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Exploring the therapeutic potential of *Heliotropium strigosum* leaves through biological activity evaluation

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Abstract: **Background**: *Heliotropium strigosum* is a traditionally used medicinal plant with reported therapeutic potential, particularly in the management of liver disorders and oxidative stress-related diseases. However, its pharmacological properties require systematic scientific validation. **Objectives**: This study aimed to evaluate the antioxidant, hepatoprotective and cytotoxic potential of *Heliotropium strigosum* leaf extract using *in-vitro* and *in-vivo* experimental models. **Methods**: Phytochemical screening was performed to identify bioactive constituents of the plant extract. Antioxidant activity was assessed using the DPPH assay at concentrations of 50, 100, 200, 500 and 1000 µg/mL. Cytotoxic potential was evaluated using the MTT assay to determine cancer cell viability following treatment with the extract. Hepatoprotective activity was assessed in a CCl_4-induced hepatotoxicity model in rabbits through biochemical analysis and histopathological examination. Data were expressed as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni post hoc test, with significance set at $p < 0.05$. **Results**: The extract contained key phytochemicals associated with antioxidant and hepatoprotective activities. In the DPPH assay, the extract exhibited dose-dependent free-radical scavenging activity. Cytotoxic analysis using the MTT assay revealed reduced cancer cell viability in treated groups compared to untreated controls. *In-vivo*, the extract exhibited significant hepatoprotective effects, as evidenced by improvement in liver biomarkers and restoration of normal hepatic architecture in histopathological analysis. **Conclusion**: The findings suggest that *Heliotropium strigosum* possesses significant antioxidant, hepatoprotective and cytotoxic properties, supporting its traditional medicinal use. Further studies focusing on isolation of active compounds, detailed mechanistic investigations and clinical trials are required to validate its therapeutic potential.

Keywords: Antioxidant; Cytotoxic; *Heliotropium strigosum*; Hepatoprotective; *In-vitro*; *In-vivo*

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INTRODUCTION

Heliotropium strigosum (HS), locally known as Bhangra (family Boraginaceae), is a medicinal plant widely distributed in arid and semi-arid regions of South Asia, including Pakistan (Singh and Sharma, 2019). Members of the genus *HS* are known to contain a diverse range of secondary metabolites, including flavonoids, phenolic acids, terpenoids and alkaloids, which contribute to their reported antioxidant, anti-inflammatory and hepatoprotective properties (Fayed, 2021). Several *HS* species have been investigated for biological activities; however, scientific evidence regarding the pharmacological potential of *HS* remains limited and fragmentary.

Oxidative stress plays a vital role in the pathogenesis of liver injury, particularly in chemical-induced hepatotoxicity models, such as carbon tetrachloride (CCl_4) exposure (Unsal *et al*., 2021). Natural products rich in antioxidants can attenuate hepatic damage by reducing lipid peroxidation, restoring endogenous antioxidant enzymes and preserving liver histoarchitecture (Firdous *et al*., 2025). Although *HS* has been traditionally used for several ailments (Cartuche *et al*., 2022), its antioxidant and hepatoprotective potential has not been systemically evaluated using integrated phytochemical, *in-vitro* and *in-vivo* approaches.

There is a lack of comprehensive experimental studies correlating the phytochemical profile of *HS* with its antioxidant and hepatoprotective effects, supported by biochemical, histopathological and cell-based assays. The present study aimed to characterize the phytochemical composition of the ethanolic leaf extract of *HS,* to evaluate its antioxidant activity using the DPPH assay and to investigate its hepatoprotective potential against CCl4-

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induced liver injury. An *in-vitro* HepG2 (human hepatocellular carcinoma) cell line was used, as well as *in-vivo* (rabbit) models. Cytotoxic effects were evaluated using cell viability assays to assess the safety and biological activity of the extract.

MATERIALS AND METHODS

Plant collection and extraction

Fresh *HS* plant leaves were collected from the local area of Bahawalpur. *HS* was washed and stored on a shady surface for 15 days at room temperature after collection. Throughout this time period, moisture content was evaporated from the *HS*. The plant was identified and authenticated by a qualified botanist, Dr. Ghulam Sarwar, at the Department of Botany, The Islamia University of Bahawalpur. Specimens as well as reference numbers were deposited at the botany department for reference and voucher numbers obtained were Ref/Botany/353 (*HS*). For future reference, voucher specimens were deposited in the botany department at the Islamia University of Bahawalpur.

Extract preparation

Fresh aerial parts of *HS* were shade-dried at ambient temperature (25-30 °C) for 15 days until a constant weight was achieved. The dried material was coarsely powdered and extracted using cold maceration with 70% ethanol (plant material: solvent 1:5 w/v) for 72 hours with intermittent shaking. The extract was filtered and concentrated under reduced pressure. After being dried, the plant was crushed, processed into a powder and homogenized. This powder was placed in a Soxhlet apparatus, which was then sealed. Ethanolic extraction was performed using Soxhlet apparatus following standard protocols as previously reported in the literature (Harborne, 1998; Alara, 2019). A rotary evaporator operating at lower pressure was used to concentrate the extract.

Removal of solvent from extract

Following the extraction procedure, a concentrated extract was obtained by evaporating the ethanol at a particular temperature using a rotary evaporator. To accelerate solvent evaporation, the water bath temperature was maintained at 80°C and the raw ethanol extract from *HS* was placed in the rotary evaporator tank. The procedure was repeated until the extract was completely free of ethanol. Ultimately, a glass jar with the purified extract was collected separately. After solvent evaporation, the percentage yield of the crude ethanolic leaf extract of *HS* was 12% w/w (120 g from 1kg dried plant material).

Phytochemical screening

Saponins (foam test), alkaloids (Wagner's test), steroids (Salkowski test), flavonoids (alkaline reagent test) and tannins (ferric chloride test) were analyzed according to standard qualitative methods (Evans, 2009).

GC-MS analysis

Using a mass spectrometer detector (MSD-5977 A) and GC B-7890 (Agilent Technology), GC–MS analysis was carried out. The HP-5MS column, which had a length of 30 meters, an internal diameter of 0.25 mm, a film thickness of 0.25µm and a stationary phase of 100% dimethyl polysiloxane, was used to separate the compounds. The injector's temperature was set at 250°C. One milliliter per minute of helium was utilized as the carrier gas. For the GC-MS analysis, two microliters of aqueous ethanolic extracts were utilized (Ullah and Alqahtani, 2022).

Antioxidant assay (DPPH)

The free radical scavenging capacity of the plant extract was assessed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. In this method, the plant extract reacts with the stable DPPH radical and the reduction in color intensity is measured spectrophotometrically. A greater decrease in absorbance reflects higher antioxidant activity (Baliyan *et al*., 2022). A 75 µM DPPH solution was prepared by dissolving 2.95 mg of DPPH in 100 mL of ethanol. For the assay, 0.5 mL of the plant extract (prepared in ethanol at different concentrations) was mixed with 2.5 mL of the DPPH solution in test tubes. The mixture was incubated in the dark at room temperature for 90 minutes, after which the absorbance was recorded at 517 nm. Appropriate blanks were included and the residual DPPH concentration was calculated. All experiments were performed in triplicate. Quercetin and ascorbic acid were used as standard antioxidants. The 50% inhibitory concentration (IC_{50}), representing the concentration required to scavenge 50% of DPPH radicals, was determined using regression analysis.

The radical scavenging activity (RSA) was calculated using the following equation:

%RSA= Abs of control − Abs of sample/Abs. of control × 100.

RSA: Radical Scavenging Activity, Abs is the absorbance of DPPH radical + ethanol, Abs sample is the absorbance of DPPH radical + Test sample

Catalase assay (CAT)

Catalase activity was measured following a previously described method (16). Briefly, secretome samples from different experimental groups were transferred to a 96-well plate, each containing 31.25 mM H_2O_2 and 12.5 mM KH_2PO_4 buffer (pH 7.0). The optical density was recorded at 240 nm against a blank after exposure to light for 45–60 seconds. Results were expressed as units of catalase activity.

Glutathione assay (GSH)

Intracellular glutathione levels were determined according to Ellman's method (22), using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) as the chromogenic reagent.

Absorbance was measured at 340 nm with a spectrophotometer and the results were expressed as µmol/g tissue.

In-vitro hepatoprotective activity

Liver cells were seeded at a density of 1 × 104 cells/cm2 on 96-well plates (Corning) and exposed to 8 mM carbon tetrachloride as part of the treatment. Four groups of liver cells were created: the first group was left untreated, the second group received CCl4, the third group received CCL4 + *silymarin* and the fourth group received CCl4 + plant extract treatment (*HS*). All experimental groups were assessed after a uniform incubation period of 24 hours. After that, testing for antioxidants and cell viability was performed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (MTT), Catalase (CAT) and Reduced glutathione (GSH) (Hadi *et al*., 2020).

In-vivo hepatoprotective activity

The central animal house facility of the pharmacy department at Islamia University of Bahawalpur provided the male rabbits used for the hepatoprotective activity and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) assigned the rabbits a registration number CPCSEA/IUB/353. The rabbits were kept at 25 ± 2°C with a 12-hour light/dark cycle. They have unrestricted access to water and a typical pellet diet. The CPCSEA ethics committee accepted the study and all experimental procedures were conducted in full adherence to ethical standards.

Composition of pellet diet

Crude protein content ranging from 16-18%, crude fiber 12-16%, crude fat 2-3%, ash 8-10% and moisture content ≤ 10% with vitamins and minerals provided in adequate amounts according to National Research Council (NRC) guidelines for laboratory rabbits (Table 1).

Drugs and dosing schedule

The experimental animals were randomly divided into seven groups. Each group consisted of n = 6 rabbits. Rabbits were selected due to their larger liver size, ease of biochemical sampling and established use in hepatotoxicity studies. Group I served as the negative control and received distilled water (10 mL/kg body weight). Group II was administered only carbon tetrachloride (CCl_4) and thus served as the toxic control. Animals in Group III were treated with CCl_4 in combination with silymarin suspension (10 mg/kg body weight, intraperitoneally). Groups IV, V, VI and VII received CCl_4 along with aqueous ethanolic extract of *HS* at oral doses of 250, 500, 750 and 1000 mg/kg body weight, respectively (Table 2).

For induction of hepatotoxicity, animals in Groups II–VII were administered a single daily subcutaneous dose of 50% (v/v) CCl_4 in olive oil at 2 mL/kg body weight for four consecutive days. Treatment with silymarin or plant extract was continued for 28 days.

Analysis of serum

Animals were anesthetized with ketamine (35 mg/kg, intramuscular) to minimize pain and distress, followed by euthanasia through an intravenous overdose of sodium pentobarbital (150 mg/kg), in accordance with approved ethical guidelines for animal experimentation. On the fourteenth day, a central ear artery was used to draw blood and centrifugation was used to separate the serum at 3000 rpm for 15 minutes at 30°C. A number of biochemical measures, such as CBC, LFTs and RFTs, were then examined. Blood was extracted from the heart on day 28. Following the course of treatment, all of the subject animals were put to sleep and killed. Their serum was separated by centrifugation at 3000 rpm for 15 minutes at 30°C after blood was extracted from the heart. A number of biochemical measures, such as CBC, LFTs and RFTs, were then examined.

Histopathology

Liver tissues were fixed in 10% neutral buffered formalin, processed and embedded in paraffin. Thin sections of 5 µm thickness were prepared and stained with hematoxylin and eosin. The slides were examined under a light microscope to assess hepatic architecture, cellular regeneration, necrosis and inflammatory cell infiltration.

Cytotoxic activity

Cell line culturing and subculturing

The liquid nitrogen cylinder's cryovials were thawed. The liver cell line was then cultivated in the culturing flask with 10% fetal bovine serum (FBS) and DMEM-HG. It was further supplemented with 100 mg/mL of penicillin G (Sigma) and 100 U/mL of streptomycin (Sigma). The cultures were maintained at 37°C in a humidified 5% CO2 incubator. Three replicates of the experiments were conducted. Subculturing of the cultivated liver cells was performed when they reached 70–80% confluence. The adherent cells attached to the culture flask walls were detached by first washing them with 1× phosphate-buffered saline (PBS), then incubating with 0.05% trypsin–EDTA until the cells detached from the flask surface. Cell detachment was confirmed by observing the flask under an inverted microscope. A few drops of fetal bovine serum (FBS) were then added to neutralize the trypsin and the contents were gently mixed. The cell suspension was transferred to a 15 mL centrifuge tube and centrifuged at 2000 rpm for 5 minutes. Following centrifugation, the pellet was suspended and the supernatant was collected (Hadi *et al*., 2020).

Cytotoxicity assays

MTT assay

The MTT assay was used to measure cell viability (Sigma Aldrich, USA). For every experimental group, the assay

was performed three times. Using a 96-well plate, the MTT test was used to assess the liver cells' capacity to proliferate following therapy. After being washed with phosphate-buffered saline (PBS), the cells were cultured for 2 hours in 100 µl of serum-free DMEM and 25 µl of MTT solution (5 mg/ml). Following the appearance of the purple formazan crystals, they were dissolved in 10% sodium dodecyl sulfate (SDS) or dimethyl sulfoxide (DMSO) and the absorbance was measured at 570 nm (Hadi *et al*., 2020). Percentage viability was calculated using the previously described method (Irshad *et al*., 2013).

% Cell viability = Experimental (OD570)/Control (OD570) × 100

Crystal violet assay (CV)

Additionally, cell viability was assessed using the CV assay (Sigma-Aldrich, USA). Another staining technique for evaluating the cellular viability of liver cell lines is the crystal violet assay. Liver cells from each experimental group were collected, pooled in a 96-well plate and the growth medium was removed; the cells were then washed with PBS. To stain the liver cells, 0.1% crystal violet dye was combined with 2% ethanol and added to the wells on the plate. The cells were then incubated at room temperature for 15 minutes. Following the incubation period, the dye was removed and the cells inside the wells were gently cleaned to prevent them from leaking out. The crystal violet stain was dissolved for 10 minutes by adding 100 µL of 1% sodium dodecyl sulfate (SDS) to each well. For each experimental group, the assay was performed three times. The absorbance of cell suspensions was then measured using a spectrophotometer on a microtiter plate at 595 nm (Hadi *et al*., 2020).

Trypan blue assay

Trypan blue, a dye that stains both live and dead cells, was used to determine the percentage of dead cells. After three PBS washes, the cells from the various experimental groups were incubated for five minutes in trypan blue (Invitrogen Inc., USA). Following three PBS washes, the cells were examined under a microscope. Trypan blue-stained cells were regarded as dead (Maqbool *et al*., 2019).

Statistical analysis

All experiments were performed in triplicate (n = 3) and the results are expressed as ± standard deviation. Statistical comparisons were performed using one-way analysis of variance (ANOVA), followed by Bonferroni's post hoc test to determine significance. A p-value ≤ 0.05 was considered statistically significant. GraphPad Prism software version 9.0 was used for all statistical analyses and graphical representations.

RESULTS

Characterization of plants

Preliminary phytochemical analysis of *HS* extract confirmed the presence of several bioactive compounds. Flavonoids were indicated by a color change from green to yellow upon sodium hydroxide treatment, which turned colorless after the addition of sulfuric acid. The formation of persistent foam in aqueous solution confirmed the presence of saponins. Alkaloids were detected by a reddish-brown coloration with Wagner's reagent, while steroids produced a dark reddish color upon sulfuric acid treatment. A reddish-black coloration confirmed the presence of tannins after ferric chloride application (Table 3).

Quantitative phytochemical analysis

Quantitative phytochemical analysis of the ethanolic extract of *HS* revealed substantial levels of bioactive secondary metabolites. The total phenolic content was 78.4 ± 2.1 mg GAE/g extract, indicating a high abundance of phenolic compounds, which are known to contribute significantly to antioxidant and hepatoprotective activities.

The total flavonoid content measures 41.6 ± 1.8 mg QE/g extract, suggesting a moderate concentration of flavonoids that may play a role in free radical scavenging and cytoprotective mechanisms. Alkaloids were quantified as 2.3 ± 0.2% (w/w), confirming their presence in measurable amounts and supporting the pharmacological relevance of the plant. Additionally, the total saponin content was 19.7 ± 1.3 mg DE/g extract, which may contribute to membrane-stabilizing and hepatoprotective effects (Table 4).

The quantitative findings provide stronger scientific evidence than qualitative color-based assays alone and support the biological activities observed in subsequent antioxidant, hepatoprotective and cytotoxic evaluations.

List of natural compounds found in HS after GCMS analysis

Gas chromatography-mass spectrometry (GC-MS) analysis of the plant extract led to the tentative identification of 27 phytochemical constituents based on comparison of their mass spectra with the NIST library database. For clarity and biological relevance, only compounds with documented antioxidant and hepatoprotective potential are presented and discussed.

The identified bioactive compounds predominantly belong to sesquiterpenes, triterpenoids, fatty acids, esters and oxygenated hydrocarbons, which are widely reported to contribute to antioxidant defense and hepatic protection. Major constituents included caryophyllene, α-bisabolol, α-amyrin, cyclolanostane derivatives and unsaturated fatty acids esters, all of which have been previously associated with attenuation of oxidative stress, modulation of inflammatory pathway and stabilization of hepatocellular membranes (Fig. 1, Table 5). Minor constituents lacking direct evidence for liver-related bioactivity were excluded from further interpretation to avoid speculative claims.

These findings suggest that the extract contains multiple phytoconstituents with established antioxidant and hepatoprotective relevance, which may collectively contribute to the observed biological effects.

Antioxidant activity by DPPH

The DPPH radical-scavenging test showed that the HS extract has potent antioxidant activity that varies with concentration. Tests were conducted at five different concentrations: 50, 100, 200, 500 and 1000 µg/ml. The findings are summed up as follows:

Antioxidant activity of HS by DPPH summary

The antioxidant potential of *HS* was evaluated using the DPPH radical-scavenging assay, with ascorbic acid as the reference standard. Ethanolic extracts of the leaves were tested at concentrations ranging from 50 to 250 µg/mL and the results revealed a clear dose-dependent increase in radical scavenging activity. At the lowest concentration (50 µg/mL), the extract showed an absorbance of 0.348, corresponding to 28.98% scavenging activity. In contrast, at the highest tested concentration (250 µg/mL), absorbance decreased to 0.008, with radical scavenging activity reaching 98.37%. The calculated IC_{50} value for *HS* was 123 µg/mL, demonstrating considerable antioxidant capacity. These findings indicate that the ethanolic extract of *HS* leaves exhibits strong free-radical scavenging activity, suggesting its potential as a natural antioxidant source.

The control (DPPH solution without plant extract) exhibited a stable absorbance of 0.49 at 517 nm, which was used to calculate the percentage radical-scavenging activity (Fig. 2, Table 6).

Hepatoprotective activity

In-vitro hepatoprotective activity through MTT assay

Five distinct doses of *H strigosum* extract (50 µg/ml, 100 µg/ml, 200 µg/ml, 500 µg/ml and 1000 µg/ml) were used to assess MTT activity. The MTT assay compares untreated and treated cancer cells to quantify changes in cell viability and assess proliferation after treatment with a plant extract. The values were expressed using mean ± SEM. A one-way ANOVA with Bonferroni was used to assess all column pairs and a *** denoted a significant difference between the treated and untreated groups when $P < 0.05$ (Fig. 3A). Five distinct doses of *H strigosum* extract 50 µg/ml, 100 µg/ml, 200 µg/ml, 500 µg/ml and 1000 µg/ml were utilized to assess MTT activity. The percentages for each concentration are given (Fig. 3B).

CAT assay

The CAT assay results demonstrated cell viability in both the treated and untreated groups. Viability is considerably lower after treatment with *H strigosum* extract than after the control, as the bar graph illustrates. $P < 0.05$, with a significant difference between the untreated and treated groups shown by a ***. The mean ± SEM was used to obtain the values (Fig. 4A). A proportion showing that the viability of the treated group was lower than that of the untreated group (Fig. 4B).

The GSH assay results demonstrated cell viability in both the untreated and treated groups. Viability is considerably lower after treatment with *HS* extract than after the control, as the bar graph illustrates. $P < 0.05$, with a significant difference between the untreated and treated groups denoted by a ***. The mean ± SEM was used to obtain the values (Fig. 5A). A percentage indicating that the treated group's viability was lower than that of the untreated group was provided (Fig. 5B). HepG2 (human hepatocellular carcinoma) cell line was used cells treated with extract had higher levels of CAT and GSH than the untreated CCl_4-stressed group, indicating a cytoprotective mechanism through oxidative stress modulation.

Biochemical serum markers

In the *in-vivo* model, CCl_4 intoxication produced marked hepatic, renal and hematological disturbances, as evidenced by elevated serum transaminases, ALP, GGT, bilirubin, urea and creatinine, along with reduced protein levels, hemoglobin, RBCs, hematocrit and platelets and altered leukocyte profiles. Treatment with *HS* extract (250–1000 mg/kg) produced a clear dose-dependent protective effect, with higher doses (750 and 1000 mg/kg) showing the greatest efficacy. Liver function markers and bilirubin levels were substantially normalized (Figs. 6-10), protein synthesis indices were significantly improved (Figs. 11-13), renal function indices were restored toward baseline (Figs. 14 and 15) and hematological parameters improved with stabilization of differential leukocyte counts (Figs. 16-28). These findings demonstrate that *HS* possesses significant hepatoprotective, nephroprotective and hematological benefits against CCl_4-induced toxicity.

Serum biomarkers (LFTs, RFTs and CBC) summary

CCl_4 intoxication produced marked hepatic dysfunction, reflected by elevated AST, ALT, ALP, GGT and bilirubin, along with reduced total protein, albumin and globulin. Administration of *HS* extract ameliorated these alterations in a dose-dependent manner. Higher doses (750–1000 mg/kg) restored liver enzymes and bilirubin levels near to silymarin-treated and normal values, while protein synthesis indices were significantly improved, indicating protection of hepatocellular integrity. Similarly, renal markers (urea and creatinine), which were markedly increased in the CCl_4 group, were significantly reduced by extract treatment, with the highest dose achieving near-normal levels, suggesting nephroprotective activity.

Table 1: Summary of the pellet diet's composition

Parameter	Crude protein	Crude fiber	Crude fat	Ash	Moisture	Vitamins and Minerals
Content (%)	16 – 18	12 – 16	2 – 3	8 – 10	≤ 10	Adequate as per NRC*

*Note: NRC = National Research Council guidelines for laboratory rabbits (Clarke, *et al* 1977).

Table 2: Animal groups with their treatment plan

Group	Induction phase (Days 1–4)	Treatment phase (Days 5–32)	Dose and Route	Duration
G0	No CCl_4	Distilled water	10 ml/kg, oral	28 days
G1	CCl_4 (50% v/v in olive oil), 2 ml/kg/day, s.c. × 4 days	No treatment	—	—
G2	CCl_4 (50% v/v in olive oil), 2 ml/kg/day, s.c. × 4 days	Silymarin suspension	10 mg/kg/day, i.p.	28 days
G3	CCl_4 (50% v/v in olive oil), 2 ml/kg/day, s.c. × 4 days	*H. strigosum* extract	250 mg/kg/day, oral	28 days
G4	CCl_4 (50% v/v in olive oil), 2 ml/kg/day, s.c. × 4 days	*H. strigosum* extract	500 mg/kg/day, oral	28 days
G5	CCl_4 (50% v/v in olive oil), 2 ml/kg/day, s.c. × 4 days	*H. strigosum* extract	750 mg/kg/day, oral	28 days
G6	CCl_4 (50% v/v in olive oil), 2 ml/kg/day, s.c. × 4 days	*H. strigosum* extract	1000 mg/kg/day, oral	28 days

In this table, G0 = Negative control; G1 = CCl_4 only; G2 = CCl_4 + Silymarin; G3 = CCl_4 + *HS* (250 mg/kg); G4 = CCl_4 + *HS* (500 mg/kg); G5 = CCl_4 + *HS* (750 mg/kg); and G6 = CCl_4 + *HS* (1000 mg/kg). From G1 to G6, CCl_4 (50% v/v in olive oil) was administered subcutaneously at 2 ml/kg/day for 7 days.

Table 3: Phytochemical analysis of selected medicinal plants

Sr No.	Name	Alkaloids	Flavonoids	Saponins	Tannin's	Terpenoids
1	*Heliotropium strigosum*	+	+	+	+	+

+ Sign shows presence of following phytochemicals while – sign shows absence of following phytochemicals

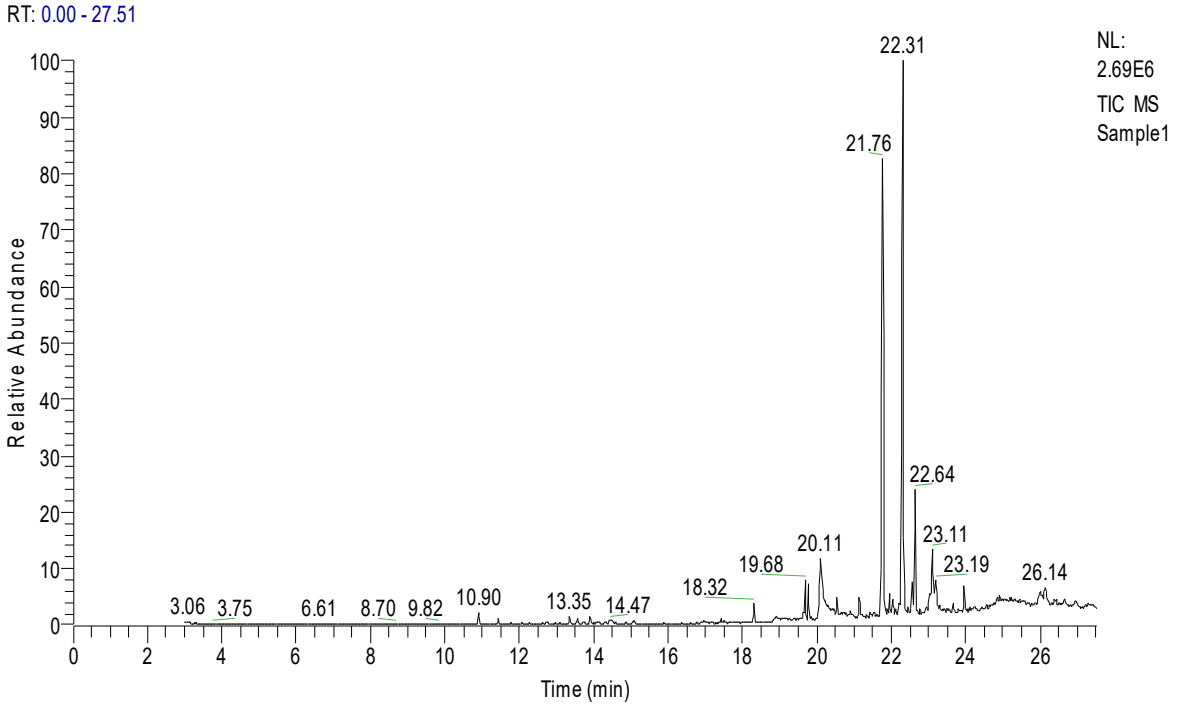

Fig. 1: GC-MS photogram of ethanolic extract of *HS*

Table 4: Quantitative phytochemical composition of *HS* ethanolic extract

Phytochemical constituents	Method employed	Expression of results	Content (Mean ± SD)
Total phenolic content (TPC)	Folin-ciocalteu assay	mg gallic acid equivalent/g extract (mg GAE/g)	78.4 ± 2.1
Total flavonoid content (TFC)	Aluminum chloride colorimetric method	mg quercetin equivalent/g extract (mg QE/g)	41.6 ± 1.8
Total alkaloid content (TAC)	Acid-base extraction (gravimetric)	percentage yield (% w/w)	2.3 ± 0.2
Total saponin content (TSC)	Vanillin-sulfuric acid assay	mg diosgenin equivalent/g extract (mg DE/g)	19.7 ± 1.3

Values are expressed as mean ± standard deviation (n = 3)

Table 5: GCMS analysis of *Heliotropium strigosum*

Sr. No.	Compound name	Molecular weight (g/mol)	Molecular formula	RT (min)	Reported pharmacological relevance	References
1.	Caryophyllene	204.35	$C_{15}H_{24}$	10.90	Antioxidant, Hepatoprotective, Anti-inflammatory,	(Unsal *et al*., 2021)
2.	α-Guaiene	204.36	$C_{15}H_{24}$	12.74	Antioxidant, Cytotoxic potential	(Maqbool *et al*., 2019)
3.	Diepicedrene-1-oxide	220.35	$C_{15}H_{24}O$	13.35	Antioxidant, Neuroprotective	(Cartuche *et al*., 2022)
4.	δ-Selinene	204.36	$C_{15}H_{24}$	13.90	Antioxidant, Cytotoxic potential	(Maqbool *et al*., 2019)
5.	α-Bisabolol	222.37	$C_{15}H_{26}O$	15.08	Antioxidant, Anti-inflammatory, Hepatoprotective,	(Unsal *et al*., 2021)
6.	Pentadecanoic acid, 14-methyl-, methyl ester	270.45	$C_{17}H_{34}O_2$	18.32	Antioxidant, Metabolic protective effects	(Christopher, 2022)
7.	9,12,15-Octadecatrienoic acid, methyl ester (Z,Z,Z)	292.46	$C_{19}H_{32}O_2$	19.71	Antioxidant, Lipid peroxidation inhibition	(Baliyan *et al*., 2022)
8.	Linoleic acid ethyl ester	308.49	$C_{20}H_{36}O_2$	20.11	Antioxidant, Membrane stabilization	(Unsal *et al*., 2021)
9.	α-Amyrin	426.70	$C_{30}H_{50}O$	26.10	Antioxidant, Anti-inflammatory, Hepatoprotective,	(Firdous *et al*., 2025)
10.	9,19-Cyclolanost-24-en-3-ol, acetate (3α)	468.75	$C_{32}H_{52}O_2$	26.96	Antioxidant, Hepatoprotective	(Hadi *et al*., 2020)

Table 6: Antioxidant activity of *HS* by DPPH

Plant extract (µg/mL)	Sample name	Ascorbic acid	2nd Absorbance	%RSA	IC-50
50	*H. strigosum*	0.49	0.348	28.98	2.406594539
100	*H. strigosum*	0.49	0.291	40.61	5.268807602
150	*H. strigosum*	0.49	0.209	57.35	8.131020665
200	*H. strigosum*	0.49	0.115	76.53	10.99323373
250	*H. strigosum*	0.49	0.008	98.37	13.85544679

Hematological disturbances, including anemia, thrombocytopenia and leukocyte imbalance, were also corrected, as hemoglobin, RBCs, hematocrit and platelet counts improved, while leukocyte profiles normalized toward baseline. Collectively, these findings demonstrate that *HS* confers hepatoprotective, nephroprotective and hematological benefits, consistent with reports on phytochemical-enriched medicinal plants mitigating oxidative and toxin-induced organ damage.

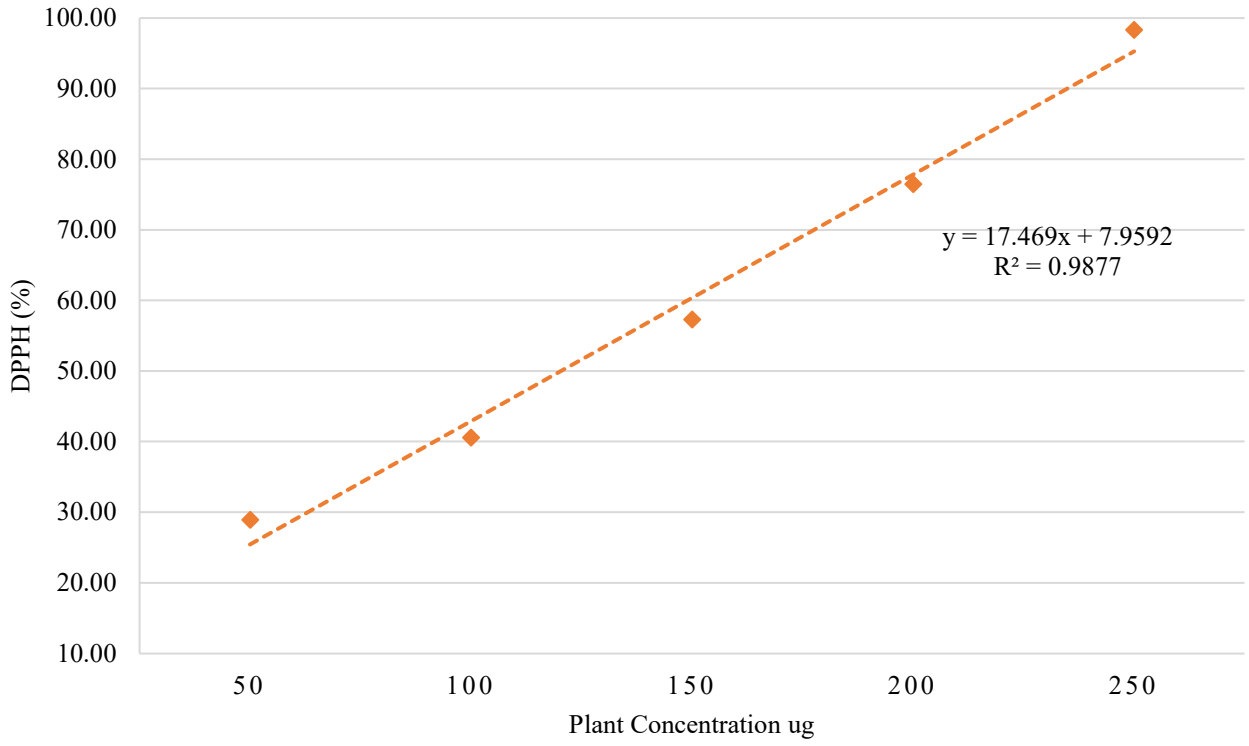

Fig. 2: Antioxidant activity of *HS* by DPPH

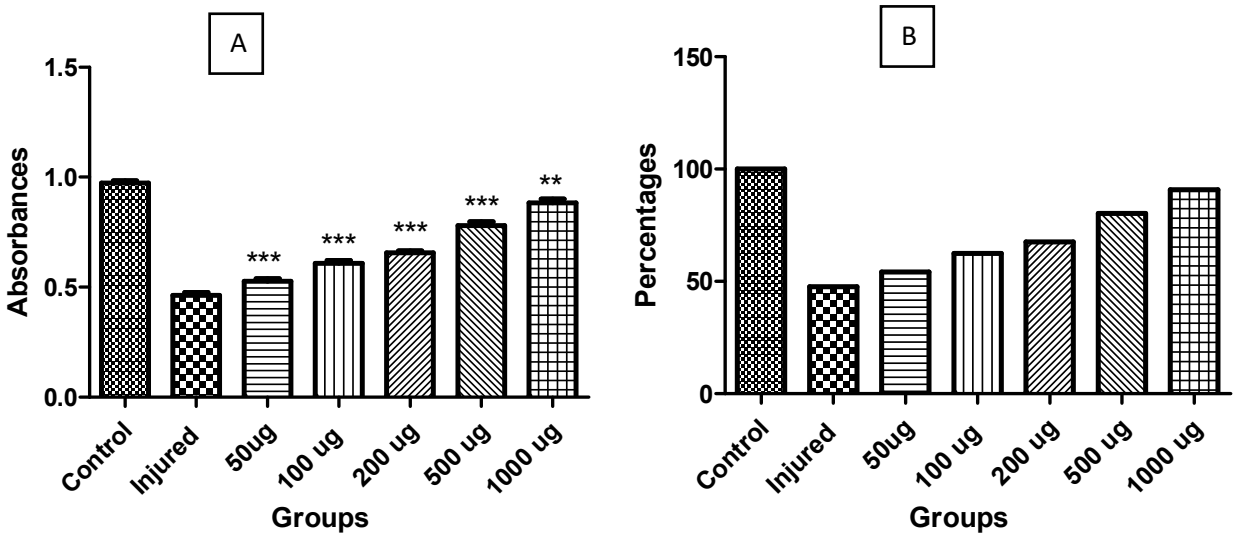

Fig. 3: *In vitro* hepatoprotective activity through MTT Assay. (A) Absorbance and (B) percentages of *HS* through MTT Assay with p values P<0.0001. Five distinct doses of *HS* extract 50 µg/ml, 100 µg/ml, 200 µg/ml, 500 µg/ml, and 1000 µg/ml were utilized to assess MTT activity. The MTT assay compares untreated and treated cancer cells that display different cell viability features in order to quantify the proliferation of cancer cells after treatment with plant extract. The values were expressed using mean ± SEM. A one-way ANOVA with bonferroni was used to assess all column pairs, and a *** denoted a significant difference between the treated and untreated groups when P < 0.05 (Fig. 3A). Five distinct doses of *HS* extract 50 µg/ml, 100 µg/ml, 200 µg/ml, 500 µg/ml and 1000 µg/ml were utilized to assess MTT activity. The percentages for each concentration are given (Fig. 3B).

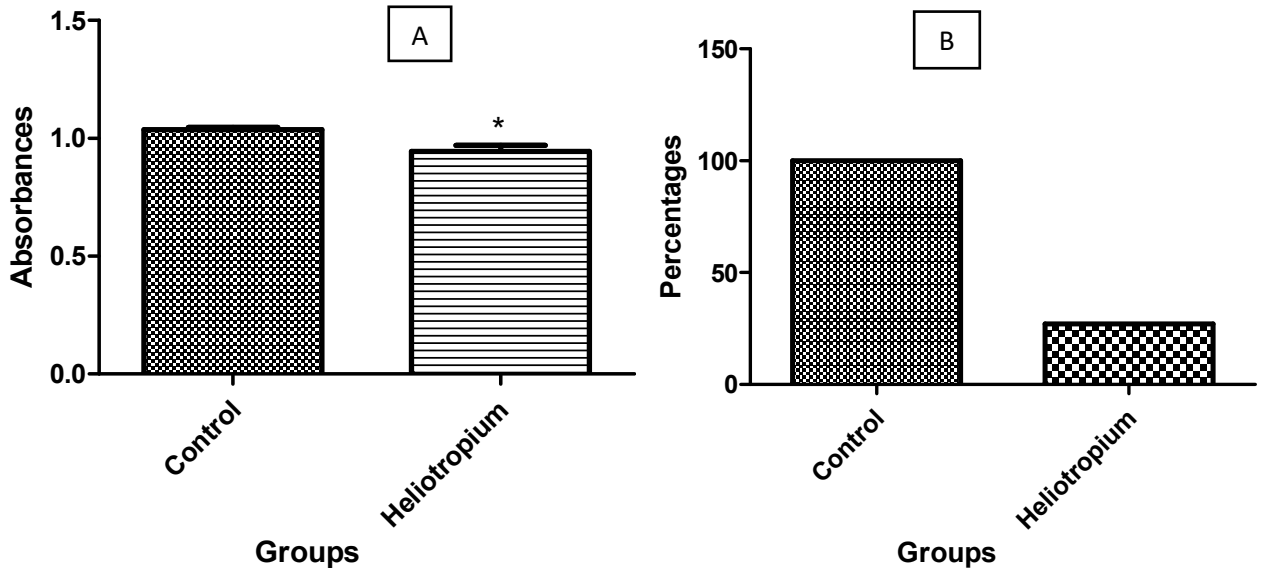

Fig. 4: Chloramphenicol acetyltransferase (CAT) assay (A) Absorbance and (B) percentages. The CAT assay results demonstrated cell viability in both the treated and untreated groups. Viability is considerably lower after treatment with *HS* extract than after the control, as the bar graph illustrates. $P < 0.05$, with a significant difference between the untreated and treated groups shown by a ***. The mean ± SEM was used to obtain the values (Fig. 4A). A proportion showing that the viability of the treated group was lower than that of the untreated group (Fig. 4B).

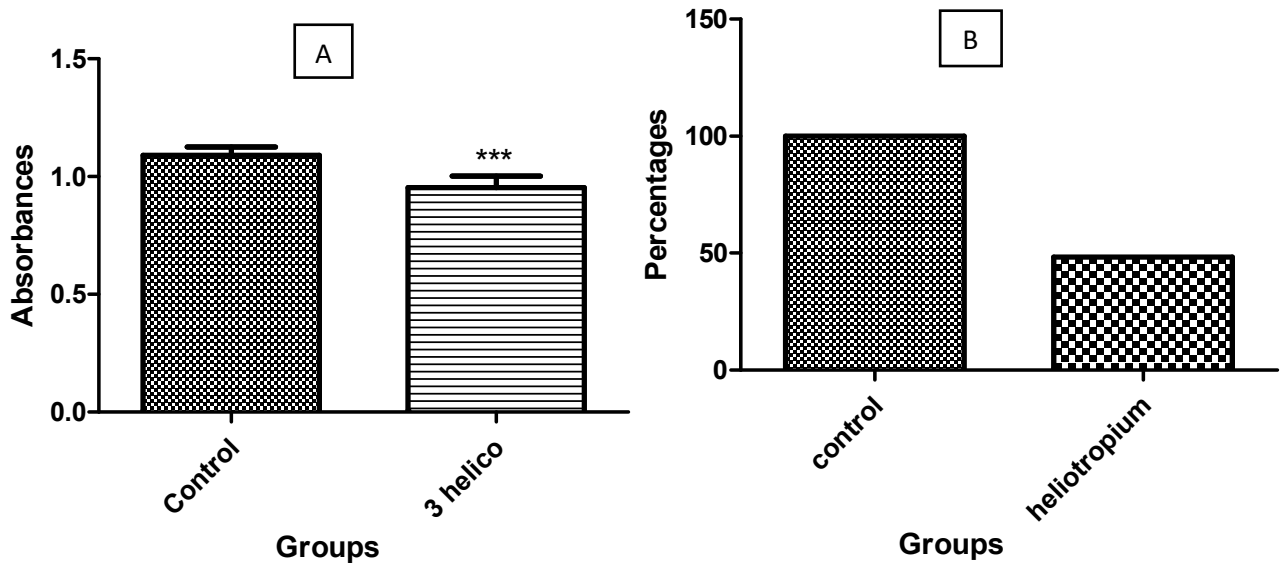

Fig. 5: *In vitro* hepatoprotective activity through GSH Assay. (A) Absorbance and (B) percentages. The GSH assay results demonstrated cell viability in both the untreated and treated groups. Viability is considerably lower after treatment with *HS* extract than after the control, as the bar graph illustrates. $P < 0.05$, with a significant difference between the untreated and treated groups denoted by a *** (Fig. 5A). The mean ± SEM was used to obtain the values. A percentage indicating that the treated group's viability was lower than that of the untreated group was provided (Fig. 5B). HepG2 (human hepatocellular carcinoma) cell line was used cells treated with extract had higher levels of CAT and GSH than the untreated CCl_4-stressed group, indicating a cytoprotective mechanism through oxidative stress modulation.

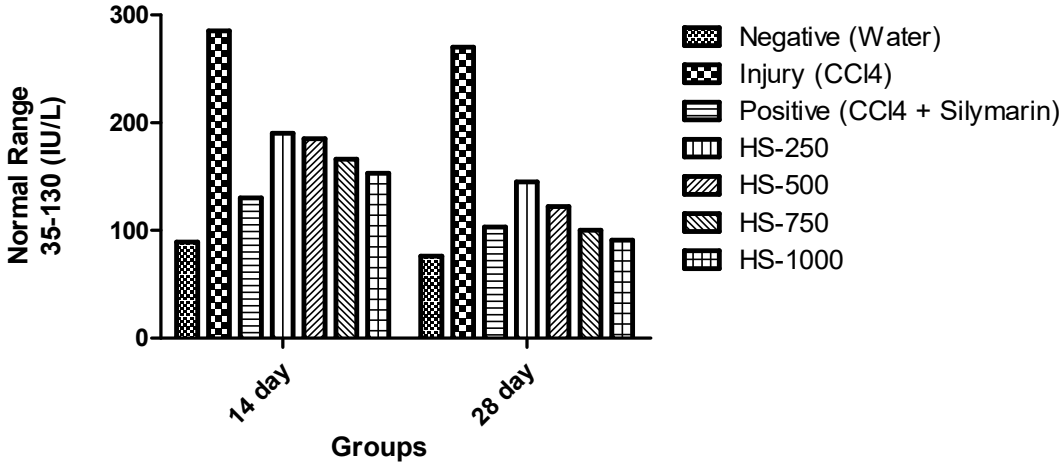

Fig. 6: Value of Aspartate transaminase after 14 and 28 days with p value P<0.0004

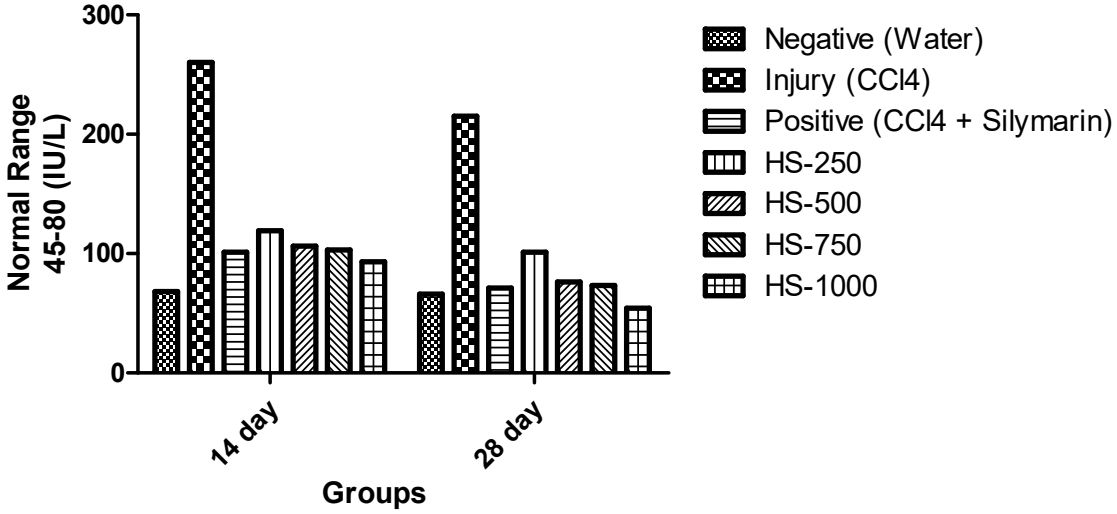

Fig. 7: Value of Alanine transaminase after 14 and 28 days with p value P<0.0001

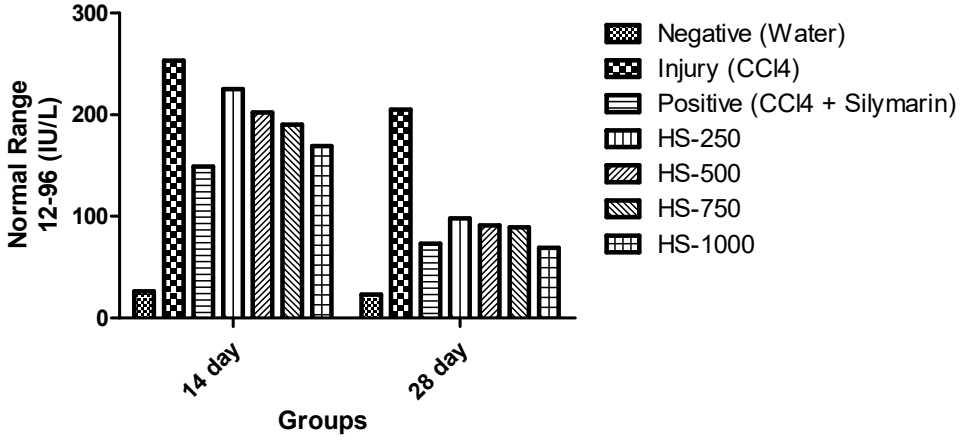

Fig. 8: Value of Alkaline phosphatase-ALP after 14 and 28 days with p value P<0.0106

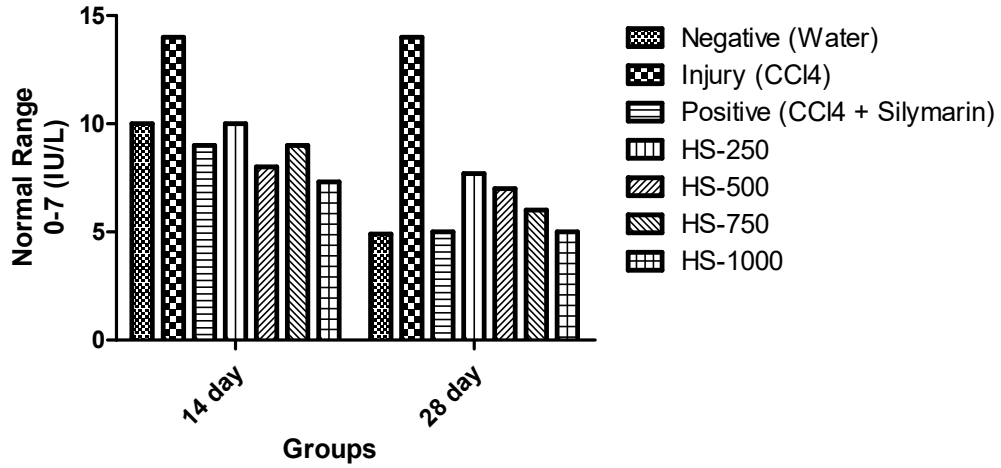

Fig. 9: Value of Gamma-glutamyl transferase-GGT after 14 and 28 days with p value P<0.0081

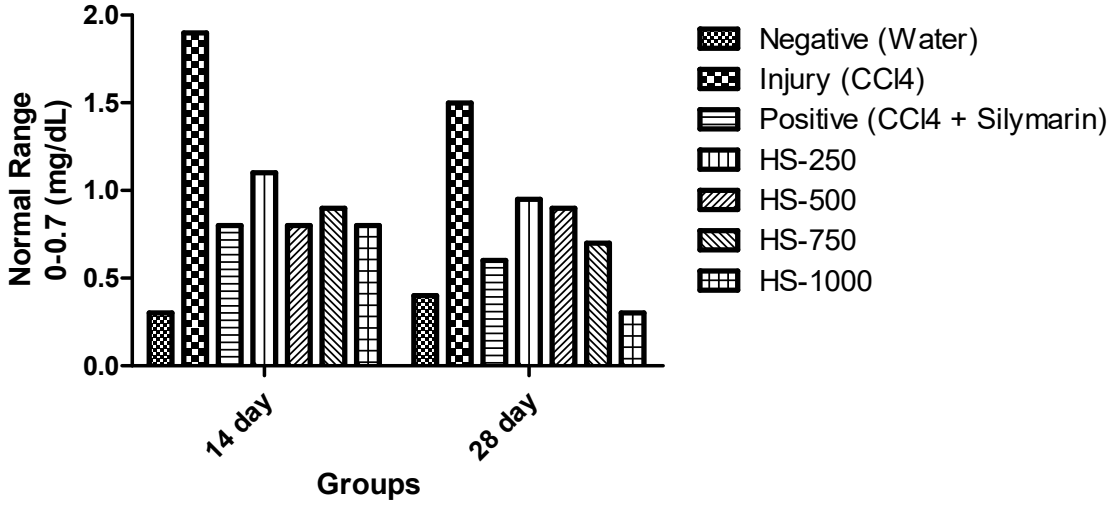

Fig. 10: Value of Total Bilirubin after 14 and 28 days with p value P<0.0021

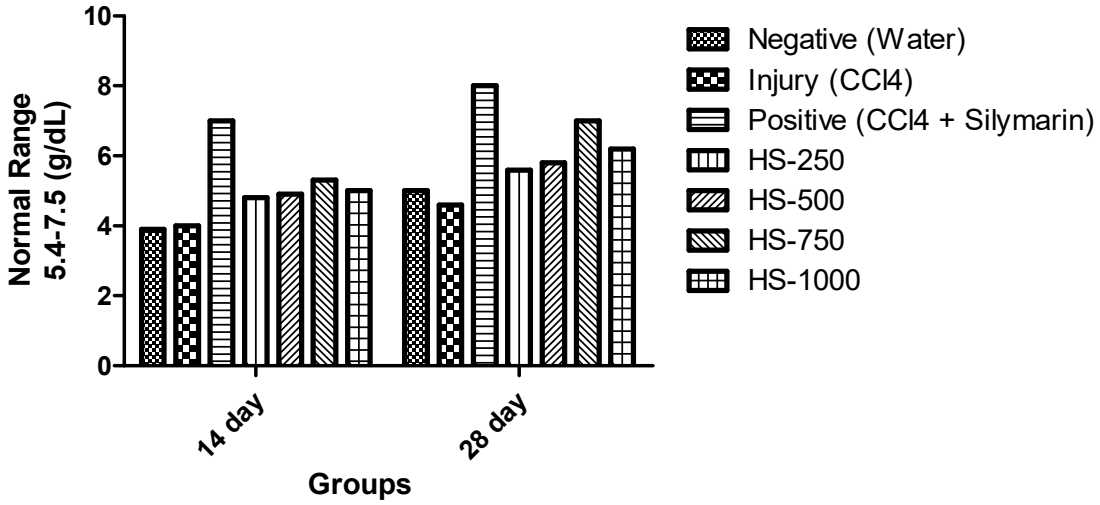

Fig. 11: Value of Total Protein after 14 and 28 days with p value P<0.0002

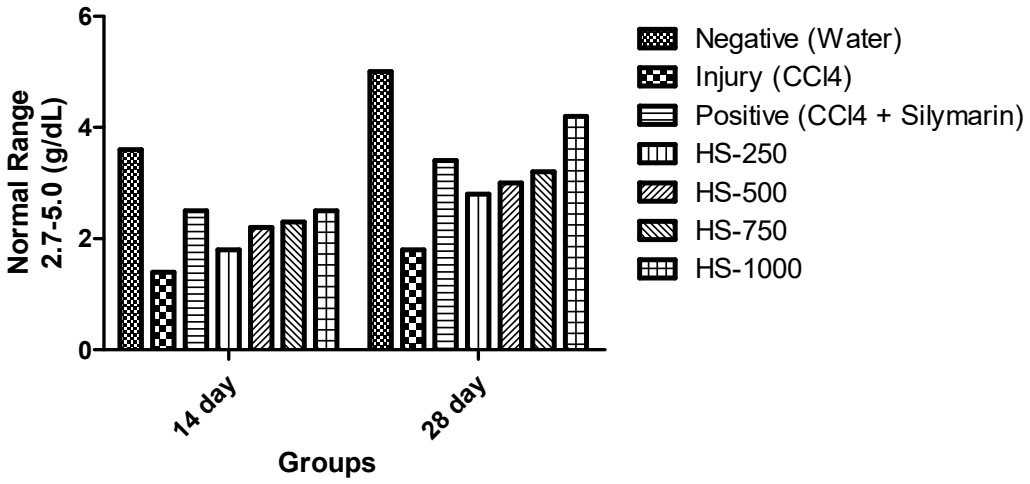

Fig. 12: Value of Albumin after 14 and 28 days with p value P<0.0018

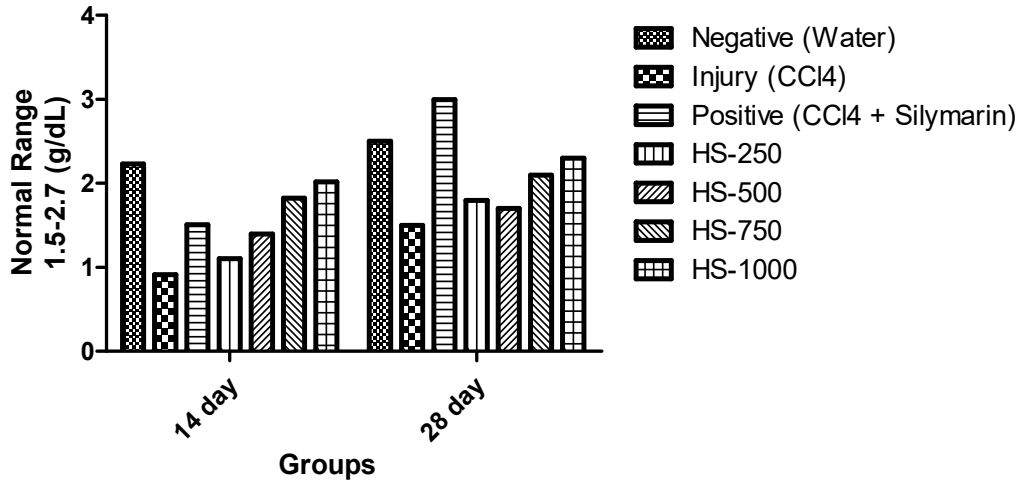

Fig. 13: Value of Globulin after 14 and 28 days with p value P<0.0469

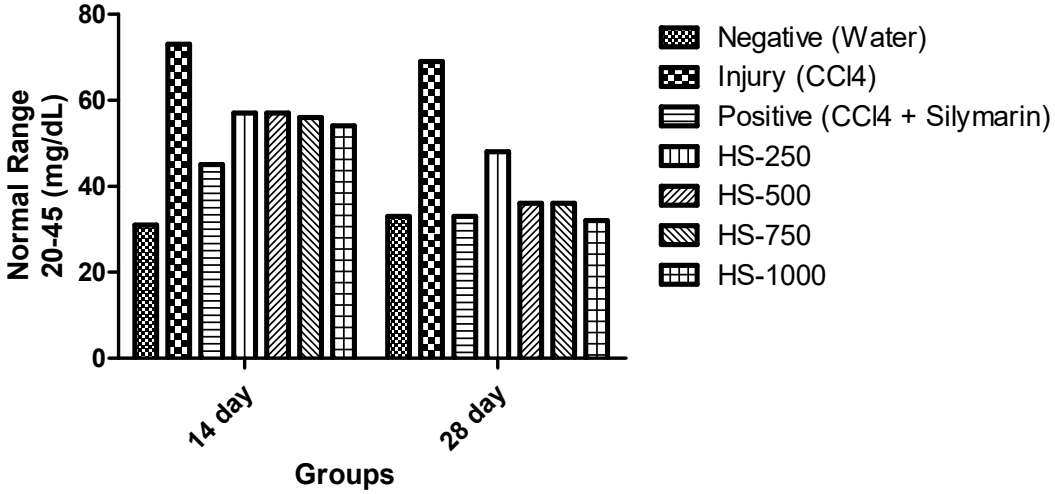

Fig. 14: Value of Urea after 14 and 28 days with p value P<0.0155

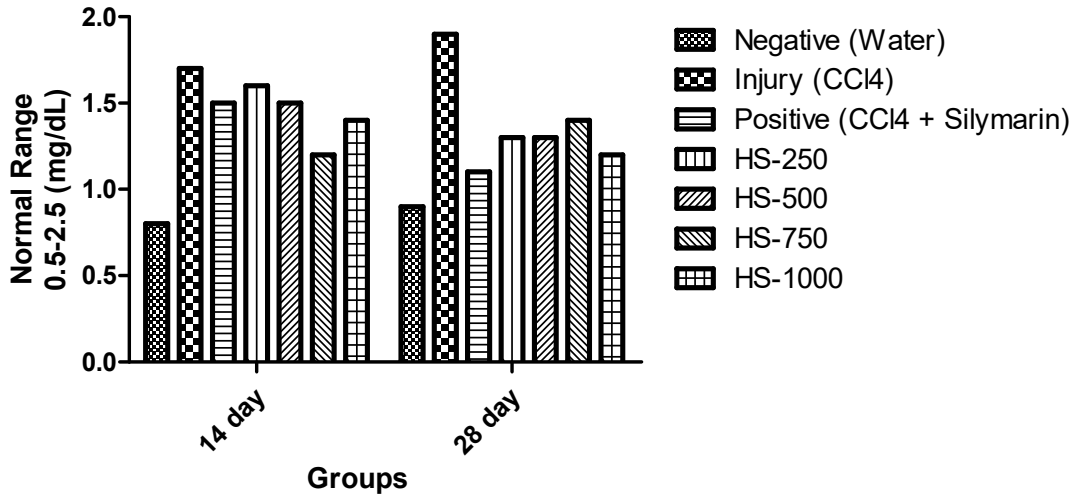

Fig. 15: Value of Creatinine after 14 and 28 days with p value P<0.0336

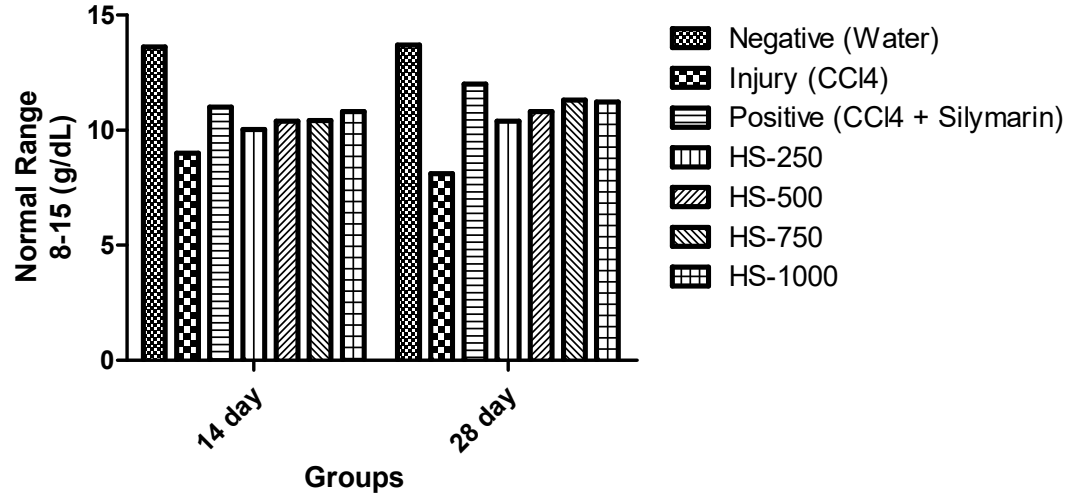

Fig. 16: Value of Hemoglobin after 14 and 28 days with p value P<0.0006

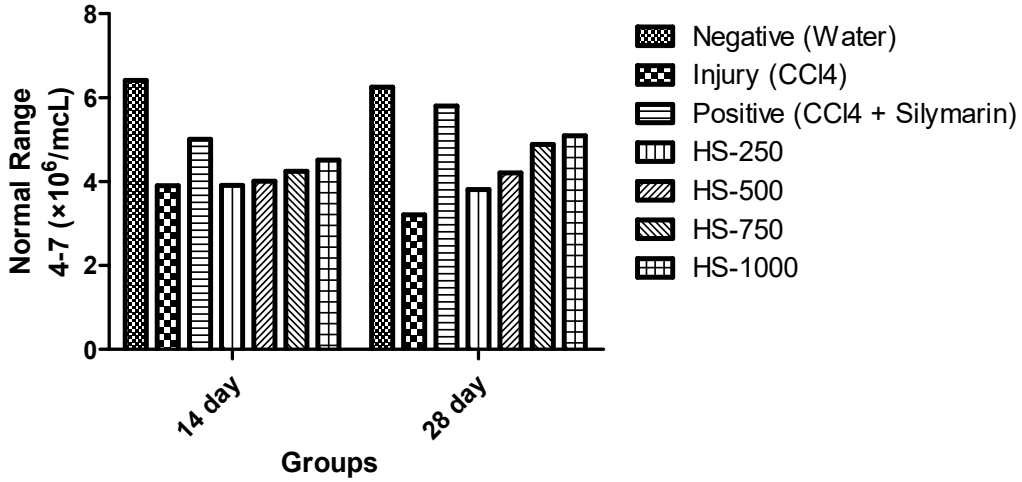

Fig. 17: Value of Red Blood Cells after 14 and 28 days with p value P<0.0032

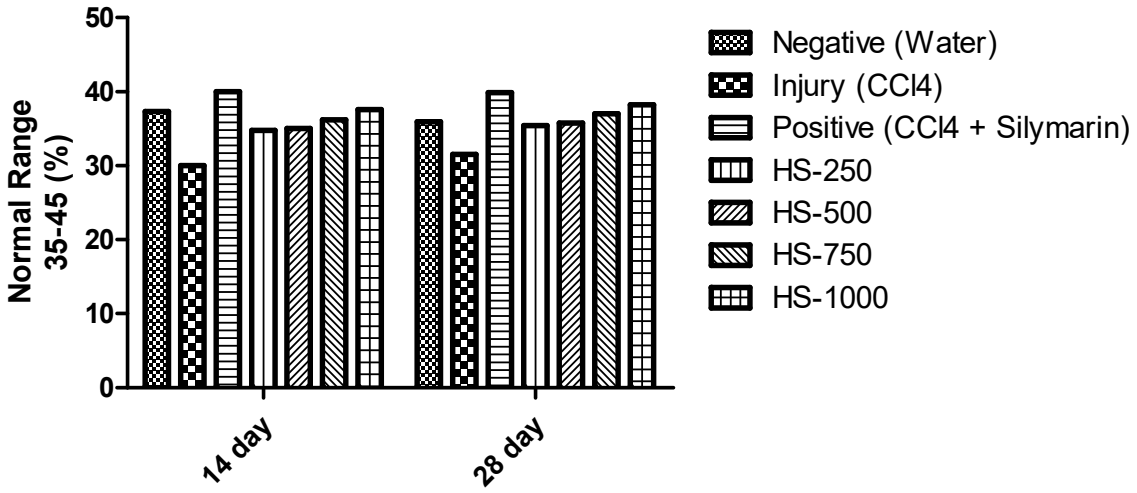

Fig. 18: Value of Hematocrit after 14 and 28 days with p value P<0.0002

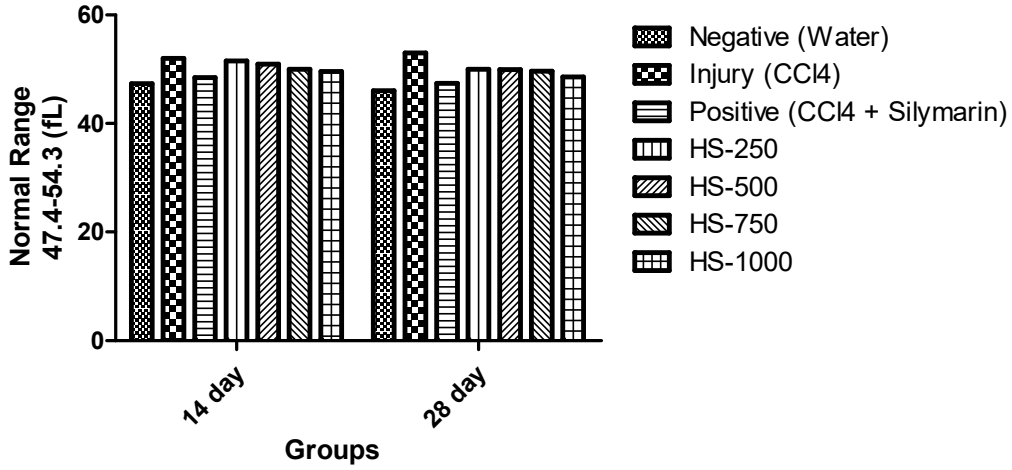

Fig. 19: Value of Mean corpuscular volume after 14 and 28 days with p value P<0.0009

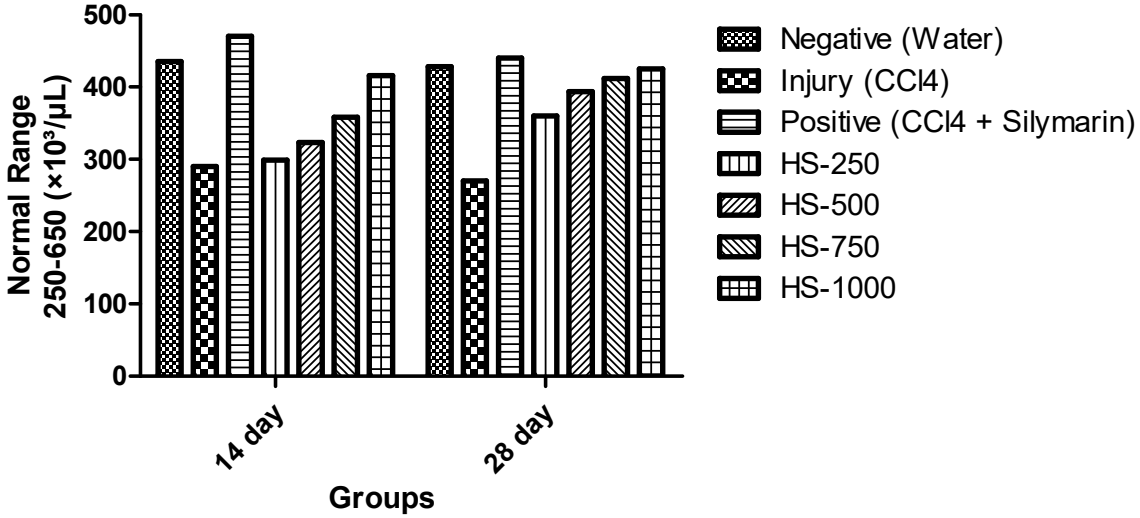

Fig. 20: Value of Platelets after 14 and 28 days with p value P<0.0090

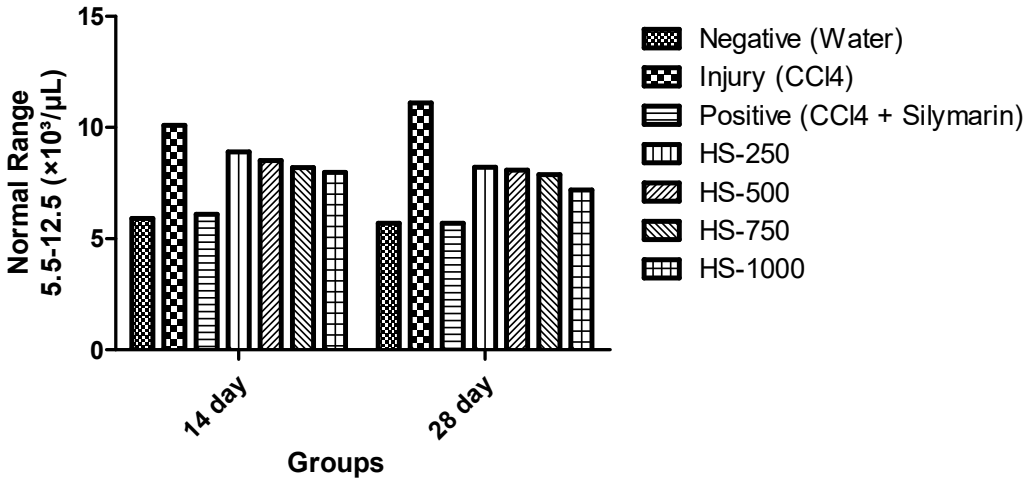

Fig. 21: Value of Leukocytes after 14 and 28 days with p value P<0.0003

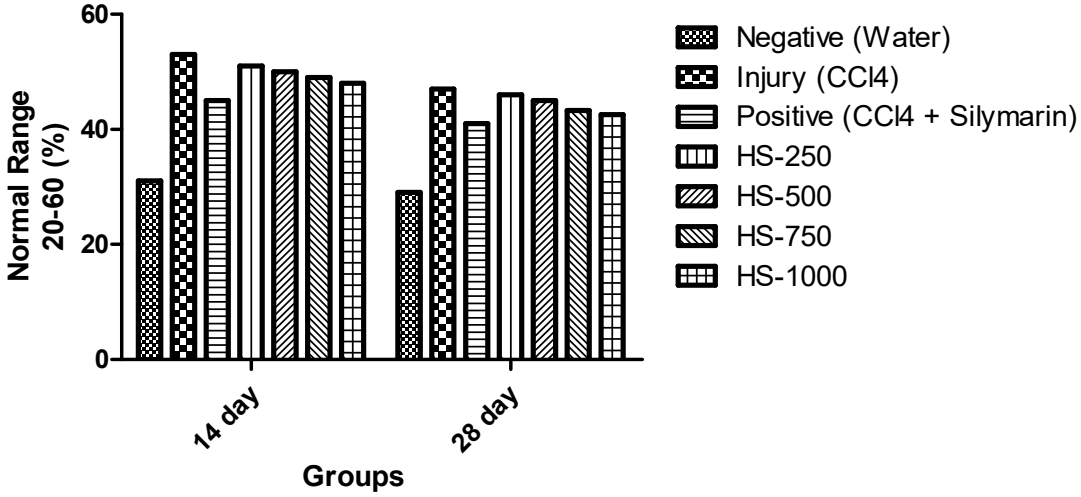

Fig. 22: Value of Neutrophils after 14 and 28 days with p value P<0.0001

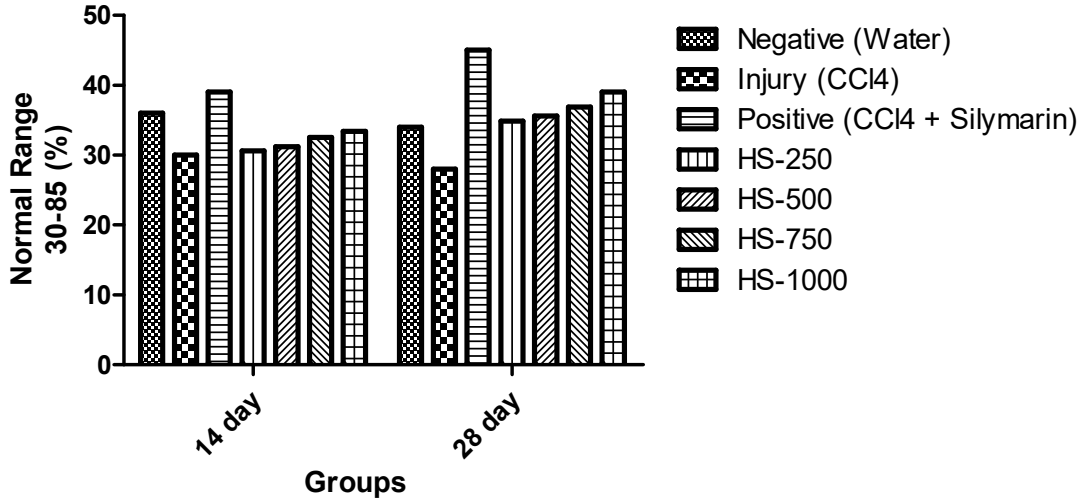

Fig. 23: Value of Lymphocytes after 14 and 28 days with p value P<0.0318

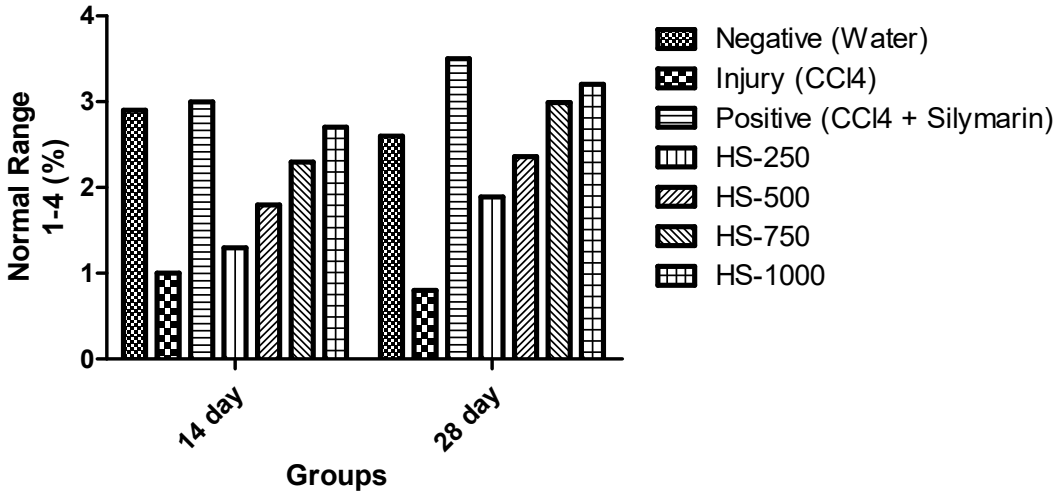

Fig. 24: Value of Monocytes after 14 and 28 days with p value P<0.0016

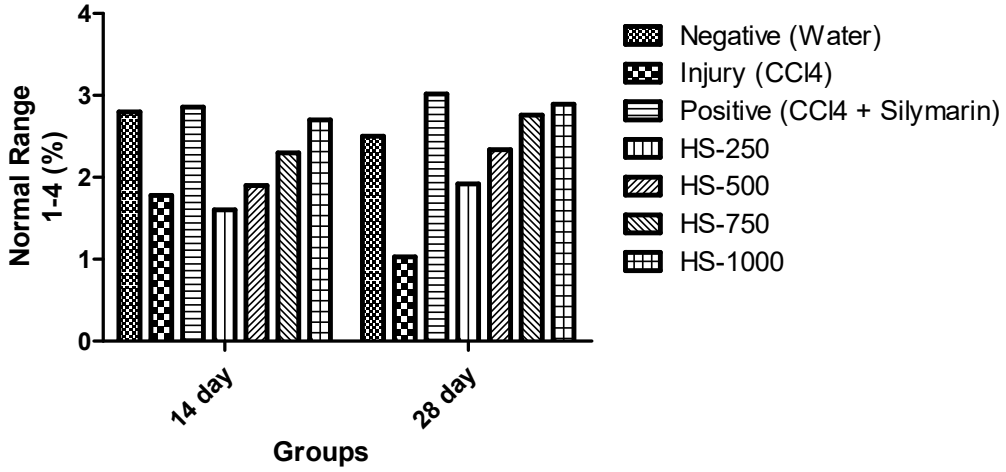

Fig. 25: Value of Eosinophil's after 14 and 28 days with p value P<0.0185

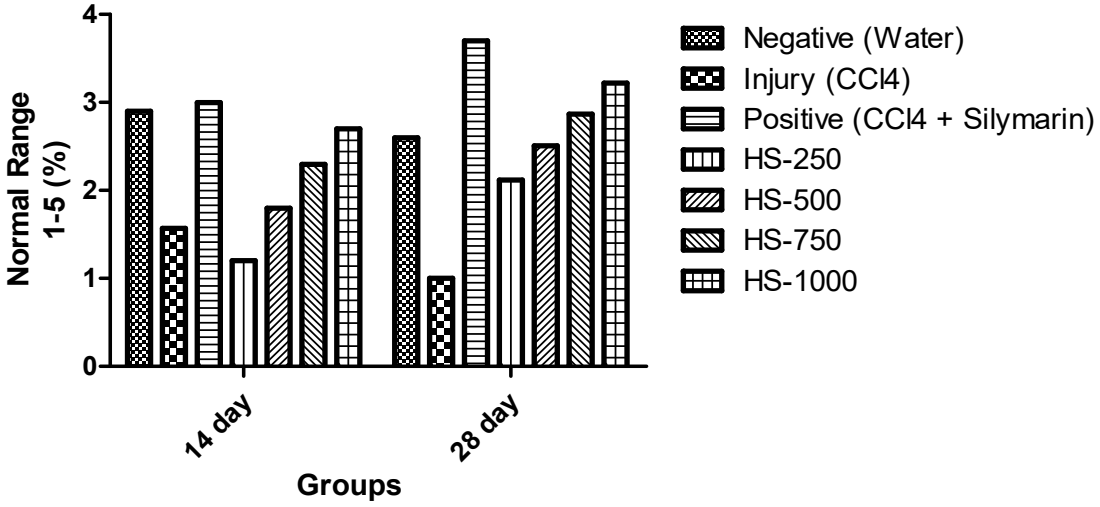

Fig. 26: Value of Basophils after 14 and 28 days with p value P<0.0178

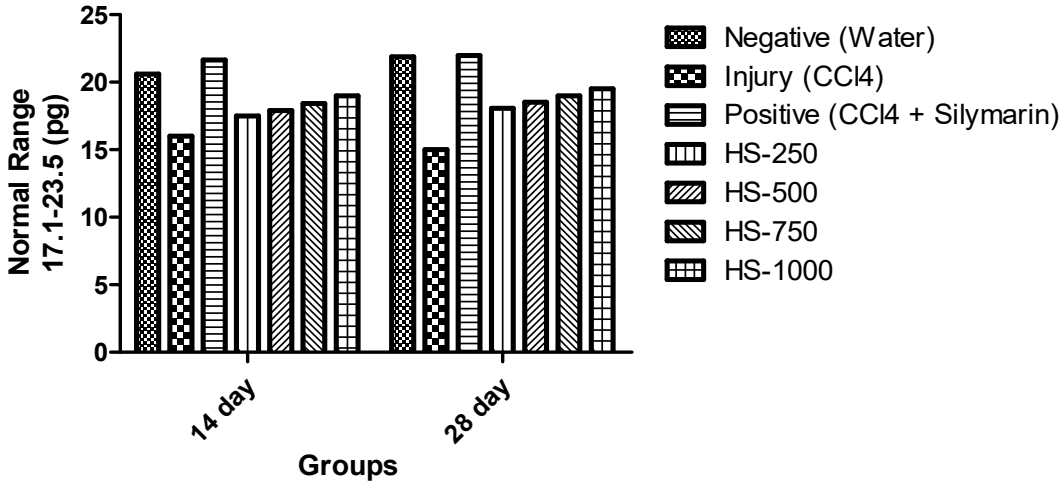

Fig. 27: Value of Mean corpuscular hemoglobin after 14 and 28 days with p value P<0.0002

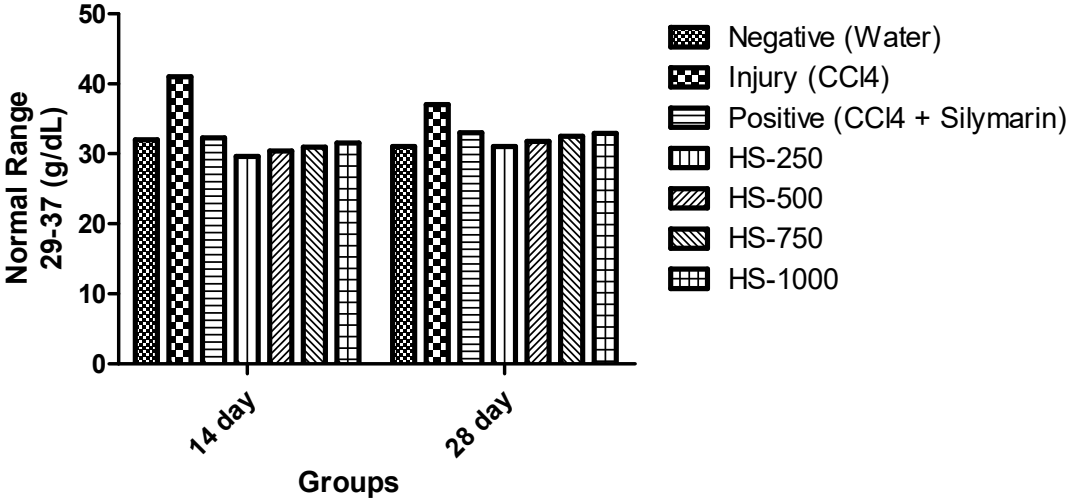

Fig. 28: Value of Mean corpuscular hemoglobin concentration after 14 and 28 days with p value P<0.0120

Pathological findings

After 28 days of the experiment, macroscopic inspection of the CCL_4-intoxicated rabbits' liver revealed hypertrophy and damage to the liver architecture. The liver in the group treated with *HS* extract had normal and intact architecture. The normal control group liver was also found to have a normal histological structure (A). The liver of those intoxicated with CCL_4 showed signs of focal hepatic necrosis (red star), cholangitis (blue star) and fibroplasia in the portal triad (black arrow) (B). Mild hydropic degeneration of hepatocytes (black arrow) (C). *HS*250mg extract reduced hepatic necrosis (red star) (D). Hepatic sinusoidal congestion was somewhat elevated in the group treated with *HS* 500mg extract (light green asterisk) (E). Rabbits treated with *HS*750mg extract showed a small increase in Kupffer cells (green arrow) and binucleation of hepatocytes (light green arrow) in their liver (F). Rabbits treated with *HS*1000mg extract showed fibroplasia in the portal triad (black arrow) while mild degeneration of hepatocytes (blue arrow) (G) (Fig 29).

Cytotoxicity assays

MTT assay

The MTT assay showed that *HS* extract significantly reduced MCF-7 cell viability in a dose-dependent manner at concentrations of 50-1000 µg/ml ($P < 0.0001$). The IC_{50} value was 633.33 µg/ml, indicating moderate cytotoxic activity (Fig. 30).

Crystal violet assay

The crystal violet assay showed that cell viability was significantly reduced in the *HS* extract-treated group compared to the control ($P < 0.05 \pm SEM$), indicating decreased viability after treatment (Fig. 31).

Trypan blue assay

The trypan blue assay showed increased cell death in the *HS*-treated group compared to the untreated control, indicating a higher percentage of non-viable cells after treatment (Fig. 32).

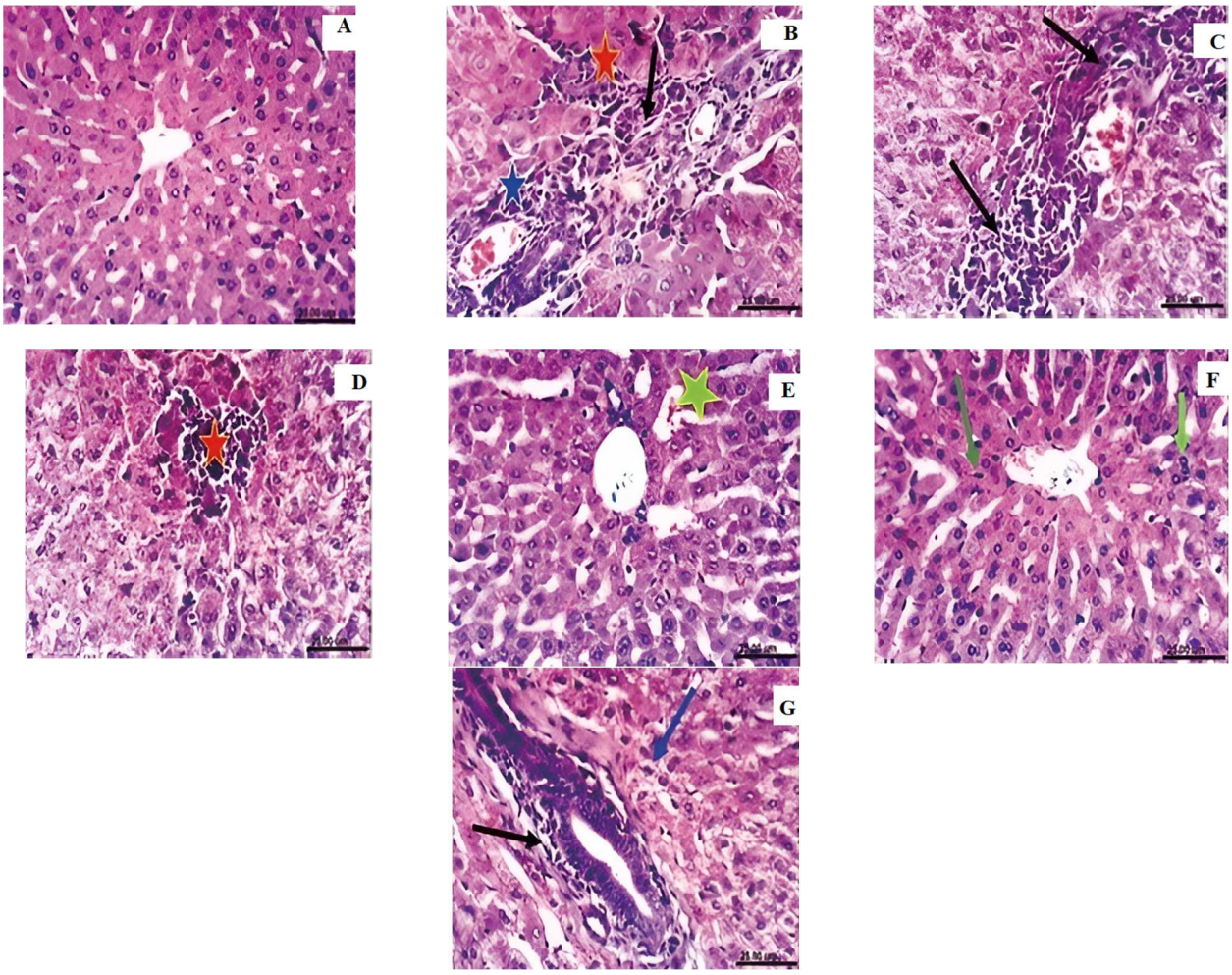

Fig. 29: Histopathological findings. (A) The normal control group histopathological photograph of the liver section, (B) CCL_4 intoxicated rabbit's group photograph, (C) CCL_4 + Silymarin treated rabbits photograph, (D) photograph of 250mg of *HS* on the rabbit's liver section, (E) photograph of 500mg of *HS* on the rabbit's liver section, (F) photograph of 750mg of *HS on* rabbits liver section and (G) photograph of 1000mg of *HS on* rabbits liver section.

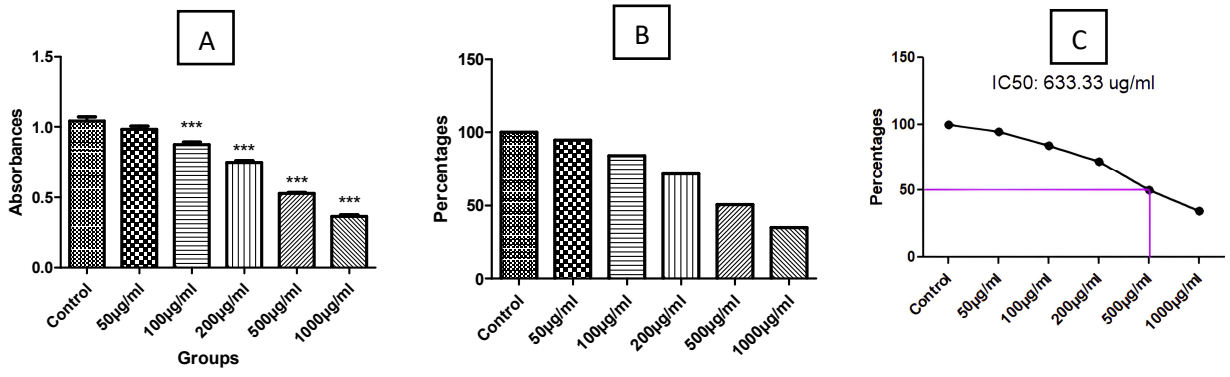

Fig. 30: Cytotoxic activity of *HS* through MTT assay. (A) Evaluation of absorbance, percentage and IC_{50} through MTT assay with p value P<0.0001. MTT assay was conducted to evaluate the cytotoxic potential of *HS* extract at concentrations of 50, 100, 200, 500, and 1000 µg/ml. (B) A dose-dependent reduction in MCF-7 cancer cell viability was observed, with statistically significant differences ($P \leq 0.05$) between treated and untreated groups. (C) The IC_{50} value was determined as 633.33 µg/ml, indicating moderate antiproliferative activity.

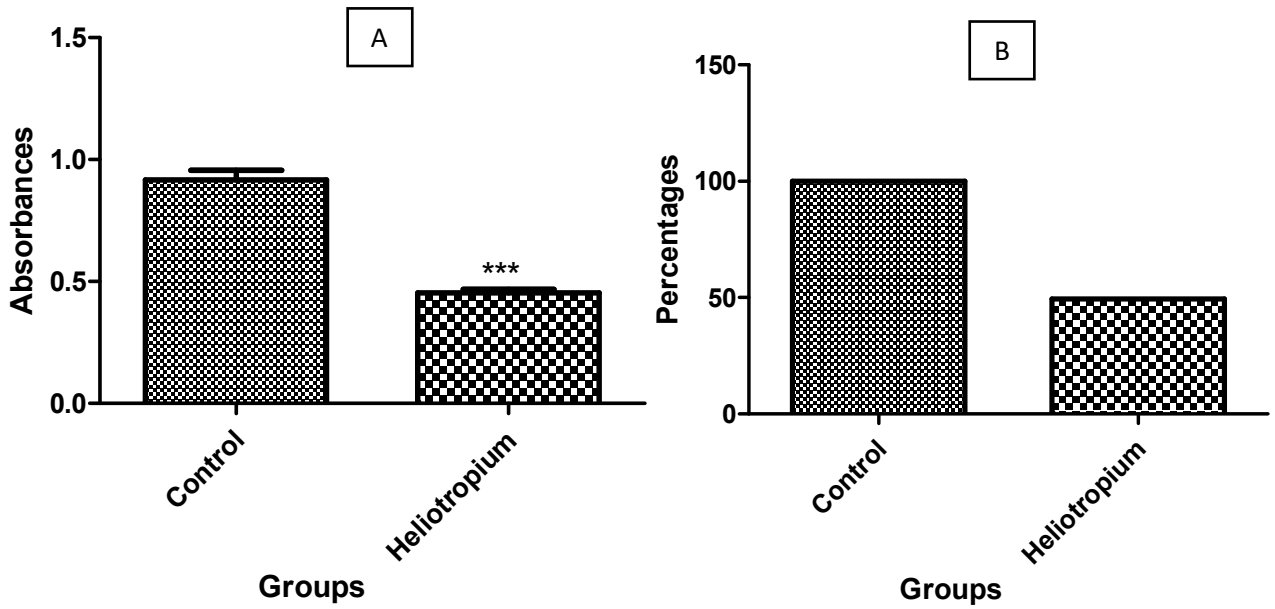

Fig. 31: Crystal Violet Assay for Studying Plant Extract Treatment. (A) The crystal violet assay results showed that cells from the treated and untreated groups were viable. The bar graph shows that, in comparison to the control, there is a marked decrease in viability following treatment with *HS* extract. $P < 0.05$, where *** indicates a significant difference between the untreated and treated groups, and values were calculated as mean ± SEM; (B) The results of the crystal violet assay showed that the treated and untreated groups' cells were viable. A percentage indicating that the treated group's viability was lower than that of the untreated group was provided.

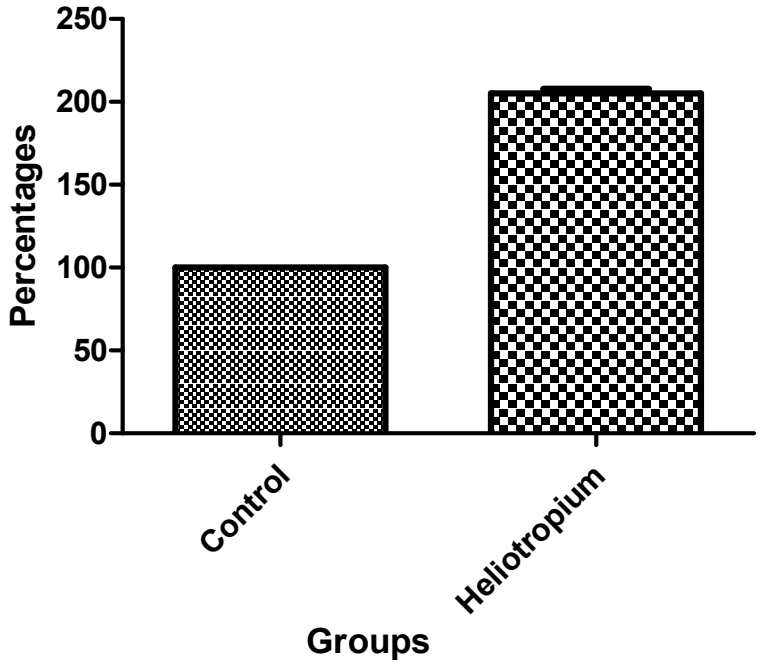

Fig. 32: Dead cells detection (Trypan Blue Assay) by plant extract treatment (Trypan blue assay percentages of *HS*). Trypan blue assay result indicated cell death between untreated (control) and treated (*Heliotropium strigosum*) groups. Percentages have been given which showed increased cell death in treated group compared to untreated group

DISCUSSION

The present study evaluated the antioxidant, hepatoprotective and cytotoxic potential of *HS* extract through *in-vitro* and *in-vivo* assays. The findings provide substantial evidence supporting the therapeutic relevance of this medicinal plant and are consistent with earlier reports on phytochemical-enriched botanicals in mitigating oxidative stress, liver injury and cancer progression. The extract demonstrated strong free radical scavenging activity, confirming its antioxidant potential. This activity is attributable to the presence of phenolic and flavonoid constituents, which have been widely reported to neutralize reactive oxygen species (ROS) and prevent oxidative damage. Similar results have been documented for other *Heliotropium* species and related medicinal plants, where flavonoid-rich extracts enhanced cellular antioxidant defense mechanisms (Basyal *et al*., 2021). The current findings align with these reports, suggesting that *HS* acts as a natural source of bioactive compounds with potent antioxidant activity. *In-vitro* assays showed a protective effect of *HS* against hepatotoxic insults, while *in-vivo* experiments demonstrated significant improvements in liver function biomarkers altered by CCl_4 toxicity. The extract normalized elevated transaminases and bilirubin levels while restoring protein synthesis, indicating preserved hepatocellular function. These results are in agreement with previous studies on plant-derived hepatoprotective agents, such as *Silybum marianum* and *Albizia lebbeck*, which showed restoration of biochemical and histopathological parameters following toxin-induced liver injury (Baig, 2011). The observed effects are consistent with earlier findings that phytoconstituents, particularly flavonoids and terpenoids, exert hepatoprotective effects through antioxidant and membrane-stabilizing properties.

In addition to hepatoprotection, the extract attenuated elevations in urea and creatinine, suggesting nephroprotective activity. These findings corroborate earlier reports that flavonoid-containing plants mitigate oxidative renal damage (Pingili *et al*., 2019). Similarly, the correction of hematological parameters in the present study is aligned with studies showing that phytochemical-rich extracts improve hematopoiesis and normalize leukocyte profiles under toxin-induced stress conditions (Christopher, 2022). The MTT assay demonstrated dose-dependent cytotoxicity of *HS* extract against cancer cells, with an IC_{50} of 633.33 µg/ml. Although moderate, this antiproliferative activity supports its potential role as a supplementary cytotoxic agent. Previous investigations on *Heliotropium indicum* and related taxa have reported comparable cytotoxic effects, attributed to alkaloids, flavonoids and phenolic compounds (Sarkar *et al*., 2021). Thus, the present findings are consistent with earlier studies indicating that *HS* species contain bioactive compounds that inhibit cancer cell growth. Collectively, the results confirm that *HS* exhibits broad-spectrum protective effects through antioxidant, hepatoprotective, nephroprotective, hematological and cytotoxic mechanisms. These findings align with earlier reports on medicinal plants rich in phenolics and flavonoids and underscore the therapeutic value of *HS* as a candidate for further pharmacological development.

CONCLUSION

HS demonstrated significant antioxidant, hepatoprotective and cytotoxic effects, supporting its traditional use and highlighting its potential as a source of natural therapeutics. These findings advance understanding of its pharmacological value and provide a foundation for future research aimed at isolating active compounds, clarifying mechanisms of action and evaluating clinical applications. Further studies, particularly clinical trials and pharmacokinetic investigations, will be essential to establish its efficacy, safety and potential role in developing novel treatments for oxidative stress–related disorders, liver diseases and cancer.

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Authors' contributions
S.R., FZ and MO: Conceptualization, collected samples of *HS and SR*, conducted research and wrote the original draft; G.S., MF, GM and MA: Supervised the research, reviewed and edited the manuscript, funding acquisition and final approval; F.H, BH and HMS: Supervision, reviewed and edited the original draft. All authors have read and agreed to the published version of the manuscript.

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Data availability statement
All data generated or analyzed during this study are included in this published article.

Ethical approval
The study protocols were approved by the animal ethical committee (DRC-IUB-362) of the Islamia University of Bahawalpur. This study was performed in adherence with the ARRIVE guidelines. See supplementary file for the ARRIVE checklist.

Conflicts of interest

The authors declare no conflict of interest.

Supplementary data

https://www.pjps.pk/uploads/2026/06/SUP1781939784.pdf

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