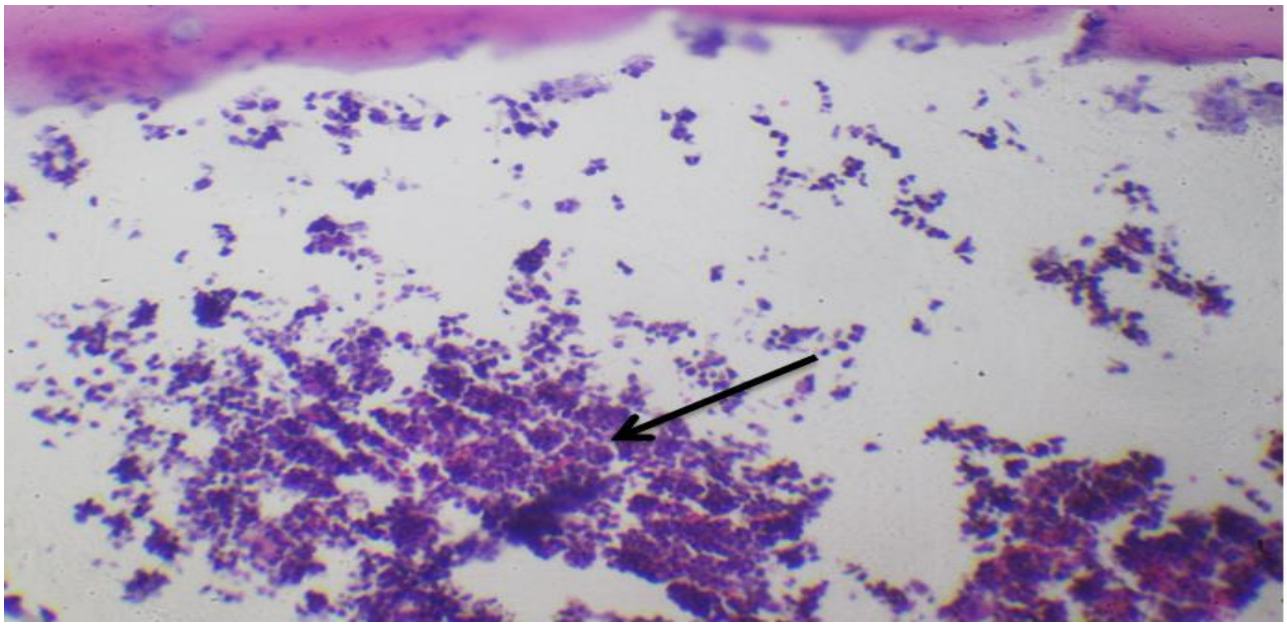


Figure (4-15) Photomicrograph of rats bone marrow section for Aloe Vera treated showing normal hemopoitic tissue formation , normal bone marrow cellularity (black arrow).(H and E,10X).

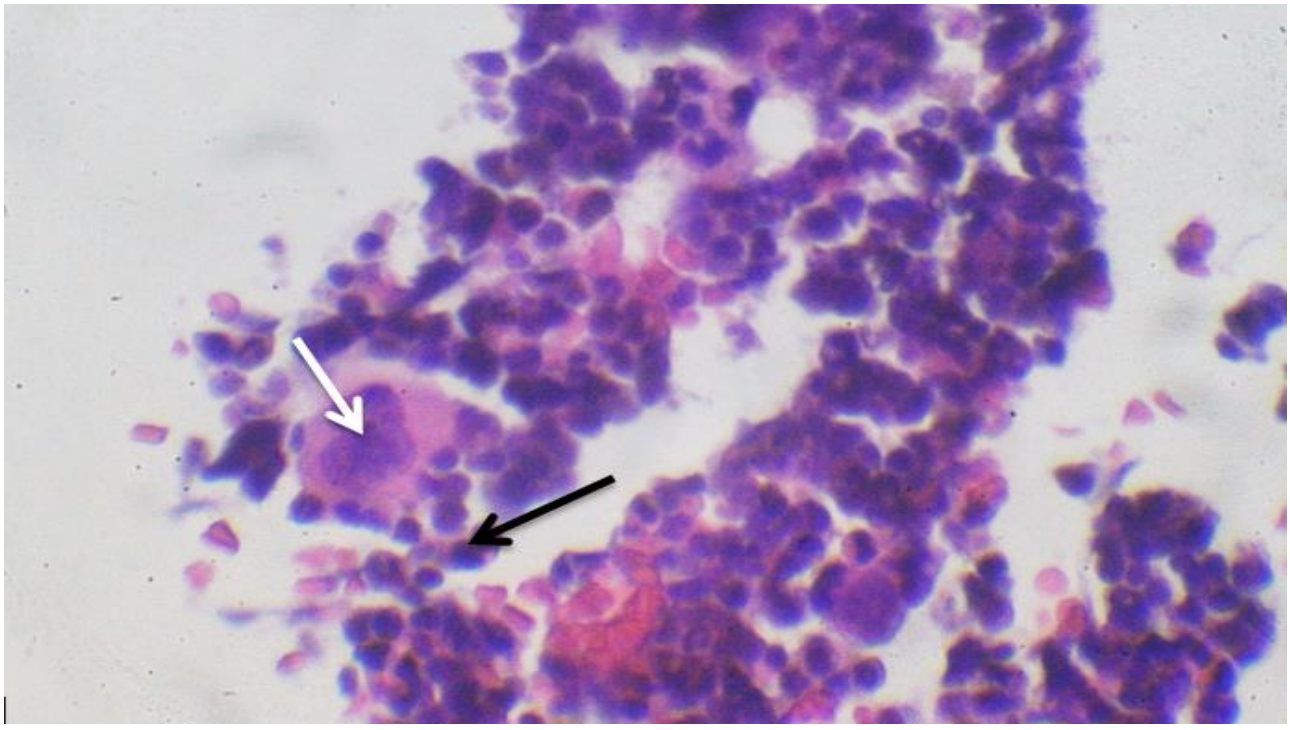


Figure (4-16) Photomicrograph of rats bone marrow section for Aloe Vera treated showing normal hemopoitic tissue formation , normal bone marrow cellularity, normal megakaryocytes (white arrow), normal erythrocytes (reticulocytes) (black arrow).(H and E,40X).

Histological examination of bone marrow section of azathioprine group were staind with haematocylin and eosin , as showed a significant decrease in hemopoitic tissue formation with marked less cellularity bone marrow and remarkable reduction of main cellular components of bone marrow , some enlargments of hollow spaces ,

Azathioprine was used to address these changes in the biochemical and hematological modifications , The outcomes showed that the drug's toxicity was what caused free radical generation and oxidative damage in organs and tissues , These findings demonstrated the role of reactive oxygen species as a potential initiator of oxidative stress and as a potential contributor to the formation of certain metabolites , This is due to the toxicity of 6-mercaptopurine and the lowered ATP levels caused by the production of reactive oxygen species **(Thompson *et al*., 2014).**

According to our findings, the azathioprine may be to blame for the changes seen in the bone marrow histology analysis a steady drop in the blood's RBC and WBC levels, a sign of active bone marrow suppression brought on by the drug azathioprine's side effects , Following exposure to cytostatic drugs that cause the loss of bone marrow cells, lymphocytes and lymphomas rise in the bone marrow and peripheral blood **(Grzechocińska *et al*., 2023).** The immunosuppressive drug azathioprine, a purine antimetabolite, can cause pancytopenia and myelosuppression, especially in patients with some TPMT (thiopurine methyltransferase) activity , A wide range of conditions can cause pancytopenia, including congenital and acquired bone marrow suppression, infections, cytotoxic treatments like chemotherapy and radiotherapy, lesions that occupy space in the bone marrow, nutritional deficiencies, destruction, and sequestration **(Pagarin, 2023).**

A useful diagnostic for identifying the underlying cause of pancytopenia, particularly in hematologic illnesses, is bone marrow aspiration and biopsy , Aplastic anemia, vitamin or mineral deficiencies like folate, B12, or copper, impaired hematopoiesis like myelodysplastic syndromes, bone marrow infiltration like myelofibrosis, metastatic cancer, storage diseases, hematologic malignancies Pancytopenia can be brought on by a number of medications, including immunosuppressant's like azathioprine **(Cheng *et al*., 2020).** This is frequently observed in bone marrow failures from various causes, such as medication toxicity and aplastic anemia , Azathioprine is a purine-mimic antimetabolite

immunosuppressive drug that functions as an antagonist of purine metabolism , It is a 1-methyl-4-nitro-5-imidazolyl derivative of thioguanine **(Ghalamkari *et al*., 2019).** It prevents the creation of DNA, RNA, and proteins , Thiopurine s-Methyltransferase (TPMT) and hypoxanthine phosphoribosyl transferase are the two key enzymes involved in the drug's metabolism , Infection, bone marrow suppression, and gastrointestinal intolerance are among azathioprine's most frequent adverse effects **(Asadov *et al*., 2017).**

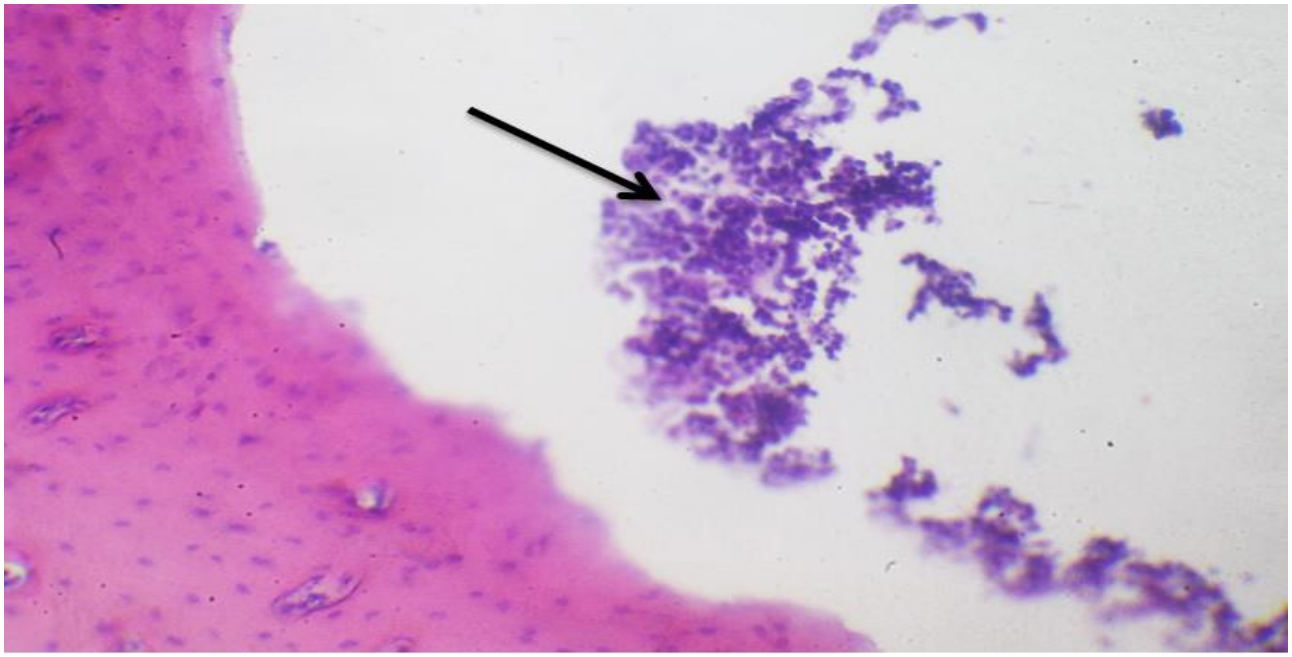


Figure (4-17) Photomicrograph of rats bone section of AZA treated showing significant decrease in hemopoitic tissue formation, marked less cellularity bone marrow (black arrow).(H and E,10X).

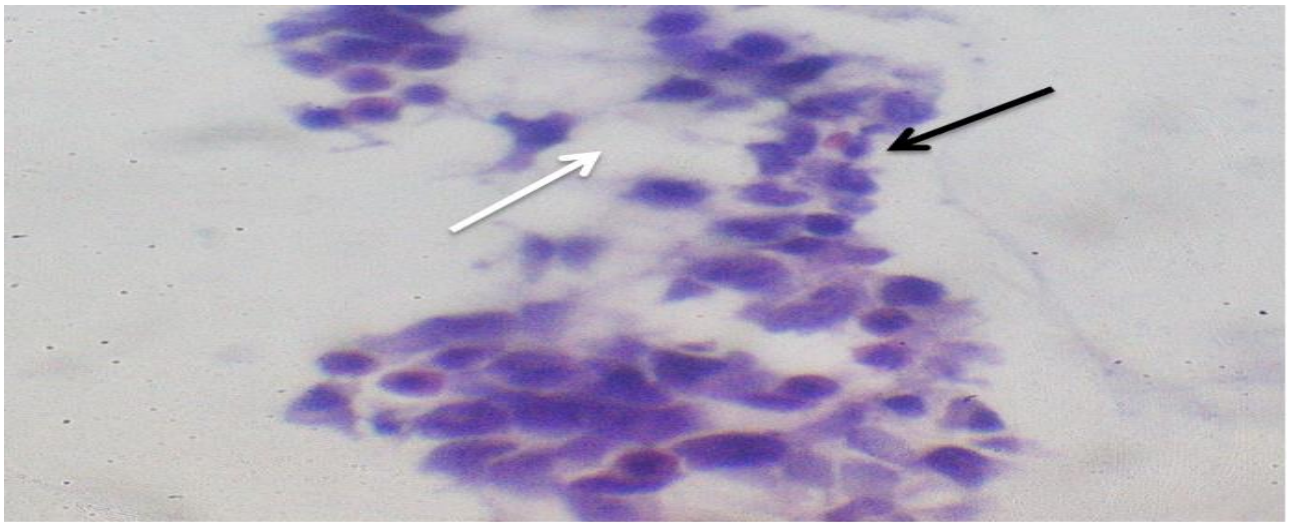


Figure (4-18) Photomicrograph of rats bone section of AZA treated showing significant decrease in hemopoitic tissue formation, remarkable reduction of main cellular components of bone marrow (black arrow) , some enlargments of hollow spaces (white arrow) .(H and E,40X).

Histological examination of bone marrow section of combination azathioprine and aloe vera group were staind with haematocylin and eosin , as showed a significant and sever hyperplasia in hemopoitic tissue , noticeable increase of bone marrow cellularity with noticeable hyperplasia in bone marrow cellularity, significant large Megakaryocytes and presence of erythroid islands.

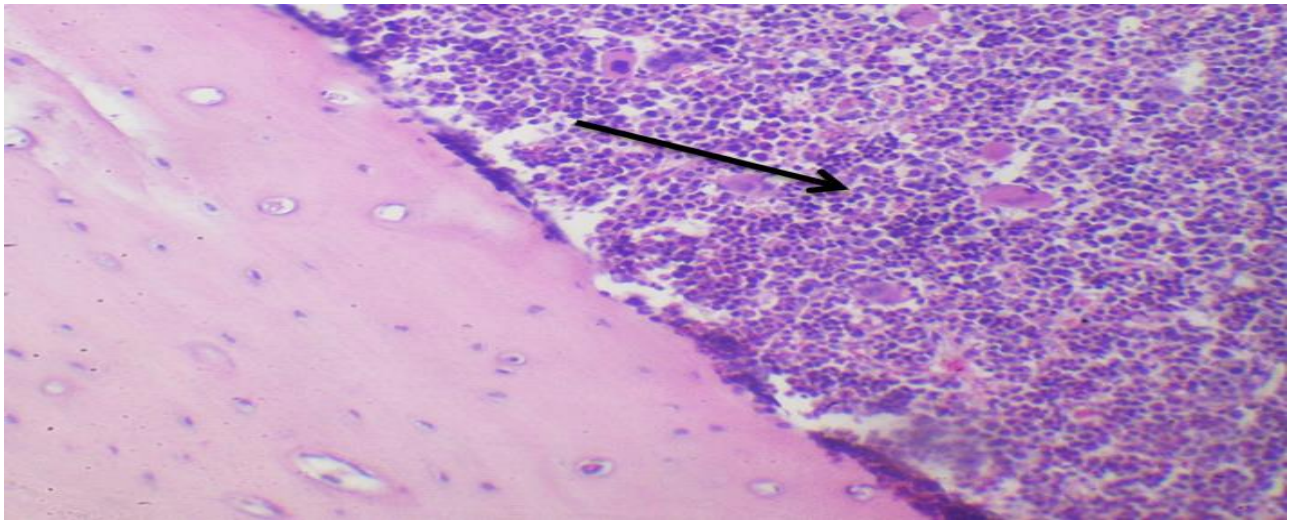


Figure (4-19) Photomicrograph of rats bone marrow section of combination (AZA and Aloe Vera) treated showing significant and sever hyperplasia in hemopoitic tissue , noticeable increase of bone marrow cellularity (black arrow).(H and E,10X).

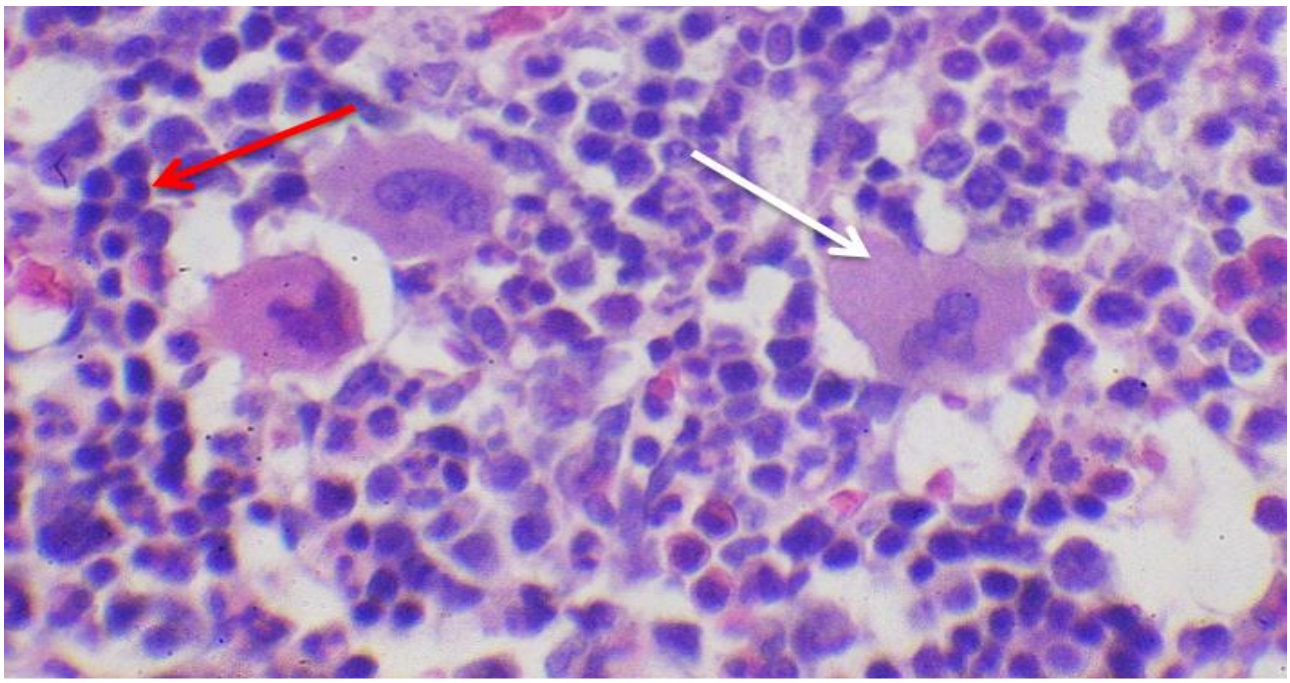


Figure (4-20) Photomicrograph of rats bone section of combination (AZA and Aloe Vera) treated showing noticeable hyperplasia in bone marrow cellularity, significant large Megakaryocytes (white arrow) and presence of erythroid islands (red arrow).(H and E,40X).

Azathioprine and aloe vera when combined showed as opposed to the control group, histopathological findings showed obvious alterations in bone tissue , The plural form was used to remedy these modification alterations , The outcomes showed that free radical generation was restored and that the drug's toxicity-induced oxidative damage to organs and tissues started to be repaired Because the detoxification of 6-mercaptopurine and the creation of reactive oxygen species increased ATP levels, it also demonstrated the re-source of reactive oxygen species following oxidative stress, which was linked to the production of some metabolites **(Alrekabi *et al*., 2020).**

Toxicities during hepatocyte metabolism and bone marrow regeneration cause mitochondrial repair, and dead cell regeneration , Our findings suggested that azathioprine may be to blame for the alterations seen in bone marrow tissue, including a gradual drop in the number of RBCs and WBCs in the blood, which indicated active bone marrow suppression due to azathioprine toxicity **(Elbaghdady *et al*., 2018).** However, all of these changes were reversed after the administration of aloe vera along with azathioprine It caused a biological increase in fat cells in the bone marrow and a comparable increase in blood-forming cells **(Alok Kumar, 2012).** Additionally, as a result of the loss of cytostatic substances, which stimulates the production of new bone marrow cells, lymphocytes and lymphomas rise in the bone marrow and peripheral blood **(Alrekabi *et al*., 2020).**

Chapter five : Conclusions and Recommendations

5. Conclusions and Recommendations

5.1. Conclusions

1) Hepatotoxicity, or liver damage from immunosuppressive medications like azathioprine, is a possible side effect**.**

2) Azathioprine has negative effects on the liver, including drug-induced hepatotoxicity, which is characterized by the generation of reactive oxygen species (ROS)**.**

3) Histological sections, which are the best predictor of liver disease, clearly demonstrate the tissue of the liver has multiple changes ranging from minor alterations to severe damage and necrosis.

4) As a hepatoprotective agent, aloe vera extract with a dosage of 500 mg/kg has superior results.

5.2. Recommendations

1) Using additional plant materials from aloe vera, such as the flowers.

2) Purification of the active ingredient, which is utilized to lessen drug adverse effects and act as a hepatoprotective**.**

3) Application of Aloe vera gel extract to treat the side effects of Azathioprine or the medications combined on bone marrow suppression is advised to the Ministry of Health.

4) A more thorough investigation into the functions of aloe vera gel and how they affect the body, including healing wounds and obliterating burn scars, is needed.

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Appendices

Appendices I

Procedure of Complete blood count

was obtained automatically by using a device **Swelab Alfa ,** The sample is collected drawing the blood into a tube containing an anticoagulant typically (EDTA) to stop it from clotting The testing is typically performed by an automated analyzer Analysis begins when a well-mixed blood sample is placed on a rack in the analyzer. The instrument utilizes flow cells, photometers and apertures analyze different elements in the blood. On board the analyzer, the sample is diluted and aspirated into at least two different channels, one of which is used to count red blood cells and platelets, the other to count white blood cells. Additional channels may be used for differential white blood cell counts and specialized measurements of platelets.

Calculation:

Blood cell counts occurs by flow cytometry, in which a very small amount of the specimen is aspirated, diluted and passed through an aperture and a flow cell. Sensors count and identify the number of cells passing through the aperture using two main principles: electrical impedance and light scattering Impedance-based cell counting operates on the Coulter principle, which measures the drop in current as cells pass through an aperture to count cells and calculate their sizes. Because red blood cells, white blood cells and platelets have different average sizes, this technique allows the three types of cells to be differentiated. Light scattering techniques direct a laser at individual cells and determine cellular size and complexity by measuring the amount of light scattered at different angles. Forward scatter, which refers to light scattered between 0 and 10 degrees of the beam's axis, correlates with cellular size, while side scatter (light scattered at a 90-degree angle) correlates with cellular complexity. White blood cells, red blood cells and platelets, as well as individual types of white blood cells, can be distinguished based on light scattering characteristics.

Figure (1-1) Swelab Alfa

Appendices II

Procedure of Reticulocyte Count

1) Take 2-3 drops of dye solution in a test tube.

2) Add 2-4 drops of well-mixed blood sample and mix.

3) Stopper the tube and incubate at 370C for 10-15 minutes.

4) After incubation, mix well and make a thin smear of stained blood.

5) When dry, examine the films without fixing or counterstain.

6) Count 1000 RBCs and note the number of reticulocytes among them. A dark blue reticulum or network will present in reticulocytes.

Appendices III

Procedure of Examination of bone marrow smears

a process where a little sample of bone marrow is taken, typically from the hip, or thigh bone. Anesthetic is used to numb the surface of the bone beneath a small patch of skin. Then, a specific, wide needle is inserted into the bone. Bone marrow sections should always be stained with reticulin using a silver impregnation procedure and hematoxylin and eosin (H&E).

Appendices IV

Procedure of Arginase I (ARG1)

1. Determine wells for diluted standard, blank and sample. Prepare 7 wells for standard, 1 well for blank. Add 100 μL each of standard working solution (read Reagent Preparation), or 100 μL of samples into the appropriate wells. Cover with the Plate sealer. Incubate for 80 minutes at 37°C.

2. Remove the liquid of each well. Aspirate the solution and wash with 200 μL of 1× Wash Solution to each well and let it sit for 1-2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Totally wash 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.

3. Add 100 μL of Biotinylated Antibody working solution to each well, cover the wells with the plate sealer and incubate for 50 minutes at 37°C.

4. Repeat the aspiration ,wash process for total 3 times as conducted in step2.

5. Add 100 μL of Streptavidin-HRP working solution to each well, cover the wells with the plate sealer and incubate for 50 minutes at 37°C.

6. Repeat the aspiration,wash process for total 5 times as conducted in step2.

7. Add 90 μL of TMB Substrate Solution to each well. Cover with a new Plate sealer. Incubate for 20 minutes at 37°C (Don't exceed 30 minutes). Protect from light. The liquid will turn blue by the addition of TMB Substrate Solution.

8. Add 50 μL of Stop reagent to each well. The liquid will turn yellow by the addition of Stop reagent. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing. The insertion order of the Stop reagent should be the same as that of the TMB Substrate Solution.

9. Remove any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450 nm immediately.

Appendices V

Procedure of Alanine aminotransferase (ALT/GPT)

1. Pre incubated working reagent, samples and controls to reaction temperature.
2. Set the photometer to 0 absorbance with distilled water.
3. Pipetted into a cuvette: working reagent (1.0 mL) and sample (50 mL).
4. Mixed gently by inversion. Inserted cuvette into the cell holder and starting stopwatch.
5. Incubated for 1 minute and recorded initial absorbance reading.
6. Repeated the absorbance readings exactly after 1, 2 and 3 minutes.
7. Calculated the difference between absorbances.
8. The results to obtained the average change in absorbance per minute (□A/min)

Appendices VI

Procedure of Aspartate aminotransferase (AST/GOT)

1. Pre incubated working reagent, samples and controls to reaction temperature.
2. Set the photometer to 0 absorbance with distilled water.
3. Pipetted into a cuvette: working reagent (1.0 mL) and sample (50 mL).
4. Mixed gently by inversion. Insert cuvette into the cell holder and started stopwatch.
5. Incubated for 1 minute and recorded initial absorbance reading.
6. Repeated the absorbance readings exactly after 1, 2 and 3 minutes.
7. Calculated the difference between absorbance

8. Calculated the mean of the results to obtain the average change in absorbance per minute (□A/min).

Appendices VII

Procedure of Determination of Serum Malondialdehyde Level (MDA) Concentration (μ mol/L)

Principle:

This method quantifies lipid peroxides by measuring aldehyde breakdown products of lipid peroxidation. A basic principle of the method is the reaction of one molecule of malondialdehyde and two molecules of thiobarbituric acid to form a red MDA-TBA complex which can be measure at 535 nm.

Stock TCA – TBA – HCl Reagent

It was prepared by dissolving 15% W/V trichloroacetic acid and 0.375% W/V thiobarbituric acid and 0.25 N HCl to make 100 ml (2.1 ml of concentrated HCl in 100 ml). This solution was mildly heated to assist in the dissolution of TBA. Dissolved 15 gm TCA and 0.375 mg thiobarbituric acid in 0.25 N HCl and volume was made up to 100 ml with 0.25 N HCl.

Procedure:

To 0.4 ml of serum, 0.6 ml TCA-TBA-HCl reagents were added. It was mixed well and kept in boiling water bath for 10 minutes. After cooling 1.0 ml freshly prepared 1N NaOH solution was added to eliminate centrifugation. This absorbance of pink color was measured at 535 nm against blank which contained distilled water in place of serum. In blank 0.4 ml distilled water and 0.6 ml TCA-TBA-HCl reagent was mixed and boiled. Blank was always taken.

Calculation:

extinction coefficient of MDA at 535 nm is = 1.56 × 105

MDA concentration = χ / 0.0624 nmol / ml.

Appendices VIII

Procedure of Estimation of serum Glutathione-S-transferase (GST) concentration

principle

This ELISA kit uses Sandwich-ELISA as the method. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to GSTω1. Standards or samples are added to the appropriate micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for GSTω1 and Avidin-Horseradish Peroxidase (HRP) conjugate is added to each micro plate well successively and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain GSTω1, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of GSTω1. You can calculate the concentration of GSTω1 in the samples by comparing the OD of the samples to the standard curve.

Assay procedure

Bring all reagents and samples to room temperature before use. Centrifuge the sample again after thawing before the assay. All the reagents should be mixed thoroughly by gently swirling before pipetting. Avoid foaming. It’s recommended that all samples and standards be assayed in duplicate.

1. Add Sample: Add 100μL of Standard, Blank, or Sample per well. The blank well is added with Reference Standard & Sample diluent. Solutions are added to the bottom of micro ELISA plate well, avoid inside wall touching and foaming as possible. Mix itgently. Cover the plate with sealer we provided. Incubate for 90 minutes at 37℃.

2. Biotinylated Detection Ab: Remove the liquid of each well, don't wash. Immediately add 100μL of Biotinylated Detection Ab working solution to each well.

Cover with the Plate sealer. Gently tap the plate to ensure thorough mixing. Incubate for 1 hour at 37°C.

3. Wash: Aspirate and wash each well and repeat the process three times. Wash by filling each well with Wash Buffer (approximately 350μL) (a squirt bottle, multi-channel pipette, manifold dispenser or automated washer are needed). Completing the removal of liquid at each step is essential. After the last wash, remove remained Wash Buffer by aspirating or decanting. Invert the plate and pat it against thick clean absorbent paper.

4. HRP Conjugate: Add 100μL of HRP Conjugate working solution to each well. Cover with the Plate sealer. Incubate for 30 minutes at 37°C.

5. Wash: Repeat the wash process for five times as conducted in step 3.

6. Substrate: Add 90μL of Substrate Solution to each well. Cover with a new Plate sealer. Incubate for about 15 minutes at 37°C. Protect the plate from light. The reaction time can be shortened or extended according to the actual color change, but not more than 30minutes. When apparent gradient appeared in standard wells, user should terminate the reaction.

7. Stop: Add 50μLof Stop Solution to each well. Then, the color turns to yellow immediately. The order to add stop solution should be the same as the substrate solution.

8. OD Measurement: Determine the optical density (OD value) of each well at once, using a micro-plate reader set to 450 nm. User should open the micro-plate reader in advance, preheat the instrument, and set the testing parameters.

9. After experiment, put all the unused reagents back into the refrigerator according to the specified storage temperature respectively until their expiry.

Appendices IX

Procedure of Serum reduced glutathione concentration (GSH)

Catalase activity was assessed by incubating the enzymes ample in 1.0 ml substrate (65 mmol/ml hydrogen peroxide in 60 mmol/l sodium–potassium phosphatebuffer, pH7.4)at37 °C for three minutes. There action was stopped with ammonium

molybdate. Absorbance of the yellow complex of molybdate and hydrogen peroxide is measured at374nm against the blank.

Reagents

1. Sodium, potassium phosphate buffer (50mM,pH7.4): this buffer isprepared by dissolving 1.1g of Na2HPO4 and 0.27g of KH2PO4 in 100ml distilled water.

2. H2O2 (20 mM) in 50mmol/l sodium, potassium phosphate buffer: this solution is freshly diluted and standardized daily using a molar extinction coefficient of 43.6M_1 cm_1 at 240nm.

3. Ammonium molybdate (32.4mmol/l).

Table 1

Reagents	Test	Control-test*	Standard	Blank
Serum	100 µl	100 µl	–	–
D.W.	–	1000 µl	100 µl	1100 µl
Hydrogen peroxide	1000 µl	–	1000 µl	–
Mix with vortex and incubate at 37 °C for 3 min, after that, add:				
Ammonium molybdate	4000 µl	4000 µl	4000 µl	4000 µl
After that, the tubes were kept at room temperature. Changes in absorbance were recorded at 374 nm against the reagent blank.				

4. Calculation The rate constant of a first-order reaction (k) equation is used to determine catalase activity:

t: time.

S°: absorbance of standard tube

S: absorbance of test tube.

M: absorbance of control test (correction factor).

Vt: total volume of reagents in test tube. Vs: volume of serum.

Appendices X

Procedure of Histological study

with aid of the light microscope as the following steps:

1) Fixation :- The specimen fixated in the formalin 10 % for 24 – 48 hours.

2) Washing and dehydration :- After fixation the specimens washed with water to remove the fixative in order to avoid the interaction between the fixative and staining

materials used later. By dehydration the water had been completely extracted from fragments by bathing them successively in a graded series of of ethanol and water (70 %, 80 %, 90 %, and 100 % ethanol)

3) Clearing :- Bathing the dehydrated fragments in solvent (xylene) for 30–60 minutes, this step was repeated 3 times. As the tissues clearing, they generally became transparent.

4) Infiltration and Embedding :- Once the tissue fragments were impregnated with the solvent, they were placed in melted paraffin in an oven, typically at 52℃. The heat causes the solvent to evaporate and the space within the tissues becomes filled with paraffin.

5) Sectioning :- After holds from the oven, the specimen let at room temperature to be solid and removed from their containers in order to sectioning they were put in the rotary microtome and were sliced by the microtome, a steel blade into sections 5 micrometers thick. The sections were floated on water bath (50–55oC), then transferred into glass slides coated with Mayers albumin as adhesive substance and left to dry.

6) Staining :- The histological sections of the studied organs were stained with Hematoxylin - Eosin stain.

الخلاصة:-

كان الهدف من الدراسة الحالية هو تقييم النشاط لمستخلص الصبار في المعايير الدموية والكيميائية الحيوية والتأثيرات الجانبية لأكسدة الكبد والإنزيمات المضادة للأكسدة الناجمة عن **الازوثابويرين** مع الفحص النسيجي المرضي للكبد ونخاع العظام. استخدمت التجربة 40 فأرًا ذكرًا. تم تقسيم الحيوانات إلى أربع مجموعات: - المجموعة الضابطة (10 فئران ذكور) التي تلقت جرعة يومية واحدة من الماء المعقم تعطى عن طريق الفم لمدة أربعة أسابيع ومجموعة الصبار (10 فئران ذكور) التي تلقت الصبار (500 ملجم / كجم من وزن الجسم) منفردة الجرعة اليومية تعطى عن طريق الفم لمدة أربعة أسابيع ومجموعة **الازوثابويرين** (10 فئران ذكور) التي تلقت **الازوثابويرين** (50 ملغم / كغم من وزن الجسم) جرعة يومية واحدة تعطى عن طريق الفم لمدة أربعة أسابيع ومجموعة الصبار مع **الازوثابويرين** (10 فئران ذكور) التي تلقت الصبار (500 ملغم/كغم من وزن الجسم) مع **الازوثابويرين** (50 ملغم/كغم من وزن الجسم) جرعة واحدة يومياً تعطى عن طريق الفم لمدة أربعة أسابيع. تم إعطاء الفئران مخدر الكلوروفورم لغرض تخديرها، ثم تم أخذ عينات دم من القلب لفحص فحوصات الدم وإنزيمات الكبد والمؤكسدات ومضادات الأكسدة، وتم تشريح الحيوانات للحصول على الكبد ونخاع العظام لإجراء فحص نسيجي. أظهرت الدراسة الحالية انخفاض معنوي في **WBC(4.19±0.10c),RBC(4.74±0.15b),PCV(30.76±0.45c)** في مجموعة **الازوثابويرين** مقارنة بمجموعة السيطرة ومجموعات الصبار بينما كان هناك ارتفاع في **WBC(9.68±0.37b),RBC(7.14±0.24a),PCV(37.10±1.40b)** في مجموعة الصبار **الازوثابويرين** مقارنة مع مجموعة **الازوثابويرين** . أظهرت الإضافة انخفاضا معنويا في **MCV(50.09±0.45b),MCH(14.42±0.52a),MCHC(30.78±0.42b)** في مجموعة **الازوثابويرين** مقارنة مع مجموعة السيطرة ومجموعة الصبار. كما حدثت زيادة ملحوظة في **MCV(58.53±0.78a),MCH(22.65±0.47c),MCHC(37.48±0.95c)** في المجموعة المعالجة بالصبار بالإضافة إلى **الازوثابويرين** مقارنة بمجموعة **الازوثابويرين**. بينما أظهر فحص عدد Reticulocyte Count (18.00±0.19b) في مجموعة **الازوثابويرين** زيادة معنوية مقارنة مع مجموعة السيطرة ومجموعات الصبار بينما أظهرت مجموعة **الازوثابويرين** مع الصبار انخفاضاً معنوياً (11.00±0.08c) مقارنة بمجموعة الآزوثيوبرين. بينما أظهر فحص bone marrow smears in mylocyte (29.00±0.75a), promylocytes (12.00±1.50a) في مجموعة **الازوثابويرين** زيادة معنوية في الخلايا النقوية بينما أظهر انخفاضاً معنوياً في neutrophil (14.00±0.50c) , eosinophil (7.00±0.50b) and lymphocytes (17.00 ±1.50a) بالمقارنة مع مجموعة السيطرة والصبار، كما كان هناك انخفاض معنوي في mylocyte (22.00±0.75b) and promylocytes (10.11±1.25a). بالإضافة إلى ذلك كانت هناك زيادة معنوية في neutrophil (16.00±1.15ab) , eosinophil (9.00±0.50a) and lymphocyte (28.00±1.00c)

في المجموعة المعالجة بالصبار بالإضافة إلى مجموعة الآزويثوبرين مقارنة بمجموعة الآزوثيوبرين. في هذه الدراسة أظهرت زيادة معنوية في مستوى AST (128.00±3.9c) , ALT (40.61±2.3c) , Arginase I level (9.33±0.33b) في مجموعة **الازوثابويرين** مقارنة بمجموعة السيطرة. ومن ناحية أخرى لوحظ انخفاض معنوي في مستوى AST (100.00±2.3d) , ALT (34.81±0.4d), Arginase I level (6.65±0.30c) في توليفة الصبار **الازوثابويرين** مقارنة مع مجموعة **الازوثابويرين**. بينما أظهر إنزيم أكسدة الكبد ومضاد الأكسدة في مجموعة **الازوثابويرين** زيادة معنوية في MDA (16.52±1.4b) وانخفاض معنوي في GSH (22.52±1.7b) and GST (9.16±0.8c) بالمقارنة مع مجموعة السيطرة والصبار، كما كان هناك انخفاض معنوي MDA (11.21±1.1c) and وزيادة معنوية في GSH (35.81±2.1c) and GST (15.98±0.76d) في مجموعة السيطرة. تعاملت المجموعة مع الألوة فيرا بالإضافة إلى **الازوثابويرين** مقارنة بمجموعة **الازوثابويرين**. أظهر الفحص النسيجي لقسم الكبد من مجموعة الآزويثوبرين توسعًا شديدًا في الوريد المركزي، وتغيرات تنكسية كبدية كبيرة، وارتشاح ملحوظ للخلايا الالتهابية النووية متعددة الأشكال حول الأوعية الدموية، وخلايا الكبد مع نوى كبيرة مدورة وتوسع الوريد المركزي. كما أظهر الفحص النسيجي لقسم النخاع العظمي لمجموعة **الازوثابويرين** صبغ الهيماتوسيلين والأيوسين، كما أظهر انخفاضاً معنوياً في تكوين الأنسجة المكونة للدم مع انخفاض ملحوظ في خلوية نخاع العظم وانخفاض ملحوظ في المكونات الخلوية الرئيسية لنخاع العظم، وبعض التوسعات في الفراغات المجوفة. كما أظهر مستخلص الصبار فعاليته كحماية الكبد والحماية من الآثار الجانبية **الازوثابويرين** مع ظهور خلايا الكبد بشكل طبيعي ولا توجد تغييرات في الوريد المركزي الطبيعي لخلية الكبد ولا تغييرات في خلايا الكبد. أظهر الفحص النسيجي لقسم نخاع العظم من مجموعة الصبار تكوينًا طبيعيًا للأنسجة المكونة للدم، وخلوية نخاع العظم طبيعية مع خلايا نواة طبيعية وخلايا دم حمراء طبيعية (خلايا شبكية) مع بانيات عظمية طبيعية، وخلايا عظمية، وخلايا عظمية مرئية في أنسجة نخاع العظم ذات السمحاق السميك. تم عكس كل هذه التغيرات النسيجية في مجموعة الصبار **الازوثابويرين** حيث أظهرت تنكس خفيف لخلايا الكبد ووجود الوريد المركزي طبيعي مع تورم كبدي خفيف وارتشاح خلايا الالتهاب الكبدي. اضافة إلى ذلك، في الفحص النسيجي لقسم نخاع العظم المكون من مجموعة **الازوثابويرين** والصبار أظهر تضخماً كبيراً وحاداً في الأنسجة المكونة للدم، وزيادة ملحوظة في خلوية نخاع العظم مع تضخم ملحوظ في خلوية نخاع العظم، وخلايا نواة كبيرة ووجود جزر كريات الدم الحمراء.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة كربلاء / كلية الطب البيطري

فرع الفسلجة والكيمياء الحياتية والادوية

الدور الوقائي لهلام الصبار على التغييرات الوظيفية لنقي العظام و الكبد المسببة بواسطة الازوثابويرين في ذكور الجرذان

رسالة مقدمة إلى

مجلس كلية الطب البيطري جامعة كربلاء وهي جزء من متطلبات درجة الماجستير في الطب البيطري / الفسلجة

من قبل

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