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Taofeeq Oduola
Department of Chemical Pathology, School of Medical Laboratory Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria

Abdulahi Abuubakar Ngaski
Department of Chemical Pathology, School of Medical Laboratory Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria

Safiyya Idris Aminu
Department of Chemical Pathology, School of Medical Laboratory Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria

Abdurrasheed Muhammed Ola
Department of Histopathology, School of Medical Laboratory Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria

Samuel Ayooye Ajayi
Department of Morbid Anatomy and Forensic Medicine, Usmanu Danfodiyo University Teaching Hospital, Sokoto, Nigeria

Fatimah Ayelabegan
Department of Chemical Pathology, School of Medical Laboratory Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria

Ganiyat Busayo Oduola
Department of Chemical Pathology, Ladoke Akintola University of Technology Teaching Hospital, Osogbo, Nigeria

Correspondence
Taofeeq Oduola
Department of Chemical Pathology, School of Medical Laboratory Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria

Use of *Gliricidia Sepium* leaf extract in the management of sickle cell disease: evaluation of possible adverse effect on liver functions in wistar rats

Taofeeq Oduola, Abdulahi Abubakar Ngaski, Safiyya Aminu Idris, Abdurrasheed Muhammed Ola, Samuel Ayooye Ajayi, Fatimah Aiyelabegan and Ganiyat Busayo Oduola

Abstract
Gliricidia sepium aqueous leaf extract is being used in parts of Nigeria by herbalists in the management of sickle cell disease. However, its safety for human consumption have not been evaluated, hence this study was designed to investigate the effect of ingestion of the extract on the liver functions in Wistar rats. The acute oral toxicity (LD_{50}) and subchronic toxicity studies of the leaf extract was determined in Wistar rats according to the procedures of Organization for Economic Cooperation and Development (OECD 423 Limit test) and OECD 407 respectively. At the end of the procedures, the rats were sacrificed; blood samples and liver were taken for liver function profile and histological examination respectively using standard techniques. In both acute oral and subchronic toxicity studies, there was no significant difference ($p>0.05$) in the biochemical and histology results of both experimental and control rats. *Gliricidia sepium* aqueous leaf extract is relatively non-toxic and unlikely to induce liver damage.

Keywords: *Gliricidia sepium*, sickle cell disease, liver function profile, liver histology

Introduction
Sickle cell disease (SCD) is caused by substitution of valine for glutamic acid at amino acid 6 in the β-globin chain of haemoglobin A (HbA), which produces haemoglobin S (HbS). Deoxygenation of HbS results in its polymerization which leads to decreased deformability of red blood cells (RBCs) [1, 2]. Through a complex interplay of adhesive events among blood cells, these altered erythrocytes can obstruct the vasculature, producing episodes of pain, hemolytic anemia, organ injury, and early mortality. Although the molecular basis of SCD is well characterized, the complex mechanisms underlying vaso- occlusion (VOC) have not been fully elucidated [2].

The liver performs important functions in the maintenance and regulation of body homeostasis. Liver is one of the most vital organs that functions as a centre of metabolism for nutrients such as carbohydrates, proteins and lipids; and excretion of waste metabolites. It is also involved in the biochemical pathways to growth, immune response, nutrient supply, energy provision and reproduction and excretion of drugs and other xenobiotic from the body [3].

Traditional herbal medicines are naturally occurring plant-derived substances with minimal or no industrial processing that have been used to treat illness within local or regional healing practices. Traditional herbal medicines are getting significant attention in global health debates. According to WHO, 80% of African populations use some form of traditional herbal medicine [4, 5]. and about 70% of the world population currently uses medicinal herbs as complementary or alternative medicine [5].

Gliricidia sepium (Jacq.) steud is a fast growing, medium size, tropical, semi-deciduous leguminous tree that belongs to the family Fabeacae, subfamily Faboideae(Papilionoideae) [6]. It originated from Central America, and different parts of the plant are used in many tropical and sub-tropical countries for different purposes [7]. Different parts of *Gliricidia sepium* plant including its stem bark, leaves, roots, fruits and seeds have been used in the treatment of various infectious diseases such as diarrhea, dysentery, helminthes and other gastrointestinal tract infections, skin diseases, wound infections [8]. Oduola *et al* [9] reported the use of *Gliricidia sepium* leaf extract in the management of sickle cell disease. The plant material contains different chemicals which might be toxic and even life threatening to the body at certain concentrations. This toxicity of herbal products will definitely affect the body system, particularly some vital organs such as the liver and the kidneys [8]. According to previous studies, liver injury from herbal preparation ranges from mild elevation of liver enzymes to

fulminant hepatic failure requiring liver transplantation, hence knowledge about the potential adverse effect of herbal remedies should be increased [10]. This study is therefore, designed to investigate the effect of intake of *Gliricidia sepium* leaf extract on liver functions in Wistar rats.

2. Materials and methods

2.1. Plant collection and identification

Fresh leaves of *Gliricidia sepium* were collected from Osogbo, Osun state, Nigeria. The plant was identified and authenticated at the Herbarium unit of Botany Department by Mr. G.A Ademoriyo, Obafemi Awolowo University, Ile Ife, by comparing with established Herbarium specimen with voucher number: IFE/17460 reference number which was kept at the Herbarium.

2.2 Preparation and extraction

Fresh leaves of *Gliricidia sepium* were collected and air dried at room temperature over a period of 6 weeks. The dried leaves were ground manually using mortar and pestle. One gram (1 g) of the grinded plant material was soaked in 5 mls of 80% methanol for 24 hours on a mixer to ensure maximum extraction by maceration technique at room temperature. This is followed by periodic stirring.[11] Resulting crude extract was filtered using Whatman number 1 filter paper and the filtrate was concentrated in an oven at 40℃ to obtain 63.5 g of green crude extract.

2.3 Experimental animals

Adult wistar rats of both sexes, weighing 150 g to 170 g purchased from the animal house of the Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, Nigeria were used for the study. The rats were housed in well aerated cages under hygienic conditions in the animal house of Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto for the duration of the study. They were allowed to acclimatize for a period of 2 weeks before the commencement of the study. The animals were fed with pelletized growers feed (Vital®), obtained from Grand Cereal Soil Mills Limited, Jos, Nigeria. They were also allowed access to clean drinking water *ad libitum* throughout the experimental period. Cleaning of the animal cages was carried out on a regular basis. The animals were maintained as described by Aniagu *et al.* [12] in a clean metabolic cage-sand, placed in a well-ventilated room with a temperature of 26˚C to 28˚C, photoperiods of 12 hours light and 12 hours darkness. All the experimental protocols were in compliance with our Institutional Animal Ethics Committee guidelines as well as internationally accepted practices for use and care of laboratory animals as contained in US guidelines [13] and also in accordance with the recommendations of the International Association for the Study of Pain (IASP) [14].

2.4 Experimental Design

2.4.1 Acute toxicity study

Acute oral toxicity study, Limit Test, was performed in accordance with the procedures outlined by the Organization for Economic Co-operation and Development Guidelines 423(OECD) [15]. Eighteen Wistar rats of both sexes were used for this study. The rats were randomly divided into three groups of six rats per group with the first group serving as control. The extract was administered to the rats in groups 2 and 3 in single oral doses of 2000 mg/kg and 5000 mg/kg body weight respectively, by intra gastric gavage using oral cannula (a feeding needle), one animal per day starting with group 2. The animals were observed within the first 4 hours and subsequently 24 hours for toxic symptoms. Also, behavioural parameters and mortality were closely monitored for 14 days. Lethal dose in 50% of total population (LD_{50}) was determined using OECD method as described by Aniagu *et al.* [12]

2.4.2 Sub-chronic Toxicity Study

Sub-chronic toxicity study was carried out following OECD 407 guidelines (OECD) [16]. Forty Wistar rats of both sexes were divided into five groups of eight rats per group. Group 1 served as the control and received distilled water as vehicle. Graded doses of crude extract were administered orally to the rats in groups 2, 3, 4 and 5. The doses given to the groups were 250 mg/Kg, 500 mg/Kg, 750 mg/Kg and 1000 mg/Kg body weight respectively daily for 28 days. All the rats had free access to feed and water throughout the period of the experiment and they were observed daily for general symptoms of toxicity and mortality.

2.4. Technique for obtaining blood and serum samples

Blood was collected by cardiac puncture from chloroform anaesthetized rats into heparinized bottles. The rats were later sacrificed using lumbar dislocation and their livers were removed. The blood samples collected were centrifuged at 4000 revolution per minute (4000 rpm) for 10 minutes. The plasma of each sample was separated and transferred into cryovial and stored (frozen) at -20℃ until required for analysis [12].

2.5 Histopathology

The livers of the animals were fixed in 10% formalin in labelled bottles after which the tissues were processed routinely using automatic tissue processor. This was immediately followed by embedding in paraffin wax. Sections of 5-6 µm thick were cut, stained with haematoxylin and eosin and examined under the light microscope [17].

2.6. Determination of biochemical parameters

The biochemical analyses were performed at the Chemical Pathology Laboratory of Usmanu Danfodiyo University, Sokoto. The separated Plasmas were used for the assays of ALT and AST [18] GGT [19] ALP [20] TB and CB [21] TP [22] and ALB [23].

2.7 Statistical analyses

The results obtained from the study were expressed as mean ± standard deviation. Statistical differences were compared with independent student t-test. P values < 0.05 were considered to be significant. All statistical tests were carried out using statistical package for social science (SPSS) for windows, version 20.0 (SPSS Inc., Chicago, IL, USA).

3.0 Results

3.1 Acute toxicity

Table 1 shows the result of acute oral toxicity (LD_{50} determination) in wistar rats. The result showed that no behavioural changes or death was recorded in both the control and the treated groups after 24 hours and up to 14 days. This indicates that the LD_{50} is greater than 5000 mg/kg.

Table 1: Acute Oral Toxicity (LD_{50}) of *Gliricidia sepium* leaf extract in Wistar Rats

Groups	Dosage/kg body weight	Observation period	Behavioural changes	Mortality
Group 1 (Control) (n=6)	**Distilled water**	**Up to 48 hours**	**None**	**None**
Group 2 (n=6)				
Day 1:1st rat	2000mg/kg	Up to 72 hours	None	None
Day 2:2nd rat	2000mg/kg	Up to 72 hours	None	None
Day 3:3rd rat	2000mg/kg	Up to 72 hours	None	None
Group 3(n=6)				
Day 1:1st rat	5000mg/kg	Up to 72 hours	None	None
Day 2:2nd rat	5000mg/kg	Up to 72 hours	None	None
Day 3:3rd rat	5000mg/kg	Up to 72 hours	None	None

Keys: n= Number of rats per group

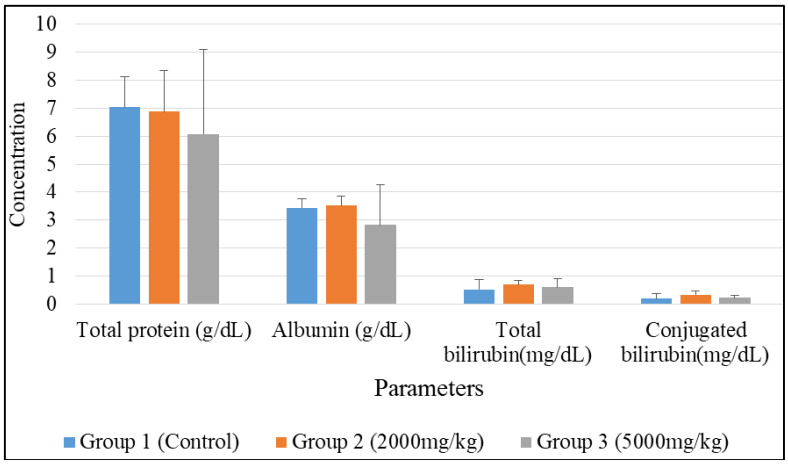

Fig 1: Liver Function Profile of Wistar Rats Exposed to *Gliricidia sepium* Leaf Extract in Acute Oral Toxicity Study

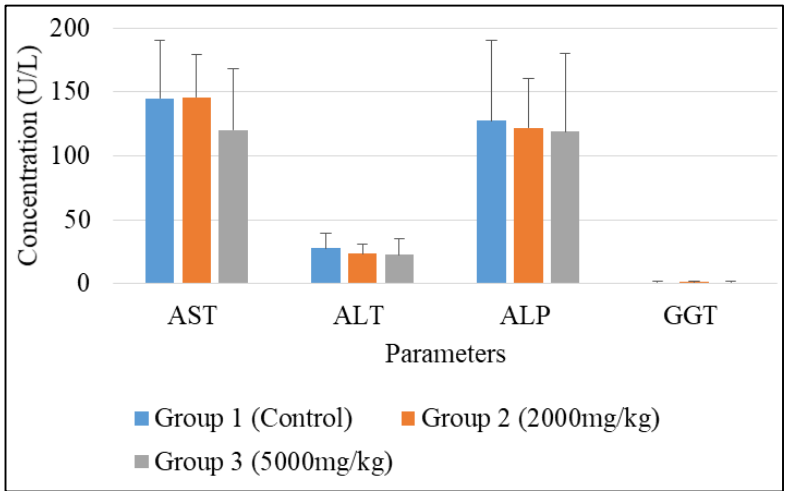

Fig 2: Liver Function Profile of Wistar Rats Exposed to *Gliricidia sepium* Leaf Extract in Acute Oral Toxicity Study

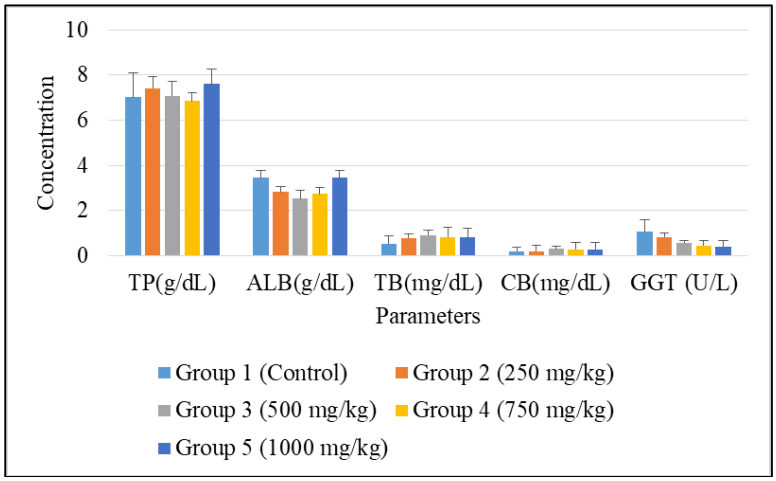

Fig 3: Liver Function Profile in Wistar Rats Exposed to *Gliricidia sepium* Leaf Extract in Sub-chronic Toxicity Study.

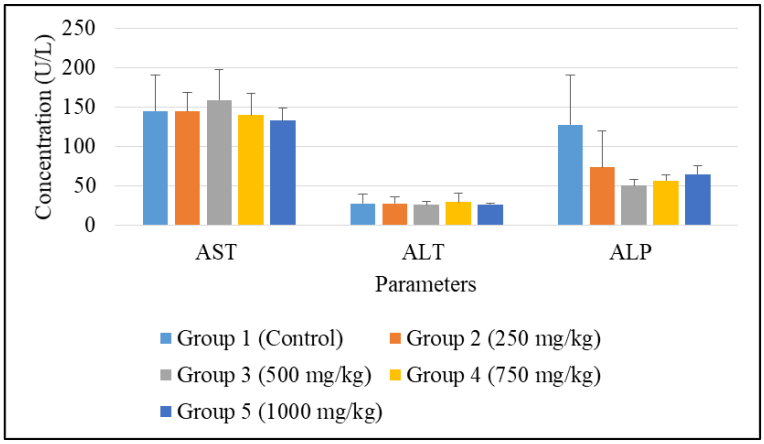

Fig 4: Liver Function Profile in Wistar Rats Exposed to *Gliricidia sepium* Leaf Extract in Sub-chronic Toxicity Study.

3.3 Histological Results

Figures 5, 6 and 7 represents the liver cells (H & E X100) of Groups 1, 2 and 3 in acute oral toxicity study.

Figures 8, 9, 10, 11 and 12 represents the liver cells (H & E X100) of Groups 1, 2, 3, 4 and 5 in sub-chronic toxicity study.

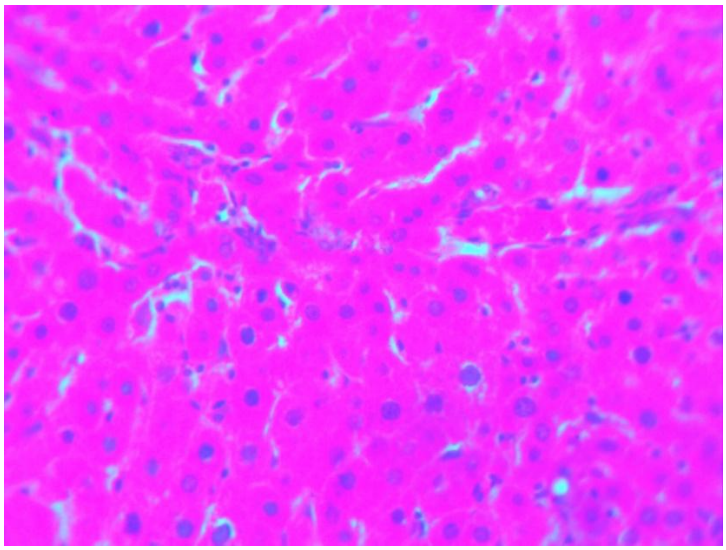

Fig 5: Group 1 (control) showing photomicrograph of liver cells (H & E X100) in oral acute toxicity study

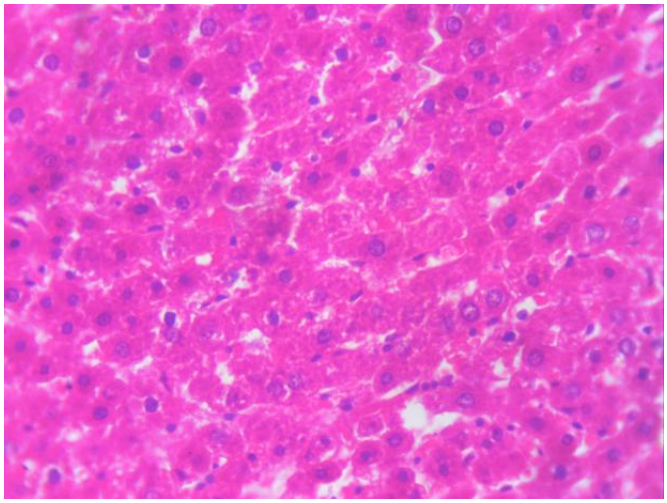

Fig 6: Group 2 (2000mg/kg) showing photomicrograph of liver cells (H & E X100) in oral acute toxicity study

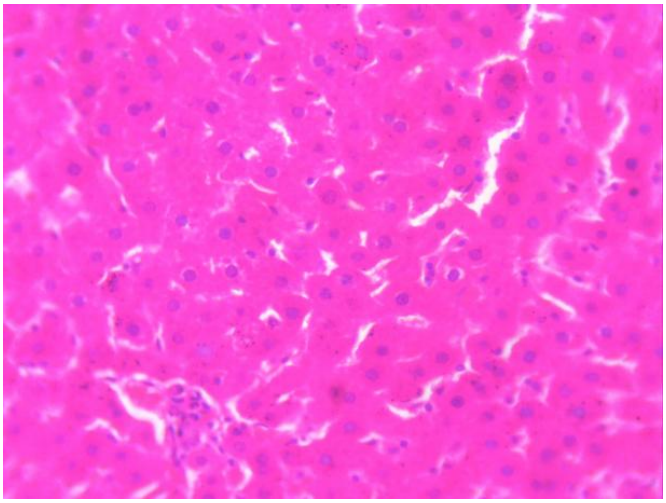

Fig 7: Group 3 (5000mg/kg) showing photomicrograph of liver cells (H & E X100) in oral acute toxicity.

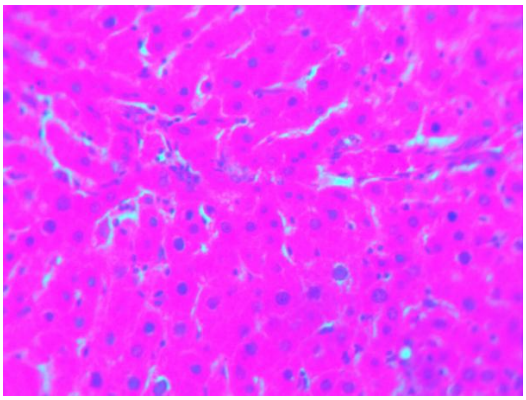

Fig 8: Group 1 (control) showing photomicrograph of liver cells (H & E X100) in sub-chronic toxicity study.

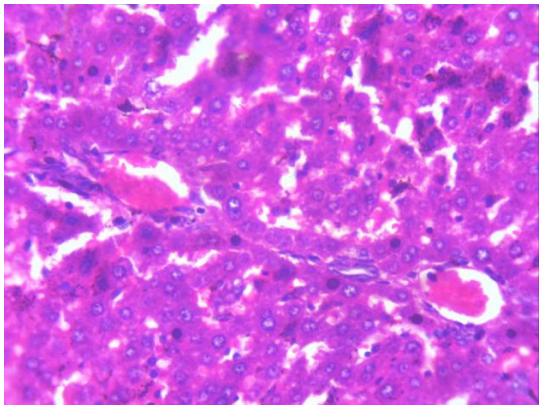

Fig 9: Group 2 (250mg/kg) photomicrograph of liver cells (H & E X100) in sub-chronic toxicity study

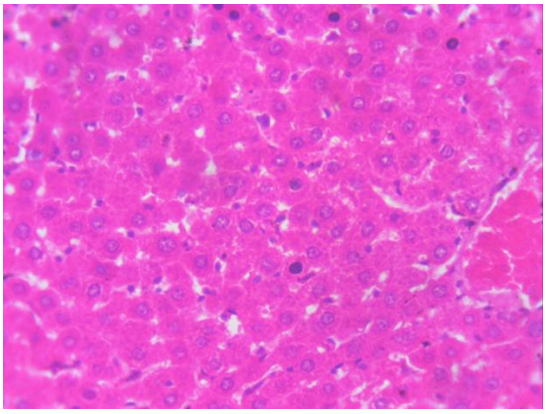

Fig 10: Group 3 (500mg/kg) photomicrograph of liver cells (H & E X100) in sub-chronic toxicity study

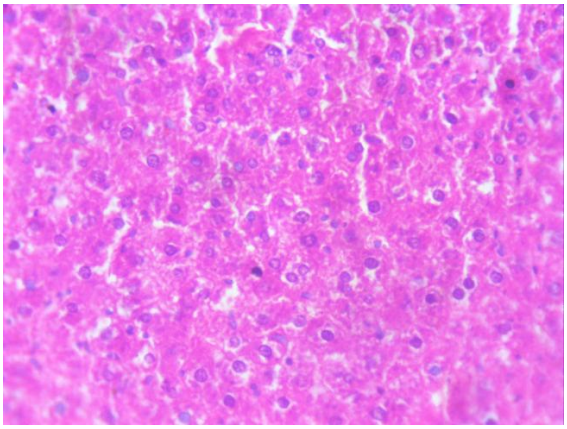

Fig 11: Group 4 (750mg/kg) photomicrograph of liver cells (H & E X100) in sub-chronic toxicity study

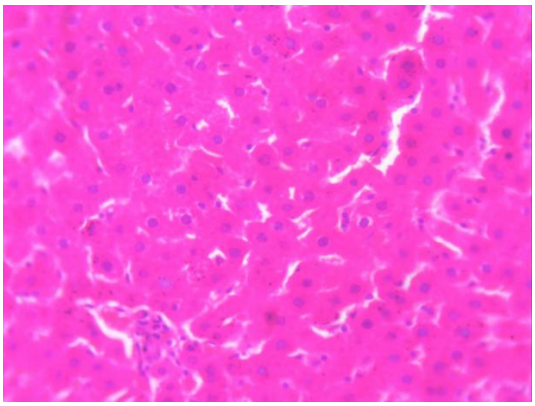

Fig 5: Group 5 (1000mg/kg) photomicrograph of liver cells (H & E X100) in sub-chronic toxicity study

4. Discussion

Herbal products, due to their natural origin, are used to be considered as safe for human consumption. However, some reports suggest potential risks involved with the use of such plants [24]. Adetuyi [25] established the presence of some active phytochemical compounds in *Gliricidia sepium* such as flavonoids, sterols, alkaloids, glycosides, tannins, saponins, medicarpin, coumarin, and coumaric acid.

In this study, no death or sign of toxicity was recorded in the experimental animals, group 2 (2000mg/kg) and group 3 (5000mg/kg) in acute oral toxicity after 24 hours and up to 14 days. This indicates that the LD_{50} of *Gliricidia sepium* leaf extract is higher than 5000mg/kg, hence it is relatively safe. This finding is in agreement with the work of Clarke and Clarke [26] who reported that any compound or drug with oral LD_{50} estimates greater than 1000 mg/kg body weight could be considered to be of low toxicity and safe. Also according to Globally Harmonised System of Classification and Labelling of Chemicals (GHS), a chemical is not classified as toxic, fatal or harmful if the LD_{50} is greater than 5000mg/kg (GHS,) [27]. However, Zbinden and Roversi [28] suggested that variables such as animal species, strain, age, gender, diet, bedding, ambient temperature, caging conditions, and time of the day can all affect the LD_{50} values obtained and as such are considerable uncertainties in extrapolating the LD_{50} obtained for species to other species.

In the acute oral toxicity study, there was no significant difference ($p > 0.05$) in all the parameters between the experimental and control rats. Hepatocellular damage is often assessed by measuring plasma level of ALT and AST. In this study, there was no significant difference ($P>0.05$) in the plasma concentration of AST and ALT of experimental and control rats in both acute and sub-chronic toxicity studies, an indication that the intake of *Gliricidia sepium* aqueous leaf extract did not cause hepatocellular damage in Wistar rats.

ALP and GGT are markers of cholestasis and are most often measured in the assessment of hepatobiliary disease. Elevations of ALP and GGT are associated with hepatobiliary obstruction. They are highest in cases of intrahepatic or posthepatic biliary obstruction, reaching activities of 5 to 30 times the upper reference limit [29] In this study, there was no significant difference ($P>0.05$) in the plasma concentration of ALP and GGT of experimental and control rats in acute oral toxicity study, but their values were significantly reduced ($P<0.05$) in experimental rats than the control rats in sub-chronic toxicity study. Although reason for this observation cannot be explained, it is the elevation of these enzymes that is associated with liver disease. This observation is an inference that the extract did not induce cholestasis in the rats.

Albumin values were significantly decreased ($p < 0.05$) in group 2 (250mg/kg), group 3 (500mg/kg) and group 4 (750mg/kg) compared to the control group whereas group 5 that were administered with the highest dose (1000mg/kg), the albumin values were not significantly different from the control group. Hence there was no dose-response relationship. Total protein values of experimental rats did not show significant difference ($P>0.05$) compared to the control rats, hence synthetic function of the liver was not impaired. Total bilirubin and conjugated bilirubin values did not show any significant difference ($P>0.05$) between the control and experimental rats, hence the detoxification function of the liver was not affected.

Histopathological examination of liver tissues of control and experimental rats reveal no lesion or malignancy. The liver cells were essentially the same, an indication that aqueous *liricidia sepium* leaf extract did not induce any lesion in the liver cells of the rats.

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6. Competing interests

Authors have declared that no competing interests exist.

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