CHAPTER 5

TOXICOLOGICAL EFFECTS OF ORAL ADMINISTRATION OF AQUEOUS STEM BARK EXTRACT OF *STRYCHNOS HENNINGSII* GILG IN WISTAR RATS

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Toxicological effects of oral administration of aqueous bark extract of *Strychnos henningsii* Gilg bark in male Wistar rats

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Abstract

Strychnos henningsii Gilg is widely used in southern African traditional medicine for the treatment of various ailments. However, no safety studies have been conducted on its toxicological profiles. The effect of oral administration of aqueous bark extract of the plant at 250, 500 and 1000 mg/kg body weight was investigated on haematological and biochemical parameters in Wistar rats for 28 days. Treatment with the plant extract did not significantly ($P>0.05$) alter the levels of haemoglobin, red blood cell, haematocrit and mean corpuscular haemoglobin concentration. Large unstained cell index and organs body weight ratio of the kidneys, lungs and hearts were also unaffected. The levels of eosinophils, total bilirubin, albumin, urea, creatinine, sodium as well as calcium were also not significantly ($P >0.05$) different from the control. However, the concentration of platelets, monocytes, basophils and white blood cells significantly decreased while those of mean corpuscular haemoglobin, alanine and aspartate aminotransferases were increased. The levels of conjugated bilirubin, mean corpuscular volume and liver body weight ratio were significantly increased at specific doses while lymphocyte was decreased at the dose of 250 mg/kg. The levels of cholesterol and triacylglycerol as well as chlorine were decreased at the higher dose (1000 mg/kg). The results obtained from this study suggest that sub-acute administration of *S. henningsii* bark

extract may not be completely safe as an oral remedy due to the impairment observed on the normal functioning of white blood cells and platelets.

Keywords: *Strychnos henningsii*; biochemical; haematological; serum lipids; toxicity.

INTRODUCTION

The use of medicinal plants for healing purposes is very common in developing countries especially in the rural areas. It has been estimated that 80 % of the population of developing countries relied heavily on the herbal medicine for their primary health care (WHO, 1991). This is probably due to the perceived beneficial and lower adverse side effect of natural products that are extracted from the plants (Leonardo et al., 2000). However, most medicinal plants are used indiscriminately without knowing their possible adverse effect. Over the past decades, several reports in both developed and developing countries indicated adverse effects allegedly arising from the use of medicinal plants (Elvin-Lewis, 2001). Some of these effects include abortion of pregnancy, dizziness, vomiting, diarrhea, abdominal pain, fast heart beat, death, ulcer and loss of appetite (Gessler et al., 1995). These effects could be attributed to the presence of phytotoxic compounds in the plant extracts and lack of actual dosage necessary for the treatment of diseases (Azaizeh et al., 2003). In order to minimize this, there is need for a thorough scientific investigation on the toxicological effect of these plants at different doses. In addition, the World Health Organization has recommended that traditional plants used for the treatment of diseases warrant further evaluation for their toxicological properties (WHO, 1980).

Strychnos henningsii Gilg (Loganiaceae) is a small evergreen tree or shrub with leathery leaves, mostly distributed in east and southern African (Leeuwenberg, 1969). The decoction or infusions of the plant are widely used in southern African traditional medicine for the treatment of various ailments. These include gynaecological complaints, abdominal pain, snake bite, gastrointestinal pain, rheumatism, malaria and diabetes mellitus (Bisset, 1970; Hutchings, 1989). To the best of our understanding, there was no or little scientific report on the biological and pharmacological activities of *S. henningsii* to substantiate its traditional usage. However, previous phytochemical screenings conducted on *S. henningsii*

bark extract have demonstrated the presence of tannins, saponins, alkaloids, phenolics and flavonoids with strong antioxidant activities (Chapter 4). Plants containing tannins and alkaloids have been reported to have toxic side effects in man (Lacomblez, 1989). For instance, strychnine and retuline alkaloid among other compounds isolated by Sandberg and Kristianson (1970) have shown to be toxic and exert some adverse effects on mice.

Despite the traditionally usage of this plant in folk medicine, there was no information in the scientific literature to validate its acclaimed pharmacological properties and the possible toxicological profiles. Therefore the present study was undertaken to assess the safety of aqueous stem bark extract of *S. henningsii* at certain doses using haematology, serum chemistry, liver and kidney functional indices in animal model after acute and sub-acute administration.

MATERIALS AND METHODS

Plant Material

The bark of *S. henningsii* was collected in February, 2009 from a thick forest in Amathole District (Eastern Cape, South Africa). The plant was identified by its vernacular name and later authenticated by Prof. D.S. Grierson of Botany Department, University of Fort Hare. Voucher specimen (Sun MED 2009) was deposited at the Giffen herbarium of the University.

Chemicals and assay kits

The reagents used were of analytical grade and were supplied by Merck Chemicals (Pty) Ltd., Bellville, South Africa. All other assay kits used for the analysis of creatinine,

urea, calcium, sodium, chloride, albumin, cholesterol, triglycerides, alanine and aspartate aminotransferases were obtained from Roche Diagnostic GmbH, Mannheim, Germany.

Preparation of plant extract

The bark material was air-dried at room temperature in the laboratory. The dried bark was thereafter grounded into powdery form using an electric blender (Waring Products Division, Torrington, USA). About 80 g of the powdered plant material was extracted in 1000 ml of cold distilled water maintained on a mechanical shaker (Stuart Scientific Orbital Shaker, UK) for 48 h. The extract was filtered using a Buchner funnel and Whatman No.1 filter paper. The filtrate was quickly frozen at -40 ^{o}C and dried for 48 h using a freeze dryer (Savant Refrigerated vapor Trap, RV T41404, USA) to give a yield of 10.6 g of dry extract. The resulting extract was reconstituted in distilled water to give the doses of 250, 500 and 1000 mg/kg body weight used in this study.

Acute toxicity

Male rats (*Rattus novergicus*) of Wistar strain weighing 159.00 ± 7.20g were obtained from the animal house of the Agricultural and Rural Development Research Institute (ARDRI), University of Fort Hare. They were kept in aluminum cages placed in well ventilated house conditions (temperature 23 ± 1 ^{o}C; photoperiod: 12 h light and 12 h dark cycle; humidity: 40-45%). The animals were allowed free access to rat pellets (Balanced Trusty Chunks, Huguenot, South Africa) and distilled water. The acute toxicity of the plant extract was evaluated in rats (6 rats per group) by preparing five different doses (100, 500, 1000, 2000 and 4000 mg/kg), and administered orally using gavages. Animals were kept without food for 18 h prior to dosing and were monitored continuously on a daily basis for 3

days after dosing for any sign of toxicity. Animals showing any symptom of toxicity were immediately sacrificed. The experiment was carried out after the approval from the Ethics Committee on the Use and Care of Experimental Animals of the University of Fort Hare. LD_{50} value of the extract was calculated arithmetically using the method described by Hamilton et al. (1977).

Sub -acute toxicity

Twenty four rats were randomized into four groups, consisting of six animals in each group. Group 1: control rats administered with drinking water (0.5 ml) on daily basis for 28 days. Groups 2, 3 and 4 received 0.5 ml of the extract corresponding to 250, 500 and 1000 mg/kg body weight respectively. The use of male rats in this study was basically due to animal availability. The toxic manifestation such as body weight was measured every fifth day of the experiment. All the animals from each group were sacrificed at 28 days after their last daily dose of the extract or distilled water. The rats were thereafter quickly dissected to remove the liver, heart, lung and kidney and then transferred into ice-cold 0.25 M sucrose solution. The organs were freed of fat, blotted with clean tissue paper and then weighed. The dosages of the extract were chosen from the result obtained from the acute toxicity study as well as translation of traditional dosage using the equation:

$$\text{Dosage (mg/kg)} = \frac{\text{Volume of extract (ml)} \times \text{Concentration of extract (mg/ml)}}{\text{Weight of animal (kg)}}$$

Preparation of serum

The procedure described by Yakubu et al. (2005) was adopted for the preparation of serum. An aliquot (2 ml) of the blood was collected from the tail vein of the animals into sample bottles containing EDTA (BD Diagnostics, Preanalytical Systems, Midrand, USA) for

the haematological analysis while another 5 ml was allowed to clot at room temperature for 10 min. This was centrifuged at 1282 g x 5 min using Hermle Bench Top Centrifuge (Model Hermle, Z300 Hamburg, Germany). The sera were later aspirated with Pasteur pipettes into sample bottles and used within 12 h of preparation for the assay of biochemical parameters.

Haematological analyses

The full blood count (FBC) including red blood cells (RBC), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), large unstained cell (LUC), red cell distribution width (RCDW), haematocrit, white blood cell (WBC), basophils, neutrophils, eosinophils platelet and monocytes were determined using a semi-automated hematology analyzer (Horiba ABX 80 Diagnostics, ABX Pentra Montpellier, France).

Biochemical parameters

Alanine transaminases (ALT) determination

A volume of 23 µl of the serum sample was added to 250 µl of reagent A (16 mmol/L α- ketoglutarate, 0.18 mmol/LNADH, 500 mmol/L alanine, LDH > 2300 IU/L) with 8 µl of reagent B (Tris buffer 97 mmol/L) and allowed to react for 2 min at room temperature. The activity of the enzyme was determined by measuring changes in absorbance at 340 nm. The ALT reagents were used to measure the enzyme activity by a kinetic rate method (Henry, 1991). The enzyme catalyzes the reversible transamination of L-alanine and alpha ketoglutarate to pyruvate and L-glutamate. The resulting pyruvate is then reduced to lactate in

the presence of Lactate dehydrogenases (LDH) with concurrent oxidation of reduced β - NADH to NAD.

Aspartate transaminases (AST) determination

The reagents of AST were used to measure the activity of the AST by enzymatic rate method (Tietz et al., 1994). In the assay reaction, the AST catalyzed the reversible transamination of L-aspartate and alpha ketoglutarate to oxaloacetate and L- glutamate. The oxaloacetate is then reduced to malate in the presence of malate dehydrogenase (MDH) with concurrent oxidation of β-nicotinamide adenine dinucleotide (NADH) to β- NAD. To 23 µl of serum sample was added 242 µl of reagent A (16 mmol/L α- ketoglutarate, 218 mmol/L L-aspartate, 0.18 mmol/L NADH) and 8 µl of reagent B (Malate > 600 IU/L).

Gamma glutamyl transferases (GGT) determination

A precise volume of 13 µl serum sample was added to 237 µl of reagent A (4.4 mM γ-glutamyl-p- nitroaniline) and 23 µl of reagent B (150 mM glycylglycine) after reaction the change in absorbance was measured at 410 nm to determine enzyme activity. In the reaction, γ-GGT catalyzes the transfer of GGT from the colourless substrate (γ- glutamyl-p-nitroaniline) to the receptor glycylglycine to produce p- nitroaniline.

Alkaline phosphatases (ALP) determination

The ALP reagents were used to measure ALP activity by a kinetic rate method using a 2-amino-2- methyl-1-propanol (AMP) buffer (Tietz et al., 1987). 5 µl of serum sample was mixed with 228 µl of AMP (pH 10.3) and 22 µl of p-nitrophenyl phosphate, allowed to react together for 2 min at room temperature. The enzyme (ALP) catalyzes the hydrolysis of p-

nitrophenyl phosphate (colourless) to p-nitrophenol (yellow). The calculation was performed by the system to produce the final result.

Total bilirubin determination

Serum sample (10 µl) was mixed together with 310 µl of reagent A (442745) and 10 µl reagent B (476861) was allowed to react for 48 s at room temperature as described by the manufacturer. The absorbance was measured at 560 nm.

Estimation of Urea contents

Serum sample (3 µl) was added to 285 µl of reagent A containing 2.9 mmol/L, 0.35 mmol/L NADH, and 1.3 KIU/L of glutamate dehydrogenase with reagent 15 µl B (24 KIU/L of urease) to determine urea concentration in the sample by an enzymatic rate method (Tietz, 1995). The system monitors the changes in absorbance at 340 nm. This change in absorbance was directly proportional to the concentration of urea in the sample. Urea in the reaction was hydrolyzed by urease to CO_2 and ammonia. Glutamate dehydrogenase catalyzes the condensation of ammonia and alpha ketoglutarate to glutamate with the concomitant oxidation of NADH to NAD.

Estimation of Creatinine content

A precise volume (20 µl) of serum sample was reacted with 175 µl of picric acid (8.1 mmol/L) and 44 µl of buffered pH 13.3. In the reaction, creatinine combined with picrate in an alkaline solution to form a creatinine picrate- complex. The complex formation was measured by changes in absorbance at 520 nm to determine the concentration of creatinine in the sample using the method described by Tietz (1995).

Estimation of Cholesterol content

The reagents were used to measure cholesterol in the sample by time-end point method as described by Henry (1991). Serum sample (5 µl) was mixed with 290 µl of reagent A (211 IU/L cholesterol esterase, 216 IU/L of cholesterol oxidase, 6667 IU/L peroxidase) with 10 µl reagent B (0.28 mmol/L 4- aminoantipyrine, 8.06 mmol/L phenol). They are allowed to mix together at room temperature for 2 min. The absorbance was measured at 510 nm to determine cholesterol level in the sample. In the reaction, cholesterol esterase hydrolyzed cholesterol esters in the sample to free cholesterol and fatty acid. Cholesterol oxidase acted on the free cholesterol to cholestene-3-one and hydrogen peroxidase. The enzyme peroxidase catalyzes the reaction of hydrogen peroxide and 4-aminoantipyrine to quinoneimine which is then measured.

Estimation of HDL and LDL contents

To 3 µl of serum sample was added 210 µl of reagent A (211 IU/L cholesterol esterase, 216 IU/L of cholesterol oxidase, 6667 IU/L peroxidase) with 70 µl of reagent B (0.28 mmol/L 4- aminoantipyrine, 8.06 mmol/L phenol). The absorbance was measured at 560 nm to determine both HDL and LDL by a timed- end point method as described above in cholesterol assay.

Estimation of triglyceride content

Serum sample (3µl) was reacted with 285 µl of reagent A (glycerol kinase, glycerol phosphate oxidase with 15 µl of reagent B (961 IU/L horseradish peroxidase). The method by a timed out end point was used to measure concentration of triglycerides in the sample

(NCCLS, 1998). Triglyceride in the sample was hydrolyzed to glycerol and free fatty acid by the action of lipase.

Total protein and albumin

Serum sample (6 µl) was mixed together with 300 µl of reagent A (0.28 mmol/L Bromocresol purple). The change in absorbance was measured at 560 nm and 600 nm to determine the concentration of total protein and albumin respectively.

Electrolytes

A precise volume of 40 µl of the serum sample mixed together with 3.3 ml of ISE electrolyte buffer (467915) and 1.3 ml of ISE buffer (467935) to determine the concentration of calcium, potassium, chlorine and sodium in the sample using indirect potentiometry (ISE) method provided by the manufacturer (Beckmann Coulter). The system monitors the absorbance at 410, 470, 600 and 700 nm for sodium, potassium, chlorine and calcium with different assay principles.

Statistical analysis

Data were expressed as means ± standard deviation of six replicates and were statistically analyzed using one way analysis of variance (ANOVA). Means were separated by the Duncan multiple test using SAS. Values were considered significant at $P < 0.05$.

RESULTS

Acute toxicity studies

The result of the toxicity test of *S. henningsii* extract for 3 days did not show any clinical adverse side effect of substance-related toxicity on the animals such as restlessness, haematuria, diarrhea and muscle coordinated movement. Similarly, there was no mortality or morbidity observed at any tested doses except at 4000 mg/kg. The LD_{50} value of the extract was found to be 3666.66 mg/kg (Table 1).

Table 1: Determination of LD_{50} value by arithmetic method of Hamilton, 1977.

Group	**Dose (mg/kg) Control**	**No of animals dead**
1	100	0
2	500	0
3	1000	0
4	2000	0
5	4000	2

Group	**Dose difference (a)**	**Mean mortality (b)**	**Probit (a×b)**
5	(4000-2000) = 2000	1	2000

Sum of the product = 2000

LD_{50} = Least lethal dose in a group- $\frac{\sum(a \times b)}{N}$

$LD_{50} = 4000 - \frac{2000}{6}$

$= 4000 - 333.3$

$= 3666.66$

Sub-acute toxicity studies

The body weight of the animals treated with the extract at the doses of 250, 500 and 1000 mg/kg and the control group were appreciably increased throughout the experimental periods (Figure 1). There was no significant changes (P >0.05) observed in the organs body weight ratio of the kidney, lung, liver and heart as compared with the control group (Figure 2). Unfortunately, the extract caused the death of two rats, one on the 21st and the other on the 27th day during the experimental period. The observed symptoms before death were respiratory distress, starry hair coat, motionlessness and slow response to external stimuli.

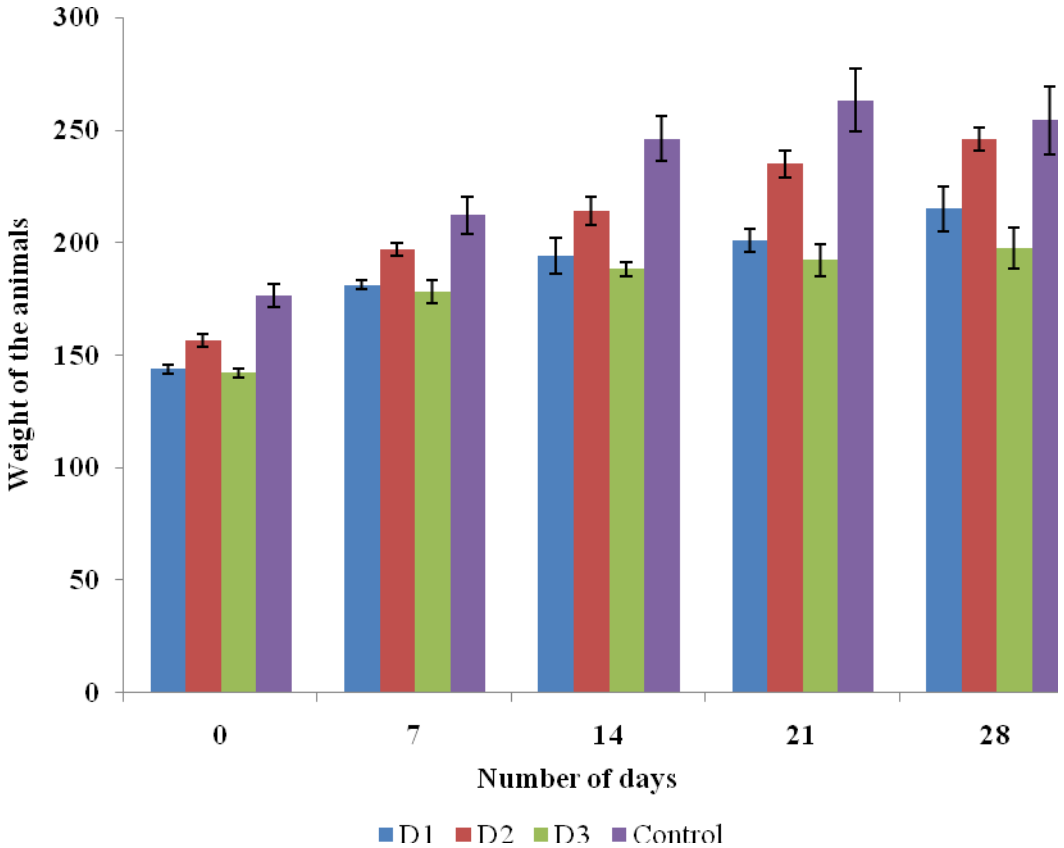

Figure 1: The effect of aqueous extract of *S. henningsii* on the body weight of Wistar rats. Values are mean ± SD of 6 rats in each group. D1 = animals fed with 250 mg/kg body weight of extract, D2 = animals fed with 500 mg/kg body weight of extract, D3 = animals fed with 1000 mg/kg body weight of extract, control = animals receiving only distilled water.

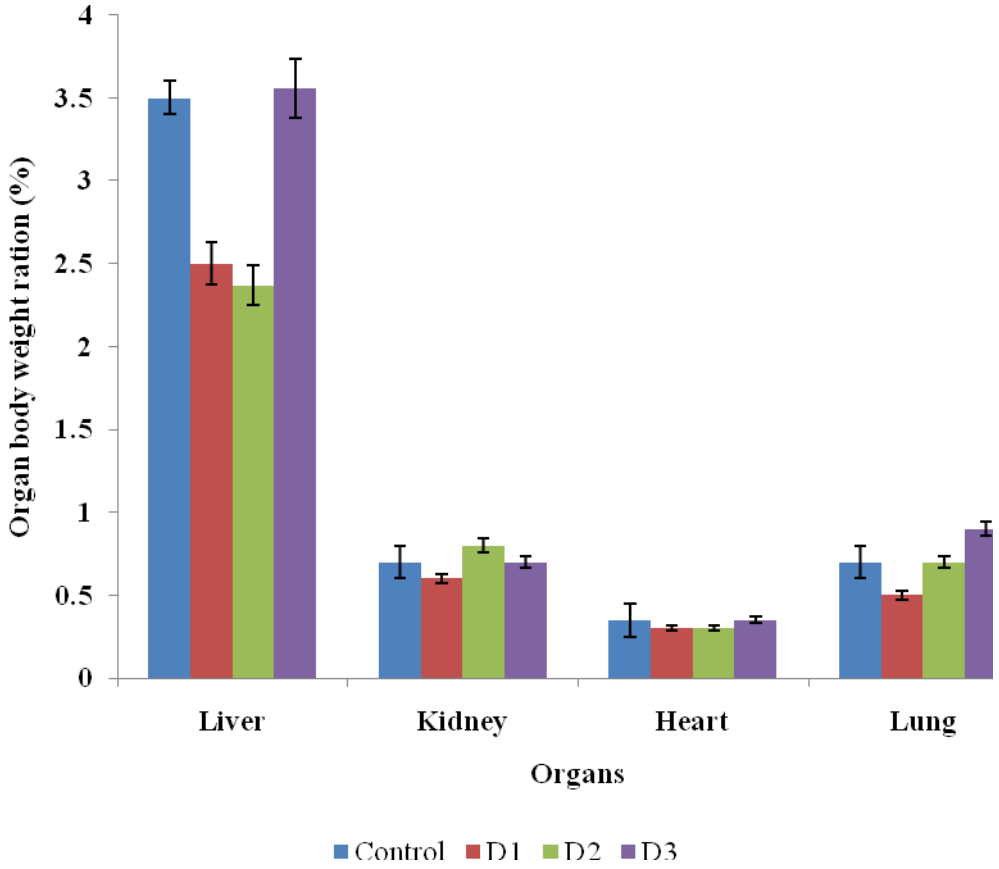

Figure 2: The effect of aqueous extract of *S. henningsii* (SH) on the organ body weight ratio of rats. Values are mean ± SD of 6 rats in each group. D1 = animals fed with 250 mg/kg body weight of extract, D2 = animals fed with 500 mg/kg body weight of extract, D3 = animals fed with 1000 mg/kg body weight of extract, control = animals receiving only distilled water.

Haematological parameters

The effects of oral administration of aqueous bark extract of *S. henningsii* at the doses investigated on red blood cells and its functional indices in male Wistar rats for 28 days are shown in (Table 2). The extract did not significantly alter the levels of Hb, RBC, haematocrit, MCHC and LUC while those of MCV, RCDW and MCH were significantly increased at certain doses.

Biochemical parameters

The plant extract showed varied effect on the kidney and its functional indices (Table 5). The levels of sodium, calcium, urea and creatinine were not significantly affected when compared with the control animals. Contrarily, the level of chlorine ion was decreased drastically at 500 mg/kg body weight while slight increase was apparent in the remaining doses. The extract did not alter the level of total bilirubin and albumin a vital markers of assessing liver damage (Table 6). The concentration of conjugated bilirubin significantly ($P < 0.05$) increased after the treatment with the plant extract at 250 and 500 mg/kg but increased only at the highest dose. The data did not show any notable ($P > 0.05$) changes in serum concentration of AST and ALT.