ANTIOXIDANT AND TOXICOLOGIC PROPERTIES OF METHANOL LEAF EXTRACT OF *STEPHANIA DINKLAGEI* IN WISTAR ALBINO RAT

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TITLE PAGE

ANTIOXIDANT AND TOXICOLOGIC PROPERTIES OF METHANOL LEAF EXTRACT OF *STEPHANIA DINKLAGEI* IN WISTAR ALBINO RAT

A PROJECT REPORT

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR AWARD OF DEGREE OF MASTER OF SCIENCE (M.Sc.) IN MEDICAL BIOCHEMISTRY, UNIVERSITY OF NIGERIA, NSUKKA

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CERTIFICATION

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DEDICATION

This research work is dedicated to Almighty God for his protection, good health and immeasurable grace throughout the duration of this work.

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ABSTRACT

Stephania dinklagei is used extensively in South East Nigeria for the traditional treatment of malaria and other associated ailments in form of decoction, in which unspecified quantities are usually consumed without due regards to toxicologic and other adverse effects. In this study, the phytochemicals were assessed as well as the effects of the antioxidant and toxicologic properties of methanol leaf extract of *stephania dinklagei* in Wistar albino rat. The rats were administered with graded doses of the extract twice daily for three weeks and the control administered with distilled water. Four rats each from the control and test groups were sacrificed every seven days and blood samples collected for analysis. The percentage yield of *stephania dinklagei* methanol leaf extract was 5.5%. Preliminary phytochemical screening showed that the methanol leaf extract contained alkaloids, flavonoids, tannins, steroids, terpenoids, carotenoids, glycosides, anthocyanins and saponins. Anthraquinone was not detected. The quantitative phytochemical analysis showed that the extract contains alkaloids (29.70 $\pm$ 0.15mg/g), flavonoids (25.30 $\pm$ 0.10mg/g), steroids (69.70 $\pm$ 0.10mg/g), saponins (13.57 $\pm$ 0.21mg/g), tannins (64.21$\pm$ 0.21mg/g) cardiac glycosides (1.45 $\pm$ 0.09mg/g), terpenoids (44.30 + 0.26mg/g), carotenoids (5.88 $\pm$ 0.52mg/g) and anthocyanin (15.40 $\pm$ 0.26mg/g). The vitamin content of the leaf extract was found to be vitamin A (44.8 $\pm$ 0.42mg/100g), vitamin C (27.85 $\pm$ 0.07mg/100g) and vitamin E (12.7 $\pm$ 0.28mg/100g). The acute toxicity test of the leaf extract showed no toxicity up to 5000mg/kg body weight as observed over a period of 48 hrs for signs of acute toxicity. The extract was found to moderately scavenge the DPPH and superoxide anion radical in a dose dependent manner compared with their respective standards. The extract however, highly scavenged the hydroxyl radical when compared with the standard, Ŭ-tocopherol. There were no significant differences ($p > 0.05$) in serum MDA level in all the groups in week I but significantly increased ($p<0.05$) in group 4 (week 2) when compared with that of their control. The serum SOD activity showed a significant decrease ($p<0.05$) in all the groups of 1st, 2nd and 3rd weeks of the experiment when compared with that of their respective controls. Serum CAT also decreased significantly ($p<0.05$) in group 3 and 4 in week 3 compared with the control but no significant difference ($p<0.05$) was observed in all the groups in week 1 and 2. Serum ALP activity increased significantly ($p<0.05$) throughout the duration of the experiment when compared with that of their controls. Serum ALT level increased significantly ($p<0.05$) only in group 4 in the 1st, 2nd and 3rd weeks of the experiment. The same trend was observed with the AST level when compared with those of their controls. Creatinine showed a non-significant increase ($p>0.05$) in groups 2 and 3 but significantly decreased ($p<0.05$) in group 4 (week 1). There were also non-significant difference ($p>0.05$) in all the groups in week 2 when compared with that of their control but in week 3, there was non-significant increase ($p>0.05$) in groups 2 and 3 and a non-significant decrease in group 4. Urea level significantly increased ($p<0.05$) in all the groups throughout the duration of the experiment. Serum Na^+ increased significantly ($p<0.05$) in week 1, 2 and 3 compared with those of their respective controls. Serum Cl^- level showed non-significant difference ($p>0.05$) in week 1 and 2 but however, increased significantly ($p<0.05$) in week 3 compared with the control. Histological examination of the liver cells of the treated rats revealed widespread hepatocellular vacuolar degeneration with hypertrophy of kupffer cells in the periportal areas and moderate infiltration of mononuclear leucocytes into the periportal area as against that of their control. The histopathology result corroborates the results of the serum biochemical parameters. The kidney showed no significant changes in the treated groups compared with that of their control. These results suggest that *Stephania dinklagei* leaf extract had a significant *in vitro* antioxidant activity. However, long term consumption of the extract at the doses studied could be hepatotoxic but not nephrotoxic.

TABLE OF CONTENTS

Title Page		i
Certification		ii
Dedication		iii
Acknowledgment		iv
Abstract		v
Table of Contents		vi
List of Tables		x
List of Figures		xi
List of Plates		xii
List of Abbreviations		xiii
CHAPTER ONE: INTRODUCTION		
1.0	Background of the study	1
1.1	*Stephania dinklagei*	1
1.2.0	Phytochemicals	3
1.2.1	Alkaloids	3
1.2.2	Saponins	4
1.2.3	Steroids	4
1.2.4	Flavonoids	5
1.2.5	Tannins	6
1.2.6	Anthocyanins	6
1.2.7	Cardiac glycoside	7
1.2.8	Terpenoids	8
1.2.9	Carotenoids	8
1.3	Antioxidants and free radicals	8
1.3.1	Hydroxyl anion radical	10
1.3.2	Superoxide anion radical	11
1.4	Dietary antioxidants	11
1.5.0	Oxidative stress	12
1.6.0	Oxidative stress marker	13
1.6.1	Malondialdehyde	13
1.7	Antioxidant enzymes	14
1.7.1	Catalase	14
1.7.2	Superoxide dismutase	14
1.8	Liver function tests	15
1.8.1	Aspartate transaminase	15
1.8.2	Alkaline phosphatase	16
1.8.3	Alanine aminotransferase	17
1.9.0	Renal function tests	17
1.9.1	Urea	18
1.9.2	Creatinine	18
1.10	Electrolytes	18
1.10.1	Sodium	19
1.10.2	Chloride	19
1.11.0	Histopathology	20
1.12	Aim of the study	20
1.13.0	Specific objectives of the research	20

CHAPTER TWO: MATERIALS AND METHODS

2.1 Materials 22
2.1.1 Plant material 22
2.1.2 Animals 22
2.1.3 Equipment 22
2.1.4 Chemicals and reagents 23
2.2 Methods 24
2.2.1 Preparation of plant material 24
2.2.2 Extraction of plant material 24
2.2.3 Preparation of reagents 25
2.2.4 Preparation of sample for microscopic analyses 25
2.2.5 Qualitative phytochemical analysis of the leaves of *Stephania dinklagei* 27
2.2.5.1 Test for alkaloids 28
2.3.5.2 Test for flavonoids 28
2.3.5.3 Test for glycosides 28
2.3.5.4 Test for saponins 28
2.3.5.5 Test for terpenoids and steroids 29
2.3.5.6 Test for tannins 29
2.3.5.7 Test for anthocyanin 29
2.3.5.8 Test for anthraquinone 29
2.2.6 Quantitative phytochemical analysis of leaves of *Stephania dinklagei* 29
2.2.6.1 Alkaloid determination 29
2.2.6.2 Flavonoid determination 30
2.2.6.3 Steroid determination 30
2.2.6.4 Saponin determination 30
2.2.6.5 Tannin determination 31
2.2.6.6 Glycoside determination 31
2.2.6.7 Terpenoid determination 31
2.2.6.8 Carotenoid determination 32
2.2.7 Antioxidant activity assays 32
2.2.7.1 DPPH radical scavenging assay 32
2.2.7.2 Hydroxyl radical scavenging assay 33
2.2.7.3 Superoxide radical scavenging assay 34
2.2.8 Determination of vitamin contents of methanol extract of *Stephania dinklagei* leaves 34
2.2.8.1 Vitamin A 34
2.2.8.2 Vitamin E 35
2.2.8.3 Vitamin C 35
2.2.9 Acute toxicity test of the methanol leaf extract of *Stephania dinklagei* 35
2.2.10 Experimental design 35
2.2.11 Antioxidant enzyme assay of the rats treated with methanol leaf extract of *Stephania dinklagei* 36
2.2.11.1 Assay of superoxide dismutase activity 36
2.2.11.2 Assay of catalase activity 37
2.2.11.3 Determination of malondialdehyde concentration 37
2.2.12 Effects of methanol leaf extract of *Stephania dinklagei* on the activities of some Liver marker enzymes 38
2.2.12.1 Assay of aspartate aminotransferase activity 38

2.2.12.2	Assay of alanine aminotransferase activity	38
2.2.12.3	Assay of alkaline phosphatase activity	39
2.2.13	Effect of methanol leaf extract of *Stephania dinklagei* on the concentrations of some renal function markers	39
2.2.13.1	Determination of urea concentration	39
2.2.13.2	Determination of creatinine concentration	
2.2.14	Determination of some electrolyte concentrations	40
2.2.14.1	Determination of sodium ion concentration	40
2.2.14.2	Determination of chloride ion concentration	40
2.2.5	Histological examination of the liver and kidney of the rats	41
2.3	Statistical analysis	44

CHAPTER THREE: RESULTS

3.1	Percentage yield of methanol leaf extract of *Stephania dinklagei*	45
3.2	Qualitative phytochemical constituents of the methanol leaf extract of *Stephania dinklagei*	46
3.3	Qualitative phytochemical constituents of methanol extract of *Stephania dinklagei*	47
3.4	Vitamin contents of methanol extracts of *Stephania dinklagei* leaf	48
3.5	Acute toxicity (LD_{50}) test of methanol leaf extract of *Stephania dinklagei* on the rats	49
3.6	Effect of methanol leaf extract of *Stephania dinklagei* on DPPH radical scavenging activity	50
3.7	Effect of methanol leaf extract of *Stephania dinklagei* on hydroxyl radical scavenging activity	51
3.8	Effect of methanol leaf extract of *Stephania dinklagei* on superoxide anion radica scavenging activity	52
3.9	Anti-radical power of the leaves of *Stephania dinklagei*	53
3.10	Effect of methanol leaf extract of *Stephania dinklagei* on serum MDA of the rats	54
3.11	Effect of methanol leaf extract of *Stephania dinklagei* on serum catalase	55
3.12	Effect of methanol leaf extract of *Stephania dinklagei* on serum superoxide dismutase	56
3.13	Effect of methanol leaf extract of *Stephania dinklagei* on serum aspartate aminotransferase (AST)	57
3.14	Effect of methanol leaf extract of *Stephania dinklagei* on serum alanine aminotransferase (ALT)	58
3.15	Effect of methanol leaf extract of *Stephania dinklagei* on serum alkaline phosphatase (ALP)	59
3.16	Effect of methanol leaf extract of *Stephania dinklagei* on serum urea	60
3.17	Effect of methanol leaf extract of *Stephania dinklagei* on serum creatinine	61
3.18	Effect of methanol leaf extract of *Stephania dinklagei* on serum sodium	62
3.19	Effect of methanol leaf extract of *Stephania dinklagei* on serum chloride	63
3.20	Histopthololological evaluation of methanol leaf extract of *Stephania dinklagei* on the rats liver	64
3.21	Photomicrograph of test group treated with 200mg/kg b.w. of *S. dinklagei*	65
3.22	Photomicrograph of test group treated with 400mg/kg b.w. of *S. dinklagei*	66
3.23	Photomicrograph of test group treated with 600mg/kg b.w. of *S. dinklagei*	67
3.24	Histopathological evalution of methanol leaf extract of *Stephania dinklagei* on the rats kidney	68

3.25 Photomicrograph of the kidney showing group treated with 600mg/kg b.w. of methanol leaf extract of *S. dinklagei* 69
3.26 Histopathology explanations 70

CHAPTER FOUR: DISCUSSION
Discussion 71
Conclusion 75
Suggestions for Further Studies 75
References 76
Appendices 89

LIST OF TABLES

Table 1 Percentage yield of methanol leaf extract of *Stephania dinklagei* 44
Table 2 Preliminary phytochemical screening of methanol leaf extract of *Stephania dinklagei* 45
Table 3 Quantitative phytochemical constituents of methanol leaf extract of *Stephania dinklagei* 46
Table 4 Vitamin contents of methanol leaf extract of *Stephania dinklagei* 47
Table 5 Acute toxicity (LD_{50}) test of *Stephania dinklagei* on albino mice 48

LIST OF FIGURES

Fig. 1	*Stephania dinklagei* leaves	2
Fig. 2	The basic skeleton of a steroid with standard stereo orientation	
Fig. 3	Production and elimination of ROS	9
Fig. 4	Mechanism of oxidative stress	13
Fig. 5	Effect of methanol leaf extract of *Stephania dinklagei* on DPPH radical scavenging activity	49
Fig. 6	Effect of methanol leaf extract of *Stephania dinklagei* on superoxide anion radical scavenging activity	50
Fig. 7	Effect of methanol leaf extract of *Stephania dinklagei* on hydroxyl anion radical scavenging activity	
Fig. 8	Anti-radical power of the leaves of *Stephania dinklagei*	51
Fig. 9	Effect of methanol leaf extract of *Stephania dinklagei* on on serum MDA of the rats.	52
Fig. 10	Effect of methanol leaf extract of *Stephania dinklagei* on serum catalase	53
Fig. 11	Effect of methanol leaf extract of *Stephania dinklagei* on serum superoxide dismutase (SOD) of the rats.	54
Fig. 12	Effect of methanol leaf extract of *Stephania dinklagei* on serum aspartate aminotransferase.	55
Fig. 13	Effect of methanol leaf extract of *Stephania dinklagei* on serum alanine aminotransferase (ALT).	56
Fig. 14	Effect of methanol leaf extract of *Stephania dinklagei* on serum alkaline phosphatase (ALP) of the rats	57
Fig. 15	Effect of methanol leaf extract of *Stephania dinklagei*on serum urea	58
Fig. 16	Effect of methanol leaf extract of *Stephania dinklagei* onserum creatinine	59
Fig. 17	Effect of methanol leaf extract of *Stephania dinklagei* on serum sodium ion concentration	60
Fig. 18	Effect of methanol leaf extract of *Stephania dinklagei* on serum chloride ion concentration	61

LIST OF PLATES

Plate 1: Photomicrograph of section of liver organ of control rats 64

Plate 2: Photomicrograph of test group treated with 200mg/kg b.w. of methanol leaf extract of *Stephania dinklagei* 65

Plate 3: Photomicrograph of test group treated with 400mg/kg b.w. of methanol leaf extract of *Stephania dinklagei* 66

Plate 4: Photomicrograph of test group treated with 600mg/kg b.w. of methanol leaf extract of *Stephania dinklagei* 67

Plate 5: Photomicrograph of the kidney showing the control group 68

Plate 6: Photomicrograph of the kidney showing group treated with 600mg/kg b.w. of methanol leaf extract of *Stephania dinklagei* 69

LIST OF ABBREVIATIONS

AGE: Advanced glycation end products

ALE: Advanced lipoxidation end products

ALP: Alkaline phosphatase

ALT:	Alanine aminotransferase
ANOVA:	Analysis of Variance
AOAC:	Association Official Analytical Chemists
AST:	Aspartate aminotransferase
BDCP:	Bionesources Development and Conservation Programme
BUN:	Blood urea nitrogen
CAT:	Catalase
DMAPP:	Dimethylallyl pyrophosphate
DMSO:	Dimethyl sulfoxide
DPPH:	2,2 Diphenyl -1- picryl-hydrazyl
EDTA:	Ethylenediaminetetra acetic acid
$Fecl_3$:	Ferric chloride
GFR:	Glomerular filtration rate
GPP:	Geranyl pyrophosphate
IPP:	Isopentenyl pyrophosphate
LD:	Lethal Dose
MDA:	Malondialdehyde
NBT:	Nitro blue tetrazolium
NO_2^-:	Nitrite ion
PAL:	Phynyalanine ammonialyase
PBS:	Phosphate-buffered saline
PUFA:	Poly unsaturated fatty acid
ROS:	Reactive oxygen species
SNP:	Sodium nitroprusside
SOD:	Superoxide dismutase
SPSS:	Statistical Package for Service Solutions
TBARS:	Thiobarbituric acid reactive substances
TCA:	Trichloroacetic acid

CHAPTER ONE

INTRODUCTION

The Southern inhabitants of Nigeria are known for their high consumption of vegetables. Some of these vegetables form part of foods consumed on special conditions, including ill health and times of convalescence. This stresses the role of plants in the life of man (Nwangwu *et al*., 2009). The use of plant parts in traditional medical practice has a long drawn history and remains the mainstay of primary health care in most of third world countries (Prescott-Allen, 1982). Medicinal plants are believed to be an important source of some secondary metabolites with potential therapeutic benefits (Farnsworth, 1989). In treatment of diseases, the use of herbs has gained grounds world wide, making traditional medicine an inevitable global discuss. This practice calls for research into pharmacological activities of plants secondary metabolites and has improved modern pharmaco therapeutics around the world (Nwaogu *et al*., 2007). Though, some medicinal plants serve as food, they contain secondary metabolites that influence biological processes and reverse disease states (Ugochukwu and Badaby, 2002).

In normal or pathological cell metabolism, free radicals which have one or more unpaired electrons are produced. Reactive Oxygen Species (ROS) react easily with free radicals such as superoxide anion radical (O_2^-) and hydroxyl anion (OH^-) as well as non-free radical species (H_2O_2) and the singlet oxygen ($^1O_2$) (Yildrim *et al*., 2002). Also excessive generation of ROS induced by various stimuli and which exceed the antioxidant capacity of the organism leads to a variety of pathophysiological processes such as inflammation, diabetes, genotoxicity and cancer (Kourounakis *et al*., 1999). A great number of medicinal plants contain chemical compounds that exhibit antioxidant properties (Gulcin *et al*., 2002). Sources of natural antioxidants are primarily plant phenolics that may occur in all parts of plant such as fruits, vegetables, nuts, seeds, leaves and barks (Pratt and Hudson, 1990).

1.1 *Stephania dinklagei*

Stephania dinklagei is a herbaceous shrub which belongs to the family of plants called menispermaceae (Iwu, 1993). In Eastern Nigeria, it is called ñOgwu-Ogburugbuò by Aku people of Enugu State.

Figure 1: *Stephania dinklagei* leaves

A large number of alkaloids have been isolated from *Stephania dinklagei,* it contains corydine (sedative drug agent) and Stephanini (analgesic drug agent) (Goren *et al*., 2003). Its constituent Liriodenine exhibits antiprotozoal and cytotoxic activities against the protozoa *Leishmania donovania* and *Plasmodium falciparum* (Camacho *et al.,* 2000). An infusion made from its young leaves is immediately given to children before it thickens to relieve them from stomach aches (Goren *et al*., 2003). Its leaves are taken to treat impotency in men and also act as an aphrodisiac (Burkill, 1997). Given its uses in the traditional setting and the emerging reports on its pharmacological actions, it is therefore necessary to evaluate its antioxidant properties, toxicity and its effect on liver and kidney marker enzymes in Wistar albino rats.

1.2 Phytochemicals

Phytochemicals are chemical compounds formed during the plants normal metabolic processes. These chemicals are often referred to as secondary metabolites which include alkaloids, flavonoids, coumarins, glycosides, gums, polysaccharides, phenols, tannins, terpenes and terpenoids (Harborne, 1973; Okwu, 2004). These can act as agents to prevent undesirable side effects of the main active substances or to assist in the assimilation of main substances (Anonymous, 2007). Phytochemicals are present in a variety of plants utilized as important components of both human and animal diets. These include fruits, seeds, herbs and vegetables (Okeke and Elekwe, 2003). Most of these phytochemical constituents are potent bioactive compounds found in medicinal plant parts which are precursors for the synthesis of useful drugs (Sofowora, 1993).

1.2.1 Alkaloid

Alkaloids are group of naturally occurring low molecular weight nitrogenous chemical compound that contain mostly basic nitrogen atoms (Manske, 1965). They are found primarily in plants and are especially common in certain families of flowering plants (Herbert, 1999). Large variety of organisms produce alkaloids, these include bacteria, fungi, plants and animals and are part of the group of natural products called secondary metabolites (Baldwin and Ohnmeiss, 1993). Most alkaloids contain oxygen in their molecular structure, those compounds are usually colourless crystals at ambient conditions (Lewis and Elvin-Lewis, 1977). Oxygen-free alkaloids, such as nicotine or coniine are typically volatile, colourless, oily liquids (Abuo-Donia *et al*., 1992) some alkaloids are coloured, like berberine (yellow) and sanguinarine (orange) (Akhtar *et al*., 2003). Most alkaloids are weak bases, but some, such as theobromine and theophylline are amphoteric (Ali and Khan, 2008). Many alkaloids dissolve poorly in water but readily dissolve in organic solvents such as diethyl ether, chloroform or 1, 2-dichloroethane. Caffeine, cocaine, codeine and nicotine are water soluble (Ashihara *et al*., 2008). Biological precursors of most alkaloids are amino acids such as ornithine, lysine, phenylalanine, tyrosine, tryptophan, histidine, aspartic acid and anthranilic acid (Berkov *et al*., 2007). Alkaloid biosynthesis are too numerous and cannot be easily classified (Blankenship *et al*., 2005).

Most of the known functions of alkaloids are related to protection. For example, aporphine alkaloid ï liriodenine produced by the tulip tree protects it from parasitic mushrooms. Many alkaloids are used in medicine: Atropine, Codeine, Nicotine and Quinine reserpine are used as anticholinergic, stimulant; antipyretics and antihypertensives respectively (Ashihara *et al*., 2008).

1.2.2 Saponin

Saponins are amphipathic glycosides grouped, in terms of phenomenology, by the soap-like foaming they produce when shaken in aqueous solutions (Francis *et al*., 2002). Saponins consist of a polycyclic aglycones attached to one or more sugar side chains. The aglycone part, which is also called sapogenin is either steroid (C27) or a triterpene (C30) (Skene, 2006). The foaming ability of saponins is caused by the combination of a hydrophobic (fat-soluble) sapogenin and a hydrophilic (water soluble) sugar part. Saponins have a bitter taste. Some saponins are toxic and are known as sapotoxin (XU *et al*., 1996). They are found in most plants, vegetables, beans and herbs (Francis *et al*., 2002). Studies have illustrated the beneficial effects on blood cholesterol levels, cancer, bone health and stimulation of the immune system (XU *et al.*, 1996). It has also shown that saponins have anti tumor and anti-mutagenic activities and can lower the risk of human cancers by preventing cancer cells from

growing. It was found that saponins may help to prevent colon cancer and as shown in their article ñSaponins as anti-carcinogensò published in the Journal of Nutrition (1995).

1.2.3 Steroid

Steroids are organic compounds that contain a characteristic arrangement of four cycloalkane rings that are joined to each other (Kuzuyama and Seto, 2003). Examples of steroids include the dietary fat cholesterol, the sex hormones estradiol and testosterone and anti-inflammatory drug dexamethasone (Rosier, 2006). Steroids are found in plants, animals and fungi. All steroids are made in cells either from the sterols lanosterol (animals and fungi) or from cycloartenol (plants). Both lanosterol and cycloartenol are derived from the cyclization of the triterpene squalene (Kuzuyama and Seto, 2003). Steroids have a chemical structure that contains the core of gonane or a skeleton derived there from. Usually, methyl groups are present at the carbons C-10 and Cï 13 ï an alkyl side-chain at carbon Cï 17 may also be present (Dubey, *et al*., 2003).

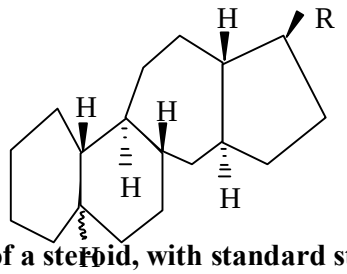


Fig. 2: The basic skeleton of a steroid, with standard stereo orientation

The three cyclohexane rings form the skeleton of phenanthrene, the last ring of the gonane has a cyclopentane structure. Hence, together they are called cyclopentaphenanthrene (Hanukoglu, 1992). Steroid biosynthesis is an anabolic metabolic pathway that produces steroids from simple precursors. A unique biosynthetic pathway is followed in animals compared to many other organisms, making the pathway a common target for antibiotics and anti-infective drugs (Hanukoglu, 1992). In addition, steroid metabolism in humans is the target of cholesterol lowering drugs such as statins. In humans and other animals, the biosynthesis of steroids follows the mevalonate pathway that uses acetyl-CoA as building blocks to form dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP) (Dubey et al, 2003). In subsequent stages, DMAPP and IPP are joined to form geranyl

pyrophosphate (GPP), which in turn is used to synthesize the steroid lanosterol, lanosterol can then be converted into other steroids such as cholesterol and ergosterol (Rosier, 2006).

1.2.4 Flavonoid

Flavonoids are water soluble polyphenolic molecules containing 15 carbon atoms. Flavonoids consist of 6 major subgroups: Chalcone, flavone, flavonol, flavanone, anthocyanins and isoflavonoids. Together with carotenes, flavonoids are also responsible for the colouring of fruits, vegetables and herbs (Galeotti *et al.,* 2008).

Flavonoids are synthesized by the phenyl-propanoid metabolic pathway in which the amino acid phenylalanine is used to produce 4-coumaroyl-CoA (Cazarolli *et al*., 2008; Friedman, 2007). This can be combined with malonyl-CoA to yield the true backbone of flavonoids, a group of compounds called chalcones, which contain two phenyl rings. Conjugate ring-closure of chalcones results in the familiar form of flavonoids, the three-ringed structure of a flavone (Friedman, 2007). The metabolic pathway continues through a series of enzymatic modifications to yield flavanones Ÿ diydroflavonolsŸ anthocyanins. Along this pathway, many products can be formed, including the flavonols, flavan-3-ols, proanthocyanidins (tannins) and a host of other various polyphenolics (Verueridis *et al.,* 2007). *In vitro* studies show that flavanoids also have anti-allergic, anti-inflammatory, anti-microbial, anti-cancer and anti-diarrheal activities (Cushnie and Lamb, 2011). Research has shown that flavonoids are poorly absorbed in the human body (less than 5%), with what is absorbed being quickly metabolized and excreted (Williams *et al*., 2004).

1.2.5 Tannins

Tannin is an astringent, bitter plant polyphenolic compound that binds to and precipitates proteins and various other organic compounds including amino acids and alkaloids (Muller-Harvey and McAllan, 1992). Tannin compounds are found mainly in plants where they play a role in protection from predation, and perhaps also as pesticides (Giner-Chavez, 1996). Tannins play an important role in the ripening of fruit and the aging of wine. There are three large classes of secondary metabolites in plants: Nitrogen containing compounds, terpenoids and phenolics (Mole, 1993). Tannins belong to the phenolics class. All phenolic compound (primary and secondary) are, in one way or another formed via the shikimic acid pathway, also known as the phenylpropanoid pathway (Reed, 1995). The same pathway leads to the formation of other phenolics such as isoflavones, coumarins, lignins and aromatic amino acids (tryptophan, phenylalnie and tyrosine). Typically, tannin molecules require at least twelve hydroxyl groups and at least five phenyl groups to function as protein binders.

Tannins are important ingredient in the process of tannin of leather. Tannins produce different atoms with ferric chloride according to the type of tannin (Souza *et al*., 2006).

1.2.6 Anthocyanin

Anthocyanins are water-soluble vacuolar pigments that belong to a parent class of molecules called flavonoids synthesized via the phenylpropanoid pathway, they are odourless and nearly flavourless (Stafford, 1994). Anthocyanins occur in all tissues of higher plants including leaves, stems, roots, flowers and fruits (Wada and Ou, 2002). Anthocyanins have been shown to act as a ñsunscreenò, protecting cells from high-light damage by absorbing blue-green and ultraviolet light, thereby protecting the tissues from photo inhibition, or high-light stress (Lieberman, 2007). Anthocyanins can be used as pH indicators because their colour changes with pH; they are pink in acidic solutions, purple in neutral solutions, greenish-yellow in alkaline solutions and colourless in very alkaline solutions where the pigment is completely reduced (WU *et al.,* 2004). They are found in cell vacuole. The anthocyanins are subdivided into the sugar-free anthocyanidin aglycones and the anthocyanin glycosides. As of 2003, more than 400 anthocyanins had been reported (Lieberman, 2007). While more recent literature (early 2006) puts number at more than 550 different anthocyanins. The difference in chemical structure that occurs in response to changes in pH is the reason why anthocyanins are often used as pH indicators, as they change from red in acids to blue in bases (De-Rosso *et al.,* 2008). In anthocyanin biosynthetic pathway, L-phenylalanine is converted to naringenin by phenylalanine ammonialyase (PAL), Cinnamate-4-hydroxylase (C4H), 4-Coumarate CoA Ligase (4CL), chalcone synthase (CHS) and chalcone isomerase (CHI). And then, the next pathway is catalysed by the formation of complex aglycone and anthocyanin composition by flavone 3 ï hydroxylase (F3H), flavonoid 3^1 ï hydroxylase ($F3^1H$), dihydroflavonol 4 ï reductase (DFR). Anthocyanidin synthase (ANS). UDP ï glucoside: flavonoid glucosyltransferase (UFGT) and methyl transferase (MT). Among these, UFGT is divided into UF3GT and UF5GT, which are responsible for the glucosylation of anthocyanin to produce stable molecules (WU *et al*., 2004). Although anthocyanins are powerful antioxidants *in vitro,* this antioxidant property is unlikely to be conserved after the plant is consumed (Stafford, 1994).

1.2.7 Cardiac Glycoside

Cardiac glycosides are glycosides of mostly C23 ï steroidal compounds. They have a characteristic 5 ï or 6 ï membered lactone ring (Wang *et al*., 2008). They are called cardiac glycosides because they modify heart action (Brower *et al.,* 1972). Cardenolides inhibit the Na^+ - K + - ATPase pump in mammals. This group of compounds is found in a large number

of families many of which are unrelated. A number of toads and frogs make cardiac active compounds that are steroidal but not glycosidic in nature (Wang *et al*., 2008). Cardenolides are derived from steroidal precursors, probably cholesterol via the intermediacy of pregnenolone or progesterone intermediates (Jungreis *et al*., 1997). Most members of the family Asclepiadaceae contain cardiac glycosides (Dussourd, 1986). Cardiac glycosides are drugs used in the treatment of congestive heart failure and cardiac arrhythmia (Dussourd, 1986). Drugs such as Ouabain and digoxin are cardiac glycosides. Digoxin from the foxglove plant is used clinically, whereas Ouabain is used only experimentally due to its extremely high potency (Dussourd, 1986). Normally, sodium-potassium pumps in the membrane of cells (in this case, cardiac myocytes) pump potassium ions in and sodium ions out. Cardiac glycosides inhibit this pump by stabilizing it in the E2 ï P transition state, so that sodium cannot be extruded: intracellular sodium concentration increases. A second membrane ions exchanger, NCX, is responsible for pumping calcium ions out of the cell and sodium ion in (3Na/Ca): raised intracellular sodium levels inhibit this pump, so calcium ions are not extruded and will also begin to build up inside the cell (Jungreis *et al.*, 1997).

1.2.8 Terpenoid

Terpenoid are large and diverse class of naturally occurring organic chemicals similar to terpenes derived from five-carbon isoprene units assembled and modified in thousands of ways (Yousefbeyk *et al*., 2014). Most are multicyclic structures that differ from one another not only in functional groups but also in their basic carbon skeletons (Wolinsky, 1973). These lipids can be found in all classes of living things and are the largest group of natural products (Gimelli, 2001). The steroids and sterols in animals are biologically produced from terpenoid precursors. Sometimes terpenoids are added to proteins, e.g. to enhance their attachment to the cell membrane, this is known as isoprenylation (Maarse, 1991). Terpenoids can be thought of as modified terpenes, wherein methyl groups have been moved or removed or oxygen atoms added (Swan, 1967). Just like terpenes, the terpenoids can be classified according to the number of isoprene units used. There are two metabolic pathways of creating terpenoids: Mevalonic acid pathway and MEP/DOXP pathway (Wolinsky, 1973).

1.2.9 Carotenoid

Carotenoids are organic pigments that are found in the chloroplasts of plants and some other photosynthetic organisms like algae, some bacteria and some fungi (Armstrong and Hearst, 1996). Carotenoids can be produced from fats and other basic organic metabolic building blocks by all these organisms (Brian, 1991).

Carotenoids are split into two classes, xanthophylls (which contain oxygen) and carotenes (which are purely hydrocarbons and contain no oxygen) (Unlu *et al.*, 2005; Brian, 1991). The major role of carotenoid in plants and algae is that they absorb light energy for use in photosynthesis and they protect chlorophyll from photo damage (Kidd, 2011). Carotenoids belong to the category of tetraterpenoids, structurally, carotenoids take the form of a polyene hydrocarbon chain which is sometimes terminated by rings and may or may not have additional oxygen atoms attached (Unlu, *et al*., 2005). The most common carotenoids include lycopene and the vitamin A precursor, B-carotene. In plants, the xanthophylls lutein is the most abundant carotenoid and its role in preventing age-related eye disease is currently under investigation (Alija, *et al*., 2004).

1.3 Antioxidants and free radicals

Antioxidants are molecules which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged (Vertuani *et al*., 2004). It is therefore an oxidation reaction (Davies, 1995). Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions (Stohs and Bagchi, 1995). Antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols (Valko *et al*., 2007). Free radicals are atoms or groups of atoms with an odd (unpaired) number of electrons and can be formed when oxygen interacts with certain molecules (Knight, 1998; Stohs and Bagchi, 1995). Once formed, these highly reactive radicals can start a chain reaction. Their chief danger comes from the damage they can do when they react with important cellular components such as DNA, or the cell membrane (Valko *et al*., 2004). Cells may function poorly or die if this occurs. To prevent free radicals damage, the body has a defense system of antioxidants (Benzie, 2003; Davies, 1995). A paradox in metabolism is that, while the vast majority of complex life on earth requires oxygen for its existence, oxygen is highly reactive molecule that damages living organisms by producing reactive oxygen species (Valko *et al*., 2007). Consequently, organisms contain a complex network of antioxidants metabolism and enzymes that work together to prevent oxidative damage to cellular components such as DNA, proteins and lipids (Jha *et al*., 1995). In general, antioxidant systems either prevent these reactive species from being formed or remove them before they can damage vital components of the cell (Sies, 1997; Davies, 1995). However, reactive oxygen species also have useful cellular functions, such as redox signaling. Thus, the function of antioxidant systems is not to remove oxidants entirely but instead to keep them at an optimum level. The reactive oxygen species produced in cells include hydrogen peroxide (H_2O_2), hypochlorous acid (HClO) and free radicals such as the hydroxyl radical ($^{\cdot}OH$) and the superoxide anion

(O_2^-) (Hirst *et al.,* 2008). The hydroxyl radical is particularly unstable and will react rapidly and non-specifically with most biological molecules (Jha *et al.,* 1995). This species is produced from hydrogen peroxide in metal-catalyzed redox reactions such as the Fenton reaction (Sies, 1997). These oxidants can damage cells by starting chemical chain reactions such as lipid peroxidation, or by oxidizing DNA or proteins (Vertuani *et al*., 2004). Damage to DNA can cause mutations and possibly cancer, if not reversed by DNA repair mechanisms (Knight, 1998) while damage to protein causes enzyme inhibition, denaturation and protein degradation (Davies, 1995).

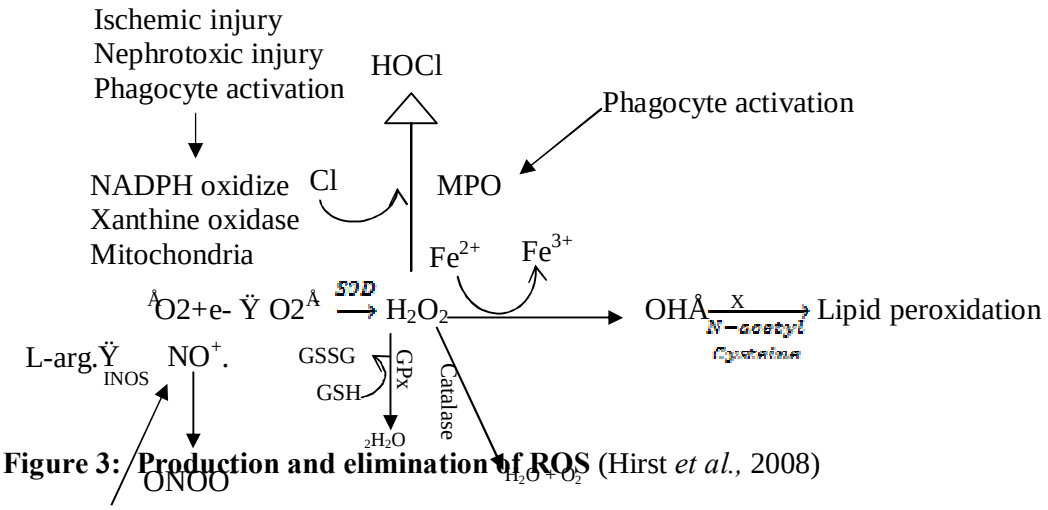


Figure 3: Production and elimination of ROS (Hirst *et al.,* 2008)

1.3.1 Hydroxyl radical

The hydroxyl radical, ·HO, is the neutral form of the hydroxide ion (HO^-). Hydroxyl radicals are highly reactive and consequently short-lived; however, they form an important part of radical chemistry (Sies, 1993). Hydroxyl radicals are produced from the decomposition of hydroperoxides (ROHO) or atmospheric chemistry by the reaction of excited atomic oxygen with water (Reiter and Caneiro, 1997), Hydroxyl radicals are also produced during UV-light dissociation of H_2O_2 and likely in Fenton chemistry, where trace amounts of reduced transition metals catalyze peroxide ï mediated oxidations of organic compounds (Sunil *et al*., 2013; Reiter and Caneiro, 1997). The hydroxyl radical is often referred to as the ñdetergentò of the troposphere because it reacts with many pollutants often acting as the first step to their removal (Sies, 1993). It has an important role in eliminating some greenhouse gases like methane and ozone (Storey *et al*., 1981). The first reaction with many volatile organic

compounds (VOCs) is the removal of an hydrogen atom, forming water and an alkyl radical ($R^{Å}$) (Sies, 1993) $^{Å}HO + RH$ Ÿ $H_2O + R^{Å}$

The alkyl radical will typically react rapidly with oxygen forming a peroxy radical $R^{Å} + O_2$ Ÿ RO_2 (Sunil *et al.,* 2013). Hydroxyl radicals can occasionally be produced as a byproduct of immune action. Macrophages and microglia most frequently generate this compound when exposed to very specific pathogens, such as certain bacteria (Sies, 1993; Sunil *et al.,* 2003).

The destructive action of hydroxyl radicals has been implicated in several neurological autoimmune diseases such has HAND when immune cells become over-activated and toxic to neighbouring healthy cells (Sies, 1993). The hydroxyl radical can damage virtually all types of macromolecules (Sies, 1993; Storey *et al*., 1981). Unlike superoxide, which can be detoxified by superoxide dismutase, the hydroxyl radical cannot be eliminated by an enzymatic reaction (Storey *et al*, 1981). Mechanisms for scavenging peroxyl radicals for the protection of cellular structures includes endogenous antioxidants such as melatonin and glutathione and dietary antioxidants such as mannitol and vitamin E. (Reiter and Carneiro, 1997).

1.3.2 Superoxide anion radical

It has the chemical formula $O_2^{Å-}$. It is the product of one-electron reduction of molecular (O_2), which occurs widely in nature (Holleman and Wibers, 2001). Superoxide is biologically quite toxic and is deployed by the immune system to kill invading microorganisms (Muller *et al*., 2007). In phagocytes, superoxide is produced in large quantities by the enzyme NADPH oxidase for use in oxygen-dependant killing mechanisms of invading pathogens (Miller and Fridovich, 1986). Mutations in the gene coding for the NADPH oxidase cause an immune deficiency syndrome called chronic granulomatous disease (Muller *et al*., 2007; Rapoport *et al*., 1994) characterized by extreme susceptibility to infection, especially catalase positive organisms (Rapoport *et al.,* 1994). Because superoxide is toxic, nearly all organisms living in the presence of oxygen contain isoforms of the superoxide scavenging enzyme, superoxide dismutase (SOD) (Holleman and Wibers, 2011). SOD is an extremely efficient enzyme that catalyzes the neutralization of superoxide nearly as fast as the two can diffuse together spontaneously in solution (Rapoport *et al*., 1994).

1.4 Dietary antioxidants

Dietary antioxidants vitamins C, E and beta carotene are among the most widely studied vitamins and are group of organic substance present in minute amounts in foods stuffs that are essentially for normal metabolism (Bender, 2003; Kutsky, 1973; Halliwel 1991). Vitamins also directly scavenge ROS and upregulate the activities of antioxidant enzymes

(Topinka *et al.,* 1989). Vitamin C is considered the most important water-soluble antioxidant in extracellular fluids. It is capable of neutralizing ROS in the aqueous phase before lipid peroxidation is initiated (Sies, 1997). Vitamin E is one of the most important antioxidants; it inhibits ROS ï induced generation of lipid peroxyl radicals thereby protecting cells from peroxidation of PUFA in membrane phospho-lipids from oxidative damage of plasma very low density lipoprotein, cellular proteins, DNA and from membrane degeneration (Aruoma, 1998). Consequently, a dietary deficiency of vitamin E reduces the activities of hepatic catalase, GSH peroxidases and glutathione reductase (Fischer ï Nielson *et al.,* 1992). Vitamin C has been cited as being capable of regenerating vitamin E (Sies, 1997). Beta carotene and other carotenoids are believed to provide antioxidant protection to lipid ï rich tissues. Research suggests beta carotene may work synergistically with vitamin E (Sies, 1997). A diet that is excessively low in fat may negatively affect beta carotene and vitamin E absorption as well as other fat-soluble nutrients. Fruits and vegetables are major sources of vitamin c and carotenoids, while whole grains and high quality properly extracted and protected vegetable oils are major sources of vitamin E (Sies, 1997).

1.5.0 Oxidative stress

As remarkable as our antioxidant defense system is, it may not always be adequate. Oxidative stress reflects an imbalance between the systematic manifestation of reactive oxygen species and a biological systemôs ability to readily detoxify the reactive intermediates or to repair the resulting damage (Sies, 1997; Finkel and Holbrook, 2000). Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids and DNA (Bjelakovic *et al*., 2007; Benzie, 2003). Oxidative stress can cause disruptions in normal mechanisms of cellular signaling. Reactive oxygen species can be beneficial as they are used by the immune system as a way to attack and kill pathogens (Stohs and Bagchi, 1995). Short-term oxidative stress may also be important in prevention of aging by induction of a process named mitohormesis (Bjelakovic *et al.,* 2007). Oxidative stress is associated with increased production of oxidizing species or a significant decrease in the effectiveness of antioxidant defenses, such as glutathione (Vertuani *et al*., 2004). The effects of oxidative stress depend upon the size of these changes, with a cell being able to overcome small perturbations and regain its original state (Valko *et al*., 2004). Oxidative damage in DNA can cause cancer. Several antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxide, glutathione reductase, glutathione S-transferase etc. protect DNA from oxidative stress. It has been proposed that polymorphisms in these enzymes are associated with DNA damage and subsequently the

individualôs risk of cancer susceptibility (Valko *et al*., 2004; Sies, 1997). The inflammatory response that occurs after strenuous exercise is also associated with oxidative stress (Knight, 1998). During this process, free radicals are produced by neutrophils to remove damaged tissue. As a result, excessive antioxidant levels may inhibit recovery and adaptation mechanisms (Davies, 1995). Antioxidants supplements may also prevent any of the health gains that normally come from exercise, such as increased insulin sensitivity (Knight, 1998; Benzie, 2003).

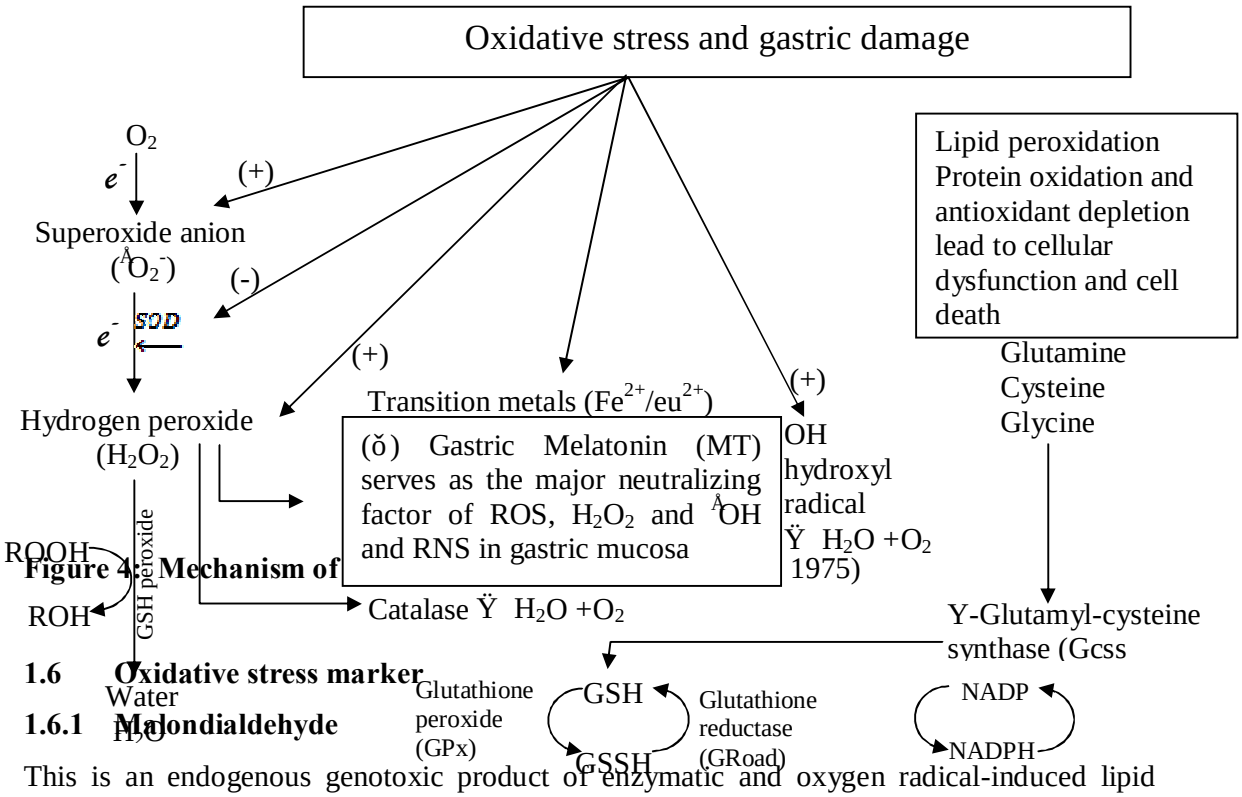


Figure 4: Mechanism of

1.6 Oxidative stress marker

1.6.1 Malondialdehyde

This is an endogenous genotoxic product of enzymatic and oxygen radical-induced lipid peroxidation whose adducts is known to exist in DNA isolated from healthy human beings (Marnett, 1999). MDA can interact with several functional groups on proteins and lipoproteins, altering their chemical behavior and possibly contributing to carcinogenesis and mutagenesis (Del-Rio *et al.,* 2005). Due to its highly reactive nature, MDA also functions as an eletrophile that can cause toxic stress within the cell and is therefore a potent marker for measuring the overall level of oxidative stress within an organism (Nair and Oôneil, 2008). Reactive oxygen species (ROS) degrade polyunsaturated lipids forming malondialdehyde (Pryor and Stanley, 1975). This compound is a reactive aldehyde and is one of the many eletrophile species that cause toxic stress in cells and form covalent protein adducts referred to as advanced lipoxidation end products (ALE), in analogy to advanced glycation end

products (AGE). The production of this adehyde is used as a biomarker to measure the level of oxidative stress in an organism (Del-Rio *et al*., 2005). Human aldehyde dehydrogenase 1 family, member A1 (ALDH1A1) is capable of oxidizing malondialdehyde (Marnett, 1999).

1.7 Antioxidant Enzymes

In addition to dietary antioxidants, the body relies on several endogenous defense mechanisms to help protect against free radical-induced cell damage. The antioxidant enzymes ï catalase and superoxide dismutase (SOD) ï metabolize oxidative toxic intermediates and require micro-nutrient cofactors such as selenium, iron, copper, zinc and manganese for optimum catalytic activity (Duthie *et al.,* 1996).

1.7.1 Catalase

Catalases are enzymes that catalyse the conversion of hydrogen peroxide to water and oxygen, using either an iron or manganese cofactor (Chelikani *et al*., 2004). This protein is localized to peroxisomes in most eukaryotic cells (Machly and Chance, 1954). Catalase is an unusual enzyme since although hydrogen peroxide is its only substrate, it follows a ping-pong mechanism. Here, its cofactor is oxidized by one molecule of hydrogen peroxide and then regenerated by transferring the bound oxygen to a second molecule of substrate (Shroeder *et al*., 1969; Isobe *et al*., 2006). Despite its apparent importance in hydrogen peroxide removal, humans with genetic deficiency of catalase ï acatalasemia ï or mice genetically engineered to lack catalase completely, suffer few ill effects (Brioukhanov *et al*., 2006; Cao, *et al*., 2003).

1.7.2 Superoxide Dismutase

Superoxide dismutase (SOD) is a class of closely related enzymes that catalyze the breakdown of the superoxide anion into oxygen and hydrogen peroxides (McCord and Fridovich, 1988). SOD enzymes are present in almost all aerobic cells and in extracellular fluids (Borgstahl *et al*., 1992). Superoxide dismutase enzymes contain metal ion cofactors that depending on the isozyme can be copper, zinc, manganese or iron. In humans, the copper/zinc SOD is present in the cytosol while manganese SOD is present in the mitochondrion (Alscher *et al*., 2002). There also exists a third form of SOD in extracellular fluids which contains copper and zinc in its active sites (Barondeau *et al*., 2004). The mitochondrial isozyme seems to be the most biologically important of these three since mice lacking this enzyme die soon after birth (Brioukhanov *et al*., 2006; Alscher *et al*., 2002).

1.8 Liver Function Tests

These are groups of clinical biochemistry laboratory blood assays designed to give information about the state of a patientôs liver. The parameters measured are albumin,

bilirubin (direct and indirect) etc. Liver transaminases (AST and ALT) are useful biomarkers of liver injury in a patient with some degree of intact liver function (Pincus and Abraham, 2011). These tests are performed on a patientôs serum or plasma sample. Some tests are associated with functionality (e.g. albumin). Some with cellular integrity (e.g. transaminase) and some with conditions linked to the biliary tract (gramma-glutamyl transferase and alkaline phosphatase (Johnson, 1999; McClatchy, 2002).

1.8.1 Aspartate transaminase (AST)

Aspartate transaminase (AST) is a pyridoxal phosphate (PLP) ï dependent transaminase enzyme that catalyzes the reversible transfer of an Ŭ-amino group between aspartate and glutamate (Muriana *et al.,* 1991). It is an important enzyme in amino acid metabolism (Muriana *et al*., 19991; McPhalen *et al*., 1992). It is found in the liver, skeletal muscle, kidneys and red blood cells and it is commonly measured clinically as a marker for liver health (McPhalen *et al*., 1992). It is also called aspartate amino ï transferase or serum glutamic oxaloacetic transaminase (SGOT) (Kochlar and Christen, 1992).

Aspartate (ASP) + Ŭ-ketoglutarate aspartate Ÿ Oxaloacetate + glutamate.

AST relies on PLP as a cofactor to transfer the amino group from aspartate or gulatate to the corresponding keto acid (Muriana *et al*., 1991). The cofactor shuttles between PLP and the pyridoxamine phosphate (PMP) form in the process (Kochlar and Christen, 1992). In amino acid degradation following the conversion of Ŭ-ketoglutarate to glutamate, glutamate subsequently undergoes oxidative deamination to form ammonium ions which are excreted as urea.

In the reverse reaction aspartate may be synthesized from oxaloacetate which is key intermediate in the citric acid cycle (Gaze, 2007). Aspartate transaminase as with all transaminases, operates via dual substrate recognition; that is, it is able to recognize and selectively bind two amino acids (ASP and GLU) with different side chains (Kirsch *et al.,* 1984). In either case, the transaminase reaction consists of two similar half-reactions that constitute what is referred to as a ping-pong mechanism. In the first half-reaction, amino acid 1 (eg. L-ASP) reacts with the enzyme ï PLP complex to generate keto acid 1(oxaloacetate) and the modified enzyme ï PMP (Hirotsu *et al.,* 2005). In the second half-reaction, keto acid 2 (Ŭ-ketoglutarate) reacts with enzyme ï PMP to produce amino acid 2 (L-Glu), regenerating the original enzyme ï PLP in the process (Kirsch *et al*., 1984; Gaze, 2007). Formation of a recemic product (D-Glu) is very rare (Hirotsu *et al*., 2005).

AST is similar to alanine transaminase (ALT) in that both enzymes are associated with liver parenchymal cells (Muriana *et al*., 1991). The difference is that ALT is found predominantly

in the liver, with clinically negligible quantities found in the kidneys, heart and skeletal muscle while AST is found in the liver, heart, skeletal muscle, kidneys, brain and red blood cells. As a result, ALT is a more specific indicator of liver inflammation than AST, as AST may be elevated also in diseases affecting other organs (Hirotsu *et al.,* 2005; Kirsch *et al.,* 1984).

1.8.2 Alkaline phosphatase (ALP)

Alkaline phosphatase is a non-specific metalloenzyme which hydrolyses many types of phosphate esters at an alkaline pH in the presence of zinc and magnesium ion (Kim and Wyckoff, 1991). It is non-specific because corticosteroids (exogenous or endogenous ñstressò) induce increases in this enzyme (Kim and Wyckoff, 1991; Martin, 2011). There are two isoenzymes (product of different genes) and several isoforms (produced from post translational modification of isoenzymes) of ALP (Maxam and Gilbert, 1980). The isoenzymes are produced from intestinal and tissue non-specific ALP genes and differ in amino acid sequence, isoforms differ in catalytic sites and activity, immunogenicity and electrophoretic mobility (Wanner and Patrick, 1980). ALP is anchored to cell membranes by glycophophatidylinositol (GPI) proteins. Cleavage of these proteins by bile acids, phospholipase D and proteases releases ALP from membranes resulting in increased ALP levels in serum/plasma (Kim and Wyckoff, 1991).

The primary importance of measuring alkaline phosphatase is to check the possibility of bone disease or liver disease (Ammerman and Azam, 1985). Since the mucosal cells that line the bile system of the liver are the source of alkaline phosphatase, the free flow of bile through the liver and down into the biliary tract and gall-bladder are responsible for maintaining the proper level of this enzyme in the blood (Maxam and Gilbert, 1980). When the liver, bile ducts or gall bladder system are not functioning properly or are blocked, this enzyme is not excreted through the bile and alkaline phosphatase is released into the blood stream (Martin, 2011). Thus the serum alkaline phosphatase is a measure of the integrity of the hepatobiliary system and the flow of bile into the small intestine (Maxam and Gilbert, 1980).

1.8.3 ALT (Alanine Aminotransferase)

ALT is found in plasma and in various body tissues but is most commonly associated with the liver (Wang *et al.,* 2012). It catalyzes the transfer of an amino group from L-alamine to Ŭ-ketoglutarate, the products of this reversible transamination reaction being pyruvate and L-glutamate (Ghouri *et al*., 2010). L-glutamate + pyruvate Ÿ Ŭ-ketoglutarate + L-alanine.

ALT (and all transaminases) require the coenzyme pyridoxal phosphate which is converted into pyridoxamine in the first phase of the reaction when an amino acid is converted into a

keto acid (Liver Function Test, 2012). It is measured to determine liver health. However, elevated levels of ALT do not automatically mean that medical problems exist. Fluctuation of ALT levels is normal over the course of the day and they can also increase in response to strenuous physical exercise (Wang *et al*., 2012).

1.9 Renal function tests

Renal function is an indication of the state of the kidney and its role in renal physiology (Stevens *et al.,* 2006). Glomerular filtration rate (GFR) which is the flow rate of filtered fluid through the kidney and creatinine clearance (CrCl) which is the volume of blood plasma that is cleared of creatinine per unit time may be calculated by comparative measurements of substances in the blood and urine (Guyton and Hall, 2006). The results of these tests are important in assessing the excretory function of the kidneys (National Kidney Foundation, 2002). Plasma concentrations of the waste substances of creatinine and urea as well as electrolytes can as well be used to determine renal function, (Levey *et al*., 2006). However, blood urea nitrogen (BUN) and creatinine will not be raised above the normal range until 60% of total kidney function is lost (Guyton and Hall, 2006).

1.9.1 Urea

The liver produces urea in the urea cycle as a waste product of the digestion of protein (Landry and Basari*,* 2011). Blood urea nitrogen (BUN) is an indication of renal health. The main causes of an increase in BUN are: high protein diet, decrease in glomerular filtration rate (GFR) and in blood volume (hypovolemia), congestive heart failure, gastrointestinal hemorrhage, fever and increased catabolism (BUN: The Test, 2009).

1.9.2 Creatinine

Creatinine is a breakdown product of creatine phosphate in muscle and is usually produced at a fairly constant rate by the body depending on muscle mass (Gross *et al.,* 2005). Serum creatinine is an important indicator of renal health because it is an easily measured byproduct of muscle metabolism that is excreted unchanged by the kidneys (Rule *et al.,* 2004). Creatine is synthesized primarily in the liver from the methylation of glycocyamine by S-adenosyl methionine. It is then transported through blood to other organs, muscle and brain where through phosphorylation, it becomes the high-energy compound phosphocreatine (Waikar and Bonventre, 2009). During the reaction, creatine and phosphocreatine are catalyzed by creatine kinase and a spontaneous conversion to creatinine may occur (Rule *et al.,* 2004). Creatinine is removed from the blood chiefly by the kidneys primarily by glomerular

filtration but also by proximal tubular secretion (Mehta *et al*., 2007). Little or no tubular reabsorption of creatinine occurs if the filtration in the kidney is deficient, creatinine blood level rises. Therefore creatinine levels in blood and urine may be used to calculate the creatinine clearance (CrCl) which correlates with the glomerular filtration rate (GFR) (Myers *et al*., 2006). A rise in blood creatinine level is observed only with marked damage to functioning nephrons, as a result, it is unsuitable for detecting early stage kidney disease, better estimation of kidney function is given by calculating the estimated glomerular filtration rate (eGFR) (Katie *et al*., 2014).

1.10 Electrolytes

They are positively and negatively charged particles (ions) that are found when mineral or other salts dissociate in water. They include sodium (Na^+), potassium (K^+), chloride (Cl^-) ions etc. Since electrolytes carry a charge, they can conduct electrical current in water (Estevez *et al*., 2008). This characteristic of electrolytes is important because the current enables electrolytes to regulate how and where fluids are distributed throughout the body, which includes keeping water from floating freely across cell membranes (Kamil *et al*., 2011). To control fluid passage across cell membranes, cells regulate the movement of electrolytes into and out of them which causes water to follow the charged particles around wherever they go (Estevez *et al*., 2008). These actions help maintain a state of fluid balance. The difference in electrical balance inside and outside of cells also allows for transmission of nerve impulses contraction or relaxation of muscles, blood pressure control and proper gland functioning. The presence of electrolytes determines the acidity or pH of some fluids especially blood (Kamil *et al*., 2011). The concentrations of these ions in the blood stream remain fairly constant and an imbalance can lead to hypernatremia and hyponatremia ï too much or too little sodium and hyperkalemia and hypokalemia or excessive and insufficient levels of potassium (Kamil *et al*., 2011; Estevez *et al*., 2008).

1.10.1 Sodium

Sodium (Na^+) is the major positive ion (cation) in fluid outside of cells (Reynolds *et al*., 2006). Excess sodium such as that obtained from dietary sources is excreted in the urine. Sodium ion regulates the total amount of water in the body and the transmission of sodium into and out of the individual cells also plays a role in critical body functions (Rosner and Kirven, 2006). Many processes in the body especially in the brain, nervous system and muscles require electrical signals for communication (Schrier, 2010; Reynolds *et al*., 2006). The movement of sodium ion is critical in the generation of these electrical signals (Adrogue and Madias, 2000). Too much or too little of sodium ion therefore can cause cells to

malfunction and extremes in the blood sodium ion level can be fatal (Reynolds *et al.,* 2006). Increased sodium ion (hypernatremia) in the blood occurs whenever there is excess sodium in relation to water while decreased concentration of sodium (hyponatremia) occurs whenever there is a relative increase in the amount of body water relative to sodium (Adrogue and Madias, 2000).

1.10.2 Chloride

Chloride, a major anion is important in the maintenance of the cation/anion balance between intra and extra cellular fluids (Zumdahl, 2009). It is essential to the control of proper hydration, osmotic pressure and acid/base equilibrium. Low serum chloride values are found with extensive burns, excessive vomiting, intestinal obstruction, nephritis, metabolic acidosis and in Addisonian crisis (Cambier *et al.,* 1998; Zumdahl; 2009). Elevated serum chloride values may be seen in dehydration, hyperventilation, congestive heart valve and prostatic or other types of urinary obstruction (Tietz, 1976; White, 1970).

1.11 Histopathology

Histopathology refers to the microscopic examination of tissues in order to study the manifestation of disease. Specifically, in clinical medicine, histopathology refers to the examination of a biopsy or surgical specimen by a pathologist, after the specimen has been processed and histological sections have been placed onto glass slides. This is the most important tool of the anatomical pathologist in routine clinical diagnosis of cancer and other diseases.

Histopathological examination of tissues starts with surgery, biopsy or autopsy. The tissue is removed and then placed in a fixative which stabilizes the tissue to prevent decay. The most common fixative is formalin (10% formaldehyde in water). The histological slides are examined under a microscope by a pathologist (Drury *et al.,* 1967).

1.12 Aim of the study

This study was aimed at investigating the antioxidant and toxicologic effects of the methanol leaf extract of *Stephania dinklagei* in Wistar male albino rat with a view to discovering its antioxidant and toxic potentials.

1.13 Specific objectives of the research

- To determine qualitatively and quantitatively the phytochemical constituents of the methanol leaf extract of *Stephania dinklagei* which could help to suggest the reason for its use as a medicinal plant.

- To determine quantitatively the vitamin contents of the methanol leaf extract of *Stephania dinklagei.*
- To determine the antioxidant activities of the methanol leaf extract of *Stephania dinklagei.*
- To determine the acute lethal dose (LD_{50}) of the extract.
- To assay for the effect of the extract on serum liver marker enzymes.
- To assay for the effect of the extract on the serum concentration of the kidney marker
- To assay for the effect of the extract on oxidative stress markers (Malondialdehyde)
- To assay for the effect of the extract on some antioxidant enzyme activity (superoxide dismutase and catalase).
- To assay for the histopathological effects of the leaf extract on the liver and kidney of the rat which will complement the results of liver and kidney enzymes

CHAPTER TWO
MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant material

The leaves of *Stephania dinklagei* were used for this study. They were collected from Aku in Igbo-Etiti L.G.A of Enugu State and were identified by Mr. Alfred Ozioko of the Bioresources Development and Conservation Programme (BDCP) Research centre, Nsukka, Enugu State.

2.1.2 Animals

Forty-eight (48) male adult albino rats (118-232g) and thirty six (36) male albino mice (37-48 g) were used for the acute toxicity (LD_{50}) study. All the animals were purchased from the animal house of the Department of Zoology, University of Nigeria, Nsukka. The animals were housed in well ventilated cages for the period of the experiment. They were fed with standard growerôs mash rat pellets (Grand Cereals LTD) and given water *ad libitum*.

2.1.3 Equipment

The equipment used were those of the Department of Biochemistry, University of Nigeria, Nsukka. They include:

Equipment	Manufacturer
Centrifuge	Vickas Ltd (England)
Colorimeter	E Scientific Co. (India)
Measuring cylinder	Pyrex (England)
Weighing balance	Vickas Ltd (England)
Spectrophotometer (E312 model)	Jenway (UK)
Electron microscope	Vickas Ltd (England)
Refrigerator	Thermocool (England)
Oven	Gallenkamp (England)
Water bath	Gallenkamp (England)
Separating funnel	Pyrex (England)
Micropipette	Pyrex (England)
Pasteur pipette	Pyrex (England)
Stop watch	Hanna (China)
Cuvette (2ml)	

2.1.4 Chemicals and Reagents

The chemicals and reagents used were of analytical grade and include:

Absolute ethanol	BDH (England)
Absolute methanol	BDH (England)
Anticoagulant (EDTA)	Randox (USA)
Chloride reagent	Merck Darmstadt (Germany)
Turkôs solution (20% glacial acetic acid)	Merck Darmstadt (Germany)
Sodium Dodecyl Sulphate	BDH (England)
Trichlordacetic acid	Sigma Aldrich (Germany)
2, 4-dinitrophenyl hydrazine	Merck Darmstadt (Germany)

Combined colour reagent	QCA (Spain)
Molischôs reagent	QCA (Spain)
Mayerôs reagent	BDH (England)
Wagnerôs reagent	Randox (USA)
Picric acid	Merck Darmstadt (Germany)
Aluminium chloride solution	BDH (England)
Thiobarbituric acid	BDH (England)
DPPH	Sigma Aldrich
Sulfanilamide	Sigma Aldrich
Methanol	Sigma Aldrich
Glacial acetic acid	Fison
HCL	BDH (England)
Sodium chloride	LOBA chemie
Ferric chloride	LOBA chemie
Sulphuric acid	LOBA chemie
Fehling solution B	Riedel-de-Haen
Ethyl acetate	Sigma-Aldrich
Na_2C0_3	J.T. Baker
Phosphoric acid	Sigma Aldrich
Potassium iodide	BDH (England)
Lead acetate	M&B
Acetone	BDH (England)

2.2.0 Methods

2.2.1 Preparation of plant material

Fresh leaves of *Stephania dinklagei* were washed with clean water to remove dirt and sand and then drained. They were dried under shade for twelve (12) days and then pulverized into fine powder.

2.2.2 Extraction of plant material

A quantity, 800 g of the ground leaves of *Stephania dinklagei* was macerated in 2.5 litres of methanol for 48 hours. The solution was filtered with Whatman filter paper and the filtrate concentrated to a semi-solid residue in an oven at 60^0C and the extract obtained as a greenish

black gummy solid. The yield was 44.55 g after which the residues were transferred into pre-weighed sample containers and stored in a refrigerator for further experiments.

2.2.3 Preparation of samples for microscopic analyses

The rats (4 per group) were anaesthetized and sacrificed after the 7^{th}, 14^{th} and 21^{st} day of feeding with the extract of *Stephenia dinklagei* leaves. Thereafter, the abdomen was opened and livers and kidneys taken and perfused with 10% normal saline for 48 hours. The biopsy materials were dehydrated in alcohol beries, processed in xylene and then embedded in paraffin. Sections taken with microtome at 0.4-0.6 micron thickness from each specimen were stained with Hematoxylene and Eosin. All sections were then evaluated under the microscope at X100 and X400 magnification and pictures taken.

2.2.4 Preparation of reagents

50% (W/V) Ferric chloride solution

Ferric chloride (5.0 g) was dissolved and made up to 100 ml with distilled water.

Dilute sulphuric acid

Concentrated sulphuric acid (10.9 ml) was mixed with 5 ml of distilled water and made up to 100 ml

Wagner's reagent.

Iodine crystal (2.0 g) and potassium iodide (3.0 g) were dissolved in minimum amount of water and then made up to 100 ml with distilled water.

Mayer's reagent

Mercuric chloride (13.5 g) was dissolved in 50 ml of distilled water. Also, 5.0 g of potassium iodide was dissolved in 20 ml of distilled water. The two solutions were mixed and the volume make up to 100 ml with distilled water.

Dragendorff's reagent

Bismuth carbonate (0.85 g) was dissolved in 100 ml of glacial acetic acid and 40 ml of distilled water to give solution A. Another solution called solution B was prepared by dissolving 8.0 g of potassium iodide in 20 ml of distilled water. Both solutions were mixed to give a stock solution.

Molisch reagent

α-naphthol (1.0 g) was dissolved in 100 ml of absolute ethanol

2% (V/V) Hydrochloric acid.

Concentrated hydrochloric acid was diluted with some distilled water and made up to 100 ml.

Preparation of normal saline

Normal saline was prepared by dissolving 0.9 g of sodium chloride in distilled water and made up to 100 ml

Preparation of 25% Trichloroacotic Acid (TCA)

TCA (25 g) was dissolved in 0.3% NaoH and made up to the 100 ml mark with NaoH in a measuring cylinder.

Preparation of 1% Thiobarbituric acid (TBA) in 3% NaOH

TBA (1 g) was dissolved in distilled water and made up to the 100 ml mark with distilled water in a measuring cylinder

Preparation of 0.3% sodium hydroxide (NaOH)

A known weight, (0.3 g) NaOH was dissolved in a little amount of water and made up to 100 ml mark with distilled water in a measuring cylinder.

Preparation of 20% Sodium Dodecyl Sulphate (SDS)

SDS (20 g) was dissolved in some quantity of distilled water and made up to 100 ml mark with distilled water in a measuring cylinder.

Preparation of 2% Glacial acetic acid

Glacial acetic acid was dissolved in distilled water and made up to 100 ml with distilled water in a measuring cylinder.

Preparation of phosphate buffer pH 7.4

Phosphate buffer was prepared by dissolving 1.20 g of dihydrogen phosphate and 0.885 g hydrogen phosphate in 1 litre volume of distilled water and the pH adjusted.

Ferric chloride ($FeCl_3$aq) 5%:

A known weight, 2.3 g of $FeCl_3$ was weighed with the scale and dissolved in 50 ml of dH_20

Ammonium solution (NH_4^+):

A known weight, 37.5 ml of NH_3 (aq) + 62.5 ml dH_20

45% methanol:

A quantity, 45 ml absolute methanol was mixed with 55 ml dH_20

Dil. H_2SO_4 (aq):

A quantity, 10.4 ml conc. H_2SO_4 was mixed with 5 ml dH_20 and made up to 100 ml

2% HCl:

Concentrated HCl (2 ml) was mixed with 98 ml of distilled water.

Na_2CO_3: Sodium trioxocarbonate IV (6 g) dissolved in distilled water and made up to 100 ml.

Sodium acetate: a quantity, 5 g of sodium acetate dissolved in distilled water and made up to 100 ml

Fehling solution A: $CuSo_4.5H_20$ (34.6 g) was mixed with 500 ml of distilled water.

1.0% TBA: Thiobarbituric acid, TBA (1 g) was dissolved in 0.3% NaoH

25% TCA: Trichloroacetic acid, TCA (25 g) was dissolved in 100 ml of distilled water

0.3% NaoH: Sodium hydroxide, NaOH (3 g) was dissolved in 100 ml of distilled water

2.2.5 Qualitative Phytochemical Analysis of Leaves of *Stephania dinklagei*

The phytochemical analysis of the leaves of *Stephania dinklagei* were carried out according to the method of Harborne (1973) and Evans (2002) to identify its active constituents.

2.2.5.1 Test for alkaloids

The sample (0.2 g) was boiled with 5ml of 2% HCl on a steam bath. The mixture was filtered and 1ml of the filtrate was treated with 2 drops of the following reagents.

i. Dragendroffôs reagent: An orange precipitate indicated the presence of alkaloids
ii. Mayerôs reagent: A creamy-white precipitate indicated the presence of alkaloids

2.2.5.2 Test for flavonoids

The sample (0.2 g) was heated with 10ml ethyl acetate in boiling water for 3 minutes. The mixture was filtered and the filtrate was used for the following tests.

i. Ammonium test: 4 ml of the filtrate was shaken with 1ml of dilute ammonium solution to obtain two layers. The layers were allowed to separate. A yellow precipitate observed in the ammonium layer indicated the presence of flavonoids.
ii. Aluminium chloride test: 4ml of the filtrate was shaken with 1ml of 1% aluminium chloride solution and observed for light yellow colouration that indicated the presence of flavonoids.

2.2.5.3 Test for glycosides

The sample (0.2 g) was mixed with 30 ml of distilled water and 15 ml of dilute sulphuric acid respectively and heated in a water bath for 5 minutes. The mixtures were filtered and the filtrates used for the test.

To 5 ml of each of the filtrate, 0.3 ml of Fehlingôs solutions A and B was added until it turned alkaline (tested with litmus paper) and heated on a water bath for 2 minutes. A brick-red precipitate indicated the presence of glycosides.

2.2.5.4 Test for saponins

The sample (0.1 g) was boiled with 5 ml of distilled water for 5 minutes. The mixture was filtered while still hot. The filtrate was used for the following tests.

i. Emulsion test: 1 ml of the filtrate was added to two drops of olive oil. The mixture was shaken and observed for the formation of emulsion
ii. Frothing test: 1 ml of the filtrate was diluted with 4 ml of distilled water. The mixture was shaken vigorously and then observed on standing for a stable froth.

2.2.5.5 Test for terpenoids and steroids

Ethanol (9 ml) was added to 1 g of the sample and refluxed for a few minutes and filtered. The filtrate was concentrated to 2.5 ml on a boiling water bath, and 5 ml of hot water was added. The mixture was allowed to stand for 1 hour, and the waxy matter filtered off. The filtrate was extracted with 2.5 ml of chloroform using a separating funnel. To 0.5 ml of the chloroform extract in a test tube was carefully added 1 ml of concentrated sulphuric acid to form a lower layer. A reddish-brown interface showed the presence of steroids. Another 0.5 ml aliquot of the chloroform extract was evaporated to dryness on a water bath and heated with 3 ml of concentrated sulphuric acid for 10 minutes in water. A grey colour indicated the presence of terpenoids.

2.2.5.6 Test for tannins

The sample (0.5 g) was boiled in 20 ml of distilled water in a test tube and then filtered. A few drops of 0.1% of ferric chloride was added and it was observed for brownish green or blue-black coloration.

2.2.5.7 Test for anthraquinone

The sample (5 g) was shaken with 10 ml of benzene, filtered and 5 ml of 10% ammonia added to the filtrate. The presence of pink or violet colour in the ammoniacal (lower) layer indicates the presence of anthraquinone.

2.2.6 Quantitative Phytochemical Analysis of the Leaves of *Stephania dinklagei*

2.2.6.1 Determination of Alkaloid Content

The determination of alkaloid content as described by Harborne (1973) was used. A portion (5 g) of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added, covered and allowed to stand for 2 hours. The mixture was filtered and

the extract was concentrated on a water bath to one quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract till a precipitate was formed. The precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue was the alkaloid, which was dried and weighed.

2.2.6.2 Determination of Flavonoids

This was determined according to the method of Harbone (1973). A quantity, 5 g of the sample was boiled in 50 ml of 2M HCl solution for 30 minutes under reflux. It was allowed to cool and then filtered. A measured volume of the extract was treated with equal volume of ethyl acetate starting with a drop. The solution was filtered into a weighed crucible. The filtrate was heated to dryness in an oven at 60^0C. The dried crucible was weighed again and the difference in the weight gave the quantity of flavonoid present in the sample.

2.2.6.3 Determination of Steroids

This was determined by the method described by Edeoga *et al* (2005) as summarized herein. A known weight of each sample was dispersed in 100 ml freshly distilled water and homogenized in a laboratory blender. The homogenate was filtered and the filtrate was eluted with normal ammonium hydroxide solution (pH 9). The elute (2 ml) was put in test tube and mixed with 2 ml of chloroform. Ice-cold acetic anhydride (3ml) was added to the mixture in the flask and 2 drops of conc. H_2SO_4 were cautiously added. Standard sterol solution was prepared and treated as described above the absorbances of standard and prepared sample were measured in a spectrophotometer at 420 nm.

2.2.6.4 Determination of Saponins

Saponin was determined according to the method by Obadoni and Ochuko (2001). The sample (10 g) was weighed into a 250 ml conical flask and 100 ml of 20% ethanol added to it. The sample was heated over a water bath for 4hours at 55 °C. The mixture was filtered and the residue re-extracted with 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml over water bath set at 90°C. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added and the solution shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml 5% sodium chloride solution. The remaining solution was heated in a water bath and evaporated to dryness after which it was dried to constant mass and saponin content calculated as percentage.

2.2.6.5 Determination of Tannins

The sample (1 g) was extracted with 300 ml diethyl ether for 20 hours at room temperature. The residue was boiled for 2 hours with 100 ml distilled water, cooled and filtered. The extract was adjusted to a volume of 100 ml in a volumetric flask. Then, the tannin content of the sample was determined colorimetrically using Folin-Denis reagent by measuring the solutionôs absorbance at 760 nm, using tannic acid a standard.

2.2.6.6 Determination of Glycosides

The method of Onwuka (2005) was used in the determination of glycoside content.

Extraction of cyanide: the sample (5 g) was weighed into a conical flask and 50 ml of distilled water was added after which the flask was corked. The mixture was allowed to stand overnight. The extract was then filtered for glycoside determination.

Procedure:

The sample (1 ml) filtrate was transferred into a test tube and 4 ml alkaline picrate was added, then placed in a water bath for 5 mins. After colour development (reddish-brown colour), the absorbance was read with a spectrophotometer at 490 nm. The blank solution was prepared using 1ml distilled water and 5 ml alkaline picrate solution. The concentration of glycoside was determined using a standard.

$$\text{Concentration of glycoside} = \frac{\text{Absorbance of test sample}}{\text{Absorbance of standard}} \times \frac{\text{Concentration of standard}}{\text{Weight of sample used}}$$

2.2.6.7 Determination of Terpenoids

This was carried out using the Subhadhirasakul and Pechpongs method 2005. The sample (20 g) was macerated with chloroform for seven days. The macerate was filtered and the process repeated. The filtrates were evaporated to dryness. The dried mass give the chloroform extract. The material was dried in the open air and then macerated with methanol using the same procedure as described above to get the methanol extract. The methanol extract gives the terpenoids. These are calculated as percentages on dry mass bases.

2.2.6.8 Determination of Carotenoids

The sample (5 g) was homogenized in 1% methanol solution using a lab blender. The homogenate was filtered to obtain the initial crude extract. 20 ml of ether was added to the filtrate to take up the carotenoid. 20 ml of distilled water was also added and the mixture shaken very well in a separating funnel. The ether layer was recovered and evaporated to dryness at low temperature 30-50 degrees Celsius. The dry extract was then saponified with

ethanoic potassium hydroxide and left overnight in a dark cupboard. The next day, the carotenoid was taken up in 20 ml ether and then washed with two portions of 20 ml distilled water. The carotenoid extract (ether layer) was dried in desiccators. It was then treated with a light petroleum spirit and allowed to stand overnight in a freezer (-10°C). The next day, the precipitated steroid was removed by centrifugation and the carotenoid extract was evaporated to dryness in a weighed evaporating dish. This was cooled in a desiccator. Weighed and carotenoid expressed as a percentage of the sample weight.

2.2.7 Determination of *In vitro* Antioxidant Potential of the Extract

2.2.7.1 2, 2 Diphenyl 1- picryl- hydrazyl (DPPH) radical-scavenging assay

Scavenging activity of the etract on DPPH free radicals was assessed according to the method reported by Gyamti, *et al* (1999) with slight modifications Awah *et al* (2010).

Principle: This method was based on the reduction of DPPH in methanol solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H, which was measured spectrophotometrically at 518nm.

Method: The extract solution (2.0 ml) diluted at different concentrations (25-250 ug/ml) in methanol was mixed with 1.0 ml of 0.3 mM DPPH in methanol. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 25 min. Blank solutions were prepared with each test sample solution (2.0 ml) and 1.0 ml of methanol while the negative control was 1.0 ml of 0.3 mm DPPH solution plus 2.0 ml of methanol. L-Ascorbic acid was used as the positive control. Thereafter, the absorbance of the assay mixtures was measured at 518 nm against each blank. Lower absorbance of the reaction mixture indicated high radical scavenging activity.

Calculation: DPPH radical scavenging activity was calculated using the equation:

% DPPH scavenging activity = 100 ï $\left(\frac{Abs\ sample - Abs\ blank}{Abs\ control}\right)$

Abs = Absorbance

The EC_{50} value (total antioxidant necessary to decrease the initial DPPH radical concentration by 50%) was calculated from the equation of line obtained by plotting a graph of concentration versus percentage inhibition. The anti-radical power (ARP) of extract was calculated as: $ARP = 1/EC_{50}$.

2.2.7.2 Hydroxyl radical (OH) scavenging assay

Principle: The sugar, deoxyribose, on exposure to hydroxyl radical generated by the Fenton reaction model system degrades into fragments and generates a pink chromogen on heating with TBA at low pH and then measured spectrophotometrically at 532 nm.

Method: The 2^1-deoxyribose assay was used to determine the scavenging effect of the extract on the hydroxyl radical ($^{\cdot}OH$) as reported by Halliwell *et al*. (1987), with minor modifications (Awah *et al.,* 2010). Each reaction mixture contained the following final concentrations of reagents in a final volume of 1.0 ml: 2-deoxyribose (2.5 µm), potassium phosphate buffer (pH 7.4, 20 mM), $FeCl_3$ (100 µm), EDTA (104 µm), H_20_2 (1 mM) and L-ascorbic acid (100 µm). Solutions of $FeCl_3$ and L-ascorbic acid were prepared immediately before use in distilled water. The mixtures were incubated for 1h at 37^0C followed by addition of 1.0ml of 1% (W/V) thiobarbituric acid (TBA) in 0.05 M NaOH and 1.0 ml of 2.8% (W/V) trichloroacetic acid (TCA). The resulting mixture was heated for 15 mins at 100^0C. After cooling on ice, absorbance was measured at 532 nm.

Calculation:

Inhibition of 2-deoxyribose degradation expressed in percentage was calculated using the following equation.

$$\% \text{ inhibition} = 100\% \times \frac{A_O - A_S}{A_O}$$

Where A_O is the absorbance of the control and A_S is the absorbance of the tested sample. All determinations were carried out in triplicates and the mean calculated.

The EC_{50} value was calculated from the equation of line obtained by plotting a graph of concentration versus percentage inhibition. The anti-radical power (ARP) of extract was calculated as: $ARP = 1/EC_{50}$.

2.2.7.3 Superoxide radical scavenging assay

The assay was based on the capacity of the extract to inhibit the photochemical reduction of nitro- blue tetrazolium (NBT) (Beauchamp and Fridovich, 1971) and the method of Martinez *et al*., (2001) to determine superoxide radical as modified by Awah *et al* (2010). Each 3.0 ml reaction mixture contained 0.05 M phosphate-buffered saline (PBS) (pH) 7.8), 13 mM methionine, 2 µm riboflavin, 100 µm EDTA, NBT (75 µm) and 1.0 ml of test sample solutions (10-250 µg/ml). The tubes were kept in front of a florescent light and absorbance was read at 560 nm after 20 minutes. The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes containing reaction mixture were kept in the dark and served as blanks. The percentage inhibition of superoxide generation was estimated by comparing the absorbance of the control and those of the reaction mixture containing test sample as per the equation.

$$\% \text{ inhibition} = 100\% \times \frac{A_O - A_S}{A_O}$$

Where A_0 is the absorbance of the control and A_S is the absorbance of the tested sample.

The EC_{50} value was calculated from the equation of line obtained by plotting a graph of concentration versus percentage inhibition. The anti-radical power (ARP) of extract was calculated as: $ARP = 1/EC_{50}$.

2.2.8 Determination of Vitamin Contents of the *Stephania dinklagei* Leaves

The vitamin contents were determined by the method of Pearson (1976).

2.2.8.1 Vitamin A

One gramme (1 g) of ground sample was macerated with 20 ml of petroleum ether. It was then decanted into a test tube and evaporated to dryness. Chloroform-acetic anhydride (0.2 ml) was added to the residue in a (1:1 v/v) ratio. TCA-chloroform (0.2 ml) was added to the resulting solution 1:1 ratio (v/v) and absorbance was measured at 620 nm. Vitamin A standard was prepared in like manner and the absorbance also read at 620 nm. The concentration of vitamin A in the sample was extrapolated from the standard curve.

2.2.8.2 Vitamin E

One gram (1 g) of the sample was macerated with 20 ml of ethanol and then filtered. Ferric chloride in ethanol 0.2% and 1 ml of 0.5% $\propto -\propto$ - dipyridine were added to 1ml of the filtrate. This was diluted to 5 ml with distilled water. The absorbance was read at 520 nm. The standard solutions were prepared similarly and the concentration of vitamin E extrapolated from the standard curve.

2.2.8.3 Vitamin C

The sample (1 g) was macerated with 20 mls of 0.4 % oxalic acid. The mixture was filtered. To 1 ml of the filtrate was added 9 ml of indolephenol reagent. The standard solution of vitamin C was prepared similarly and the absorbances of the sample and the standard solutions were read at 520 nm. The concentration of vitamin C was extrapolated from the standard curve of vitamin C.

2.2.9 Acute Toxicity text of the methanol leaf extract of *Stephania dinklagei*

The method of Lorke (1983) was used for the acute toxicity test of the leaves of *Stephania dinklagei.* Thirty six (36) albino mice were used in this study. The test involved two stages. In first stage, the animals were divided into three (3) groups of three mice each and were given 10,100 and 1000 mg/kg body weight of the extracts respectively. In the second stage, 1600, 2900 and 5000 mg/kg body weight of the extracts were orally administered to the animals.

2.2.10 Experimental Design

Forty-eight (48) male Wister albino rats of weighing between 118-232 g were used for the study. They were acclimatized for fourteen (14) days with free access to feed and water. After acclimatization, they were evenly distributed into four groups of twelve rats each. Group 1 was the control group administered with distilled water while the other groups 2, 3 and 4 were administered with 200, 400 and 600 mg/kg respectively of the methanol extracts of *Stephania dinklagei* twice daily for three weeks (21 days). At the 7^{th}, 14^{th} and 21^{st} days of administration of extract, four rats from the control and test groups were sacrificed. Organs including liver and kidney were excised and fixed in 10% formyl saline for histological examination. Whole blood was collected by cardiac puncture with sterile tubes and allowed to clot for 2 hrs. Clotted blood was centrifuged for 10 mins and the serum obtained was pooled and the various parameters determined. The route of administration of extracts was via oral route with the aid of an oral intubation tube. The groups and doses administered are summarized below.

Group 1: control rats administered (distilled water, 100 mg/kg)

Group 2: 200 mg/kg body weight of methanol leaf extract

Group 3: 400 mg/kg body weight of methanol leaf extract

Group 4: 600 mg/kg body weight of methanol leaf extract

2.2.11 Antioxidant Enzyme Assay of Rats Treated with Methanol Leaf Extracts of *Stephania dinklagei*

2.2.11.1 Assay of Superoxide Dismutase (SOD Activity

This was determined according to the method described by Xin *et al.* (1993).

Principle: SOD accelerates the dismutation of the toxic superoxide radical ($0_2^{Å}$), produced during oxidative energy processes, to hydrogen peroxide and molecular oxygen.

The assay employed xanthine and Xanthine oxidase (XOD) to generate superoxide radicals which reacted with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. The superoxide dismutase activity was then measured by the degree of inhibition of this reaction. One unit of SOD was that which causes a 50 % inhibition of the rate of reduction of INT under the conditions of the assay.

Xanthine $\underline{\text{SOD}}$ Uric acid + $0_2^{Å}$

I.N.T. $\underline{0_2^{Å}}$ formazan dye

Method: Three test tubes, sample diluents, standards and diluted sample were labeled respectively. To the sample diluents tube, 0.05 ml of Ransod sample diluents was added, to

the standard test tube, 0.05 ml of standard SOD was added and 0.05 ml of diluted sample was added into the diluted sample test tube. 1.7 ml of mixed substrate was added into all the test sample tubes. The solutions in the test tubes were well mixed thoroughly and 0.25 ml of xanthine oxidase added to all the test tubes and mixed. The initial absorbances A_1 was read after 30 seconds. The final absorbance A_2 was read after 3 minutes.

Calculation: % inhibition = $\frac{A_{O-A_S}}{3}\ 100 - \frac{(\Delta A\,sample/min \times 100)}{(\Delta ASi/min)}$

2.2.11.2 Assay of Catalase Activity

This was done according to the method of Aebi (1983)

Principle: The ultra violet absorption of hydrogen peroxide can be easily measured at 240 nm. On the decomposition of hydrogen peroxide (H_2O_2) by catalase, the absorption decreased with time and from the decrease, catalase activity was measured.

Method: Two milliliters (2 ml) of hydrogen peroxide and 2.5 ml of phosphate buffer were added to a beaker. Again, 0.5 ml of the sample was also added and mixed. 1 ml portion of the reaction mixture was added to 2 ml of dichromate acetic acid reagent. The absorbance was read at 540 nm at a minute interval into four places. Catalase activity was calculated using the following equation.

Catalytic concentration (u/L) = $\frac{1.og[abs1/abs2]x0.23}{0.00693}$

2.2.11.3 Determination of Malonialdehyde Concentration

Lipid peroxidation was determined by measuring spectrophotometrically the lipid peroxidation product, malondialdehyde (MDA) as described by Wallin *et al.,* (1993).

Principle: Malondialdchyde (MDA) reacted with thiobarbituric acid to form a pink coloured complex which in acid solution absorbs maximally at 532 nm.

MDA + 2TBA ---------- MDA: TBA adduct + H_2O

Method: The serum (0.1 ml) was mixed with 0.9 ml of water in a beaker. 0.5 ml of 25% trichloroacetic acid and 0.5 ml of 1 % thiobarbituric acid in 0.3 % NaOH were also added to the mixture. The mixture was boiled for 40 minutes in water-bath and then cooled in cold water. Then 0.1 ml of 20 % sodium dodecyl sulfate (SDS) was added to the cooled solution and mixed properly. The absorbance was read at wavelength 532 nm and 600 nm against a blank.

% TBARS = $\frac{A532-A600\ X\ 100}{0.5271\ X\ 0.1}$ (mg/dl).

2.2.12 Effects of Methanol Leaf Extracts of *Stephania dinklagei* on the Activities of Some Liver Marker Enzymes.

The parameters were determined using assay kit from Randox Laboratories Ltd, United Kingdom according to the method of Reitman and Frankel (1957).

2.2.12.1 Assay of Aspartate Aminotransferase (AST) Activity

Principle: AST was measured by monitoring the concentration of oxaloacetate hydrazone formed with 2,4-dinitrophenyl hydrazine. The colour intensity was measured against the blank at 546 nm.

Method: The reagent blank and sample test tubes were set up in duplicates. Serum (0.1 ml) was pipetted into the sample test tubes and 0.5 ml of reagent 1 was pipetted into both sample and blank tubes. The solution were thoroughly mixed and incubated for 30 minutes at 370^0C and pH 7.4. 0.5 ml of Reagent 2 containing 2,4-dinitrophenyldrazine was added into all the test tubes followed by 0.1 ml of sample into the blank tubes. The tubes were thoroughly mixed and incubated for 20 minutes at 25^0C. Sodium hydroxide solution (5.0 ml) was then added to each tube and mixed. The absorbance was read against the blank after 5 mins at 546 nm.

Calculation: The activity of AST was read up from table shown in the appendix.

2.2.12.2 Assay of Alanine (ALT) Aminotransferase Activity

Principle: ALT was measured by monitoring the concentration of pyruvate hydrazone formed with 2,4-dinitrophenylhydrazine. The colour intensity was measured against the blank at 540 nm.

Method: The blank and sample test tubes were set up in duplicates. The serum (0.1 ml) was pipetted into the sample tubes. To these solutions were added 0.5ml buffer solution containing phosphate buffer, L-alanine and α-oxoglutarate. The mixtures were thoroughly mixed and incubated for 30 minutes at 370^0C and pH 7.4. 0.5 ml of reagent containing 2,4-dinitrophenylhydrazine was later added to both tubes while 0.1 ml of sample was added to sample blank tube. The tubes were mixed thoroughly and incubated for 20 minutes at 25^0C. 5ml of sodium hydroxide solution was then added to each tube and mixed. The absorbance was read against the blank after 5 minutes at 540 nm.

Calculation: The activity of ALT was read up from table shown in the appendix

2.10.3 Assay of Alkaline Phosphatase (ALP) Activity

Principle: The estimation of ALP activity was based on the method by Williamson, ALP activity was measured by monitoring the concentration of P-nitrophenol formed when ALP reacts with P-nitrophenylphosphate (PNPP) at 405 nm.

P-nitrophenylphosphate + H_20 ALP phosphate + p-nitrophenol.

Method: The blank and sample test tubes were set up in duplicates and 0.05 ml of sample was pipetted into the sample test tubes. Distilled water (0.05 ml) was pipetted into the blank tube. Substrate (3.0 ml) was pipetted into each tube respectively, which was then mixed and the initial absorbance read at 405 nm. The stop watch was started and the absorbance of the sample and the blank read again three more times at one minute interval.

Calculation: Alkaline phosphatase activity was calculated using the following formulae.

ALP Activity = $\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 3300$ (U/L)

2.2.13 Effects of Methanol Leaf Extract of *Stephania dinklagei* on the Concentrations of Some Renal Function Markers.

2.2.13.1 Determination of Urea Concentration

Principle: Urea in serum was hydrolysed to ammonia in the presence of urease. The ammonia was then measured photometrically by Berthelotôs reaction.

Urea + $H_20 \xrightarrow[\text{Urease}]{} 2NH_3 + CO_2$

NH_3 + hypochlorite + phenol ---------- indophenol (blue compound)

Method: The blank, standard and sample test tubes were set up in duplicates. 10 εl of the sample was added to the sample tubes, 10 εl of the standard was added into the standard test tubes while 10 εl of distilled water was added to the blank test tubes. 10 εl of reagent 1 containing sodium nitroprusside and urease were added into all the test tubes. They were mixed and incubated at 37^0C for 10 minutes. Thereafter, 2.50 ml of reagent 2 containing phenol was added into all the test tubes. Two and half milliliters (2.50 ml) of reagent 3 containing sodium hypochlorite was again added into all the test tubes. The solutions were mixed and incubated at 37^0C for 15 mins. The absorbance of the sample (A sample) and standard (A standard) were read against the blank at 546 nm.

Calculation: The urea concentration was calculated using the formulae:

Urea concentration = $\frac{\text{A sample}}{\text{A standard}} \times \text{standard concentration (mg/L)}$

2.2.13.2 Determination of Creatinine Concentration

Principle: Creatinine in alkaline solution reacts with picric acid to form a coloured complex. The amount of the complex formed is directly proportional to the creatinine concentration.

Method: Test tubes for standard (macro and semi-micro) and sample (macro and semi-micro) were all set in duplicates. Working reagent (2.0 ml and 1.0 ml) (picric acid and sodium hydroxide mixed in equal volumes) were added to the standard (macro and semi-micro) and sample (macro and semi-micro) respectively. Standard solution (2.0 ml and 1.0 ml) were added to the standard (macro and semi-micro) tubes respectively. Again, 0.2 ml and 0.1 ml of the sample were added to the sample test tubes respectively. The solutions were mixed and the absorbance of the standard and sample A_1 were read after 30 seconds. At exactly 2 minutes later, the absorbance A_2 of standard and sample were read at 492 nm.

Calculation: Concentration of creatinine in serum was calculated using the following formulae:

A_2 ï A_1 = æA sample or æA standard

$$\frac{\Delta A\ sample}{\Delta A\ standard} \times \text{standard concentration (mg/L)}$$

2.2.14 Determination of Some Electrolyte Concentrations

The electrolyte concentrations were determined using method of Tietz (1994) as outlined in Teco Diagnostic kit.

2.2.14.1 Determination of Sodium Ion (Na^+) Concentration

Principle: The method was based on modifications of that first described by Maruna and Trinder in which sodium was precipitated as the triple salt, sodium magnesium uranyl acetate, with the excess uranium then reacted with ferrocyanide, producing a chromophore whose absorbance varies inversely as the concentration of sodium in the test specimen.

Method: Test tubes were set up in duplicates for blank, standard, control and sample. Filtrate reagent (uranyl acetate and magnesium acetate in ethyl alcohol, 1 ml) was pipetted into all the test tubes. The sample (50 ε l) were added into all the tubes and distilled water to the blank. All the test tubes were vigorously mixed and centrifuged for 10 minutes. Another set of test tubes were set up and labeled corresponding to the above filtrate tubes. The acid reagent (dilute acetic acid, 1.0 ml) was pipetted to all the tubes. Supernatant (50 ε l) was added to the respective tubes and mixed, colour reagent (potassium-ferrocyanide, reactive stabilizers and fillers, 50 ε l) were added to all tubes and mixed. The absorbance was read at 550 nm.

Calculation: To calculate the concentration, the formulae below were used:

$$\frac{\text{Abs of Blank} - \text{Abs of sample}}{\text{Abs of Blank} - \text{Abs of standard}} \times \text{standard concentration (mEq/l)}$$

2.2.14.2 Determination of Chloride Ion (Cl^-) Concentration

Principle: Chloride ions formed a soluble, non-ionized compound, with mercuric ions and displaced thiocyanate ions from non-ionized mercuric thiocyanate. The released thiocyanate ions reacted with ferric ions to form a coloured complex that absorbed light at 480 nm. The intensity of the colour produced was directly proportional to the chloride concentration.

$Hg(SCN)_2 + 2Cl$ ---------- $Hgcl_2 + 2SCN^-$

$3SCN^- + Fe^{3+}$ ---------- 4 $Fe(SCN)_3$ red complex

Method: Test tubes labeled blank, calibrator and sample were set up in duplicates, and 1.5 ml of chloride reagent was added into each test tube. Calibrator (0.01 ml) or sample (0.01 ml) were added to respective tubes, mixed and was then incubated at room temperature for 5 minutes and the absorbance read at 480 nm.

Calculation: The concentration was calculated as follows:

$$\frac{\text{Abs of Unknown}}{\text{Abs of Calibrator}} \times \text{concentration of} = \text{concentration of calibrator chloride (meq/l)}$$

2.2.14 Histological Examination of the Methanol Leaf Extract of *Stephania dinklagei* by the method of Drury *et al.* (1967)

A. Slicing, fixation and washing

A thin section of the tissue (1cm in diameter) was trimmed with a sharp razor blade. Formalin was used as the fixative agent and for the purpose of preservation. The small pieces of the tissues were placed in 10% formyl saline, the container was shaken gently several times to make sure that the fluid has reached all surfaces and the pieces were not sticking to the bottom. This was incubated for 24 hours to allow proper fixing. The fixed tissue pieces were washed with running water for 24 hours to free them from excess fixatives.

B. Dehydration

All water was removed from the tissue before embedding the tissue in paraffin. The dehydration was achieved by immersing the thin section of the tissue in automatic tissue processor containing 12 jars. The first three jars contained 70 %, 90 % and 90 % absolute alcohol respectively. This was done to remove the water content in the tissues. The absolute alcohol reduced the shrinking that occurred in the tissue. The time for each step was 30 minutes. A second change of absolute alcohol was included to ensure complete removal of water. This was achieved in the second three jar of the automatic tissue processor. This is called a well-refining step.

C. Clearing

Xylene solution was used for clearing the tissue sections. This step was achieved in the third three jars of the automatic tissue processor. This was because the alcohol used for dehydration would not dissolve or mix with molten paraffin, the tissue was immersed in xylene solution which was miscible with both alcohol and paraffin before infiltration could take place. Clearing remove opacity from dehydration tissue, making them transparent. A period of 15 minutes was allowed to elapse before the tissue was removed from the solution for infiltration with paraffin.

D. Infiltration with Paraffin

Paraffin wax with a melting point of 50 to 52^0C range was used to infiltrate the tissue. The tissue was transferred directly from the clearing to a bath containing melted paraffin. After 30 minutes to 1hour incubation in the first bath, the tissue was then removed to a fresh dish of paraffin contained in the fourth three jars of the automatic tissue processor for a similar length of time.

E. Embedding (Blocking) with Paraffin

As soon as the tissue was thoroughly infiltrated with paraffin, paraffin was allowed to solidify around and within the tissue. The tissue was then placed in a small container already filled with melted paraffin and the whole fixture was cooled rapidly with water which embedded the tissue sections.

F. Paraffin Sectioning

The embedded blocks were trimmed into squares and fixed in the microtome knives for sectioning after which the sections were floated on a water bath.

G. Mounting

Glass slides were thoroughly cleaned and a thin smear of albumen fixative was made on the slides. The albumenized slide was used to collect the required section from the rest of the ribbon in the water. The section on the glass slide was kept moist before staining.

H. Staining with hematoxylin

The slides were passed through a series of jars containing alcohols of decreasing strength and various section staining solutions in the following order.

Xylene	3 minutes

Absolute alcohol	3 minutes
95% alcohol	2 minutes
70% alcohol	2 minutes
Lugolôs solution	3 minutes
Running water	3 minutes
50% sodium thiosulphare	3 minutes
Running water	5 minutes
Delafield hematoxylin	5 minutes
Running water	3 minutes
Scott solution	9 minutes
Running water	3 minutes

The counterstaining of the tissue with eosin was followed in the order as stated below:

70% alcohol	1 dip
95% alcohol	2 dips
Absolute alcohol	3 minutes
Absolute alcohol-xylene (1:1)	3 minutes
Xylene	3 minutes
Mounting medium	The section was kept with xylene while cover glass was added on the glass slide.

I. Microscopic observation of slide

The slides prepared were mounted on a photomicroscope, one after the other and were then viewed at different magnification powers (X100 and X400) of the microscope . Photographs of each of the slides were taken.

2.3 Statistical Analysis

The data obtained were analysed using statistical package for service solutions (SPSS) version 20 and the results expressed as mean $\pm$ standard error of mean. Significant differences of the result were established by one-way and two-way ANOVA and the acceptance level of significance was $p<0.05$ for all the results.

CHAPTER THREE

RESULTS

3.1 Percentage Yield of Methanol Extracts of *Stephania dinklagei* Leaves

Table 1 represents the percentage yield of the ethanol extracts of the sample. The result shows that 800 g of the ground sample of the leaves of *Stephania dinklagei* after extraction with, methanol gave 44.55 g (5.57 %).

Table 1 Percentage yield of methanol leaf extracts of *Stephania dinklagei*

Leave (g)	**Yield after extraction (g)**	**Percentage yield**
800.00	44.55	5.57

3.2 Qualitative Phytochemical Contents of Methanol Leaf Extracts of *Stephania dinklagei*

Table 2 shows the results of the qualitative phytochemistry of sample. The result below showed that the leaves of *Stephania dinklagei* contain alkaloids, tannins, flavonoids, steroids, terpenoids, carotenoids, glycosides, authocyanins and Saponins. Anthraquinone were not detected.

Table 2 qualitative phytochemical contents of methanol extract of *Stephania dinklagei* Leaves

Phytochemicals	Relative Abundance
Alkaloids	+ +
Flavonoids	+ + +
Tannins	+ +
Steroids	+ + +
Terpenoids	+ + +
Carotenoids	+
Glycosides	+
Anthocyanins	+
Saponins	+ +
Anthraquinone	ND

Key: + slightly present , ++ moderately present, +++ highly present, ND not detected.

3.3 Quantitative Phytochemical Constituents of Methanol Extracts of *Stephania dinklagei* Leaves.

Table 3: Quantitative phytochemical constituent of methanol extract of *Stephania dinklagei* leaves.

Phytochemical constituents	**Relative Abundance (mg/g)**
Alkaloids	29.07± 0.15
Flavonoids	25.30 ± 0.10
Steroids	69.70 ± 0.10
Saponins	13.57 ± 0.21
Tannins	64.21± 0.12
Cardiac glycosides	1.45 ± 0.09
Terpenoids	44.30 ± 0.26
Carotenoids	5.88 ± 0.52
Anthraquinone	15.40 ± 0.26

3.4 Vitamin Contents of Methanol Leaf Extract of *Stephania dinklagei*

Table 4 shows the vitamin contents in the samples. The vitamin contents in the leaves were found to be; vitamin A was found to have the highest concentration of the three vitamins.

Table 4 Vitamin contents of methanol extract of *Stephania dinklagei* leaves.

Vitamins	Contents (mg/100g)
A	44.80 ± 0.42
C	27.85 ± 0.07
E	12.7 ± 0.28

3.5 Acute Toxicity (LD_{50}) Test of Methanol Extract of *Stephania dinklagei* Leaves

As shown in Table 5, the acute toxicity test of methanol extract of *Stephania dinklagei* leaves indicated no death up to 5000 mg/kg body weight, suggesting that the leaves could be tolerated and hence not toxic up to 5000 mg/kg.

Table 5 Phase I and II of the acute toxicity (LD50) test

	Dosage mg/kg body weight	Mortality
Phase I		
Group 1	10	0/3
Group 2	100	0/3
Group 3	1000	0/3
Phase II		
Group 1	1600	0/3
Group 2	2900	0/3
Group 3	5000	0/3

3.6 Effect of Methanol Extract of *Stephania dinklagei* Leaves on DPPH Radical Scavenging Activity

Figure 5 shows the DPPH radical scavenging activity of *Stephania dinklagei* and the standard (ascorbic acid). The data represents the percentage DPPH inhibition. The activity was observed to increase with increasing concentrations of both extract and reference standard. At 250 ε g/ml, the percentage inhibitions of DPPH were 66.52 and 77.13 % for extract and standard (ascorbic acid) respectively, suggesting that the extract has good scavenging activity. EC_{50} value of 80.00 ε g/ml and 36.24 ε g/ml for the plant extract and ascorbic acid respectively.

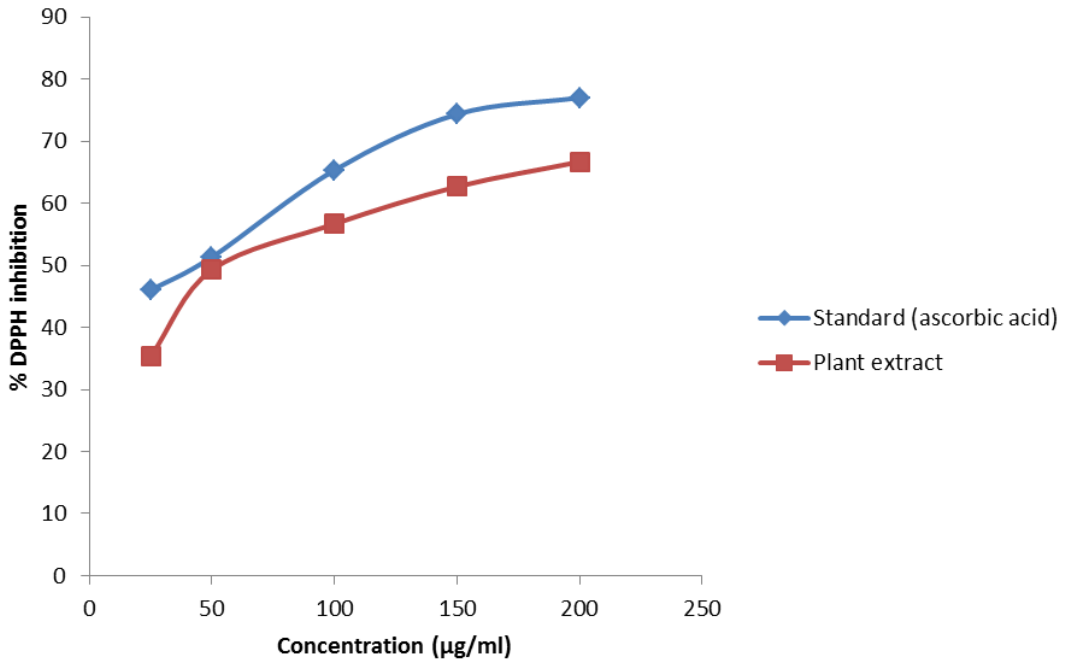


Fig 5: Effect of the scavenging activity of *Stephania dinklagei* methanol leaf extract and the standard (ascorbic acid) on DPPH ion.

3.7 Effect of Methanol Extract of *Stephania dinklagei* Leaves on Superoxide Anion Radical Scavenging Activity

As observed in Figure 6, the data represents the percentage superoxide anion inhibition. Each value represents mean ± S.D (n =3). The result of the superoxide anion radical activity was increased with increasing concentration. At 200 ε g/ml, the plant extract and ascorbic acid scavenge superoxide radical by 44.12 and 50.14 % respectively, indicating the high scavenging potency of the extract. The plant extract and the standard (ascorbic acid) have EC_{50} value of 241.08 and 196.83 ε g/ml respectively.

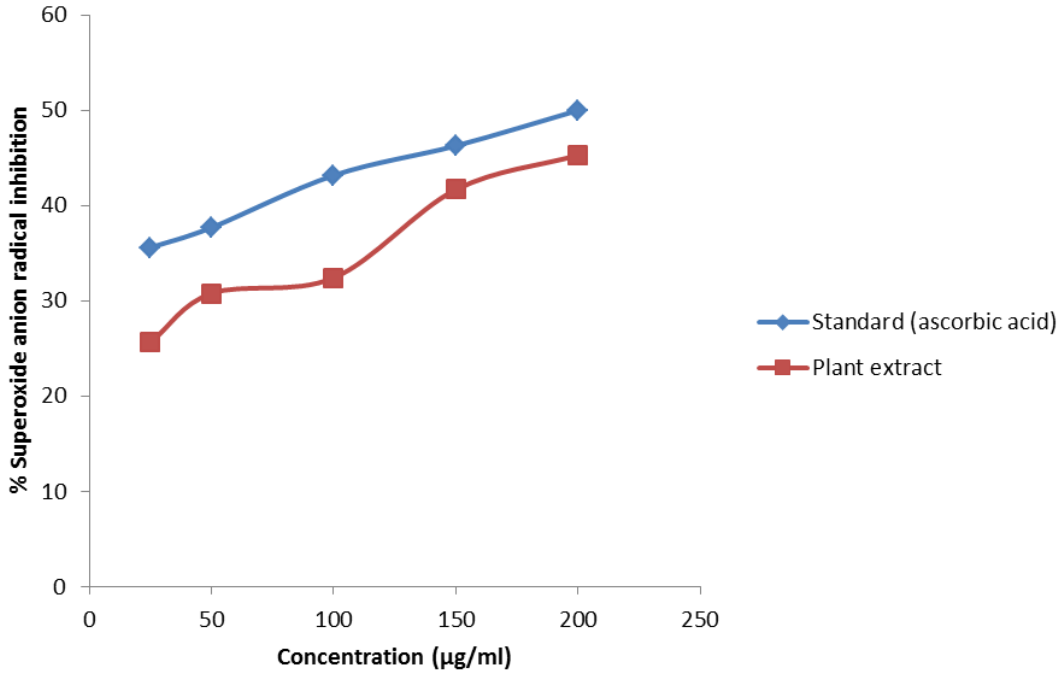


Fig 6: Effect of the scavenging potency of methanol extract of *Stephania dinklagei* leaves and the standard (ascorbic acid) on superoxide ion

3.8: Effect of Methanol Leaf Extract of *Stephania dinklagei* on Hydroxyl Anion Radical Scavenging Activity

Shown in fig. 7 is the scavenging action of *Stephania dinklagei* and the standard $(\propto -tocopherol)$. The data represents mean ± S.D (n=3) percentage inhibition. The activity was observed to increase in a dose dependent manner in both extract and the standard. At 200 ε g/ml, the percentage inhibitions of the hydroxyl anion radical were 90.24 and 97.14 % for extract and standard respectively. EC_{50} value of 74.72 and 41.52 ε g/ml for the plant extract and Ŭ-tocopherol respectively. These, suggests that the extract is a powerful quencher of ^{Å}OH radical.

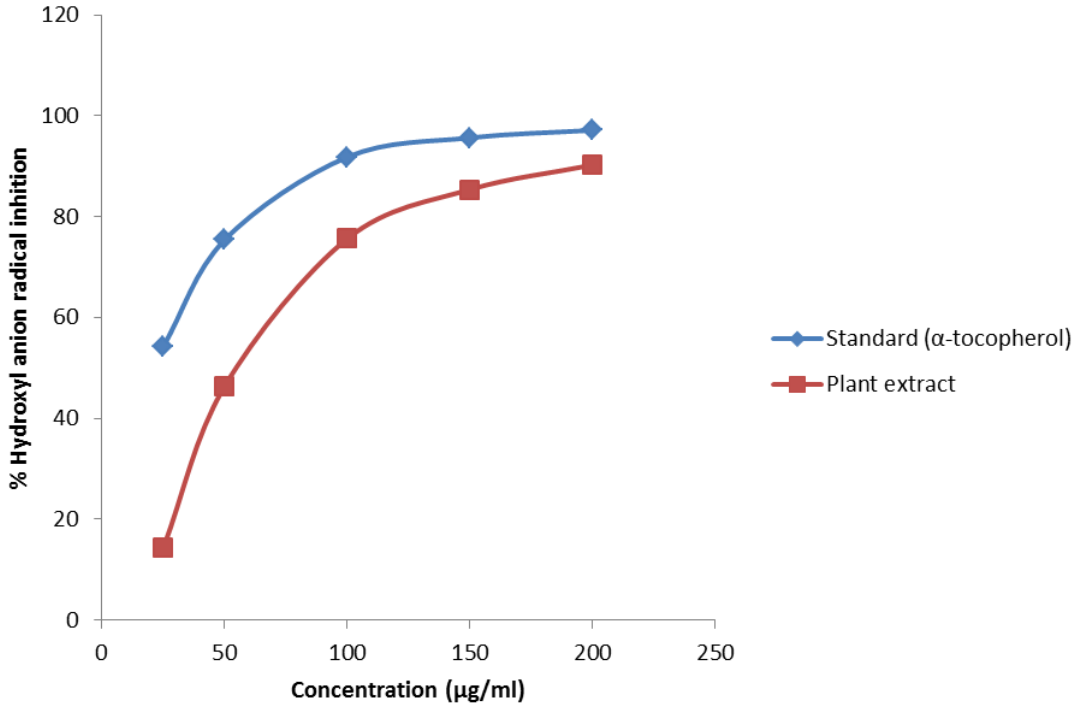


Fig 7: Effect of the scavenging activity of methanol extract of *Stephania dinklagei* leaves and the standard $(\propto -tocopherol)$ on hydroxyl radical

3.9 Anti-Radical Power of Methanol Extract of *Stephania dinklagei* Leaves

Fig 8 shows the hydroxyl anion radical and the DPPH as observed have high antiradical activity as against the superoxide anion.

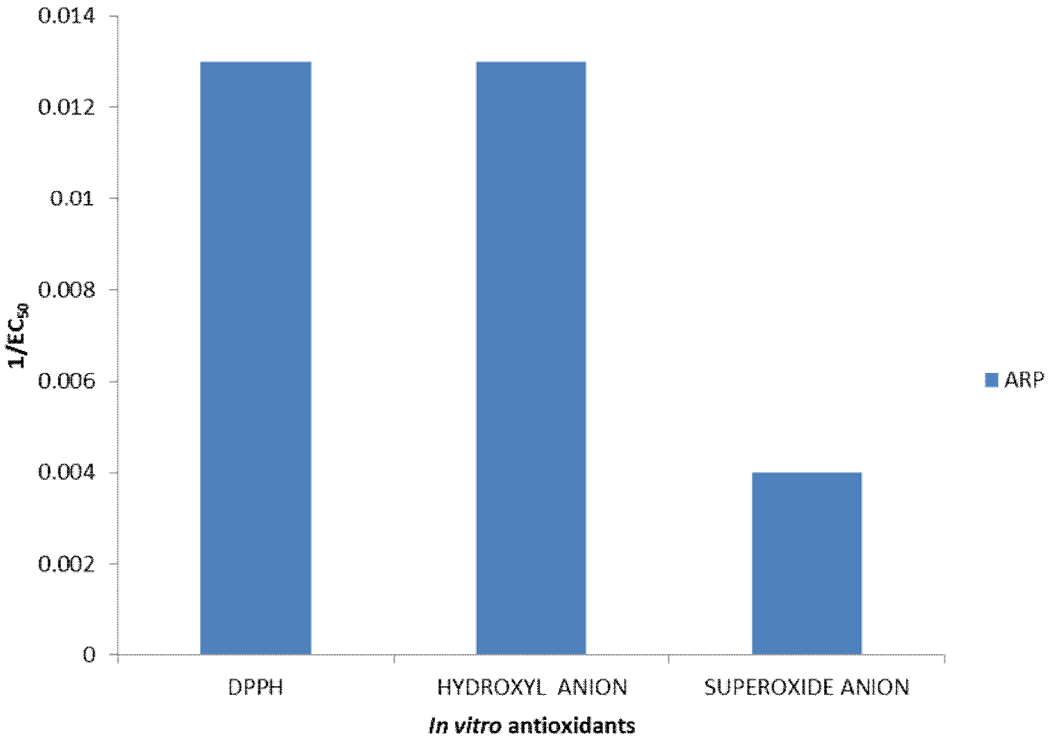


Fig 8: The antiradical power of the *in vitro* antioxidant studies

3.10 Effect of Methanol Extract of *Stephania dinklagei* Leaves on Serum Malondialdehyde (MDA) Concentration in Rats

As shown in Fig. 9 is the serum MDA concentration of the rats treated with methanol extract of *Stephania dinklagei* leaves. The result indicated that in the first week, there was non-significant increase ($p>0.05$) in the test groups 2, 3 and 4 compared to that of the control (group 1). In weeks two (2), there was significant increase ($p<0.05$) in groups 2, 3 and 4 compared to that of the control (group 1). The same trend was observed in week 3.

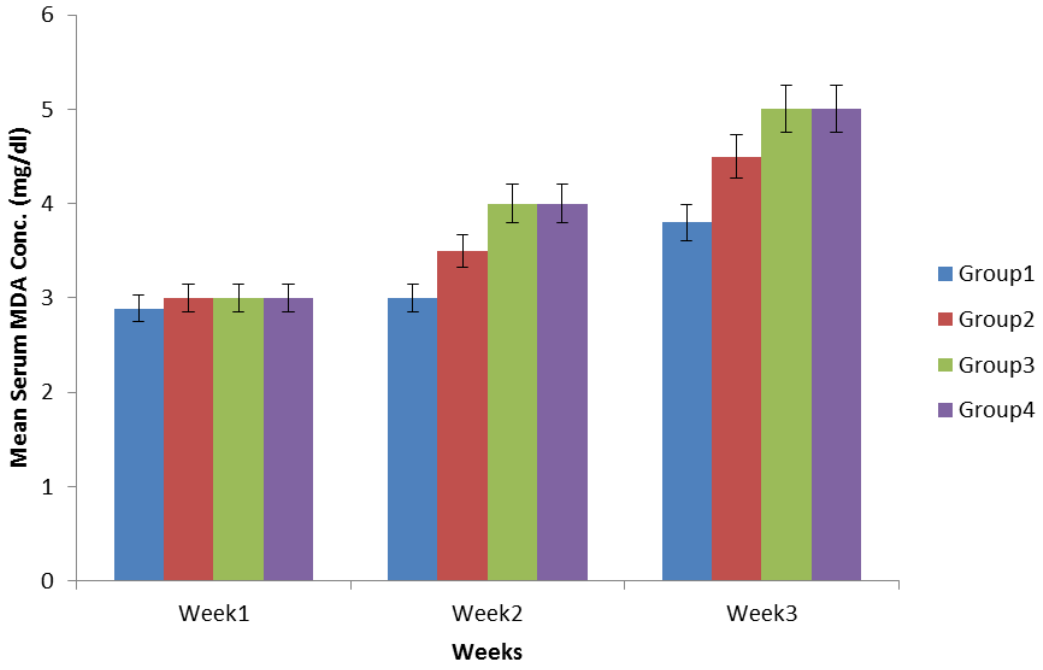


Figure 9: Effect of the methanol extract of *Stephania dinklagei* leaves on serum MDA concentration in rats

Group 1: Control (Normal rats)
Group 2: 200 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*
Group 3: 400 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*
Group 4: 600 mg/kg body weight of methanol leaf extact of *Stephania dinklagei*

3.11 Effect of Methanol Extract of *Stephania dinklagei* Leaves on Serum Catalase Activity in Rats

There was a non-significant difference ($p>0.05$) in group 2 compared to group 1 and significant decreases in groups 3 and 4 compared to the control (group 1) in the first week as shown in fig. 10. In week two, there was a non-significant decrease ($p>0.05$) and a significant decrease ($p<0.05$) in groups 3 and 4 respectively, compared to that of group 1. In week three (3), there was a non-significant increase in group two and significant decreases ($p<0.05$) in groups 3 and 4 compared to that of group 1.

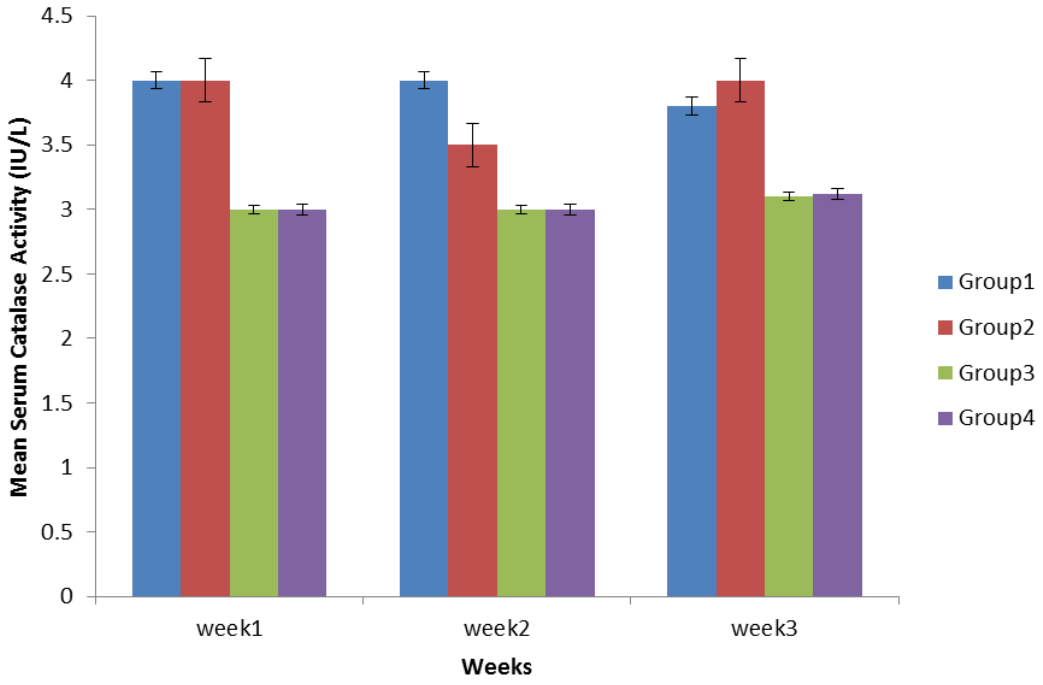


Figure 10: Effect of the methanol extract of *Stephania dinklagei* leaves on serum catalase activity in rats

Group 1: Control rats
Group 2: 200 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*
Group 3: 400 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*
Group 4: 600 mg/kg body weight of methanol leaf extract of *Stephania dinklagei* leaf

3.12 Effect of Methanol Extract of *Stephania dinklagei* Leaves on Serum Superoxide Dismutase (SOD) Activity in Rats

Figure 11 shows the activity of the serum SOD of the rats treated with methanol extract of *Stephania dinklagei* leaves in rats. The results of the 1st week treatment showed that there was non-significant decrease in group 2 while in groups 3 and 4, the decreases were significant ($p<0.05$) compared with that of their controls. In week two (2) and three (3), there were significant decreases ($p<0.05$) in groups 2, 3 and 4 compared with their controls (groups 1).

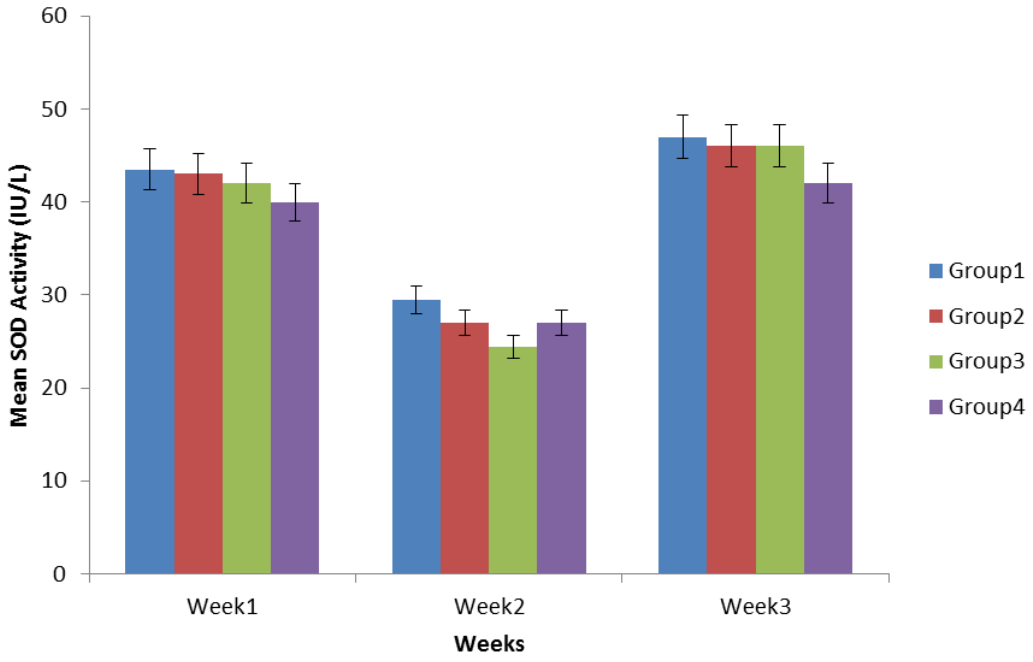


Figure 11: Effect of methanol extract of *Stephania dinklagei* leaves on serum SOD activity in rats

Group 1: Control rats
Group 2: 200 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*
Group 3: 400 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*
Group 4: 600 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*

3.13 Effect of the Methanol Extract of *Stephania dinklagei* Leaves on Serum Aspartate Aminotransferase Activity of Rats

Fig. 12 shows the serum aspartate aminotransferase activity of the rats treated with methanol extract of *Stephania dinklagei* leaves in rats. In the first week, there was a non-signficant ($p>0.05$) difference in group 2 compared to group 1 and significant increases in groups 3 and 4 compared to group 1. In weeks 2 and 3, there were non-significant difference ($p>0.05$) in groups 2 and 3 and significant increase ($p<0.05$) in group 4 when compared to those of their respective controls.

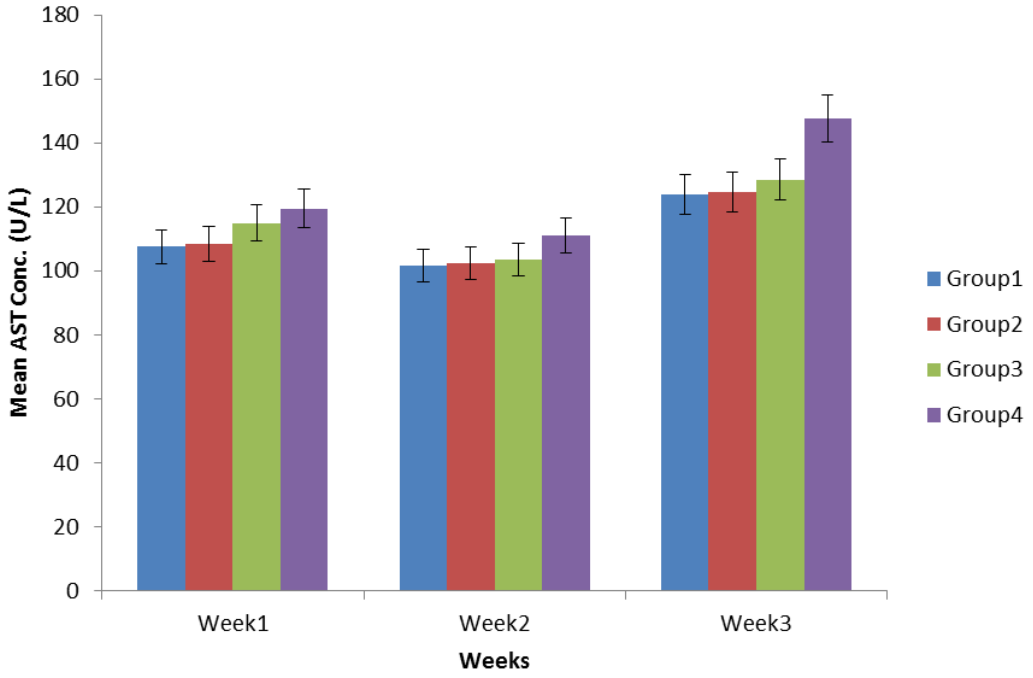


Figure 12: Effect of the methanol extract of *Stephania dinklagei* leaves on serum aspartate aminotransferase activity

Group 1: Control rats
Group 2: 200 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*
Group 3: 400 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*
Group 4: 600 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*

3.14 Effect of Methanol Extract of *Stephania dinklagei* Leaves on Serum Alanine Aminotransferase (ALT) Activity in Rats

Figure 13 shows the serum ALT concentration of the rats treated with *Stephania dinklagei* leaf extract. In weeks 1, 2 and 3, there were non significant difference ($p>0.05$) in groups 2 and 3 compared to those of their controls. However, there were significant increases in group 4 in all the weeks compared to those of their respective controls. The ALT concentration in the third week was observed to increase most.

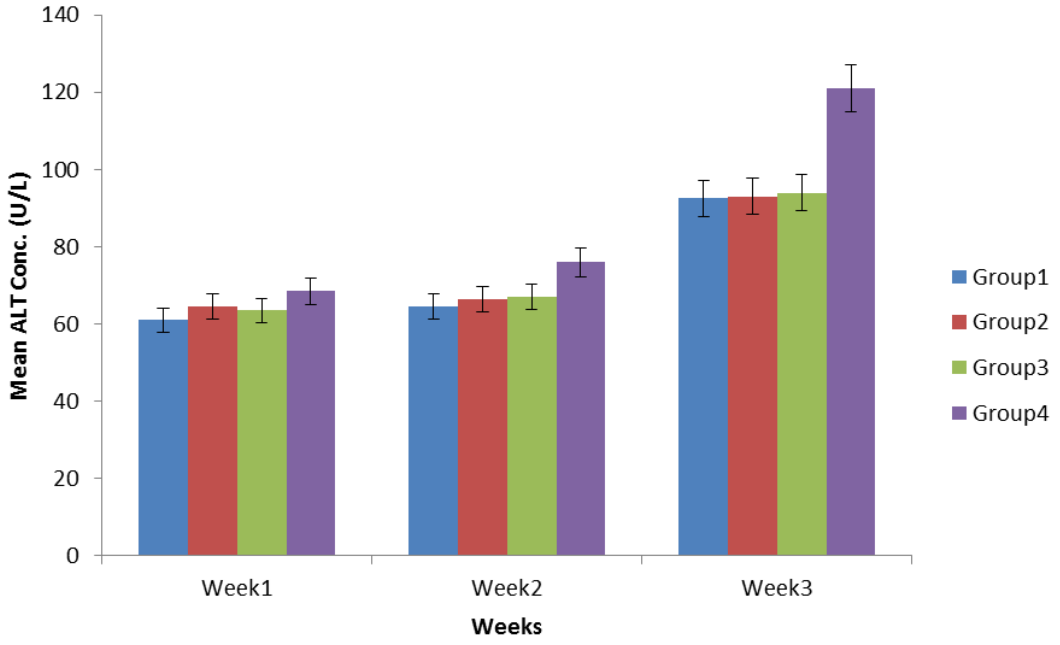


Figure 13: Effect of the methanol leaf extract of *Stephania dinklagei* on serum ALT concentration in rats

Group 1: Control rats
Group 2: 200 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*
Group 3: 400 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*
Group 4: 600 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*

3.15 Effect of Methanol Leaf Extract of *Stephania dinklagei* on Serum Alkaline Phosphatase (ALP) Activity in Rats

Figure 14 shows the serum ALT level of the rats treated with *Stephania dinklagei* leaf extract. In week one (1) there was non-significant difference ($p>0.05$) in groups 2 and 3 and a significant increase ($p<0.05$) in group 4 compared with the control (group 1). In week two (2) and three (3) there were non-significant difference ($p>0.05$) in group 2 compared with group 1 and significant increases in groups 3 and 4 compared with their respective controls, suggesting 600mg/kg leaf extract treatment might have produced some effect on the liver cells.

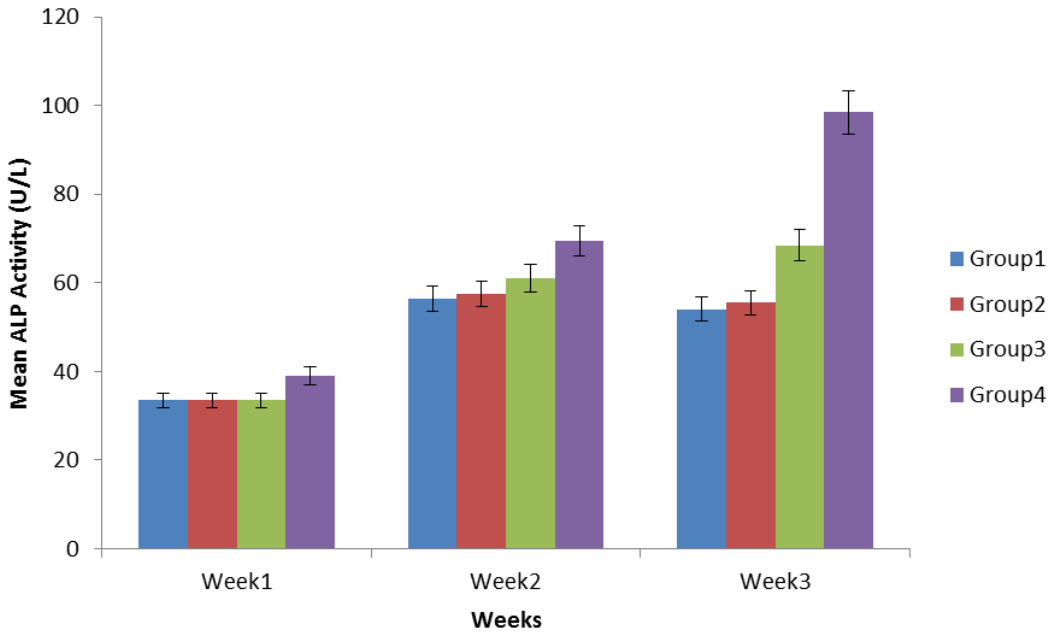


Figure 14: Effect of methanol leaf extract of *Stephania dinklagei* on serum alkaline phosphatase activity

Group 1: Control rats
Group 2: 200 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*
Group 3: 400 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*
Group 4: 600 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*

3.16 Effect of Methanol Leaf Extract of *Stephania dinklagei* on Serum Urea

Figure 15 shows the serum urea of the rats treated with *Stephania dinklagei* leaf extract. The result of the first week from the bar chart showed that there was a significant increase ($p<0.05$) in groups 2, 3 and 4 compared with group 1. In week 2, there was a non-significant difference ($p>0.05$) in groups 2, 3 and 4 compared with group 1. In week 3, there was significant increases ($p<0.05$) in groups 2, 3 and 4 compared with group 1

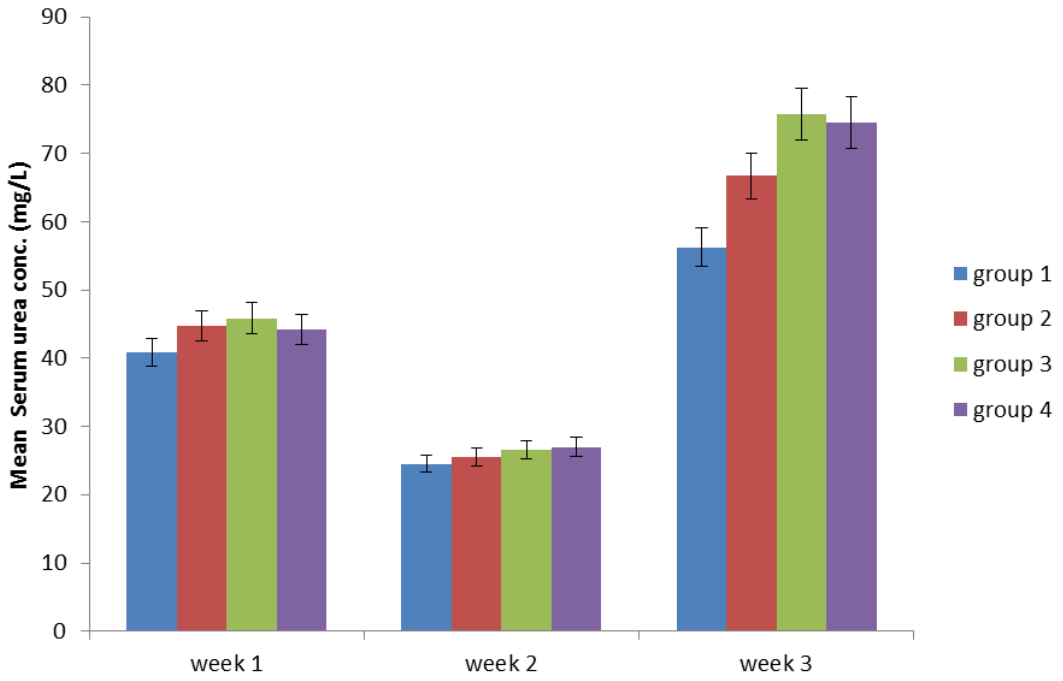


Figure 3.11: Effect of methanol leaf extract of *Stephania dinklagei* on serum urea Concentration of rats

Group 1: Control rats
Group 2: 200 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*
Group 3: 400 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*
Group 4: 600 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*

3.17 Effect of Methanol Extract of *Stephania dinklagei* Leaves on Serum Creatinine

Figure 16 shows the serum creatinine. In week 1, there was non-significant difference ($p>0.05$) in groups 2 and 3 while significant decrease ($p<0.05$) occured in group 4 compared with that of their control. In week 2, there was non-significant difference in groups 2, 3 and 4 compared to that in group 1. In week 3, significant increases were observed in groups 2 and 3 while in group 4, the decrease was significant compared to that of their controls.

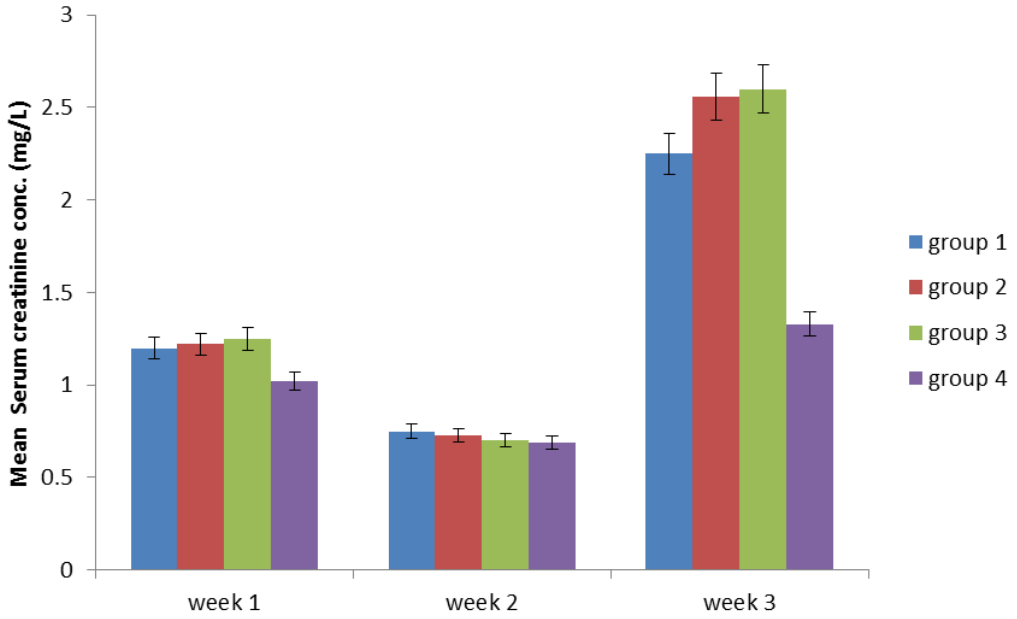


Figure 16: Effect of methanol leaf extract of *Stephania dinklagei* on serum creatinine

Group 1: Control rats
Group 2: 200 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*
Group 3: 400 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*
Group 4: 600 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*

3.18: Effect of Methanol Leaf Extract of *Stephania dinklagei* on Serum Sodium Ion Concentration

Figure 17 shows the serum sodium of the rats treated with *Stephania dinklagei* leaf extract. In weeks 1, 2 and 3, we observed significant increases ($p<0.05$) in groups 2, 3 and 4 compared with that of their controls (group 1).

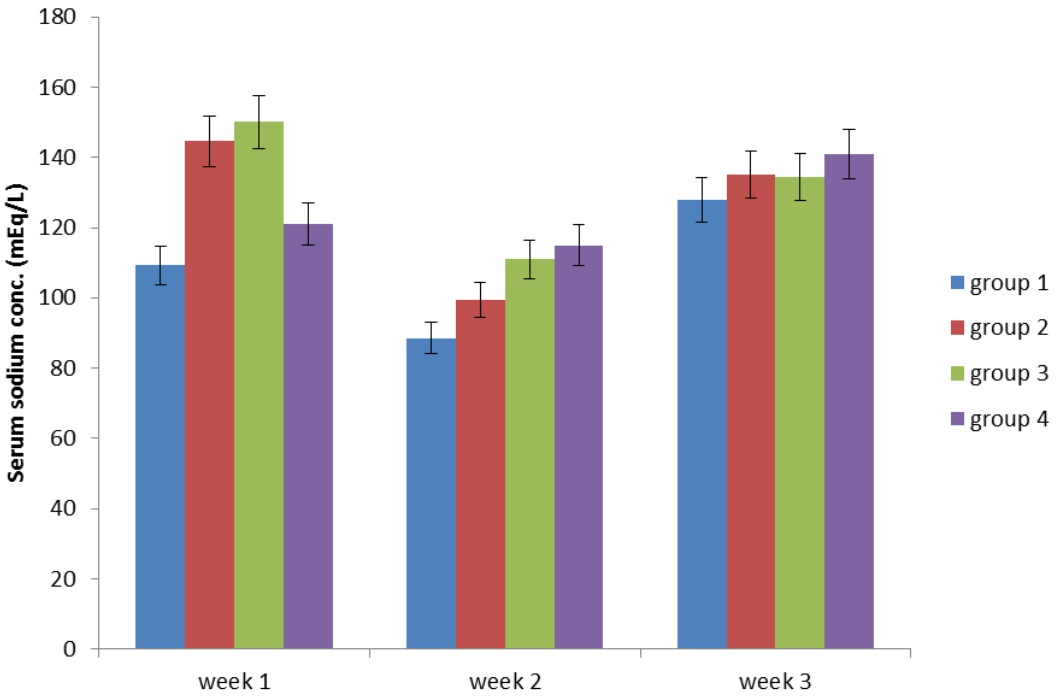


Figure 17: Effect of the methanol leaf extract of *Stephania dinklagei* on serum sodium ion concentration

Group 1: Control rats
Group 2: 200 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*
Group 3: 400 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*
Group 4: 600 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*

3.19: Effect of Methanol Leaf Extract of *Stephania dinklagei* on Serum Chloride Ion Concentration

Figure 18 shows the serum chloride of the rats treated with *Stephania dinklagei* leaf extract. In weeks 1 and 2, there was non-significant difference ($p>0.05$) in groups 2, 3 and 4 compared with that of their controls (group 1). In week 3, significant increases ($p<0.05$) occurred in groups 2 and 3 but no change in the ion concentration in group 4 compared to the control.

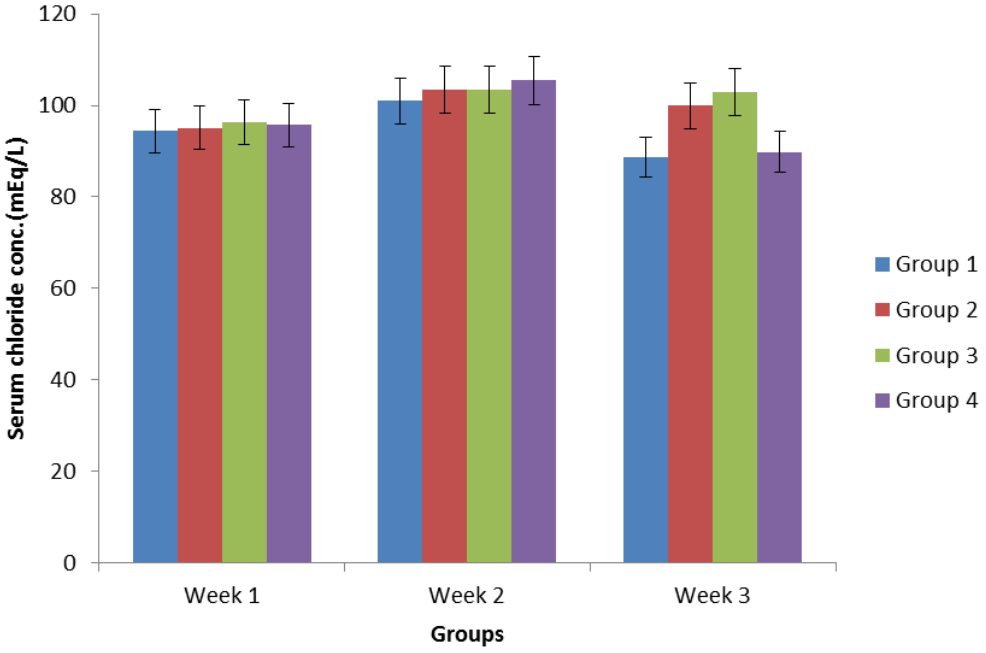


Figure 18: Effect of methanol leaf extract of *Stephania dinklagei* on serum chloride ion concentration

Group 1: Control rats
Group 2: 200 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*
Group 3: 400 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*
Group 4: 600 mg/kg body weight of methanol leaf extract of *Stephania dinklagei*

3.20 Histopathological Evaluation of Methanol Extract of *Stephania dinklagei* Leaves on the Rat Liver Cells

3.26 Histopathology Explanation

Microscopic examination of sections of liver organs from control (Plate 1) showed Lobules with radiating cords of normal hepatocyes around a central vein (CV), with bile ducts, hepatic artery and portal vein at the periphery of the lobules, however, (Plate 2) group 2 administered with 200 mg/kg showed a widespread hepatocellular vacuolar degeneration (arrowhead) with hypertrophy of kupffer cells in the periportal areas and moderate infiltration of mononuclear leucocytes (Arrow) into the periportal areas (P). The liver of rats treated with 400 mg/kg (group 3) showed a more severe diffuse hepatocellular vacuolar degeneration with kupffer cell hypertrophy and mild to moderate periportal infiltration of mononuclear leucocytes (arrow) (Plate 3). Liver section from group 4 treated with 600mg/kg body weight of the leaf extract showed a widespread hepatocellular vacuolar degeneration, kupffer cell hypertrophy and a mild to moderate, multifocal (varying from periportal to midzonal and paracentral infiltration of mononuclear leucocytes (arrow) as shown in Plate 4. Plate 5 and Plate 6 respectively showed the kidney of the control and the treated groups. Plate 5 (control) showed normal glomerulus in its bowmanôs capsule (arrow), with normal renal tubules (RT) and renal interstitium. Plate 6 showed no significant changes in the treated group with no deviation from normal kidney architecture, Bowmanôs capsule (arrow) and renal tubules (RT), suggesting that at the doses studied, the extract is hepatotoxic but not nephrotoxic.

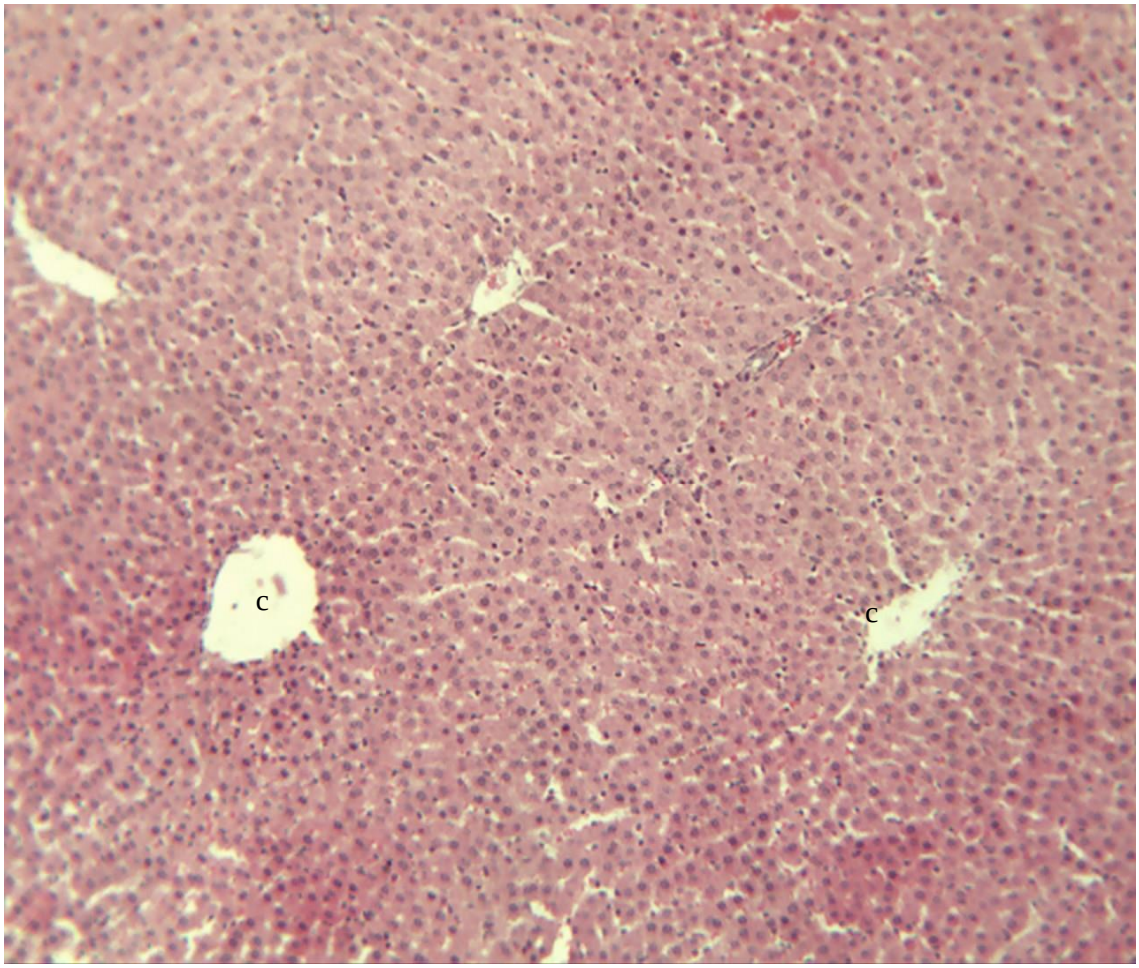


Plate 1 shows the photomicrograph of section of liver organ of control rats with normal hepatocytes around the central vein (CV). H & E × 100

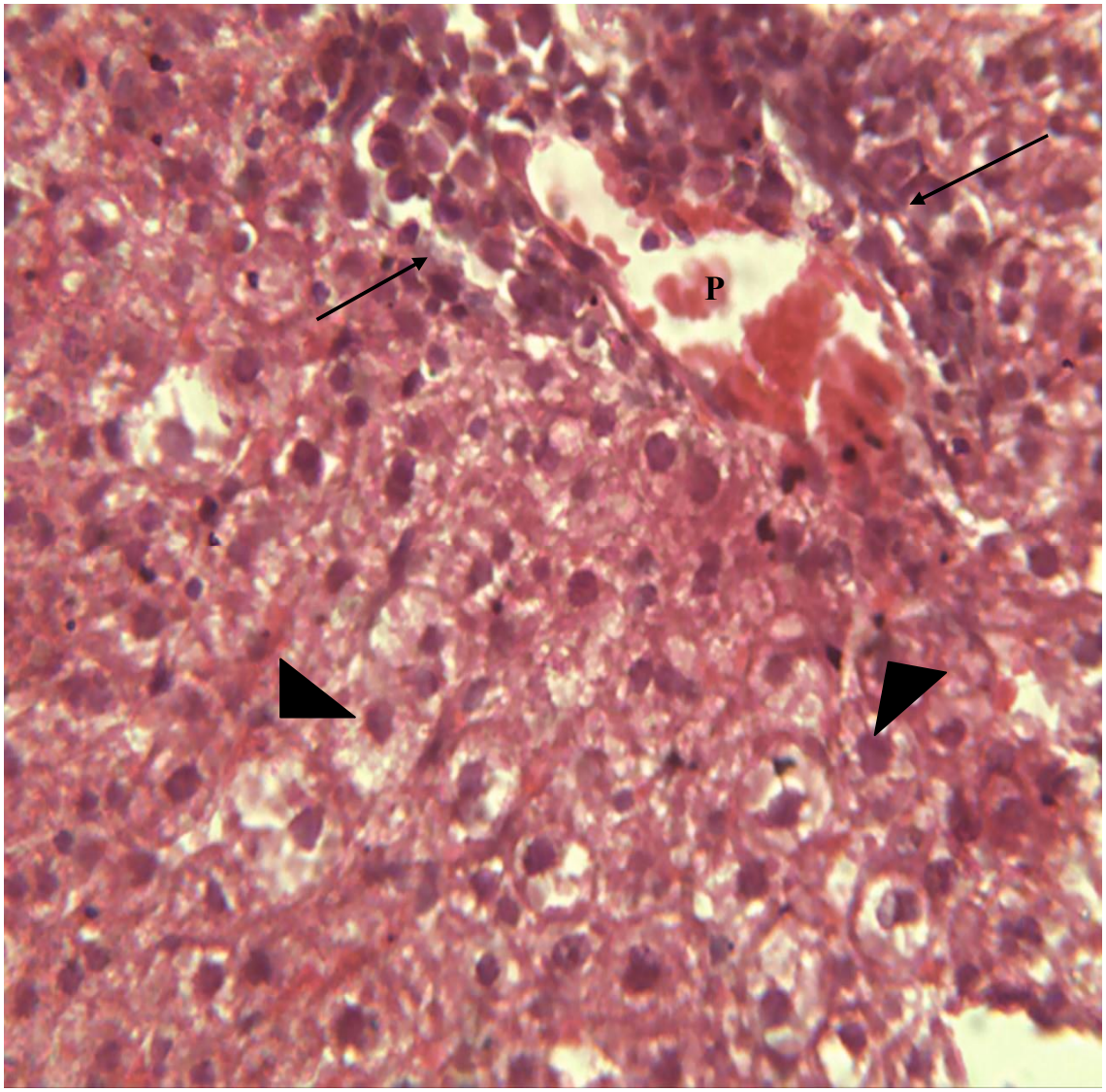


Plate 2: Photomicrograph of test group treated with 200mg/kg b.w. of methanol leaf extract of *Stephania dinklagei* on the rat liver. **H & E × 400**

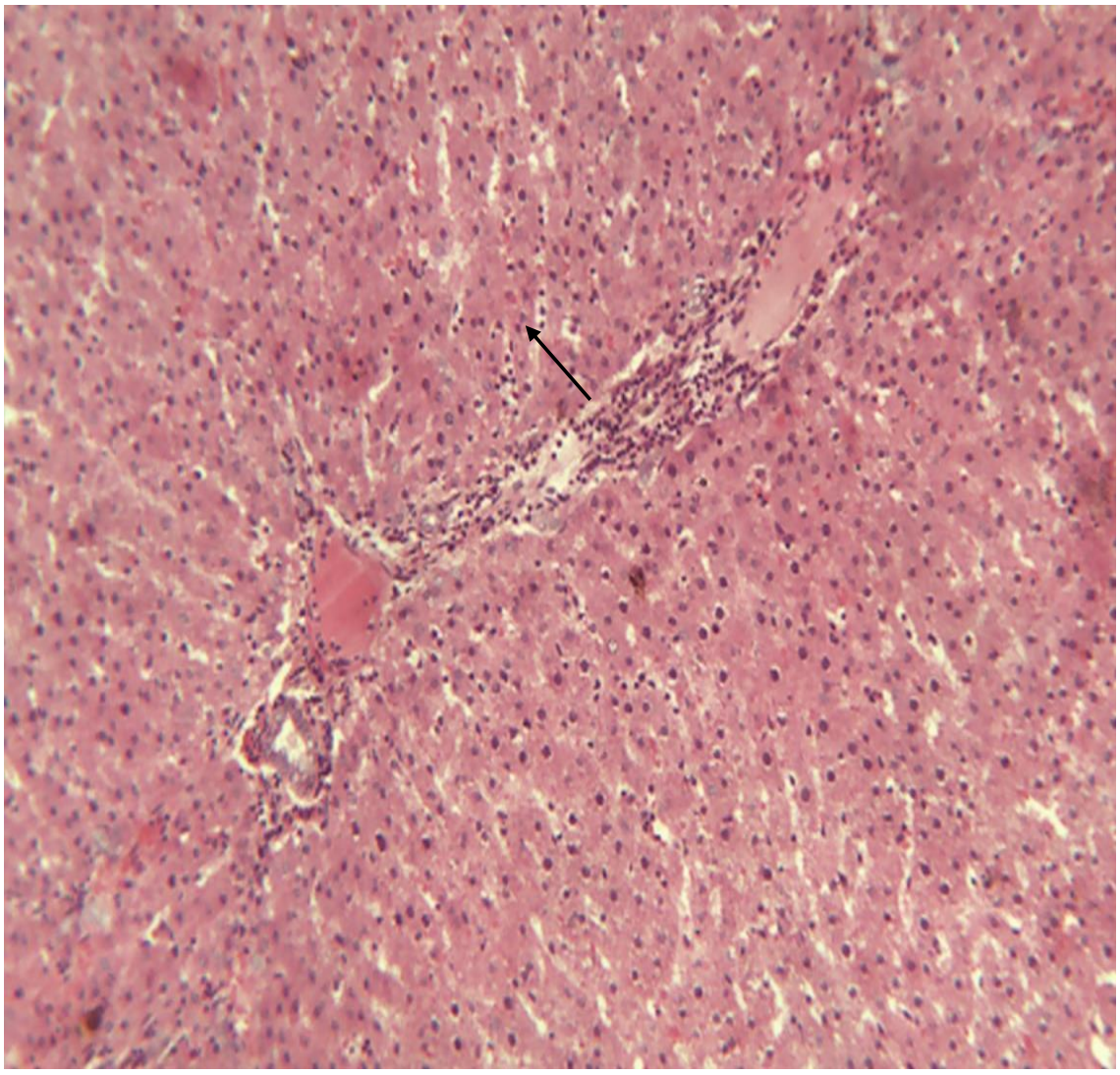


Plate 3: Photomicrograph of test group treated with 400mg/kg b.w. of methanol leaf extract of *Stephania dinklagei* on the rat liver. **H & E × 100**

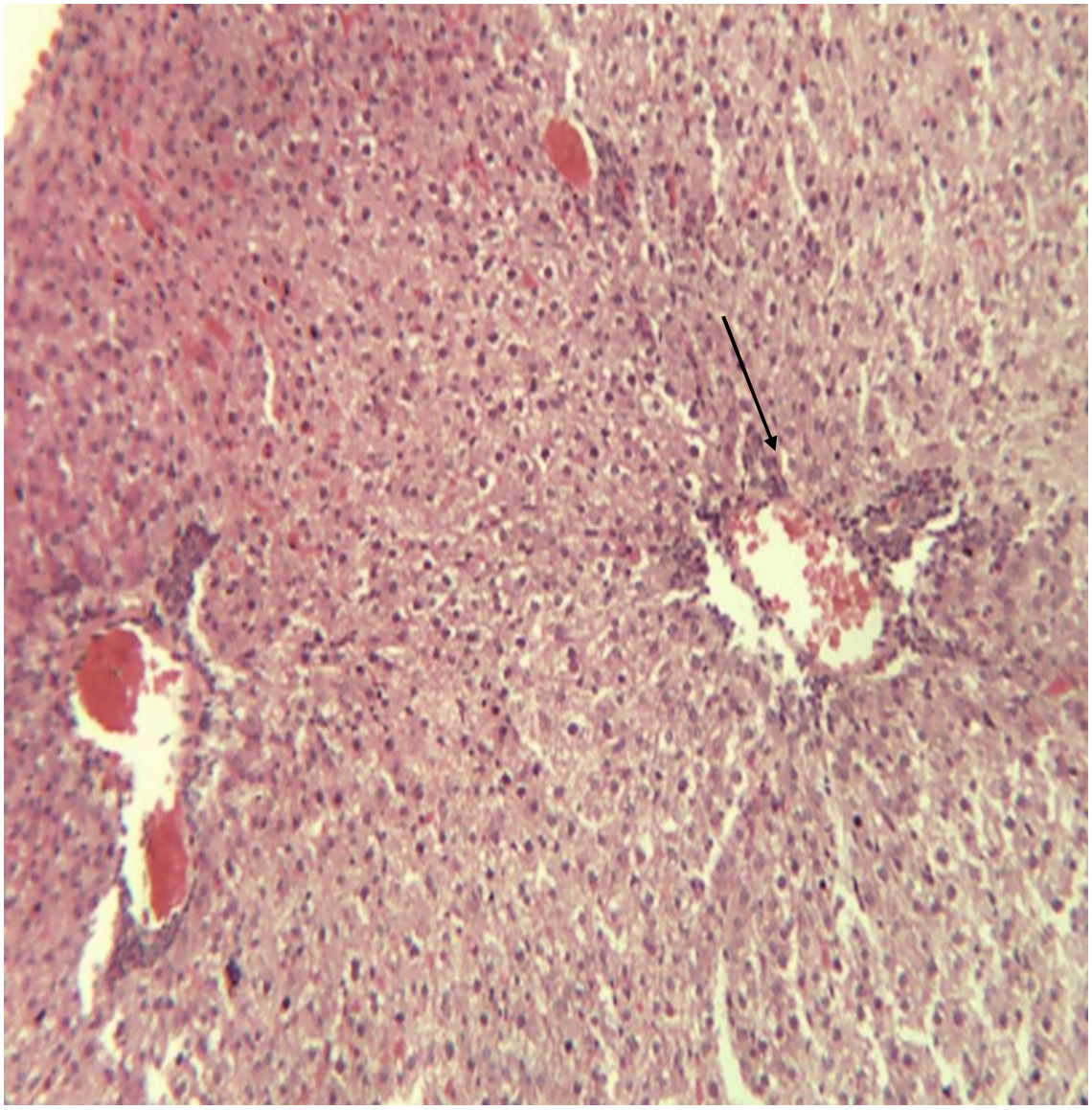
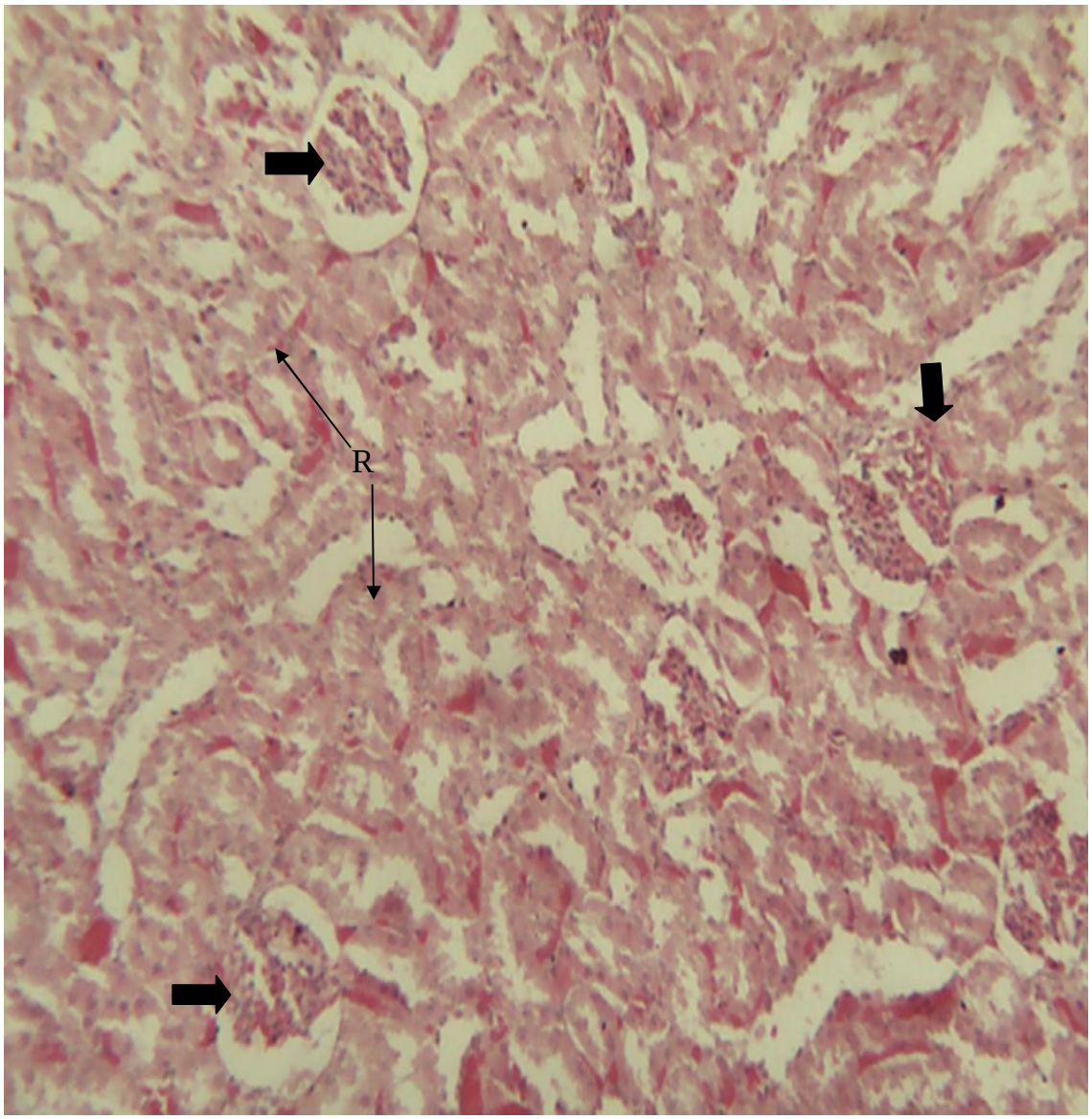


Plate 4: Photomicrograph of test group treated with 600 mg/kg b.w. of methanol extract leaves of *Stephania dinklagei* on the rat liver. **H&E × 400**

3.24 Histopathological Evaluaton of Methanol Leaf Extract of *Stephania dinklagei* on Rat Kidney Cells



Photomicrograph of the kidney showing the control group. **H & E × 100**

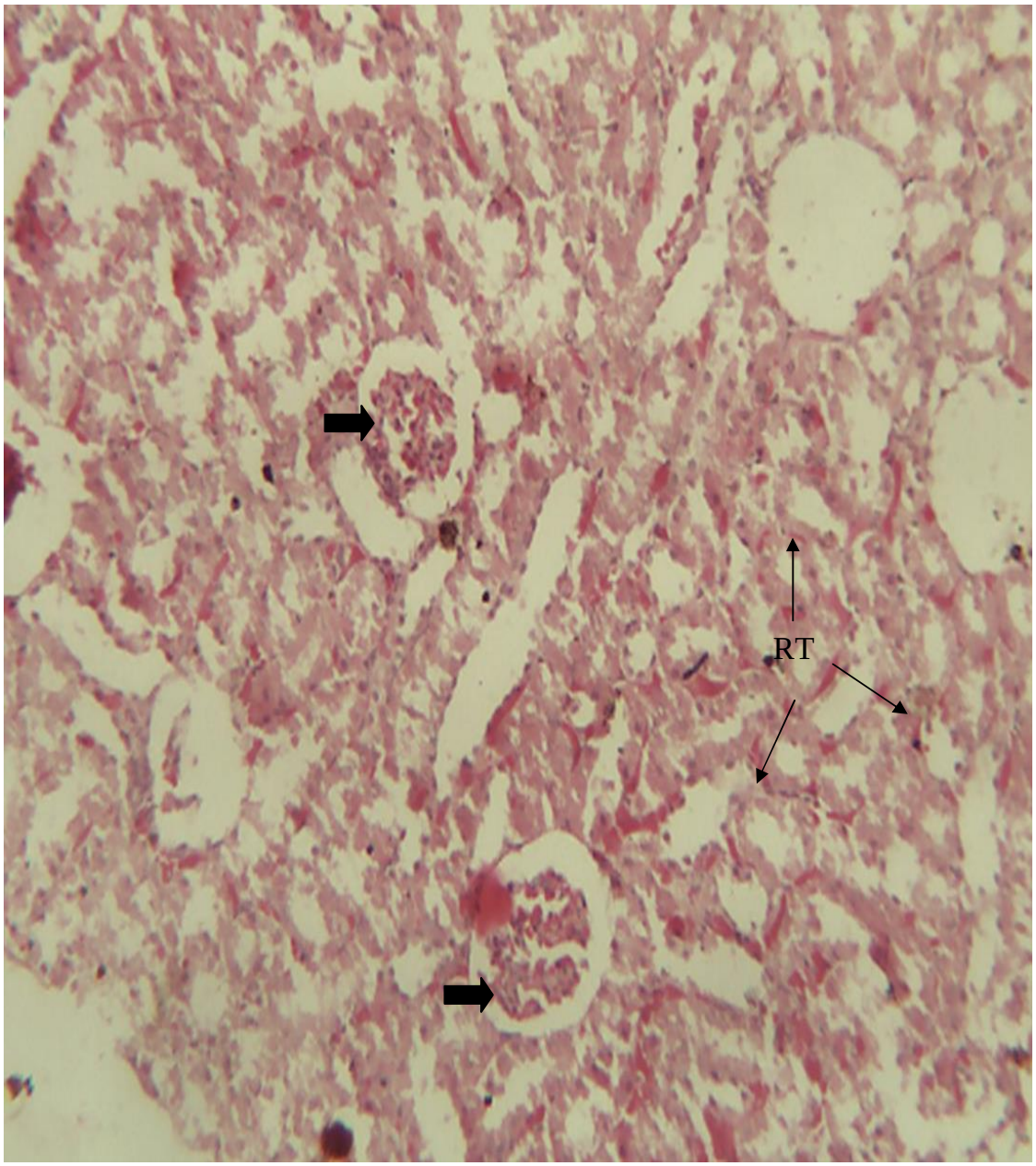


Plate 6: Photomicrograph of the kidney showing group treated with 600mg/kg b.w. of methanol leaf extract of *Stephania dinklagei*. **H&E × 100**

CHAPTER FOUR
DISCUSSION

The preliminary phytochemical analysis revealed the presence of alkaloids, flavonoids, tannins, steroids, terpenoids, carotenoids, glycosides, anthocyanins and saponins in the leaf extract. Anthraquinone was not detected. This result is consistent with the findings of Habibur-Rahman *et al.,* (2011). The presence of alkaloids in the leaves indicates that this plant could be effective antimalarial as alkaloids consist of quinine secondary metabolite (Vinson *et al.,* 1995). The presence of saponins supports the fact that *Stephania dinklagei* leaves have cytotoxic effects such as permealization of the intestine as saponins are cytotoxic (Okwu and Okwu, 2004). The extract of a similar plant, *Stephania japonica* showed significant cytotoxicity which may be due to the fact that both plants contains isotrilobine and trilobine alkaloids which was reported to possess multidrug-resistance-reversing activity in human breast cancer cell line (Andrea and Chang, 1997). The cardiac glycoside therapeutically has the ability to increase the force and power of the heart beat without increasing the amount of oxygen needed by the heart muscle. They can thus increase the efficiency of the heart and at the same time steady excess heart beats without strain to the organ (David, 1983). The presence of both qualitative and quantitative phytochemicals as observed in this study suggest that the plant possesses some bio-active compounds which could serve as potential sources of drugs and that the secondary metabolites could exert some biological activities when taken by animals (Okafor, 1983). Polyphenolic compounds like flavonoids, tannins and phenolic acids commonly found in plants have been reported to have multiple biological effects including antioxidant activity (Brown and Rice-Evans, 1998; Vinson *et al*., 1995). Fangchinoline and cepharanthine isolated from a similar plant *Stephania rotunda* showed antioxidant activity in different *in vitro* model (Gulcin *et al.,* 2010).

The high contents of antioxidant vitamins (vitamins A, C and E) suggest that the leaves of this test plant could be used as antioxidants that could scavenge ROS and upregulate the activities of some antioxidant enzymes as vitamin C exhibit protective effects against free radical induced oxidative damage while vitamin E inhibits ROS induced generation of lipid peroxyl radicals (Topinka *et al.,* 1989).

The acute toxicity (LD_{50}) test of the methanol leaf extract of *Stephania dinklagei* shows that the plant extract was not toxic up to 5000 mg/kg body weight. The observed non toxicity of the plant extract in this study is in consonance with the findings of Liang *et al.,* (2010) on a similar plant, *Stephania tetrandra*.

The effect of the concentration of antioxidant compounds on the DPPH radical was observed as a function of reducing power, a direct correlation between antioxidant capacity and reducing power of certain plant extracts has been reported (Sharma *et al.,* 2000). The antioxidant property of the studied plant extract was evaluated through scavenging activity on DPPH radicals (examined by the capacity to decrease the absorbance at 517 nm of DPPH solution). The result showed that the free radical scavenging activity increased with increasing concentration of the extract indicating the concentration dose dependency of antioxidative activities (figure 5). This observation concur with that of Banerjee (2012) who also noted a similar trend of antioxidative activities dose dependency and associated it with the presence of reductones that are reported to be the terminators of free radical chain reactions. Similar studies showed that the extracts of *Stephania japonica* and *Stephania hernandifolia* strongly scavenged DPPH radicals (Andrea and Chang, 1997; Sharma *et al.,* 2010). The effective concentration (EC_{50}) of the leaves of *Stephania dinklagei* required to scavenge 50% of DPPH was found to be 80.00 µg/ml compared with the standard, EC_{50} = 36.24 µg/ml. The lower the $EC_{50,}$ the better it is able to scavenge radicals particularly peroxyl radical which are propagators of the oxidation of lipid molecules and thus halt the free radical chain reaction (Lee *et al.,* 2003).

Flavonoids and catechins are reported to be effective scavengers of the superoxide anion ($O_2^{\cdot}$) radical (Robak and Gryglewski, 1988). The plant extract inhibited the formation of reduced NBT in a dose dependent manner. As shown in figure 6, *Stephania dinklagei* showed high $O_2^{\cdot}$ anion scavenging activity (EC_{50} = 241.08 µg/ml) compared to ascorbic acid (EC_{50} = 196.83 µg/ml). The $O_2^{\cdot}$ anion scavenging effect of the extract could culminate in the prevention of hydroxyl anion radical formation since superoxide and hydrogen peroxide are required for hydroxyl radical generation.

The hydroxyl radical ($^{\cdot}OH$) is the most reactive radical known to initiate lipid peroxidation and damage of biomolecules (Halliwell, 1994). The effect of the extract on $^{\cdot}OH$ generated by Fe^{3+} ions was measured by determining the degree of deoxyribose degradation. The extract scavenged $^{\cdot}OH$ radical in a concentration-dependent manner (fig. 7). The extract was found to be a very good quencher of $^{\cdot}OH$ radical thereby preventing the propagation of lipid peroxidation (EC_{50} = 74.72 µg/ml) compared to Ŭ-tocopherol (EC_{50} = 41.52). At very high concentration of the extract, lower activities was observed. The antioxidant in the plant extract competed with deoxyribose against the $^{\cdot}OH$ radical generated from the Fe^{3+} - dependent system. The antioxidant in this plant extract could be acting as chelators of the Fe^{3+} ions in the system, thereby preventing them from complexing with the deoxyribose or

simply donating hydrogen atoms and accelerating the conversion of H_2O_2 to H_2O (Wang *et al.,* 2007). The observed ability of the extract to scavenge the ^{Å}OH radical indicates that the extract could inhibit lipid peroxidation since ^{Å}OH radicals are highly implicated in peroxidation.

Antiradicals are whole antioxidant which helps the body to neutralize excess free radicals by providing them with extra electrons (Barnard, 2004). The presented graph (fig. 8) of antiradical power (ARP) shows the DPPH and ^{Å}OH having high antiradical activity comparable to some medicinal plant extract (Sroka, 2006). The antiradical power of superoxide anion radical was however, low.

On a comparative basis, the extract showed better activity in quenching ^{Å}OH anion radical with an EC_{50} value of 74.72 µg/ml followed by DPPH radical with an EC_{50} value of 80.00 µg/ml and then $O_2^{Å}$ anion with an EC_{50} value of 241.08 µg/ml.

Oxidative stress is the disequilibrium between pro-oxidants and antioxidants in biological systems (Gordon, 1990). In the *in vivo* antioxidant study therefore, an increase in lipid peroxidation observed in fig. 9 could be due to significant reduction in the activities of enzymatic antioxidant such as catalase, superoxide dismutase and gluthione peroxidase or any other oxidants. Superoxide dismustase protects cells from oxidative damage by breaking down a potentially hazardous free radical, superoxide ($O_2^{Å}$) to hydrogen peroxide (H_2O_2) and oxygen (O_2) (Nagai *et al.,* 2003). The H_2O_2 produced can then be decomposed enzymatically by catalase (Nagai.,, 2003). Thus, significant reduction in the enzyme activities of catalase and superoxide dismutase (fig 10 & 11) could be responsible for increased lipid peroxidation (fig 3.5) observed in this study.

Liver damage is assessed by the determination of serum concentrations of its enzymes such as ALT and AST (Dobbs *et al.,* 2003). High concentrations of AST and ALT indicate liver damage, cardiac infarction and muscle injury. However, ALT is more specific to the liver and is thus a better parameter for detecting liver injury. Serum ALP concentration on the other hand is related to the function of hepatic cell. Increase in serum concentration of ALP is due to increased synthesis of the enzyme in the presence of increasing biliary pressure (Moss and Butterworth, 1974). Generally, an increase in these liver enzymes indicates injury or toxicity to the organ (Ghadi, 2000). The results of this study (figure 12, 13 and 14) showed that the serum concentrations of AST, ALT and ALP increased significantly ($P < 0.05$). These increases were roughly most significant on the fourteen (week 2) and twenty one (week 3) days at 400 and 600 mg/kg body weight of leaf extract administered. The results obtained agrees with the study of Liang *et al.*, (2010) which showed significant increases in ALT, AST

and ALP activities of rats in serum. These increases could be clear demonstrations of cellular leakage and loss of functionality of membrane integrity (Saraswat *et al.,* 1993). The presence of xenobiotics in the form of extract in experimental rats could cause derangement of biochemical processes (Uboh *et al.,* 2010), increasing or decreasing the activities of AST and ALT which are indicators of liver injuries (Edet *et al.,* 2011). These injuries could have been caused by free radicals and peroxidants which are implicated in the pathogenesis of toxic liver injuries (Jalalpure *et al.,* 2003). Creatinine and urea are major catabolic products of carbohydrate and protein metabolism respectively. Increases in serum urea concentration observed (figure 15) however, do not always indicate kidney problems but instead may reflect dehydration or increased protein uptake. Urea concentration raised out of proportion to the creatinine may indicate a prerenal problem such as volume depletion (Afolayan and Yakubu, 2009). The creatinine concentration (fig 16) was seen to increase and decrease significantly and non-significantly. Creatinine is regarded as the most useful endogenous marker in the diagnosis and treatment of kidney disease. The results obtained suggest that the plant extract is not nephrotoxic. The loss of body fluids containing less sodium and water intake restriction, or excessive intake of sodium may lead to a rare condition of hypernatremia (Kang *et al.,* 2002). This does not agree with the results of this work where water was given *ad libitum* and as such could not have been responsible for the increased sodium ion concentration shown in figure 17. The extract may have compromised the ability of the kidney to excrete adequate sodium ion from tubular fluids (Saba *et al.,* 2009). The increase in serum chloride ion observed in week three (3) was not different in pattern when compared with serum sodium ion concentration seen in this work, suggesting similar mechanism as both are known to have similar transmembrane transport (fig. 18).

The two vital organs (livers and kidneys) removed from the test groups at the end of the sub-chronic study were carefully observed microscopically and revealed observable gross lesions when compared with that of the control group. Evidence of liver damage usually manifest as a result of architectural disarray, vascular congestion, hepatocytotic necrosis or inflammatory cell infiltration in either acute or chronic conditions. Some of these features were observed in the rats administered with the extract as observed by Eroschenko (2000). The liver of the control group showed lobules with radiating cords of normal hepatocytes around a central vein (CV), with bile ducts, hepatic artery and portal vein at the periphery of the lobules while the groups treated with 200, 400 and 600mg/kg body weight of leaf extract showed widespread hepatocellular vacuolar degeneration with hypertrophy of kupffer cells in the periportal areas and moderate infiltration of mononuclear leucocytes into the periportal areas

indicative of liver abnormality. The kidneys of both the control and the treated groups showed no significant change and have normal glomerulus in its Bowmanôs capsule with normal renal tubules and renal interstitium in both. The result showed that the extract had no histological change on the kidney of the treated rats. The results on histopathology corroborates the observed increase in concentration of serum biochemical parameters studied and further suggests hepatotoxicity of the leaves extract at the doses studied.

4.2 Conclusion

In developing countries of the world, most of people depend on herbal medical care (Edeoga *et al.,* 2005). In most part of Nigeria, traditional medicine has been claimed to be vital in prevention and treatment of various diseases, thereby playing an important role in the health services of the state especially among the low socio-economic class. But most often, these medicinal plants are consumed in unspecified quantities in form of decoction without due regard to its toxic effects. This study showed that the extract possesses antioxidants and cytotoxic substance and as well possess potential toxic effects as shown by the effects it caused on the serum biochemical parameters. Thus, it could be said that the plant extract showed hepatotoxic but no nephrotoxic effects at the doses studied and as a result, care should be taken in prolonged use of the plant extract in treating various ailments it is suggested to treat.

4.3 Suggestions for further studies

- The plant should be investigated for the elucidation of the active principles.
- The plant extract was toxic to the liver during the period of treatment; therefore, more studies are needed to authenticate its toxicity as well as prolonged treatment of the extract is suggested to ascertain its effect on the kidney.
- The pharmacological validation in terms of modern medicine will be of great medicinal importance in future.

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APPENDICES

Table 8: Absorbance and activity of ALT in the serum

Absorbance	U/L	Absorbance	U/L
0.03	4	0.28	48
0.05	8	0.30	52
0.08	12	0.33	57
0.10	17	0.35	62
0.13	21	0.38	67
0.15	25	0.40	72
0.18	29	0.43	77
0.20	34	0.45	83
0.23	39	0.48	88
0.25	43	0.50	94

Table 9: Absorbance and activity of ALT in the serum

Absorbance	U/L	Absorbance	U/L
0.02	7	0.12	47
0.03	10	0.13	52
0.04	13	0.14	59
0.05	16	0.15	67
0.06	19	0.16	76
0.07	23	0.17	89
0.08	27		
0.09	31		
0.10	36		
0.11	51		

Post Hoc Tests

Multiple Comparisons

Dependent Variable	(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error
MDA LSD WEEK1	Control	200mg/kg b.w. Leaf Extract	-.11500	.08132
		400mg/kg b.w. Leaf Extract	-.11500	.08132
		600mg/kg b.w. Leaf Extract	-.11500	.08132
	200mg/kg b.w. Leaf Extract	Control	.11500	.08132
		400mg/kg b.w. Leaf Extract	.00000	.08132
		600mg/kg b.w. Leaf Extract	.00000	.08132
	400mg/kg b.w. Leaf Extract	Control	.11500	.08132
		200mg/kg b.w. Leaf Extract	.00000	.08132
		600mg/kg b.w. Leaf Extract	.00000	.08132
	600mg/kg b.w. Leaf Extract	Control	.11500	.08132
		200mg/kg b.w. Leaf Extract	.00000	.08132
		400mg/kg b.w. Leaf Extract	.00000	.08132

	Dunnett t (2-sided)[a]	Control	600mg/kg b.w. Leaf Extract	-.11500	.08132	
		200mg/kg b.w.Leaf Extract	600mg/kg b.w. Leaf Extract	.00000	.08132	
		400mg/kg b.w.Leaf Extract	600mg/kg b.w. Leaf Extract	.00000	.08132	
MDA Week2	LSD	Control	200mg/kg b.w.Leaf Extract	-.500	.354	
			400mg/kg b.w.Leaf Extract	-1.000*	.354	
			600mg/kg b.w. Leaf Extract	-1.000*	.354	
		200mg/kg b.w.Leaf Extract	Control	.500	.354	
			400mg/kg b.w.Leaf Extract	-.500	.354	
			600mg/kg b.w. Leaf Extract	-.500	.354	
		400mg/kg b.w.Leaf Extract	Control	1.000*	.354	
			200mg/kg b.w.Leaf Extract	.500	.354	
			600mg/kg b.w. Leaf Extract	.000	.354	
		600mg/kg b.w. Leaf Extract	Control	1.000*	.354	
			200mg/kg b.w.Leaf Extract	.500	.354	
			400mg/kg b.w.Leaf Extract	.000	.354	
	Dunnett t (2-sided)[a]	Control	600mg/kg b.w. Leaf Extract	-1.000	.354	
		200mg/kg b.w.Leaf Extract	600mg/kg b.w. Leaf Extract	-.500	.354	
		400mg/kg b.w.Leaf Extract	600mg/kg b.w. Leaf Extract	.000	.354	
MDA Week3	LSD	Control	200mg/kg b.w.Leaf Extract	-.7000	.3808	
			400mg/kg b.w.Leaf Extract	-1.2000*	.3808	
			600mg/kg b.w. Leaf Extract	-1.2000*	.3808	
		200mg/kg b.w.Leaf Extract	Control	.7000	.3808	
			400mg/kg b.w.Leaf Extract	-.5000	.3808	
			600mg/kg b.w. Leaf Extract	-.5000	.3808	
		400mg/kg b.w.Leaf Extract	Control	1.2000*	.3808	
			200mg/kg b.w.Leaf Extract	.5000	.3808	
			600mg/kg b.w. Leaf Extract	.0000	.3808	
		600mg/kg b.w. Leaf Extract	Control	1.2000*	.3808	
			200mg/kg b.w.Leaf Extract	.5000	.3808	
			400mg/kg b.w.Leaf Extract	.0000	.3808	
	Dunnett t (2-sided)[a]	Control	600mg/kg b.w. Leaf Extract	-1.2000	.3808	
		200mg/kg b.w.Leaf Extract	600mg/kg b.w. Leaf Extract	-.5000	.3808	
		400mg/kg b.w.Leaf Extract	600mg/kg b.w. Leaf Extract	.0000	.3808	

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Multiple Comparisons

iable	(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence
						Lower Bound

	Control	200mg/kg b.w.Leaf	.500	.354	.230	-.48
		400mg/kg b.w. Leaf Extract	1.000*	.354	.047	.02
		600mg/kg b.w. Leaf Extract	1.000*	.354	.047	.02
	200mg/kg b.w.Leaf	Control	-.500	.354	.230	-1.48
		400mg/kg b.w. Leaf Extract	.500	.354	.230	-.48
		600mg/kg b.w. Leaf Extract	.500	.354	.230	-.48
SD	400mg/kg b.w. Leaf Extract	Control	-1.000*	.354	.047	-1.98
		200mg/kg b.w.Leaf	-.500	.354	.230	-1.48
		600mg/kg b.w. Leaf Extract	.000	.354	1.000	-.98
	600mg/kg b.w. Leaf Extract	Control	-1.000*	.354	.047	-1.98
		200mg/kg b.w.Leaf	-.500	.354	.230	-1.48
		400mg/kg b.w. Leaf Extract	.000	.354	1.000	-.98
unnett t (2-sided)[b]	Control	600mg/kg b.w. Leaf Extract	1.000	.354	.103	-.28
	200mg/kg b.w.Leaf	600mg/kg b.w. Leaf Extract	.500	.354	.445	-.78
	400mg/kg b.w. Leaf Extract	600mg/kg b.w. Leaf Extract	.000	.354	1.000	-1.28
	Control	200mg/kg b.w.Leaf	-.20000	.18114	.331	-.7029
		400mg/kg b.w. Leaf Extract	.70000*	.18114	.018	.1971
		600mg/kg b.w. Leaf Extract	.67500*	.18114	.020	.1721
	200mg/kg b.w.Leaf	Control	.20000	.18114	.331	-.3029
		400mg/kg b.w. Leaf Extract	.90000*	.18114	.008	.3971
		600mg/kg b.w. Leaf Extract	.87500*	.18114	.008	.3721
SD	400mg/kg b.w. Leaf Extract	Control	-.70000*	.18114	.018	-1.2029
		200mg/kg b.w.Leaf	-.90000*	.18114	.008	-1.4029
		600mg/kg b.w. Leaf Extract	-.02500	.18114	.897	-.5279
	600mg/kg b.w. Leaf Extract	Control	-.67500*	.18114	.020	-1.1779
		200mg/kg b.w.Leaf	-.87500*	.18114	.008	-1.3779
		400mg/kg b.w. Leaf Extract	.02500	.18114	.897	-.4779
unnett t (2-sided)[b]	Control	600mg/kg b.w. Leaf Extract	.67500*	.18114	.046	.0197
	200mg/kg b.w.Leaf	600mg/kg b.w. Leaf Extract	.87500*	.18114	.019	.2197
	400mg/kg b.w. Leaf Extract	600mg/kg b.w. Leaf Extract	-.02500	.18114	.998	-.6803

fference is significant at the 0.05 level.

sts treat one group as a control, and compare all other groups against it.

Multiple Comparisons

Dependent Variable		(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error
SOD Week1	LSD	Control	200mg/kg b.w.Leaf Extract	.500	.354
			400mg/kgbw Leaf Extract	1.500*	.354
			600mg/kg b.w.Leaf Extract	3.500*	.354
		200mg/kg b.w.Leaf Extract	Control	-.500	.354
			400mg/kgbw Leaf Extract	1.000*	.354
			600mg/kg b.w.Leaf Extract	3.000*	.354
		400mg/kgbw Leaf Extract	Control	-1.500*	.354
			200mg/kg b.w.Leaf Extract	-1.000*	.354
			600mg/kg b.w.Leaf Extract	2.000*	.354
		600mg/kg b.w.Leaf Extract	Control	-3.500*	.354
			200mg/kg b.w.Leaf Extract	-3.000*	.354
			400mg/kgbw Leaf Extract	-2.000*	.354
	Dunnett t (2-sided)[b]	Control	600mg/kg b.w.Leaf Extract	3.500*	.354
		200mg/kg b.w.Leaf Extract	600mg/kg b.w.Leaf Extract	3.000*	.354
		400mg/kgbw Leaf Extract	600mg/kg b.w.Leaf Extract	2.000*	.354
SOD Week2	LSD	Control	200mg/kg b.w.Leaf Extract	2.45000*	.84945
			400mg/kgbw Leaf Extract	5.07500*	.84945
			600mg/kg b.w.Leaf Extract	2.45000*	.84945
		200mg/kg b.w.Leaf Extract	Control	-2.45000*	.84945
			400mg/kgbw Leaf Extract	2.62500*	.84945
			600mg/kg b.w.Leaf Extract	.00000	.84945
		400mg/kgbw Leaf Extract	Control	-5.07500*	.84945
			200mg/kg b.w.Leaf Extract	-2.62500*	.84945
			600mg/kg b.w.Leaf Extract	-2.62500*	.84945
		600mg/kg b.w.Leaf Extract	Control	-2.45000*	.84945
			200mg/kg b.w.Leaf Extract	.00000	.84945
			400mg/kgbw Leaf Extract	2.62500*	.84945
	Dunnett t (2-sided)[b]	Control	600mg/kg b.w.Leaf Extract	2.45000	.84945
		200mg/kg b.w.Leaf Extract	600mg/kg b.w.Leaf Extract	.00000	.84945
		400mg/kgbw Leaf Extract	600mg/kg b.w.Leaf Extract	-2.62500	.84945

*. The mean difference is significant at the 0.05 level.

b. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Multiple Comparisons

Dependent Variable		(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error
week1 AST	LSD	Control	200mg/kg b.w. Leaf Extract	-1.000	.935
			400mg/kg b.w. Leaf Extract	-7.500*	.935
			600mg/kg b.w. Leaf Extract	-12.000*	.935
		200mg/kg b.w. Leaf Extract	Control	1.000	.935
			400mg/kg b.w. Leaf Extract	-6.500*	.935
			600mg/kg b.w. Leaf Extract	-11.000*	.935
		400mg/kg b.w. Leaf Extract	Control	7.500*	.935
			200mg/kg b.w. Leaf Extract	6.500*	.935
			600mg/kg b.w. Leaf Extract	-4.500*	.935
		600mg/kg b.w. Leaf Extract	Control	12.000*	.935
			200mg/kg b.w. Leaf Extract	11.000*	.935
			400mg/kg b.w. Leaf Extract	4.500*	.935
	Dunnett t (2-sided)[b]	Control	600mg/kg b.w. Leaf Extract	-12.000*	.935
		200mg/kg b.w. Leaf Extract	600mg/kg b.w. Leaf Extract	-11.000*	.935
		400mg/kg b.w. Leaf Extract	600mg/kg b.w. Leaf Extract	-4.500*	.935
week2 AST	LSD	Control	200mg/kg b.w. Leaf Extract	-1.000	.935
			400mg/kg b.w. Leaf Extract	-2.000	.935
			600mg/kg b.w. Leaf Extract	-9.500*	.935
		200mg/kg b.w. Leaf Extract	Control	1.000	.935
			400mg/kg b.w. Leaf Extract	-1.000	.935
			600mg/kg b.w. Leaf Extract	-8.500*	.935
		400mg/kg b.w. Leaf Extract	Control	2.000	.935
			200mg/kg b.w. Leaf Extract	1.000	.935
			600mg/kg b.w. Leaf Extract	-7.500*	.935
		600mg/kg b.w. Leaf Extract	Control	9.500*	.935
			200mg/kg b.w. Leaf Extract	8.500*	.935
			400mg/kg b.w. Leaf Extract	7.500*	.935
	Dunnett t (2-sided)[b]	Control	600mg/kg b.w. Leaf Extract	-9.500*	.935
		200mg/kg b.w. Leaf Extract	600mg/kg b.w. Leaf Extract	-8.500*	.935
		400mg/kg b.w. Leaf Extract	600mg/kg b.w. Leaf Extract	-7.500*	.935
week3 AST	LSD	Control	200mg/kg b.w. Leaf Extract	-.500	2.208
			400mg/kg b.w. Leaf Extract	-4.500	2.208
			600mg/kg b.w. Leaf Extract	-23.500*	2.208
		200mg/kg b.w. Leaf Extract	Control	.500	2.208
			400mg/kg b.w. Leaf Extract	-4.000	2.208
			600mg/kg b.w. Leaf Extract	-23.000*	2.208

	400mg/kg b.w. Leaf Extract	Control	4.500	2.208
		200mg/kg b.w. Leaf Extract	4.000	2.208
		600mg/kg b.w. Leaf Extract	-19.000*	2.208
	600mg/kg b.w. Leaf Extract	Control	23.500*	2.208
		200mg/kg b.w. Leaf Extract	23.000*	2.208
		400mg/kg b.w. Leaf Extract	19.000*	2.208
Dunnett t (2-sided)[b]				
Control		600mg/kg b.w. Leaf Extract	-23.500*	2.208
200mg/kg b.w. Leaf Extract		600mg/kg b.w. Leaf Extract	-23.000*	2.208
400mg/kg b.w. Leaf Extract		600mg/kg b.w. Leaf Extract	-19.000*	2.208

*. The mean difference is significant at the 0.05 level.

b. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Multiple Comparisons

iable	(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidenc Lower Bound
	Control	200mg/kg b.w. Leaf Extract	-3.500	1.369	.063	-7.30
		400mg/kg b.w. Leaf Extract	-2.500	1.369	.142	-6.30
		600mg/kg b.w. Leaf Extract	-7.500*	1.369	.005	-11.30
	200mg/kg b.w. Leaf Extract	Control	3.500	1.369	.063	-.30
		400mg/kg b.w. Leaf Extract	1.000	1.369	.506	-2.80
SD		600mg/kg b.w. Leaf Extract	-4.000*	1.369	.043	-7.80
	400mg/kg b.w. Leaf Extract	Control	2.500	1.369	.142	-1.30
		200mg/kg b.w. Leaf Extract	-1.000	1.369	.506	-4.80
		600mg/kg b.w. Leaf Extract	-5.000*	1.369	.022	-8.80
	600mg/kg b.w. Leaf Extract	Control	7.500*	1.369	.005	3.70
		200mg/kg b.w. Leaf Extract	4.000*	1.369	.043	.20
		400mg/kg b.w. Leaf Extract	5.000*	1.369	.022	1.20
	control	600mg/kg b.w. Leaf Extract	-7.500*	1.369	.012	-12.45
unnett t (2-sided)[b]	200mg/kg b.w. Leaf Extract	600mg/kg b.w. Leaf Extract	-4.000	1.369	.095	-8.95
	400mg/kg b.w. Leaf Extract	600mg/kg b.w. Leaf Extract	-5.000*	1.369	.049	-9.95
	Control	200mg/kg b.w. Leaf Extract	-2.000	1.118	.148	-5.10
		400mg/kg b.w. Leaf Extract	-2.500	1.118	.089	-5.60
		600mg/kg b.w. Leaf Extract	-11.500*	1.118	.001	-14.60
	200mg/kg b.w. Leaf Extract	Control	2.000	1.118	.148	-1.10
		400mg/kg b.w. Leaf Extract	-.500	1.118	.678	-3.60
SD		600mg/kg b.w. Leaf Extract	-9.500*	1.118	.001	-12.60
	400mg/kg b.w. Leaf Extract	Control	2.500	1.118	.089	-.60
		200mg/kg b.w. Leaf Extract	.500	1.118	.678	-2.60
		600mg/kg b.w. Leaf Extract	-9.000*	1.118	.001	-12.10
	600mg/kg b.w. Leaf Extract	Control	11.500*	1.118	.001	8.40
		200mg/kg b.w. Leaf Extract	9.500*	1.118	.001	6.40
		400mg/kg b.w. Leaf Extract	9.000*	1.118	.001	5.90
	control	600mg/kg b.w. Leaf Extract	-11.500*	1.118	.001	-15.54
unnett t (2-sided)[b]	200mg/kg b.w. Leaf Extract	600mg/kg b.w. Leaf Extract	-9.500*	1.118	.002	-13.54
	400mg/kg b.w. Leaf Extract	600mg/kg b.w. Leaf Extract	-9.000*	1.118	.003	-13.04
	Control	200mg/kg b.w. Leaf Extract	-.500	1.275	.715	-4.04
		400mg/kg b.w. Leaf Extract	-1.500	1.275	.305	-5.04
		600mg/kg b.w. Leaf Extract	-28.500*	1.275	.000	-32.04
	200mg/kg b.w. Leaf Extract	Control	.500	1.275	.715	-3.04
SD		400mg/kg b.w. Leaf Extract	-1.000	1.275	.477	-4.54
		600mg/kg b.w. Leaf Extract	-28.000*	1.275	.000	-31.54
	400mg/kg b.w. Leaf Extract	Control	1.500	1.275	.305	-2.04
		200mg/kg b.w. Leaf Extract	1.000	1.275	.477	-2.54
		600mg/kg b.w. Leaf Extract	-27.000*	1.275	.000	-30.54

		control	28.500*	1.275	.000	24.96
	600mg/kg b.w. Leaf Extract	200mg/kg b.w. Leaf Extract	28.000*	1.275	.000	24.46
		400mg/kg b.w. Leaf Extract	27.000*	1.275	.000	23.46
	control	600mg/kg b.w. Leaf Extract	-28.500*	1.275	.000	-33.11
unnett t (2-sided)[b]	200mg/kg b.w. Leaf Extract	600mg/kg b.w. Leaf Extract	-28.000*	1.275	.000	-32.61
	400mg/kg b.w. Leaf Extract	600mg/kg b.w. Leaf Extract	-27.000*	1.275	.000	-31.61

fference is significant at the 0.05 level.

sts treat one group as a control, and compare all other groups against it.

Multiple Comparisons

Dependent Variable		(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error
week1 ALP	LSD	Control	200mg/kg b.w. Leaf Extract	.000	.612
			400mg/kg b.w. Leaf Extract	-2.000*	.612
			600mg/kg b.w. Leaf Extract	-5.500*	.612
		200mg/kg b.w. Leaf Extract	Control	.000	.612
			400mg/kg b.w. Leaf Extract	-2.000*	.612
			600mg/kg b.w. Leaf Extract	-5.500*	.612
		400mg/kg b.w. Leaf Extract	Control	2.000*	.612
			200mg/kg b.w. Leaf Extract	2.000*	.612
			600mg/kg b.w. Leaf Extract	-3.500*	.612
		600mg/kg b.w. Leaf Extract	Control	5.500*	.612
			200mg/kg b.w. Leaf Extract	5.500*	.612
			400mg/kg b.w. Leaf Extract	3.500*	.612
	Dunnett t (2-sided)[b]	Control	600mg/kg b.w. Leaf Extract	-5.500*	.612
		200mg/kg b.w. Leaf Extract	600mg/kg b.w. Leaf Extract	-5.500*	.612
		400mg/kg b.w. Leaf Extract	600mg/kg b.w. Leaf Extract	-3.500*	.612
week2 ALP	LSD	Control	200mg/kg b.w. Leaf Extract	-1.000	.935
			400mg/kg b.w. Leaf Extract	-4.500*	.935
			600mg/kg b.w. Leaf Extract	-13.000*	.935
		200mg/kg b.w. Leaf Extract	Control	1.000	.935
			400mg/kg b.w. Leaf Extract	-3.500*	.935
			600mg/kg b.w. Leaf Extract	-12.000*	.935
		400mg/kg b.w. Leaf Extract	Control	4.500*	.935
			200mg/kg b.w. Leaf Extract	3.500*	.935
			600mg/kg b.w. Leaf Extract	-8.500*	.935
		600mg/kg b.w. Leaf Extract	Control	13.000*	.935
			200mg/kg b.w. Leaf Extract	12.000*	.935
			400mg/kg b.w. Leaf Extract	8.500*	.935
	Dunnett t (2-sided)[b]	Control	600mg/kg b.w. Leaf Extract	-13.000*	.935
		200mg/kg b.w. Leaf Extract	600mg/kg b.w. Leaf Extract	-12.000*	.935
		400mg/kg b.w. Leaf Extract	600mg/kg b.w. Leaf Extract	-8.500*	.935

riable	(I) Groups	(J) Groups	Mean	Std. Error	Sig.	95% Confidenc
week3 ALP	LSD	Control	200mg/kg b.w. Leaf Extract	-1.500	.935	
			400mg/kg b.w. Leaf Extract	-14.500*	.935	
			600mg/kg b.w. Leaf Extract	-44.500*	.935	
		200mg/kg b.w. Leaf Extract	Control	1.500	.935	
			400mg/kg b.w. Leaf Extract	-13.000*	.935	
			600mg/kg b.w. Leaf Extract	-43.000*	.935	
		400mg/kg b.w. Leaf Extract	Control	14.500*	.935	
			200mg/kg b.w. Leaf Extract	13.000*	.935	
			600mg/kg b.w. Leaf Extract	-30.000*	.935	
		600mg/kg b.w. Leaf Extract	Control	44.500*	.935	
			200mg/kg b.w. Leaf Extract	43.000*	.935	
			400mg/kg b.w. Leaf Extract	30.000*	.935	
	Dunnett t (2-sided)[b]	Control	600mg/kg b.w. Leaf Extract	-44.500*	.935	
		200mg/kg b.w. Leaf Extract	600mg/kg b.w. Leaf Extract	-43.000*	.935	
		400mg/kg b.w. Leaf Extract	600mg/kg b.w. Leaf Extract	-30.000*	.935	

*. The mean difference is significant at the 0.05 level.

b. Dunnett t-tests treat one group as a control, and compare all other groups against it.

		Difference (I-J)			Lower Bound	
Control	200mg/Kg Leaf Extract	-4.9783*	.44603	.000	-5.9501	
	400mg/Kg Leaf Extract	-8.8667*	.44603	.000	-9.8385	
	600mg/Kg Leaf Extract	-8.0283*	.44603	.000	-9.0001	
200mg/Kg Leaf Extract	Control	4.9783*	.44603	.000	4.0065	
	400mg/Kg Leaf Extract	-3.8883*	.44603	.000	-4.8601	
	600mg/Kg Leaf Extract	-3.0500*	.44603	.000	-4.0218	
400mg/Kg Leaf Extract	Control	8.8667*	.44603	.000	7.8949	
	200mg/Kg Leaf Extract	3.8883*	.44603	.000	2.9165	
	600mg/Kg Leaf Extract	.8383	.44603	.085	-.1335	
600mg/Kg Leaf Extract	Control	8.0283*	.44603	.000	7.0565	
	200mg/Kg Leaf Extract	3.0500*	.44603	.000	2.0782	
	400mg/Kg Leaf Extract	-.8383	.44603	.085	-1.8101	
Control	200mg/Kg Leaf Extract	-.1000	.13742	.481	-.3994	
	400mg/Kg Leaf Extract	-.1150	.13742	.419	-.4144	
	600mg/Kg Leaf Extract	.3900*	.13742	.015	.0906	
200mg/Kg Leaf Extract	Control	.1000	.13742	.481	-.1994	
	400mg/Kg Leaf Extract	-.0150	.13742	.915	-.3144	
	600mg/Kg Leaf Extract	.4900*	.13742	.004	.1906	
400mg/Kg Leaf Extract	Control	.1150	.13742	.419	-.1844	
	200mg/Kg Leaf Extract	.0150	.13742	.915	-.2844	
	600mg/Kg Leaf Extract	.5050*	.13742	.003	.2056	
600mg/Kg Leaf Extract	Control	-.3900*	.13742	.015	-.6894	
	200mg/Kg Leaf Extract	-.4900*	.13742	.004	-.7894	
	400mg/Kg Leaf Extract	-.5050*	.13742	.003	-.8044	
Control	200mg/Kg Leaf Extract	-17.7833*	.85175	.000	-19.6391	
	400mg/Kg Leaf Extract	-23.2700*	.85175	.000	-25.1258	
	600mg/Kg Leaf Extract	-17.1000*	.85175	.000	-18.9558	
200mg/Kg Leaf Extract	Control	17.7833*	.85175	.000	15.9275	
	400mg/Kg Leaf Extract	-5.4867*	.85175	.000	-7.3425	
	600mg/Kg Leaf Extract	.6833	.85175	.438	-1.1725	
400mg/Kg Leaf Extract	Control	23.2700*	.85175	.000	21.4142	
	200mg/Kg Leaf Extract	5.4867*	.85175	.000	3.6309	
	600mg/Kg Leaf Extract	6.1700*	.85175	.000	4.3142	
600mg/Kg Leaf Extract	Control	17.1000*	.85175	.000	15.2442	
	200mg/Kg Leaf Extract	-.6833	.85175	.438	-2.5391	
	400mg/Kg Leaf Extract	-6.1700*	.85175	.000	-8.0258	
Control	200mg/Kg Leaf Extract	-4.8333*	.58120	.000	-6.0997	
	400mg/Kg Leaf Extract	-6.2083*	.58120	.000	-7.4747	
	600mg/Kg Leaf Extract	-2.2850*	.58120	.002	-3.5513	

200mg/Kg Leaf Extract	Control	4.8333*	.58120	.000	3.5670	
	400mg/Kg Leaf Extract	-1.3750*	.58120	.036	-2.6413	
	600mg/Kg Leaf Extract	2.5483*	.58120	.001	1.2820	
400mg/Kg Leaf Extract	Control	6.2083*	.58120	.000	4.9420	
	200mg/Kg Leaf Extract	1.3750*	.58120	.036	.1087	
	600mg/Kg Leaf Extract	3.9233*	.58120	.000	2.6570	
600mg/Kg Leaf Extract	Control	2.2850*	.58120	.002	1.0187	
	200mg/Kg Leaf Extract	-2.5483*	.58120	.001	-3.8147	
	400mg/Kg Leaf Extract	-3.9233*	.58120	.000	-5.1897	