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Research Paper

Hepatoprotective Activity of Poly Herbal Formulation in Against Azathioprine-Induced Liver Injury in Rats

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ABSTRACT

Drug-induced liver injury (DILI) is a significant clinical problem and a major cause of hepatic dysfunction worldwide. It occurs due to the toxic effects of various pharmacological agents, herbal products, and environmental chemicals, often leading to oxidative stress, hepatocellular damage, and, in severe cases, liver failure. The liver, being the primary organ responsible for drug metabolism, is highly susceptible to such toxic insults. Among hepatotoxic drugs, azathioprine is widely used as an experimental model to induce liver injury due to its ability to generate reactive oxygen species (ROS), deplete antioxidant defenses such as glutathione (GSH), and elevate liver enzymes, resulting in cellular damage and necrosis.The present study focuses on evaluating the hepatoprotective potential of a polyherbal formulation consisting of Glycyrrhiza glabra, Ginkgo biloba, and Amaranthus spinosus against azathioprine-induced liver injury in rats. These medicinal plants are rich in bioactive phytoconstituents such as flavonoids and phenolic compounds, which exhibit strong antioxidant, anti-inflammatory, and cytoprotective properties. The polyherbal combination is expected to exert a synergistic effect by scavenging free radicals, restoring antioxidant enzyme levels, and improving biochemical and histopathological parameters associated with liver damage. The study involves assessment through liver function tests, hematological parameters, oxidative stress markers, and histopathological examination Overall, this research aims to establish the effectiveness of the selected polyherbal formulation as a safer and natural therapeutic alternative for the management of drug-induced hepatotoxicity, while also providing insights into its underlying protective mechanisms.

INTRODUCTION

The liver is a vital organ responsible for numerous physiological functions including metabolism,

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detoxification, synthesis of proteins, and regulation of biochemical processes essential for homeostasis. Due to its central role in drug metabolism and detoxification, it is highly vulnerable to damage caused by toxic substances. Hepatotoxicity refers to liver injury induced by chemicals such as drugs, alcohol, herbal products, and environmental toxins, and can range from mild biochemical alterations to severe liver failure[1–4].

Drug-induced liver injury (DILI) is one of the most common causes of liver disease and represents a major challenge in clinical practice[5]. It may occur either as a predictable (intrinsic) reaction or as an unpredictable (idiosyncratic) response depending on the nature of the drug and individual susceptibility[6,7]. The underlying mechanisms involve the formation of reactive metabolites, oxidative stress, mitochondrial dysfunction, and activation of immune-mediated pathways leading to hepatocyte apoptosis or necrosis[8,9].

Azathioprine, an immunosuppressive agent, is widely used to induce experimental hepatotoxicity. It is metabolized into 6-mercaptopurine, which leads to depletion of glutathione and generation of reactive oxygen species, resulting in lipid peroxidation, mitochondrial damage, and disruption of cellular integrity. These biochemical alterations are associated with elevated liver enzymes such as ALT, AST, and ALP, along with histopathological changes in liver tissues[10–12].

In recent years, there has been growing interest in herbal medicines for the prevention and treatment of liver disorders due to their safety, affordability, and therapeutic efficacy. Polyherbal formulations, in particular, offer synergistic effects by targeting multiple pathways involved in disease progression. Medicinal plants such as *Glycyrrhiza glabra*, *Ginkgo biloba*, and *Amaranthus spinosus* are known for their rich antioxidant content and diverse pharmacological activities, including hepatoprotective effects[13–15].

These plants contain bioactive compounds such as flavonoids, polyphenols, and terpenoids that help neutralize free radicals, enhance antioxidant defenses, and protect hepatocytes from oxidative damage. The combination of these plant extracts is hypothesized to produce enhanced therapeutic efficacy through synergistic mechanisms, offering improved protection against drug-induced liver injury[16,17].

Therefore, the present study is designed to evaluate the hepatoprotective activity of a polyherbal formulation comprising *Glycyrrhiza glabra*, *Ginkgo biloba*, and *Amaranthus spinosus* against azathioprine-induced liver injury in rats, using biochemical, hematological, and histopathological parameters as evaluation tools.

1. RESEARCH PROTOCOL

1.1 Aim of the study

The present work is about to evaluate the Hepatoprotective activity of Glycyrrhiza glabra (Liquorice) and Ginkgo biloba (Living fossil) Amaranthus spinosus (Spiny Amaranth or Pig weed) leaves extract combination against Azathioprine-induced liver injury in rats.

2.2 Objectives of the study

To investigate the Hepatoprotective potential of liquorice, ginkgo biloba, and Amaranthus spinosus in mitigating Azathioprine-induced liver injury in rats, through comprehensive analysis of liver function tests, histophathological examinations, and oxidative stress parameters.

- Induction of liver injury in experimental animal by using Azathioprine as a model.
- Extraction of test drug Glycyrrhiza glabra (Liquorice) ,Ginkgo biloba (Living fossil) Amaranthus spinosus (Spiny Amaranth or Pig weed) leaves To evaluate the Hepatoprotective effect of test drugs 1 + 2+3 extract combination in Azathioprine-Induced liver injury in rats.

- To determine the synergistic effect of extract combination of test drugs.
- To determine the hematological findings, biochemical parameter and histophathological changes in liver section of rats.

2.3 Scope of study

As we know that the Drug-induced liver injury (DILI) is a disorder whereby drugs including pharmacological therapies, traditional medicines, herbal and dietary supplements cause liver injury. In this study we are going to evaluate the Hepatoprotective effects of Glycyrrhiza glabra (Liquorice) and Ginkgo biloba (Living fossil) Amaranthus spinosus (Spiny Amaranth or Pig weed) leaves in combination.

2.4 Phytochemical available in all test compounds

These are the rich source of antioxidants. Radicals are unstable oxygen compounds with an unpaired electron react with other molecules to trap electron far from them. As a consequence, these molecules become radicals and begin a series response are amazing antioxidants which can neutralize the impact of free radicals. Flavonoids and phenolic derivate, present play also an important role as antioxidants. Extract formulation trap reactive oxygen species (ROS) which includes singlet oxygen, superoxide radical, hydroxyl radical, nitric oxide, peroxynitrite and nitrogen dioxide, for this reason reducing damage to proteins, lipid membranes and nucleic acid in cellular-free systems. [18,19]

Combination of drugs shows synergistic effects. It could lead to an increased understanding of complex disease Pathophysiology and design of better remedy for the disease. Drug Combinations can enhance healing efficacy ideally synergistic results for rising and reemerging viruses, also lessen the resistant arises due to the mutation of host mobile protein. It produces effective therapeutics capacity for drug–drug interaction with drug combination remedy. The identity of powerful, synergistic drug combinations may want to lead to an accelerated and provide effective, better treatment for complex ailment Pathophysiology of disease. Drug combination reduces the development of drug resistance so that the multiple targeting mechanisms of the drug combination. It has been also reported that incomplete hindrance of a small number of targets could be more efficient than totally hindrance of a single target. [20,21]

2.5 Rationale of study

Animals (Adult, male, Wistar albino rats weighing between 150-200g) will be used for this study. The animals will be kept in polypropylene cages, with stainless steel wire mesh cover on the top having 6 animals in per cage, temperature 25±2°C, relative humidity 60±5%, standard pellet diet and water ad libitum to animals so that they familiar with us throughout the study.

3. MATERIAL & METHODS

3.1 Chemicals and Equipment's required:

Silymarin (standard) 50mg/kg(w/w), Azathioprine (model) 50mg/kg(w/w) obtained from local market. Methanol, Tween 20 (10%), 4% paraformaldehyde, paraffin, hematoxylin eosin (H&E), homogenizer, metabolic cages, centrifuge. All the chemicals used are analytical grade and obtained from approved vendor.

3.2 Plant material required: Glycyrrhiza glabra (Liquorice) and Ginkgo biloba (Living fossil) Amaranthus spinosus (Spiny Amaranth or Pig weed) leaves extract purchased and COA provided by Kshipra biotech.

3.3 Methodology:

- Silymarin 50mg/kg (w/w) **standard drug**

- Azathioprine 50mg/kg (w/w**)** **Inducing model**
- Glycyrrhiza glabra (Liquorice) leaves **Test Compound 1**
- Ginkgo biloba (Living fossil) leaves **Test Compound 2**
- Amaranthus spinosus (Spiny Amaranth or Pig weed) leaves **Test Compound 3**

3.3.1Extraction procedures for test drug 1, drug 2, drug 3

Extraction Process and Preparation of Polyherbal Formulation

1. Collection and Authentication of Plant Materials:

-Liquorice (Glycyrrhiza glabra): Collect the leaves.

- Ginkgo biloba: Collect the leaves.

-Amaranthus spinosus: Collect the leaves.

- Authenticate each plant material by a botanist or a herbarium to ensure correct identification.

2. Drying and Grinding:

- Wash the plant materials thoroughly with distilled water to remove any dirt and contaminants.

- Dry the plant materials in a shaded, well-ventilated area at room temperature for several days until they are completely dry.

- Grind the dried plant materials separately into a fine powder using a mechanical grinder.

3. Preparation of Extracts:

- Solvent Selection: Use distilled water as the solvent for extraction due to its safety and efficacy.

- Aqueous Extraction:

1. Liquorice:

- Weigh 100g of liquorice leaves powder and add it to 1000ml of distilled water.

- Boil the mixture for 30 minutes and then allow it to cool to room temperature.

- Filter the extract using muslin cloth followed by Whatman No. 1 filter paper.

2. Ginkgo biloba:

- Weigh 100g of ginkgo biloba leaves powder and add it to 1000ml of distilled water.

- Boil the mixture for 30 minutes and then allow it to cool to room temperature.

- Filter the extract using muslin cloth followed by Whatman No. 1 filter paper.

3. Amaranthus spinosus:

- Weigh 100g of Amaranthus spinosus leaves powder and add it to 1000ml of distilled water.

- Boil the mixture for 30 minutes and then allow it to cool to room temperature.

- Filter the extract using muslin cloth followed by Whatman No. 1 filter paper.

4. Concentration of Extracts:

- Concentrate each of the filtered extracts separately using a rotary evaporator at a temperature not exceeding 40°C to obtain a thick, viscous liquid.

- Further dry the concentrated extracts in a freeze dryer to obtain a powdered extract.

5. Preparation of Polyherbal Formulation:

- Formulation Ratio: Decide on the ratio of the extracts based on previous studies or preliminary experiments (e.g., equal parts of each extract or a specific ratio depending on their individual potency and synergistic effects).

- Weigh the dried extracts according to the chosen ratio. For example, if using equal parts, weigh 33.3g of each extract.

- Mix the extracts thoroughly to ensure uniform distribution using a mortar and pestle or a mechanical mixer.

4. PROCUREMENT OF ANIMALS

Animals obtained from lovely institute of pharmaceutical sciences Phagwara, (Adult, male, Wistar albino rats weighing between 150-200g) kept in animal House Facility of St. Soldier institute of Pharmacy, Jalandhar, Punjab, India before one month from the study started in polypropylene cages, temperature 25±2°C, relative humidity 60±5%, standard pellet diet and water ad libitum to animals so that they familiar with us throughout the study.

5. EXPERIMENTAL PROTOCOLS

5.1 Induction of disease: Azathioprine-induced liver injury is used as a model for this study. Azathioprine is among the oldest pharmacologic immunosuppressive agents in use today. It suppresses the immune system and causes elevation in liver enzymes, depletion of hepatic reduced glutathione (GSH), catalase (CAT) and glutathione peroxidase (GPx), accumulation of oxidized glutathione (GSSG), and elevation of lipid peroxides (LPO) expressed as Malondialdehyde (MDA), reduction of the hepatic total antioxidant activity (TAA), decrease serum total proteins and elevation of liver protein carbonyl content. Significant rises in liver tumor necrosis factor-alpha (TNF-) and caspase-3 levels were noticed in AZA-intoxicated rats. Azathioprine 50mg/kg (w/w) i.p is given to group II (negative control group) on 8 day as a single day, as a result induction of liver injury within 12 days[22,23].

5.2 Experimental protocol according to the days required for study as described in Table

In experimental protocol animals were divided into 5 groups, six rats in each group. i.e., **Group I** (Control group) which is a normal control group which received normal saline on the daily basis by orally. **Group II** (Disease group) which is a disease group treated with drug model Azathioprine 50mg/kg (w/w) i.p route to induce disease. **Group III** (standard group) which is a standard drug group and the rats are treated with drug Silymarin 50mg/kg (w/w) p.o **Group IV** Test drug low dose 300mg/kg body weight by p.o route. **Group V** Test drugs high dose 600 mg/kg body weight by p.o route**. As shown in Table**

Groups	Treatment	Dose	Route of administration	Animals required
I (control)	Normal saline	Daily basis	p. o	6
II (Disease group)	Azathioprine	50mg/kg (w/w)	i.p	6
III (Standard Group)	Silymarin+ Azathioprine	50mg/kg (w/w)+ 50mg/kg (w/w)	p. o	6
IV	Polyherbal Preparation Low dose + Azathioprine	300 mg/kg +50mg/kg (w/w)	p. o	6
V	Polyherbal Preparation High dose + Azathioprine	600 mg/kg, p.o +50mg/kg (w/w)	p. o	6

Representation of experimental protocol and study design which shows that treatment schedule for animals according to days required for the study, we have to observe the different parameters and physical examination for all 21 days.

Group I is normal control group, Azathioprine single dose on 8th day will be given to group II. In

group III standard drugs will be given. In group IV, 1-7th day Polyherbal Preparation 1, 8th day Azathioprine, 9-21th test drug 1 will be given. In group V, 1-7th day Polyherbal Preparation 2, 8th day Azathioprine, 9-21th test drug 2 will be given and evaluated the different parameters like Biochemical parameters, CBC, Histophathological assessment of liver.

No of days required for study: 21 days

Number of groups of animals (N): 5 groups

No of animals required (n): 30 (6 animals in each group)

Species and Strain: male, adult, Wistar Albino rats

Poly Herbal Preparation 1: extract of all 3 test compound (300mg/kg) p.o.

Poly Herbal Preparation 2: extract of all 3 test compound (600mg/kg) p.o.

6. EVALUATION OF DIFFERENT PARAMETERS

6.1 Complete blood count: Total number of erythrocytes (RBCs), total number of leukocytes (WBCs), differential leukocyte count, platelet count, packed cell volume (PCV) %, and hemoglobin (Hb) concentration were estimated by adopting standard procedures. Blood smears were prepared as soon as possible after blood collection on a glass slide and quickly dried and stained with Giemsa and May-Grunwald stain for the differential blood count. Erythrocyte indices like mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated according to standard formulas. [23]

6.2 Biochemical parameters: Estimation of biochemical parameters in serum i.e the levels of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphate (ALP), with the help of biochemistry analyzer kit with corresponding reagents according to standard protocols. [24]

6.3 Histopathological assessment of liver: The tissue of liver fixed in 4% paraformaldehyde will embedded in paraffin, sectioned into 5 μm thickness, and stained with hematoxylineosin (H&E) for evaluation of histopathological changes. The histopathological changes of stained liver slices were observed under a bright-field microscope. [22,24–26]

6.4 Statistical analysis: Statistical analysis will be carrying out by one-way analysis of variance (ANOVA) for significant value. Data will express as mean±SEM (standard error mean).

7. RESULTS (TABLES & GRAPHS)

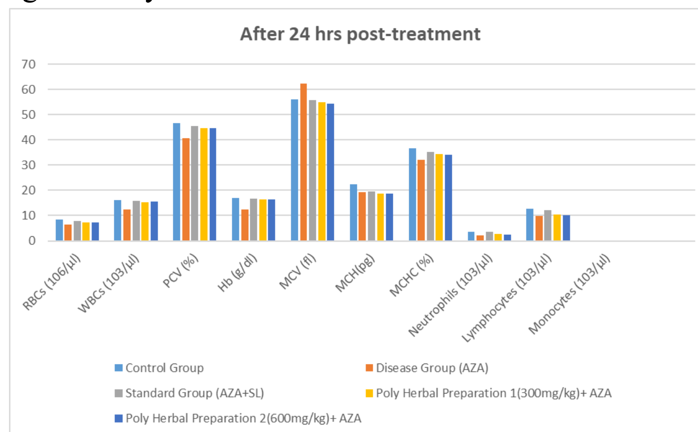
7. 1. Hematological findings

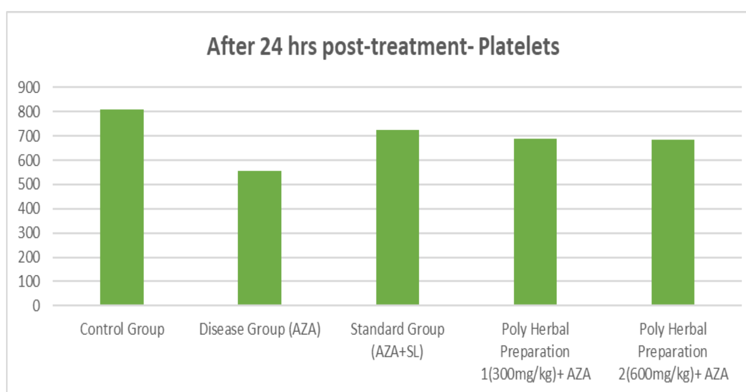
1.1 After 24 hrs post-treatment (table)

TEST NAME	**CONTROLGROUP**	**DISEASEGROUP (AZA)**	**STANDARD GROUP (AZA+SL)**	Poly Herbal Preparation 1(300mg/kg)+ **AZA**	Poly Herbal Preparation 2(600mg/kg)+ **AZA**
RBCs (10^6/µl)	8.32±0.2	6.46±0.2^a	7.96±0.6^b	7.22±0.6 ^{a,b}	7.24±0.6^b
WBCs (10^3/µl)	16.81±0.6	12.28±0.4^a	15.82±0.5^b	15.22±0.4^{a,b}	15.62±0.2^b
Platelets (10^3/µl)	808.6±36.4	556.3±86.6^a	726.22±11.8^{a,b}	688.24±10.6^{a,b}	682.25±10.4^{a,b}
PCV (%)	46.62±1.4	40.82±1.2^a	45.58±1.6^b	44.72±1.4^{a,b}	44.54±1.2$^{a.b}$
Hb (g/dl)	16.92±1.2	12.34±0.2^a	16.82±1.2^b	16.32±1.4^{,b}	16.44±1.6^b
MCV (fl)	56.16±0.2	62.47±2.4^a	55.82±2.5^{a,b}	54.98±2.4^b	54.36±2.2^b
MCH(pg)	22.55±1.8	19.27±0.4	19.46±3.9^{a,b}	18.72±1.8^{a,b}	18.68±1.6^b

MCHC (%)	36.68±2.8	32.06±2.2[a]	35.22±2.4[a,b]	34.28±2.4 [a,b]	34.12±2.2[a,b]
Neutrophils (10^3/µl)	3.62±0.6	2.18±0.2[a]	3.62±0.5[b]	2.72±0.3[b]	2.56±0.7[b]
Lymphocytes (10^3/µl)	12.82±0.9	9.96±0.2[a]	12.22±0.6[a,b]	10.34±0.3[b]	10.16±0.6[b]
Monocytes (10^3/µl)	0.28±0.1	0.18±0.1[a]	0.28±0.3[a,b]	0.26±0.2[b]	0.24±0.2[b]

All the data given in table as means ± S.D with dissimilar superscript letters (significantly differ at $p < 0.05$) (a) means significantly differ from control value. (b) means significantly differ from AZA group.



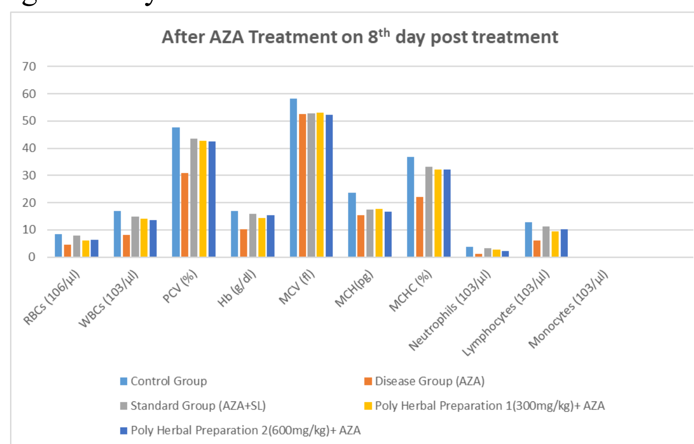


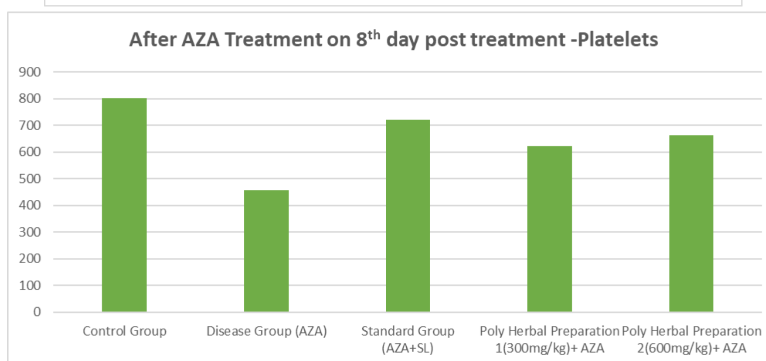
As shown in figure there were significant reduction in RBCs, WBCs, Platelets, PCV, Hb, MCHC, Neutrophils, Lymphocytes, Monocytes in the AZA group of rats (disease group) after 24hrs of single dose of AZA i.p route as compared to control group but there is significant elevation in the value of MCV. On the other hand when the treatment was given it shows the protective effect against AZA induced reduction in the values of RBCs, WBCs, Platelets, PCV, Hb, MCHC, Neutrophils, Lymphocytes, and Monocytes. MCV value also significantly reduced than AZA treated group.

7.2 After AZA Treatment on 8th day post treatment (table)

TEST NAME	CONTROLGROUP	DISEASEGROUP (AZA)	STANDARD GROUP (AZA+SL)	Poly Herbal Preparation 1(300mg/kg)+ AZA	Poly Herbal Preparation 2(600mg/kg)+ AZA
RBCs (10^6/µl)	8.30±0.24	4.46±0.22^a	7.90±0.42^b	6.22 ±0.6 ^{a,b}	6.24±0.6^b
WBCs (10^3/µl)	16.82±0.42	8.28±0.42^a	14.82±0.6^b	14.22±0.4^{a,b}	13.62±0.2^b
Platelets (10^3/µl)	802.6±38.6	456.3±16.6^a	722.22±10.8^{a,b}	622.24±10.6^{a,b}	662.25±10.6^{a,b}
PCV (%)	47.62±1.8	30.82±1.2^a	43.58±1.6^b	42.72±1.4^{a,b}	42.54±1.2$^{a.b}$
Hb (g/dl)	16.98±1.6	10.34±1.2^a	15.82±1.4^b	14.32±1.4^{,b}	15.44±1.6^b
MCV (fl)	58.16±0.2	52.47±2.4^a	52.82±2.6^{a,b}	52.98±2.4^b	52.36±2.2^b
MCH(pg)	23.56±1.6	15.27±1.4	17.46±3.8^{a,b}	17.72±1.8^{a,b}	16.68±1.8^b
MCHC (%)	36.88±2.8	22.06±2.2^a	33.22±2.4^{a,b}	32.28±2.4 ^{a,b}	32.12±2.2^{a,b}
Neutrophils (10^3/µl)	3.68±0.4	1.18±1.2^a	3.22±0.6^b	2.70±0.3^b	2.16±0.8^b
Lymphocytes (10^3/µl)	12.82±0.6	5.98±0.2^a	11.22±0.6^{a,b}	9.34±0.4^b	10.12±0.6^b
Monocytes (10^3/µl)	0.16±0.2	0.11±0.1^a	0.18±0.6^{a,b}	0.22±0.2^b	0.22±0.2^b

All the data given in table as means ± S.D with dissimilar superscript letters (significantly differ at $p < 0.05$) (a) means significantly differ from control value. (b) means significantly differ from AZA group.



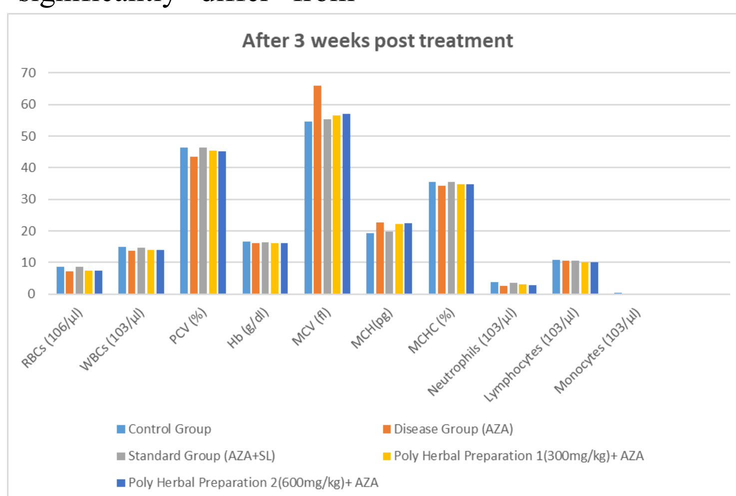


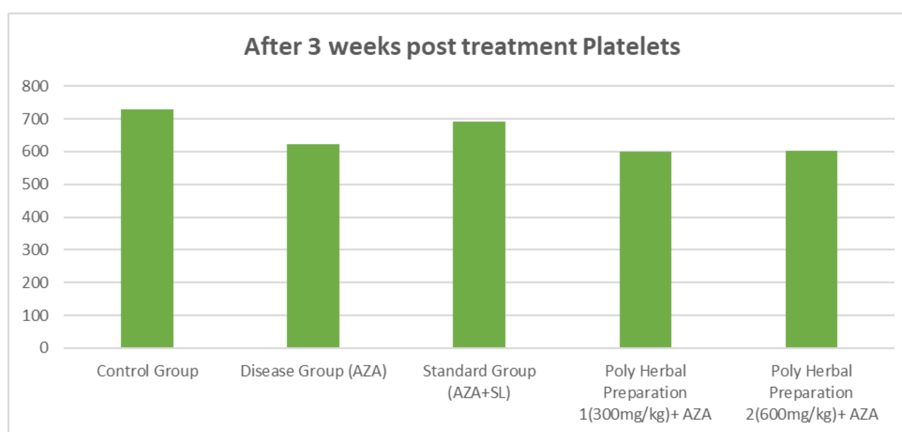
As shown in figure there were significant reduction in RBCs, WBCs, Platelets, PCV, Hb, MCHC, Neutrophils, Lymphocytes, Monocytes in the AZA group of rats (disease group) after 24hrs of single dose of AZA i.p route as compared to control group but there is significant elevation in the value of MCV. On the other hand when the treatment was given it shows the protective effect against AZA induced reduction in the values of RBCs, WBCs, Platelets, PCV, Hb, MCHC, Neutrophils, Lymphocytes, and Monocytes. MCV value also significantly reduced than AZA treated group.

7.3 After 3 weeks post-treatment (table)

TEST NAME	CONTROL GROUP	DISEASE GROUP (AZA)	STANDARD GROUP (AZA+SL)	Poly Herbal Preparation 1(300mg/kg)+ AZA	Poly Herbal Preparation 2(600mg/kg)+ AZA
RBCs (10^6/µl)	8.58±0.24	7.28±0.6^a	8.62±0.8^b	7.48±0.6^b	7.42±1.2^b
WBCs (10^3/µl)	14.86±0.9	13.62±1.6^a	14.72±0.8^b	13.98±0.6^b	13.92±1.2^b
Platelets (10^3/µl)	730.22±28.5	623.33±28.1^a	692.32±12.4^{a,b}	598.96±12.6^{a,b}	601.24±12.2^{a,b}
PCV (%)	46.44±1.6	43.42±1.8^a	46.28±1.8^b	45.52±1.8^{a,b}	45.22±1.6^b
Hb (g/dl)	16.54±1.4	16.26±0.6^a	16.44±1.4^b	16.12±1.8^b	16.18±1.6^b
MCV (fl)	54.52±2.5	65.88±2.4^a	55.22±2.2^b	56.44±2.6^{a,b}	56.98±2.5^{a,b}
MCH(pg)	19.38±0.1	22.56±2.5^a	19.82±1.8^{a,b}	22.16±1.8^{a,b}	22.34±1.4^{a,b}
MCHC (%)	35.61±2.5	34.36±2.6	35.52±2.0^{a,b}	34.81±2.9^{a,b}	34.78±3.1^{a,b}
Neutrophils (10^3/µl)	3.81±0.4	2.72±0.5	3.62±0.7^b	2.98±1.3^{a,b}	2.81±0.8^b
Lymphocytes (10^3/µl)	10.75±0.9	10.49±1.3	10.69±0.3^{a,b}	10.01±0.5^{a,b}	10.13±0.2^{a,b}
Monocytes (10^3/µl)	0.32±0.4	0.28±1.5^a	0.31±0.6^{a,b}	0.30±0.7^b	0.23±0.7^b

All the data given in table 10 as means ± S.D with dissimilar superscript letters (significantly differ at $p < 0.05$) (a) means significantly differ from control value. (b)means significantly differ from AZA group.





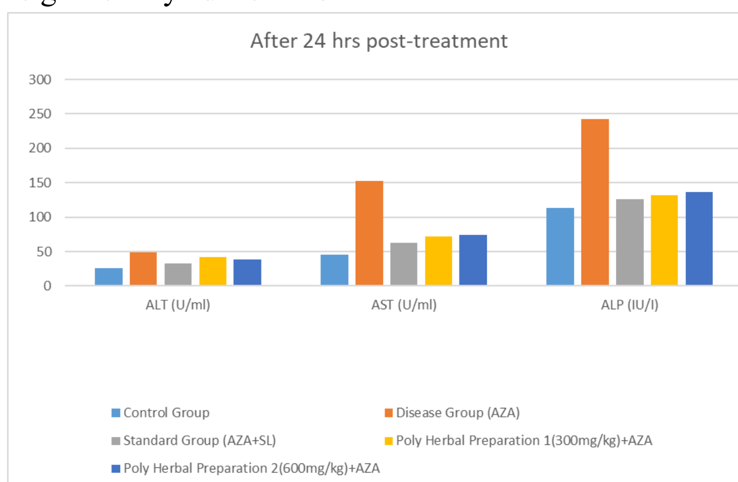
As shown in figure after 2 weeks of post treatment there were significant reduction was seen in RBCs, WBCs, Platelets, Neutrophils, Monocytes in the AZA group of rats (disease group) after 24hrs of single dose of AZA i.p route as compared to control group but there is significant elevation in the value of MCV and MCH while PCV, Hb, MCHC, Lymphocyteswere comparable with control group. On the other hand when the treatment was given it shows the protective effect and returned to normal values. But there is significant reduction in neutrophils and elevation in lymphocytes as compared to normal control value.

7.4. Biochemical findings

7.4.1 After 24hrs post treatment (table)

TEST NAME	CONTROL GROUP	DISEASE GROUP	STANDARD GROUP	Poly Herbal Preparation 1(300mg/kg)+ **AZA**	Poly Herbal Preparation 2(600mg/kg)+ **AZA**
ALT (U/ml)	26.28±2.2	49.42±3.6[a]	32.26±3.2[ab]	41.42±3.4[a,b]	38.28±2.6[a,b]
AST (U/ml)	45.22±2.4	152.42±3.6[a]	62.12±2.8[ab]	72.24±2.8[a,b]	74.22±3.2[a,b]
ALP (IU/I)	112.91±1.4	242.22±1.9[a]	126.22±1.6[a,b]	132.12±2.2[a,,b]	136.22±2.2[a,b]

All the data given in table 11 as means ± S.D with dissimilar superscript letters (significantly differ at p <0.05) (a) means significantly differ from control value. (b)mean significantlyNG9, differ from AZA group.

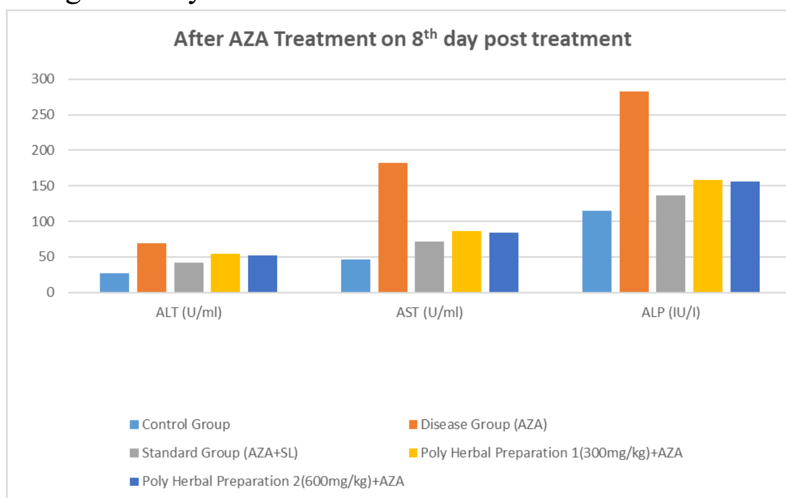


As shown in figure, after 24 hrs of post treatment of AZA groupthe level of serum hepatic enzymesALT, AST, ALP was significantly increased as compared to the control values. On the other hand, when the treatment is given to the group of rats the combination significantly decreased the value of ALT, AST, ALP as compared to AZA group but the values of AST, ALP remains significantly increased than control value.

7.4.2 After AZA Treatment on 8th day post treatment (table)

TEST NAME	CONTROL GROUP	DISEASE GROUP	STANDARD GROUP	Poly Herbal Preparation 1(300mg/kg)+ **AZA**	Poly Herbal Preparation 2(600mg/kg)+ **AZA**
ALT (U/ml)	27.28±2.4	69.44±4.6[a]	42.26±3.4[ab]	54.42±3.6[a,b]	52.28±2.8[a,b]
AST (U/ml)	46.22±2.6	182.46±4.6[a]	72.12±2.6[ab]	86.24±2.8[a,b]	84.22±3.4[a,b]
ALP (IU/I)	114.91±1.6	282.26±2.8[a]	136.22±1.4[a,b]	158.14±2.4[a,,b]	156.22±2.4[a,b]

All the data given in table 11 as means ± S.D with dissimilar superscript letters (significantly differ at p <0.05) (a) means significantly differ from control value. (b)mean significantlyNG9, differ from AZA group.

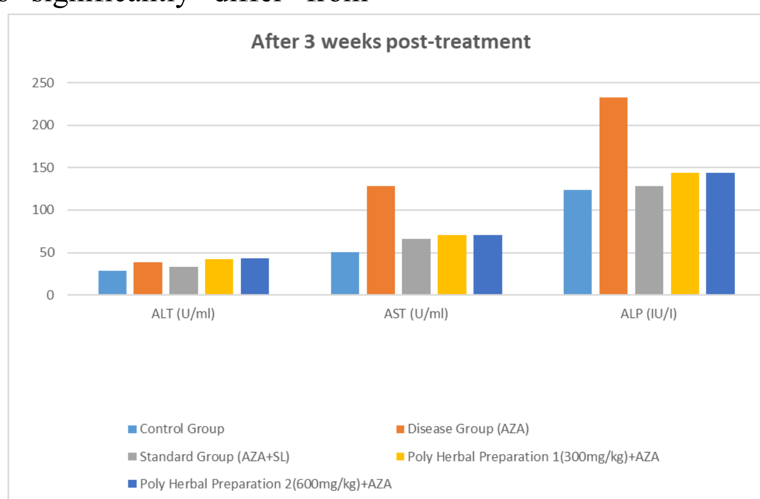


As shown in figure, after 24 hrs of post treatment of AZA group the level of serum hepatic enzymes ALT, AST, ALP was significantly increased as compared to the control values. On the other hand, when the treatment is given to the group of rats the combination significantly decreased the value of ALT, AST, ALP as compared to AZA group but the values of AST, ALP remains significantly increased than control value.

2.3 After 3 weeks post-treatment (table)

TEST NAME	CONTROL GROUP	DISEASE GROUP	STANDARD GROUP	Poly Herbal Preparation 1(300mg/kg)+ **AZA**	Poly Herbal Preparation 2(600mg/kg)+ **AZA**
ALT (U/ml)	28.38±0.8	38.62±2.6[a]	32.68±3.6[a,b]	42.18±3.2[a,b]	43.34±2.2[a,b]
AST (U/ml)	50.22±1.5	128.22±3.5[a]	66.22±3.2[a,b]	70.68±3.6[a,b]	70.88±3.8[a,b]
ALP (IU/I)	123.88±2.8	232.38±2.5[a]	128.22±2.2[a,b]	144.22±2.5[a,b]	144.22±2.2[a,b]

All the data given in table 12 as means ± S.D with dissimilar superscript letters (significantly differ at $p < 0.05$) (a) means significantly differ from control value. (b) Means significantly differ from AZA group.



As shown in figure, after 2 weeks of post treatment of AZA group, the level of serum hepatic enzymes ALT, AST, ALP was significantly increased as compared to the control values. On the other hand, when the treatment is given to the group the combination significantly restore the value of ALT, ALP as compared to AZA treated group except AST whose value was remains significantly increased than control value.

3. Histophathological findings

Histopathological findings in hepatotoxicity (drug-induced liver injury/DILI) vary widely but commonly include hepatocellular necrosis, inflammation, apoptosis (acidophil bodies), and cholestasis. Key features often show a zonal pattern (e.g., zone 3 centrilobular necrosis), ballooning degeneration, steatosis (micro- or macrovesicular), and sometimes fibrosis.

Key Histopathological Patterns of Hepatotoxicity:

- **Necroinflammatory Changes**: Characterized by hepatocellular necrosis, spotty lobular inflammation, and portal inflammation, with apoptosis (acidophil bodies) often present.
  - **Hepatocellular Necrosis/Apoptosis**:
    - **Centrilobular (Zone 3) Necrosis:** Common in toxic liver injury (e.g., acetaminophen overdose).
    - **Interface Hepatitis:** Lymphocytes/plasma cells destroy hepatocytes at the limiting plate, common in DILI.
    - **Apoptosis:** Formation of acidophil bodies.

- **Steatosis:**
  - **Microvesicular Steatosis:** Associated with mitochondrial injury (e.g., tetracycline, valproic acid).
  - **Macrovesicular Steatosis:** Often seen in alcoholic or metabolic liver damage.
- **Cholestasis:** Bile plugging within hepatocytes and canaliculi, indicating biliary obstruction/damage, commonly seen with anabolic steroids or amoxicillin-clavulanate.
- **Ballooning Degeneration:** Hepatocytes appear swollen with pale cytoplasm, often with Mallory-Denk bodies.
- **Fibrosis:** Occurs in chronic injury scenarios, such as with amiodarone or chronic alcohol consumption.
- **Specific Findings:** Granulomas (in cases of hypersensitivity), sinusoidal dilatation, and Kupffer cell hyperplasia.

These findings, along with clinical history, are crucial for diagnosing drug-induced liver injury, although some patterns can mimic viral or autoimmune hepatitis.

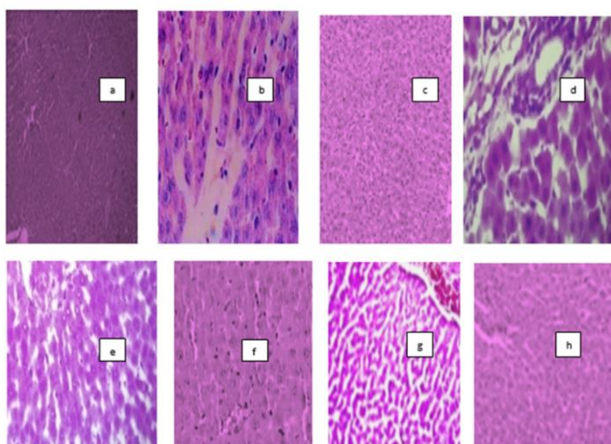


Figure : liver section of rat, the control group of rats showed the normal histopathology of liver i.e normal structure of central vein which is surrounded by hepatocytes having no alteration (fig a).After 24 hrs AZA alone group showed some inflammatory cell infiltration in the portal area and diffusion in Kupffer cell proliferation is also detected in cytomegalic hepatocytes (fig b).After24 hrs of AZA and treatment group showed normal histopathology (fig c) after 2 weeks AZA alone group showed inflammatory cell infiltration in the portal area with dilation in bile duct, cytomegalic and fatty changes were also detected (fig d). After 2 week of AZA and treatment group showed normal histopathology (fig e).The standard group shows normal histopathology after 2 week (fig f). There is little alteration and swelling but normal appearance of hepatocytes (fig g and h).

CONCLUSION

The present study demonstrates that the polyherbal formulation comprising *Glycyrrhiza glabra*, *Ginkgo biloba*, and *Amaranthus spinosus* exhibits significant hepatoprotective activity against azathioprine-induced liver injury in Wistar albino rats. Azathioprine administration resulted in marked hepatic damage, as evidenced by altered hematological parameters, elevated liver enzyme levels (ALT, AST, ALP), and pronounced histopathological changes such as inflammation, cellular degeneration, and necrosis.

Treatment with the polyherbal formulation at both low (300 mg/kg) and high (600 mg/kg) doses

effectively mitigated these toxic effects. The formulation significantly restored hematological indices (RBCs, WBCs, Hb, PCV, platelets), reduced elevated liver enzymes toward normal levels, and improved liver architecture, as confirmed by histopathological examination. The higher dose showed comparatively better protective effects, indicating a dose-dependent response.

The hepatoprotective effect of the formulation can be attributed to the synergistic action of its phytoconstituents, particularly flavonoids and phenolic compounds, which possess strong antioxidant and free radical scavenging properties. These compounds likely act by reducing oxidative stress, enhancing endogenous antioxidant defenses, stabilizing hepatocellular membranes, and preventing lipid peroxidation.

Overall, the findings suggest that the selected polyherbal formulation offers a promising, natural, and safer therapeutic approach for the management of drug-induced liver injury. However, further studies, including molecular mechanism exploration and clinical trials, are recommended to validate its efficacy and safety in humans.

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CONFLICTS OF INTERESTS

There are no conflicts of interest.

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