Ind. J. Pharm. Edu. Res., 2025; 59(1s):s229-s242.
https://www.ijper.org

Original Article

Ziziphus hamur Engl., a Rare Medicinal Plant Modulates Cytokine and Hematological Levels during Sub-Acute Toxicity Studies in Wistar Rats

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ABSTRACT

Introduction: *Ziziphus hamur* Engl. (Vernacular name: *Xamur gob*) is a thorny shrub native to Ethiopia, Kenya and Somalia. The plant is extensively used in the traditional system of the Somali region in the treatment of jaundice and mental illness. Lack of prior experimental studies and wide usage in traditional medicine demands the toxicological evaluation of *Ziziphus hamur* Engl. The aim of this study was to evaluate the safety of *Ziziphus hamur* root bark by sub-acute toxicity study in male albino wistar rats. Investigation of cytokine levels were done to check for cytokine modulatory role of *Ziziphus hamur*, thereby providing insights on the toxicity profile and for the therapeutic property of *Ziziphus hamur*. **Materials and Methods:** The aqueous extract of *Z. hamur* root bark was used for this study. The sub-acute toxicity study was carried out as per OECD guidelines. 24 albino Wistar rats (only males) were grouped and subjected to sub-acute toxicity study with orally administered extract doses of 100, 200 and 400 mg/kg body weight for 28 days. The biochemical parameters for hepatic and renal injury and hematological parameters were assessed. The evaluation of cytokines IL-6, TNF-α, INF-γ, IL-2 and IL-10 were done by ELISA to document the toxic responses *in vivo*. The histological studies of the liver and renal tissues were performed to check tissue damage caused. **Results:** We noticed no mortality, no behavioural changes and physical changes in sub-acute toxicity experimental rats proving the preliminary evidence about the safety of the *Z. hamur* root bark extract. No noticable changes in water and food intake among the experimental rats proved the non-toxic nature of *Z. hamur* root bark extract. Body weight gain in the test group and control group of experimental rats were similar without significant difference. The biochemical analysis for liver toxicity, renal toxicity and lipid profile parameters also proved the evidence for non-toxic nature and healthier effect of *Z. hamur* by significant decrease in marker enzyme activities, TGL, LDL, VLDL and significantly higher level of HDL. The hematological parameters assessment showed significant beneficial effects in the treatment of the extract at the dose of 400 mg/kg body weight. The levels of IL-6 (7.68% decrease), TNF-α (7.58% decrease), IL-2 (3.46% increase) and IFN-γ (7.79% decrease) were altered without statistical significance and IL-10 was significantly increased (50.32%; $p<0.001$) compared to the control suggesting that *Ziziphus hamur* is non-toxic and safe for the use of various pathological treatments. **Conclusion:** Results of this study concluded that *Z. hamur* has no toxicity, revealed by biochemical, haematological and histopathological parameters, up to a dose of 400 mg/kg body weight in experimental animals. Changes in the cytokine levels in sub-acute toxicity studies supported the cytokine modulatory effect of *Ziziphus hamur* that could benefit towards its therapeutic potential.

Keywords: Traditional medicine, Biochemical, Histological, Hematology, Cytokines.

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Received: 28-05-2024;
Revised: 01-07-2024;
Accepted: 22-10-2024.

DOI: 10.5530/ijper.20257420

Publishing Partner : Manuscript Technomedia.[www.mstechnomedia.com]

INTRODUCTION

Ethiopia is gifted with knowledge of traditional medicinal practices. Like other Asian countries, there is a widespread medicinal plant species in Ethiopia.[1-4] Their protective activities on malaria, jaundice, mental illness and efficacy against many other pathological conditions were reported.[2,3] The practice of herbal medicinal treatment has seen a great upsurge globally due to cultural acceptability, availability and cost and it is assumed to be safer than allopathic medicines. After the pandemic COVID-19 the use of traditional herbal medicine has spiked.[5] In recent times, there has been a growing query about their safety. This is due to studies about the adverse effects of traditional medicine.[6] Plants that are well known for their medicinal properties in traditional medicine are also known for their toxic or poisonous nature. This includes *Atropa belladonna, Datura* spp. and *Digitalis* spp.[7] Many plants used in traditional medicine or used as food have demonstrated some toxicity (mutagenic and carcinogenic) effects.[8] These reports emphasize the need for research on the toxicity of traditional medicine. Toxicological studies of Ethiopian traditional plants have been conducted for many decades to prove the safety of traditional medicine and to determine the non-toxic dosage.[9] Medicinal properties and toxicity of plants are due to secondary metabolites, the phytochemicals produced by the plants. The phytochemical analysis of plants is widely studied to aid the discovery of single chemical components from plant extract and this needs a lot of research inputs.[1]

Ziziphus hamur Engl. (Vernacular name: *Xamur gob*) is a thorny shrub native to Ethiopia, Kenya and Somalia. It is widely used in the traditional system of the Somali region in the treatment of jaundice and mental illness. There is no scientific data reported to date documenting toxicology studies of *Ziziphus hamur*.[4] This is the first study to explore the safeness of *Ziziphus hamur.* It is mandatory to evaluate the safety aspects of the *Ziziphus hamur* Engl. because it is orally consumed for 7 to 40 days at a dose of three cups a day (depending on the severity of illness) in the Somali region for jaundice and mental illness. This work is designed to assess the safety dosage of *Ziziphus hamur* and bridge the gap between molecular mechanisms and medical efficacy. The sub-acute toxicity tests were done to check the toxicological effects of *Ziziphus hamur.* The toxicity tests were conducted as per OCED guidelines (OECD 423 guidelines for sub-acute toxicity studies, 2001).[10] The toxicity studies provide precise information about the toxicity dosage and are used to prove the safety of plant extract.[9] The toxicity evaluation of *Ziziphus hamur* was done by the analysis of biochemical, histological and hematological parameters of rats subjected to acute studies. In addition to the safety assessment of *Ziziphus hamur* its therapeutic effect was investigated by evaluating the cytokine levels in acute toxicity experimental studies. Cytokines play a major role in the prognosis, diagnosis and treatment of various pathological conditions.[11-13] Plant extracts were documented for the treatment of various pathological conditions by modulation of cytokine levels.[14,15] An increase in anti-inflammatory cytokine and decrease in pro-inflammatory cytokine also ensures the safeness of the plant extract.[16,17]

MATERIALS AND METHODS

Laboratory

The present experimental studies were conducted in Addis Ababa University, Addis Ababa and Haramaya University, Haramaya situated in Ethiopia. The study period was from February 2020 to January 2022.

Chemicals and Kits

All chemicals used in this study were of analytical grade. The chemicals used were phenol, sodium nitroprusside, sodium hydroxide, sodium hypochlorite, phosphotungstic acid, paraffin, formalin, heamtoxylin [Sigma Aldrich USA (H3136)] and eosin [Sigma Aldrich USA (45260)]. The kits used were total Cholesterol Assay Kit [Cell Biolabs, Inc. USA (Catalog Number STA-384)], triglycerides assay kit [Cayman chemicals, USA (item no 10010303)], ALP colour endpoint kit [ID labs Canada (SUP6003-C)], AST assay kit [Cayman chemicals, USA (Item no: 701640)], ALT assay kit [Cayman chemicals, USA (item no 700260)]. The ELISA kits used were as follows: Quantikine Rat IL-6 Immunoassay (RB006. R&D systems, USA), Quantikine Rat TNF-alpha Immunoassay (RTA00, R&D systems, USA), Quantikine Rat IL-10 Immunoassay (R1000, R&D systems, USA), Quantikine Rat IL-2 Immunoassay (R2000, R&D systems, USA), Quantikine Rat IFN-gamma Immunoassay (RIF00. R&D systems, USA).

Collection of Plant material

The root bark of *Ziziphus hamur* Engl was collected from the Somali region in Shabelay woreda, Fafan Zone, Ethiopia in February 2020. The plant was identified and authenticated by a plant biologist.

Preparation of Plant Extract

Ziziphus hamur root barks were washed with clean water and shade-dried for 7 days. After drying, they were ground into fine powder using a mechanical homogenizer. The *Ziziphus hamur* root bark powder was kept in a clean, dry container and stored at room temperature. *Ziziphus hamur* root bark powder was soaked in distilled water (240 g in 1.5 L) for 24 hr. It was filtered and concentrated to dryness in a water bath (50°C) for 3 days. The dried extract was dispensed into an airtight sterile container and stored at 4°C in the refrigerator. This was used for our further research studies.

Experimental animals

A formal approval letter was obtained from Jigjiga university Research ethical review committee (RERC/018/2020 dated 06/07/2020). All animal studies were conducted according to the standard guidelines of Jigjiga University for the care and use of laboratory animals. Ethical approval for our study was given by the Ethical Review Committee of the College of Medicine and Health Science Jigjiga University, Ethiopia.

The experimental animals, twenty-four weaned male albino rats of 6 weeks old of the same stock were procured from the Ethiopian Public Health Institute, Traditional and Modern Medicine Drug Research Directorate, Addis Ababa. The animals were housed in a wooden cage and were allowed to drink water freely *ad libitum* till the end of the experiment in the laboratory. The animals were maintained in normal 12 hr light and dark cycles under tropical weather conditions. The experimental rats were examined and allowed to accustom for 2 weeks before commencement of experiments.

Experimental design for sub-acute toxicity test of *Z. hamur* root bark

The sub-acute toxicity study was conducted as per the OECD guidelines 423.[10] The experimental rats were randomly divided into four groups of 6 animals per group, one group served as control and the other three were administered with a graded dosage of *Z. hamur* root bark extract solution as follows:

Group I: These were given 0.25 mL of water.

Group II: These were given with100 mg *Z. hamur* root bark extract per kg body weight.

Group III: These were given with 200 mg *Z. hamur* root bark extract per kg body weight.

Group IV: These were given with 400 mg *Z. hamur* root bark extract per kg body weight.

All the rats were allowed free access to food and water and observed for a period of 28 days for signs of sub-acute toxicity. The animals were observed for behavioral changes and physical changes throughout the experimental period.

Sample collection for sub-acute toxicity test of *Z. hamur* root bark

After treatment with plant extract, the experimental rats of all 4 groups were fasted overnight, anesthetized with chloroform and sacrificed. Blood samples were collected by cardiac puncture. Blood samples from each experimental animal were collected into dry sample bottles for clinical chemistry analysis and EDTA (Ethylene Diamine Tetra Acetic acid) container for hematological examination. The sample bottles with the whole blood were allowed to stand for 15 min and centrifuged at 5,000 rpm for 20 min for serum separation and used for measurement of biochemical parameters. The liver and kidney tissues were kept in 10% formalin saline and used for histopathological analysis.

Biochemical assay for renal toxicity

Biochemical investigations for renal toxicity done by urea,[18] creatinine[19] quantification. In Chaney and Marbach method, urea is digested with urease. Then followed by a reaction with combined phenol and sodium nitroprusside; combined sodium hydroxide and sodium hypochlorite solutions. The resulted coloured compound was measured in a spectrophotometer at 578 nm. The creatinine in the sample reacted with picric acid in an alkaline medium resulting in the production of orange coloured product, which is measured spectrophotometrically at 520 nm. Using a standard curve, the levels of urea and creatinine were determined.

Biochemical assay for liver toxicity

Biochemical investigations of Alanine aminotransferase (ALT), Aspartate aminotransferase (AST),[20] and Alkaline Phosphatase (ALP)[21] were done in collected samples. AST activity was calculated from the rate of NADH oxidation which was measured by the rate of decrease in absorbance at 340 nm. ALP activity was determined by hydrolysis of the substrate thymolphthalein monophosphate released thymolphthalein, which was measured spectrophotometrically at 590 nm. The values were calculated as per the formula given in the respective methods.

Lipid profile assessment

Serum Total Cholesterol (TC),[22] TGL,[23] HDL-C,[24] LDL-C[25] and VLDL-C[25] were done. The principle of cholesterol estimation was the free cholesterol generated by cholesterol oxidase was converted to cholest-4-en-3-one and hydrogen peroxide, the later formed red-coloured chinoimine derivative on reaction with phenol and 4-amino antipyrine in the presence of peroxidase. The resulting product was read in a spectrophotometer at an absorbance of 500 nm. The estimation of triglycerides was based on the principle that serum triglycerides release glycerol upon hydrolysis by lipase. The glycerol generated hydrogen peroxide in a reaction catalysed by glycerol kinase and L-alpha-glycerol-phosphate oxidase in a system. The hydrogen peroxide produced chromogen in the presence of horseradish peroxidase with 3,5-dichloro-2-hydroxybenzenesulfonic acid/4-aminophenazone. The chromogen is read at 510nm spectrophotometrically. The determination of HDL-C was by precipitating all lipoproteins except HDL using phosphotungstic acid. The HDL-C estimation was the same as that of total cholesterol. The LDL-C and VLDL-C levels were calculated as per the formula of Friedewald *et al.*[25]

Hematological parameters

Serum hematological parameters-White Blood Cell count (WBC), Red Blood Cells (RBC), Hemoglobin level (Hb), red

blood cell indices (MCV, MCH and MCHC), Platelets (PLT) were analyzed using an automated hematology cell counter.

Histopathological Studies

The liver and kidney tissues were removed and fixed in 10% formalin saline in labelled sample bottles. The tissues were processed with graded alcohol and embedded in paraffin wax. Tissue sections of 5 µm thickness were cut and stained with hematoxylin and eosin.[26] Processed sections were examined under a microscope by an experienced pathologist.

Quantification of IL-6 in serum

Quantikine Rat IL-6 Immunoassay (RB006 R&D systems, USA), was used for quantification of IL-6. It is based on quantitative sandwich enzyme immunoassay technique. To the IL-6 monoclonal antibody coated plate was provided samples (standards, control and tests) were added. The plates were washed with provided buffer, then enzyme-linked polyclonal antibody specific for rat IL-6 was added. The plates were washed and the provided substrate solution was added, followed by this stop solution was added. The resulting product was read in a plate reader at 450 nm. The concentration of IL-6 was calculated as per the instruction provided along with the kit.

Quantification of TNF-α in serum

Quantikine Rat TNF-alpha Immunoassay (RTA00-1, R&D systems, USA) was used for quantification of I TNF-α. It is based on quantitative sandwich enzyme immunoassay technique. To the TNF-α monoclonal antibody coated plate was provided samples (standards, control and tests) were added. The plates were washed with provided buffer, then enzyme-linked polyclonal antibody specific for rat TNF-α was added. The plates were washed and the provided substrate solution was added, followed by this stop solution was added. The resulting product was read in a plate reader at 450 nm. The concentration of TNF-α was calculated as per the instruction provided along with the kit.

Quantification of IL-10 in serum

Quantikine Rat IL-10 Immunoassay (R1000, R&D systems, USA) was used for quantification of IL-10. It is based on quantitative sandwich enzyme immunoassay technique. To the IL-10 monoclonal antibody coated plate was provided samples (standards, control and tests) were added. The plates were washed with provided buffer, then enzyme-linked polyclonal antibody specific for rat IL-10 was added. The plates were washed and the provided substrate solution was added, followed by this stop solution was added. The resulting product was read in a plate reader at 450 nm. The concentration of IL-10 was calculated as per the instruction provided along with the kit.

Quantification of IL-2 in serum

Quantikine Rat IL-2 Immunoassay (R2000, R&D systems, USA) was used for quantification of IL-2. It is based on quantitative sandwich enzyme immunoassay technique. To the IL-2 monoclonal antibody coated plate was provided samples (standards, control and tests) were added. The plates were washed with provided buffer, then enzyme-linked polyclonal antibody specific for rat IL-2 was added. The plates were washed and the provided substrate solution was added, followed by this stop solution was added. The resulting product was read in a plate reader at 450 nm. The concentration of IL-2 was calculated as per the instruction provided along with the kit.

Quantification of IFN-γ in serum

Quantikine Rat IFN-gamma Immunoassay (RIF00. R&D systems, USA), was used for quantification of IFN-γ. It is based on quantitative sandwich enzyme immunoassay technique. To the IFN-γ polyclonal antibody coated plate was provided samples (standards, control and tests) were added. The plates were washed with provided buffer, then enzyme-linked polyclonal antibody specific for rat IFN-γ was added. The plates were washed and the provided substrate solution was added, followed by this stop solution was added. The resulting product was read in a plate reader at 450 nm. The concentration of IFN-γ was calculated as per the instruction provided along with the kit.

Statistical Analysis

Statistical analysis was performed using standard method to know the variation between control and experimental groups. (Values are expressed as mean±standard deviation; n=6).

RESULTS

Effect of *Z. hamur* root bark extract on body weight

The oral administration of the aqueous extract to rats up to a maximum dose (400 mg/kg) under sub-acute toxicity test conditions had no negative effect on the body weight of the rats. There was no significant difference in the body weight of rats among the control and study groups. We also observed no apparent behavioural changes and adverse effects like diarrhoea, immobility, excitement, refusal of food and tremor in the plant-extract-treated animal groups. The body weight was measured and results were analyzed statistically and the variation was found to be insignificant (Table 1).

Effect of *Z. hamur* root bark extract on food and water consumption

Effects of *Z. hamur* root bark extract on food and water consumption were not deleterious. Food as well as water intake were monitored in all the groups daily and the results were analyzed. There were no significant changes in the amount of

food or water intake between the groups and the variations were found to be insignificant (data not shown).

Effect of *Z. hamur* root bark extract on hepatic and renal biochemical parameters

The effect of extract of *Z. hamur* root bark on renal biochemical parameters-urea, creatinine, hepatic biochemical parameters Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT) and Alkaline Phosphatase (ALP) are depicted in Table 2. Our findings revealed that hepatic biochemical parameters in the *Z. hamur* root bark extract-treated group of rats were similar to those of the control group (Table 2). There was a negligible decrease in AST and ALP levels with an increase in the dosage of plant extract which was statistically insignificant. (p<0.05) In our study, no adverse impact on urea and creatinine levels was observed in the plant extract-treated group and levels were similar to that of the control group of animals (Table 3). There was a slight increase in the level of urea but it decreased with an increase in dosage and the 400 mg/kg dosage was similar to that of urea level in the control group. The results were represented as mean±SD. The difference in levels of renal parameters among the experimental group of rats was statistically insignificant. (p<0.05).

Effect of *Z. hamur* root bark extract on lipid profile

Sub-acute toxicity studies revealed that *Z. hamur* root bark had no significant effect at a dosage of 100, 200 and 400 mg *Z. hamur* root bark per kg body weight. The levels of lipid profile parameters were almost similar to those of the control group rats (Table 4). The serum lipid profile analysis showed that there was no change significant change in total cholesterol and LDL. There was a slight increase in HDL level compared to that of the control in *Z. hamur* root bark-treated group of animals with the increase in plant extract dosage. In *Z. hamur* root bark-treated group of animals, there was a decrease in TGL and a negligible decrease in VLDL. The results were represented as mean±SD. The statistical analysis showed that variations in levels of lipid profile parameters among the group of animals were statistically insignificant. (p<0.05).

Effect of *Z. hamur* on hematological parameters

The effects of acute toxicity of aqueous extract of *Z. hamur* root bark on the hematological parameters of all 4 groups of rats were reported in Table 5. The hematological parameters such as White Blood Cell count (WBC), Red Blood Cells (RBC), Haemoglobin (Hb), red blood cell indices (mean corpuscular volume-MCV, Mean Corpuscular Hemoglobin-MCH and Mean Corpuscular Haemoglobin Concentration-MCHC) and Platelets (PLT) in

Table 1: Effect of *Z. hamur* root bark on body weight in Wistar rats.

Groups	Initial weight (g)	Final weight (g)	Percentage change (g)
Group I (Control)	214.68±25.22	243.46±32.56	13.41%
Group II (100 mg/kg)*	211.64±32.4	242.64±30.63	14.65%
Group III (200 mg/kg)*	213.98±28.82	246.94±26.86	15.4%
Group IV (400 mg/kg)*	215.42±30.61	249.17±31.94	15.67%

Number of animals per group=6 (n=6); *dosage of plant extract; Values are expressed as mean±SD.

Table 2: Effect of *Z. hamur* root bark on hepatic markers in Wistar rats.

Groups	ALT (U/L)	AST (U/L)	ALP (U/L)
Group I (Control)	139.00±1.79	61.67±1.03	187.83±1.17
Group II (100 mg/kg)*	129.17±0.98$^{\$}$	65.83±1.47$^{\$}$	180.00±1.41$^{\$}$
Group III (200 mg/kg)*	125.67±1.86$^{\$}$	69.33±2.16$^{\$}$	180.33±1.37$^{\$}$
Group IV (400 mg/kg)*	130.50±1.05$^{\$}$	61.67±1.63	180.17±0.75$^{\$}$

Number of animals per group=6 (n=6); *dosage of plant extract; Values are expressed as mean±SD. "#" p<0.05; "@" p<0.01; "$" p<0.001.

Table 3: Effect of *Z. hamur* root bark on renal functions in Wistar rats.

Groups	Urea mg/dL	Creatinine mg/dL
Group I (Control)	29.67±1.03	0.78±0.12
Group II (100 mg/kg)*	33.00±1.26$^{\$}$	0.85±0.05
Group III (200 mg/kg)*	30.50±1.87	0.82±0.08
Group IV (400 mg/kg)*	28.50±1.38$^{\#}$	0.82±0.08

Number of animals per group=6 (n=6); *dosage of plant extract; Values are expressed as mean±SD. "#" p<0.05; "$" p<0.001.

Table 4: Effect of *Z. hamur* root bark on lipid profile in Wistar rats.

Groups	TC mg/dL	TGL mg/dL	HDL-C mg/dL	LDL-C mg/dL	VLDL mg/dL
Group I (Control)	120.17±2.23	100.67 ±2.16	43.67±2.25	40.33±1.86	20.13± 0.43
Group II (100 mg/kg)*	119.00±2.97	93.67±4.76$	48.83± 1.72$	39.08 ±1.02	18.73±0.95$
Group III (200 mg/kg)*	116.00±1.26$	91.67±3.93$	49.40±1.14$	38.80±1.11#	18.33±0.79$
Group IV (400 mg/kg)*	119.17±1.47	89.67±1.63$	51.50±1.38$	38.33±1.83#	17.93±0.33$

Number of animals per group=6 (n=6); *dosage of plant extract; Values are expressed as mean±SD (Total Cholesterol (TC), Triglycerides (TGL), High Density Lipoprotein Cholesterol (HDL-C), Low Density Lipoprotein Cholesterol (LDL-C), very low-density lipoprotein cholesterol (VLDL-C)). "#" p<0.05; "@" p<0.01; "$" p<0.001.

Table 5: Effect of *Z. hamur* root bark on hematological parameters.

Groups	WBC X(10^3/µL)	RBC x10^6	Hb (g/dL)	MCV (fl)	MCH (pg)	MCHC (g/dL)	PLT X(10^3/µL)
Group I (Control)	7.07±0.82	6.56±0.62	13.71±0.50	62.92±1.14	19.97±0.94	31.88±0.63	207.50±4.23
Group II (100 mg/kg)*	8.18±0.64@	7.52±0.71@	13.92±0.59	62.13±1,77	19.23±0.79	31.23±0.55	211.00±1.79#
Group III (200mg/kg)*	8.56±0.42$	7.39±0.46@	14.05±0.54	63.97±2.27	20.63±1.04	32.02±1.35	210.00±3.52
Group IV (400mg/kg)*	11.78±1.27$	8.02±0.57$	14.59±0.69@	64.72±1.63@	20.78±0.29@	32.50±0.50#	215.17±3.97$

Number of animals per group=6 (n=6); *dosage of plant extract; Values are expressed as mean±SD; white Blood Cell Count (WBC), Red Blood Cells (RBC), Haemoglobin level (Hb), red blood cell indices (MCV, MCH and MCHC), Platelets (PLT). "#" p<0.05; "@" p<0.01; "$" p<0.001.

extract treated animals were not adversely affected. The group II, III and IV showed almost similar levels of hematological parameters compared to the control group I. However, there is a fringe difference in WBC levels seen in group IV extract-treated rats compared to the group I control rats. There was a slight increase in platelet count and it increased with the increase in dosage of plant extract in groups II, III and IV.

Effect of *Z. hamur* on histopathological profile

In this study, the microscopic examination of liver and kidney tissues of group II, III and IV animals stained with hematoxylin and eosin displayed neither histopathological changes nor lesions. The group I control showed normal hepatic and renal architecture. The hepatic cellular architecture was the same in the treated group and control (Figure 1). The cells were intact, with an undisturbed vascular structure. The kidney tissue section showed the same features as that of the control. There was neither inflammation nor necrosis (Figure 2). Hence, the aqueous extract of *Z. hamur* root bark was found to be safe for the liver and kidneys of oral-treated rats with doses up to the maximum of 400 mg/kg under acute toxicity conditions.

Effect of *Z. hamur* on cytokine modulation

The quantification of serum cytokines IL-6, IL-2, IL-10, TNF-α and IFN-γ were done by ELISA (Table 6). The control showed basal level of these cytokines. The serum IL-6 and TNF-α were lowered on treatment and higher the dosage of plant extract, lower the levels of cytokines. The levels of IL-10 were elevated at a higher dosage of 400 mg/kg body weight. The levels of IL-2 were increased at a dosage of 400 mg/kg body weight. The IFN-γ levels were decreased and the decrease was in parallel to that of decrease of IL-2. The root bark extract of *Z. hamur* increased IL-10; decreased IL-6, IL-2, TNF-α and IFN-γ. Thus *Z. hamur* modulated the levels of cytokine in acute toxicity study in experimental rats.

DISCUSSION

Plants have been the primary resource for the existence of life. Plants are not only a food source but also a repository of compounds with medicinal properties. This has been realised by humans and well used for a very long time in almost all continents of the world. They have played a vital role in the promotion and restoration of human health. This has made plants form a vital component of Traditional Medicine (TM). The use of TM for the maintenance

Table 6: Effect of *Z. hamur* root bark on cytokine levels.

Groups	IL-6 (pg/mL)	IL-2 (pg/mL)	IL-10 (pg/mL)	TNF- (pg/mL)	IFN- (pg/mL)
Group I (Control)	84.6±9.2	285.9±17.4	329.5±53.1	246.6±25.7	635.7±54.2
Group II (100 mg/kg)*	85.2±3.6	274.1±18.6	358.3±63.7	243.9±40.2	617.9±93.5
Group III (200 mg/kg)*	79.5±3.5	278.4±22.5	421.7±57.3$	238.6±47.5	603.7±49.3
Group IV (400 mg/kg)*	78.1±9.4	295.8±31.6	495.3±38.4$	227.9±36.6	586.2±69.7

Number of animals per group=6 (n=6); *dosage of plant extract; Values are expressed as mean±SD. "#" p<0.05; "$" p<0.001.

of health and well-being of human society is a common practice in all African societies.[27] TM or plant-based medicines fill the medicinal demand of nearly 80% of the world population that too in rural countries. Especially in Africa, 90% of the population relies on traditional medicine healers, which makes Africa a hot spot for new plant-based drug discovery.[28] Ethiopia is known for plant-based TM, 95% of medicines are plant-based.[29] Ethiopia has well-structured hospital healthcare, but still TM plays a major role in healthcare.[30,31] The healthcare practice based on medicinal plants is well established in southwest, south, central, north and north-western parts of Ethiopia.[30-33] Eastern Ethiopia has a lot of medicinal plants but recorded data regarding these indigenous plants are very less.[34-36]

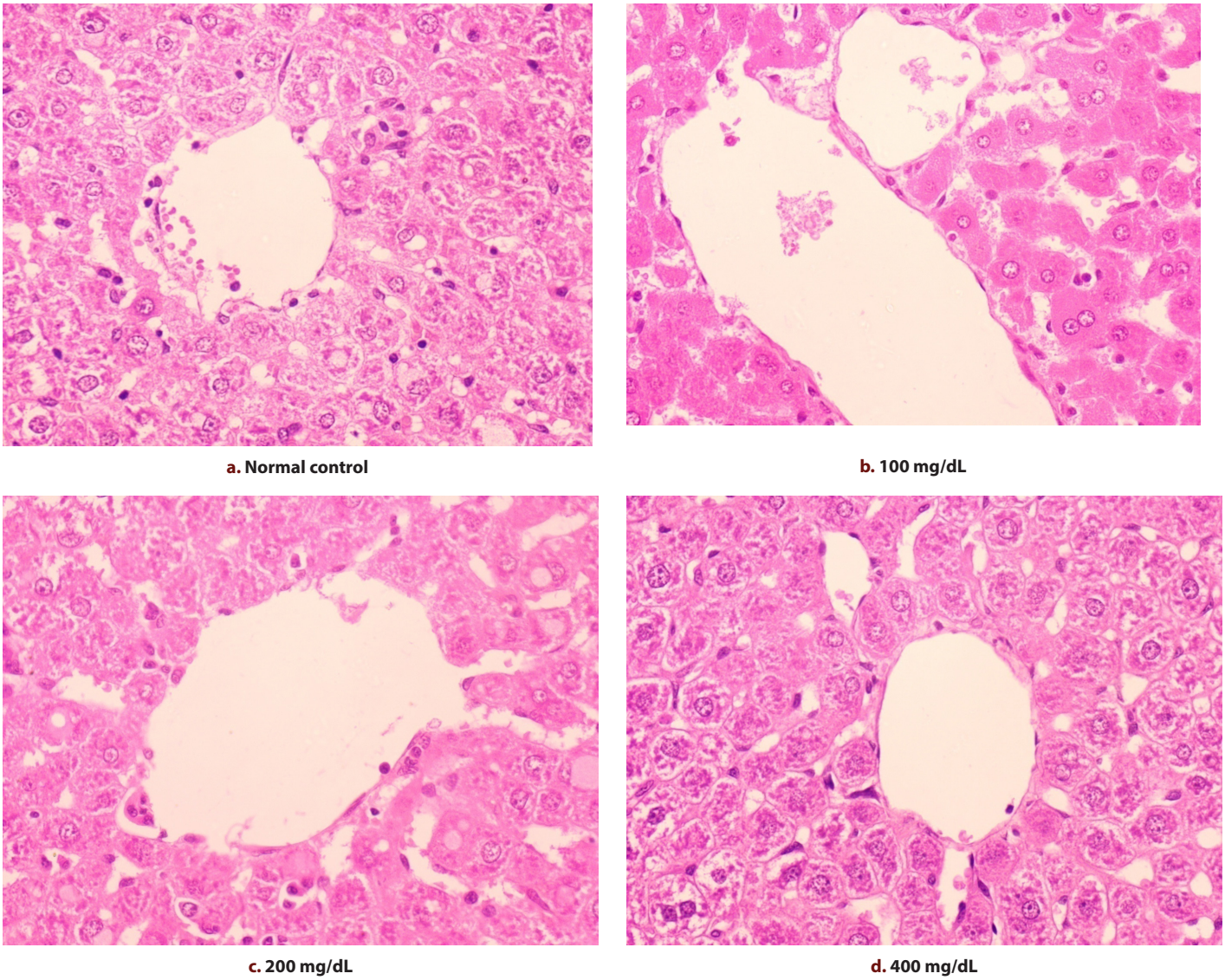
Medicinal plants contain bioactive compounds the phytochemicals responsible not only for medicinal properties but also the toxicity.[1] There is a variation in the type and content of phytochemicals. This suggests plant has both medicinal and toxic properties. The dosage and solvent preference of medicine preparation in TM plays a vital role. So, plants used in traditional medicine can be toxic due to their intrinsic toxicity. Some toxic plants are also human as medicines Datura (tropane alkaloids) and Digitalis (cardiac glycosides). Medicinal plants have demonstrated toxicity in laboratory studies and field observations. Many plants used in traditional have demonstrated some toxicity (mutagenic and carcinogenic) effects.[8] Along with potential therapeutic potential medicinal plants also harbours toxicity and worldwide reports are there for toxicological risk of medicinal plants.[37,38] Medicinal plants *Atropa belladonna, Datura* spp. and *Digitalis* spp. were reported for toxicological risks.[39] The toxicological risk involved in plant-based medicine demands the biochemical studies of these plants in TM.[40] Now there is an increase in toxicology studies along with efficacy studies[40] to ensure the safety of medicinal plants. The validation of toxicity is very important for safety.[41-43] Toxicological studies are useful in extending the therapeutic potential of plant-based remedies and for further drug development.[44,45] Further, the selected plant *Z. hamur* has not hitherto been explored for any bioactive studies to validate its therapeutic claim and for toxicological evaluation at different doses. Hence, the current study evaluated the toxicity effects of *Z. hamur* which is used in the traditional system of the Somali region and Ethiopia against jaundice and mental illness. The study on cytokine modulation by plant extracts and traditional medicinal formulation provides more information on underlying molecular mechanisms. Cytokine modulation by plant extract also ensures the safety of usage in long-term treatment.[46,47]

Z. hamur had no signs of toxicity in rats

The results of acute toxicity not only serve as experimental methods for studying accidental poisoning with a chemical but also experimental designs for chronic toxicity studies.[48] The rate of mortality plays a major role in acute toxicity tests. Bodyweight measurement serves as an indicator in toxicity studies.[10] Consumption of some plant-based drugs may induce weight gain in animals.[49] Alterations in body weight is a significant indicator for the examination of initial symptoms of drug and chemical-induced toxicity.[50] In acute toxicity studies, the toxic effects produced by a single large dose of a compound lasting no longer than 24 hr were investigated. The biological effects produced were studied to understand the extent of toxicity to the organism. The development of tolerance was revealed by an acute exposure. The absence of adverse effects in acute toxicity in our study ruled out the toxicity of *Z. hamur* root bark aqueous extract up to a dosage of 400 mg/kg. The absence of behavioural and physical changes supports the non-toxicity of *Z. hamur* and thus it could be a potential therapeutic candidate.

Z. hamur does not affect food and water consumption

The alteration or adverse effect in food intake or water drinking pattern in an organism due to the administration of a compound indicates the toxic impact on metabolism.[51] The administration of *Z. hamur* extract showed no adverse effect on food intake and water drinking, very slight changes seen were statistically insignificant. This indicated that the *Z. hamur* extract is safe for long-term administration. This revealed that *Z. hamur* root bark extract had not induced any alteration in the metabolism of experimental animals.



a. Normal control

b. 100 mg/dL

c. 200 mg/dL

d. 400 mg/dL

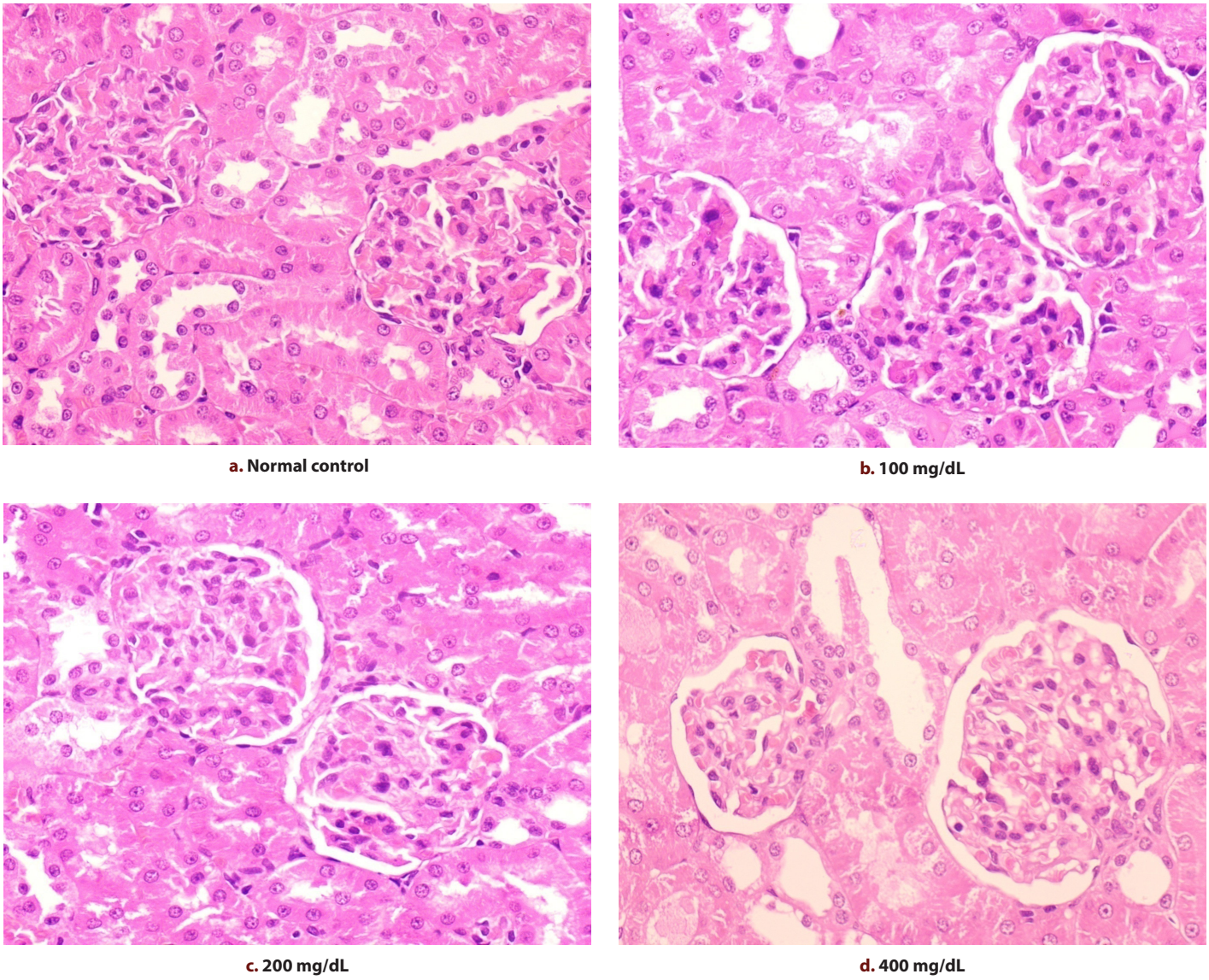
Figure 1: Histopathology of liver sections-H&E staining (40X), a. control showing normal hepatic structure; b, c, d administered with 100 mg/dL, 200 mg/dL, 400 mg/dL plant extract also showed intact cellular structure with normal hepatic vascular structure.

Hepatic and renal non-toxic nature of *Z. hamur*

Hepatotoxicity is diagnosed by an increase in the activities of aminotransferases due to their in-blood stream which leads to hepatic dysfunction and hepatocellular necrosis (OECD, 1998). The analysis of the level of enzymes in the serum serves as a vital quantitative marker of hepatocellular injury and indicates the type of injury.[52] The insignificant changes in levels of liver marker enzyme in toxicity studies are an indicator of the safeness of plant extract.[53] There was a negligible decrease in AST and ALP levels with an increase in the dosage of plant extract which suggests its hepatoprotective role. The abnormal levels of urea and creatinine are indicators of nephrotoxicity.[54] Absence of adverse effects on urea and creatinine levels confirms that plant extract is not renal toxic. This plays a vital role in the toxicology study of plant extract. Elevation in creatinine is an indicator of glomerular dysfunction and elevation in urea is an indicator of kidney disease.[53] In our study the slight increase in serum urea level may be due to an increase in protein metabolism. When a plant extract is introduced, the body achieves homeostasis as treatment is prolonged. The nil adverse impact on urea and creatinine indicates the renal non-toxicity of *Z. hamur*. Our study findings in the current study, the doses used for the test seem to be safe in rats and this supports the nontoxic property of *Z. hamur* root bark. However, histopathological studies were done to prove the integrity of liver and renal tissues thus ensuring absolute safety of plant extract.

Z. hamur does not affect lipid profile

Serum lipid profile examination which encompasses the measurement of Total Cholesterol (TC), Triglycerides (TGL),



a. Normal control **b.** 100 mg/dL

c. 200 mg/dL **d.** 400 mg/dL

Figure 2: Histopathology of kidney sections-H&E staining (40X), a. control showing normal renal structure; b, c, d administered with 100 mg/dL, 200 mg/dL, 400 mg/dL plant extract also shows intact cellular structure with non-eccentric nuclei and granular cytoplasm.

High-Density Lipoprotein Cholesterol (HDL-C) and Low-Density Lipoprotein cholesterol (LDL-C) provides important information on atherosclerosis as well as other related coronary heart diseases.[55] HDL plays major role in removal of excess cholesterol, inhibiting oxidation of LDL by transition metal ions and prevention of formation of lipid hydroperoxide by mediated 12-lipoxygenase. Thus, HDL prevents the generation of ROS and reduces the risk of atherosclerosis. Our studies showed a slight increase in HDL and not a drastic decrease suggesting that plant extract may increase the HDL concentration at higher dosage. High levels of LDL and TGL leads to the development of various cardiovascular diseases. High LDL and TGL accompanied by low HDL increase the risk of cardiovascular disease.[56] In our study the serum lipid parameter showed a pattern of increased HDL; low LDL, VLDL and TGL making it prominent that there is no impairment in lipid metabolism. The usage of *Z. hamur* has no negative effect on lipid metabolism in rats. Our results suggest that it is safe to use *Z. hamur* and further studies with induced toxicity and different dosages can prove the effectiveness of *Z. hamur* in the treatment of lipid metabolism-related disorders.

Z. hamur had no effect on haematological parameters except WBC

The hematological parameters namely Packed Cell Volume (PCV), Hemoglobin level (Hb), White Blood Cell count (WBC), platelets and red blood cell indices-MCV, MCHC and MCH in *Z. hamur* extract treated animals were not adversely and the levels were similar with that of the control group. A fringe difference in WBC was exhibited by extract-treated rats when compared to the control rats in the highest dose of 400 mg/kg body weight. The hematological system is the most important indicator of health status. The blood is the mode of transport of almost all

compounds, so it is an easy target for toxic compounds.[57] There are also reports of alteration of hematological parameters on the administration of toxic plant extract.[58] Blood cells along with cytokines play a major role in various pathological conditions. The effect of plant extract on these blood cell levels indicates the role of plant extract on cytokine levels.[54] Both increase and decrease of hematological parameters are associated with pathological conditions or toxicity. For example, decreased WBC is an indicator of low immunity; on the other hand, higher WBC is associated with stress and inflammation. RBC and its indices play a key role in hypoxia, toxicity and renal carcinoma.[59] WBC is associated with inflammation and platelet homeostasis of the immune system. Platelets and their indices play a key role in the detection of atherosclerosis and ischemic heart disease.[60] A plant extract is safe for use when it does not alter the hematological parameters in toxicology studies. This also requires an assessment of other pathology-related parameters.[59] There was a slight elevation in WBC and platelet in the present study and it increased with the dosage of *Z. hamur* extract. This suggests further study with various dosages and periods. The elevation of WBC along with the increase in plant extract dosage suggests the role of cytokines. Other species of *Ziziphus* have shown attenuation of pathological conditions by regulating pathways involved in cytokine production thereby possessing immunomodulatory function. *Z. hamur* extract showed an effect on WBC levels suggesting an immunomodulatory effect of *Z. hamur* that needs to be further investigated.[61] Our results showed no adverse effects indicating that the *Z. hamur* extract is nontoxic to the hematopoietic system.

Z. hamur has no adverse effect on the histopathological profile

To assess the liver function, both histological and biochemical examination are generally used in the case of biochemical parameters, the hepatic function is assessed by measuring the levels of intracellular serum liver toxicity marker enzymes. Chemical or drug-induced liver alteration is also investigated by histological examination.[62] The presence of hepatic lesions, fibrosis and disruption of the vascular system are key microscopic features of hepatic cellular damage induced by toxicity. RBC accumulation in the hepatic vein is a characteristic feature of hepatic damage.[63] However; this was not seen in our study. The renal sections showed no necrosis and intact cellular structure. The major features of damaged kidney cells are eccentric nuclei and the absence of granules. These were not observed in our study. The both control and treated group showed non-eccentric nuclei and granular cytoplasm. The absence of lesions and alteration in liver and kidney sections proved that plant extract is nontoxic to the hepatic and renal system. The dosage of up to 400 mg of *Z. hamur* root bark per kg body weight is safe to use. These results were also in line with the biochemical screening results which indicated the absence of biochemical alterations in serum liver toxicity marker enzymes and hepatotoxicity markers in treated rats. Hence, the aqueous extract of *Z. hamur* root bark was found to be safe for the liver and kidneys with a dosage of up to 400 mg of *Z. hamur* per kg body weight.

Z. hamur is a cytokine modulator

Liver disease in most cases is characterised by involvement of T-cells, natural killer cells and proinflammatory and anti-inflammatory cytokines. Study of cytokines can be a key event in understanding disease progression and alleviation by various agents. Cytokines are signalling molecules that play a major role in the homeostasis of almost every organ function.[11] Cytokine plays major role not only in inflammation progression but also in tissue regeneration. Modulation of cytokine activity is a vital property of therapeutic agents.[14] The cytokines IL-6, IL-2, IL-10, TNF-α and IFN-γ plays major role in liver disease[13] and mental illness.[64] Increased levels of cytokines are associated with nervous system pathophysiology. Elevated levels of major cytokines are associated with schizophrenia, major depressive disorder and bipolar disorder.[65] Decrease in levels of these molecules makes antidepressants a promising therapeutic strategy.[66]

IL-6 family cytokines play a major role in liver pathophysiology.[67] IL-6 can be detrimental to liver tissues and can also help in regeneration.[68] The levels of IL-6 and TNF- are higher in case of obstructive jaundice.[69,70] Increased IL-6 and TNF-α levels were associated with liver disease and its progression.[71] In most cases of psychiatric illness and mental illness, IL-6 and TNF-α levels were increased.[72] IL-6 is a potential activator of acute phase proteins, thus aiding inflammation progression. Interestingly, IL-6 also acts as hepatocyte regenerator, it was proved by studies in knockout mice, as the restoration of IL-6 caused an increase in hepatocyte proliferation.[73] The activation of TNF-α is a key event that activates IL-6 cytokine family and eventually activates cell cycle via STAT3 pathway.[74] The decrease of TNF-α and absence of any behavioural changes suggest a positive effect of the cytokine on nervous system. This can be clearly explained by the fact that TNF-α has a negative effect on neuron and can activate apoptosis.[75] *Zizipus jujuba* fruit extract showed down regulating effect on serum levels of proinflammatory cytokines IL-6, TNF-α and NFκB expression *invitro*. Inhibition of IL-6 mRNA expression but not TNF-α *Zizipus jujuba* suggested independent inhibition of IL-6.[15] A well known toxic plant Nerium oleander caused an increase in serum IL-6 and TNF-α.[16] The increase in TNF-α by plant extract questions its safety in treatment, as seen in case of Tetrorchidium didymostemon.[76] Decrease in levels of TNF-α is a key factor supporting the safeness of plant extract usage.[17] The absence of an increase in IL-6 and TNF-α levels in this experimental study at a dosage of 400 mg/kg body weight suggests the non-toxicity of *Z. hamur*. Histopathological studies clearly showed that *Z. hamur* had no effect on hepatic tissue architecture and this supports the above statement.

IL-10 is the most important anti-inflammatory cytokine, its anti-inflammatory property has been explored using knock-out mice studies.[77] IL-10 inhibited TNF-α, NFκB signalling independently and IFN-γ synthesis.[78] IL-10 is used for treatment of neuroimmune disease due to its above mentioned cytokine inhibitory properties.[79] IL-10 is highly considered for the treatment of hepatic inflammation.[80] IL-10 deficiency is involved in enhanced fibrosis and neurodegenerative diseases.[81] In murine macrophage, IL-10 downregulated the expression of proinflammatory cytokines by macrophages, thereby acting as antifibrotic agent in CCl_4 induced toxicity. In our studies IL-10 increase by *Z. hamur* supports the hepatoprotective role of *Z. hamur*.[80] IL-10 was increased by *Melissa officinalis* treatment in stress induced rat models.[82] A natural flavonoid pinostrobin has been proven for its hepatoprotective role by increasing IL-10 and decreasing IL-6 and TNF-α.[83] *Hypericum triquetrifolium* extract mediated anti-inflammatory properties by increasing IL-10 and decreasing IL-6 and TNF-α.[84] Increased IL-10 in our study, at a dosage dependent manner supports the hepatoprotective and neuroprotective role of *Z. hamur*.

IL-2 plays major role in T-cell regulatory mechanism and activation of proinflammatory cytokines. IL-2 level was increased in acute liver injury of wistar rat models.[85] The Hepatoprotective effect of *Centella asiatica* in dimethylnitrosamine was documented by decreased IL-2, IL-6, TNF-α and IFN-γ levels.[86] Anti-inflammatory property of *Z. jujuba* was documented by its IL-2 inhibiting activity in Jurkat T-cells. IL-2 decrease by *Z. hamur* proves the anti-inflammatory role of the same. This also suggests immunosuppressive role of *Z. hamur*.[87] The decreased IL-2 by *Z. hamur* might be due to increased B-lymphocyte and decreased T-cells as seen in case of silymarin treated poultry animals. Silymarin almost diminished IL-2 activity and it was supported by other liver parameters in that study.[88] The levels of IL-2 were decreased in our acute toxicity by *Z. hamur* but not drastically, supporting the absence of inflammation.

IFN-γ is known for its antiproliferative effect on liver cells.[89] It also acts as a potent immunomodulator in liver cell proliferation and regeneration. It is a fate determinator of liver cell.[90] Increased levels of IFN-γ are seen in acute liver injury and decrease of this level by hepatoprotective agent reversed disease condition.[91] Increased levels of IFN-γ are also seen in case of obstructive jaundice.[70] In IFN-γ deficient mice models the extra-hepatic bile duct obstruction and inflammation were prevented even though there was no delay in onset of jaundice. Strikingly there was a recurrence of obstruction and inflammation on administration of recombinant IFN-γ.[92] Hepatoprotective agents like silymarin exhibited its role by decreasing IFN-γ levels. Moreover, lower doses inhibited the T-cells and at higher dosage increased inflammation.[88] IFN-γ levels were higher in case of patients with depressive disease, to a level it could be a marker for diagnosis along with other few parameters.[93] Similarly increased IFN-γ levels along with IL-10 were associated with the severity of schizophrenia.[94,95] The TH-1 cells being the major source of IL-2 and IFN-γ.[96] The decrease in IL-2 and IFN-γ may be due to a decrease in T-cells. In our study, the levels of IFN-γ were decreased compared to control in all the three-dosage supporting the non-toxicity of *Z. hamur*. The histopathological results also support the non-toxicity of *Z. hamur*. Overall, changes in cytokine levels suggest that *Z. hamur* is capable of modulating cytokine expressions in a positive way. The histological, biochemical and hematological parameters also support the non-toxic effect of cytokine modulation by *Z. hamur*.

CONCLUSION

Our study findings showed that the aqueous extract of *Z. hamur* root bark is non-toxic at all three-dosage used in this study. Oral administration of the aqueous extract of *Z. hamur* root bark had no mortality and behavioural changes up to a dosage of 400 mg/kg in experimental animals. Higher dosages of plant extract could be studied for acute toxicity studies. The sub-acute toxicity studies proved the non-toxicity of *Z. hamur* root bark as it showed no adverse effect on the hepatic system, renal system and overall body homeostasis. There was an increase in HDL-C suggesting the non-deleterious effect on lipid metabolism. Studies using higher dosage of the plant were recommended. Alteration in the haematological parameters that were noticed in the research also needs to be considered for further investigations. The histological study results correlated with biochemical parameters assuring the safety of *Z. hamur* root bark. The cytokine modulatory property of *Z. hamur* suggested further investigation at higher dosages and longer periods. We also recommend that further pharmacological investigations are needed to know the efficacy of *Z. hamur* in the treatment of various diseases.

ACKNOWLEDGEMENT

This research project was supported by research funding from Jigjiga University, Ethiopia (Funding No. JJU-DRPTT/O25/2020). Also, this project was supported by Researchers Supporting Project number (RSP2024R177) King Saud University, Riyadh, Saudi Arabia. Infra-structure facilities provided by Addis Ababa University and Haramaya University were acknowledged.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

***Z. hamur*:** *Zizipus hamur*; **ALT:** Alanine transaminase; **AST:** Aspartate transaminase; **ALP:** Alkaline phosphatase; **TC:** Total cholesterol; **TGL:** Triglycerides; **HDL-C:** High density lipoprotein cholesterol; **LDL-C:** Low density lipoprotein cholesterol; **VLDL-C:** Very low density lipoprotein cholesterol; **WBC:** White blood cells; **RBC:** Red blood cells; **Hb:** Haemoglobin; **MCV:** Mean

corpuscular volume; **MCH:** Mean corpuscular haemoglobin; **MCHC:** Mean corpuscular Haemoglobin concentration; **PLT:** Platelets; **IL-6:** Interleukin-6; **IL-2:** Interleukin-2; **IL-10:** Interleukin-10; **TNF-:** Tumor necrosis factor-; **IFN-:** Interferon-.

SUMMARY

Research findings of this study showed that the aqueous extract of *Z. hamur* root bark is non-toxic in nature. Oral administration of the aqueous extract of *Z. hamur* root bark had no mortality and behavioural changes up to a dosage of 400 mg/kg in experimental animals. The sub-acute toxicity studies proved the non-toxic nature of *Z. hamur* root bark as it showed no significant adverse effect on the hepatic system, renal system and overall body homeostasis.

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Cite this article: Ameer K, Ali M, Muleta CO, Yasothkumar N, Balakrishnan S, Geethanjali K, *et al. Ziziphus hamur* Engl., a Rare Medicinal Plant Modulates Cytokine and Hematological Levels during Sub-Acute Toxicity Studies in Wistar Rats. Indian J of Pharmaceutical Education and Research. 2025;59(1s):s229-s242.