Acute and sub-chronic toxicity studies of an aqueous stem bark extract of *Sclerocarya birrea* using a rat model.

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Abstract

Background: *Sclerocarya birrea,* a plant that is commonly available in many communities, is used as a source of food and for ethnomedicinal and cultural practices. Stem-bark toxicity studies for the plant are however lacking. This study was therefore conducted in an effort to determine its toxic effects using a rat model.

Methods: Acute toxicity was performed using a single oral administration of 50, 100, 200, 400, 800, 1000 and 2000mg/kg body weight of *S. birrea* to determine the lethal dose. Test animals in the sub-chronic study received 50, 100, 200, 400, 800, 1000 and 2000mg/kg body weight of *S. birrea* for 28 days.

Results: No animal mortality occurred during acute toxicity testing, suggesting that the lethal dose is probably higher than 2000mg/kg body weight. Behavioural and posture changes were however observed with the higher doses. During the sub-chronic test, animals in the 1000mg/kg and 2000mg/kg groups showed a significantly ($p<0.05$) smaller growth rate as compared to the animals in the other groups. Significant organ to body weight ratio changes were observed only in the livers and kidneys of animals that received 800, 1000mg/kg and 2000mg/kg body weight of *S. birrea* suggesting possible toxicity. This was supported by increases in direct bilirubin, total protein, albumin, AST and ALT. In addition, histopathological changes to the liver and kidneys were observed with increasing dosages of the plant extract.

Conclusions: The results of the study indicate that at doses of ≥1000mg/kg, the extract affects growth rate as well as liver and kidney function. Therefore, high doses should be used with caution.

Key words: Acute toxicity, *Sclerocayra birrea*, Stem bark, Sub-chronic toxicity.

Introduction

A large percentage of the population in developing countries relies heavily on traditional medicine practitioners and herbal plants to meet their primary healthcare needs. The high cost of medicines, and concurrent shortage of drugs, has necessitated the need for many people in several African countries to go back to the old ways of using traditional herbal concoctions and decoctions for their ailments [1]. The disadvantage however, is that most of the traditional medicinal plants have never been exposed to toxicological tests such as those required for modern pharmaceutical compounds [2,3], and little to no research has been carried out based on WHO guidelines on the safety and efficacy of herbal medicines. The reason for this is that medicinal plants are often assumed to be safe based on their past traditional use, and they also provide a cheaper alternative as compared to modern medicine [4].

Phytochemical studies by numerous researchers have however, shown that a large number of these plants have *in vitro* toxic, mutagenic and/or carcinogenic effects [5,6]. The toxicity and adverse effects of African medicinal plant use may arise due to a number of factors or issues including inherent poisonous phytochemicals, adulteration of the medicines, contamination with various chemicals and heavy metals, herb-drug interactions, and poor quality control of herbal products [7]. As a result, there has been increased emphasis on research and development focused on the safety, efficacy and quality of medicinal plants as they might be developed into medicinally useful drugs.

Sclerocarya birrea, a tree that grows abundantly in southern Africa, is an example of a plant commonly used by local communities whose toxicity profile is still lacking. Numerous communities use the plant as a source of food, and for ethnomedicinal and cultural practices [8]. The tree is one of the greatest trees indigenous to Africa, with different species occurring from Ethiopia in the north, to KwaZulu-Natal Province in the south [9]. Every part of the plant is utilised either for nutrition or medicinally. The stem bark, roots and leaves of *S birrea* are used traditionally to treat malaria and fevers, diarrhoea and dysentery, headaches, toothache, backache and body pains, schistosomiasis, epilepsy, general musculo-skeletal system disorders, diabetes mellitus, circulatory

system ailments, and so on [10-12]. Considerable medical significance however, is attached to the stem bark from *S. birrea*. It is traditionally assumed that the mature bark on the trunk of the tree is the most potent medicinally; and it's believed to be more potent than other parts of the plant [13]. Toxicological studies involving *S. birrea* stem bark however, have not been reported yet. This study was thus conducted in an effort to determine the toxicological effects, if any, of *S. birrea* stem-bark using a rat model.

Methodology

Ethics

Experimental protocols and procedures used in this study were approved by the Joint Research and Ethics Committee (JREC) of the University of Zimbabwe and conformed to *"Handbook of laboratory animal management and welfare"* [14].

Collecting the samples

The stem bark of *Sclerocarya birrea* was collected from Plumtree in Zimbabwe. The plant specimen were identified and authenticated by a botanist from the Harare botanical gardens.

Preparation of *Sclerocarya birrea* stem bark aqueous extract

One kilogram of fresh stem bark was air-dried at room temperature ($26\pm1°C$) for two weeks. The dried stem bark was then milled into a fine powder, and macerated in 2.5litres of distilled water with occasional shaking, for 48 hours at room temperature ($26\pm1°C$). A rotary evaporator was used to concentrate the aqueous extract by drying it at $60\pm1°C$. Aliquot portions of the plant's crude stem bark extract residue were weighed and dissolved in distilled water (at room temperature) for use on each day of the experiments.

Animals

48 Sprague Dawley rats were used in the experiments. The animals were acclimatised to the laboratory conditions for at least two weeks prior to the experiments. Animals were housed in cages, each containing six animals, and these were labeled: A-control; B-50mg/kg; C-100mg/kg; D-200mg/kg; E-400mg/kg; F-800mg/kg; G-1000mg/kg; H-2000mg/kg and I-acute test [Ii)-Ivii)] depending on the treatment being given. Groups A to H were used for the sub-chronic study and group I was used for the acute toxicity study.

Acute toxicity studies

The aqueous extract of SBE 50, 100, 200, 400, 800, 1000mg/kg and 2000mg/kg body weight was administered by oral gavage to the seven groups of animals Ii-Ivii. The control group received distilled water only. Observations for any possible toxic symptoms were made and recorded. After the sample was administered, food but not water was withheld for a further 3-4 hours.

Sub-chronic Toxicity Studies

The animals were divided into seven groups with six rats in each group. Animals in Groups B, C, D, E, F, G and H were orally given extract of 50, 100, 200, 400, 800, 1000mg/kg and 2000mg/kg body weight once daily for 28 days respectively. Group A was the control group and received only distilled water by the same route. The body weight of each animal was recorded before commencing the experiments and every other day for a period of 28 days. Soon after dosing the rats were observed for a period of 10 minutes in an effort to note any changes in behavior. These included noting any changes in mobility, posture, vocalizations or any involuntary movements.

Blood sample and visceral organs collection for biochemical and histopatholigical analyses

Animals were fasted for 12 hours after the last treatment dose before being anaesthetized with chloroform. Anaesthetised animals were then weighed, slaughtered and blood was collected. Visceral organs (liver, kidneys, lungs and heart) were collected and weighed and organ to body weight ratios were calculated for each animal. The organs were then preserved in 10% formalin in air tight containers for histopathological examination.

Clinical chemistry

The collected blood was centrifuged at 3000rpm for 10 minutes to obtain sera. The analysis for the presence of different compounds was done using different methods. Serum total protein (Biuret method); albumin (Bromocresol Green (BCG) method); aspartate amino transferase (AST), alanine amino transferase (ALT), creatinine and bilirubin were determined using assay kits obtained from Randox laboratories.

Histopathologic examination

Morphological examination of the viscera was performed under the guidance of a histopathologist from the Histopathology Department (Parirenyatwa Group of Hospitals Zimbabwe). The liver tissues were cut and placed in imbedding cassettes. The cassettes were then placed in a tissue processor which was allowed to run for 16hrs. This was followed by the removal of the tissues and the preparation of the slides for observation under a light microscope and images were taken.

Statistical analyses

The collected data was analyzed using GraphPad Prism and Microsoft excel. The comparison between groups was carried out using the Scheffe multiple comparison method. Statistical significance was determined at $p \leq 0.05$. In all cases, data obtained from the plants extract 'tests' were compared with those obtained from distilled water (vehicle)-treated 'controls'. The differences between the data from 'test' and 'controls' was subjected to GraphPad Prism's (version 6.0) Student's t-test, and/or one-way analysis of variance (ANOVA; 95% confidence interval). The comparison between groups was carried out using the Dunnett and Goldsmiths (1993) *post-hoc* test. Statistical significance was determined at $p \leq 0.05$.

Results and Discussion

Acute toxicity

There were no deaths recorded in any of the groups subsequent to oral administration of *S. birrea* after the 24 hour observational period. This suggests that the LD_{50} of *S.birrea* bark extract is greater than 2000mg/kg. In addition, no significant changes in body weight were recorded for any of the animals in the groups. Behavioural changes in the form of reduced mobility were however noted in the animals given $\geq$1000mg/kg.

Table 1: Acute toxicity effects of *S. birrea*. Behavioural changes were observed with increases in concentration of *S. birrea* used.

ANIMAL NUMBER	DOSE	SIGNS OF TOXICITY OBSERVED
Group I i)-Control	0.0mg/kg	No changes in behaviour
Group I ii)	100mg/kg	No changes in behaviour
Group I iii)	200mg/kg	No changes in behaviour
Group I iv)	400mg/kg	No changes in behaviour
Group I v)	800mg/kg	No changes in behaviour
Group I vi)	1000mg/kg	Reduced movement in cages, vocalization and posture change
Group I vii)	2000mg/kg	Reduced movement in cages, vocalization and posture change

Sub-chronic toxicity

Growth rate

There was a general increase in body weight in all the groups. At the commencement of the study the mean mass of the animals ranged from 120-133g and this increased to 184-193g. The animals in the 1000mg/kg and 2000mg/kg groups showed a smaller growth rate as compared to the animals in the other groups. They had an increase in weight from 120-125g to 142-158g. The differences between the slopes of the animals in these groups and the control group were statistically significant (p<0.05). Group G and H (1000mg/kg and 2000 mg/kg) both showed a statistically smaller gradient as compared to the control group A (Fig 1).
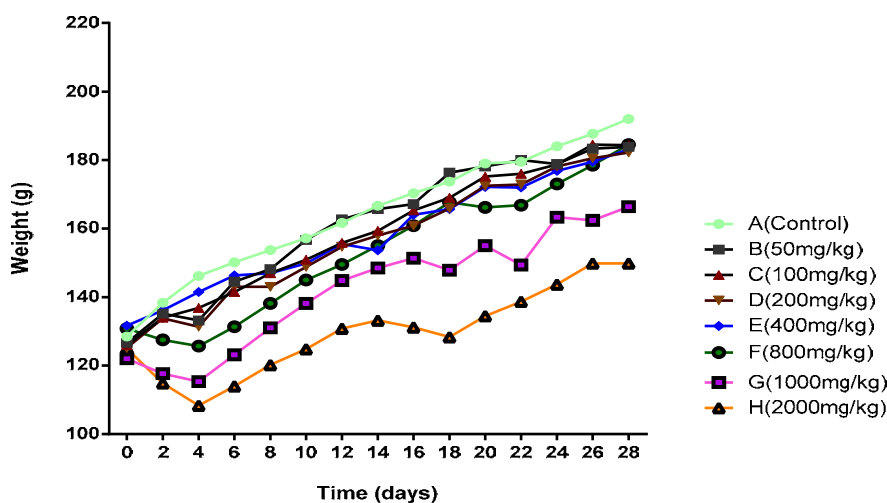

Figure 1: Effect of *Sclerocarya birrea* on the growth rate of albino rats using body weight as a parameter.

Organ weight to body weight ratios

Significant changes (p<0.05) in organ to body weight ratios were recorded only with the liver and kidney as the other organs showed no significant difference when compared to the control group (**Table 2**). The average kidney to body weight and average liver to body weight ratios for groups F, G and H showed significant differences when compared to the control group A. The percentage increase in liver weight for groups F, G and H (800, 1000, 2000mg/kg liver to body ratio were 12.6%, 16% and 17.7% respectively, relative to the control group. The percentage increase in kidney weight for groups F, G and H kidney to body weight ratio were 10%, 12% and 14.1 % relative to that of the control group A.

Table 2: Effects of *S. birrea* on organ to body weight expressed as ratios. SBE (800, 1000, 2000mg/kg) increased liver and kidney size.

ORGAN TO BODY WEIGHT RATIOS			
Group	**Liver to body weight ratio**	**Kidney to body weight ratio**	**Heart to body weight ratio**	**Lung to body weight ratio**
A(Control)	0.0350	0.0071	0.0035	0.0060
B(50mg/kg)	0.0359	0.0073	0.0040	0.0060
C(100mg/kg)	0.0381	0.0068	0.0036	0.0061
D(200mg/kg)	0.0366	0.0073	0.0039	0.0057
E(400mg/kg)	0.0367	0.0073	0.0038	0.0060
F(800mg/kg)	0.0391*	0.0077*	0.039	0.0060
G(1000mg/kg)	0.0406*	0.0079*	0.0039	0.0062
H(2000mg/kg)	0.0412*	0.0081*	0.0040	0.0061

Biochemical findings

Biochemical tests were conducted to determine the effects of *S. birrea* on the liver and kidney. Serum total protein concentration decreased with increases in *S. birrea* concentration. These values were not statistically significant for groups B-E (50-400mg/kg) relative to the control group (p>0.05) but significant for groups F-H (p<0.05-0.001) (**Figure 2**). This could possibly indicate the presence of liver or kidney diseases. Increased levels of creatinine were observed in all the animal groups (**Figure 3i**). These increases in creatinine concentration, were however not statistically significant between groups B-E (50-400mg/kg) and the control group A (p>0.05), but they were significant between groups F-H (800-2000mg/kg). Increases in creatinine may indicate kidney impairment or kidney disease. Direct bilirubin levels increased with increases in *S. birrea*. These increases were statistically significant at p<0.001 for only groups G and H versus the control (**Figure 3ii**). Increases in direct bilirubin indicate decreased elimination of bilirubin by the liver cells. AST and ALT concentration increased significantly (p<0.05) in all the groups with increases in the dose of *S. birrea* administered (**Figure 4i and 4ii**). Increases in AST and ALT indicate liver damage.
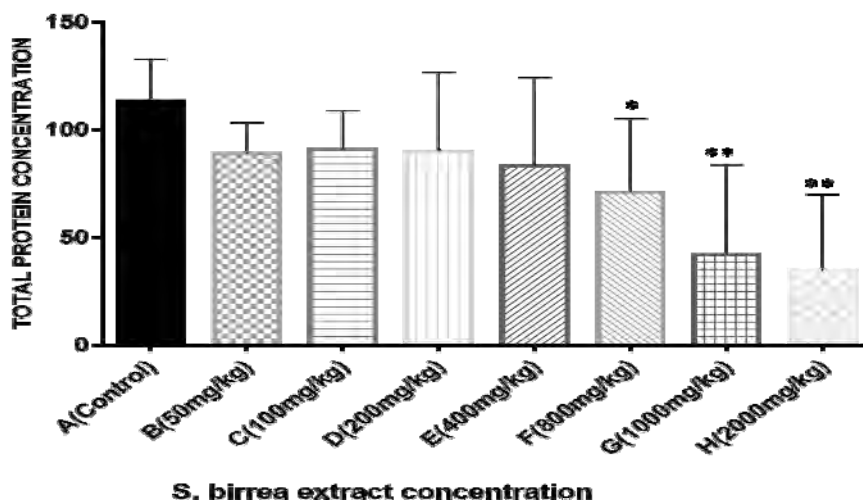

Figure 2: The effect of *S. birrea* on serum total protein. Serum total protein concentration decreased with increases in the dose of SBE administered.
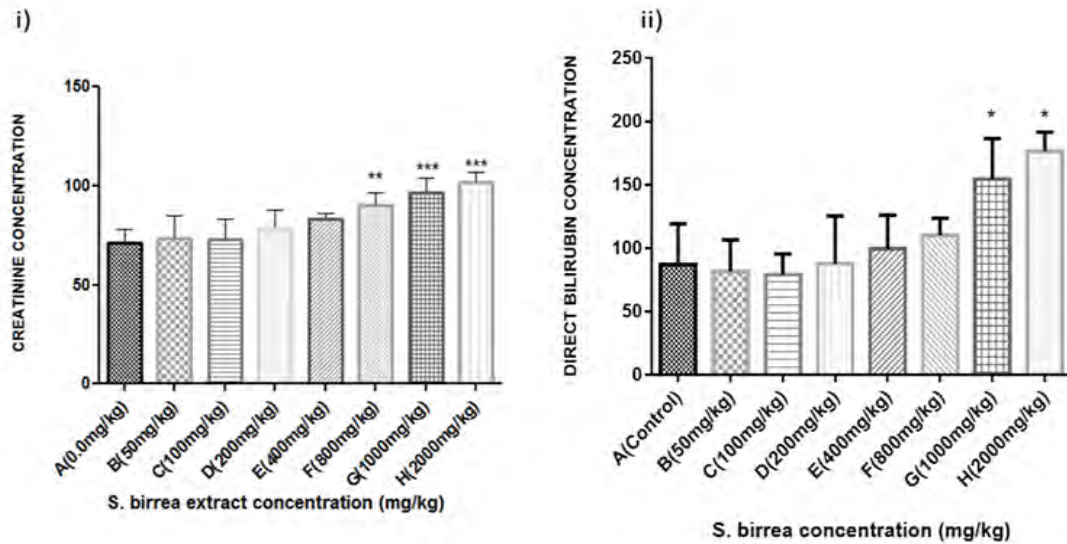
Figure 3: The effects of *S. birrea* on: i) serum creatinine concentration ii) direct bilirubin concentration. Both serum creatinine concentration and direct bilirubin increased with increases in SBE concentration.
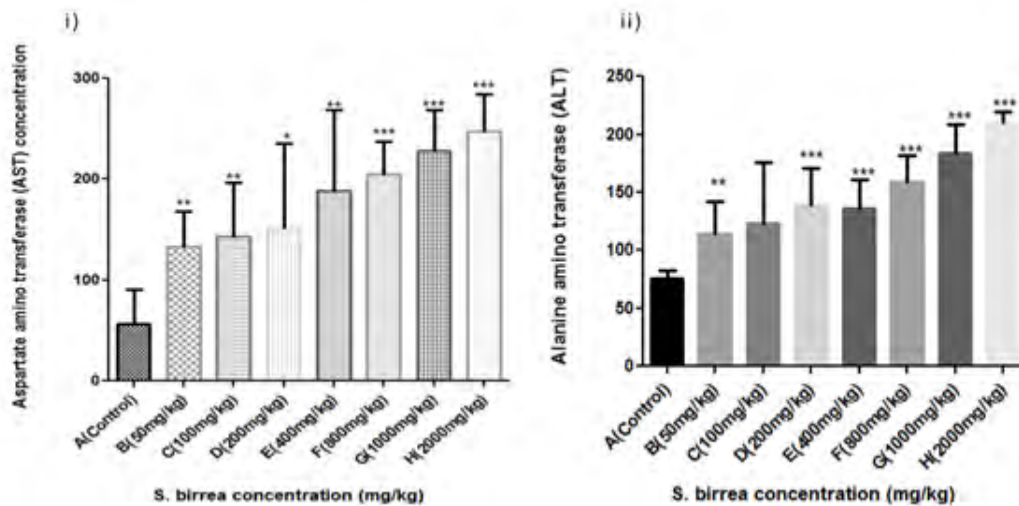

Figure 4: Effect of *S. birrea* on i) aspartate amino transferase concentration (AST) and ii) alanine amino transferase enzymes (ALT). AST and ALT levels increased significantly with increases in SBE concentration

Histo-pathological effects

Liver

There were no significant histo-pathological changes in the livers of animals in group B (50mg/kg) compared to the control group. Histopathological changes were however observed on the animals in groups C-H (100, 200, 400, 800, 1000 and 2000 mg/kg). These changes include: the presence of congested blood vessels, peritoneal inflammation, mononuclear cell infiltration, presence of fatty cell vacuoles, peri-portal inflammation, cell necrosis; and eosinophil infiltration
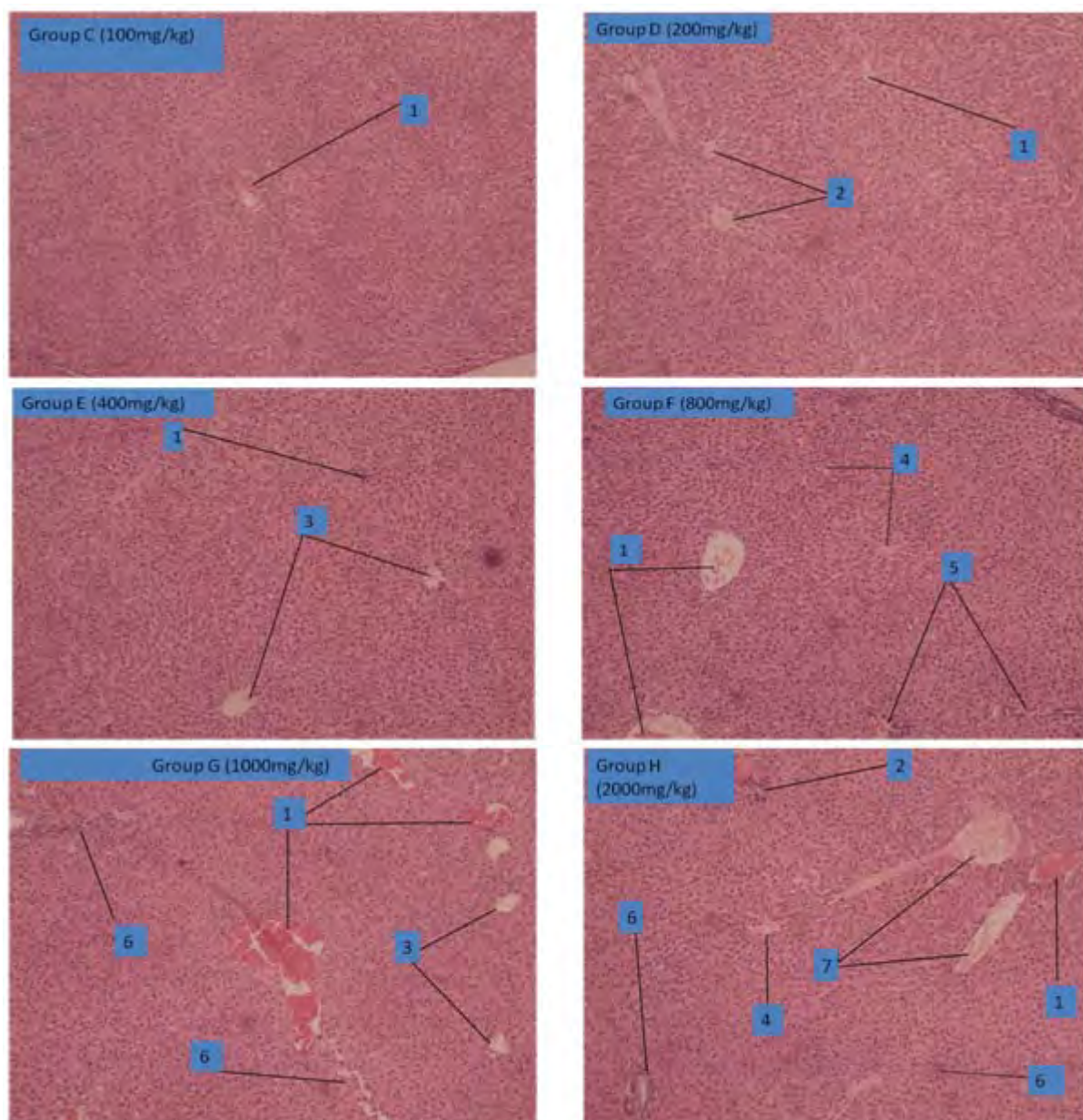
Figure 5: Effects of *S. birrea* on liver cells. The extent of liver damage increased with increases in the SBE concentration.

Key: **1**-Congested blood vessels; **2**-peritoneal inflammation; **3**-mononuclear cell infiltration; **4**-fatty cell vacuoles; **5**-periportal inflammation; **6**-cell necrosis; **7**-eosinophil infiltration. These observations indicate possible liver damage.

Kidney

There were no major histological changes observed in the animals treated with SBE 50-100mg/kg (Groups B and C). Histo-pathological changes were however observed with animals in groups D-H. These changes include: eosinophil infiltration, acute interstitial nephritis, enlargement of the subcapsular space and cell death. These results indicate possible kidney damage (**Figure 6**).
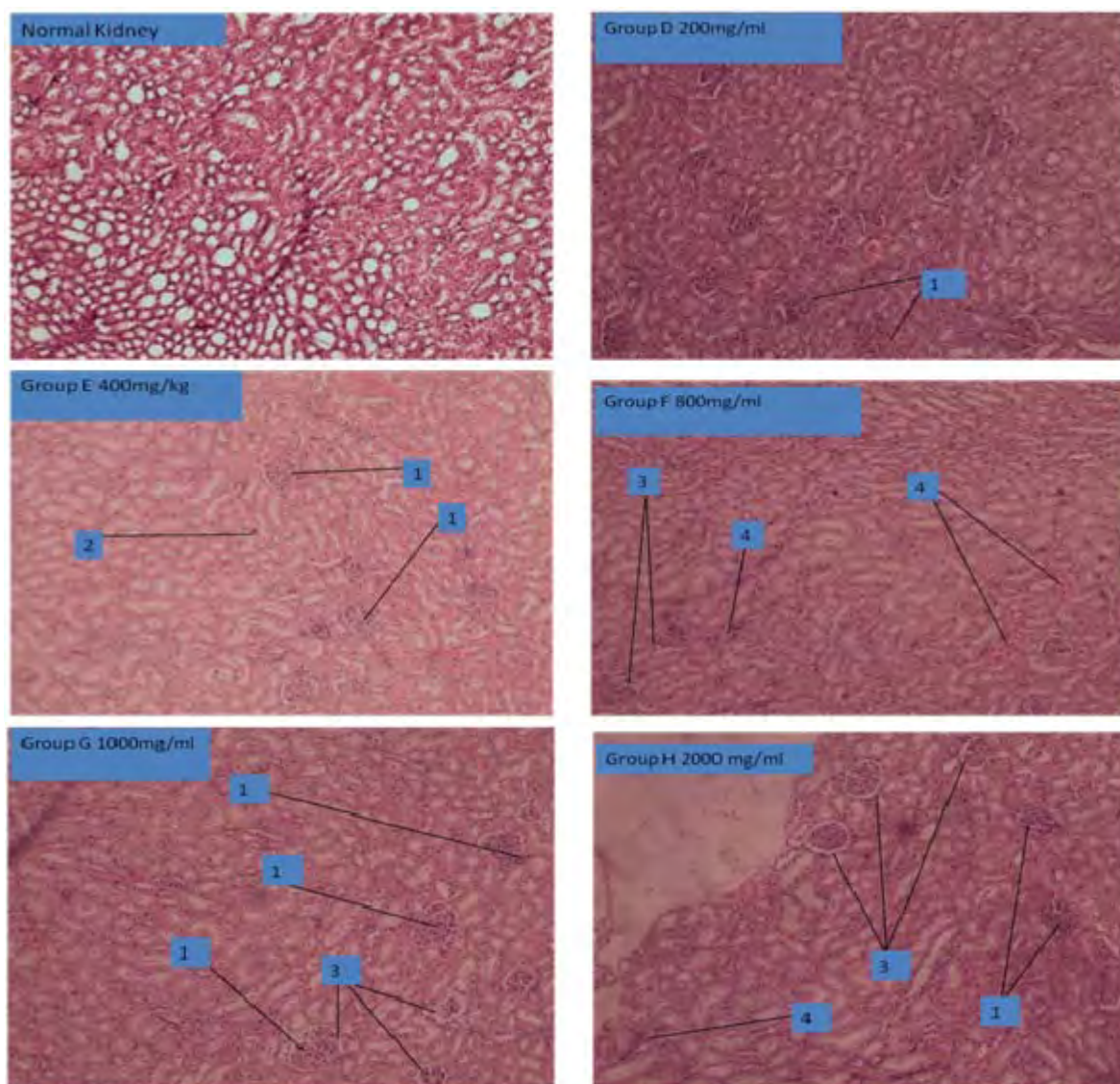
Figure 6: Effect of *S. birrea* on kidney cells

Key: 1- Eosinophil infiltration; 2- Acute interstitial nephritis; 3- Enlargement of the sub-capsular space; 4- cell death

Discussion

No lethal effects were observed during the acute toxicity tests on any of the test animals. Changes in behaviour were however noted in animals who received 1000 and 2000mg/kg of *S. birrea*. These animals reduced the frequency of their movements in the cages; decreased vocalization and posture changes were also observed. Thakur and Mengi (2005) report that locomotor activity is considered to be an index of alertness, and any decrease in locomotion can indicate sedation. Acute toxicity study results suggest that *S. birrea* might be sedating the animal when high doses are used. Therefore the estimated toxic dose might be above 800mg/kg.

Although all the test animals in the sub-chronic arm of the study gained weight throughout the experimental period, body weight results show that the growth rate of the animals that received *Sclerocarya birrea* bark was slower than that of the animals in the control group. Moreover, animals in groups B-F (50-800mg/kg) had weight gains of between 45-53% while animals in group G and H (1000-2000mg/kg) had increases of between 18-26%. This suggests that higher doses of *S. birrea* reduce weight gain.

In addition, decreases in body weight might be attributed to the phytochemicals present in *S. birrea*. Tannins, present in *S. birrea* [16], have been reported to have many anti-nutritional effects [17]. They are responsible for decreasing food intake, growth rate, decreasing food efficiency, net and metabolisable energy, and protein digestibility [17,18]. These effects are exerted through the formation of complexes with protein, starch and digestive enzymes [19]. Therefore the reduced growth rate which was observed might have been caused by the tannins present in the extract.

In toxicological experiments, comparison of organ weights between treated and untreated groups of animals have conventionally been used to evaluate the toxic effects of the test article [20,21]. This is because organ weight changes are a sensitive indicator of chemically induced changes to organs [22]. Liver tissue is analysed in toxicity tests because it can be used to evaluate or support diagnosis of hepatocellular hypertrophy from hepatic enzyme induction, peroxisome proliferation or lipidosis, while testing the kidneys helps to predict toxicity, enzyme induction, physiologic perturbations and acute injury [22].

In this study, organ to body weight ratios were significantly increased for the livers and kidneys of animals receiving the high doses. Animals in groups B-E (SBE 50-400 mg/kg) which had higher increases in body weight compared to animals in group G-H (1000, 2000 mg/kg), did not have significant increases in the size of the organs. Animals in group F however, had slight increases in organ to body weight ratios as well as increases in body weight. Animals in group G (1000mg/kg) had a 12.6% increase in the size of the liver and 11.3% in the kidney, while animals in group H (2000mg/kg) had a 16% and 14.1% increase in size for the liver and kidney respectively. According to Amacher *et al*., (2006) and Juberg *et al*., (2006), alterations in liver weight may suggest treatment-related changes including hepatocellular hypertrophy (e.g. enzyme induction or peroxisome proliferation). Greaves (2000) also reports that changes in kidney weight may reflect renal toxicity, tubular hypertrophy or chronic progressive nephropathy [25].

Results obtained in the histopathological and biochemical analysis of the liver and kidney tissues support the possibility of treatment-related changes in the organs. Histopathological findings showed that rat isolated liver cells liver cells in the animals had congested vessels, eosinophil infiltration, dark spots, fatty vacuoles and inflammation which are a suggestive of liver tissue damage. This was in line with the biochemical findings which showed elevated levels of liver enzymes ALT and AST in groups which received the high doses of the drug and an overall decrease in the amount of total serum proteins. Increased levels of direct bilirubin observed also indicate liver diseases which might be associated with the increased break down of the red blood cells. Creatinine levels seem to have increased with increased dosage of the plants extract. Elevated creatinine levels indicate either impaired kidney function or the presence of kidney disease. This was also supported by histological analysis of the kidneys which showed eosinophil infiltration, acute interstitial nephritis, enlargement of the sub-capsular space and cell death. These observed changes may be induced by the phytochemical constituents from the plant extract.

The results obtained from this study therefore indicate that at high doses, the extract has effects on growth rate, as well as liver and kidney function. As a result high doses should be used with caution. Studies on the active components and mechanism(s) of toxicity of the extract are therefore recommended.

Acknowledgements

Conflict of interest statement

We declare that there is no conflict of interest with other people or any financial organization regarding the material discussed in the manuscript

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