REPORT

Safety and toxicological evaluation of herbal formulation on rodents

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Abstract: Herbal medicines are still most popular, abundant and affordable remedies for curing various ailments. Garlina is one of the herbal formulations of Hamdard Laboratories (waqf) Pakistan used to treat cardiovascular diseases and elevated sugar level. However, there is no scientific data available regarding the potential toxicity. Therefore, the present study was to assess the acute and sub-chronic toxicity in rats. The single dose of Garlina 5000mg/kg were administered orally and observed for 14 days. A sub-chronic toxicity test was performed at 2000mg/kg of Garlina daily for 30 days. Control rats received saline. The biochemical, hematological and histopathological analysis was carried out. The acute toxicity LD_{50} was determined to be >5000mg/kg. The result of acute and sub-chronic toxicity revealed no mortality and sign of toxicity. Garlina did not elicit any significant change in body weight, hematological and histopathology analysis when compared to saline treated rats. The relative weight of organs was not affected by the treatment. While the daily dose of Garlina for humans is 20mg/kg. However, the sub-chronic toxicity at 2000mg/kg dose of Garlina exhibited significant increase in gamma glutamyltransferase while total protein significantly decreased. Results obtained from study demonstrated that there is wide margin of safety for the therapeutic use of Garlina and significant decrease in LDL, atherogenic index, GGT and bilirubin direct at the dose of 5000mg/kg further strengthen the use as hypolipidemic and hypoglycemic agent.

Keywords: Garlina, acute and sub- chronic toxicity, biochemical, hematology, histopathology.

INTRODUCTION

Cardiovascular disorders are one of the major causes of death worldwide. The world health organization estimates that a cardiovascular disorder claims lives of 3.8 million men and 3.4 million women around the world (Emslie, 2005). People suffer cardiovascular diseases due to several factors like patient's non-compliance, environmental factor and hereditary factors etc. Although several treatments for cardiovascular diseases exist, but associated adverse effects with almost every treatment limit their use. Hence, there is a constant need to search for a more active and safe therapies. Herbal products provide human safe and effective treatments against different type of diseases including cardiovascular ailments. Herbs like garlic (*Allium sativum*), onion (*Allium cepa*), kalongi/black seeds (*Nigella sativa*), *g*ugul *g*um (*Commiphora mukul*) and *g*inger (*Zingiber officinale*) are popularly used in folkloric system of medicine to treat cardiovascular diseases and risk factors.

Garlic (*Allium sativum*) possess cardioprotective properties including antioxidant, hypotensive, hypolipidemic, antiplatelet, fibrinolytic and antiatherogenic activities (Sharma *et al*., 2012). Kalongi (*Nigella sativa*) has reported for multiple beneficial effects like antioxidant, antihyperlipidemic and hypoglycemic activities. Its thymoquinone is an active constituent of *Nigella sativa* (Asgary *et al*., 2013). Gugul gum (*Commiphora mukul*) produces improvements in lipid disorders, diabetes, arthritis, hypercholesteremia, hypertention, and other cardiovascular diseases (Ramesh *et al*., 2012). Ginger (*Zingiber officinale*) possesses hypoglycemic, antiatherosclerotic, antithrombotic, anti-inflammatory and antioxidant activities. Its phenolic derivatives are active constituents of rhizome (Heeba and Abd- Elghany, 2010). Onion (*Allium cepa*) has reported for antiatherosclerotic effects and also reduces the risk of coronary heart diseases. Flavonoids of onion are responsible for its different activities (Hamauzu *et al*., 2011).

All above mentioned herbs have been combined in a herbal product known as "Garlina" which is prepared by Hamdard Laboratories (Waqf) Pakistan. Garlina claims to lower blood pressure and blood cholesterol levels. It contains garlic, onion, kalongi, gugul gum and ginger in a ratio of 250:150:50:50:10. Despite the extensive beneficial use of the individual constituents of Garlina, there is no data on the safety of this product used for these claims. Therefore, the present study was undertaken to carry out the acute and sub- chronic toxicity on rats.

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MATERIALS AND METHODS

Drug

Garlina was obtained from Hamdard Laboratories (waqf) Pakistan. It is composed of five herbal plants which are as follows:

1. Garlic (*Allium sativum*).....250 mg
2. Onion (*Allium cepa*) .....150 mg
3. Kalongi/black seeds (*Nigella sativa*) .....50 mg
4. Gugul *g*um (*Commiphora mukul*) .....50 mg and
5. Ginger (*Zingiber officinale*).....10 mg

The human dose of Garlina is one tablet two times a day i.e. 20mg/kg. The two doses of Garlina 5000mg/kg and 2000mg/kg that were 250 and 100 times greater than human dose respectively were selected for acute and chronic toxicity test. Tablets were given orally by dissolving in saline.

Chemicals

The kits for biochemical estimations were purchased from Diagnostic Merck (Germany). Hematoxylin from BDH Chemicals Ltd., Poole, England and Eosin from E. Merck. All chemicals (chloroform, xylene, ethanol and formalin) were of analytical grades.

Animals

Sprague Dawley Rats of either sex were used in this study were obtained from animal house of Dr. HMI Institute of Pharmacology and Herbal Sciences. Animals were housed under the maintained environmental condition and were fed on standard rodent pellet diet and water *ad libitum*. The handling of laboratory animals was duly approved and assessed by Hamdard University Animal Ethical Committee (HU-AEC) and follows the protocols based on internationally accepted standard guideline for animals use.

Acute toxicity test

According to the Organization for Economic Co-operation and Development (OECD) guideline 425 (OECD guideline, 2008), the limit test at one dose level of 5000mg/kg body weight in acute oral toxicity was administered to a group of 10 rats at least five per sex. The animals were kept overnight fasting prior to drug administration. After the drug administration, food was withheld for further 3-4 hours. The control group received 0.9% normal saline. Animals were closely observed for the initial 4 hours, and then daily thereafter for 14 days. Rats were weighed and observed for mortality and behavioral changes (salivation, diarrhea, ataxia, restlessness and tremors) and any sign of toxicity once daily during the period of 14 days (Sim *et al*., 2010).

Sub-chronic toxicity test

Rats were randomly allotted to 2 groups of 20 animals each (10 males and 10 females), according to the Organization for Economic Co-operation and Development (OECD) guideline 407 (OECD guideline, 2008). One group received Garlina 2000mg/kg while the control group was orally administered 0.2ml of saline for 30 consecutive days. Body weights of animals were monitored daily before drug administration for calculating the exact volume of dose as well as to observe any change in weights compared with the weight of saline treated group.

The heart, liver, spleen and kidneys of freshly killed rats after treated with Garlina 5000mg/kg and 2000mg/kg were excised and weighed immediately on a sensitive electronic balance (Sartorius BP-211-D) and were preserved in a fixation medium of 10% solution of buffered formalin for histopathological study.

Biochemical Study

At the end of the study, all animals were anesthetized with chloroform manufactured by E. Merck, administered through inhalation. Blood samples were collected via cardiac puncture into non heparinized and EDTA containing tubes for biochemical and hematological analyses respectively. Serum was separated by centrifugation by centrifuge machine (Model 80-2, No 02561, Changzhou Guohua Electric Appliance Co. Ltd., China) at a speed of 1500-2000 pm for 20 minutes. The kits for assessment of biochemical parameters like lipid, heart, liver, kidney and glucose enzyme levels were estimated spectrophotometrically by Hitachi U-2000 spectrophotometer.

Hematological parameters

A hematological analysis was performed in Shifa-ul-Mulk Memorial Hospital for Eastern Medicine, Hamdard University, by using an automatic haematological analyser (Sysmex KX-21, Japan).

Histopathology

Small pieces of liver, heart, spleen and kidney were fixed in 10% neutral formalin, dehydrated in graded (80-100%) alcohol, cleared in xylene, and embedded in paraffin wax. 4-5 um thick sections were prepared by Leica RM 2145-Rotator Microtome. The tissues were stained with hematoxylin and Eosin (H & E). The tissues were observed and photographed through Nikon's Advanced Research Microscope OPTIPHOT Model X2T-21E equipped with Nikon's Microphotography system model UFX-DX-35 and phase contrast N plan for the assessment and comparison of morphological changes between control and Garlina treated rats at the doses of 5000mg/kg and 2000mg/kg.

STATISTICAL ANALYSIS

Data was expressed as mean ± s.e.m. by independent sample t- test by using the Software of SPSS version 20. Results were considered to be significant at $p<0.05$.

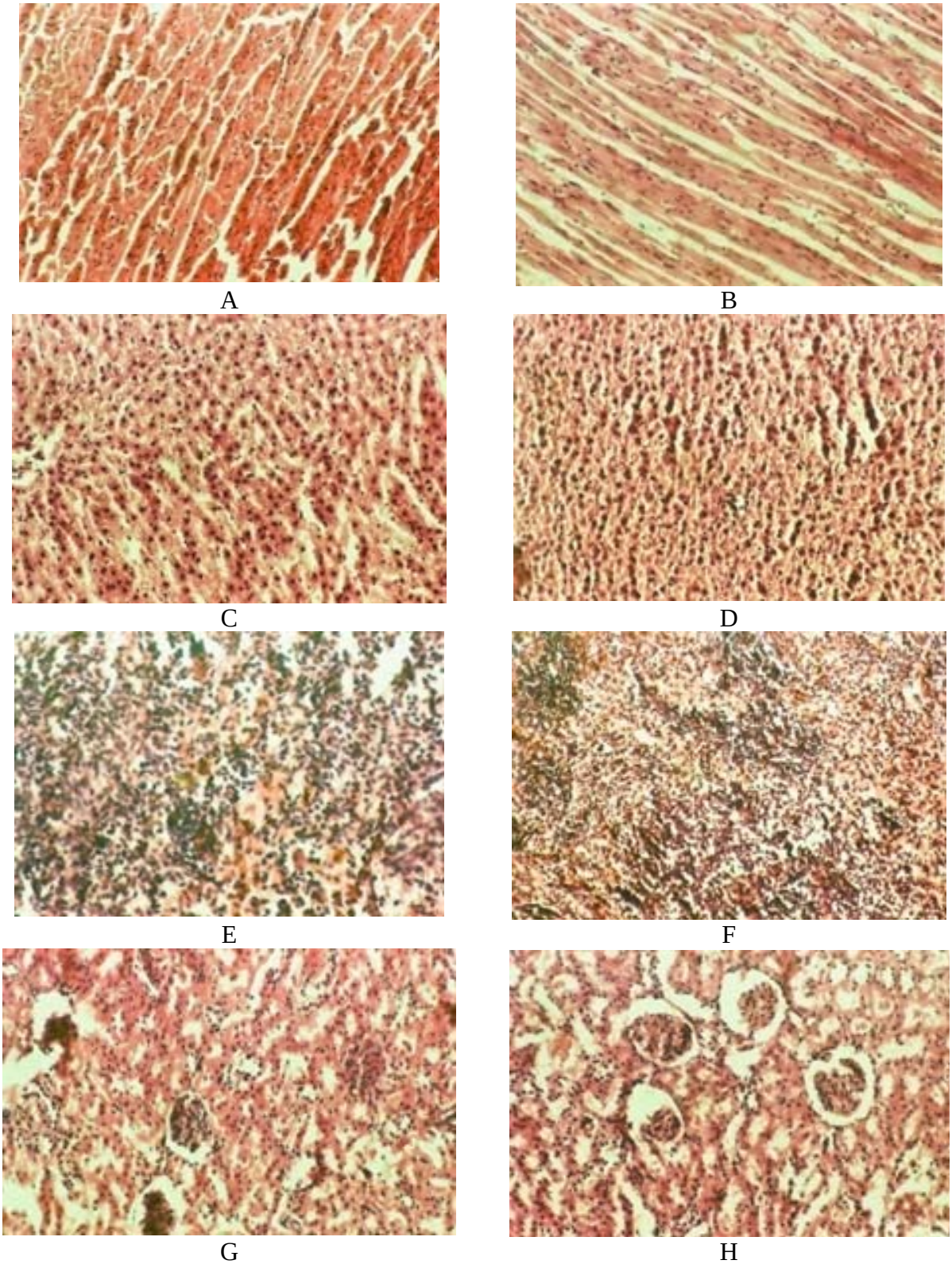

Fig. 1: Heart of control rat(X10) (A), Heart of rat treated with Garlina (5000mg/kg) (X10) (B), Liver of control rat (X10) (C), Liver of rat treated with Garlina (5000 mg/kg) (X10) (D), Spleen of control rat (X10) (E), Spleen of rat treated with Garlina (5000mg/kg) (X10) (F), Kidney of control rat (10X) (G), Kidney of rat treated with Garlina (5000 mg/kg) (X10) (H).

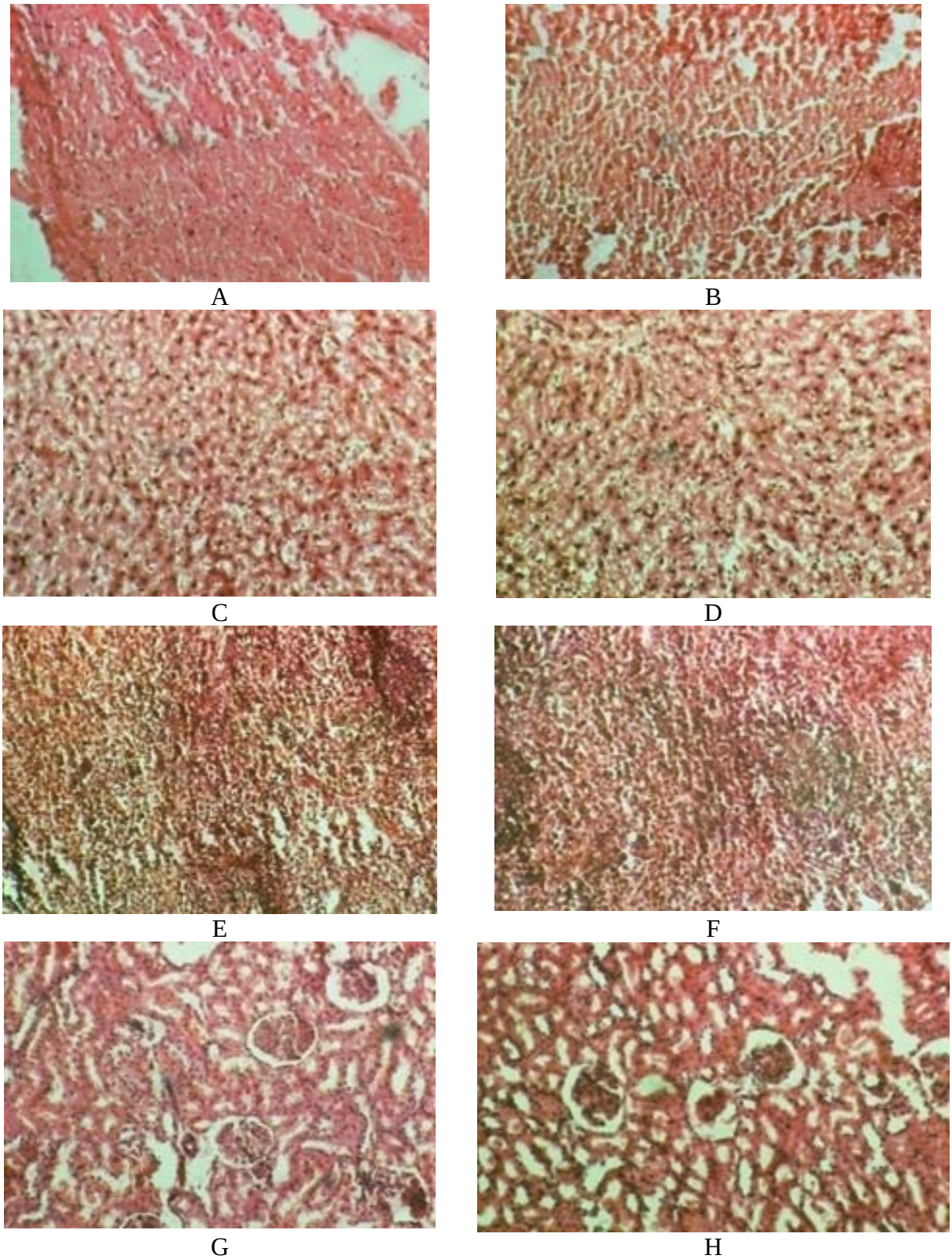

Fig. 2: Heart of control rat(X10) (A), Heart of rat treated with Garlina (2000 mg/kg) (X10) (B), Liver of control rat (X10) (C), Liver of rat treated with Garlina (2000 mg/kg) (X10) (D), Spleen of control rat (X10) (E), Spleen of rat treated with Garlina (2000 mg/kg) (X10) (F), Kidney of control rat (10X) (G), Kidney of rat treated with Garlina (2000 mg/kg) (X10) (H).

Table 1: Analysis of biochemical parameters in serum of rats treated with saline and Garlina (5000mg/kg).

Biochemical Parameters	Saline Control	Garlina 5000 mg/kg
Lipid function markers (mg/dl)		
Cholesterol	118.02±5.3	123.0±5.2
Triacylglycerol	126.8±6.2	125.6±6.3
HDL	99.3±5.4	93.5±4.3
LDL	-8.1±3.03	4.4±4.4**
VLDL	25.7±1.2	25.1±1.3
Atherogenic Index	-7.8 ±3.04	4.7 ±4.4**
Heart enzymes (u/l)		
ASAT	665.7±29.7	630.9±36.2
CK-NAC	1692.5±235.2	1654.002±259.0
LDH	15253.2±607.4	16053.2±438.7
Liver function markers		
ALAT (u/l)	778.7±42.2	726.6±34.02
Alkaline PO_4 (u/l)	443.6±11.9	447.5±24.2
Bilirubin T (mg/dl)	1.5±0.1	1.6±0.1
Bilirubin D (mg/dl)	2.8±0.2	2.2±0.2*
Gamma GT (u/l)	225.04±5.5	206.9±6.3**
Kidney function markers		
Albumin (g/dl)	5.3±0.2	5.2±0.2
Creatinine (mg/dl)	0.3±0.2	0.3±0.2
Total Protein (mg/dl)	5.3±0.2	5.1±0.2
Urea (mg/dl)	36.3±41.2	47.2±77.6
Uric Acid (mg/dl)	5.3±0.2	5.5±0.3
Glucose (mg/dl)	85.1±11.9	77.9±11.2

Values are expressed as mean ± s.e.m. of 10 animals (5/sex). Parentheses are expressed as range values for mean -95% confidence limit. *p<0.05, **p<0.025

Table 2: Hematological studies in different constituents of blood in rats treated with saline and Garlina (5000mg/kg).

Blood Picture	Saline Control	Garlina 5000 mg/kg
Hemoglobin (g/dl)	12.53±0.24	12.80±0.32
PCV (%)	-12.49±11.98	-14.47±15.05
MCV (fl)	-57.79±0.52	-58.20±0.43
MCH (pg)	-18.21±0.24	-18.56±0.14
MCHC (g/dl)	-5.59±10.35	5.36±12.83
RBCs ($x10^6$/ul)	6.88±0.10	6.91±0.21
Platelets ($x10^3$/ul)	795.20±34.04	774.14±28.33
TLC ($x10^3$ /l)	4.80±1.50	5.81±1.99
Neutrophils (%)	15.90±2.31	17.29±4.49
Lymphocytes (%)	81.90±2.58	77.86±4.85
Eosinophils (%)	1.00±0.26	1.14±0.26
Monocytes (%)	1.20±0.29	1.71±0.29

RESULTS

Acute and sub-chronic toxicity test in rats

Rats administered with Garlina at the doses of 5000mg/kg and 2000mg/kg did not show abnormal behavior and any significant change in weight, when compared to saline treated group. No mortality was observed during the study period.

On autopsy, the vital organs including heart, liver, spleen and kidneys did not show significant change in color and texture of rats treated with Garlina at both the doses.

Biochemical parameters

The detailed investigation of all the biochemical profiles i.e., lipid, cardiac, liver, kidney and serum glucose levels produced no apparent change, a part of significant decrease ($p<0.025$) in LDL, atherogenic index, GGT and ($p<0.05$) in direct bilirubin in the group treated with Garlina 5000 mg/kg compared to saline treated group (table 1). The result (table 3) obtained from sub-chronic treatment of Garlina 2000mg/kg for a period of one month showed no effect on serum biochemical levels, with the exception of significant increase ($p<0.05$) in GGT and decrease ($p<0.025$) in total protein.

Table 3: Analysis of biochemical parameters in serum of rats treated with Saline and Garlina (2000mg/kg)

Biochemical Parameters	Saline Control	Garlina 2000mg/kg
Lipid function markers (mg/dl)		
Cholesterol	162.41±13.06	151.10±15.24
Triacylglycerol	151.06±6.56	139.98±4.79
HDL	83.92±3.43	78.44±2.43
LDL	41.64±13.66	71.50±30.02
VLDL	30.21±1.31	28.00±0.96
Atherogenic Index	45.93±13.97	45.02±15.77
Heart enzymes (u/l)		
ASAT	2549.49±41.96	2604.60±53.70
CK-NAC	2667.32±71.20	2436.36±129.01
LDH	14634.44±420.28	14891.25±364.24
Liver function markers		
ALAT (u/l)	539.64±14.14	550.22±13.90
Alkaline PO_4 (u/l)	496.95±15.04	530.59±17.51
Bilirubin T (mg/dl)	1.48±0.06	1.54±0.04
Bilirubin D (mg/dl)	2.35±0.13	2.36±0.20
Gamma GT (u/l)	235.19±4.22	246.08±4.36*
Kidney function markers		
Albumin (g/dl)	4.17±0.10	4.33±0.12
Creatinine (mg/dl)	1.02±0.15	0.79±0.23
Total Protein (mg/dl)	5.89±0.16	3.47±0.12**
Urea (mg/dl)	-15.29±34.48	-17.06±16.20
Uric Acid (mg/dl)	5.42±0.19	5.40±0.18
Glucose (mg/dl)	105.63±2.91	108.58±3.99

Values are expressed as mean ± s.e.m. of 20 animals (10/sex). Parentheses are expressed as range values for mean -95% confidence limit. *p<0.05, **p<0.025

Table 4: Hematological studies in different constituents of blood in rats treated with saline and Garlina (2000mg/kg).

Blood Picture	Saline Control	Garlina 2000 mg/kg
Hemoglobin (g/dl)	9.88±1.72	9.89±1.74
PCV (%)	-13.07±7.93	-16.74±8.49
MCV (fl)	-57.65±0.51	-57.85±0.33
MCH (pg)	-18.26±0.16	-18.15±0.21
MCHC (g/dl)	-7.85±7.14	-5.36±7.75
RBCs ($x10^6$/ul)	6.71±0.12	6.60±0.66
Platelets ($x10^3$/ul)	724.21±52.87	707.77±67.28
TLC ($x10^3$/l)	6.71±1.13	5.22±1.34
Neutrophils (%)	24.84±2.19	22.65±2.62
Lymphocytes (%)	71.25±2.21	73.89±2.98
Eosinophils (%)	1.74±0.20	1.41±0.23
Monocytes (%)	2.16±0.18	1.82±0.25

Hematological Study

Acute and sub- chronic oral administration of Garlina did not cause any significant change in hematological profile as shown in tables 2 and 4.

Histopathalogical study

Histological features of heart, liver, spleen and kidney of rats treated with 5000mg/kg and 2000/kg of Garlina exhibited normal structure when examined under the microscope (figs. 1 and 2).

DISCUSSION

Rats treated with Garlina 5000mg/kg and 2000mg/kg did not show mortality and toxic signs and symptoms. There is no significant difference in the body and organ weights in comparison with saline treated rats.

Findings of serum lipid profile at the dose of 5000mg/kg showed significant decrease (p<0.025) in LDL, atherogenic index and GGT levels. The decline GGT level may reduces the risk of morbidity regarding liver and heart diseases (Kunutsor *et al*., 2016) whereas the reduction in LDL and atherogenic index confirmed that plants of Garlina have beneficial effect on hypercholesterolemia as reported by (Sharma *et al*., 2012; Hamauzu *et al*., 2011; Asgary *et al*., 2013; Ramesh *et al*., 2012; Heeba and Abd- Elghany, 2010).

The results of serum liver profile of Garlina at the dose of 2000mg/kg exhibited the increase ($p<0.05$) GGT level. The rise in Gamma glutamyltransferase (GGT) is one of the most sensitive indicators of liver and heart diseases (Kunutsor *et al*., 2016). Hence, the sub-chronic toxicity was assessed by daily oral dosing of Garlina 2000mg/kg for a period of one month which may enhance the risk of cardiac and hepatic morbidity rate (Sanyal *et al*., 2015). The decrease measure of total protein in serum kidney profile at the dose of 2000mg/kg can reflect the malnutrition state which may be due to a lack of insufficient absorption of protein. Moreover, the low levels of total protein may indicate a sign of liver disease or other acute infections (Thierry *et al*., 2011). Though, the imbalance in GGT and total protein levels is emerge at the dose of 2000mg/kg which is 100 folds than human dose however, these effects may be absent at recommended human dose.

The normal hematological and histopathological features indicate that Garlina administered orally either once at a dose of 5000 mg/kg or 2000 mg/kg/day continuously for a period of one month did not produce any significant change in normal and treatment groups.

According to the Organization for Economic Co-operation and Development (OECD) guideline 425 (OECD guideline, 2008), if no animal die as a result of a limit test at one dose level of 5000mg/kg, there is no need to test higher dosage. Therefore, the acute toxicity of Garlina can be expressed as being greater than 5000mg/kg body weight. Practically, 5000mg/kg of Garlina is the upper limit that can be administered by an oral gavage tube to the test animal.

CONCLUSION

The detailed investigation of acute and sub-chronic toxicity of Garlina in rats did not caused lethality or produced any remarkable toxic, hematological and histopathological signs. However, there is some alteration in serum biochemical parameters and these may be absent at the prescribed human dose. Therefore, it appears that Garlina is relatively safe herbal preparation for human consumption.

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