UNIVERSITY OF ZIMBABWE

In vivo toxicological study of aqueous extracts of *Vitex payos* fruits

BY

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DECLARATION

I, Felicia Nonsikelelo Dube, declare that this is unaided work carried out in the Biochemistry Department in the laboratory of Natural Products at the University of Zimbabwe from August 2019 to May 2020. I declare that this work has not been submitted before and that I have fully acknowledged through referencing the thoughts and ideas of others.

Signed : ....

Date : ....

ABSTRACT

The plant *Vitex payos* is used in folk medicine to treat several ailments. Despite *Vitex payos'*, ethno medicinal benefits, very few studies have described the fruits' potential toxicity. The aim of the present study was to evaluate the *in vivo* acute and subchronic toxicity of aqueous extracts of *Vitex payos* fruits which grow in Zimbabwe on Sprague Dawley rats. Aqueous extraction of *Vitex payos* fruits was performed by using standard routine methods. The extract administered at a single dose of 5000 mg/kg did not produce treatment related signs of toxicity or mortality in any of the treated animals during the 14-day observation period. Therefore, the Lethal dose 50% of *Vitex payos* was estimated to be more than 5000 mg/kg. In the, 90 day repeated dose oral toxicity study, twenty (20) adult Sprague Dawley rats were uniformly divided into four groups of 5 rats each. Group 1 served as a control while groups 2, 3 and 4 were respectively orally administered with 100 mg/kg body weight, 400 mg/kg body weight and 800 mg/kg body weight of the *Vitex payos* aqueous extract daily. The results revealed no significant difference in food and water consumption, body weight change and biochemical parameters, compared to the control group which received only distilled water. Histopathology examinations of the liver and kidney did not reveal morphological alteration. Analysis of these results with the information of signs, health monitoring and behaviour, could lead to the conclusion that the long-term oral administration of *Vitex payos* aqueous extract for 90 days does not cause sub chronic toxicity.

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I have no valuable words to express my gratitude but my heart is full of every assistance and support received.

DEDICATION

This project is dedicated to my mother,

Flattela Nleya.

LIST OF ABBREVIATIONS

Alb	Albumin
ANOVA	Analysis of Variation
AST	Aspartate aminotransferase
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
bw	body weight
DNEL	derived no effect level
OEL	occupational exposure limit
ADI	acceptable daily intake.
GSH	Globally harmonized system of classification and labelling of chemicals
kg	kilogram
LD_{50}	Lethal dose 50% / Median lethal dose
LC_{50}	Lethal concentration 50%
mg	milligrams
ml	milliliters
µ	micro
NOAEL	No observed adverse effect level
LOAEL	Lowest observed adverse effect level
ppm	parts per million
P Value	Probability value
Tbil	Total bilirubin
TP	Total Protein
V.payos	*Vitex payos*

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CHAPTER ONE

RESEARCH OVERVIEW

1.1 Introduction

Nutraceuticals have proven health benefits. When consumed within their recommended dietary intakes they have the potential to keep diseases at bay and therefore allow humans to maintain an overall good health. Despite the fact that, nutraceuticals have significant promise in the promotion of human health and disease prevention, health professionals, nutritionists, and regulatory toxicologists should strategically work together to plan appropriate regulations to provide the ultimate health and therapeutic benefit to mankind (Chauhan *et al*., 2013).

The high consumption of processed foods associated with sedentary lifestyle has caused an increase in the number of chronic diseases related to inadequate diet e.g. Diabetes. The World Health Organization (WHO) in 2004 launched the Global Strategy on Healthy Eating, which establishes certain guidelines with the main objective of reducing risk factors related to such diseases based on a healthy diet, physical activity and health (Das *et al*., 2012).

Recommendations included an increase in the intake of vegetables, fruits, vitamins and minerals with the principle that these foods contained bioactive compounds such as carotenoids, phytoestrogens, glycosinolates, sulfur compounds, fructooligosaccharides and flavonoids (Slavin *et al*., 1999). Approximately 80% of Africans depend on herbal medicine for their health care. The high cost, low availability, undesirable side effects and uncertainty of use during pregnancy of synthetic drugs has been some of the factors leading to a preference for drugs of plant origin, which are believed to be suitable for chronic treatments (Agrawal and Paridhavi, 2007).

In the twenty-first century era, nutraceuticals became increasingly popular in both humans and animals due to their easy access, cost-effectiveness, and tolerability with a wide margin of safety. Most nutraceuticals possess antioxidant or anti-inflammatory properties and are claimed to provide protection against many diseases if taken regularly. At the same time, toxicological studies of nutraceuticals have been limited. Therefore the safety of many nutraceuticals cannot be guaranteed. While some nutraceuticals are safe, others have a toxic potential (Gupta *et al*., 2018).

Due to a lack of pharmacological/toxicological studies a great number of nutraceuticals have no toxicity/safety data available. The safety of some nutraceuticals can be compromised via contamination with toxic plants, metals, pesticides, fertilizers, drugs of abuse, mycotoxins, etc. (Samuelsson, 2004). Knowledge of toxicokinetic/pharmacokinetic studies appears to play a pivotal role in the safety and toxicity assessment of nutraceuticals. Interaction studies are vital to determine efficacy, safety, and toxicity when nutraceuticals and therapeutic drugs are used simultaneously.

1.2 Background of research problem

Medicinal plants, either as a pure compound or extract, offer limitless opportunities for the discovery of new drugs. Most of the natural products used in folk remedy have solid scientific evidence with regard to their biological activities. However, there is little information available concerning the possible toxicity that medicinal plants may cause to the consumers (Dias and Takahashi, 1994).

1.3 Problem statement

Although *Vitex payos* has been proven to have antidiabetic properties, its toxicity profile has not been established.

1.4 Justification of study

Vitex payos fruits have the potential to provide a valuable source of nutrition to the body. They could also generate income for some people living in various rural areas and also serve as a herb in curing some ailments among the people. These benefits call for the need to establish the chemical, nutritional and toxicological properties for its increased utilization

(Ilelaboye *et. al*., 2013).

1.5 Hypothesis

Vitex payos has a toxic effect on animals when administered in high concentrations or when administered over a long period of time.

1.6 Study aims

Aim of this study is to evaluate the potential toxicity profile of aqueous extract of *Vitex payos* through the method of acute and sub chronic oral administration in Sprague Dawley rats.

1.7 Specific objectives

The aims of the study were achieved by:

- preparing an aqueous extract from the fruits of *V. payos*
- determining the acute toxicity of the aqueous extract in Sprague Dawley rats
- determining the sub chronic toxicity of the aqueous extract in Sprague Dawley rats

CHAPTER TWO

LITERATURE REVIEW AND THEORETICAL FRAMEWORK

2.1 Nutraceuticals

Nutraceutical is the hybrid of the words ‘nutrition’ and ‘pharmaceutical’. They have been proven to be natural, bioactive chemical compounds that have health-promoting, disease-preventing or general medicinal properties. The principal reasons for the growth of the nutraceutical market worldwide are the current population and the health trends. The food products used as nutraceuticals are categorized as dietary fibre, polyphenols, probiotics, prebiotics, polyunsaturated fatty acids, antioxidants and other different types of herbal/ natural foods (Coopens *et al*., 2006).

Nutraceuticals aid in combating some of the major health problems of the century such as obesity, cardiovascular diseases, cancer, osteoporosis, arthritis, diabetes, cholesterol etc. In brief, nutraceutical have led to the new era of medicine and health, in which the food industry has developed into a research oriented sector.

The term “nutraceutical” was introduced in the year 1989 by American medical doctor Stephen L. De Felice, founder and chairman of the Foundation for Innovation in Medicine (FIM) in Cranford, New Jersey. The relationship between food and medicine has been of interest and was reflected in many classical traditions, including those of ancient Greece and China. Nutraceuticals are described as products that are extracted, purified or produced from a plant, animal or marine source (e.g. antioxidants from blueberries, fish oils), or produced from dried, powdered, or pressed plant material and demonstrated to have a physiological benefit, or provide protection against chronic disease (Chauhan *et al*., 2013).

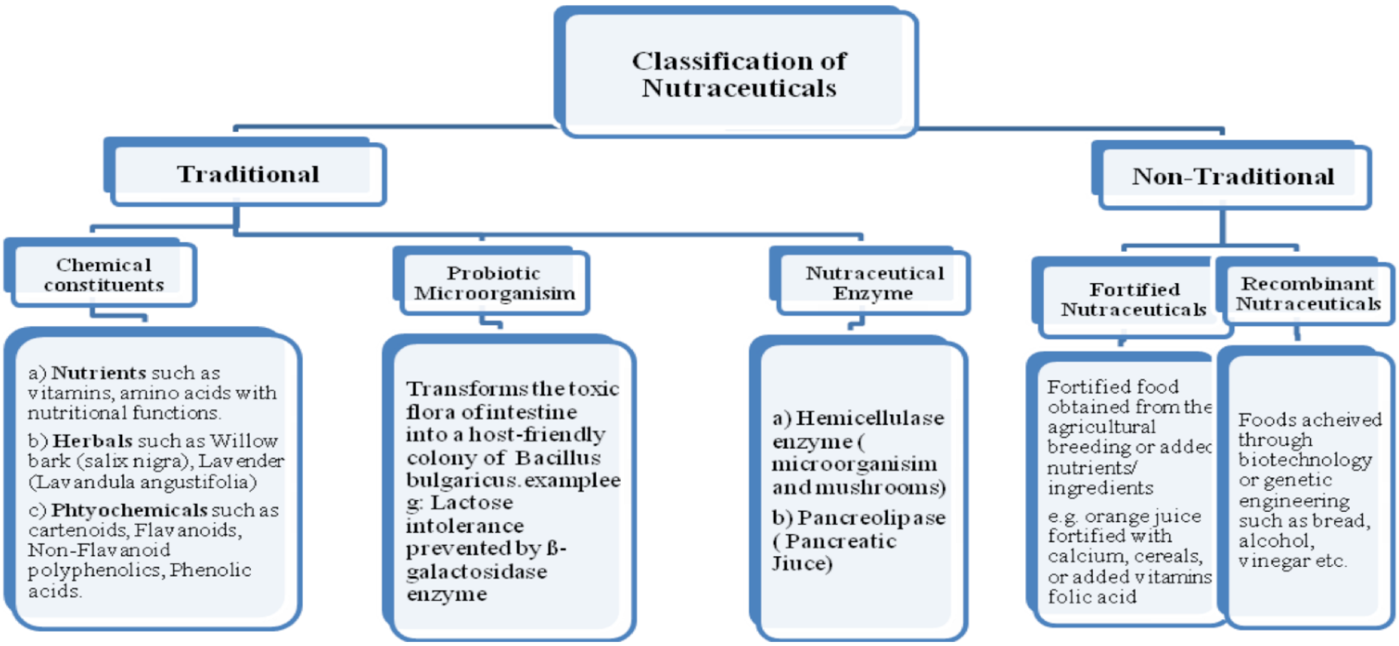
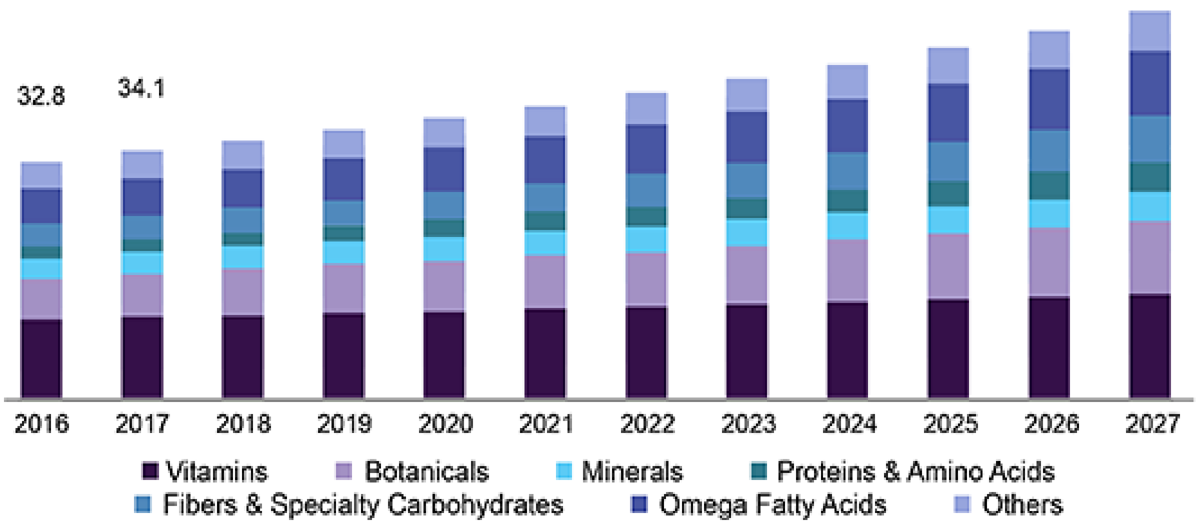


Figure 2.1: Tubular representation of classification of nutraceuticals

Regarding the promise of nutraceuticals, they should be considered in two ways:

1. Potential nutraceuticals
2. Established nutraceuticals

A potential nutraceutical is one that holds a promise of a health or medical benefit. It becomes an established nutraceutical only after there are satisfactory clinical data which demonstrate such a benefit. For instance, folic acid was a potential nutraceutical until sufficient clinical evidence for the prevention of neural tube defects was generated to make it an established one. Ginseng remains a potential nutraceutical because of a lack of such evidence. It is important to note that the overwhelming majority of nutraceutical products are in the potential category which are waiting to become established (DeFelice, 1995)



Source: www.grandviewresearch.com

Fig 2.2: United States of America nutraceuticals Market Size, by product, 2020 - 2027

The global nutraceutical market size in 2019 was valued at US$382.51 billion. This market is said to grow at a compound annual growth rate of 8.5 % percent. Asia Pacific accounted for the major market share of 31.01 % in 2019 and is likely to maintain its lead over the forecast period. Rising levels of disposable income, changing lifestyle, and shift in preferences for healthier dietary intake in emerging economies are expected to drive the demand for nutraceuticals in Asia Pacific. Brazil, China, and India are likely to expand at the fastest CAGR over the forecast period.

Growing consumer interest in a healthy diet has led to a thriving food and beverage market in the recent past and the trend is expected to continue over the forecast period. Demand for functional foods is on the rise as they are believed to impart exceptional health benefits owing to their nutrient content. This is further expected to boost the growth of the market for nutraceuticals

(Danaei *et al.*, 2011).

The positive mentality of consumers towards functional foods mainly on account of the added health and wellness benefits offered by these nutraceutical products is fueling the market growth. Growing geriatric population, increasing healthcare costs, changing lifestyles, food innovations, and expectations regarding their higher prices have aided the overall growth of the nutraceuticals industry. It has been reported that nine out of ten adults consume 55 % minerals and over 50 % vitamins in their daily diets (WHO, 2005). The global nutraceuticals industry has been witnessing key developments in terms of product innovation and portfolio expansion over the past few years. Public companies have been proactive in initiating strategies to gain a competitive advantage in the nutraceuticals industry.

Industrialization has triggered high levels of atmospheric pollution. Vast resources of water, soil and food are contaminated due to the extensive use of various chemicals and hazardous products which lead them to be potentially harmful to human beings. The elevated demands for health care have dramatically escalated the cost of medical care. Thus, people have tried to achieve a better quality of life by consuming more vegetables, fruits, dietary supplements or nutraceuticals. Development of better characterized and research-proven products will help enhance consumer confidence in nutraceutical products in the rest of the world (Ganesh *et al*., 2015).

Phytochemicals of nutraceutical importance are bioactive constituents that sustain or promote health and occur at the intersection of food and pharmaceutical industries. These range from isolated nutrients, dietary supplements and specific diets to genetically engineered designer foods, herbal products, processed foods and beverages.

Phytochemicals are broadly described as phytoestrogens, terpenoids, carotenoids, phytosterols, glucosinolates, polyphenols, flavonoids, isoflavonoids and anthocyanidins (Middleton *et* al., 2000).

Majority of foods, such as whole grains, beans, fruits, vegetables and herbs contain phytochemicals of nutraceutical importance. These phytochemicals have tremendous therapeutic potential in curing various diseases.

Advantages of nutraceuticals

- They are obtained from natural sources and can be trusted to have little or no side effects in comparison to pharmaceuticals.
- Lifestyle (diet), related chronic diseases/disorders are a major burden on health care costs particularly with ageing population.
- Offer low cost and early control of risk factors for subsequent disease e.g. heart disease
- Offer control of moderately elevated risk factors which current health care systems fail to control

Disadvantages

- They take time to work
- Lack of public awareness as people are still skeptical if old age wisdom will exhibit any benefit
- There is a problem of quality control, which deals with issues such as contamination by toxins and by harmful alternatives

The nutraceutical market is driven by new trends in popular healthcare, such as the shift towards preventative therapies, self-medication and an increase in disposable income. However, nutraceuticals have different legal definitions according to various state laws. This disparity often confuses customers regarding the original use, potency and safety of the product.

Thus, there is an absolute need for a uniform nomenclature for this booming nutraceutical market across the globe. Nutraceuticals are not regulated in the same way that drugs are. Instead, they are classified according to different food categories. This is a notable benefit for animal and fish farmers, as they can use cheaper nutraceuticals in appreciable amounts to prevent diseases, which is also beneficial for drug companies. Along with the knowledge that nutrition has a role in preventing many diseases, the use and trade of nutraceuticals has boomed in recent years, across the entire globe. As the population ages, there is a marked increase in healthcare spending, especially in developed countries. The regulation of these products is one of the boldest challenges for any government as the people get ever more health conscious (Coopens *et al*., 2006).

In turn, nutraceuticals are proving to be more and more popular with consumers as they offer a safe and easy healthcare option. This requires careful formulation and the implementation of government legislation. As nutraceuticals blur the line between food and drugs, it is often difficult by legal definition to distinguish between nutrients, food additives, drugs and nutraceuticals. When their dosages exceed those of a normal diet, the bioactivity of a nutrient can be drug-like. For instance, at the levels present in foodstuffs, tryptophan is a nutrient; but at higher doses, its activity can be drug-like. It is the precursor of serotonin, which is extensively used to treat insomnia (Degnan, 2003).

Legal definitions are predominantly based on two aspects: one is the origin or source; the other is the benefit(s) that it can provide to the consumer. The nomenclature of nutraceuticals also varies in different countries: the US describes them as "Dietary Supplements," in Canada they're referred to as "Natural Health Products" and Japan lists them as "Foods for Special Health Use (FOSHU)". These definitions can be general or specific. There are distinct definitions and regulations for dietary supplements and functional foods in the US, Canada and Europe, whereas in Japan, both dietary supplements and functional foods are governed under the same set of regulations (Wallace *et al*., 2013).

A drawback is that quality control, unethical and criminal elements exist in the industry. There is no provision to offer financial protection to the innovator companies who are ready for this long-term challenge. Less-strict regulations offer advantages to both drug companies and animal farmers since drug companies are always eager to capitalize on quick financial returns. In this respect, nutraceuticals offer significant advantages compared with the long development times and high manufacturing cost of drugs. Moreover, nutraceuticals do not require costly clinical trials.

Regulation presents significant challenges to the globalisation of nutraceuticals in many ways. Primarily, the regulatory variation between countries hinders global trade and marketing. Therefore, there is an explicit need to study nutraceutical dose-related issues. It is essential to test and regulate the use of nutraceuticals in a cost-effective and efficient manner with regard to farm animals. This can offer a better health, not only to those cultured animals, but to human beings (Ganesh *et al*., 2015).

In general, the goals of nutraceutical regulation have been focused on safety and labeling with a lesser emphasis as compared to pharmaceuticals on product claims and intended use. The consumer has to spot the label, claims made on the label, ingredient and nutritional information and, most importantly the instructions for use.

Consumers often check out the price first, with a belief that the higher the price, the better the quality. This is accomplished through Good Manufacturing Practice (GMP) regulations and a recent increase in enforcement. Food and Drug Administration (FDA) is authorized to take action against any unsafe product after it reaches the market. Manufacturers have to make sure that the information on the product label is truthful and not misleading, but they are not obliged to register their products with the FDA nor get FDA approval before producing or selling nutraceutical (Taylor, 2004)

Medicinal plant preparations may contain high levels of toxic chemical constituents to potentially cause serious harm to animals and/or humans. Thus, toxicity studies are important to assess the toxic effects of plant derived products.

2.2 Toxicology

Toxicology is a branch of science that deals with the study of poisons and toxins. More comprehensively, it is the identification and quantification of adverse outcomes associated with exposures to physical agents, chemical substances and other conditions (Levine, 2002). The man considered to be the 'father' of this discipline is Philippus Aureolus Theophrastus Bombastus von Hohenheim, also known as Paracelsus. It is said that Paracelsus meant 'equal to Celsus'. This was referring to the Roman encyclopaedist Aulus Cornelius Celsus.

The change in his name was meant to be an indication of Paracelsus' desire to rival ancient medical authorities such as Celsus and Galen. One of Paracelsus' contributions to the field of medicine was the idea that pathological changes were caused by both internal factors and external factors. These included cosmic influences differing with climate, as well as toxic matter originating in food (Coopens *et al*., 2006).

Paracelsus proposed that all natural substances have two types of influences a helpful one and a harmful one. This led to one of Paracelsus' most famous proverb, which is also the fundamental principle of classical toxicology, "*Alle Dinge sind Gift und nichts ist ohne Gift, allein die Dosis macht es, dass ein Ding kein Gift ist."* meaning "All things are poison and nothing is without poison; only the dose makes a thing not a poison." This has often been shortened to "The dose makes the poison" (Grandjean, 2016)

Toxicology provides important information for both medicine and epidemiology in understanding aetiology. It provides information as to the plausibility of observed associations between exposures, including occupations, and disease. Toxic effects are generally categorized according to their site. Sometimes the effect may occur at only one site (Wooley, 2008).

This site is referred to as the specific target organ. In other cases, toxic effects may occur at multiple sites. This is referred as systemic toxicity. The different types of systemic toxicity are; Acute Toxicity, Subchronic Toxicity, Chronic Toxicity, Carcinogenicity, Developmental Toxicity and Genetic Toxicity (somatic cells).

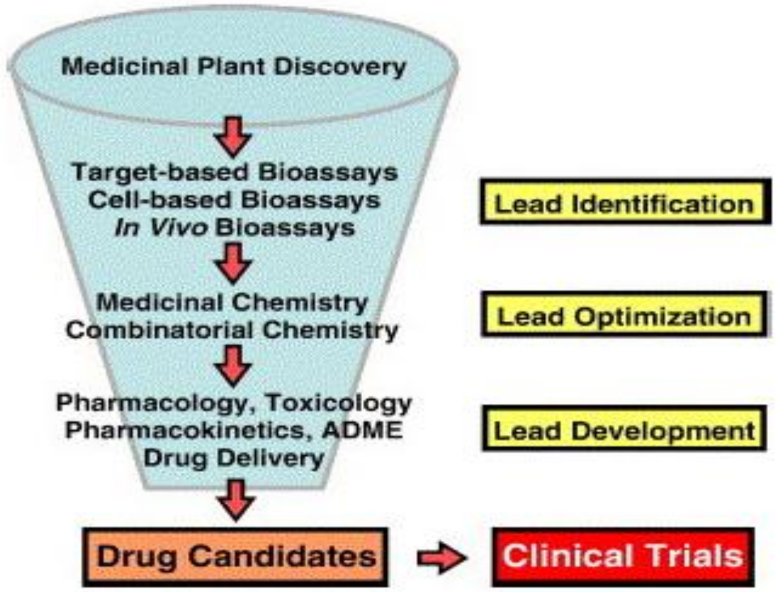


Figure 2.3: Schematic representation of a typical medicinal plant drug discovery process and development (Balunas and Kinghorn, 2005).

Toxicological screening is very important for the development of new drugs and for the extension of the therapeutic potential of existing molecules. The US Food and Drug Administration (FDA) states that it is essential to screen new molecules for pharmacological activity and toxicity potential in animals (Butler, 2004; Koehn and Carter, 2005). Recently, rediscovering natural products has been of interest. It is clear that nature has played and will continue to play, a vital role in the drug discovery process (Cragg and Newmann, 2005).

Drug discovery from medicinal plants has evolved to include numerous fields of inquiry and various methods of analysis. The process typically begins with a botanist, ethnobotanist, ethnopharmacologist, or plant ecologist who collects and identifies the plant(s) of interest. Collection may involve species with known biological activity for which active compound(s) have not been isolated (i.e traditionally used herbal remedies) or may involve taxa collected randomly for a large screening program (Baker *et al.*, 1995).

Natural product chemists prepare extracts from the plant material, subject these extracts to biological screening in pharmacologically relevant assays, and commence the process of isolation and characterization of the active compound(s) through bioassay-guided fractionation. Molecular biology has become essential to medicinal plant drug discovery through the determination and implementation of appropriate screening assays directed towards physiologically relevant molecular targets. Pharmacognosy encapsulates all of these fields into a distinct interdisciplinary science (Balunas and Kinghorn, 2005).

2.2.1 Acute Toxicity

Acute toxicity describes the adverse effects of a substance that result either from a single exposure or from multiple exposures in a short period of time (usually less than 24 hours). It is a short term assessment and evaluation of potential hazard test substance or consequences of a single dose of a test substance. Acute toxicity testing may be used in risk assessments of chemicals for humans and non-target environmental organisms. It provides guidelines on the dose to be used in more prolonged studies and it also provides the basis for other testing programs to be designed. This test is carried out in each species of animal as the same route as intended to be used in treatment (Wooley, 2008).

Acute toxicity effects were seen in the Bhopal disaster in India in the year 1984 which was considered to be among the world's worst industrial disasters. Over 500 000 people were exposed to methyl isocyanate (MIC) gas. Within a few hours 5000 people died and 30 000 were permanently disabled. The initial effects of exposure were coughing, severe eye irritation, feeling of suffocation, burning in the respiratory tract, stomach pains inducing vomiting. The primary causes of death were choking, reflexogenic circulatory collapse and pulmonary oedema.

2.2.1.1 Importance of acute toxicity testing

- To identify the target organ of toxicity
- To provide safety measures and monitoring guild lines for workers involved in the development and testing of test substances
- To provide information needed for the dose selection in prolonged toxicity studies
- To produce data containing the adverse effects of a substance on human and animal health as well as the environment
- For classification, labelling and transportation of chemical agents

A common dose-response descriptor for acute toxicity is the LD_{50} (Lethal Dose 50 %). This is a statistically derived dose at which 50 % of the individuals will be expected to die. The LC_{50} (Lethal Concentration 50 %) is used for inhalation toxicity whereby air concentrations are used for exposure values. LD_{50} and LC_{50} values are used to describe the relative acute toxicity of a chemical substance. Acute toxicity tests in animals use mortality as the main observational endpoint.

The units of LD_{50} and LC_{50} are as follows:

- LD_{50}: mg/kg/bw. mg/kg bw/d stands for mg of substance per kg of body weight administered per day.
- LC_{50}: mg/L. mg/L is the estimated air concentration of a substance administered via inhalation route.

2.2.1.2 Dose Descriptor for Acute Toxicity

LD_{50}/LC_{50} from acute toxicity studies are mainly used for Globally Harmonized System of classification and labelling of chemicals for acute toxicity classification, qualitative risk assessment and dose selection for repeated dose toxicity studies. They cannot be used to derive No-observed-adverse-effect-level (NOAEL).

	Category 1	Category 2	Category 3	Category 4	Category 5
Symbol	Skull and Crossbones	Skull and Crossbones	Skull and Crossbones	Exclamation Mark	No symbol is used
Signal word	Danger	Danger	Danger	Warning	Warning
Hazard statement --Oral	Fatal if swallowed	Fatal if swallowed	Toxic if swallowed	Harmful if swallowed	May be harmful if swallowed
--Dermal	Fatal in contact with skin	Fatal in contact with skin	Toxic in contact with skin	Harmful in contact with skin	May be harmful in contact with skin
--Inhalation	Fatal if inhaled	Fatal if inhaled	Toxic if inhaled	Harmful if inhaled	May be harmful if inhaled

Table 1. Acute toxicity label elements

2.2.2 Subchronic Toxicity

Subchronic Toxicity results from repeated exposure for several weeks or months. This is a common human exposure pattern for some pharmaceuticals and environmental agents. This study is basically to determine the organs affected and to check whether the drug is potentially carcinogenic or not.

Subchronic toxicity effect where observed by the ingestion of coumadin tablets for several weeks as a treatment for venous thrombosis which resulted in internal bleeding. Coumadin medication is used to treat blood clots (such as in deep vein thrombosis or pulmonary embolus) and/or to prevent new clots from forming in your body and reduce the risk of a stroke or heart attack (Sireeratawong *et al*., 2013).

The primary goal of repeated dose toxicity studies is to characterize the adverse toxicological effects occurring as a result of repeated daily dosing with, or exposure, to a substance for a specified period up to the expected lifespan of the test species (usually from 3 weeks up to 2 years in animal studies). Effects include changes in morphology, physiology, growth or life span, clinical chemistry and behaviour.

2.2.2.1 Importance of subchronic toxicity testing

- Essential for the quantitative risk assessment of chemical substances.
- Studies yield information on general characteristics of the toxicity to specific target organs
- Provide the dose response relationship, responses to toxic metabolites formed in the organism, delayed responses and cumulative effects
- Provide information on reversibility/irreversibility of the toxic effect.

2.2.2.2 Dose Descriptor for Repeated Dose Toxicity

Typically, a **NOAEL** or **LOAEL** can be obtained from repeated dose toxicity studies.

- No Observed Adverse Effect Level (NOAEL): The highest exposure level at which there are no biologically significant increases in the frequency or severity of adverse effect between the exposed population and its appropriate control; some effects may be produced at this level, but they are not considered adverse effects.
- Lowest Observed Adverse Effect Level (LOAEL)**:** The lowest exposure level at which there are biologically significant increases in frequency or severity of adverse effects between the exposed population and its appropriate control group.
- The units of NOAEL or LOAEL: mg/kg/bw/day or ppm**.** For inhalation route, the unit can be mg/L/6h/day.

NOAEL is not only used for determining the hazard classification of the specific target organ systematic toxicity of a chemical substance, but also used to derive threshold safety exposure dose to humans such as derived no effect level (**DNEL**), occupational exposure limit (**OEL**) and acceptable daily intake (**ADI**).

In repeated dose toxicity studies, usually 3 dose levels are given. The highest of three dose levels should be chosen with the aim to induce toxicity but not death. A descending sequence of dose levels should be selected to demonstrate any dose-related response and a no-observed-adverse-effect level (NOAEL) at the lowest dose level (Ibrahim *et al*., 2016)

2.2.3 Importance of toxicity testing

It should be noted that toxicity testing is paramount in the screening of newly developed drugs before they can be used on humans. The principle of toxicity testing is not just to check the safety of a test substance is; but to characterize the possible toxic effects it can produce. The guiding principles of toxicity testing are to check the effect of the test substances on laboratory animals and direct toxic effects in humans. Secondly, exposure of laboratory animals to high doses in order to evaluate its possible hazard on humans that are exposed to a much lower dose. The use of animals in toxicity testing is most likely to continue for the foreseeable future because of the benefits they offer in examining a whole functioning organism (Parker *et al*., 2008)

All toxicity study is supported by; clinical analysis, haematological, haematochemical analysis, histopathological analysis and statistical presentation and data interpretation.

In a nutshell toxicity testing enables;

- To establish a dose response curve
- To ensure safety of novel chemicals for use as pesticides, drugs, or food additives before they are registered for general use in industry or clinics
- To establish the mode of action or mechanism for a toxic effect that may have been seen in other studies.
- To produce epidemiological studies to explain observations in the population, for instance
- To endorse new methods of testing or investigation, particularly those conducted in vitro rather than in animals.

Toxicity testing plays a crucial role in ascertaining the toxic effect and characterization of test substance. Toxicity obtained in animal studies occurs with similar incidence and severity in humans. The use of animals in toxicity testing is most likely to continue for the foreseeable future because of the benefits they offer in examining a whole functioning organism (Levine, 2002)

2.3 Vitex payos

In the dry areas, indigenous fruits become important staples when cereals harvested are inadequate to support populations. Farmers in these areas have identified many of the handicaps in domestication but there is still need for inputs from the food industry into identification of the desirable traits and characteristics of potentially novel food. Utilization of indigenous fruits is limited in drylands of East and Central Africa (ECA) where they are abundantly available because of scarce knowledge on their nutritional values, postharvest handling and lack of appreciation of the quantities available (Swai and Kimata, 2005). One such resource is *V.payos* (Lour.) Merr fruit, which most subsistence farmers collect for food consumption and for subsistence sale in local markets during the fruit ripening seasons.

2.3.1 Description, botany and history

Vitex payos, the “real” chocolate berry, commonly known as “tsubvu” in Shona or “umtshwankela” in Ndebele is a shrub or small tree in the interesting verbenaceae family. *Vitex* is one of several genera that was transferred from *Verbenaceae* to *Lamiaceae* in the 1990s as a result of phylogenetic studies of DNA sequences. It is the largest genus in the subfamily *Viticoideae* of *Lamiaceae*. Medicinally, the plant is used for stomach ailments, threadworm, skin problems, and loss of appetite. Fallen leaves are used as a mulch. The wood is very hard and suitable as poles for roofing house, furniture, stools, caring, tool handles, gunstocks, bowls, spoons, drums, guitars, beehives, firewood and charcoal production. It is also used for fuel. The fruits are good sources of minerals and vitamins, essential for human health (Saka and Msonthi, 1994). A decoction of the root is used as a remedy for stomach problems. The pounded bark is used to treat threadworm and skin problems.

The leaves are boiled and the liquid drunk by patients who have lost their appetite. They have nutritional value that could provide the necessary body requirements. There is ongoing research in the control of mosquito with compounds extracted from the plant, which inhibit the growth of the larva (Ghosh *et al*., 2012). *Vitex payos* is rich in vitamin B and helps in adding bone marrow in one's body. It has high levels of phytonutrients that add as protection against sun damage to the skin.

It has a rounded crown and grows up to 10 m in height. *Vitex* fruits are ovoid to spherical and are about 3-4cm long and 2,5cm wide with a single hard seed inside. Bark distinctive, grey-brown and deeply fissured. The leaves are densely covered with hairs are bright green (Ruffo *et al*., 2002). *V.payos* is a species of the tropics, growing in hot, low and semi-arid places with a high water table. In more arid zones it is found near rock outcrops. It grows in areas where the mean annual rainfall is within the range 650 - 850 meters.

Source: Exotic fruits of Africa

Figure 2.4: Photograph of *Vitex payos*

The chocolate brown or black fruits are sweet and edible. It can be eaten raw or cooked, or made into jams. These fruits, are a very popular food in parts of southern and eastern Africa, where local people collect them in quantity for their own use and also to sell in local markets. Fruit is sweet, with a mealy texture and is definitely an acquired taste for people who have not eaten it previously. Westerners are typically offended by the flavor, the powdery texture, the oily feel in the mouth, and the strong smell. The fruits have a rather unpleasant smell, and leave a temporary black stain on the teeth, but they are much eaten by children and herdsmen (Kimondo *et al*., 2010). The low-growing tree is attractive enough to have promise purely as an ornamental, especially when in full flower.

2.3.2 Geographic distribution

Vitex payos has a wide distribution from Angola; The Democratic Republic of Congo, Kenya, Malawi, Mozambique, Tanzania, Zambia; Zimbabwe, Burkina Faso, Cameroon, Central Africa, Côte d'Ivoire, East Africa, Egypt, Gabon, Gambia, Ghana, Guinea, Guinée, Guinea-Bissau, Ivory Coast, Mali, Nigeria, North Africa, Senegal, Sierra Leone, Southern Africa, Sudan and Uganda. It is widely distributed in most parts of Zimbabwe, occurring in open woodlands, on termite mounds on loamy soils and at times near rivers on alluvial soils.

2.3.3 Nutritional information

Nutritional Value of *V. payos* fruit pulp per 100g of raw edible portion	
Energy	63Kj
Water %	70.6
Carbohydrates	27.4 g
Fibre	27g
Protein	0.7g
Ash	5.5g
Fat	0.8g
Vitamin C	1.96mg
Calcium	34mg
Iron	2.7mg
Phosphorus	50mg

2.3.4 Phytochemical composition

Very little is known about the phytochemical composition of *Vitex payos*

Diet rich in nutraceuticals along with regular exercise, stress reduction and maintenance of healthy body weight will maximise health and reduce disease risk. As the old proverb goes, "You can lead a horse to the water, but you cannot make it drink", likewise you can rave about the very many benefits of nutraceuticals and lead people to them but you cannot persuade them.

CHAPTER THREE

METHODOLOGY

3.1 Collection and preparation of *Vitex payos* fruits

Vitex payos fruits were identified and collected from Nehuimba village in Rusape. The fruits were then transported to the Biochemistry department at the University of Zimbabwe and stored at -20 °C.

Preparation of the fruits began with thawing in order to easily peel off the skin. The fruit seeds were separated from the pulp by hand scrapping using a knife. The pulp was dried in an oven at 55 °C for 48 hours. The dried pulp was pulverized into a fine powder at the Institution of mining and research, University of Zimbabwe.

3.1.2 Preparation of aqueous extract

The pulverized sample was extracted in water whereby, 30 g of the *Vitex payos* powder was dissolved in 120 ml of distilled water. Five samples (A, B, C, D, E) were prepared and placed on a shaker for 72 hours. The samples were centrifuged at a speed of 3500 rpm (J.P. Selecta, Centronic, Spain) for 15 minutes. The supernatants produced were mixed and stored. Re-extraction was done by respectively collecting the different obtained pellets and macerating in 120 ml of distilled water and placing on a shaker for 48 hours. The samples were centrifuged to obtain more supernatant. Supernatants 1 and 2 were combined and freeze dried (Edwards-Freeze Dryer Modulyo, EF4) for 48 hours at the Faculty of Veterinary Science, University of Zimbabwe. The freeze dried sample was used to make the stock solution which would be used to make the respective concentrations required to administer to the rats.

3.2 Preparation of rats

Male and female adult Sprague Dawley rats (120-150 g) were used for the sub chronic toxicity profiling and female nulliparous rats (190-250 g) were used for the acute toxicity studies. The animals were obtained from the animal house of school of medicine, University of Zimbabwe. They were fed on commercial rat pellets obtained from the Zimbabwe National foods. Drinking water was provided to the rats *ad libitum* from tap water. The animals were maintained under standard conditions of temperature and 12 hour light/darkness cycle.

Relevant procedures on handling and caring of rats were laid out by the animal house staff as the rats were kept at the animal house. The rat cages were bedded with wood shavings which had to be changed regularly in order to maintain a clean environment. The animals were allowed to acclimatize for 5 days in their different allocated cages before the commencement of the study.

3. 3 Acute toxicity study

The acute oral toxicity (LD_{50}) test of the aqueous extract of fruits of *Vitex payos* was evaluated in Sprague Dawley rats following Lorke's method which involved two phases (Garrido-Acosta O, 2014).

The first phase was conducted by dividing nine rats into three groups of three rats. Following an overnight fast, the rats were weighed and the dose was calculated in reference to their body weight and the freeze dried samples were dissolved in distilled water. The first, second and third group received 10, 100 and 1 000 mg/kg body weight of the aqueous extract respectively. The animals were observed keenly for about 30 mins for any signs of toxicity or mortality. Further observations were made every hour for 24 h after administration of the extract. The absence of death of any animals in this phase necessitated the conduct of the second phase.

In the second phase, three rats were divided into three respective groups. The three groups received 1 500, 3 000 and 5 000 mg/kg body weight of the aqueous extract respectively. The rats were observed for any signs of toxicity or mortality within 24 h. Further observation of all the rodents was made for a period of 14 days.

The final LD_{50} value was determined from Lorke's formula as follows:

$LD_{50} = \sqrt{a \times b}$

Where *a* is the highest dose at which no death occurred in the second phase and *b* is the least dosage at which death occurred in the second phase. The extract was classified using the LD_{50}. In each case, the LD50 value is expressed as the weight of chemical administered per kilogram body weight of the animal and it states the test animal used and route of exposure or administration; e.g., LD50 (oral, rat) - 5 mg/kg, LD50 (skin, rabbit) - 5 mg/kg. So, the example "LD50 (oral, rat) 5 mg/kg" means that 5 milligrams of that chemical for every 1 kilogram body weight of the rat, when administered in one dose by mouth, causes the death of 50 % of the test group.

3.4 Sub chronic toxicity study

The subchronic toxicity test was performed following the protocol described by the OECD guideline 408 for testing chemicals. Twenty rats of both sexes were randomly assigned into four groups: a control group and three treatment groups. The *V.payos* aqueous extract was administered orally on a daily basis for 90 days at single doses of 100, 400, and 800 mg/kg, while the control group received only distilled water.

The extract was freshly prepared on a daily basis. The rats were weighed twice a week. Visual observations for mortality, behavioral pattern (salivation, lethargy, sleep and fur), changes in physical appearance, injury, pain and signs of illness were conducted once daily during that period. The first day of dosing was considered D_0 whereas the day of sacrifice was designated D_{90}.

3.5 Mortality and Clinical Signs

During the 90 day period, all animals were observed on a daily basis for likely clinical signs and mortality patterns once before dosing and after dosing.

3.6 Gross pathology and microscopic examination

On day 90 of the dosing period, the animals were starved for 24 hours. On day 91 all animals were sacrificed. After cardiac puncture, the rat organs were excised and examined microscopically.

Principal vital organs (liver and kidney) were preserved in a fixation medium of 10 % buffered formalin for histopathological study. The tissues were dehydrated using various concentrations of alcohol (70 %, 90 %) and a final bath of 100 % alcohol to ensure total elimination of moisture. Tissues were embedded in paraffin blocks for the process of infiltration and sectioned at approximately 3-5 μm thickness. Staining of tissues was performed using iron hematoxylin and eosin. Stained sections were examined for histological changes using a Leitz Flouvert microscope at X10 and X40 (Leitz, Germany) and representative images were captured with a camera fitted onto the microscope. The pictures were analyzed using a Future Win Joe software for windows. Histopathological analysis was done at the department of Anatomy, University of Zimbabwe.

3.7 Preparation of serum samples

Blood samples were collected via cardiac puncture into sterilized blood collecting tubes for biochemical and haematological analyses, respectively. Approximately 5 mls of whole blood was collected and allowed to stand for 30 minutes to ensure complete clotting. The clotted blood was centrifuged at a speed of 3000rpm for 15 minutes. Serum was transferred into Eppendorf tubes and stored at -4 - -8 °C for 24 hours before analysis.

3.8 Serum Biochemistry

All blood biochemical parameters were determined by automated photometric systems at the Veterinary Haematology Laboratory, Faculty of Veterinary Science, University of Zimbabwe using a Mindray Chemistry analyzer (BS-120, China). The parameters which were determined included total protein (TP), albumin (Alb), Alkaline phosphatase (ALP), aspartate phosphatase (AST), alanine transferase (ALT) and total bilirubin (Tbil). The manufacturer's instructions for each biochemical parameter were strictly adhered to in the course of the study.

3.9 Statistical Analysis

The results of the study are expressed as the mean ± S. E. M. The results were analysed using Graph Pad Prism version 6 software. Comparison in all the groups was made using one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison test. A 95% level of significance ($p \leq 0.05$) was used in the analysis.

CHAPTER FOUR

RESULTS

4.1 Acute toxicity studies

Effect of plant extract on acute toxicity studies

The *V.payos* aqueous extract at a dose of 5000 mg/kg had no adverse effect on the behavioural responses of the treated rats up to 14 days of observation. Physical observations indicated no signs of changes in the fur, skin, eyes mucous membrane, tremous, behaviour patterns, salivation, and diarrhoea of the rats as shown in **Tables 1** and **2** for the first and second phases of toxicity testing respectively. There was no mortality observed at the tested dose

There was no mortality observed in the first and second phase of acute toxicity study as shown in **Table 3.** The LD_{50} of this plant was therefore estimated to be more than 5000 mg/kg.

Table 4.1: Phase I clinical manifestations.

Sr. No	Response	Animals			
		Before Treatment	**After Treatment (10 mg/kg)**	**After Treatment (100 mg/kg)**	**After Treatment (1000 mg/kg)**
1	Mortality	Absent	Absent	Absent	Absent
2	Alertness	Normal	Normal	Normal	Normal
3	Sleep	Normal	Normal	Normal	Normal
4	Tremors	Absent	Absent	Absent	Absent
5	Pain response	Normal	Normal	Normal	Normal
6	Touch response	Normal	Normal	Normal	Normal
7	Lethargy	Absent	Absent	Absent	Absent
8	Gait	Normal	Normal	Normal	Normal
9	Circling	Absent	Absent	Absent	Absent
10	Salivation	Normal	Normal	Normal	Normal
11	Lacrimation	Absent	Absent	Absent	Absent
12	Fur	Normal	Normal	Normal	Normal
13	Convulsions	Absent	Absent	Absent	Absent
14	Slow response to stimuli	Absent	Absent	Absent	Absent
15	Food Consumption	Normal	Normal	Normal	Normal
16	Diarrhoea	Absent	Absent	Absent	Absent

Table 4.2: Phase II clinical manifestations.

Sr. No	Response	Animals			
		Before Treatment	**After Treatment (1500 mg/kg)**	**After Treatment (3000 mg/kg)**	**After Treatment (5000 mg/kg)**
1	Mortality	Absent	Absent	Absent	Absent
2	Alertness	Normal	Normal	Normal	Normal
3	Sleep	Normal	Normal	Normal	Normal
4	Tremors	Absent	Absent	Absent	Absent
5	Pain response	Normal	Normal	Normal	Normal
6	Touch response	Normal	Normal	Normal	Normal
7	Lethargy	Absent	Absent	Absent	Absent
8	Gait	Normal	Normal	Normal	Normal
9	Circling	Absent	Absent	Absent	Absent
10	Salivation	Normal	Normal	Normal	Normal
11	Lacrimation	Absent	Absent	Absent	Absent
12	Fur	Normal	Normal	Normal	Normal
13	Convulsions	Absent	Absent	Absent	Absent
14	Slow response to stimuli	Absent	Absent	Absent	Absent
15	Food Consumption	Normal	Normal	Normal	Normal
16	Diarrhoea	Absent	Absent	Absent	Absent

Table 4.3: Result of Oral Acute toxicity studies of aqueous extract of *Vitex payos* fruits on Sprague Dawley Rats

Dose mg/kg	Number of animals used	Number of dead animals	% Mortality
	Phase 1		
10	3	0	0
100	3	0	0
1000	3	0	0
	Phase 11		
1500	1	0	0
3000	1	0	0
5000	1	0	0

4.2 Subchronic Toxicity studies

Effect of plant extract on subchronic toxicity.

4.2.1 Serological parameters

From the present study it was seen that there was no significant change in the haematological and biochemical parameters in the *V. payos* extract treated rats compared to the normal control rats.

4.2.1.1 Total Protein

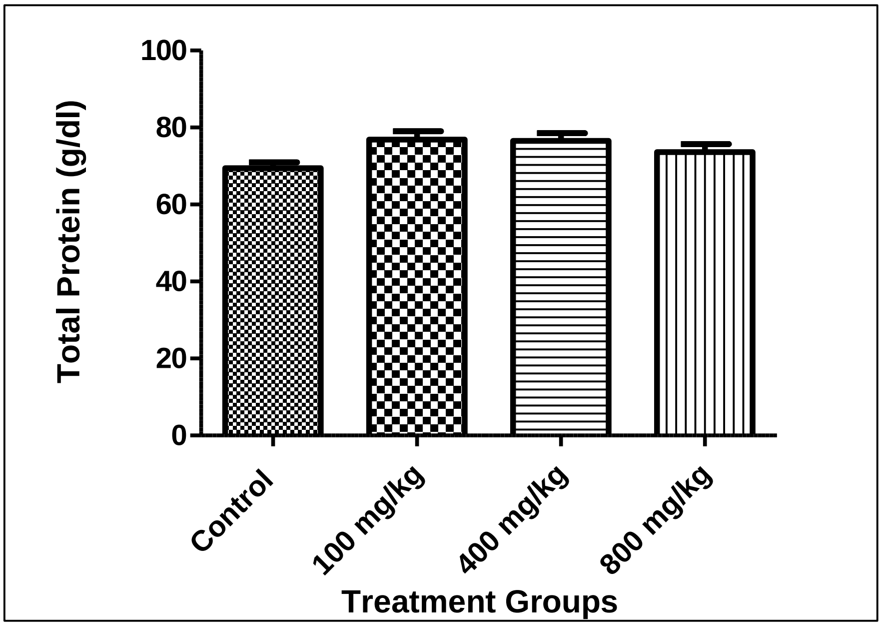


Figure 4.1: Mean total protein concentrations

The mean concentrations for total protein were 69.5, 76.9, 76.6 and 73.6 g/dl for the control and treatment groups of rats treated with 100, 400 and 800 mg/kg extract respectively. As shown in Figure 1, rats treated with 100 mg/kg dose of *V. payos* extract had the highest total protein level followed by rats treated with 400 mg/kg and 800mg/kg of extract, and the control rats respectively. There were no statistically significant differences between the control and treatment groups ($p \geq 0.05$).

4.2.1.2 Albumin

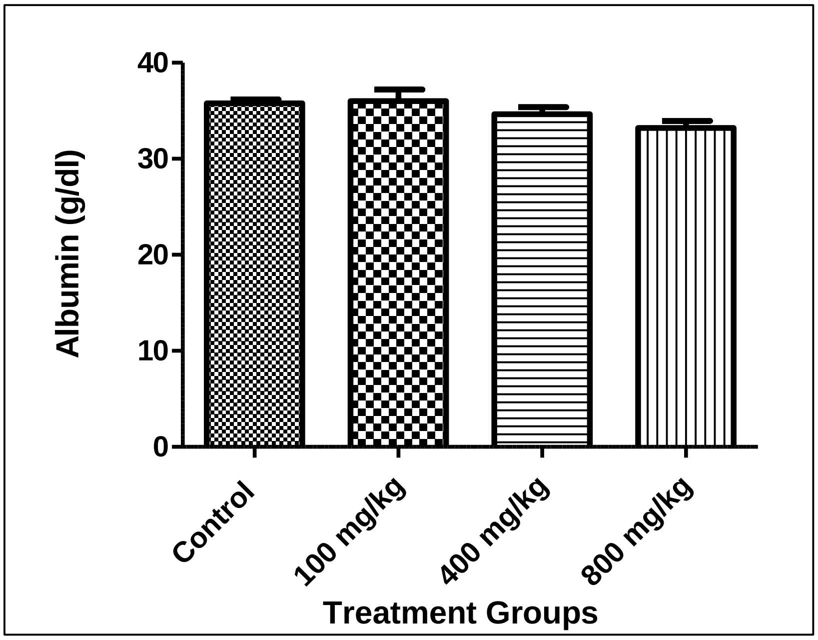


Figure 4.2: Mean Albumin concentrations

The mean concentrations for albumin were 35.8, 36.0, 34.6 and 33.2 g/dl for the control and treatment groups of 100, 400 and 800 mg/kg of extract respectively. As illustrated in Figure 2, rats treated with 100 mg/kg had the highest albumin content followed by the control then 400 mg/kg and 800 mg/kg treated rats respectively. No statistically significant differences in albumin content were observed between the control and treated rats ($p \geq 0.05$).

4.2.1.3 Alkaline phosphatase

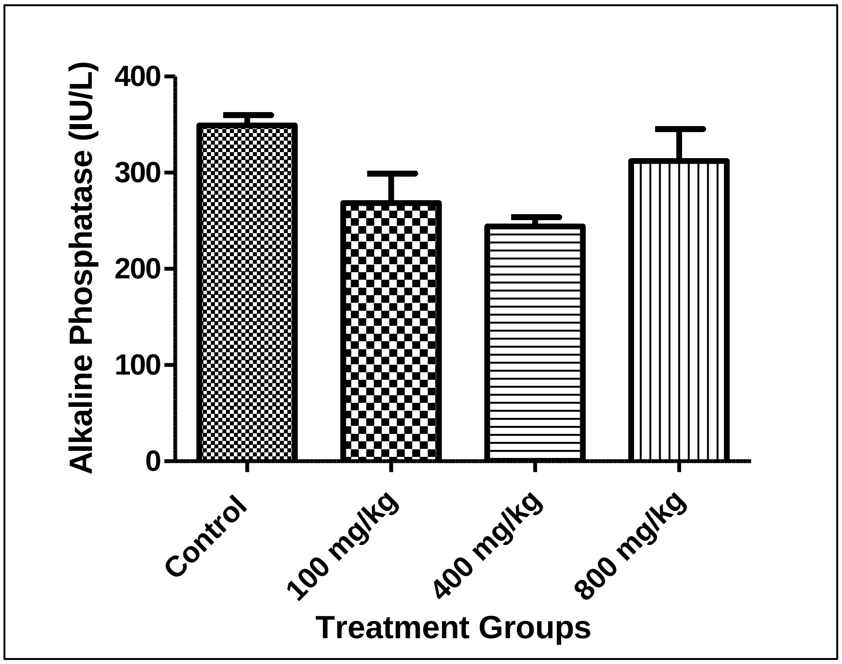


Figure 4.3: Mean alkaline phosphatase enzyme concentrations

The mean concentrations for alkaline phosphatase were 349.3, 268.6, 244.1 and 312.1 IU/L for the control and treatment groups 1-3 respectively. As shown in Figure 3, the control rats had the highest alkaline phosphatase concentration followed by 800 mg/kg, 100 mg/kg and 400 mg/kg treated rats respectively. There were no statistically significant differences between the control and treatment groups ($p \geq 0.05$).

4.2.1.4 Aspartate transaminase

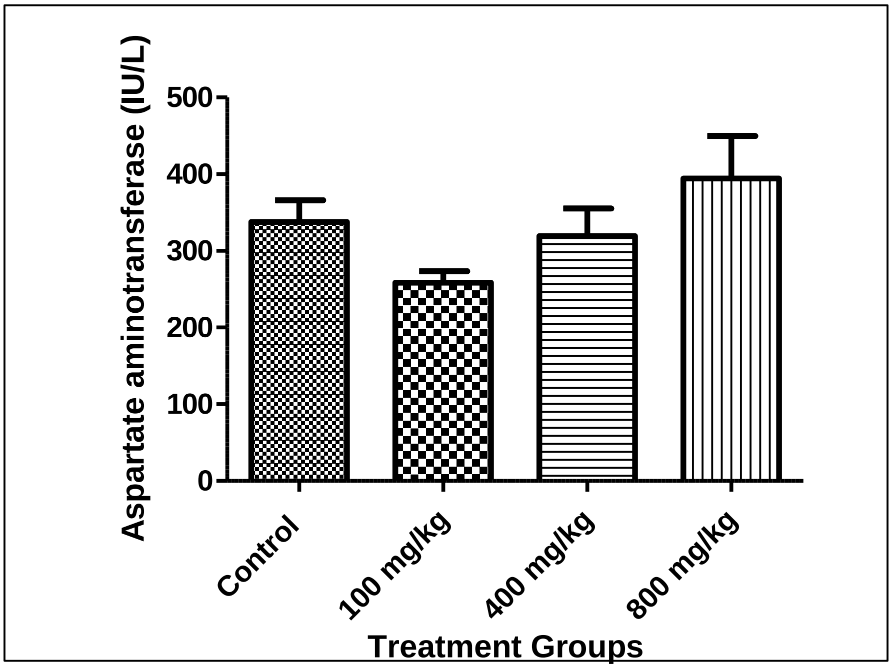


Figure 4.4: Mean aspartate aminotransferase enzyme concentrations

The mean concentrations for aspartate aminotransferase were 337.5, 258.4, 319.4 and 394.5 IU/L for the control and treatment groups 1-3 respectively. No statistically significant difference was observed between the control and treatment groups ($p \geq 0.05$). Rats treated with 800 mg/kg had the highest AST content followed by the control rats then rats treated with 400 mg/kg and 100 mg/kg of extract respectively. There were no significant differences between rats treated with *V.payos* aqueous extract at the doses of 100, 400 and 800 mg/kg body weight when compared with those of the control group as shown in Figure 4.

4.2.1.5 Alanine aminotransferase

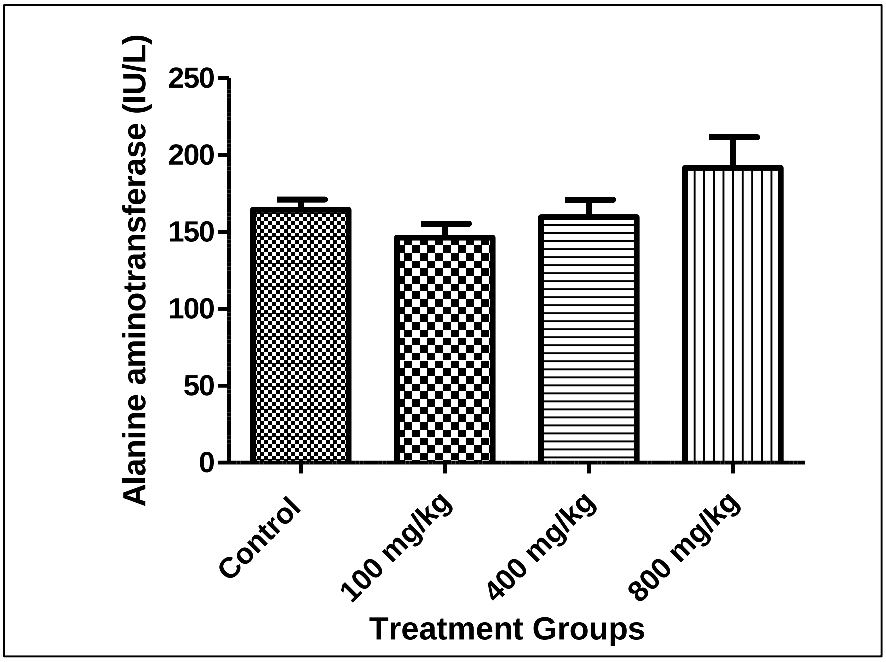


Figure 4.5: Mean Alanine aminotransferase enzyme concentrations

The mean concentrations for alanine aminotransferase were 164.6, 146.3, 159.7 and 191.7 IU/L for the control and treatment groups 1-3 respectively, as shown in Figure 5. There were no significant differences between rats treated with *V.payos* aqueous extract at the doses of 100, 400 and 800 mg/kg body weight when compared with those of the control group ($p \geq 0.05$).

4.2.1.6 Total bilirubin

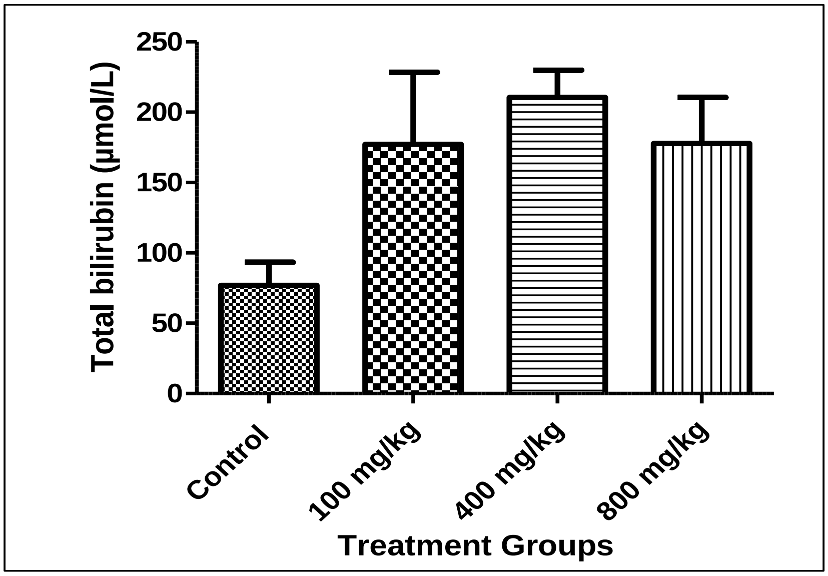


Figure 4.6: Mean total bilirubin concentrations

The mean concentrations for total bilirubin were 77.0, 177.2, 210.4 and 177.7 µmol/L for the control and treatment groups of rats treated with 100, 400 and 800 mg/kg extract respectively. No statistically significant differences were observed between the control and treated ($p \geq 0.05$). As shown in Figure 6, rats treated with 400 mg/kg had the highest total bilirubin level followed by 800 mg/kg, 100 mg/kg treated rats and the control rats respectively. There were no statistically significant differences between the control and treatment groups ($p \geq 0.05$).

4.2.2 Histopathology

Gross examination for histopathological evaluations of the various organs stained with haematoxylin and eosin revealed no significant differences between rats in the control group and rats treated with different concentrations of *V.payos* extract.

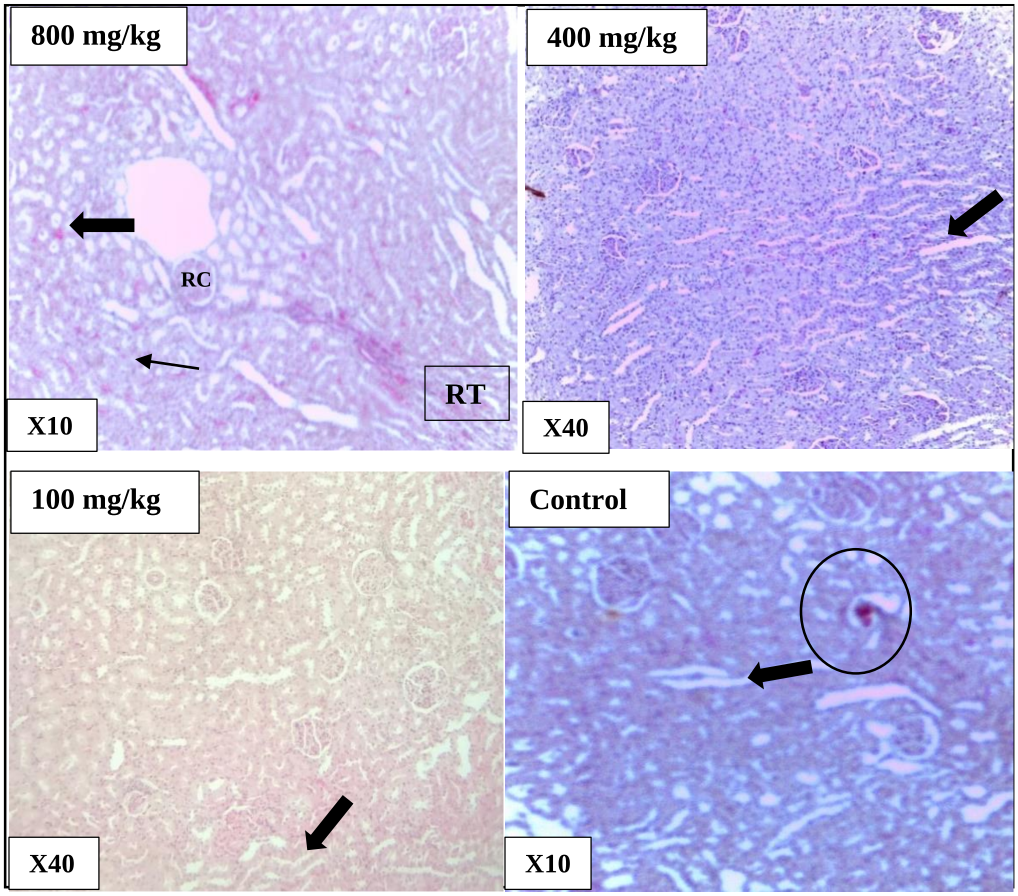


Figure 4.7: Photomicrographs of rat kidney sections in control and treated groups.

The photomicrographs shown above are to compare kidneys of normal rats (control) and rats treated with 100 mg/kg, 400 mg/kg and 800 mg/kg of *Vitex payos* extract. All photomicrographs showed same features. The 800 mg/kg photomicrograph also illustrates a typical normal renal capsule (RC) and renal tubules (RT). The 400 mg/kg shows disruption of tubular structure (arrow). The 100 mg/kg photomicrograph shows dilated renal tubules. The control is showing a contracted glomeruli (circle) and disruption of tubular order.

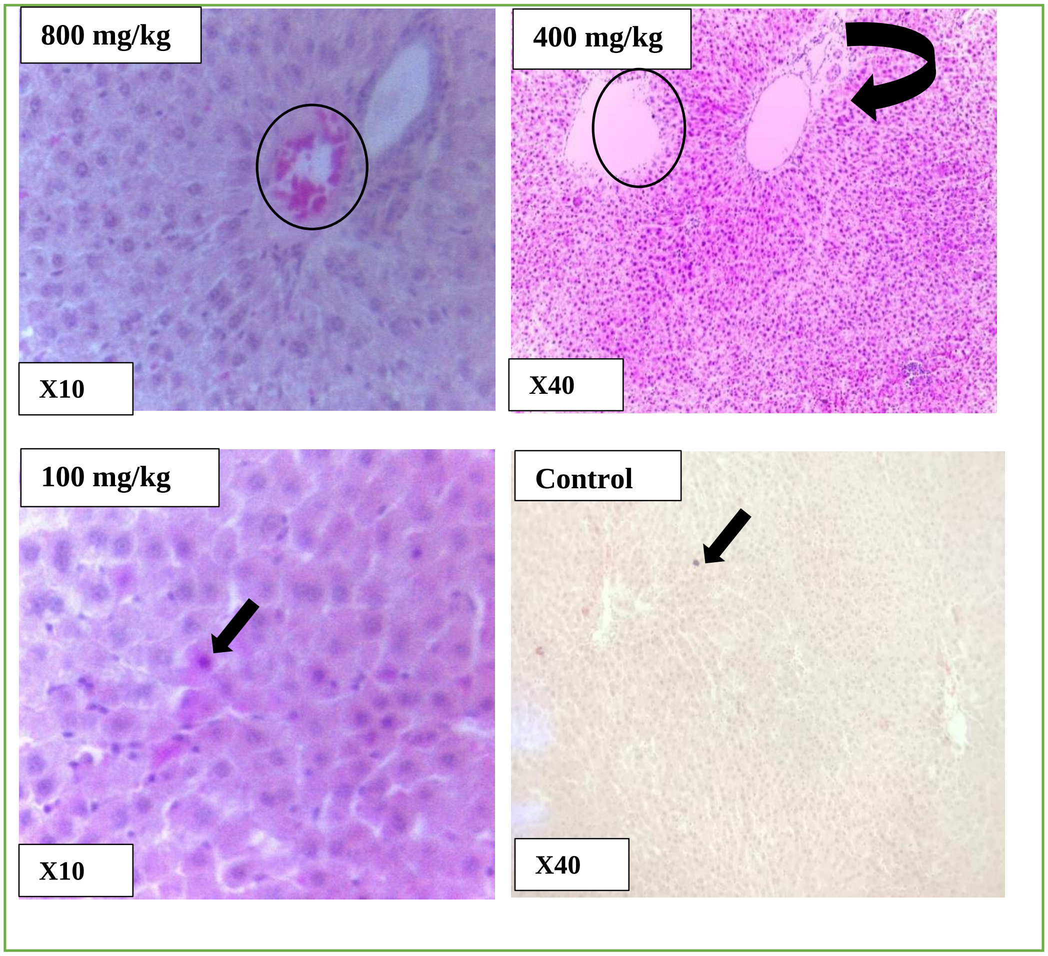


Figure 4.8: Photomicrographs of rat liver sections in control and treated groups.

The photomicrographs shown above are to compare livers of normal rats (control) and rats treated with 100 mg/kg, 400 mg/kg and 800 mg/kg of *Vitex payos* extract. The photomicrographs had similar features. The 800 mg/kg photomicrograph illustrates congested vessels shown by pink staining. The 400 mg/kg shows the portal vein (CV) with radiating cords of hepatocytes and portal triad (curved arrow). The 100 mg/kg photomicrograph shows degenerating cells (arrow). The control is showing eosinophilic degeneration (arrow).

CHAPTER FIVE

DISCUSSION

Natural products, such as medicinal plants have been the basis for the treatment of various ailments for centuries. The initial step in screening natural products for their pharmacological activity involves, assessment and evaluation of the toxic characteristics of the natural products fraction, extract, or compound. Regardless of the beneficial effects of *Vitex payos*, detailed knowledge about the toxicology of this plant is lacking.

The principal aim of evaluating the safety of any medicinal plant is to identify the nature and significance of adverse effects as well as to establish the exposure level at which this effect is observed (Ibrahim *et al*., 2016). The results of the acute toxicity study indicate that the aqueous extract of *Vitex payos* administered through oral route to Sprague Dawley rats at 10, 100, 1000, 1500, 3000 and 5000 mg/kg did not produce any signs of toxicity and death in the animals. The acute toxicity study showed that the LD_{50} of the extract was greater than 5000 mg/kg. According to OECD criteria under its Globally Harmonised Classification System (GHS) for chemical substances and mixtures, substances with LD_{50} > 2000–5000 mg/kg are categorised as unclassified or category 5 (Organization for Economic Development, 2001). This suggests that the oral LD_{50} of the plant being greater than 2000 mg/kg may be safe.

The crude extract of *Vitex payos* extract was assigned class 5 status (LD_{50} > 5000 mg/kg), which was the lowest toxicity class according to the chemical labelling and classification of acute systemic toxicity recommended by OECD. According to the study by Ibrahim *et al*., 2016 substances with LD_{50} values higher than 5000 mg/kg by oral route are regarded as being safe or practically nontoxic.

It has been reported that many medicinal plants may be toxic to both humans and animals. It should then be emphasized that the traditional use of any plant for medicinal purposes, guarantees the safety of the plant. Furthermore, the data of the acute and subchronic toxicity studies on medicinal plants or preparations derived from them should be obtained in order to increase their safety to humans, particularly for use in the development of pharmaceuticals (Ambadasu *et al.*, 2013).

During the evaluation of the toxic characteristics of medicinal plants, the determination of LD_{50} is usually an initial step to be conducted. Data from the acute toxicity study may (a) serve as the basis for classification and labelling; (b) provide initial information on the mode of toxic action of a substance; (c) help arrive at a dose of a new compound; (d) help in dose determination in animal studies; and (e) help determine LD_{50} values that provide many indices of potential types of drug activity (Ambadasu *et al*., 2013). If a high dose e.g, 5000 mg/kg is found to be survivable, no further acute testing will be conducted (Harbone, 1993).

Subchronic studies assess the undesirable effects of continuous or repeated exposure of plant extracts or compounds over a portion of the average life span of experimental animals. Specifically, they provide information on target organ toxicity and are designed to identify no observable adverse effects. Subchronic evaluation can also help to determine appropriate dose regimens for long-term studies. In our subchronic toxicity study, there were no significant adverse effects detected on body weight, mortality, clinical chemistry, gross pathology or histopathology.

The role of liver and kidney functions are important for survival of animals. Their functionality can be measured by serum biochemical analysis, which are crucial in the toxicological evaluation of xenobiotics (Bariweni *et al*., 2018). Serum liver function tests provide information about the status of the liver.

The liver enzymes (aminotransferases; ALT and AST) describe its cellular integrity, while albumin and total protein levels describe its functionality. AST and ALT are principally produced by the liver cells and any assault to the liver may lead to an increase in the serum level of these enzymes (Adedapo et al., 2004). High levels of liver enzymes are signs of hepatocellular toxicity (Brautbar and Williams, 2002), whereas a decrease may indicate enzyme inhibition. However, ALT is the most sensitive marker of liver damage or toxicity since AST is also found in abundance in kidneys, testes, cardiac and skeletal muscle (Bariweni *et al*., 2018).

Tukey's multiple comparison test, also called Tukey’s honestly significant difference (HSD) test is one of several tests that can be used to determine which means amongst a set of means differ from the rest. The correct way to do the analysis is to use a one-way analysis of variance (ANOVA) to evaluate whether there is any evidence that the means of the populations’ differ. The test compares the difference between each pair of means with appropriate adjustment for the multiple testing.

The values of biochemical parameters of liver and kidney functions in this study are shown in Table 4. No significant difference was observed in AST, ALT, and ALP of rats treated with *V.payos* aqueous extract at the doses of 100, 400 and 800 mg/kg body weight when compared with those of the control group. A reduction in serum levels of total proteins, bilirubin and albumin depicts reduced synthetic function, which is evident in liver damage or diseases. An increase in these parameters is usually seen in cancerous conditions, or following high protein diet. Statistical analysis was done between the control animals and all *V. payos* treated animals in order to either accept or reject the null hypothesis- *Vitex payos* has a toxic effect on animals when administered in high concentrations or when administered over a long period of time.

Our study detected no significant difference between the control and all *V.payos* treated groups therefore we reject the null hypothesis

All biochemical parameters investigated did not show a trend. Serum enzymes did not increase with an increase in extract dose instead the values were inconsistent. The control rats would have results similar to the highest dosed rats of even higher results. The Tukey's multiple comparison test compared all groups against each other in pairs. The results were clear that there was no statistical difference between the control rats and the extract treated rats.

The reference ranges of AST, ALT, ALP, were 50 to 150, 10 to 40 and 30 to 130 and IU/L respectively (Sharp and La Regina, 1998). The reference ranges for total bilirubin, albumin and total protein are 4 to 16 µmol/L, 3 to 5 and 5 to 7 g/dL. The functionality of the liver was assessed by the serum total protein, bilirubin and albumin.

Administration of extracts of *V. payos* for 90 days produced no clinical signs for toxicity or mortality. The treated rats did not show any significant alteration in food or water consumption during the 90-day treatment period when compared to a control group in this study. Furthermore, the diets and water were well accepted by the rats treated with *V. payos* extract suggesting the extract did not possibly cause any alterations in carbohydrate, protein or fat metabolism in these treated animals. Also, the *V. payos* extract did not interfere with the nutritional benefits, such as weight gain and stability of the appetite, which are expected for animals that are continually supplied with food and water *ad libitum*. The extract is safe by oral route in relation to its folkloric practice in the administration of medicinal herbs through oral route to treat patients. Therefore, *V. payos* extract can be considered as non-toxic up to 5000 mg/kg.

The microscopic examinations of the organs of rats treated with various doses of *V. payos* extract did not show any changes in colour compared with control group rats' organs. Hypertrophy is the enlargement of an organ due to the increased size of the constituent cells. It is the first hand indication of toxicity of chemicals or biological substances. In the study, no hypertrophy of organs was observed amongst all the groups studied. In addition, both organs from the extract treated and control rats showed some alteration in cell structure and unfavourable effects when viewed under the light microscope. Some of the photomicrographs showed different colours due to stain distribution. The stain loses concentration as you stain more slides hence the slides with fresh stain produced vivid colored slides under the microscope. Another reason for different coloured slides is the filter used in order to get a clear image. Photography through the microscope is complicated by a wide spectrum of unexpected color shifts and changes that affect how the image is rendered on the film emulsion or digital electronic image capturing device. These unexpected imaging results are caused by a number of factors ranging from incorrect color balance between the light source and the film emulsion to optical artifacts (Abramowitz and Davidson, 2018).

CHAPTER SIX

CONCLUSION

The oral LD_{50} of aqueous extract of *V. payos* has been shown to be greater than 5000 mg/kg and is generally considered safe. It did not cause death or any abnormal changes in both acute and subchronic toxicity studies.

With knowledge of these findings, we may conclude that *V. payos* extract is not toxic in all the doses studied. The extract did not produce any toxic signs or evident symptoms at acute and subchronic oral toxicity. The *V. payos* extract did not cause any lethality or produce any significant histopathological signs or serum chemical alterations. The results suggest promising alternatives for exploring therapeutic and pharmaceutical interest in *V. payos* extract with a reduction of possible adverse effects.

Biochemical and histopathological investigations clearly demonstrates that single oral administration up to 5000 mg/kg in acute toxicity study and daily oral administration of the *V.payos* for 90 days up to 800 mg/kg in subchronic toxicity study caused no significant adverse changes in the liver and kidney

The study provided significant data on the toxicity profile of *V.payos* which may be valuable in the clinical study of the medicinal plant. Thus, the aqueous extract of *V.payos* may be used as one of the ingredients in traditional formulation or any other pharmaceutical formulations. The study on the interaction of this plant with other herbs in multi herbal formulation may be useful for assessment of efficacy of the formulations.

Therefore the Null hypothesis is rejected.

CHAPTER SEVEN

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CHAPTER EIGHT

APPENDIX

Acute toxicity assay

<u>Phase I</u>

Treatment group	Initial weight (g)	Weight in 1 day (g)	Weight in 7 days (g)	Weight 14 days (g)
Control				
A	193.3	195.6	223.7	224.2
B	198.2	200.9	227.1	223.6
C	190	194.2	215.4	220.4
Group 1 (10 mg/kg)				
A	207	209.2	232.1	233.6
B	174.6	176.8	204.9	209.4
C	180.8	184.7	194.7	199.7
Group 2 (100 mg/kg)				
A	168.7	169.9	181.5	185.9
B	166.6	168.3	176.6	179.8
C	172.5	178.9	189.3	190.7
Group 3 (1000 mg/kg)				
A				
B	249.3	252.9	259.3	266.7
C	247.8	249.7	253.4	253.4
	246.6	248.8	251.3	253.9

Phase II

Treatment group	**Initial weight (g)**	**Weight after 24 hours (g)**	**Weight after 48 hours (g)**
A (1500 mg/kg)	230.2	233.5	235.7
B (3000 mg/kg)	249.1	250.3	251.6
C (5000 mg/kg)	236.2	237.1	239.7

1. **Water extract concentration**

Beaker	**Mass of sample (g)**	**Volume of distilled water (ml)**	**Concentration (mg/ml)**
1	1	10	100
2	1.2	10	120
3	1.2	10	120

Concentration = $\frac{mass}{volume}$ **x 1000**

Mean concentration = $\frac{100+120+120}{3}$

= 113 mg/ml*

2. **Water extract concentration**

Beaker	**Mass of sample (g)**	**Volume of distilled water (mg/ml)**	**Concentration (mg/ml)**
1	1.3	10	130
2	0.8	10	80
3	0.7	10	70

Concentration = $\frac{mass}{volume}$**x 1000**

Mean concentration = $\frac{130+80+70}{3}$

= 93.3 mg/ml*

***The obtained concentrations were used as the stock solution for the smaller extract doses (100 mg/ml and 400 mg/ml).**

Preparation of Extract doses

Each rat was administered with 1 ml of extract dose according to its body weight

Control group was administered with 1ml distilled water.

Group 1 – 1000 g rat gets 100 mg/kg

Group 2 – 1000 g rat gets 400 mg/kg

Group 3 – 1000 g rat gets 800 mg/kg

8 g of freeze dried pulp was dissolved in 40ml distilled water (for 800mg/ml group)

Calculation = $\frac{8}{40}$ x 100

= 200 mg/ml **STOCK SOLUTION**

Calculation of how much extract pipetted from stock solution for each rat according to its body weight and concentration was done as follows:

Group 1.

If rat A weighs 127 g

$$\frac{127\ x\ 100}{1000} = 12.7\ mg$$

127 g rat gets 12.7 mg

$M_1\ V_1 = M_2V_2$

M_1 = Stock solution

M_2= Mass

V_1= Volume of extract

V_2= Final Volume

200 mg/ml x V_1 = 12.7 mg x 1 ml

V_1 = 0.0635 ml / **63.5 µl**

Group 2

If rat A weighs 112.9 g

$$\frac{112.9\ x\ 400}{1000} = 45.16\ mg$$

$M_1 V_1 = M_2 V_2$

200 mg/ml x V_1 = 45.16 mg x 1 ml

V_1 = 0.2258 ml / **225.8 µl**

Group 3

If rat A weighs 157.3 g

$$\frac{157.3\ x\ 800}{1000} = 125.8\ mg$$

$M_1 V_1 = M_2 V_2$

200 mg/ml x V_1 = 125.8 mg x 1 ml

V_1 = 0.2258 ml / **225.8 µl**

NB - Highlighted values were pipetted from stock solution and marked up to 1 ml using distilled water. The above formulae were used for individual rats in their respective groups.

Subchronic toxicity study

Sprague Dawley rats were weighed twice a week on Mondays and Thursdays

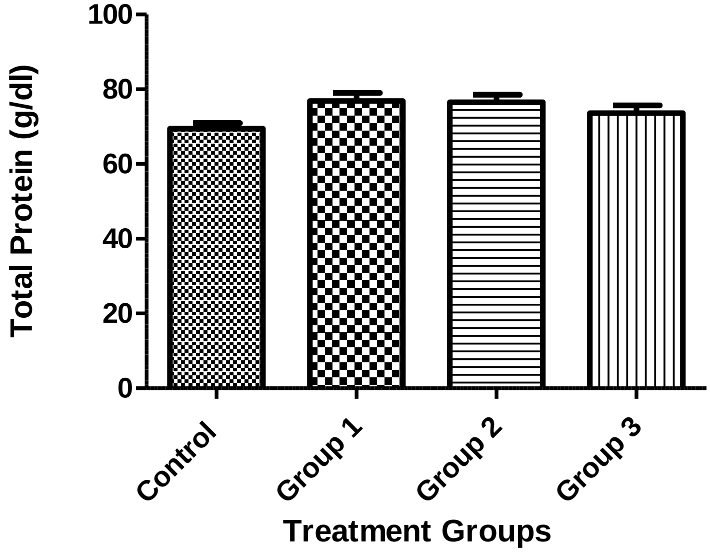
Group	Week 1	Week 1	Week 2	Week 2	Week 3	Week 3	Week 4	Week 4
C1	164	168.7	176.2	187.2	193.3	201	206.8	216.7
C2	182.3	197.4	199	200.3	210.8	217.4	224.4	231.4
C3	193	200.7	203.5	211.5	217.1	210.1	226.5	234.8
C4	175.2	180.5	189.6	192.3	196.7	203.6	211.2	216.7
C5	179.7	185.8	192.1	198.6	201.7	209.6	212.4	224.4
G1A	147.8	153.8	157.3	160.2	165.7	167.7	174.7	175.3
G1B	142	148.2	157.3	161.7	165.2	175.3	179.7	176.2
G1C	144.4	157.2	163.2	169.8	173	174.6	186.8	182.7
G1D	163.3	169.9	175.6	181.9	189.9	189.6	193.9	194.6
G1E	151.8	163.5	167.1	171.6	178	177.5	176.6	182.7
G2A	139.1	141.1	146.6	150.9	156.2	161.5	159.5	165.8
G2B	151.1	153.4	157.2	161.7	166.6	169.6	167.6	175.6
G2C	136.8	144.4	150.5	159	167.2	174.1	173.7	182.6
G2D	129.8	132.4	137.5	141.3	148.2	152.5	156.8	165.6
G2E	139.4	142.2	145.2	151.8	157.1	158.9	157.1	164.6
G3A	167.8	171.3	173.9	181.1	185.3	200.7	217.2	222.2
G3B	128.9	136.8	148.4	153.7	158.7	171.2	185.1	191.4
G3C	165.8	169.1	174.5	189.4	195.4	222.4	225.1	229.6
G3D	129.9	137.5	139.6	143.4	158.7	189.2	199.4	211.7
G3E	153.9	155.1	160	167.9	172.5	197.5	217.2	216.3

Group	Week 5	Week 5	Week 6	Week 6	Week 7	Week 7	Week 8	Week 8
C1	222.5	230.3	236.6	239.1	243.6	244.7	256.8	258.4
C2	237.9	242.6	243.6	246.5	255.8	257.1	273.1	280.6
C3	242.1	242.7	250.5	257.8	263.4	261.3	279.1	284.5
C4	222.4	220.3	218.4	224.8	227.6	235.3	253.2	254.6
C5	228.9	230	231.2	233.5	236.3	239.5	255.5	252.7
G1A	176.5	182.7	190.9	194.5	194.9	191.3	201.2	203.1
G1B	181.6	182.6	185.5	195.2	190.6	186.4	202.5	202
G1C	183.6	186.2	188.6	190.8	195.3	198.2	206.5	208.8
G1D	197.9	192.2	192.7	193.6	197.8	201.4	213.3	214.9
G1E	186.2	187.3	190.1	190.9	193.2	204.6	216.7	207.2
G2A	170.9	171.5	173.5	172.7	182.4	173.2	193.7	195.9
G2B	179.1	172.3	181.4	184.3	195.3	191.6	196.2	198.9
G2C	184.5	181.7	188.5	181.8	193.7	185.7	202.4	200.5
G2D	165.3	165.4	162.5	165.5	173.9	169.7	181.2	181.3
G2E	170.1	164.6	172.2	175	182.4	172.5	184.1	182.6
G3A	234.6	236.2	244.9	240.7	252.5	254.7	268.4	260.9
G3B	201.8	203.3	206.9	203.3	213.3	214.8	231.5	226.8
G3C	218.3	223.3	231.5	235.2	241.7	249	263.7	263.6
G3D	236.7	240.4	245.6	247.2	247.8	248.2	253.6	251.6
G3E	231.7	232.5	241.9	248.5	252.8	265.2	266.4	268.3

Group	Week 9	Week 9	Week 10	Week 10	Week 11	Week 11	Week 12	Week 12
C1	260.7	265.4	273.2	270.7	271.3	274.5	277.2	285.7
C2	278.6	282.3	288.6	289.3	294.5	297.7	303.2	312.3
C3	286.3	290	294.4	304.4	296.1	301.3	306.4	311.8
C4	258.3	261.8	268.7	277.2	274.7	278.8	281.9	289.6
C5	258.6	259.2	267.2	273.1	270.4	267.9	274.7	281.6
G1A	203.5	204.1	209.2	213.3	210.5	213.1	215.1	219.3
G1B	203.3	205.4	206.6	208.4	202.5	208.6	209.9	215.5
G1C	209.4	210.1	214.5	221.5	209.7	217.5	220.8	225.8
G1D	212.6	214.8	215.4	217.5	219.6	222.8	224.7	231.5
G1E	205.1	209.6	211.2	215.8	212.7	230.7	234.2	236.4
G2A	195.3	197.5	199.3	199.6	199.3	208.6	209.4	211.6
G2B	202.3	205.6	208.7	209.6	210.2	216.2	211.7	219.8
G2C	202	203.7	203.6	208.9	205.6	214.2	210.6	216.4
G2D	183.2	183.8	184.2	192.2	186.3	189.4	193.4	200.9
G2E	182.4	185.6	182.7	190.1	193.6	192.5	197.7	203.2
G3A	273.3	276.4	281.6	283.9	286.8	293.2	291.4	302.3
G3B	235.4	236.9	240.6	245.5	243.7	252.5	258.5	265.9
G3C	274.2	276.8	278.9	285.7	284.8	287.3	298.2	302.2
G3D	261.5	263	264.7	266.7	265.4	279.4	273.9	286.3
G3E	271.3	273.2	275.9	279.7	283.6	291.9	297.4	304.3

Table 4: Serum biochemistry in Sprague Dawley rats treated with 100, 400 and 800 mg/kg body weight of *V.payos* fruits aqueous extract for 90 days as compared to the control group.

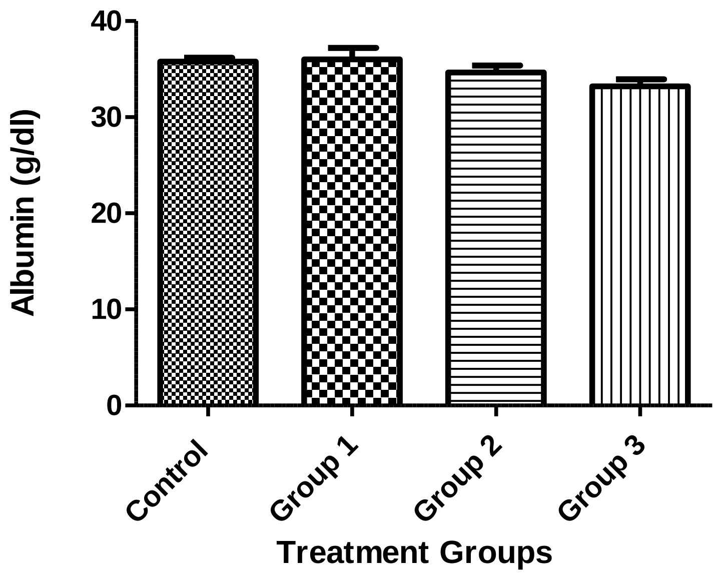
	g/dl		IU/L			μmol/L	
	TP	**Alb**	**ALP**	**AST**	**ALT**	**Tbil**	**Comment**
C1	68.1	35.7	372.8	258.7	153.4	63.7	Haemolysed
C2	72.1	37.1	375.6	433.7	165.0	90.8	Haemolysed
C3	66.4	34.7	326.3	339.0	178.5	44.5	Haemolysed
C4	66.8	35.9	344.6	344.4	179.7	51.8	Haemolysed
C5	74.0	35.5	327.0	311.8	146.2	134.4	Haemolysed
G1A	74.8	33.6	249.1	273.5	130.8	243.1	Haemolysed
G1B	79.9	32.5	195.2	263.8	120.7	297.4	Haemolysed
G1C	71.1	37.8	329.4	261.3	167.3	45.9	Haemolysed
G1D	75.3	38.0	351.4	201.0	152.2	63.8	Haemolysed
G1E	83.4	38.1	217.8	292.3	160.7	235.9	Haemolysed
G2A	83.3	36.7	272.1	321.8	151.6	220.4	Haemolysed
G2B	77.3	35.0	220.6	256.8	156.9	226.6	Haemolysed
G2C	76.4	35.5	237.9	332.2	203.0	183.3	Haemolysed
G2D	71.6	33.5	260.8	241.9	140.9	153.8	Haemolysed
G2E	74.2	32.5	229.0	444.5	146.3	268.0	Haemolysed
G3A	76.7	34.1	345.6	237.8	158.2	106.8	Haemolysed
G3B	78.0	34.4	348.3	332.3	168.4	213.4	Haemolysed
G3C	74.1	31.6	179.5	533.0	259.6	289.2	Haemolysed
G3D	66.1	31.3	331.3	363.0	157.2	136.9	Haemolysed
G3E	73.3	34.7	356.0	506.6	215.1	142.3	Haemolysed



Mean total protein concentrations

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
C vs G1	-7.420	3.835	No	ns	-15.25 to 0.4079
C vs G2	-7.080	3.659	No	ns	-14.91 to 0.7479
C vs G3	-4.160	2.150	No	ns	-11.99 to 3.668
G1 vs G2	0.3400	0.1757	No	ns	-7.488 to 8.168
G1 vs G3	3.260	1.685	No	ns	-4.568 to 11.09
G2 vs G3	2.920	1.509	No	ns	-4.908 to 10.75

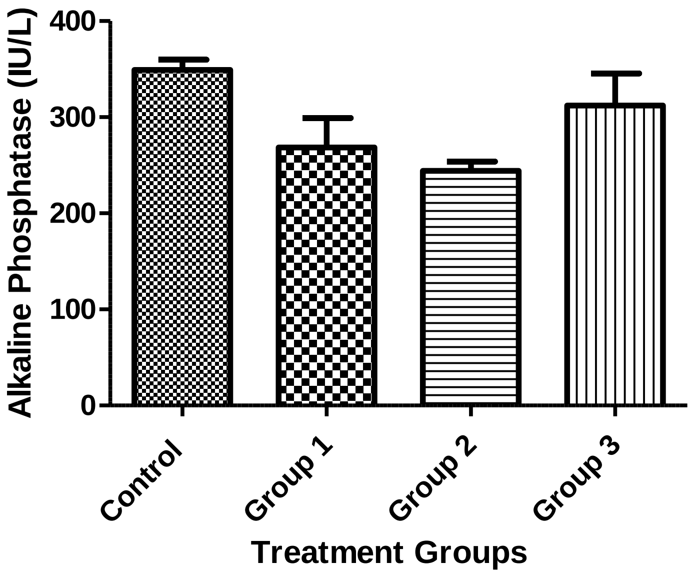
Statistical analysis of total protein



Mean albumin concentration

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
C vs G1	-0.2200	0.2670	No	ns	-3.554 to 3.114
C vs G2	1.140	1.383	No	ns	-2.194 to 4.474
C vs G3	2.560	3.107	No	ns	-0.7742 to 5.894
G1 vs G2	1.360	1.650	No	ns	-1.974 to 4.694
G1 vs G3	2.780	3.373	No	ns	-0.5542 to 6.114
G2 vs G3	1.420	1.723	No	ns	-1.914 to 4.754

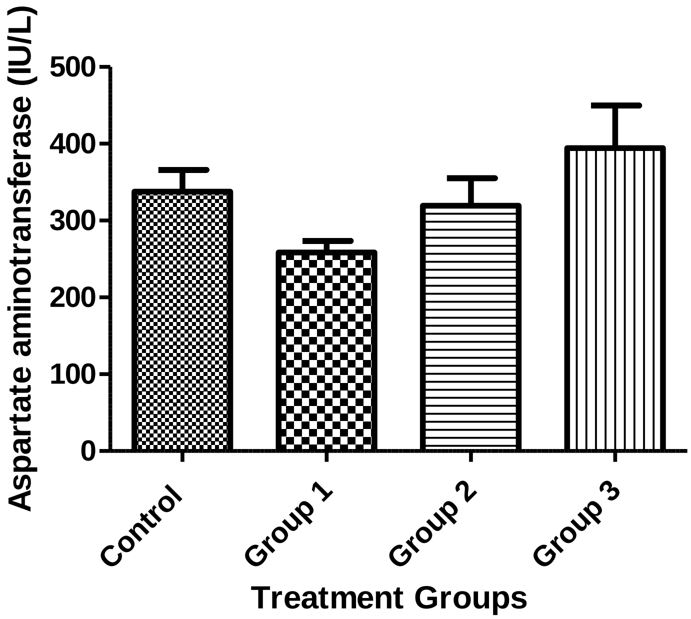
Statistical analysis of albumin



Mean ALP concentrations

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
C vs G1	80.68	3.387	No	ns	-15.69 to 177.1
C vs G2	105.2	4.416	Yes	*	8.810 to 201.6
C vs G3	37.12	1.558	No	ns	-59.25 to 133.5
G1 vs G2	24.50	1.029	No	ns	-71.87 to 120.9
G1 vs G3	-43.56	1.829	No	ns	-139.9 to 52.81
G2 vs G3	-68.06	2.857	No	ns	-164.4 to 28.31

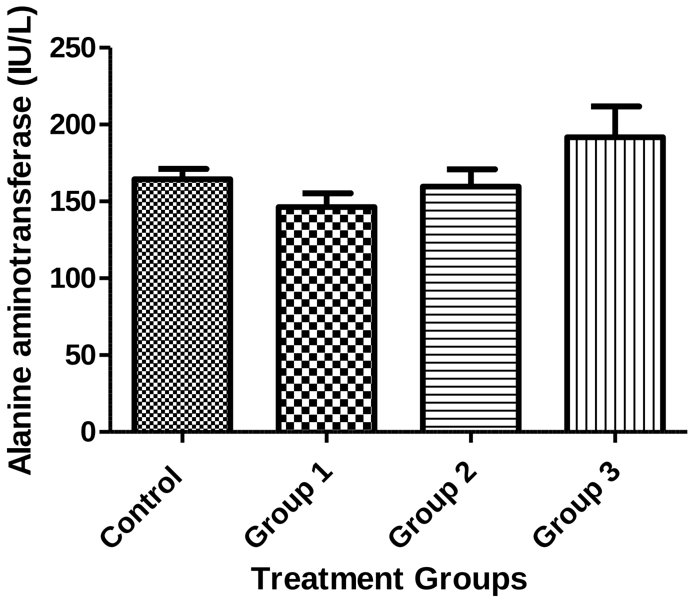
Statistical analysis of ALP



Mean AST concentrations

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
C vs G1	79.14	2.156	No	ns	-69.38 to 227.7
C vs G2	18.08	0.4925	No	ns	-130.4 to 166.6
C vs G3	-57.02	1.553	No	ns	-205.5 to 91.50
G1 vs G2	-61.06	1.663	No	ns	-209.6 to 87.46
G1 vs G3	-136.2	3.709	No	ns	-284.7 to 12.36
G2 vs G3	-75.10	2.046	No	ns	-223.6 to 73.42

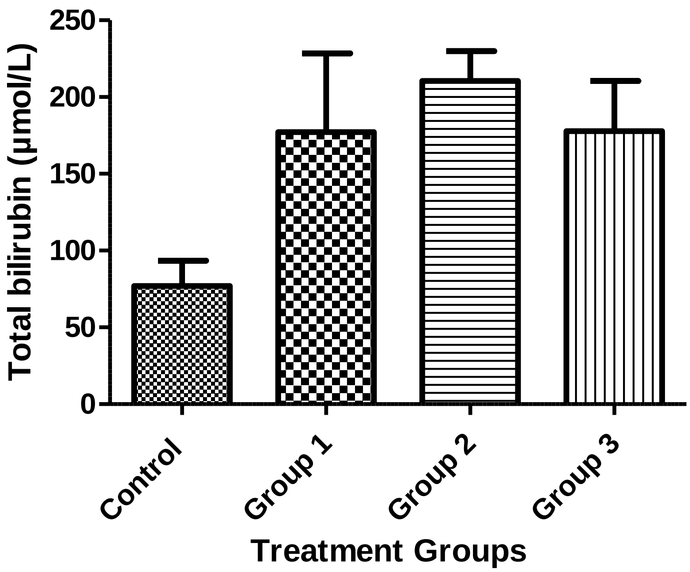
Statistical analysis of AST



Mean ALT concentrations

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
C vs G1	18.22	1.432	No	ns	-33.27 to 69.71
C vs G2	4.820	0.3787	No	ns	-46.67 to 56.31
C vs G3	-27.14	2.132	No	ns	-78.63 to 24.35
G1 vs G2	-13.40	1.053	No	ns	-64.89 to 38.09
G1 vs G3	-45.36	3.564	No	ns	-96.85 to 6.135
G2 vs G3	-31.96	2.511	No	ns	-83.45 to 19.53

Statistical analysis of ALT

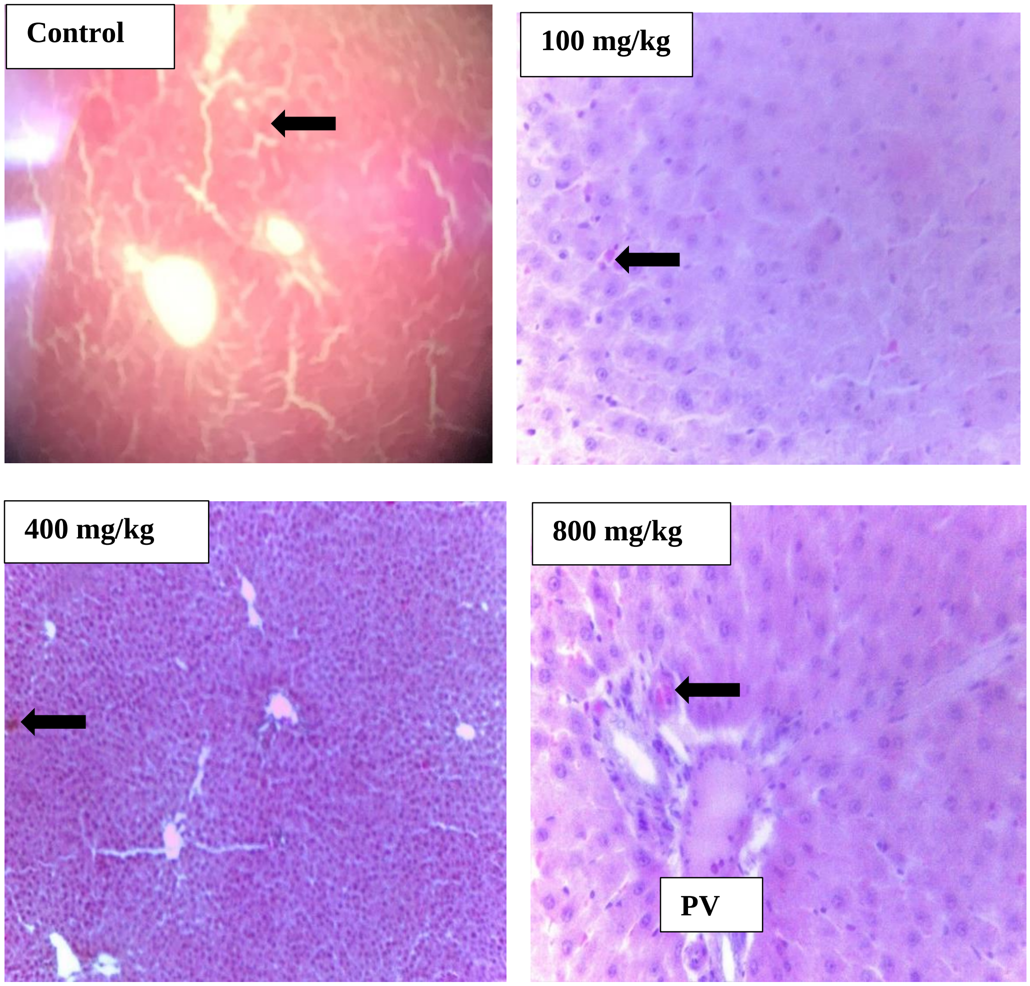


Mean total bilirubin concentrations

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
C vs G1	-100.2	3.039	No	Ns	-233.6 to 33.21
C vs G2	-133.4	4.046	No	Ns	-266.8 to 0.01432
C vs G3	-100.7	3.054	No	Ns	-234.1 to 32.71
G1 vs G2	-33.20	1.007	No	Ns	-166.6 to 100.2
G1 vs G3	-0.5000	0.01517	No	Ns	-133.9 to 132.9
G2 vs G3	32.70	0.9918	No	Ns	-100.7 to 166.1

Statistical analysis of total bilirubin

Photomicrographs of rat liver sections



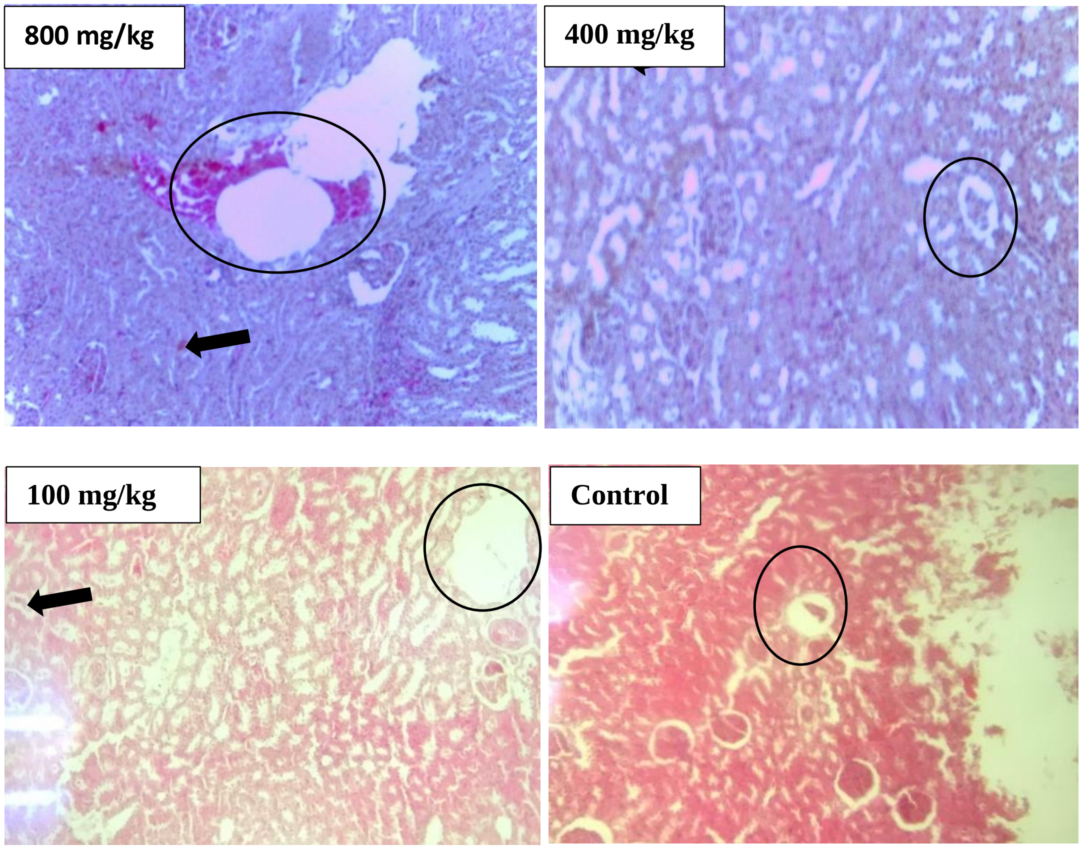
a) The control photomicrograph is showing eosinophilic degeneration (arrow).

b) The 100 mg/kg photomicrograph is showing mild degeneration represented by eosinophilic bodies (arrow).

c) The 400 mg/kg photomicrograph is showing early stage degeneration (arrow). Cells with no nucleus.

d) The 800 mg/kg photomicrograph is showing the portal vein (PV) with radiating cords of hepatocytes and portal triad. Eosinophilic bodies (arrow).

Photomicrographs of rat kidney sections



a) The 800 mg/kg photomicrograph is showing integrity of vessel dilated (circle), cells with highly eosinophilic cytoplasm (arrow).

b) The 400 mg/kg photomicrograph is showing disrupted glomeruli (circle), dilated renal tubules (arrow).

c) The 100 mg/kg photomicrograph is showing dilated vein (circle). Contracted glomeruli (arrow).

d) The control photomicrograph is showing contracted glomeruli (circle).