ACUTE TOXICITY AND HISTOLOGICAL SUDY OF NALONGO'S RENAL/HEPATIC DISORDER POTION IN RATS

By

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DECLARATION

I, GEORGE F.0. ONYANGO hereby declare that the research work entitled:

"Acute toxicity and histological study of Nalongo's renal/hepatic disorder herbal potion in rats"

Is the result of my own investigation and research and that it has not been submitted in part or in full for any other degree or to any other university. Where use of the work of others was made, it is duly acknowledged in the text.

Student: George F. O. Onyango
Signature.....G.F.O.Onyango 03/03/2014.....

Supervisor: Mr. Ezeonwumelu Joseph
Signature.....

DEDICATION

I dedicate this work to ALMIGHTY GOD, to my mother Mrs. Rose Onyango, to my dear wife Jacqueline and to my siblings Charles and Catherine Onyango.

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First and foremost I thank God Almighty for being my shepherd on the way of my academic achievements and scientific discovery; it is through His love and graces that I have made it this far. I also want to thank the following people whose input made this work achievable:

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Table of Contents

LIST OF TABLES:

ABSTRACT

The history of use of herbal medicine dates back to more than 4000 years. A wide range of plants have been utilized for treatment of multiple disorders of the liver. The utilization has been as extracts of single plants and also compound preparations of more than one-plant type. Herbal medicine has been categorically employed for a variety of medical problems and modem trends have helped in extracting the active ingredients which have been classed into many chemical groups such as alkaloids, glycosides, resins and tannins. Africa is faced by a ''double burden'' of communicable and non communicable diseases (NCD), the latter of which includes kidney diseases. Kidney disease means the kidneys are damaged and can no longer remove wastes and extra water from the blood as they should. Kidney damage is most often caused by diabetes or high blood pressure, whereas liver disease refers to a type of damage to or disease of the liver, such as is seen in hepatitis (inflammation of the liver), alcoholic liver disease (due to excess consumption of alcohol), fatty liver disease (hepatic stenosis), non-alcoholic fatty liver disease (associated with obesity), liver cancer, among many others. Liver and kidney problems afflict many people in the world at large and particularly in Uganda the death toll due to kidney problems seems to be on the rise according to data published by the WHO in 2011, due to lack of specialized systems to diagnose and manage patients with kidney and liver problems early. Herbal remedies have proven beneficial to several patients with liver and kidney disorders, yet the main stream medical practice does not put much emphasis on such findings. This was an experimental study that involved the testing of Nalongo's renal/hepatic disorder potion for acute and sub-chronic toxicity on rats and histological effects on various organs; liver, kidney, heart, intestine, lungs. The study was conducted at Mbarara and Bushenyi Districts. The extract tested positive for terpenoids, saponins, tannins, flavonoids, diterpenes and phenolic compounds. There was no mortality observed during acute and sub-chronic toxicity studies, suggesting a relatively high safety margin of the extract on experimental animals. However, some pathological changes were noted in tissues of the heart, liver and intestines of the experimental animals. As such, recommendations are that, more work needs to be carried out to ascertain the efficacy and dosing regimen of the potion, whilst machinery needs to be put in place by the relevant authorities to moderate use of herbal products in Uganda.

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CHAPTER ONE

1.0 INTRODUCTION

1.1Background

The liver is the largest solid organ in the body and it has a number of functions like producing bile for digestion, storing glucose and converting it back into energy, producing blood clotting factors, producing amino acids, manufacturing cholesterol required for fat transport, and metabolizing medicine in the body. Liver diseases occur when there is a disturbance of the liver functions.

The kidney is a gland situated on either side of the vertebral column in the upper posterior abdominal cavity of the human being. Its main function is secretion of urine, which flows into the ureters. Healthy kidneys process approximately 200 quarts of blood every 24 hours and, from that, produce about 2 quarts of urine (VanDeGrift, 2013)

Kidney disease means the kidneys are damaged and can no longer remove wastes and extra water from the blood as they should. Kidney damage is most often caused by diabetes or high blood pressure, whereas liver disease refers to a type of damage to or disease of the liver, such as is seen in hepatitis (inflammation of the liver), alcoholic liver disease (due to excess consumption of alcohol), fatty liver disease (hepatic stenosis), non-alcoholic fatty liver disease (associated with obesity), liver cancer, among many others.

Africa is faced by a ''double burden'' of communicable and non communicable diseases (NCD), the latter of which includes kidney diseases. In Sub- Saharan Africa the spectrum of renal diseases differs from that of developed countries, with infectious diseases contributing an enormous portion to this problem (Naicker, 2003). With lack of specialized systems to diagnose and manage patients with kidney problems early, many progress to end stage renal disease (ESRD). In Uganda 717,970 people were estimated to have renal disease by the end of 2004 (US

Census Bureau). According to the latest WHO data published in April 2011 kidney disease deaths in Uganda reached 3,463 or 0.93% of total deaths.

The history of use of herbal medicine dates back to more than 4000 years. A wide range of plants have been utilized for treatment of multiple disorders of the liver. The utilization has been as extracts of single plants and also compound preparations of more than one-plant type. Herbal medicine has been categorically employed for a variety of medical problems and modem trends have helped in extracting the active ingredients which have been classed into many chemical groups such as alkaloids, glycosides, resins and tannins (De Smet, 1997).

Liver disease in Greece-Arab era was recognized only by the symptomatic jaundice. This was classed as green and black depending on the colour which was imparted to the exposed parts of the patients and was treated by different types of herbs obtained from indigenous plants with promising healing effects (Samuelson, 1989).

Certain botanicals have been used by herbalists and indigenous healers worldwide for the prevention and treatment of liver disease, such as; *Silybum marianum* (milk thistle), *Picrorhiza kurroa* (kutkin), *Curcuma longa* (turmeric), *Camellia sinensis* (green tea), *Chelidonium majus* (greater celandine), *Glycyrrhiza glabra* (licorice), and *Allium sativa* (garlic).

Silybum marianum.....the active constituents are flavonolignans, including silybin, silydinin, and silychristine, collectively known as silymarin. Silybin is the component with the most biological activity. Silymarin has been remarkably used in the treatment of Amanita mushroom poisoning, which possess two powerful hepatotoxins, amanitin & phalloidin, it has also been effectively used for its hepatoprotective properties in chronic active hepatitis, chronic alcoholic liver disease, and liver cirrhosis (Salmi, Sarna; 1982, Buzzeli, Moscarella, Giust, *et al.*; 1993, Feher, Deak, Muzes; 1989). The typical adult dosage is 240 to 900 mg/day in two or three divided doses (Awang; 1993, Rumyantseva; 1991, Brown; 1994).

Picrorhiza kurroa (kutkin).....the most active constituents are the iridoid glycoside picrosides I, II, III and kutkoside, known collectively as kutkin. It is an important herb in

traditional Ayurvedic medicine and has been used to treat liver and bronchial problems (Stuppner, Wagner; 1989), viral hepatitis (Vaidya, Antarkar, Doshi, *et al.*; 1996). The usual adult dosage is 400 to 1500 mg/day (Kapoor; 1990)

Curcuma longa (turmeric).....the most well researched active ingredient is cur cumin (diferuloymethane), which has been used for its anti-inflammatory and hepatoprotective properties (Leung; 1980), as well as for its choler tic activities (Ammon, Wahl; 1991).

Camellia sinensis (green tea)..... Green tea has been found to reduce or prevent the growth of hepatic neoplasm in rodents (Cao, Xu, Chen, *et al.*, 1996). Single doses of decaffeinated green tea solids up to 4.5 g/day (equal to 45 cups of tea) have been well tolerated by humans (Sai, Kai, Umemura, *et al.*, 1998).

Glycyrrhiza glabra (licorice)..... Glycyrrhiza has been shown to have a direct hepatoprotective effect. Glycyrrhiza flavonoids provided protection to hepatocytes exposed to carbon tetrachloride (Wang, Han, 1993). The primary active constituent of Glycyrrhiza, as it relates to hepatic disorders, is the triterpene glycoside glycyrrhizin (also known as glycyrrhizic acid or glycyrrhetinic acid...Tyler, Brady, Robbers, 1988). Oral dosing of a Glycyrrhiza extract (750mg, equivalent to 7.5g of crude herb) may also be of benefit in acute and chronic hepatitis (Xianshi, Huiming, Lizhuang, *et al.*, 1984)

The problem of drug use in liver disease is that it is responsible for handling of all foreign chemicals (including drugs). In diseased state, the power of liver to perform the important function of degrading toxic substances is much reduced and the increased half life of the chemicals could not only damage the liver itself but could also affect other important organs of the body such as kidneys. Use of herbal medicine in several trials has revealed promising results in acute and chronic liver disease (Gilani, Thyagarajan; 1998)

Renal fibrosis is a common consequence of progressive renal diseases. In nearly all cases, the extent of fibrotic lesions strongly correlates with disease severity and eventual progression to end-stage renal disease (ESRD) (Yu, Noble, Border; 2002) Modern day therapy of renal disease includes dietary protein restriction, blood pressure control, angiotensin-converting enzyme

inhibitors (ACEIs), and angiotensin receptor blockers (Becker, Perkovic, Hewitson; 2001). However, little is known about the renoprotective effects of herbal medicine and as such more research needs to be conducted into the healing effects of herbal medicine, some of which are a mainstay in both ancient and modern Chinese medicine. To date, a substantial body of evidence suggests that some Chinese herbs possess a range of important pharmacological properties in retarding progressive chronic kidney disease (CKD).

The effects of Astragalus on the reduction of proteinuria and hyperlipidaemia as well as on immune modulation and renoprotection have been studied in patients and experimental animals. Most progressive renal diseases are accompanied by proteinuria and there are several studies on the use of Traditional Chinese Medicine (TCM) in its treatment (Zhang, Liang, Zhang, Lu; 2001)

Disorders of lipid metabolism (*Hyperlipidaemia of nephrotic syndrome*) may also enhance the renal injury in CKD. Most studies have shown that Astragalus alone or combined with other herbs such as Angelica, Ligusticum *(Ligusticum wallichii)* or Schizandrae not only improves oedema, increases serum albumin level and lowers proteinuria, but also reduces serum total cholesterol, triglyceride, low-density lipoprotein (LDL) and very low density lipoprotein levels (Wang, Li, Pan, Zou, Li, Zhang, *et al.*, 2002)

Tripterygium wilfordii Hook F (TwHF), a perennial vine-like member of the Celastraceae plant family has had a large number of compounds which have been identified from the extract, including diterpenoids, such as triptolide and tripdiolide, alkaloid glycosides, beta-sitosterol and tritoquinones. However, triptolide appears to be the major active component (Gu, Chen, Brandwein, McAlpine, Burres; 1995).

Many ingredients with different pharmacological actions have been isolated from the root of rhubarb, among which anthraquinone is the most important, and has been shown to slow the progression of chronic renal disease.

Despite their reported beneficial uses, herbal medicines have been reported to cause unexpected and occasional fatal outcomes. These can be attributed to the accidental use of the wrong plant

species, effects due to adulterated herbal ingredients, contamination with hazardous or toxic substances, administration of wrong quantities which could result in an overdose of any one of the active constituents of the plant or concomitant use with other medicines, and as such much research is yet to be done, but these plants appear to have a place in the treatment of liver poisoning, viral hepatitis, and cirrhosis of the liver.

Nonalcoholic fatty liver disease (NAFLD) is a frequent and potentially progressive chronic liver disease that occurs in subjects who do not abuse alcohol. NAFLD is often associated with obesity, metabolic syndrome and insulin resistance and its more aggressive form, nonalcoholic steatohepatitis (NASH) is a major cause of cryptogenic cirrhosis.
Current management of NAFLD/NASH is largely conservative and includes diet regimen, aerobic exercise, and interventions towards the associated metabolic abnormalities. The main concern is therefore to decrease liver steatosis and its progression toward steatohepatitis and fibrosis, and the risk of "cryptogenic" cirrhosis. Among the most promising medications, weight reducing drugs, insulin sensitizers and lipid-lowering agents, antioxidants, bile salts, co-factors increasing the mitochondrial transport of fatty acids are being considered. Among them, thiazolidinediones are the most promising drug family that act by activating PPARgamma nuclear receptors and by regulating both microsomal and peroxisomal lipid oxidative pathways

Some orthodox medication used in the treatment of acute renal failure are; dopamine, furosemide, mannitol, and thiazide

Modern lifesaving drugs are beyond the reach of nearly three-quarters of the third world population. As part of strategy to reduce the financial burden in developing countries, it would be prudent that the use of traditional medicine be considered. If such common herbal medicines can be made available for a plethora of liver and kidney disorders, it would help the common poor man who cannot afford costly therapies, which too have a limited role.

1.2 Problem statement

Liver and kidney problems afflict many people in the world at large and particularly in Uganda the death toll due to kidney problems seems to be on the rise according to data published by the WHO in 2011, due to lack of specialized systems to diagnose and manage patients with kidney and liver problems early. Herbal remedies have proven beneficial to several patients with liver and kidney disorders, yet the main stream medical practice does not put much emphasis on such findings. These findings could greatly bridge the gap between the poor patients and the high cost of modern medicine and overall improve the management of these disorders at affordable costs to the public at large, and reduce disease progression if diagnosed at an early stage.

1.3 Objectives of the study

1.3.1 General objective

The general objective of this study is to ascertain the effect of Nalongo's renal/hepatic disorder herbal potion on selected rat organs.

1.3.2 Specific objectives

The specific objectives of this study include the following:

1. To determine the phytochemical constituents of Nalongo's renal/hepatic disorder potion qualitatively.

2. To determine acute toxicity of Nalongo's renal/hepatic disorder potion in rats.

3. To determine the histological effect of Nalongo's renal/hepatic disorder potion on rats' liver, kidney, heart, stomach, intestine, lungs, brain, testes and/or ovaries.

1.4 Justification of the study

Herbal products seem to have beneficial effects in treatment of kidney and liver problems. They have been in use since the early days of mankind, even though there is insufficient scientific evidence to explain and validate their apparent use, safety and efficacy.

National surveillance systems to monitor and report adverse effects as well as to evaluate the efficacy of herbal treatments are not well developed in the third world countries and virtually non-existent in Uganda. It is thus important to carry out this study in order to establish acute toxicity effects of Nalongo's renal/hepatic disorder potion in rats, and to determine the histological effects on various organs such as the lungs, liver, kidney, heart and intestines.

1.4.1 Structure of organs and functions

1.4.1.1 THE LUNG

The lung is the essential respiratory organ in many air-breathing animals, including most tetrapods, a few fish and a few snails. Their principal function is to transport oxygen from the atmosphere into the bloodstream, and to release carbon dioxide from the bloodstream into the atmosphere. A large surface area is needed for this exchange of gases which is accomplished by the mosaic of specialised cells that form millions of tiny, exceptionally thin walled air sacs called alveoli.

The lungs are a pair of cone-shaped bodies that occupy the thorax. The mediastinum, the cavity containing the heart, separates the two lungs. The left and right lungs are divided by fissures into two and three lobes respectively. Each lobe of the lung is further divided into bronchopulmonary segments (each with a tertiary bronchus), which are further divided into lobules (each with a terminal bronchiole). Blood vessels, lymphatic vessels and nerves penetrate each lobe.

Each lung has the following superficial features:

- The apex and base which identify the top and bottom of the lung respectively.
- The costal surface of each lung borders the ribs (front and back)
- On the medial (mediastinum) surface where each lung faces the other lung, the bronchi, blood vessels, and lymphatic vessels enter the lung at the hilus.

The pleura is a double layered membrane consisting of an inner pulmonary (visceral) pleura, which surrounds each lung, and an outer parietal pleura, which lines the thoracic cavity, is filled with pleural fluid, a lubricant secreted by the pleura. (www.cliffsnotes.com; 2014)

1.4.1.2 THE LIVER

The liver is the largest internal organ of the body, weighing in at around 3 pounds. The liver performs many essential functions related to digestion, metabolism, immunity, and the storage of nutrients within the body. These functions make the liver a vital organ without which the tissues of the body would quickly die from lack of energy and nutrients.

In the body, the liver is situated under the diaphragm, and is further protected by the costal cartilage of the ribs. It occupies most of the right hypochondrium as well as part of the abdomen. It is for the most part covered by peritoneum and entirely by connective tissues. The upper surface of the organ fits nicely against the under surface (inferior aspect) of the diaphragm.

There are on the surface, four lobes: right, left, caudate and quadrate. The falciform ligament divides the liver into two main lobes, right and left, with the right lobe being the larger and is subdivided into the right lobe proper, the caudate lobe and the quadrate lobe.

The under surface of the liver, also known as the visceral surface, is more irregular in appearance than is the domed convex upper surface. This irregularity is caused by the fact that the inferior surface is in contact with:

- The lower esophagus
- The stomach and
- The right kidney and adrenal gland

The liver is essential for life, yet it can suffer extensive damage before malfunction becomes pronounced. It is made up of liver lobules (the functional units of the liver). Each lobule is constructed around a central vein that empties into the right and left hepatic veins, which then drain into the vena cava. The lobule is composed of cellular plates that radiate from the central vein. Each cellular plate is two cells thick and between the two cells are small bile canaliculi that empty into the terminal ducts.

Cirrhosis of the liver denotes chronic tissue degeneration in which cells are destroyed leading to the formation of fibrous scar tissue. As the cellular degeneration continues, blood, lymph, and bile channels within the liver become distorted and compressed, leading to intrahepatic congestion, portal hypertension and impaired liver function. The fibrous changes within the

organ cause it to become firmer and smaller. The surface however, becomes rough and bumpy because of the development of nodules on the surface of the organ. The nodules are regenerated hepatic cells. (www.ece.ncsu.edu/imaging/MedImg/SIMS; 2014)

1.4.1.3 THE KIDNEY

In human beings, the kidneys are paired. They are bean shaped and located between the levels of the last thoracic and third lumbar vertebrae. The right kidney is placed slightly lower than the left because of the presence of the liver.

The average size of the human kidney measures about 11 to 13cms in length, 5 to 7.5cms wide and 2.5cms thick. In adults, each kidney weighs about 150 grams and this corresponds to about 1% of the body weight. The medial border of the kidney is slightly concave and has a marked depression. A long section of the kidney shows two main regions- an outer reddish cortex and an inner pale coloured medulla.

The kidney functions to:

- Filter materials out of the blood and pass them out of the body as urine
- Regulate blood pressure and the levels of water, salts, and minerals in the body
- Produce hormones that control other body functions

Damage to the kidney can occur in people who have had diabetes for many years, particularly if the diabetes is not well controlled. (Ja Teline; 2010)

1.4.1.4 THE HEART

The heart is located in the center of the chest cavity slightly tilted towards the left. It is a hollow organ made up of cardiac muscle fibers. It is the pumping organ of the circulatory system, and has four chambers, the right and left auricles (or atria) and the right and left ventricles. A wall separates the right and left halves of the heart.

Auricles are thin-walled chambers that receive blood from the body. Each auricle passes the incoming blood to the ventricle of its own side. Ventricles are thick-walled and pump blood out of the heart.

The heart beats rhythmically throughout life. The periodic contraction and relaxation of the heart is called the heartbeat. The normal human heartbeat is 70-72 per minute. The human heart pumps about 5 liters of blood per minute, which can increase to about 20 liters per minute during strenuous exercise. (Ja Teline; 2010)

1.4.1.5 INTESTINES

The intestines are vital organs in the gastrointestinal tract of the digestive system. Their functions are to digest food and to enable the nutrients released from that food to enter into the bloodstream. The intestines consist of two major sub-divisions: the small intestine and the large intestine.

The small intestine is much smaller in diameter, but is much longer and more massive than the large intestine. The small intestine is about 1 inch in diameter and about10 feet long in a living body. It extends from the stomach to the large intestine and consists of three major regions: the duodenum, jejunum and ileum.

The large intestine is about $2^{1/2}$ inches in diameter and about 5 feet long in a living body. It receives fecal matter from the small intestine through the ileocecal sphincter. The smooth walls of the large intestine absorb water from fecal matter. These intestinal walls also absorb vitamins released from the fermentation of faeces by bacteria living in the large intestine.

Together the intestine take up most of the space within the abdominal cavity and are folded many time over to pack their enormous length into such a small area. The intestines are located interior to the stomach in the abdominal cavity. They are connected to the posterior wall of the abdomen by the mesentery, a thin vascular membrane. Blood vessels of the mesentery carry oxygenated blood to support the tissues of the intestines and carry nutrient-rich blood away from the intestines to feed the tissues of the body. (Tim Taylor; 2012)

CHAPTER TWO

2.0 LITERATURE REVIEW

Traditional medicine is the sum total of knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures that are used to maintain health, as well as to prevent, diagnose, improve or treat physical and mental illnesses. Herbal treatments are the most popular form of traditional medicine, and are highly lucrative in the international marketplace. Annual revenues in Western Europe reached US$ 5 billion in 2003-2004. In China, sales of products totaled US$ 14 billion in 2005. Herbal medicine revenue in Brazil was US$ 160 million in 2007. Natural remedies represent a $1.8 billion market in the United States, and a single herbal preparation, silymarin, which is used almost exclusively for liver diseases, amounts to $180 million in Germany alone (Breevort, 1996) In many developed countries, 70% to 80% of the population has used some form of alternative or complementary medicine (e.g. acupuncture). In some Asian and African countries, 80% of the population depends on traditional medicine for primary health care (WHO, 2008).

According to the 2007 National Health Interview (NHI) Survey in the United States, of >23,300 adults and >9400 children, 38% adults (up from 36% in 2002) and 12% children used CAM (Complementary and Alternative Medicine), with use greater among women, those with higher levels of education, those who were not poor, and those who lived in the western United States of America (Beaubrun, Gray; 2000). Liver disease causes approximately 2% of all deaths in England (Beynon, Hungerford, 2011).

In Africa, traditional healers and remedies made from plants play an important role in the health of millions of people. The relative ratios of traditional practitioners and university-trained doctors in relation to the whole population in African countries are revealing. In Ghana, for example, in the Kwahu district, there are 224 people for every traditional practitioner, compared to nearly 21,000 people for one university trained doctor. The same applies to Swaziland where the ratios are 110 people for every traditional healer and 10,000 people for every university trained doctor. It is estimated that the number of traditional practitioners in Tanzania is 30,000–40,000 in comparison to 600 medical doctors (Rukangira, 2002)

In East Africa, the Kenyan situation is not much different, and many communities especially from the poor rural areas still rely on herbal remedies. In addition, many Kenyans believe in the potency of herbal medicine, even when they can access modern medicine (Nagata *et al.*, 2011).

An herbal concoction from the wonder plant (*mtandamboo*) in Kiswahili, that was claimed to cure all ailments made news headlines from a small village called Loliondo in Tanzania. Studies carried out showed that extracts from the plant could cure various diseases. Further studies have shown the plant to contain ingredients that make it a good diuretic. Diuretics are drugs used to increase the frequency of urination to remove excess fluid in the body, a condition that comes with medical conditions such as congestive heart failure, liver and kidney disease (Kituyi, 2012).

Nigerian researchers from the Niger Delta University Bayelsa State and the University of Port Harcourt have found that that aqueous leaf extracts of scent leaf/basil (botanically called *Ocimum gratissimum*) affect the course of tubular repair after the onset of cisplatin-induced nephrotoxicity (kidney poisoning) in rats with accelerated recovery (Muanya, 2013)

More than 60% of Uganda's population depends on traditional medicine because it is accessible, affordable and culturally familiar. With an estimated traditional health practitioner for every 200-400 Ugandans (compared to 1 western trained doctor per 20,000), herbal medicine has long been used to manage a range of conditions, including liver and kidney problems (The cross-cultural foundation of Uganda, 2008). Among the herbal medicines is Nalongo's renal/hepatic disorder potion, of which there is lack of scientific evidence of its therapeutic/curative properties.

Despite the widespread use of herbal medicines globally and their reported benefits, they are not completely harmless. The indiscriminate, irresponsible or non-regulated use of several herbal medicines may put the health of their users at risk of toxicity (Nnorom, Osibanjo, Eleke; 2006). Also, there is limited scientific evidence from studies done to evaluate the safety and effectiveness of traditional medicine products and practices (WHO, 2008). Adverse reactions have been reported to herbal medicines when used alone (Oshikoya, Njokanma, Chukwura, Ojo; 2007) or concurrently with conventional or orthodox medicines (Klassen, Kipp, Jhangri, Rubaale; 2007) Despite the international diversity and adoption of traditional medicines (TM) in

different cultures and regions, there is no parallel advance in international standards and methods for its evaluation. National policies and regulations also are lacking for TM in many countries and where these are available; it is difficult to fully regulate TM products, practices and practitioners due to variations in definitions and categorizations of TM therapies. Lack of knowledge of how to sustain and preserve the plant populations and how to use them for medicinal purposes is a potential threat to TM sustenance.

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study design

This was an experimental study that involved the testing of Nalongo's renal/hepatic disorder potion for acute and sub-chronic toxicity on rats and histological effects on various organs; liver, kidney, heart, intestine, lungs.

3.2 Study setting

The study was conducted at Mbarara and Bushenyi Districts.

3.3 Study population

The animals (*Wistar* rats), were selected from a population of rats bred at Kampala International University-WC School of Pharmacy animal house. The study was conducted using a mixed population of young mature rats, weighing not less than 100 grams.

3.4 Sample size

Thirty six *Wistar* rats with a mixed population of male and female rats, weighing not less than 100 grams were used in the study. The sample size was determined according to the standard experimental methods used.

3.4.1 Inclusion criteria

Young, healthy mature adult male and female rats were used for the study.

3.4.2 Exclusion criteria

Any rat that displayed signs of ill health, pregnancy, having external wounds or a rough coat was eliminated from the study.

3.5 Sampling techniques

A random sampling technique in order to get a population of male and female rats was used.

3.6 Data collection procedures

3.6.1 Identification and acquisition of extract

The extract was identified and recommended for research and analysis by a lecturer at the School of Pharmacy, Kampala International University -Western Campus (Mr. Ezeonwumelu Joseph) and a sample was acquired from lady Nalongo in Mbarara, Western Uganda.

3.6.2 Preparation of the extract

A five litre gallon of the extract was portioned off into 5 one litre beakers and placed in a hot air oven for drying at 40°C, a process which took two weeks. At the end of the drying process, a semi-solid mass, collectively weighing 90.49g was realized and kept in the freezer in readiness for the experiment.

3.6.3 Phytochemical screening

Phytochemical screening was carried out to determine the different secondary metabolites present in the extract. Aqueous extracts of Nalongo's potion were prepared according to standard procedures as described by Trease and Evans (1983) and Kokale, Purohit & Gokhale (2010). Secondary metabolites tested for were: Saponins, Tannins, Flavonoids, Alkaloids, Cardiac glycosides, Terpenoids, Steroids, Reducing sugars, Phlabotannins, Phenolic compounds, Essential oils, Diterpenes, Amino acids.

3.6.3.1 Test for saponins

0.5g of sample was dissolved in 10ml of distilled water and heated to boil. Formation of a layer of foam indicates the presence of saponins.

3.6.3.2 Test for tannins

0.5g of sample was stirred with 10ml of distilled water and then filtered. 2 drops of ferric chloride reagent was added to the filtrate and observation of a blue-black, green or blue-green precipitate indicates the presence of tannins.

3.6.3.3 Test for flavonoids

0.2g of sample was dissolved in dilute NaOH and then HCL was added. A yellow solution that turns colourless will indicate the presence of flavonoids.

3.6.3.4 Test for Alkaloids

0.5g of sample was stirred in 5ml of 1% aqueous HCL in a steam bathe for 5 minutes. 2-3 drops of Mayer's reagent was then added by the side of the test tube. Observation of turbidity or a white precipitate indicates the presence of alkaloids,

3.6.3.5 Test for terpenoids

0.5g of sample was dissolved in 5ml of distilled water and filtered. The filtrate was then mixed with 2ml chloroform and conc. H_2SO_4 to form a layer. Observation of a reddish coloration at the interface indicates the presence of terpenoids.

3.6.3.6 Test for steroids

0.2g of sample was mixed with 2ml of conc.H_2SO_4. 2ml of acetic acid anhydride was then added to the mixture. A colour change from violet to blue or green indicates the presence of steroids.

3.6.3.7 Test for reducing sugars

0.2g of extract was shaken with 5ml of distilled water and filtered. The filtrate was boiled with 3 drops of Fehling's solution A and B for two minutes. Observation of an orange red precipitate indicates the presence of a reducing sugar.

3.6.3.8 Test for phenolic compounds (ferric chloride test)
0.2g of sample was dissolved in 5ml distilled water. Three drops of neutral 5% ferric chloride solution was then added. Observation of a dark green colour indicates the presence of phenolic compounds.

3.6.3.9 Test for essential oils
0.2g of sample was dissolved in 5ml distilled water and filtered. 2ml of the filtrate was then shaken with 0.2ml of 2M NaOH and then 2ml of 2M HCL was added. Formation of a white precipitate indicates the presence of essential oils.

3.6.3.10 Test for phlabotannins
0.2g of sample was dissolved in 5ml of distilled water and filtered. The filtrate was the boiled with 2ml 2% aqueous HCL solution. Observation of a red precipitate indicates presence of phlabotannins.

3.6.3.11 Detection of diterpenes
Extract was dissolved in distilled water and treated with 3-4 drops of copper acetate solution. Formation of an emerald green colour indicates the presence of triterpene.

3.6.3.12 Test for cardiac glycosides (Baljet's test)
About 2g of the extract was treated with picric acid solution and formation of an orange colour indicates the presence of cardiac glycosides.

3.6.3.13 Test for amino acids (Ninhydrin test)
To the extract, 0.25% $^{w}/_{v}$ Ninhydrin reagent was added and boiled for a few minutes. Formation of a blue colour indicates the presence of free amino acids.

3.7 Sources of chemicals and reagents

Equipment and chemical reagents used in phytochemical screening and toxicity studies were obtained from the school of pharmacy laboratory while those for histological preparation of tissues were obtained from the histology department of Mbarara University of Science and Technology –Mbarara, Uganda.

3.7.1 Equipment used in phytochemical screening and toxicity study

Beakers, Conical flasks, Test tubes, Weighing balance, Bunsen burner, Water bathe, Reagents, Refrigerator, Distilled water, Spatula, Feeding cannula and animal cage.

3.7.2 Chemical reagents used in phytochemical screening and toxicity study

5% Ferric chloride solution, dilute ammonia solution, concentrated sulphuric acid, 1% aqueous hydrochloric acid, Mayer's reagent, distilled water, chloroform, 2M sodium hydroxide, 2M dilute hydrochloric acid, 2% aqueous hydrochloric acid, acetic acid anhydride, Fehling's solution A and B.

3.7.3 Chemical reagents used in Histological preparation of tissue samples

10% buffered formalin, isopropyl alcohol 30% and absolute isopropyl alcohol, paraffin wax, xylene, dibutylplaticizer xylene mountant, haematoxylin and eosin stain.

3.7.4 Equipments and materials used in Histological preparation of tissue samples

Dissecting tools- (scissors, forceps, pins, dissecting tray, and scalpel), containers for holding specimen, labels, disposable blades, microtome machine (Ernst Leitz Wetzlar GMBH, Germany), automated tissue processor (Histokinate, USA), slides and cover slips, microscope, latex gloves, tissue cassettes, lead pencil, water bath (Griffin) and digital camera (Nikon, Japan).

3.8 Animal experiments

3.8.1 Acquisition and handling of laboratory animals

Male and female *Wistar* rats (not less than 100 grams) were used for the study. The animals were bred and housed at the animal house of the school of pharmacy, Kampala International University- Western Campus. Male and female *Wistar* rats both possess teats, as such, the scrotal sacs next to the last pair of teats in the males, was used for differentiation. Thirty six young adult

male and female rats were purchased and housed separately for 1 week to allow for acclimatization. The animals were kept in cages with ample space and lined with wood shavings at room temperature, with adequate ventilation and free from rodents. The room was naturally illuminated with 12 hours of light and 12 hours of darkness. They were fed on standard diet (Nuvita® Animal Feed Ltd, Jinja Uganda). They had access to clean drinking water *ad libitum*.

The animal experiment was conducted according to the National Institute of Health Guide for the care and use of laboratory animals (NIH, 1996) and ethical guidelines for investigation of experimental toxicity in animals (Zimmerman, 1983).

3.8.2 Toxicity Tests

3.8.2.1 Acute toxicity

The test was conducted in two phases according to Lorke's method (Lorke, 1983). Sixteen young adult male and female *Wistar rats* in all were used for this test.

3.8.2.2 Phase 1

Twelve young adult *Wistar rats*, grouped into 3 rats per group were used for this phase of tests. The animals were deprived of food for 16-18 hrs prior to administration of the extract and distilled water for the control group. They were weighed and grouped as follows: 3 rats in group A receiving low dose (2000mg/kg), 3 rats in group B receiving medium dose (3000mg/kg), 3 rats in group C receiving high dose (4000mg/kg) and 3 rats in control group D receiving distilled water (10mg/kg)

3.8.2.3 Phase 11

This phase involved three young adult *Wistar rats*, with one rat in each group. The animals were deprived of food for 16-18 hrs prior to administration of the extract. They were weighed and grouped as follows: 1 rat in group A receiving low dose (5000mg/kg), 1 rat in group B receiving medium dose (7500mg/kg) and 1 rat in group C receiving high dose (10000mg/kg).

3.8.3 Sub-chronic toxicity test

The experiment was carried out for 30 days using twenty young healthy *Wistar rats* which were weighed and grouped randomly into four groups as follows: The treatment group included fifteen rats, five rats per group: group A for low dose (200mg/kg), group B for medium dose

(400mg/kg) and group C for high dose (800mg/kg)).The extract was diluted using distilled water before administration and given according to their weight and dose per group. The negative control group comprised of five rats to which distilled water was administered at 10ml/kg. The rats were all given food thirty minutes after administration of the extract. This enhanced absorption of the extract by avoiding possible interaction with food. Parameters to be measured during the study were weekly weighing of animal body weight before administration of the extract in order to adjust the doses accordingly. The rats were observed daily to detect differences in appearance; discolored fur; diarrhea; bloody stool and constipation; lack of interest in food, water and surroundings.

3.8.4 Histological preparation of tissues

At the end of the treatment period, a postmortem of the animals was carried out where organ samples were removed in order to detect organ toxicity. The animals were sacrificed through use of chloroform.

Dissection was carried out using dissecting tools such as scalpel, scissors, dissecting board, pins and forceps. The rat was pinned to the dissecting board using pins to hold it in place before dissection. Prior to cutting, the structures were lifted by using forceps so that the underlying organs were not disturbed (Ningthoujam, 2010). The liver, lung, kidney, heart, and intestines were carefully dissected and removed from the abdominal region of the animals and weighed, and the relative organ weights calculated as follows:

$$\text{Relative organ weight} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of rat on sacrificed day (g)}}$$

The organ samples to be studied were immediately preserved in 10% buffered formalin in plastic sample bottles, and left overnight to allow for full absorption of formalin, to better preserve the samples and to kill the bacteria causing autolysis.

The tissue samples were then processed with an automated tissue processor, embedded and stained with Haematoxylin and Eosin stain and observed under microscope at (×100 and ×400 magnifications) for changes in structure.

3.9 Data analysis

The data generated was expressed as Mean ± Standard Error of Mean and the means were compared by using independent samples t-test.

3.10 Ethical considerations

Care was taken to ensure humane treatment of the animals and they were housed in an environment that was quiet and stress free with enough room for movement.(NIH, 1996) and ethical guidelines for investigation of experimental toxicity in animals (Zimmerman, 1983).

3.11 Limitations of the study

- Lack of equipment to carry out histology at KIU-WC.
- High cost of histology laboratory work.
- Lack of transparency by the herbalist on the plant constituents of the extract.
- Lack of a qualified laboratory technician and research assistant at KIU-WC.

CHAPTER FOUR

4.0 RESULTS

4.1Extract Yield

The percentage yield of the extract after drying in a hot air oven for a two week period was 90.49 grams (2%).

4.2 Phytochemical screening

Table 1: Secondary metabolites tested for in Nalongo's aqueous extract

TEST	RESULTS
Alkaloids	–
Terpenoids	+
Steroids	–
Carbohydrates	+
Phlabotannins	–
Cardiac glycosides	–
Saponins	+
Tannins	+
Flavonoids	+
Diterpenes	+
Amino acids	+
Phenolic compounds	+

Key: + (present) - (absent)

4.3 Acute toxicity results

No animal mortalities were recorded in both phase I and phase II. However subtle signs of toxicity were exhibited, such as sedation, increased motor activity, piloerection, lack of interest in the environment and feeding.

4.4 Effect of Nalongo's extract on weight of rats

Table 2: Mean body weight and S.E.M. values of rats (grams)

Group/Days	Day 0	Day 7	Day 14	Day 21	Day 28
Control	167.74±12.22	168.00±14.46	167.9±12.90	169.96±9.11	167.90±9.72
200mg/kg	157.62±8.86	153.02±8.66	152.22±7.26	156.66±6.95	155.02±7.51
400mg/kg	160.60±7.10	156.58±6.33	156.46±6.61	159.00±4.53	159.22±4.72
800mg/kg	164.22±9.28	160.44±8.92	159.72±7.81	164.02±6.70	162.40±5.85

N: 5; values presented as Mean ± Standard Error of Mean

Table 3: Mean percentage weight change and S.E.M. values of rats (%)

Group/Days	Day 7	Day 14	Day 21	Day 28
Control	68.15±0.13	68.00±0.08	71.27±0.52	68.00±0.42
200mg/kg	60.54±0.10	61.05±0.50	58.23±0.48	59.27±0.34
400mg/kg	63.10±0.38	63.18±0.22	61.61±0.56	61.46±0.52
800mg/kg	66.52±0.18	66.96±0.44	64.34±0.58	65.33±0.65

N: 5; values presented as Mean ± Standard Error of Mean

Weight change presented as percentage (%)

4.5 Effect of Nalongo's extract on organs of rats

Table 4: Mean organ weights and S.E.M. values of rats

Group/Organs	Lungs	Liver	Kidney	Heart	Intestine
Control	1.29±0.06	8.40±0.41	1.62±0.08	0.86±11.12	11.17±0.52
200mg/kg	1.19±0.05	7.75±0.31	1.49±0.06	0.79±0.03*	10.24±0.41
400mg/kg	1.22±0.03*	7.96±0.19	1.53±0.04*	0.81±0.02*	10.51±0.25
800mg/kg	1.25±0.04*	8.12±0.24	1.56±0.05*	0.83±0.02*	10.72±0.31

N: 5; values presented as Mean ± Standard Error of Mean

*indicates values significant at $p<0.05$

Table 5: Mean relative organ weight and S.E.M. values of rats

Organ	Control	200mg/kg	400mg/kg	800mg/kg
Lung	0.77±0.00	0.77±0.00	0.77±0.00	0.77±0.00
Liver	5.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00
Kidney	0.96±0.00	0.96±0.00	0.96±0.00	0.96±0.00
Heart	0.51±0.00	0.51±0.00	0.51±0.00	0.51±0.00
Intestine	6.65±0.05*	6.60±0.00	6.60±0.00	6.60±0.00

N: 5; values presented as Mean ± Standard Error of Mean

*indicates values not significant at $p<0.05$

Values are in grams.

Mean is not significantly different from control.

Relative organ weight = Absolute organ weight (g)/Body of rat on sacrificed day (g)*100.

CHAPTER FIVE

5.0 DISCUSSION

The phytochemical screening of Nalongo's extract tested positive for terpenoids, saponins, tannins, flavonoids, diterpenes and phenolic compounds. Acute toxicity tests showed that the extract can be considered to be relatively safe since it did not cause death in rats and there were no signs of toxicity such as alterations of the skin, mucous production and the effects on the eye, tremors and any general signs of toxicity observed after 72 hours.

Acute toxicity studies are designed to determine the dose that will produce either mortality or serious toxicological effects or high safety margins when administered once or over a few administrations. They also provide information on doses that should be used during sub-chronic toxicity testing.

In sub-chronic toxicity testing, there was no death of rats recorded throughout the duration of the study, indicating a high safety margin of Nalongo's extract on experimental animals. Increase in body weights were observed among the control group, with the highest noted on days 7 and 21, though it was not steady. The observed increase in weight could be due to the nutritive components in the animal feed. However, there was a general reduction in the mean percentage body weight in the groups treated with Nalongo's extract as compared with the control. The observed reduction in body weight was dose dependent; statistically significant ($p<0.05$) and could have been due to the suppression of appetite by the extract.

There was no significant change in the mean relative organ weights of the treated groups as compared to the control. There were however some pathological changes noted in the heart tissue of groups treated with 200mg/kg and 400mg/kg, such as; loss of striations and some nuclei, waviness of fibres and pockets of haemorrhage, suggestive of myocardial infarction. Changes were also noted in the tissues of the liver in the group treated with 400mg/kg of the extract, such as; appearance of inflammatory cells, suggestive of hepatitis/hepatotoxicity. The kidney and lung issue appeared normal as compared to control group. Therefore, even though acute and sub-chronic studies indicate a higher level of safety margins of the extract, more work and consultation with the herbalist on the preparation of the extract needs to be done in order to

ascertain the true phytochemical constituent picture of the extract, before it can be encouraged for continued consumption by the local liver and kidney patient population around Bushenyi and Mbarara Districts.

5.1 CONCLUSION

Nalongo's extract tested positive for terpenoids, saponins, tannins, flavonoids, diterpenes and phenolic compounds.

There was no mortality observed during acute and sub-chronic toxicity studies, suggesting a relatively high safety margin of the extract on experimental animals.

Some pathological changes were noted in tissues of the heart, liver and intestines of the experimental animals, as such more work needs to be carried out on the extract to ascertain its total constituent composition, before encouraging its consumption by the liver and kidney patients within Mbarara and Bushenyi Districts.

5.2 RECOMMENDATIONS

- More work needs to be carried out to ascertain the efficacy and dosing regimen of the potion.
- Work also needs to be carried out to exact the mechanism of action of the potion.
- Machinery needs to be put in place to moderate use of herbal products in Uganda.

CHAPTER SIX

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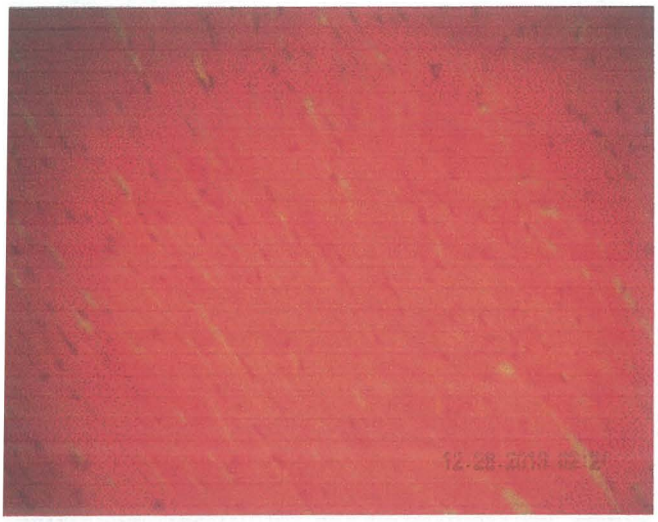
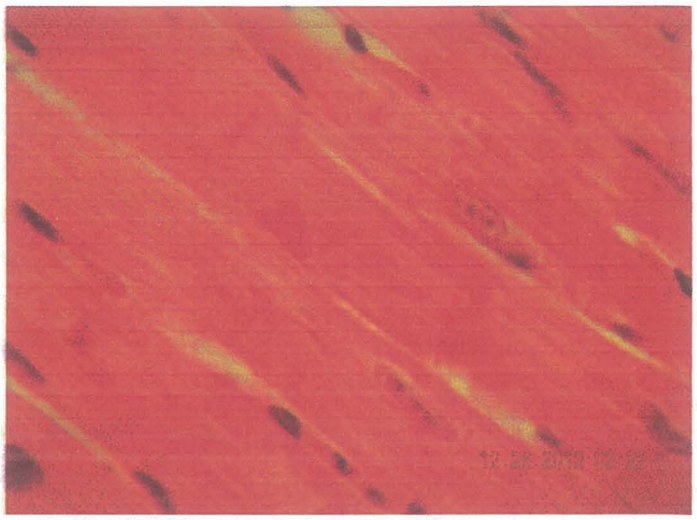
CHAPTER SEVEN

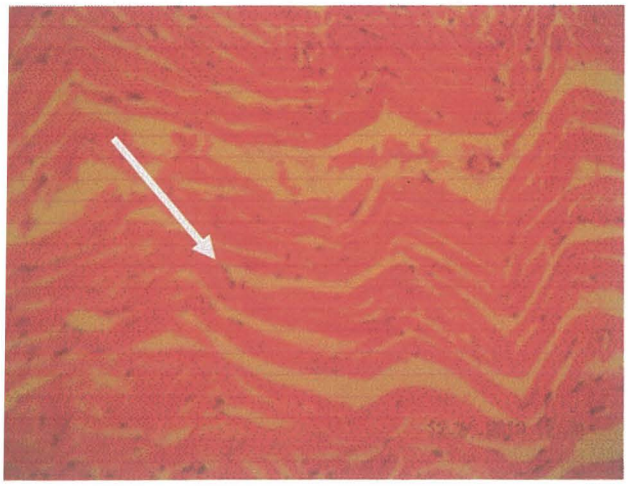
APPENDICES: Photo Micrographs of different organs.



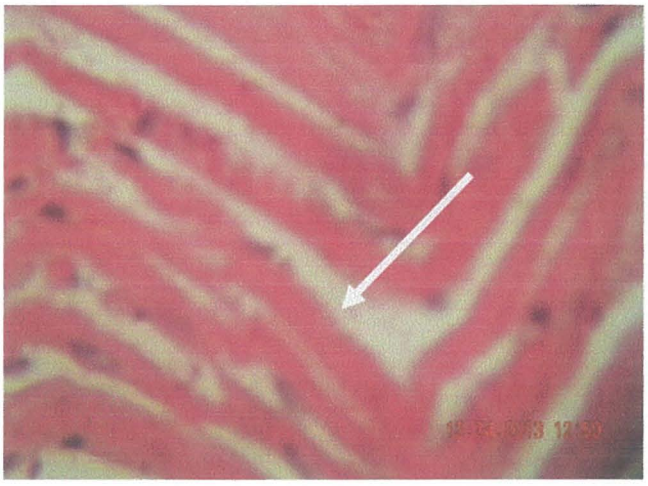
Control: Heart x 100



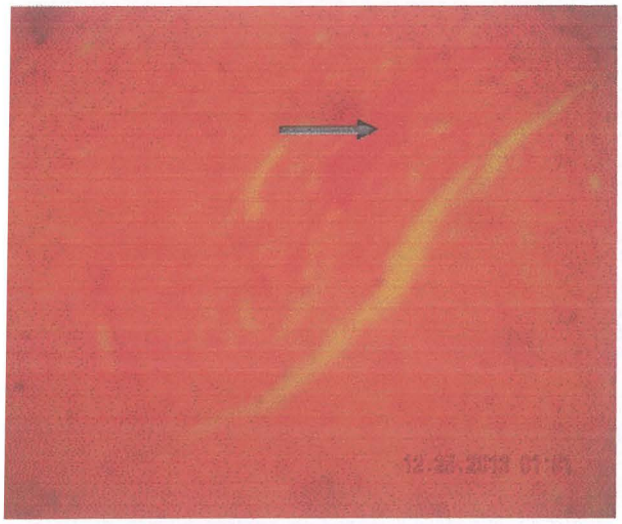
Control: Heart x 400



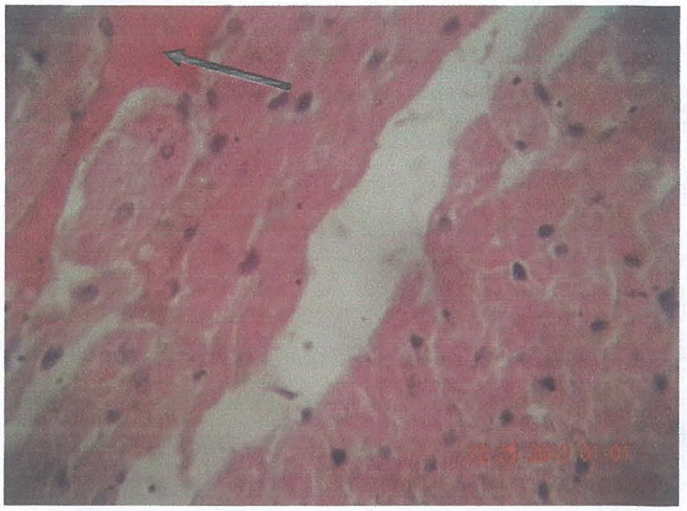
Group A: Heart×100 (arrowhead-loss of Striations)



Group A (200mg/kg): Heart×400

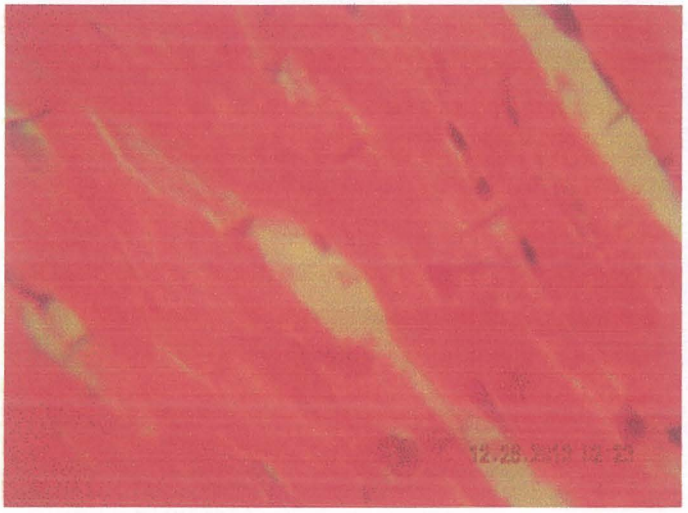


› B: Heart x 100 (arrowhead – hemorrhage)

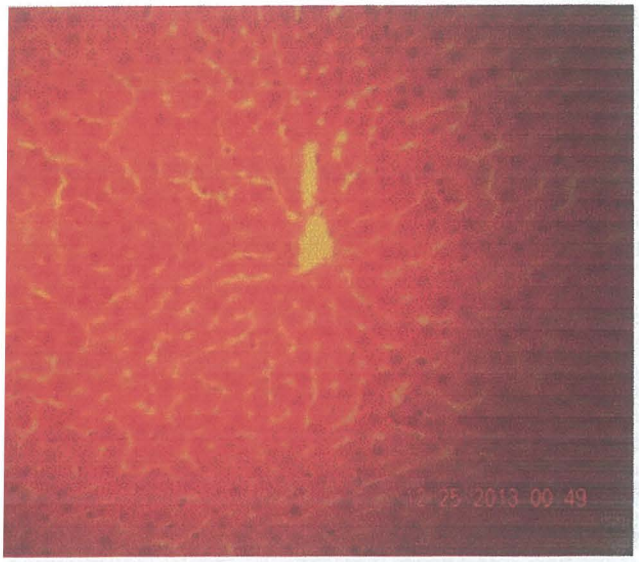


Group B (400mg/kg): Heart x 400

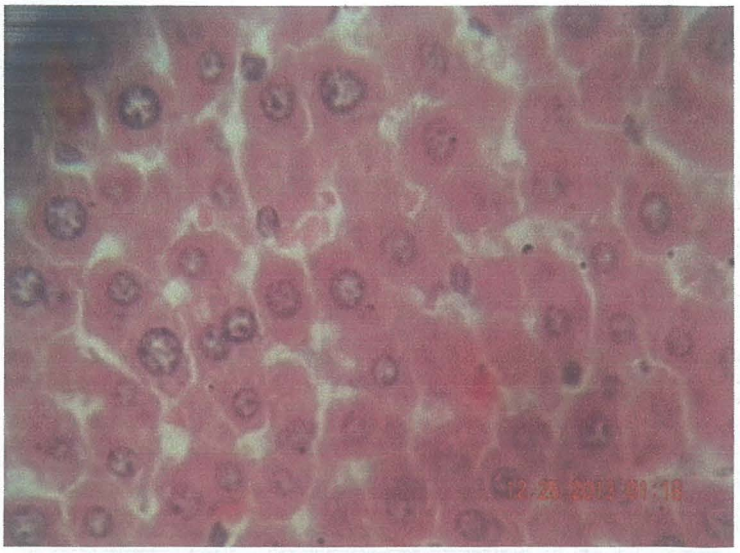
Group C (800mg/kg): Heart x 100



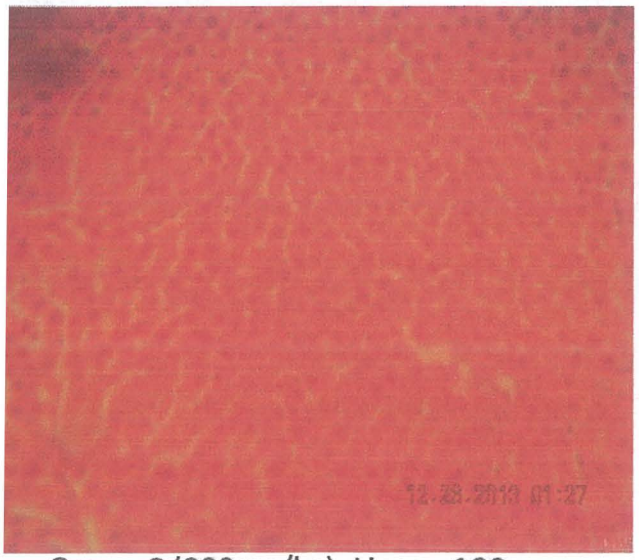
Group C (800mg/kg): Heart x 400



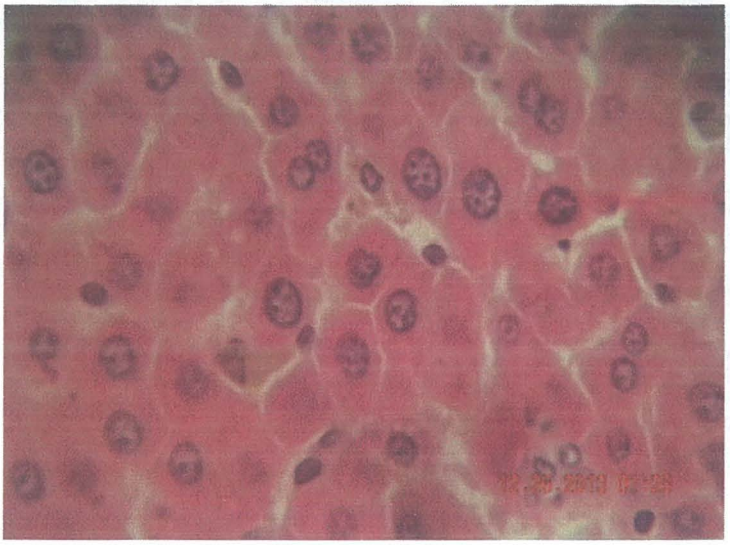
Group B (400mg/kg): Liver x 100



Group B (400mg/kg): Liver x 400



Group C (800mg/kg): Liver x 100



Group C (800mg/kg): Liver x 400