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DEPARTMENT OF PHARMACY

HPTLC fingerprinting and safety profile of *Warburgia ugandensis* Sprague (Fam. *Canellaceae)*

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A DISSERTATION SUBMITTED TO THE DIRECTORATE OF RESEARCH AND GRADUATE TRAINING IN PARTIAL FULFILMENT FOR THE AWARD OF A MASTER OF SCIENCE DEGREE IN PHARMACOGNOSY FROM MAKERERE UNIVERSITY.

OCT 2023

SUPERVISORS' APPROVAL

This is to confirm that the student carried out his research under our supervision.

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DECLARATION

This is to declare that this research dissertation entitled, **"HPTLC fingerprinting and Safety profile of *Warburgia ugandensis* (Fam. *Canellaceae*)."** is my original work, and has never been submitted to any University for the award of a degree.

Signature ..... Date 19/OCTOBER/2023

JAMES OCHAN

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DEFINITION OF KEY TERMS

Adulteration: intentional substitution with another plant species or intentional addition of a foreign substance to increase the weight or potency of the product or to decrease its cost.

Chromatogram: is the pattern formed on an adsorbent medium by the layers of components separated by chromatography

Conventional Medicine: is a health system in which medical doctors, nurses, pharmacists, and other trained healthcare professionals are licensed to practice and treat symptoms and diseases often with the use of scientifically proven pharmaceuticals and surgery.

Extract: the complex, multi-component mixture obtained after using a solvent to select for, or remove, components of the herbal material. Extracts may be in dry, liquid or semisolid form.

Fingerprint: a chromatographic pattern of pharmacologically active and or chemically characteristic constituents present in a medicinal plant extract.

Herbal Medicine/drugs/ therapies: any medicinal product that exclusively contains as active ingredients one or more botanical substances or one or more herbal preparations.

Herbal Monograph: a document that defines a botanical drug and provides information that allows for its proper identification. It contains the basic description including nomenclature, part used, constituents, range of application, contraindications and side effects, incompatibilities with other medications, dosage, use, and action of the herb.

Median lethal dose: the dose required to kill half the members of a tested population in single or multiple doses over 24-hour period following Oral, dermal or parenteral route.

Medicinal Plants: plants that possess therapeutic properties or exert beneficial pharmacological effect on the human or animal body.

Phytochemical: biologically active compounds found in plants.

Phytotoxin: a poisonous substance derived from a plant.

Retardation Factor: the ratio of the distance travelled by the centre of a spot to the distance travelled by the solvent front.

Safety: The condition of being unlikely to cause danger, risk, or injury.

Toxicity: the capacity of a substance to cause injury to a living organism.

Traditional Medicine: the sum total of the knowledge, skill, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness.

LIST OF ABBREVIATIONS AND ACRONYMS

µg/L: micrograms per Litre

µL: microliter

µmol/L: micromole per litre

ALB: Albumin

ALT: Alanine aminotransferase

ANOVA: Analysis of Variance

AST: Aspartate aminotransferase a

BALB/c: Albino, laboratory-bred strain of the house mouse

CC50:50% cytotoxic concentration

g/dl: grams per decilitre

g/L: grams per litre

GCS; Global Harmonized System

HBG: Haemoglobin

HCT: Haematocrit

HPTLC: High Performance Thin Layer Chromatography.

ICLAS: International Council for laboratory animal science

LD50: Median Lethal Dose

Mmol: Millimole per litre

NDA: National Drug Authority

OECD: Organization for Economic Co-operation and Development

PLT: Platelets

RBC: Red blood cells

R.F: Retardation factor.

SD: Standard deviation.

WBC: White blood cells

WHO: World Health Organization

WUBA: *W. ugandensis* stem bark aqueous extract.

WUBE: *W. ugandensis* stem bark ethanolic extract

WULA: *W. ugandensis* leaf aqueous extract

WULE: *W. ugandensis* leaf ethanolic extract

ABSTRACT

Background: Despite the extensive breakthrough of conventional medicine, herbal medicine use is on the increase worldwide. The continued lack of quality control measures such as proper identification, and safety evaluation data constitute major challenges affecting herbal medicine acceptance in the era of evidence-based medicine. This study sought to develop an HPTLC fingerprint profile for *W. ugandensis* leaf and stembark extracts and further investigate the acute and subacute toxicity to provide identification and safety information on the medicinal plant for purposes of drug development and regulation. **Methods**: This was an experimental study. The leaf and stembark of *W. ugandensis* were collected from Mabira forest in Central Uganda, and extracted by maceration using acetone, ethyl acetate, hexane and methanol for HPTLC analysis; and 70% ethanol and distilled water for safety assessments. A mobile phase system of hexane: acetone (70: 30) for the leaf extracts, and ethyl acetate: acetone: hexane (10:5:3) for the bark extracts was used for HPTLC analysis. OECD Tests 432 and 407 were followed for assessment of acute and sub-acute toxicity respectively of the aqueous leaf, aqueous stembark, 70% ethanolic leaf and 70% ethanolic stembark extracts. **Results:** The acetone extract of the leaf showed the highest number of bands in both white light and derivatized modes of visualization with 9 and 12 bands respectively corresponding to an RF range of 0.06 to 0.90. On the other hand, the acetyl acetate extract of the stembark produced the highest number of bands in the derivatized chromatogram with 7 corresponding to an RF range of 0.04-0.87. No mortalities were observed in the acute toxicity studies of the extracts of *W. ugandensis* with the LD_{50} estimated as 5000mg/Kg. There was no loss of weight in study animals in both the acute and subacute studies. The *W. ugandensis* extracts appeared to increase AST levels nearly two-fold compared to control group in the subacute study but neither raised creatinine nor urea levels compared to control group. A delayed enhancement of RBC count was reported in animals that received *W. ugandensis* extracts. **Conclusion:** This study demonstrated that a simple HPTLC method using vanillin as a staining agent can be used to develop fingerprints for *W. ugandensis* leaf and stem bark Extracts. The study further showed the safety of *W.ugandensis* leaf and stem bark, aqueous and 70% ethanolic extracts in acute use. However, there should be caution in the development of herbal therapies that involve repeated dosing exceeding two weeks due to possibility liver toxicity.

CHAPTER ONE: INTRODUCTION

1.1 Background

Despite the extensive breakthrough of conventional medicine, herbal medicine use is on the increase, with an expected worldwide market value of 129 billion US dollars in 2023 (WHO, 2019). This growing interest in plant remedies has been attributed to claims of effectiveness of plant medicines, preference of consumers for natural therapies, a dissatisfaction with the results from synthetic drugs, the belief that herbal medicines might be effective in the treatment of certain diseases where conventional therapies have proven to be inadequate, the high cost and side effects of most modern drugs, as well as a movement towards self-medication (Welz et al., 2018).

Herbal medicine is currently used by at least 80% of the WHO member states, with over 60% of Ugandan population relying on them for their primary health care needs as alternative or complementary medicines (WHO, 2019(Logiel et al., 2021). This use is often accompanied by the misconception that herbal products are safe because they are natural. However, Medicinal plants can be inherently toxic, this toxicity is as a result of the presence of phytotoxins (Gamaniel, 2000). Phytotoxins have varied structures and can be alkaloids, terpenes, phenylpropanoids or polyketides(Chen et al., 2022). For instance, aristolochic acid found in *Aristolochia clematitis,* was found to possess carcinogenic and nephrotoxic effects leading to the banning of the use of the plant as a component of traditional medicine in many countries (Tankeu et al., 2016). Today, comprehensive safety data of many widely used medicinal plants is still lacking (Dorato & Engelhardt, 2005) (Chen et al., 2022).

The toxic effects of herbal medicinal products are not only a function of the innate toxicity of the medicinal plants, but can also arise from deficient quality. The quality problems often come about as a consequence of inadequate quality control practices during sourcing of raw materials, leading to the use of falsified or mislabelled raw materials (Capasso et al., 2000; Pan et al., 2013). Herbal medicine raw materials can be authenticated in a cost effective manner by a simple comparison with a High-Performance Thin-Layer Chromatography (HPTLC) fingerprint of a standard preparation (Attimarad et al., 2011). HPTLC offers several advantages over other analytical methods, including its speed, cost-effectiveness, and ability to handle complex mixtures of compounds often found in herbal materials (Attimarad et al., 2011).

A number of medicinal plants are widely used in African Traditional Medicine for management of various diseases. In Uganda and other parts of East Africa, *Warburgia ugandensis* Sprague (Fam. Canellaceae*),* locally known as *Mukuzanume (*Luganda*),* is highly valued for its

medicinal applications. Various preparations of the plant are used to treat upper respiratory tract symptoms, gastrointestinal ailments , fevers, malaria, oral thrush, measles, diarrhoea and HIV opportunistic infections (Maroyi, 2014; Okello & Kang, 2019) . Some of these folk uses have been validated through pharmacological studies and attributed to the phytochemical compounds present in the plant (Kubo et al., 1976; Wube et al., 2008) (Karani et al., 2013; Maroyi, 2014). Despite its continued use, *W. ugandensis* like many medicinal plants in Africa, still suffers from the lack well established quality control methods compounded with a relatively unexplored safety profile.

The current study was therefore designed in response to the aforementioned challenges in quality control of herbal materials and the lack of comprehensive safety data to support their use. This study utilized an HPTLC method to develop a fingerprint profile for *W. ugandensis,* and, an animal model; to generate both acute and subacute safety profiles for *W. ugandensis* with a view to provide information that might find application in drug development and regulation of *W. ugandensis* derived herbal remedies.

1.2 Statement of the Problem

Often times, serious adverse reactions are reported from the use of herbal medicines. These include: anaphylactic reactions, hepatoxicity and renal toxicity (Bhagavathula et al., 2016; Ernst, 2003b; Lutoti et al., 2013; Paik & Lee, 2015). Such effects may arise from the innate toxicity of medicinal plant components, and the consumption of substituted or falsified products with similar looking but toxic herbs (Ernst, 2003a). This emphasizes the need to correctly identify active raw materials used in herbal formulations and to carry out safety assessment of such medicines to predict toxicities (Rotblatt & Ziment, 2001).

Warbugia ugandensis is widely used in East Africa for various medicinal benefits, however, the scarcity of documented safety data, and the lack of standard methods for quality control of *W. ugandensis* raw materials undermines the ability of the industry to manufacture products of sufficient quality and safety. Specifically, the lack of a cost-effective chemical identification method for the medicinal plant increases the likelihood of substitution or falsification of raw materials containing *W. ugandensis*.

A few studies have been conducted on *W. ugandensis* albeit with some gaps. For instance, (Karani et al., 2013) investigated the safety of *W. ugandensis* in mice and found an LD_{50} of 2201.207 mg/Kg of body weight. However, this study was notably limited to the single dose acute toxicity of the aqueous stembark extract whereas in practice, aqueous and ethanolic extracts of the leaf in addition to aqueous stembark extracts are being used, sometimes

repeatedly in a sub chronic manner (Maroyi, 2014). In another study, *W. ugandensis* aqueous and ethanolic leaf extracts were shown to be non-toxic to *Drosophila melanogaster* at acute exposure but toxic at chronic exposure (Ringim et al., 2017). The results of this study are however difficult to extrapolate to humans because of the use of an insect model (Olson et al., 2000).

This study therefore set out to develop an HPTLC fingerprint profile for *W. ugandensis* leaf and stembark extracts and further investigate the acute and sub-acute toxicity of *W. ugandensis* in Wistar rats to provide identification and safety information on the medicinal plant for purposes of drug development and regulation.

1.3 Objectives

1.3.1 General Objective

The general objective of this study was to develop HPTLC fingerprint and safety profiles of *Warburgia ugandensis* leaf and stembark extracts.

1.3.2 Specific Objectives

This study was guided by the following specific objectives:

i. To develop an HPTLC fingerprint profile for *W. ugandensis* leaf and stembark extracts.

ii. To assess the acute toxicity of the aqueous and 70% ethanolic leaf and stembark extracts of *W. ugandensis* in Wistar rats.

iii. To assess the sub-acute toxicity of the aqueous and 70% ethanolic leaf and stembark extracts of *W. ugandensis* in Wistar rats.

1.4 Research Questions

iv. What is the HPTLC fingerprint profile for *W. ugandensis* leaf and 70% stembark extracts?

v. What is the acute toxicity profile of *W. ugandensis* aqueous and 70% ethanolic; leaf and stembark extract on Wistar rats?

vi. What is the sub-acute toxicity profile of *W. ugandensis* aqueous and 70% ethanolic stembark extracts on Wistar rats?

1.5 Significance of Study

The HPTLC method and HPTLC fingerprints obtained from this study may be used to identify *W. ugandensis* processed active raw materials. Furthermore, the information obtained from this study may be used in drug development, for the conduct and design of clinical trials and to

direct policy and regulation of the medicinal plant and products derived from it. Lastly, the findings from this study will add to the existing scientific body of knowledge on the plant and help guide future research on the plant.

1.6 Scope of the Study

The study was conducted in the Central Region of Uganda between September and December of 2021 and involved the collection of leaves and stembark of *W. ugandensis* growing in Mabira Forest located at GPS coordinates 0.3894° N, 33.0057° E, about 60km from Kampala, the capital of Uganda. Toxicity assessment was conducted following the oral route using OECD guidelines. The extraction, and screening were done at the pharmacognosy laboratory, Pharmacy Department, College of Health Sciences of Makerere University. Toxicological tests were conducted at the Pharmaceutical, and Toxicology Research Centre for Tropical Diseases and Vector Control (RTC) laboratory at the College of Veterinary Medicine, Animal Resource and Biosecurity of Makerere University (COVAB), GPS Coordinates 0.3326° N, 32.5686° E. HPTLC analysis was conducted at the National Drug Quality Control laboratory, Mulago Hill, Kampala Uganda, GPS Coordinates: 0.335078° N, 32.576759° E. Haematological and clinical chemistry analysis of the samples from the toxicity test was conducted at the Mulago National Referral Hospital Private Laboratory.

CHAPTER TWO: LITERATURE REVIEW

2.1 Thin Layer Chromatography (TLC) analysis and Finger Printing of Medicinal Plants

Thin layer chromatography is a fast, affordable and cost-effective method for obtaining a characteristic analytical fingerprint of a plant extract (Anne & Eike, 2007) and has been used extensively for identifying medicinal plants. Thin Layer Chromatography is used to verify the identity of plant extracts by determining the chemical fingerprint of the extracts. For instance, (Eloff et al., 2011) developed three TLC solvent systems and successfully used TLC analysis with acetone as extractant and benzene: ethanol: ammonia [9:1:0.1], chloroform: ethylacetate: formic acid [5:4:1] and ethylacetate: methanol: water [10:1.35:1] as TLC solvent system to identity and verify 81 samples of more than 50 herbal preparations on the basis of the TLC chromatograms.

It is possible to determine certain classes of compounds by spraying developed plates with stains that give a colour reaction with a particular compound class. According to (Eloff et al., 2011) 44 spray reagents have been used to verify the identity of important herbal preparations. (Eloff et al., 2011) also investigated the use of several sprays i.e. 20% toluene-sulphonic acid in chloroform, 15% of 85% phosphoric acid in methanol, 0.5% vanillin in 80% ethanolic sulphuric acid, 20% perchloric acid, 5% p-anisaldehyde in 5% ethanolic sulphuric acid and 25% trichloroacetic acid in chloroform and discovered that in general using vanillin-sulphuric acid (0.1g vanillin in 28 ml methanol:1 ml sulphuric acid) on aluminium backed plates gave good results and this was selected as standard treatment for visualizing triterpenoids and other phenolic compounds. The same spray reagent was also successfully applied on TLC chromatograms from several plant extracts of *Mikania glomerata, Spilanthe sacmella, Lippia alba, Achillea millefolium, Piper regnelli, Eugenia uniflora, Arctium lappa, Tanacetum vulgare, Erythrina speciosa, Psidium guajava, Punica granatum, Sambucus canadensis* and *Plantago major* (Holetz et al., 2002).

The TLC method employs glass or aluminium plates pre-coated with the sorbent (e.g. silica gel) to varying thickness depending on the amount of the sample to be loaded. The compound mixture is loaded both in preparative or analytical plates at around 1-2 cm from the bottom of the plate and lowered in a tank containing the solvent. The mixture migrates up the plates and the compound mixture separate according to the polarity of the components.

TLC has the advantage of being a highly cost-effective qualitative technique since a large number of samples can be analysed or separated simultaneously. The few drawbacks include

poor detection and control compared to high performance liquid chromatography (HPLC) (Ferenczi-Fodor et al., 2011). However, the detection and control of TLC can be markedly improved by using an automated form of TLC called High Performance Thin Layer Chromatography (HPTLC).

Unlike TLC, HPTLC uses TLC plates with finer silica particles for improved resolution/detection, incorporates an automatic sampler for drawing and application of samples on to the TLC plate and uses an automatic development chamber for faster development time (Attimarad et al., 2011).

2.2 Overview on Toxicity of Medicinal Plants

For millennia, plants have been recognized to be a source of medicine to man to treat many ailments. The plant kingdom still provides the largest source of useful drugs, food, additives, colorants, binders, flavouring agents and lubricants to the respective industries (Gamaniel, 2000). It is estimated that by the year 2000, more than 25% of all prescribed medicines were substances derived from plants or their analogues, highlighting the need to continue studying medicinal plants used in the traditional medicine of developing societies like Africa and Asia (Gamaniel, 2000). The likes of anti-inflammatory drug aspirin first obtained from *Filipendula ulmaria*, anticancer drugs, vincristine and vinblastine obtained from *Catharantus roseus* and anti-malarial drug quinine first obtained from Cinchona bark serve as classic examples of successful plant derived conventional pharmaceuticals (Gamaniel, 2000).

The study of toxicities from medicinal plants and their products is a special branch of Toxicology termed as Ethno toxicology or Ethinology (Gamaniel, 2000). Historically, herbs have been considered to be non-toxic and have been used for treating various problems by the general public and traditional medicine practitioners worldwide (Oduola et al., 2010) . Despite the fact that numerous studies have documented several cases of toxicity resulting from the use of herbs, it is unfortunate that the potential of herbs to cause toxicity has not been recognized by the general public or by professional groups of traditional medicine (O'Hara et al., 1998).

The toxic effect of plants emanates from the presence of various secondary metabolites, which are classified into alkaloids, glycosides, proteins, oxalates, anti-vitamins, tannins among others. They act through specific mechanisms involving enzymes, receptors and genetic material in particular cells and tissues (Sekhar et al., 2012). It is therefore important to study the toxicity of medicinal plants and herbal drugs using the same methods that are used to study conventional pharmaceuticals (Rotblatt and Ziment, 2001).

2.3 Principles of Toxicity Assessment of Chemicals

Generally speaking, the toxicity of a substance can be defined as the inherent capacity to cause injury to a living organism (Camougis, 1985; Hodson & Wright, 2022; Pierre-Marie et al., 2011). A toxicity assessment is a tool used to investigate the potential for the medicinal plant extract to cause harm and is a major component of the risk assessment of a drug before clinical use(Laurence & Bacharach, 2013). Toxic effects are classified as acute, sub-acute and chronic(Laurence & Bacharach, 2013).

2.3.1 Goals of Toxicity testing of Herbal drugs

The primary goals of a toxicological assessment of any herbal drug are to identify adverse effects and to determine limits of exposure level at which such effects occur. It is also of paramount importance to note the nature and significance of the adverse effect and in addition, the exposure level where the effect is observed (Gamaniel, 2000).

Toxicity testing is also important in the detection of toxic plant extracts or compounds in the pre-clinical and clinical stages of drug discovery and development from plant sources. This facilitates the identification of toxicants which can be discarded or modified during the process and create an opportunity for extensive evaluation of safer, promising alternatives (Gamaniel, 2000).

2.3.2 Acute Toxicity

Historically, acute toxicity tests were the first tests conducted. They provide data on the relative toxicity likely to arise from a single or brief exposure, or sometimes multiple doses over a brief period of time (Laurence & Bacharach, 2013).

Acute toxicity studies are commonly used to determine the Lethal Dose 50 (LD50) of substances. The LD50 is the dose of a substance that can be expected to cause the death of 50% of the tested animals. Since a great range of concentrations or doses of various chemicals may be involved in the production of harmful effects, the LD50 has been used by some authors to devise categories of toxicity on the basis of the amounts of the chemicals necessary to produce harm. An example of such a categorization, along with the respective lethal doses, is given in Table 1.

Table 1: Classification of toxicity based on LD_{50} dose ranges by Hodge and Sterner (Frank,1992).

LD_{50} (mg/Kg)	Classification
1 or less	Extremely toxic
1 to 50	Highly toxic
50 to 500	Moderately toxic
500 to 5000	500 Slightly toxic
5000 to 15000	Practically non-toxic
More than 15000	Relatively harmless

2.3.3 Sub-acute Toxicity

Sub-acute toxicity tests are employed to determine toxicity likely to arise from repeated exposures of sub-lethal doses of a substance for a period of 14 to 28 days (Laurence & Bacharach, 2013). Sub-acute toxicity studies are used to determine effect of a drug on biochemical and haematological parameters of blood as well as to determine histopathological changes (Kunimatsu et al., 2004).

2.3.4 Chronic Toxicity

Chronic toxicity tests determine toxicity from exposure for a substantial portion of a subject's life. They are similar to the sub-chronic tests except that they extend over a longer period of time and involve larger groups of animals. In chronic toxicity studies, a drug is given in different doses for a period of 90 days to over a year to determine carcinogenic and mutagenic potential of drug (Laurence et al, 2013).

2.3.5 Routes of Administration in Toxicological Assessments

This term refers to the way in which the test substances are introduced to animals during the conduct of the toxicity assessment. It is therefore important for the route of administration used in an assessment to mimic the route used clinically as much as possible for the best comparison. Various routes are used for toxicity assessments;however, the intraperitoneal injection and the oral route are the most used modes of administration (Poole & Leslie, 1989).

2.3.5.1 Intra-peritoneal injection

This method of dosing can provide information on both local and systemic toxicity. To give drugs by intra-peritoneal injection, the animal is laid on its back and the abdomen shaved and,

using an appropriate syringe and needle, the abdominal wall is punctured (Poole and Leslie, 1989).

2.3.5.2 Oral Administration

The oral route is probably the most common means by which a chemical enters the body. The route involves the administration of a substance through the gastro intestinal (GIT) tract of the test organism. This method can produce both systemic and GIT localized effects. The oral route has the added advantage of exposing chemicals that are rapidly absorbed from the gastrointestinal tract to the liver at high concentrations, a feat not possible if other routes of administration were used (Hayes & Loomis, 1996). It follows, therefore that compounds that are known to be toxic to the liver would be expected to be more toxic following oral administration on repeated occasions than when they are administered by other routes (Loomis and Hayes, 1996).

2.3.6 Target tissue Toxicity

The degree to which an organ is susceptible to the toxic effects of a substance varies from tissue to tissue. The more highly vascularised organs like the liver and kidneys are more susceptible to toxic effects than less vascularized organs (Viala & Botta, 2005).

2.3.6.1 Hepatotoxicity

The liver is the largest internal organ in the body made of approximately a million lobules filled with hepatocytes (Abdel-Misih & Bloomston, 2010). The hepatocytes are responsible for bile secretion and also perform a variety of metabolic functions. The liver functions to destroy old or defective red blood cells, remove bacteria and foreign particles from the blood, and to detoxify toxins and other harmful substances (Mitra & Metcalf, 2009). Such functions make the liver, the first and foremost target organ for chemically induced injuries.

Indicators of hepatotoxicity include elevated liver enzymes, acute or chronic hepatitis, cholestasis, hepatic necrosis or fibrosis, cirrhosis, liver failure, and hepatic veno-occlusive disease (Bischoff et al., 2018; Ozougwu, 2017; Thakkar et al., 2018).

2.3.6.2 Nephrotoxicity

The kidneys are an essential part of the urinary system responsible for the production of urine and also serve homeostatic functions such as the regulation of electrolytes (Wallace, 1998). In producing urine, the kidneys excrete wastes such as urea, ammonia, and polar drug metabolites (Middendorf & Williams, 2000).

Kidney damage is frequently assessed in nonspecific terms such as changes in kidney weight (both increases and decreases), increases in protein content of the urine (proteinuria), or

changes in the volume of urine and increments in the content of creatinine and urea in the blood (Middendorf and Williams, 2000).

2.3.6.3 Hematotoxicity

Hematoxicty involves toxic injury to the blood cells and blood-forming tissues. Blood forms the main medium of transport in the body and, in the same capacity serves to transport many drugs. The various components of blood are thus initially exposed to significant concentrations of xenobiotics (Baker, 2012).

The bone marrow is the principal blood-forming tissue, producing stem cells that are precursors of erythrocytes, leucocytes, and platelets. The bone marrow is a major target for many toxic substances leading to anaemia and susceptibility to infections due to a total reduction in both red and white cell count (Baker, 2012). The assessment of haematological parameters is therefore relevant to the evaluation of risks from a drug.

2.3.7 Models for Toxicity Assessments

Human data on the toxicity of substances is the gold standard for safety evaluation rather than those obtained from the exposure of experimental animals. However, controlled exposures of man to hazardous or potentially hazardous substances are limited by ethical considerations, and information obtained by clinical or epidemiological methods must be relied on (WHO, 1978). Where such information is not available, data must be obtained from tests on experimental animals and other laboratory procedures. Models for toxicity assessment of compounds can be divided into two broad groups, in vitro and in vivo methods (Laurence et al, 2013).

2.3.7.1 In vivo methods for Toxicity Assessment.

These involve the use of animal models. The selection of the animal species to use depends on the nature of the test and ethical requirements. Strictly speaking, higher mammalian animal models like non-human primates and dogs provide the closest response to humans but their use is often restricted by ethical considerations and sometimes outrightly banned in many places. Therefore, rodents like rats, mice, and rabbits have emerged as a very useful model for toxicity assessment because they are highly available, inexpensive to breed and house, and have a history of producing reliable results in experiments (WHO, 1978).

2.3.7.2 In vitro Methods for Toxicity Assessment.

In vitro tests have gained prominence over the last decades fuelled by advantages such as lower cost and shorter durations of study. In vitro test systems include microorganism systems, mammalian cell culture systems, tissue preparations and organ cell cultures. In vitro tests for chromosomal effects involve exposure of cell cultures and followed by microscopic

examination of them for chromosome damage. The most commonly used cell lines are Chinese Hamster Ovary (CHO) cells and human lymphocyte (Laurence et al, 2013).

2.3.8 Standard Methods used in Toxicity Assessment of Substances.

Animal methods to evaluate toxicity have been developed for a wide variety of toxic effects. Some procedures for routine safety testing have been standardized (Ecobichon, 1997). Standardized animal toxicity tests have been reported to be highly effective in detecting toxicity that may occur in humans (Ecobichon, 1997). As noted earlier, concern for animal welfare has resulted in tests that use humane procedures and only as many animals as are needed for statistical reliability. To be standardized, a test procedure must have scientific acceptance as the most meaningful assay for the toxic effects. The OECD has published widely used standard test methods for Toxicity assessment (Combes et al., 2004; Kunimatsu et al., 2004; Toxicity–Up, 2001; Wilhelm & Maibach, 2012).

2.4 Review of the genus of *Warburgia*

The genus *Warburgia* is a member of *Canellaceae*, a dicot family that contains 16 species grouped in 6 genera(Natho, 2008). The genus was named after Dr Otto Warburg (1859– 1938), born in Hamburg, Germany, a lecturer in botany at the University of Berlin and author of several botanical papers. The genus contains four species that are of valuable medicinal importance and are found all over Africa, these include *W. ugandensis*, *Warburgia elongate Verdc, Warbugia salutaris, Warburgia stuhlmannii* (Muchugi et al., 2008).

2.4.1 Description and Distribution of *Warburgia ugandensis*

*Warburgia ugandensis i*s widespread in Central and East Africa and has been recorded in lowland rainforest, evergreen and swamp forest of DRC, Ethiopia, Kenya, Malawi, Tanzania, Uganda (Verdcourt, 1954) used for timber, firewood, building poles, charcoal, carvings and medicinal purposes (Leonard & Viljoen, 2015). *Warburgia ugandensis Sprague ssp. Longifolia Verdc*. is endemic to southern Tanzania (Verdcourt, 1956), while *W. ugandensis Sprague ssp. ugandensis* is more widespread and recorded in lowland rainforest, evergreen and swamp forest of DRC, Ethiopia, Kenya, Malawi, Tanzania, Uganda (Maroyi, 2014)).

Warburgia ugandensis is a spreading evergreen tree 4.5 metres to 30 metres tall, 70 centimetres in diameter, bark smooth or scaly, pale green or brown, slash pink; bole short and clear of branches for about three metres (Verdcourt, 1956)(Kuria et al., 2012).

2.4.2 Pictures of *Warburgia ugandensis*

Figure 1: *W. ugandensis* tree courtesy of Tropical Plants Database (2022*)*

Figure 2: *W. Ugandensis* trunk showing the characteristic bark courtesy of Tropical Plants Database (2022)

Figure 3:*W. ugandensis* leaves courtesy of Tropical Plants Database (2022)

2.4.3 Local names of *Warburgia ugandensis*

Warburgia ugandensis is known by the local names of English (pepper-bark tree, Kenya green heart, greenheart, East African greenheart, East African green wood); Luganda as *muwiya, mukuzanume* (Kokwaro, 2009).

2.4.4 Phytochemistry of *Warburgia ugandensis*

Phytochemical investigations of *W. ugandensis* have shown the presence of multiple sesquiterpernoids (Figure 4) including ugandensolide (29), ugandesidial (cinnamodial) (7), warburgin (30) and warburgiadione (31) from the heartwood (Brooks & Draffan, 1969a, 1969b).

Further studies have isolated cinnamolide (32), cinnamolide-3b-acetate (33), 11a-hydroxymuzigadiolide (34), 7a-hydroxy-8-drimen-11,12-olide (35), deacetylugandensolide (19), linoleic acid (36), mukaadial (9) , muzigadiolide (37), ugandensolide (29) , muzigadial (cannelal) (6), pereniporin B (38) , polygodial (4) and waburganal (3) from the stem bark(Brooks & Draffan, 1969b; Kubo et al., 1976; Wube et al., 2005); and monoterpenes (Kioy et al., 1990) from the leaves. Wube and others in 2005 isolated coloratane sesquiterpenes from the stem bark of *W. ugandensis.*

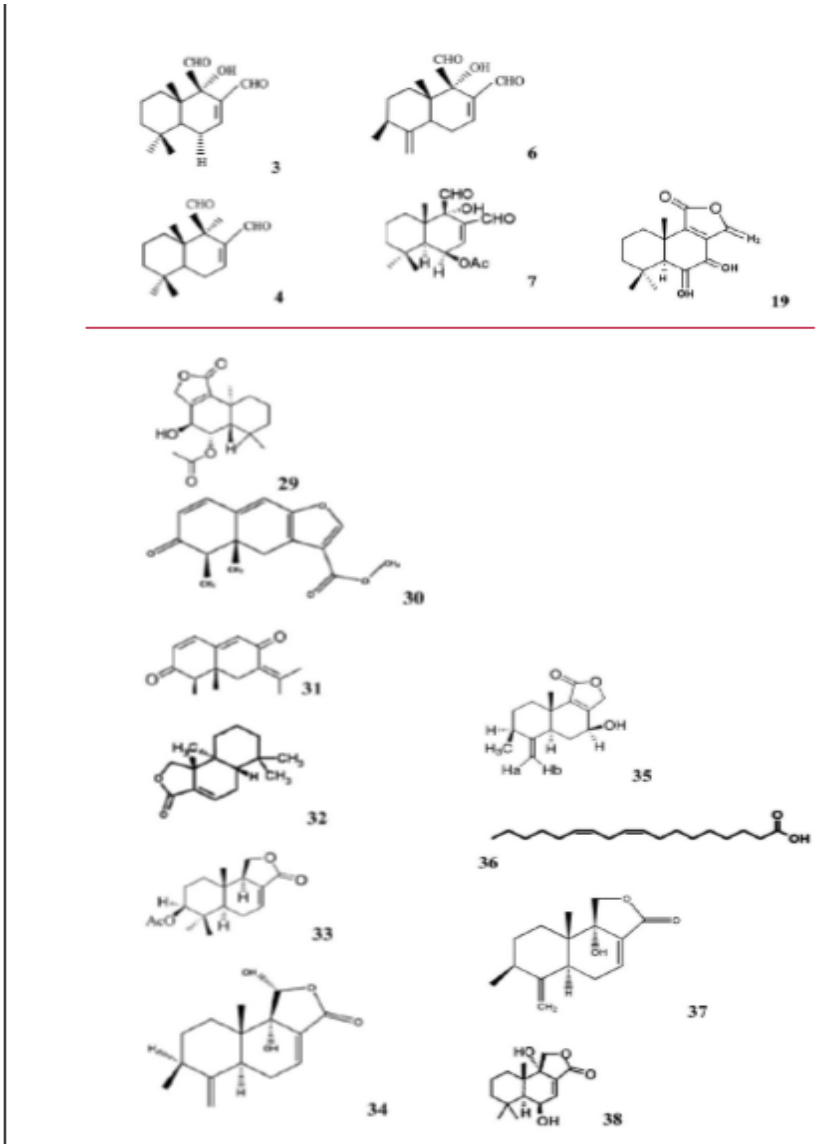


Figure 4 Structures of Selected Sesquiterpenoids isolated from *Warburgia ugandensis*

2.4.5 Traditional Medicinal uses of *Warburgia ugandensis*

Different preparations of the plant are used traditionally in East Africa to treat stomach-ache, constipation, toothache, cough, fever, muscle pains, weak joints and general body pains. Other conditions treated by the herb include; diarrhoea, several skin diseases, common cold; sinuses; malaria, HIV-related opportunistic infections, constipation, snake bites, measles and trypanosomiasis. The most frequently used preparations are decoctions of the bark (79%), leaves (11%), roots (7.5%) and stalks (2.1%) (Maroyi, 2014).

2.4.6 Reported Pharmacological activities of *Warburgia ugandensis*

2.4.7 Antibacterial activity

The ethanolic extract of the dried leaves of *W. ugandensis* was found to have antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Vibrio cholerae*, and *Bacillus (Mbwambo et al., 2009)*. In another experiment by Kuglerova et al (2011), *W. Ugandensis* stem

bark exhibited antibacterial activity with an MIC of 256 mg/ml against *Staphylococcus aureus* and 512 mg/ml against *Enterococcus faecalis*.

2.4.8 Antifungal activity

Warburgia ugandensis was demonstrated by Taniguchi et al. (1983) using a two-fold dilution method to have broad antifungal activity against yeasts and filamentous fungi; and it was highly active against *Saccharomyces cerevisiae, Candida utilis and Sclerotinia libertiana.* Olila and others in 2001 demonstrated that *W. ugandensis* ethanol extract of stem bark had antifungal activity against *Candida albicans*. Mbwambo and colleagues in 2009 demonstrated that the ethanolic extract from the dried leaves of *W. ugandensis* exhibited antifungal activity against *Candida albicans* and *Cryptococcus neoformans*. In another experiment by Kuglerova et al. (2011), *W. ugandensis* stem bark exhibited antifungal activity with an MIC of 256 mg/ml against *Candida albicans*. *Warburgia ugandensis* was also found to exhibit antifungal activity against *Candida utilis* (Kubo, 1995); Taniguchi et al., 1978).

2.4.9 Anti-mycobacterial activity

Dichloromethane extract of the stem bark of *W. ugandensis* demonstrated antimycobacterial activity against *Mycobacterium aurum, Mycobacterium fortuitum, Mycobacterium phlei* and *Mycobacterium smegmatis* (Wube et al., 2005). The active constituents showed minimum inhibitory concentration (MIC) values ranging from 4 to 128 mg/ml compared to the antibiotic drugs ethambutol and Isoniazid with had MICs ranging from 0.5 to 8 mg/ml and 1 to 4 mg/ml respectively.

2.4.10 Anti-inflammatory and antioxidant properties

Warburgia ugandensis showed anti-oxidative properties with a half maximal inhibitory concentration (IC50) of 6.59 mg/ml, which was very close to the inhibitory effect achieved by reference compound Trolox (IC50 of 3 mg/ml), suggesting strong potent anti-oxidative properties (Kuglerova et al, 2011).

2.4.11 Antiparasitic activity

The dichloromethane extract of *Warbugia* stem bark displayed strong antiplasmodial and antitrypanosomal activities with IC_{50} values of 8.10 and 1.10 µg/ml against the K1 strain of the malaria parasite and the STlB900 strain of *T. b. rhodesiense,* respectively. The drimane and coloratane sesquiterpenes possessing aldehyde groups at positions 8 and 9 showed the highest antitrypanosomal activity with IC_{50} values ranging from 0.14–1.97µg/ml (Wube et al., 2008). The anti-plasmodial activity of stem bark of *W. ugandensis* has also been demonstrated against *Plasmodium knowlesi* and *P. Berghei* (Were et al., 2010).

2.4.12 Cytotoxic, anthelmintic and antileishmanial activities

Mbwambo et al. (2009) demonstrated that ethanolic leaf extracts of *W. ugandensis* exhibited cytotoxic activity (95% CI), against brine shrimp larvae with reference to cyclophosphamide, a standard anticancer drug. Research by Xu et al. (2009), showed that the ethyl acetate extract of *W. ugandensis* bark exhibited potent cytotoxic activity on KB cell line at99% and 64% inhibition at 10 and 1 mg/ml, respectively.

Ngure et al (2009) demonstrated in vitro antileishmanial activity of *W. ugandensis* hexane extract against *Leishmania major* and *Leishmania donovani*. The hexane extract had the best activity against *L. major* promastigotes and amastigotes with IC_{50} value of 9.95 for promastigotes and 8.65 for amastigotes and MIC of 62.5 mg/ml (Ng'ang'a et al., 2009). The activity of the hexane extract on amastigotes was comparable to that of pentostam and amphotericin B. Similar results were obtained for *L. donovani* with IC_{50} values of 8.67 for promastigotes and 100-fold reduction of amastigotes in macrophage cultures.

The water and methanol extracts of the stem bark of *W. ugandensis* showed antileishmanial activity (with IC_{50} of 1.114 mg/ml against *Leishmania major*) and immunomodulative effects (Githinji et al., 2010).

2.5 Thin Layer Chromatography (TLC) analysis on members of Warburgia Species.

Thin Layer Chromatography analysis has been attempted before on some members of the genus *Warburgia*. In study by (MMUSHI, 2011), *Warburgia salutaris* TLC finger prints were successfully developed using three solvent systems and a number of spray agents. The solvent system of BEA (Benzene, Ethanol, and Ammonia) produced the best separation while the spray agent of Vanillin-Sulphuric acid produced the most intense colouration.

(ONDORA, 2016) after extensive method development, observed 18 and 16 spots from *W. ugandensis* ethyl acetate leaf and stem bark extracts respectively, with 8 spots of the leaf extract being seen with the naked eye after using mobile phase system of n-heptane: chloroform: acetone (30:20:10) and stationary phase of pre-coated silica gel F-254 plates at room temperature (22-25°C) and spraying with 0.5% anisaldhyde in sulphuric acid.

2.6 Toxicity of *Warburgia ugandensis* in Literature

A few studies have been conducted to assess the safety of *W.ugandensis.* In a study conducted by Ringim, Crespo and Khan (2017), different concentrations of aqueous and ethanol extracts of *W. ugandensis* with or without food were fed on *Drosophila melanogaster* and acute toxicity, locomotion assay, longevity study of the flies was done. Extracts of *W. ugandensis* were found to be nontoxic at acute exposure. The ethanol extract of *W. ugandensis* decreased

negative geotaxis ($P<0.01$) and so did the aqueous extract ($P<0.05$). However, longevity study showed toxicity of the ethanolic extract *of W. ugandensis* at concentration of 1% and 2%. However, it is difficult to extrapolate these studies to humans as a non-mammalian model was used.

In another study conducted by (Karani et al., 2013), the acute toxicity of *W. ugandensis* was assessed in BALB/c mice. Preliminary cytotoxic assessment of *Warbugi*a on the Growth of Vero E6 cells and the CC_{50} was determined to be greater than 250 μg/ml. The LD_{50} of the aqueous extract of *W. ugandensis* was determined to be greater than 5000 mg/kg bodyweight demonstrating the nontoxic nature of the aqueous extract at acute doses. This supports the starting dose of 2000 mg/kg for our acute studies. However, the study only investigated the aqueous extract, the subacute toxicity was not investigated and effects on the organ function and morphology was not investigated.

CHAPTER THREE: METHODS

3.1 Study Design and approach

This study involved an experimental study design using rats and the conduct of quality control tests. The Experimental design is a scientific approach to research, where one or more independent variables are manipulated and applied to one or more dependent variables to measure their effect on the latter. This design aids researchers in drawing a reasonable conclusion regarding the relationship between the two variable types. This was the ideal design to study the toxic effects of *W. ugandensis* extracts on the study animals. The approach of the study was mixed and involved the collection of both qualitative and quantitative data to better explain phenomena.

3.2 Study Setting

3.2.1 Mabira Forest

The study was conducted in the Central Region of Uganda between September and December of 2021 and involved the collection of leaves and stembark of *W. ugandensis* growing in Mabira Forest located at GPS coordinates 0.3894° N, 33.0057° E, about 60km from Kampala, the capital of Uganda.

The Central region was chosen because of the reported high rate of use of the plant by the native people (Tabuti, 2012). Considering the fact that local producers mostly wild source the plant, Mabira forest is one of the last remaining locations where sufficient numbers are growing together in the wild (under the protection of Uganda National Forests Authority) to provide the required quantity of plant material to be used in the study without jeopardizing the health of individual trees.

Mabira forest lies north of Lake Victoria with elevations ranging from 1070 to 1340 metres above sea level. The soils of Mabira forest are of two main kinds: red soil found on hill slopes and black clays in the valley bottoms often overlain by a few centimetres of peat produced by rotting swamp vegetation. These soils are rich in organic matter and nutrients with a mean annual rainfall of 1300mm, providing optimum conditions for tropical tree species like *W. ugandensis* to thrive (National Forest Authority, 2017).

3.2.1 Wistar Rats

Toxicity assessment was conducted following the oral route using OECD guidelines using Wistar rats. Wistar rats were the chosen strain because of their docility; relatively small size and easiness to handle and; uniform size. (Journal of Biotechnology and Biomedical Science,

2023). Only Female rats were used in the acute toxicity studies. According to the OECD, this is because literature surveys of conventional LD_{50} tests show that, although there is little difference in sensitivity between the sexes, in those cases where differences are observed females are generally slightly more sensitive (OECD, 2003). The subacute study involved the use of equal numbers of male and female rats as per OECD guidance in order to provide a balanced outcome.

3.3 Reagents, instruments/equipment and variables

3.3.1 Reagents

All laboratory reagents were analytical grade reagents manufactured by Sigma Aldrich and purchased from BHS laboratory suppliers Kampala Uganda. The following were the key reagents used; 99.9% analytical grade ethanol, distilled water, Acetone, Ethyl Acetate, Methanol, Hexane, Formalin and Xylene.

3.3.2 Key Instruments and equipment

The following equipment were used;

Extraction: Rotatory Evaporator, Hei-VAP model, serial number 200138825 0218 manufactured by Heidolph Instruments of Germany; Freeze Dryer, Model Free Zone 4.5L, -105^0C, serial number 77500204 manufactured by Labconco Corporation, USA.

HPTLC Analysis: Camag Automatic TLC Sampler 4, serial number 241613; Camag Automatic Developing Chamber 2, serial number 250052; Camag TLC Visualizer, serial number 230296 (white light, long-wave UV (366 nm) and short-wave UV (254)) all manufactured by Camag, Germany; Vortex Mixer, model number VM-10 manufactured by Witeg of Germany; TLC Plate Heater 2, manufactured by Camag, Germany.

Haematology and Clinical chemistry analysis: COBAS 6000 series Chemistry Analyzer manufactured by Roche Diagnostics of Switzerland and Haematology Analyzer, XN-9100, Manufactured by Sysmex Corporation of Japan.

Histology processing: Automatic benchtop Tissue Processor, model number 1020, Manual Rotatory Microtome Model number RM2235 all manufactured by Leica, Biosystems Germany.

Micrograph reading and imaging: Eclipse Ci-s microscope mounted with 4x, 10x and 20x objectives in combination with a Digital Sight -Fi1 digital camera head, serial number K16091 with 5.24-megapixel CCD all manufactured by Nikon Corporation, Japan.

3.3.3 Variables

3.3.3.1 Variables in HPTLC Analysis

Independent:

Categorical: nature of extract (leaf or bark extracts), solvents of extraction (methanol, ethyl acetate, acetone and hexane).

Dependent: Retardation Factors.

3.3.3.2 Variables in Toxicity Assessment

Dependent variable

Mortality, weight, water consumption, food consumption, weights and weight indices of liver, left and right kidneys, stomach and heart**,** levels of alanine aminotransferase (ALT), aspartate transferase (AST), albumin (ALB). creatinine (CRET), urea, sodium (Na), potassium (K), red blood cells (RBC), white blood cells (WBC), hematocrit (HCT), hemoglobin (HGB), platelets (PLT).

Independent variable

Categorical- Nature of extract i.e. *W. ugandensis* aqueous stembark extract (WUBA), *W. ugandensis* 70% ethanolic stembark extract (WUBE), *W. ugandensis* aqueous leaf extract (WULA), *W. ugandensis* 70% ethanolic leaf extract (WULE) and dose levels i.e. 250 mg/Kg 500 mg/Kg, 1000 mg/Kg.

3.4 Experimental Procedures

3.4.1 Collection and Preparation of Plant Material

Leaves (4Kg) and stem barks (4Kg) were collected from *W. ugandensis* trees growing in Mabira forest located in the Central region of Uganda. This region is known for the extensive use of the plant for its medicinal effects. A voucher specimen was prepared and subsequently identified and authenticated by botanist Ms Olivia Wanyana of the Makerere University Herbarium. The specimen was assigned a voucher number OJ001. The plant matter was collected using acceptable bio-conservation/sustainable harvesting techniques that involved harvesting from multiple trees in the same locality as opposed to one tree. The stembark was harvested as vertical narrow strips and care was taken to avoid extensive tree debarking.

3.4.2 Procedure for HPTLC analysis

3.4.2.1 Extraction Procedure for HPTLC

Powdered plant material (leaf and stem bark) (50 grams of each) were weighed in glass beakers and 500 mL of the extracting solvents (hexane, ethyl acetate, acetone and methanol) added and

extraction commenced via cold maceration at room temperature for 72 hours. The resultant extracts were filtered and concentrated with a rotary evaporator and stored at $<5^0$C.

3.4.2.2 Sample Preparation for HPTLC

Each extract was reconstituted by adding 0.5 grams of the extract to 5mL of the solvent used for extraction and vortex mixed at 1000 rpm and then filtered with a 0.45µm membrane filter pore size for HPTLC development. The reconstituted samples were put in HPTLC vials and stored in a refrigerator maintained at $<5^0$C.

3.4.2.3 Mobile phase development and optimization:

(Refer to Appendix 1: Chromatograms from Mobile Phase Optimization)

The mobile phase was initially optimized by trying 3 different mobile phase solvent systems of decreasing polarity originally developed by Kotzé et al (2002), however the genotoxic solvents i.e. benzene and chlorinated hydrocarbons chloroform, dichloro-methane used by Kotze et al (2002) were avoided because of safety concerns. After a number of trials, the following mobile phase systems were selected based on their separation ability and resolution. The hexane: acetone (70: 30) for the leaf extracts, and Ethyl acetate: Acetone: Hexane (10:5:3) for the stembark extracts.

3.4.2.4 Pre-washing and conditioning of plates*:*

The plates were prewashed in methanol and activated by placing them in an oven at 120°C for 15 minutes. The plates were initially checked for contamination in the TLC visualizer before placing them on to the automatic TLC sampler.

3.4.2.5 Sample Application

The HPTLC vials containing the sample extracts were placed on the sample port of Camag Automatic TLC Sampler. The conditioned plates were placed on the plate port of the sampler. The autosampler was programmed to apply a band of 2 microliters of each extract in triplicate to create a total of 12 tracks.

3.4.2.6 Mobile phase preparation

Thirty-five milliliters of the respective mobile phase systems were freshly prepared in a conical flask.

3.4.2.7 Chromatographic development and drying

The spotted plates were removed from the Automatic TLC sampler and placed in the chamber for automatic development with the following parameters: pre-drying enabled, saturation with pad for 20 minutes, humidity control at 33% Relative Humidity with magnesium chloride for 10 minutes, migration distance of 70mm, drying time of 5 minutes, 10ml of development solvent, 25ml of saturation solvent.

3.4.2.8 Visualization

The developed chromatograms were visualized in the Camag TLC visualizer coupled with Camag VISIONCATS software (version 3.0) in white light, UV 254nm and UV 366nm. The software was used to compute RF values for observed bands in each track.

3.4.2.9 Staining /Derivatization

Vanillin (15g) was added to 250mL ethanol and 2.5mL concentrated sulphuric acid. The freshly prepared solution was sprayed onto the developed chromatogram using a manual spray gun. The chromatogram was heated on the TLC plate heater at 105^0C degrees for 5 minutes and then visualized in white light.

3.4.3 Plant material extraction for Safety assessment

Clean stem barks (3.8 Kg) and leaves (3.8 Kg) were air-dried at room temperature under shade for 14 days and pulverized using a laboratory mill. Extraction was done by cold maceration, briefly, a hundred and fifty grams of each of the powdered plant material was soaked in 1500mLs of distilled water or 70% alcohol, shaken every morning and kept for a period of 72 hours. The extracts were initially filtered, and water extracts freeze-dried. The ethanol extracts were concentrated in a round bottomed flask using the rotary evaporator and then dried in an oven under low temperature (<45^0C) for 24 hours. The dry powders were weighed, labelled and stored in airtight bijou bottles at <4 °C.

3.4.4 Phytochemical screening of the extracts used in Toxicity Assessment.

Qualitative methods for detection of alkaloids, saponins, steroids, flavonoids, tannins and phenolic compounds originally documented by (Evans, 2009) (Sofowora, 1993)((Harborne, 1973) were used to test the extracts of WUBA, WULA, WUBE and WULE as described briefly under sections 3.4.4.1 to 3.4.4.8.

3.4.4.1 Mayer's Test for alkaloids

To one gram of each dry *W. ugandensis* extract in a test tube was added 4mLs of concentrated hydrochloric acid drop wise. The solution was divided into two portions; to the first portion was added 1mL of Mayer's reagent. Appearance of a yellow precipitate indicated presence of alkaloids.

3.4.4.2 Dragendorff"s Test for alkaloids

1mL of Dragendorff"s reagent was added to 2mLs of the extract from the second portion in a test tube, appearance of an orange red precipitate, indicated the presence of alkaloids.

3.4.4.3 Test for phytosterols and terpenoids (Liebermann–Burchard test)

One gram of each *W. ugandensis* extract was dissolved in acetic anhydride until the extract completely dissolved. To the solution was added 2mLs of chloroform. 5mLs of the mixture was pipetted into a dry test tube, then 2 drops of concentrated sulphuric was added. The appearance at the bottom of the mixture, of a green supernatant indicated the presence of steroids and triterpenoids.

3.4.4.4 Test for Tannins

One milligram of each *W. ugandensis* extract was dissolved in 1.5mLs of distilled water in a test tube and three drops of dilute ferric chloride solution was added drop wise. Appearance of a green black solution indicated the presence of catechol tannins.

3.4.4.5 Test for Saponins

To 1mg of each *W. ugandensis* extract was added 5mLs of distilled water and shaken vigorously. Appearance of a foam which persisted for more than 15 minutes indicated the presence of saponins.

3.4.4.6 Test for Flavonoid.

To 1mg of each extract was added 5mLs of distilled water, and to 1mL of the resultant solution was added 3 drops of concentrated sodium hydroxide, appearance of a white precipitate which disappeared on addition of a few drops of concentrated hydrochloric acid indicated the presence of flavonoids.

3.4.4.7 Test for Phenolic compounds.

To 1mg of each extract in a test tube, 5mLs of distilled water were added. To 1mL of the resultant solution, 3 drops of 10% lead acetate solution were added drop wise. Appearance of a white precipitate indicated the presence of phenolic compounds.

3.4.4.8 Test for Terpenoids (Salkowski's test)

To one gram of each *W. ugandensis* extract in a test tube was added 2mLs of chloroform followed by 3mLs of concentrated sulphuric acid to form a layer. A reddish colouration at the interface indicates presence of Terpenoids.

3.4.4.9 Reducing sugars (Fehling's test)

One gram of each *W. ugandensis* was shaken in 10mLs of distilled water and filtered.

1mL of the filtrate was treated with 1mL of Fehling's solution 1 followed by 1mL of

Fehling's solution 2 and boiled in a water bath for 3 minutes. Formation of a red precipitate indicated presence of reducing sugars**.**

3.4.5 Procedure for conducting acute toxicity assessment

Acute toxicity studies were conducted according to the OECD guideline for testing of chemicals 423 as described in the sections below (OECD, 2002).

3.4.5.1 Selection, housing, feeding and preparation of animals

Thirty healthy female nulliparous adult Wistar rats of age between 8 and 12 weeks sourced from COVAB were used. The rats were housed in the animal room at RTC maintained at $25^0C \pm 3^0$ temperature and, with artificial white light from an electric bulb source sequenced at 12 hours' light, 12 hours' dark. The animals were fed on a diet of fortified rice pellets with an unlimited supply of drinking water. The animals were randomly assigned, marked to permit individual identification, and kept in their cages (group-caged by dose) for at least 5 days prior to dosing to allow for acclimatisation to the laboratory conditions.

3.4.5.2 Randomization procedure

Simple randomisation was used. Each animal from a group of female animals was uniquely identified by a code and using a randomization chart generated by Microsoft Excel software Version 1808 (Build 10401.20025), 3 animals were randomly assigned to to five groups to receive either WUBA, WUBE, WULA, WULE extract or Control in the first step. In the second step, 3 animals from remaining animals were randomly assigned to a second set of five groups to receive either WUBA, WUBE, WULA, WULE extract or Control in the second step.

3.4.5.3 Preparation and administration of doses

The test substances were formulated as suspensions in distilled water. Doses were prepared shortly prior to administration. The animals were fasted prior to dosing with withholding of food but allowed water over night. Following the period of fasting, the animals were weighed. The dose volume was calculated for each individual animal, on the basis of their body weights. The test substances were administered in a single dose by oral gavage using an intubation cannula. The control group received distilled water at the same dose volume. After the test substances had been administered, food was withheld for another further 3-4 hours.

3.4.5.4 Rationale for the starting dose of 2000mg/kg

The OECD test guidelines prescribe four starting doses i.e. 5, 50, 300 and 2000mg/Kg of body weight. The highest starting dose was selected on the basis of LD50 results obtained

by Karani et al (2013) for *Warburgia ugandensis* aqueous stembark which suggested that the extract was likely to be nontoxic at the lower concentrations.

3.4.5.5 Observations

3.4.5.5.1 Clinical Observations

Animals were observed individually after dosing, at least once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours, and daily thereafter, for a total of 14 days.

3.4.5.5.2 Body weight.

Individual weights of animals were determined shortly before the test substance was administered and weekly thereafter. Weight changes were calculated and recorded. At the end of the test, all surviving animals were weighed and sacrificed.

3.4.5.5.3 Histopathology.

All surviving test animals were sacrificed. The stomach. Liver, small intestine and kidney of each sacrificed animal were harvested and fixed in 10% Formalin. Stomach, small intestine, liver and kidneys from randomly selected animals from each treatment group and control groups were sent for histology tissue preparation at the histopathology laboratory of the Department of Pathology, COVAB.

3.4.5.5.4 Histology Tissue processing and observation

Tissue processing prior to staining was conducted in the following steps using an Automatic Tissue processor: Loading onto embedding cassettes, dehydration with absolute alcohol, removal of the alcohol using xylene and infiltration of the tissue with paraffin wax. The processed tissues were then mounted on the rotary microtome and sectioned. The sections were then stained with haematoxylin and eosin and mounted on to slides. The prepared slides were observed and photographed using the Microscope Camera system to identify any changes in the microanatomy of the organs caused by the test substances.

3.4.6 Procedure for Sub-Acute Toxicity Assessment

Sub-acute toxicity was assessed following the OECD test 407 (OECD, 2008), as described in the following section.

3.4.6.1 Selection, housing, feeding and preparation of animals

The study used 170 young healthy adult Wistar rats of both sexes obtained COVAB. Females were nulliparous and non-pregnant. The animals were kept in an experimental animal room maintained at a temperature of $25^0C \pm 3^0$. White light from an electric bulb was provided with the photoperiod being 12 hours' light, 12 hours' dark. The animals

were fed on a diet of fortified rice pellets with an unlimited supply of drinking water. Animals were group housed in groups of 5 of the same sex per cage. The animals were uniquely identified and kept in their cages for at least five days prior to the start of the tests to allow for acclimatisation to the laboratory conditions.

3.4.6.2 Randomization procedure

Stratified Randomization was used. The animals were initially divided into two strata of male and female rats and each stratum was subjected to simple randomization to different test groups as follows: Every animal from each stratum was initially identified with a unique code, using a randomization chart generated by Excel software Version 1808 (Build 10401.20025). Five animals of each sex (5) were randomly assigned to each test group. A total 17 groups were created **(*Table 2*).**

3.4.6.3 Preparation and administration of doses

The test substances were formulated as suspensions in distilled water. The formulations were prepared shortly prior to administration by mixing the extracts with lukewarm distilled water. The animals were fasted prior to dosing with withholding of food but allowed water over night. Following the period of fasting, the animals were weighed. The dose volume was calculated for each individual animal, on the basis of their body weights. The animals were administered the test substances between 10:00 to 11:00 hours daily for a total of 28 days by oral gavage through a single dose using a gavage tube. The control group received distilled water while satellite groups of animals (five per sex) in the top dose group for each extract were kept. The satellite groups were kept for an extra 14 days without the administration of extracts to detect delayed occurrence, or persistence of, or recovery from toxic effects.

Table 2: Summary of Subacute Toxicity Treatment Groups

GROUP	DOSE (mg/Kg)	Number of rats (Sex)
CONTROL	N. A	10 (f=5, m= 5)
WUBA	250	10(f=5, m= 5)
	500	10(f=5, m= 5)
	1000	10(f=5, m= 5)
	Satellite 1000	10 (f=5, m= 5)
WUBE	250	10(f=5, m= 5)
	500	10(f=5, m= 5)
	1000	10(f=5, m= 5)
	Satellite 1000	10 (f=5, m= 5)
WULA	250	10 (f=5, m= 5)
	500	10 (f=5, m= 5)
	1000	10(f=5, m= 5)
	Satellite 1000	10 (f=5, m= 5)
WULE	250	10(f=5, m= 5)
	500	10(f=5, m= 5)
	1000	10(f=5, m= 5)
	Satellite 1000	10(f=5, m= 5)

Note: WUBA- aqueous stembark extract, WUBE- 70% ethanol stembark extract, WULA- aqueous leaf extract, WULE- 70% ethanol leaf extract.

3.4.6.4 Observations

3.4.6.4.1 Mortality and clinical observations

General clinical observations were made once a day between 09:00 to 10:00 hours, just before dosing for that day. The health condition of the animals was recorded. Animals were observed for morbidity and mortality.

3.4.6.4.2 Body weight and food/water consumption

All animals were weighed before dosing on the first day initially and then weekly thereafter. Measurements of food consumption was made weekly and expressed as daily food consumption.

3.4.6.4.3 Gross necropsy

At the end of the test period, all animals were sacrificed. The animals were given anaesthesia in a chloroform chamber, a longitudinal cut made to expose the heart, fresh blood was drawn from the animals and placed in red and blue top vacutainers for haematology and clinical chemistry respectively.

The stomach, liver, kidneys and heart were harvested and trimmed of any adherent tissue. The weights of the stomach, right and left kidneys, heart and liver were taken and the corresponding weight indices calculated by dividing the organ weight with the weight of the corresponding animal and multiplying by a factor of 1000.

3.4.6.4.4 Haematology

The levels of the following haematological parameters were measured at the end of the test period using a Haematology Analyzer at Mulago National Referral Hospital Private Laboratory to investigate the effects of the extracts on blood and bone marrow: haematocrit, haemoglobin concentrations, red blood cell count, white blood cell count and platelet count.

3.4.6.4.5 Clinical biochemistry

The plasma levels of clinical chemistry parameters were measured at the end of the test period using a Chemistry Analyser at Mulago National Referral Hospital Private Laboratory, to investigate major toxic effects on kidney and liver. The parameters measured included albumin, aspartate aminotransferase, alanine aminotransferase, creatinine, urea, sodium and potassium.

3.5 Statistical Analysis

The means and standard deviations of weight, food consumption, water consumption, and levels of haematological and clinical chemistry parameters were determined and presented in tables. The statistical significance of the differences observed in means of various parameters between dose groups was evaluated by one-way ANOVA and presented in the tables. Differences were considered statistically significant if $p<0.05$. A post Hoc Dunnett's test was conducted to reveal differences between treatment groups and the control group. Differences were considered statistically significant if $p<0.05$ and were presented as Compact Letter Displays (CLD) in the various tables with **A** representing groups were the observed difference

in means is statistically significant versus the control group and **B** were the observed difference in means is not statistically significant versus the control group.

3.6 Ethical Consideration

The study was approved by the School of Health Sciences Research Ethics Review Committee as under protocol version number 01. The approval letter is attached in (Appendix 2). Laboratory animals were treated humanely according to the WHO and ICLAS guidelines for the Breeding and Care of Laboratory Animals. A minimum number of rats required to obtain statistically reliable data were used in the experiments. The rats were housed in appropriate laboratory facilities and fed on a standard laboratory diet with unlimited supply of water. All surviving animals from the study were humanely sacrificed and subjected to gross necropsy as is directed by the OECD guidelines.

Laboratory Waste Management and Disposal was done according to the guidelines in the document "Prudent Practices for Disposal of Chemicals from Laboratories; Prudent Practices in the Laboratory: Handling and Disposal of Chemicals" published by the National Research Council of the American Academy of Sciences. All animal waste, body parts, syringes were incinerated using the College of Veterinary medicine incinerator.

CHAPTER FOUR: RESULTS

4.1 Preliminary Assessments

4.1.1 Percentage yields of the crude plant extracts

Extraction of 150g of *W. ugandensis* stem bark powder in water yielded a mean of 18.60 grams of a pale brown extract, giving the highest average yield of 12.40%. On the other hand, *W. ugandensis* leaf powder extracted in water gave the lowest average yield of 10.10% (**Table 3**).

Table 3: Average yield and appearance of each extract (n=7)

EXTRACT	Plant MASS (g)	Average YIELD (g) and SD	Percentage Yield (%)	Appearance
WUBA	150	18.60±0.57	12.40	Pale brown
WUBE	150	18.10±0.85	12.06	Pale brown
WULA	150	15.25±1.06	10.10	Light green
WULE	150	16.30±0.56	10.87	Light green

Note: WUBA- aqueous stembark extract, WUBE- 70% ethanol stembark extract, WULA- aqueous leaf extract, WULE- 70% ethanol leaf extract.

4.2 Phytochemical screening (Toxicity test extracts)

Warbugia ugandensis aqueous and 70% ethanolic stembark extracts, were found to contain saponins, alkaloids, flavonoids, phenols and tannins. Terpenoids were detected in all but aqueous leaf extracts (**Table 4**).

Table 4: Summary of results of phytochemical screening of *W. ugandensis* bark and leaf extracts.

Phyto-compound	**Chemical test**	**EXTRACT**			
		WUBA	WUBE	WULA	WULE
Saponins	Froth	+	+	+	-
Alkaloids	Dragendroff's	+	+	+	+
	Mayer's	+	+	+	+
Flavonoids	Alkaline reagent	+	+	+	+
Phenols	Lead acetate	+	+	+	+
Tannins	Ferric chloride	+	+	+	+
Phytosterols	Libermann-Burchard's test	+	_	-	-
Terpenoids	Salkowski's	+	+	-	+
Reducing sugars	Fehling's	+	+	+	+

Note: (**-):** negative test (absence of turbidity, flocculation or precipitation, (**+**): positive test (presence of turbidity, flocculation or precipitation). WUBA- aqueous stembark extract, WUBE- 70% ethanol stembark extract, WULA- aqueous leaf extract, WULE- 70% ethanol leaf extract.

4.3 Results of HPTLC Fingerprinting

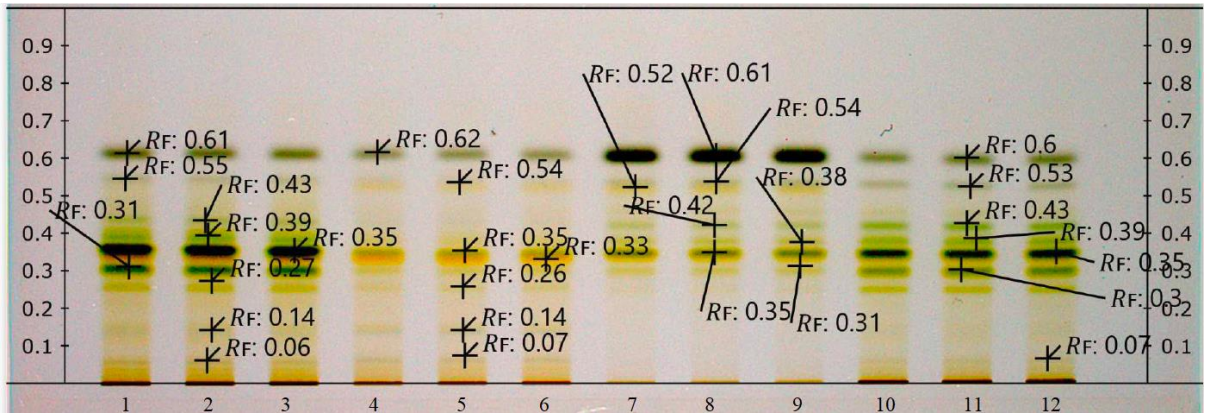
The acetone extract of the leaf showed the highest number of bands in both white light and derivatized modes of visualization with 9 and 12 bands respectively corresponding to a retardation factor (RF) range of 0.06 to 0.90. On the other hand, the acetyl acetate extract of the stembark produced the highest number of bands in the derivatized chromatogram with 7 corresponding to an RF range of 0.04-0.87. (**Table 5**).

Table 5: A summary of HPTLC analysis for the leaf and bark fingerprints in respect to extraction solvents, method of visualization and RF range.

Plant Part	Solvent	Mode of Visualization	White light	UV 254nm	UV 366nm	Derivatized
Leaf	Acetone	Number of bands	9	12	9	9
		RF range	0.06-0.61	0.14-0.71	0.06-0.70	0.15-0.90
	Ethyl acetate	Number of bands	7	11	8	8
		RF range	0.07-0.62	0.15-0.76	0.07-0.70	0.16-0.91
	Hexane	Number of bands	7	6	7	7
		RF range	0.31-0.61	0.06-0.70	0.06-0.70	0.26 -0.91
	Methanol	Number of bands	7	8	9	8
		RF range	0.07-0.6	0.20-0.61	0.06-0.70	0.27-0.91
Stem bark	Acetone	Number of bands	0	6	8	6
		RF range	N. A	0.05-0.87	0.03-0.88	0.15-0.79
	Ethyl acetate	Number of bands	0	7	9	8
		RF range	N. A	0.06-0.86	0.09-0.88	0.04-0.87
	Hexane	Number of bands	0	4	2	3
		RF range	N. A	0.06-0.87	0.79-0.88	0.07 -0.88
	Methanol	Number of bands	0	6	8	5
		RF range	N. A	0.07-0.86	0.07-0.88	0.34-0.88

Note: RF-Retardation factor. N.A- Not applicable.

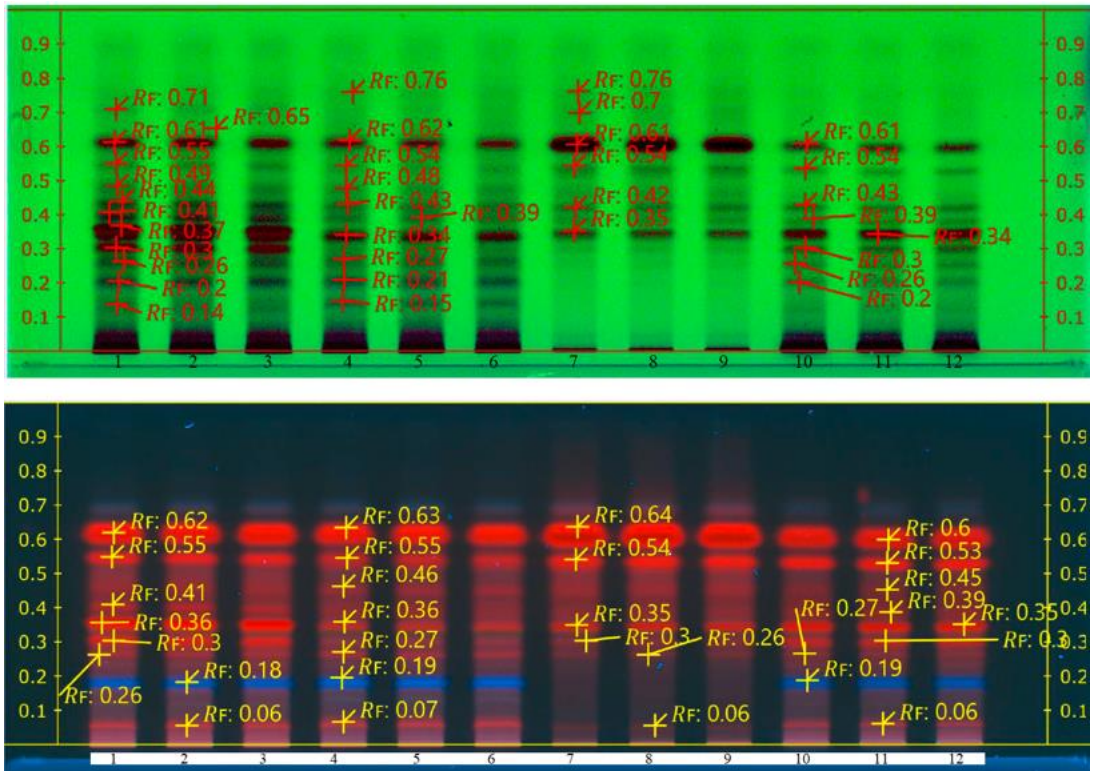
Close examination of the HPTLC leaf fingerprint in white light revealed 9 bands in acetone and 7 bands in each of ethyl acetate and methanol extract tracks. Furthermore, the acetone and methanol tracks produced the most intense colouration. The bands were in colours ranging from grey, yellow to green. (**Figure 5**).



Note: Track 1-3; acetone extract, track 4-6; ethyl acetate extract, track 7-9; hexane extract, track 10-12; methanol extract.

Figure 5: HPTLC Leaf Fingerprint in white light.

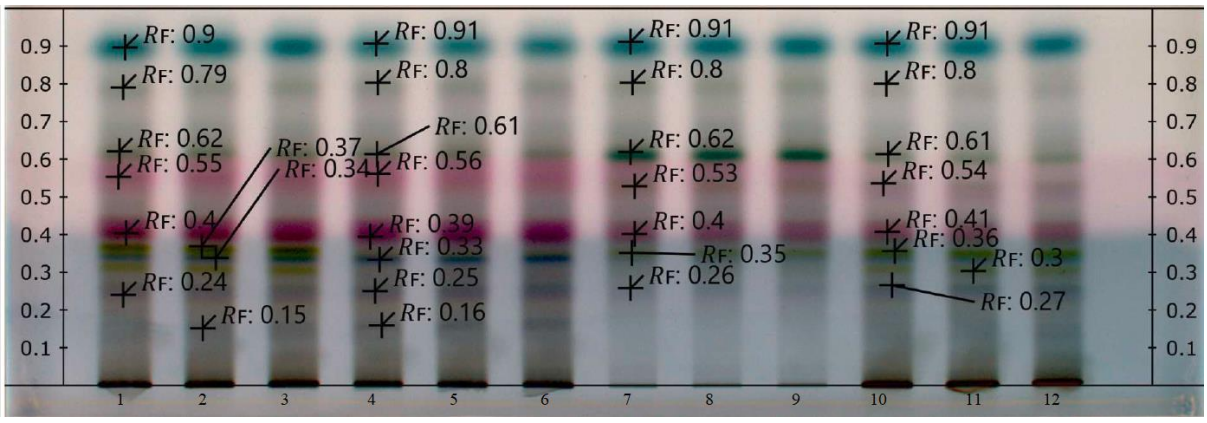
Examination of the leaf fingerprint in UV 366nm showed intensely coloured bands with 9, 8, 7 and 9 bands seen in the acetone, ethylacetate, hexane and methanol tracks respectively. On the other hand, the 254nm chromatogram showed 12, 11, 6 and 8 bands in the acetone and ethylacetate, hexane and methanol tracks respectively. (**Figure 6**).



Note: **Track 1-3;** **acetone extract, track 4-6; ethyl acetate extract, track 7-9; hexane extract, track 10-12; methanol extract**.

Figure 6: Leaf Fingerprint in UV 254nm (top) and 366nm (bottom)

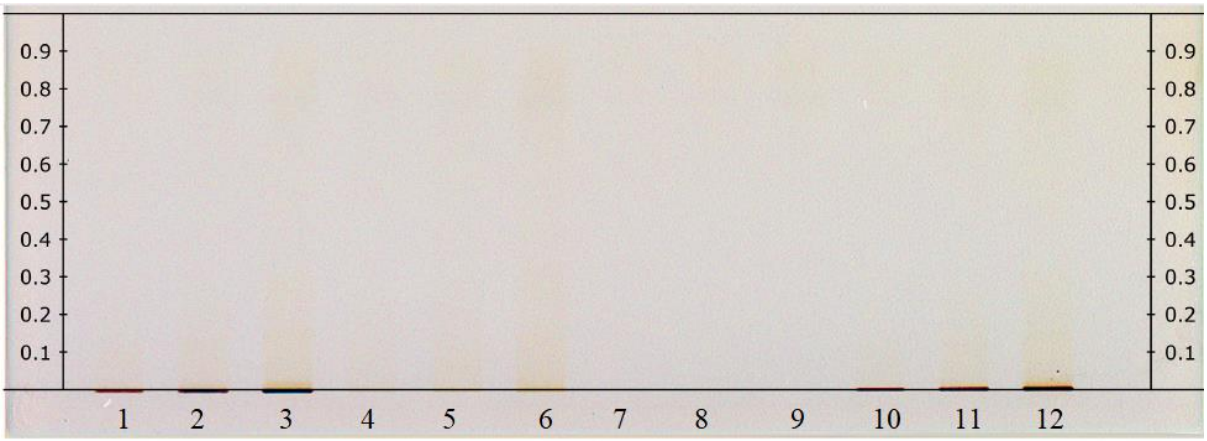
Spraying of the Leaf fingerprint produced intensely coloured bands, ranging from blue, purple to red in white light. Closer examination of the derivatized fingerprint showed 9,8,7 and 8 bands in acetone, ethyl acetate, hexane and methanol tracks respectively. (**Figure 7**).



Note: Track 1-3; acetone extract, track 4-6; ethyl acetate extract, track 7-9; hexane extract, track 10-12; methanol extract.

Figure 7: Sprayed HPTLC Leaf Fingerprint.

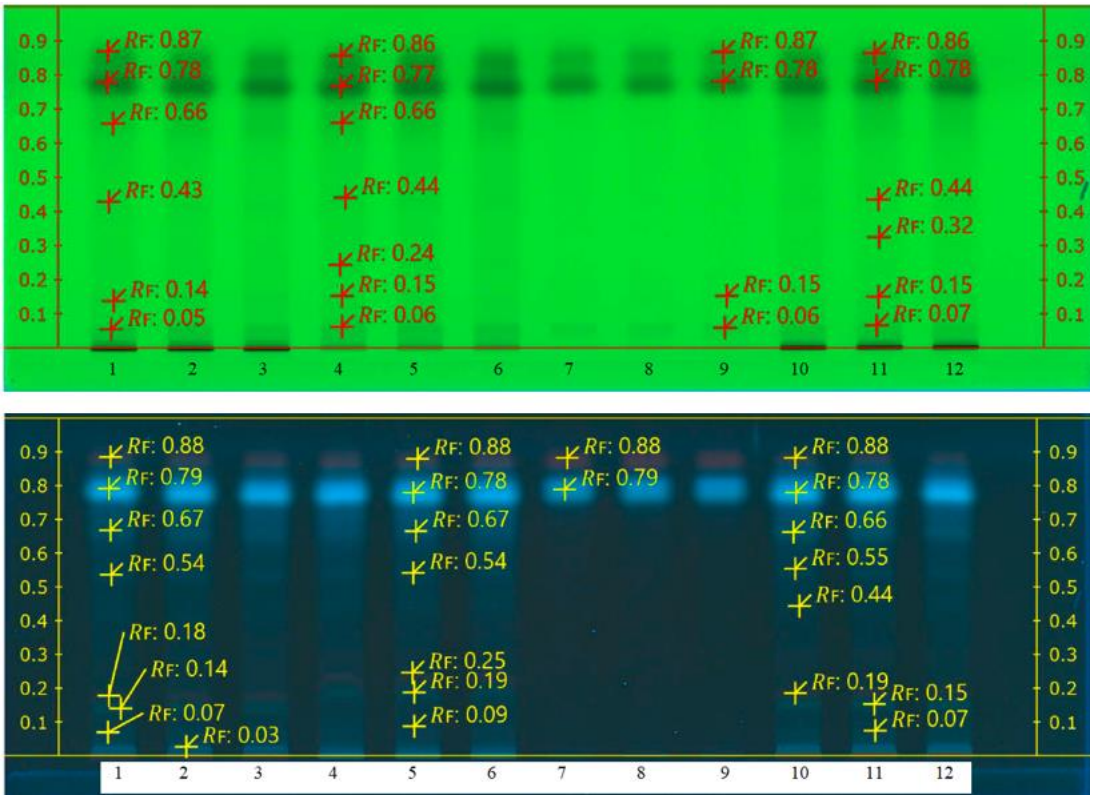
There were no bands observed on the HPTLC stembark fingerprint in white light (**Figure 8**).



Note: Track 1-3; acetone extract, track 4-6; ethyl acetate extract, track 7-9; hexane extract, track 10-12; methanol extract

Figure 8: Stembark HPTLC Fingerprint in white light.

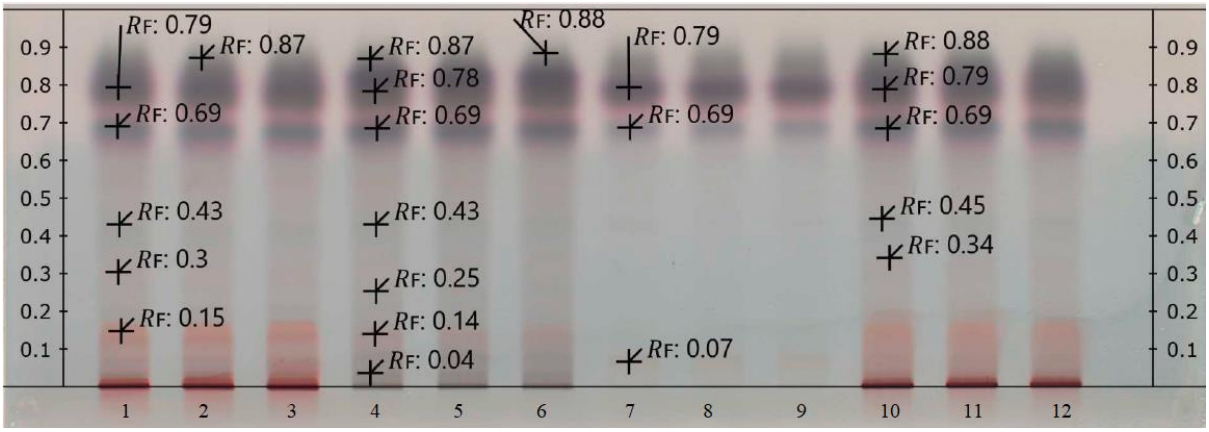
Observation in UV 254nm of the stembark finger print showed 6,7, 4 and 6 bands in the acetone, ethyl acetate, hexane and methanol tracks respectively. On the other hand, observation of the stembark finger print in 366nm UV showed 8,9,2 and 8 bands in acetone, ethyl acetate, hexane and methanol tracks respectively (**Figure 9**).



Note: **Tracks 1-3; acetone extract, track 4-6; ethyl acetate extract, tracks 7-9; hexane extract, tracks 10-12; methanol extract**.

Figure *9:* Stembark fingerprint in UV 254nm (top) and 366nm (bottom).

Examination of the derivatized/stained stembark finger print in white light showed 6,8,3 and 5 bands in the acetone, ethyl acetate, hexane and methanol tracks respectively (**Figure 10**).



Note: **Tracks 1-3; acetone extract, track 4-6, ethyl acetate extract, tracks 7-9 hexane extract, tracks 10-12: methanol extract.**

Figure 10: Derivatized Stembark HPTLC fingerprint in white light

4.4 Results of Acute Toxicity

4.4.1 Mortality and clinical observations

There were no mortalities after dosing with aqueous and 70% ethanolic leaf and stembark extracts at a dose of 2000 mg/Kg. All animals exhibited acute symptoms of raised fur and abdominal spasms within 3 hours of dosing but resolved in 24 hours. General body weakness, polyuria and increased defaecation were observed up to one week after dosing but resolved completely by the 14^{th} day.

4.4.2 Effect on animal weight

All animals given 2000mg/Kg of acute dose of *W. ugandensis* extracts i.e. WUBA, WUBE, WULA and WULE gained weight. The animals showed stable increase in body weight over two weeks. ANOVA revealed no statistically significant differences in the average weight gained amongst the treatment groups (p=0.45) (**Table 6**).

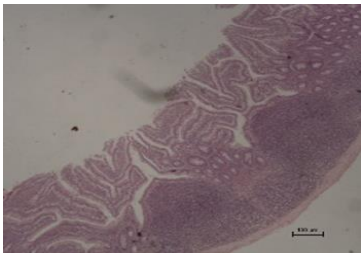
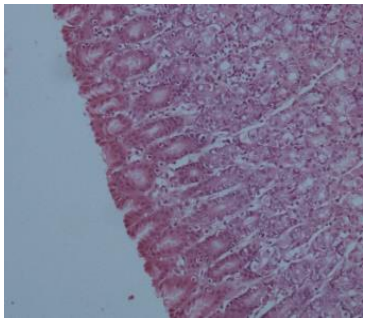
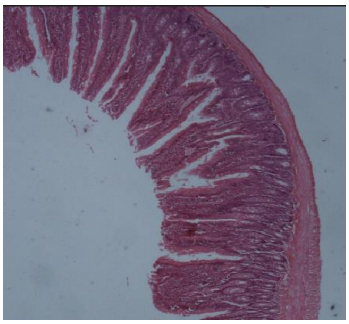
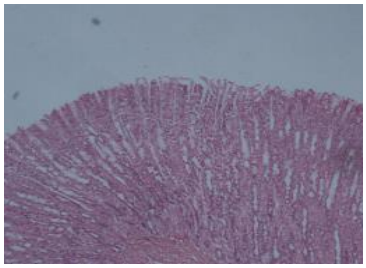
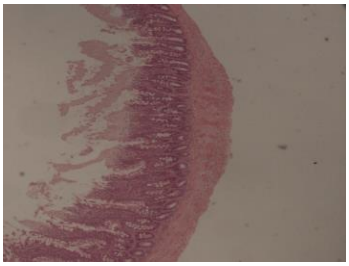
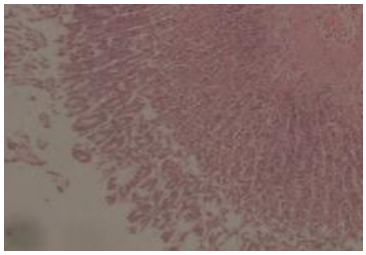
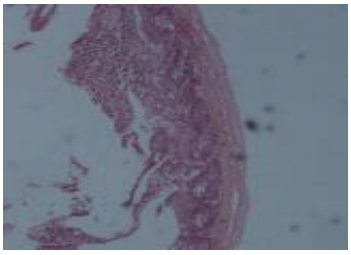
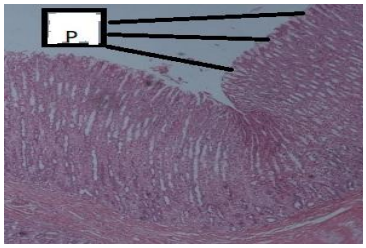
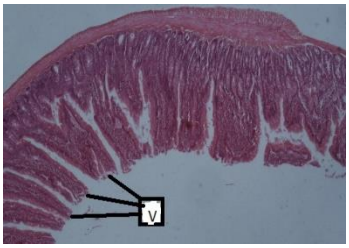
Table 6: Effect of *W. ugandensis* extracts on the average weight (g) of the animals (n=6)

GROUP (n=6)	INITIAL	WEEK 1	WEEK 2	Average Weight gain
WUBA	115.20±3.56	119.30±4.53	140.80±5.54	25.8±1.98
WUBE	116.40±5.85	118.50±5.74	138.30±6.56	22.3±1.71
WULA	108.10±5.14	111.70±5.67	130.01±5.90	21.9±2.46
WULE	116.60±5.94	120.70±6.56	142.90±6.50	26.3±2.56
Control	112.30±4.56	119.40±6.54	139.04±7.60	27.0±3.04

Note: WUBA- aqueous stembark extract, WUBE- 70% ethanol stembark extract, WULA- aqueous leaf extract, WULE- 70% ethanol leaf extract.

4.4.3 Histopathology

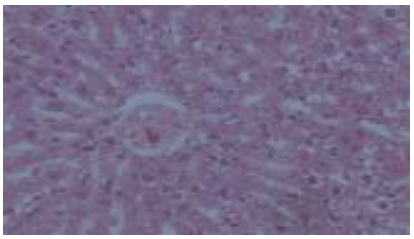
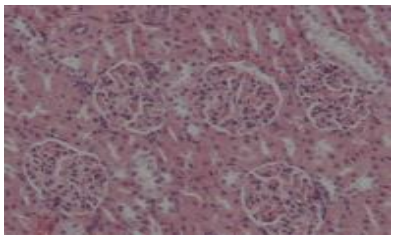
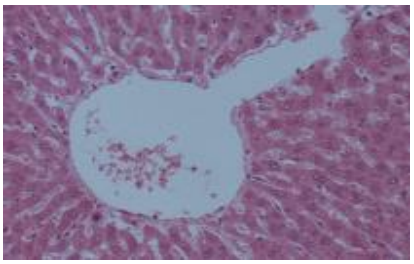
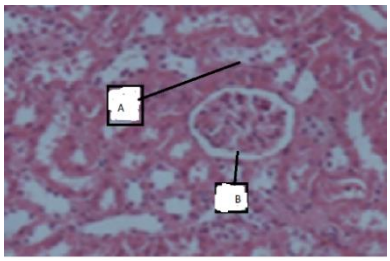
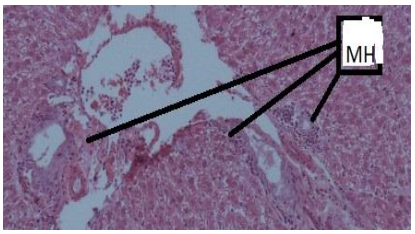
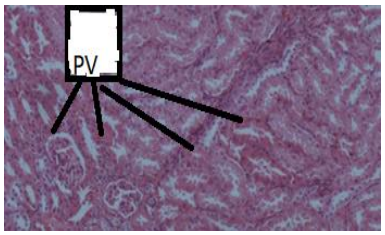
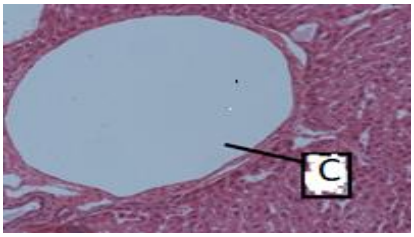
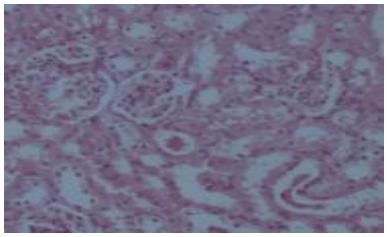
Photomicrograms of the intestine and stomach of WUBA, WUBE and WULE animals appeared normal under the microscope. However, mild epithelial sloughing and mucosal erosion were observed in photomicrographs of the stomach and intestine respectively obtained from WULA animals (**Figure11**).

	STOMACH	INTESTINE
CONTROL		
	NSL	NSL
WUBA		
	NSL	NSL
WUBE		
	NSL	NSL
WULA		
	Mild Epitherial slothing	Mucosal Erosion
WULE		
	NSL	NSL

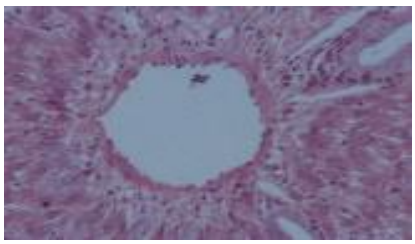
Note: WUBA- aqueous stembark, WUBE- 70% ethanol stembark, WULA- aqueous leaf, WULE- 70% ethanol leaf, P-stomach pits, v-intestine villi, NSL-No significant lesions.

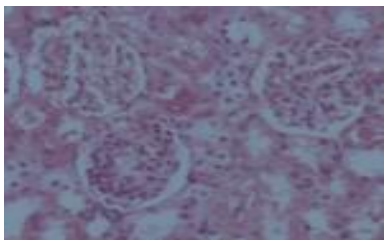
Figure 11: Photomicrographs of stomachs and intestines from acute toxicity tests.

Sections of the livers and kidneys of animals dosed with 2000mg/Kg of WUBA, WULA and WULE appeared normal under the microscope (**Figure 12**). Photomicrographs showed no significant lesions. On the other hand, mild periportal hepatitis and perivascular nephritis were observed in photomicrographs taken of the liver and kidney respectively of animals given WUBE (**Figure 12**).

	Liver	Kidney
CONTROL		
	NSL	NSL
WUBA		
	NSL	NSL
WUBE		
	Mild periportal hepatitis	Perivascular nephritis
WULA		
	NSL	NSL

WULE





NSL NSL

Note: A-intact tubules, B-intact Bowman capsule C-intact central portal vein, MH-mild periportal hepatitis, PV-perivascular nephritis, NSL- No significant lesion.

Figure 12: Photomicrographs of the livers and kidneys from acute toxicity tests.

4.5 Results of Subacute Toxicity

4.5.1 Animal Characteristics

Animal baseline characteristics are summarised in **Table 7**. The weights of the animals ranged from 100 to 135 grams. There was a total of 17 groups with each group consisting of ten animals, half of animals from each sex.

Table 7 :Summary of animal baseline characteristics for subacute tests

GROUP	DOSE (mg/Kg)	Average Weight (g) and SD
CONTROL	N.A	124.00±3.32
WUBA	250	121.20±6.35
	500	122.50±5.06
	1000	122.00±4.07
	SAT 1000	123.70±3.08
WUBE	250	125.70±5.30
	500	121.70±8.14
	1000	128.50±4.50
	SAT 1000	120.20±3.70
WULA	250	124.70±5.37
	500	125.20±4.84
	1000	122.00+9.30
	SAT 1000	122.20±2.39

WULE	250	122.90±5.23
	500	121.40±7.81
	1000	121.60±6.19
	SAT 1000	122.50±4.40

Note: WUBA- aqueous stembark extract, WUBE- 70% ethanol stembark, WULA- aqueous leaf, WULE- 70% ethanol leaf extract, F-female, M-Male, SD- Standard Deviation. SAT- Satellite.

4.5.2 Food, Water Consumption and Weight

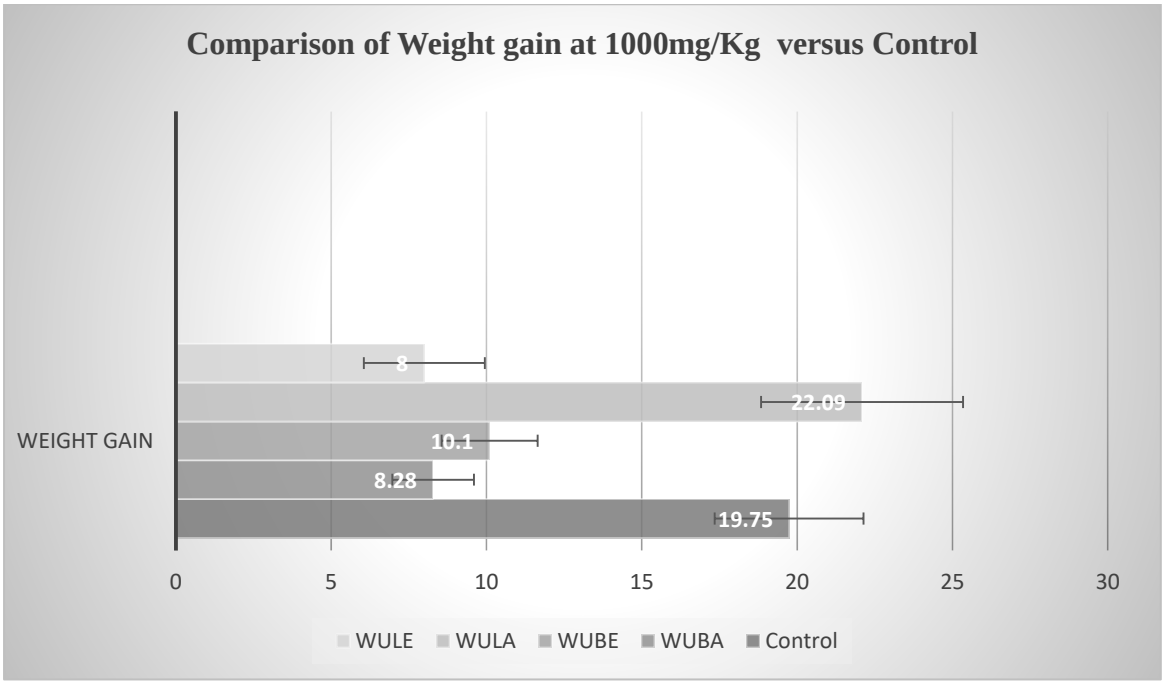
There were statistically significant differences in the average weight gained and in the daily water and food consumption between groups for all extracts of *W. ugandensis* administered. All treatment groups reported a positive weight difference over 28 days across all doses of the four extracts of *W. ugandensis*. The post hoc test revealed statistically significant differences between the control group and various treatment groups in both food consumption and weight gain. Of note, food consumption was increased at all doses in WULE and WUBE animals compared to control group but not in the corresponding satellite groups whereas WUBA, WUBE and WULE animals reported lower average weight gain compared to the control group at doses of 500 and 1000mg/Kg and in the corresponding satellite groups. (**Table 8**).

Table 8 : Effect of *W. ugandensis* extracts on food, water consumption and weight gain.

EXTRACT	Parameter	DOSE (mg/Kg) Control	250	500	1000	SAT 1000	ANOVA Test (P-value)
WUBA	Weight	19.75±6.66	19.57±5.34 [B]	15.36±1.56 [A]	8.28±1.33 [A]	13.34±6.77 [A]	**<0.001**
	Water	140.27±24.23	103.02±8.73 [A]	116.66±13.70 [A]	133.77±19.47 [B]	150.79±26.15 [A]	**<0.001**
	Food	125.94±14.79	99.63±13.31 [A]	105.51±15.29 [A]	122.97±7.71 [B]	138.34±17.53 [A]	**<0.001**
WUBE	Weight	19.75±6.66	17.89±4.13 [B]	12.55±3.64 [A]	10.10±2.44 [A]	4.44±2.34 [A]	**<0.001**
	Water	140.27±24.23	109.64±43.48 [A]	108.66±37.71 [A]	133.93±33.89 [B]	97.64±15.81 [A]	**<0.001**
	Food	125.94±14.79	138.98±21.59 [A]	148.56±17.55 [A]	167.66±21.08 [A]	120.34±9.11 [B]	**<0.001**
WULA	Weight	19.75±6.66	19.46±5.41 [B]	17.31±5.18 [B]	22.09±7.45 [B]	10.58±6.84 [A]	**<0.001**
	Water	140.27±24.23	119.54±23.14 [A]	122.23±20.21 [A]	120.04±19.82 [A]	148.95±19.08 [B]	**<0.001**
	Food	125.94±14.79	127.40±6.02 [B]	131.01±7.51 [B]	134.79±8.69 [A]	152.33±34.59 [A]	**<0.001**
WULE	Weight	19.75±6.66	11.75±.45 [A]	11.76±2.33 [A]	8.00±2.84 [A]	10.78±4.05 [A]	**<0.001**
	Water	140.27±24.23	98.66±30.90 [A]	103.57±29.37 [A]	126.88±30.43 [A]	112.98±13.25 [A]	**<0.001**
	Food	125.94±14.79	137.82±21.21 [A]	145.46±17.57 [A]	154.48±18.15 [A]	131.51±9.48 [B]	**<0.001**

Note: All values stated as mean and standard deviation. Weight in grams, water consumption in mLs per day and food consumption in grams per day. WUBA- aqueous stembark extract, WUBE- 70% ethanol stembark extract, WULA- aqueous leaf extract, WULE- 70% ethanol leaf extract. [A] Value statistically significant from control group. [B] Not statistically significant from control group**.**

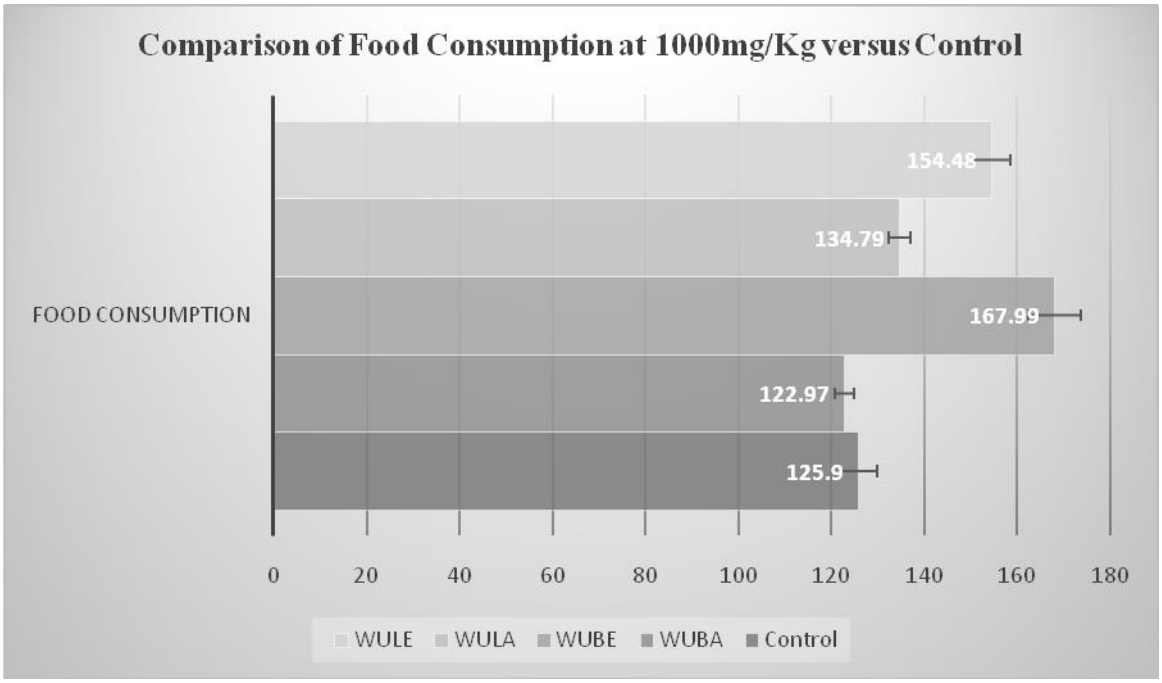
At the dose of 1000mg/Kg, the groups that received extracts of WUBA, WUBE and WULE reported less average weight gained ($p<0.05$) compared to the control group (**Figure 13**).



Note: WUBA-stembark aqueous extract, WUBE-stembark 70% ethanol extract, WULA-leaf aqueous extract, WULE-leaf 70% ethanol extract**.**

Figure 13: Comparison of weight gain at the dose of 1000mg/Kg of different extracts with the control group.

At 1000mg/Kg the groups that received extracts of WUBE, WULA and WULE reported greater average daily food consumption ($p<0.05$) compared to control group (**Figure 14**).



Note: WUBA-stembark aqueous extract, WUBE-stembark 70% ethanol extract, WULA-leaf aqueous extract, WULE-leaf 70% ethanol extract.

Figure 14: Comparison of Food Consumption rates at the dose of 1000mg/Kg for different extracts with the control group.

4.5.3 Mortality

There were two mortalities reported of one male rat each from the WULA 500mg/Kg and WUBA 500mg/Kg groups during the first week of dosing. The dead animals were observed to have physical body injuries consistent with bites.

4.5.4 Organ weights and indices

Organ weights

There were statistically significant differences reported in the average weights of the hearts, stomachs, livers and kidneys between dose groups for animals that received WUBE and WULE extracts. In animals that received WULA extracts, differences between dose groups were reported only in the masses of the livers. (**Table 9**).

Table 9: The weights of various key organs of animals dosed with extracts of *W. ugandensis* after 28 days.

EXTRACT	ORGAN	Weights (g)					
			Dose (mg/Kg)				
		CONTROL	250	500	1000	SAT 1000	ANOVA (p-value)
WUBA	Heart	0.84±0.07	1.28±2.01	0.64±0.07	0.75±0.08	0.82±0.91	0.45
	Stomach	2.33±0.20	1.72±0.12	1.66±0.15	1.80±0.27	1.86±0.41	<0.01
	Liver	8.10±1.23	4.00±2.41	5.33±1.00	5.75±0.33	5.12±1.00	0.01
	L. K	0.73±0.07	0.54±0.08	0.54±0.07	0.59±0.07	0.48±0.11	<0.01
	R. K	0.65±0.05	0.54±0.05	0.50±0.07	0.57±0.05	0.55±0.16	<0.01
WUBE	Heart	0.84±0.07	0.78±0.12	0.76±0.20	0.95±0.05	0.58±0.06	<0.01
	Stomach	2.33±0.20	1.80±0.20	1.68±0.42	2.18±0.27	1.78±0.41	<0.01
	Liver	8.10±1.23	8.24±1.82	7.76±1.19	8.99±1.47	4.47±1.16	<0.01
	L. K	0.73±0.07	0.67±0.15	0.69±0.25	0.89±0.09	0.47±0.13	<0.01
	R. K	0.65±0.05	0.65±0.12	0.67±0.25	0.84±0.12	0.51±0.16	<0.01
WULA	Heart	0.84±0.07	0.72±0.06	0.74±0.07	0.80±0.11	0.69±0.23	0.07
	Stomach	2.33±0.20	2.24±0.26	1.88±0.15	2.33±0.21	2.22±0.66	0.05
	Liver	8.10±1.23	5.69±0.82	5.78±1.20	8.33±1.54	5.28±0.77	<0.01
	L. K	0.73±0.07	0.60±0.11	0.58±0.08	0.66±0.10	0.62±0.21	0.08
	R. K	0.65±0.05	0.58±0.08	0.58±0.08	0.66±0.05	0.61±0.13	0.11
WULE	Heart	0.84±0.07	0.73±0.08	0.66±0.07	0.85±0.19	0.50±0.08	<0.01
	Stomach	2.33±0.20	1.61±0.23	1.24±0.21	1.69±0.49	2.13±0.28	<0.01
	Liver	8.10±1.23	8.27±1.40	7.03±1.19	8.92±2.15	5.07±0.50	<0.01
	L. K	0.73±0.07	0.61±0.10	0.52±0.04	0.80±0.23	0.52±0.15	<0.01
	R. K	0.65±0.05	0.62±0.08	0.51±0.06	0.83±0.28	0.55±0.13	<0.01

NB: Values are reported as mean and SD. L. K- Left Kidney, R. L- Right Kidney**.** WUBA- aqueous stembark extract, WUBE- 70% ethanol stembark extract, WULA- aqueous leaf extract, WULE- 70% ethanol leaf extract**.**

Weight indices

There were statistically significant differences in the weight indices of the heart, stomach, liver and kidney between dose groups in animals that received WUBE extracts and in those that received WULE extracts ($p<0.05$) (**Table 10**). Animals that received the WUBA extracts reported statistically significant differences between dose groups for the stomach, liver and left Kidney ($p<0.05$). Animals that received WULA extract reported differences in the weight indices among dose groups for only the stomach and liver ($p<0.05$). (**Table 10**).

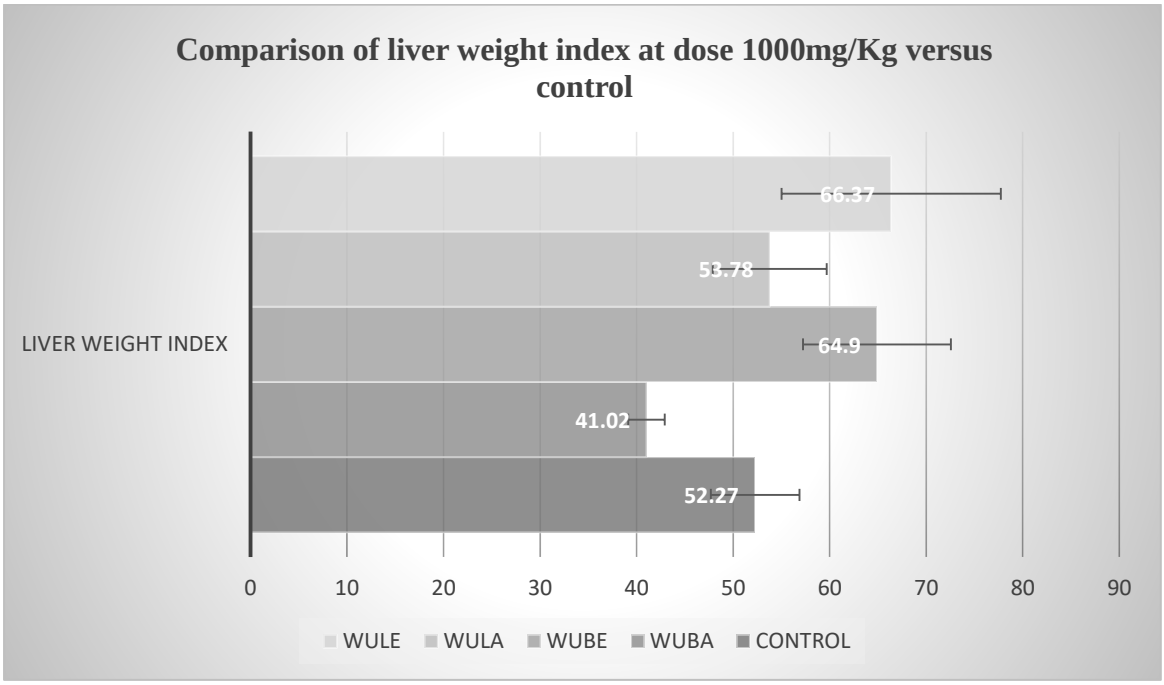
The post hoc Dunnett's test revealed statistically significant differences between animals that received the various *W. ugandensis* extracts versus the control group. Notably, the weight indices of the stomach were reduced in WUBA and WULE animals at the doses of 1000 mg/Kg and 500 mg/Kg ($p<0.05$). The effects were maintained in the satellite group of 1000 mg/Kg for WUBA extracts but not for WULE (**Table 10**).

Table 10: Weight Indices of Heart, Stomach, Liver, Kidneys after 28 days of dosing with *W. ugandensis* extracts

EXTRACT	ORGAN	Organ Weight Index (per 1000g)					
		Dose (mg/kg)					
		Control	250	500	1000	1000 SAT	ANOVA
WUBA	Heart	5.44± 0.45	5.01±0.52 B	5.35±0.64 B	3.88±1.20 B	6.01±7.43 B	0.36
	Stomach	15.07±0.97	13.67±0.61 B	12.87±1.06 A	12.84±1.93 A	13.03±2.40 A	**0.01**
	Liver	52.27±6.42	31.48±18.45 A	41.43±7.26 B	41.03±2.63 B	36.10±6.78 A	**<0.01**
	L. K	4.73±0.41	4.29±0.58 B	4.23±0.48 B	4.20±0.45 B	3.50±0.97 A	**<0.01**
	R. K	4.29±0.37	3.89±0.51 B	4.06±0.33 B	3.86±1.07 B	4.07±0.60 B	0.44
WUBE	Heart	5.44±0.45	5.44±0.89 B	5.92±1.63 B	6.86±0.39 B	5.04±0.55 A	**<0.01**
	Stomach	15.07±0.97	12.53±1.27 B	13.09±3.60 B	15.74±1.92 B	15.39±3.06 B	**0.01**
	Liver	52.27±6.42	57.14±10.77 B	60.11±9.40 B	64.90±10.70 A	38.63±8.73 A	**<0.01**
	L. K	4.73±0.41	4.67±1.09 B	5.37±1.99 B	6.43±0.64 A	4.10±1.18 B	**<0.01**
	R. K	4.21±0.35	4.53±0.87 B	5.22±2.00 B	6.06±0.81 A	4.43±1.36 B	**<0.01**
WULA	Heart	5.44±0.45	5.36±0.52 B	5.26±0.44 B	5.18±0.56 B	5.09±1.94 B	0.95
	Stomach	15.07±0.97	16.62±1.24 B	13.31±1.3 0 B	15.13±1.23 B	15.87±3.61 B	**0.01**
	Liver	52.27±6.42	42.21±4.83 A	41.01±8.94 A	53.78±8.27 B	38.22±3.70 A	**<0.01**
	L. K	4.73±0.41	4.46±0.75 B	4.09±0.59 B	4.28±0.57 B	4.40±1.19 B	0.46
	R. K	4.21±0.35	4.30±0.43 B	4.09±0.55 B	4.29±0.31 B	4.40±0.70 B	0.69
WULE	Heart	5.44±0.45	5.23±0.60 B	4.96±0.39 B	6.31±1.37 A	3.76±0.60 A	**<0.01**
	Stomach	15.07±0.97	11.54±1.69 A	9.28±1.11 A	12.55±3.58 A	16.05±2.48 B	**<0.01**
	Liver	52.27±6.42	59.37±10.71 B	52.49±5.45 B	66.37±15.91 A	38.07±3.65 A	**<0.01**
	L. K	4.73±0.41	4.37±0.69 B	3.92±0.31 B	5.93±1.61 A	3.91±1.16 B	**<0.01**
	R. K	4.21±0.35	4.44±0.55 B	3.83±0.32 B	6.14±1.94 A	4.12±0.88 B	**<0.01**

Note: Values are reported as mean and SD, L. K- Left Kidney, R. L- Right Kidney. A Value is statistically significant from control group. B Not statistically significant from control group**.** WUBA-stembark aqueous extract, WUBE-stembark 70% ethanol extract, WULA-leaf aqueous extract, WULE-leaf 70% ethanol extract.

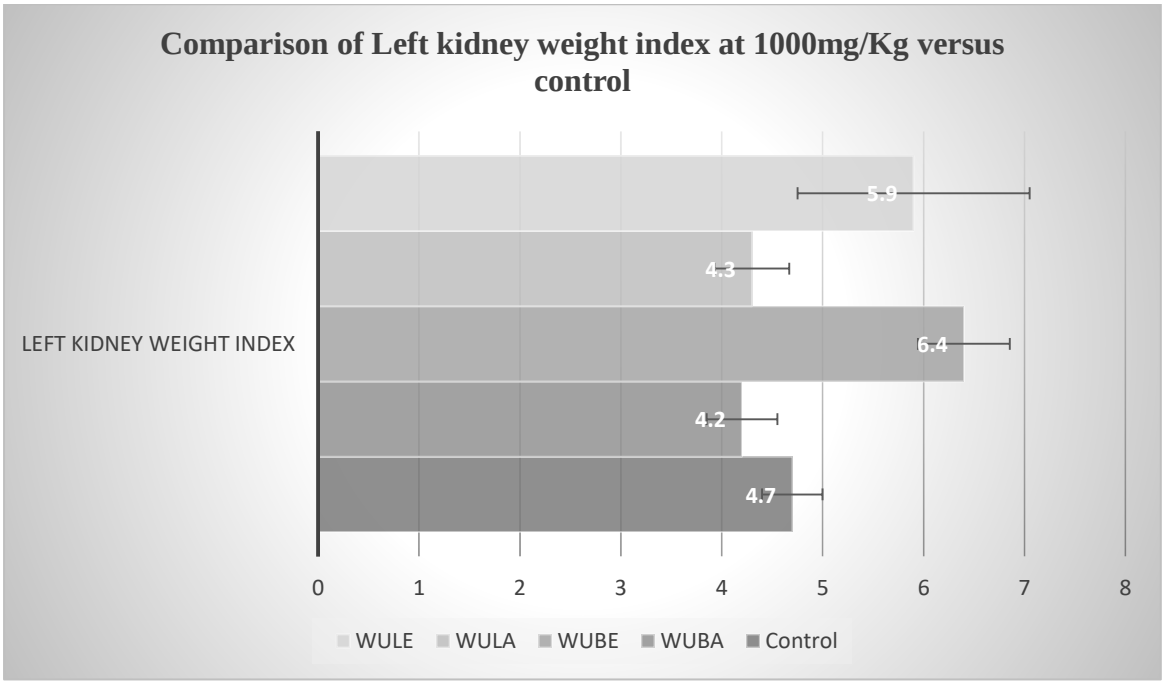
The post hoc test also revealed statistically significant differences in the weight indices of livers of the control group versus animals that various *W. ugandensis* extracts. Notably, at the dose of 1000 mg/Kg, the weight indices of the liver were increased compared to the control group in WULE and WUBE animals but not in the corresponding satellite animals (**Figure 15**).



Note: WUBA-stembark aqueous extract, WUBE-stembark 70% ethanol extract, WULA-leaf aqueous extract, WULE-leaf 70% ethanol extract.

Figure 15: Effect of *W. ugandensis* extracts on liver weight indices at the dose 1000mg/Kg.

The post hoc test also revealed statistically significant differences in the weight indices of kidneys of the control group versus groups that received various *W. ugandensis* extracts. Notably, at the dose of 1000mg/Kg, the weight index of the left kidney was increased in both WUBE and WULE animals ($p<0.05$) but not in their corresponding satellite groups (**Figure 16**).



Note: WUBA-stembark aqueous extract, WUBE-stembark 70% ethanol extract, WULA-leaf aqueous extract, WULE-leaf 70% ethanol extract.

Figure 16: Effect of *W. ugandensis* extracts on left kidney weight indices at the dose 1000mg/Kg.

4.5.5 Results of Biochemical Parameters

Liver function parameters

The mean plasma levels of liver function parameters in the different treatment groups of *W. ugandensis* extracts after 28 days of dosing ranged between 28.96 to 43.35g/l, 138.28 to 264.761µg/L and 41.74 to 168.26µg/L for albumin (ALB), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) respectively (**Table 11**). The levels of ALT in animals that received WUBE and WULE extracts were not different between dose groups.

However, compared to the control group, the serum levels of AST were significantly raised in animals that received the extracts of WUBA, WUBE, WULA and WULE at 250, 500, 1000mg/Kg and in the corresponding the satellite groups of 1000mg/Kg ($p<0.05$). The ALT levels were also raised in WUBA and WULA extracts compared to control at dose of 250, 500 and 1000mg/Kg ($p<0.05$) but not in the corresponding satellite groups of 1000mg/Kg ($p>0.05$) (**Table 11**).

Kidney function parameters

The mean plasma levels of key kidney function parameters in the various groups after 28-day dosing of extracts ranged between 35.40 to 55.67umol/L and 5.46 to 8.88mmol/L for creatinine and urea respectively (**Table 11**). Animals that received WUBA, WUBE and WULE reported no differences between dose groups in the levels of creatinine ($p>0.05$). The mean creatinine levels were however slightly lowered in WULA animals at doses of 250 and 1000mg/Kg ($P<0.05$) compared to the control group. Animals that received WUBA, WULA and WULE extracts reported no differences between dose groups in the levels of urea ($p>0.05$). In the animals that received WUBE, the post hoc Dunnett test revealed no statistically significant differences in urea serum levels between the control group and all doses of WUBE

Table 11: Results of biochemical parameters in animals dosed with different extracts of *W. ugandensis* after 28 days (reported as mean and SD).

Extract	Parameter	Dose (mg/kg)					
		Control	**250**	**500**	**1000**	**1000 SAT**	**ANOVA Test (P-value)**
WUBA	ALB	38.19±1.88	43.35±2.69 ^{A}	43.06±3.34 ^{A}	41.11±4.02 ^{B}	30.50±1.84 ^{A}	**<0.01**
	AST	122.34±40.93	231.22±46.37 ^{A}	215.30±28.04 ^{A}	238.65±63.98 ^{A}	168.61±38.03 ^{A}	**0.03**
	ALT	48.90±14.43	116.57±43.58 ^{A}	144.38±34.99 ^{A}	168.26±61.77 ^{A}	55.26±13.12 ^{B}	**<0.01**
	CRET	47.00±7.87	52.20±19.53 ^{B}	55.67±4.61 ^{B}	52.50±3.66 ^{B}	49.30±8.13 ^{B}	0.45
	UREA	6.84±1.11	5.52±1.03 ^{B}	5.46±0.73 ^{B}	6.73±0.61 ^{B}	5.85±1.45 ^{B}	0.45
	Na	145.00±3.68	149.40±3.06 ^{A}	147.22±3.99 ^{B}	148.00±2.58 ^{B}	150.40±5.40 ^{A}	**0.03**
	K	9.14±1.92	9.12±3.66 ^{B}	11.71±3.57 ^{B}	12.48±2.70 ^{A}	5.58±0.44 ^{A}	**<0.01**
WUBE	ALB	38.19±1.88	35.71±6.10 ^{B}	37.28±6.12 ^{B}	30.41±16.62 ^{B}	31.50±3.24 ^{B}	0.18
	ALT	48.90±14.43	51.60±14.36 ^{B}	52.31±11.13 ^{B}	41.74±30.23 ^{B}	70.13±21.83 ^{B}	0.14
	AST	122.34±40.93	237.58±42.24 ^{A}	204.11±50.96 ^{A}	148.73±66.17 ^{A}	202.82±62.77 ^{A}	**0.01**
	CRET	47.00±7.87	53.30±10.83 ^{B}	45.90±11.22 ^{B}	51.96±8.08 ^{B}	47.30±12.64 ^{B}	0.40
	UREA	6.84±1.11	8.25±1.20 ^{B}	8.80±1.72 ^{B}	7.70±4.31 ^{B}	5.28±0.92 ^{B}	**0.01**

	Na	145.00±3.68	128.20±6.22 A	130.50±2.99 A	148.00±2.87 B	152.50±8.21 A	**<.01**
	K	9.14±1.92	26.94±5.42 A	25.15±3.73 A	6.60±2.36 B	6.92±2.39 B	**<0.01**
WULA	ALB	38.19±1.88	34.88±5.48 B	37.09±2.99 B	38.79±1.11 B	31.40±2.32 A	**<0.01**
	ALT	48.90±14.43	84.91±35.58 A	68.38±41.23 A	112.14±32.81 A	62.87±20.44 B	**<0.01**
	AST	122.34±40.93	264.761±46.64 A	261.13±30.08 A	252.37±73.48 A	138.28±36.71 A	<0.01
	CRET	47.00±7.87	37.80±3.26 A	41.44±2.80 A	35.40±12.32 B	55.10±8.48 B	**<0.01**
	UREA	6.84±1.11	5.36±0.58 B	5.85±1.5 B	5,43±1.11 B	6.65±1.65 B	0.54
	Na	145.00±3.68	139.20±3.23 A	138.22±2.99 A	138.70±1.49 A	155.50±5.95 A	**<0.01**
	K	9.14±1.92	18.15±28.94 B	11.37±1.74 B	9.79±2.25 B	5.82±0.56 B	0.33
WULE	ALB	38.19±1.88	39.05±7.78 B	28.86±11.80 B	37.66±11.50 B	31.70±2.26 B	**0.03**
	ALT	48.90±14.43	48.95±10.87 A	29.97±22.81 A	52.66±20.24 B	65.54±16.16 B	0.17
	AST	122.34±40.93	250.95±53.05 A	175.78±72.02 A	250.31±93.45 A	171.46±18.52 A	**0.01**
	CRET	47.00±7.87	52.40±20.45 B	35.89±13.82 B	51.00±20.91 B	47.50±7.53 B	0.17
	UREA	6.84±1.11	8.47±2.20 B	6.08±2.32 B	7.55 ±2.12 B	5.79±0.84 B	0.43
	Na	145.00±3.68	139.90±2.23 B	143.10±4.46 B	141.00±3.56 B	153.70±7.63 A	**<0.01**
	K	9.14±1.92	17.50±2.54 B	14.09±4.09 B	16.58±5.42 B	5.34±1.07 B	0.13

- Units: ALB (g/L) ALT(µg/L) AST(µg/L) UREA (mmol/L) CRET (µmol/L) Na(mmol/L) K(mmol/L). [A] Statistically significant [B]-Not significant vs control.

4.5.6 Effects on hematological parameters.

The levels of haematological parameters in the different treatment groups of *W. ugandensis* extracts after 28 days of dosing ranged from 6.80 to 9.17x10^6/µL, 6.52 to 8.70x10^3/µL, 12.18 to 16.14 g/dL, 36.05 to 50.92 % and 511.30 to 942.33x10^3/µL for red blood cells (RBC), white blood cells (WBC), haemoglobin (HBG), haematocrit (HCT) and platelets (PLT) respectively (**Table 12**).

ANOVA revealed no differences between dose groups in the levels of WBC and haematocrit in animals that received WUBA extracts ($p>0.05$). Similarly, the levels of WBC in animals that received WUBE extracts were not different between dose groups ($p>0.05$). However,

differences between groups were found for serum levels of RBCs, haemoglobin and platelets for all extracts of *W. ugandensis*, p<0.05 (**Table 12**).

The post hoc Dunnett test however revealed no statistically significant differences between the control group and animals that received WUBA, WUBE, WULA and WULE extracts in mean serum levels of WBC and RBC (p>0.05) at doses of 250, 500 and 1000 mg/Kg. Interestingly, the RBC levels in the satellite groups of WUBA, WUBE and WULE were all slightly raised compared to the control group (p<0.05. Furthermore, platelet levels were decreased slightly in animals that received WUBE extract compared to control group at doses of 250, 500 and 1000 mg/Kg (P<0.05) but not in the corresponding satellite group of 1000 mg/Kg.

Table 12: Results of haematological parameters in animals dosed with different extracts of *W.ugandensis* after 28 days.

Extract	Parameter	Control	Dose (mg/Kg)				
			250	**500**	**1000**	**SAT 1000**	**ANOVA**
WUBA	RBC	7.70±0.79	8.15±0.46^{B}	7.73±0.54^{B}	7.84±0.43^{B}	8.62±0.53^{A}	**<0.01**
	WBC	9.06±2.37	8.57±3.64^{B}	7.78±2.34^{B}	8.05±4.07^{B}	7.25±3.09^{B}	0.75
	HGB	14.45±1.45	15.71±0.49^{A}	14.89±0.95^{B}	14.54±0.94^{B}	15.39±0.97^{B}	**0.04**
	HCT	48.12±5.24	50.92±1.69^{B}	50.48±2.51^{B}	49.73±3.04^{B}	48.46±3.37^{B}	0.32
	PLT	832.20±238.71	665.11±61.82^{B}	511.30±311.84^{A}	651.60±145.55^{B}	813.30±132.74^{B}	**<0.01**
WUBE	RBC	7.70±0.79	7.32±1.74^{B}	7.34±0.37^{B}	6.80±1.19^{B}	9.10±0.69^{A}	**<0.01**
	WBC	9.06±2.37	7.26±4.10^{B}	7.63±2.23^{B}	7.73±3.91^{B}	7.27±2.46^{B}	0.70
	HGB	14.45±1.45	13.36±2.06^{B}	13.40±0.74^{B}	12.18±2.01^{A}	15.73±1.06	**<0.01**
	HCT	48.12±5.24	43.54±5.96^{B}	44.66±2.50^{B}	36.05±5.27^{A}	49.19±3.26^{B}	**<0.01**
	PLT	832.20±238.71	556.11±111.23^{A}	513.40±221.46^{A}	608.10±123.24^{A}	775.22±158.30^{B}	**<0.01**
WULA	RBC	7.70±0.79	7.70±0.62^{B}	7.10±2.62^{B}	7.50±0.99^{B}	9.08±0.74^{B}	**<0.01**

	WBC	9.06±2.37	7.45±3.97 **B**	7.12±2.62 **B**	7.16±2.48 **B**	8.16±2.16 **B**	**0.04**
	HGB	14.45±1.45	13.85±1.37 **B**	14.12±1.12 **B**	14.49±0.96 **B**	16.04±1.19 **A**	**<0.01**
	HCT_	48.12±5.24	41.96±8.39 **B**	45.22±4.13 **B**	45.76±4.23 **B**	50.05±3.53 **B**	**<0.01**
	PLT	832.20±238.71	817.80±226.38 **B**	942.33±331.97 **B**	757.44±269.74 **B**	712.50±128.46 **B**	**<0.01**
WULE	RBC	7.70±0.79	7.53±0.66 **B**	7.10±0.34 **B**	7.33±0.62 **B**	9.17±0.60 **A**	**<0.01**
	WBC	9.06±2.37	6.52±1.00 **B**	8.70±3.81 **B**	6.83±2.91 **B**	6.04±1.72 **A**	**0.04**
	HGB	14.45±1.45	13.65±1.36 **B**	12.87±0.98 **A**	13.35±0.93 **B**	16.14±0.47 **A**	**<0.01**
	HCT	48.12±5.24	42.54±4.84 **A**	40.952±.36 **A**	41.563±0.66 **A**	50.26±1.64 **B**	**<0.01**
	PLT	832.20±238.71	591.00±166.89 **B**	463.00±143.16 **B**	511.60±210.87 **B**	721.40±105.47 **B**	**<0.01**

Note: RBC in $10^6/\mu L$, WBC in $10^3/\mu L$, HGB in g/dL, HCT in Percent, PLT in $10^3/\mu L$

Results reported as mean and SD. [A] Statistically significant [B]-Not significant vs control.

CHAPTER FIVE: DISCUSSION

5.1 Discussion background.

In developing countries, majority of the population has resorted to the use of herbal products (Kaggwa et al., 2022; Lutoti et al., 2013). The rise in herbal medicine use has been attributed to a number of reasons, including; preference of consumers for natural therapies, a dissatisfaction with the results from synthetic drugs, the belief that herbal medicines might be effective in the treatment of certain diseases where conventional therapies have proven to be inadequate, the high cost and side effects of most modern drugs, as well as a movement towards self-medication (Welz et al., 2018). The use of herbal medicines/natural remedies is however not without its problems. Many adverse effects including hepatoxicity, cardiotoxicity, nephrotoxicity, and food poisoning have been reported by consumers from the use of herbal products (Additives et al., 2018; Capasso et al., 2000; Deng, 1994; Dunnick et al., 2007; Ekor, 2014; Ernst, 2003b).

Adverse events from the use of herbal remedies might arise due to the innate toxicity of the medicinal plant constituents of the herbal product, conferred to the plant by the presence of various phytotoxins (Gamaniel, 2000). Phytotoxins have varied structures and can be alkaloids, terpenes, phenylpropanoids or polyketides (Chen et al., 2022). The conduction of toxicity studies in rodents helps pharmaceutical developers to predict adverse reactions from herbal products (Ecobichon, 1997). The concordance between toxicities observed in rodents to humans is reported to be up to 71% for rodent and non-rodent species (Olson et al., 2000). Once toxicities in medicinal plants are discovered, pharmaceutical developers can mitigate them by avoiding the offending plant in product formulations, using a known safe dose or designing short duration therapies to reduce the exposure to patients.

Nefarious effects of herbal products might also arise from poor quality. Insufficient quality is often present in various inputs along the herbal medicinal products value chain and can be as a result of poor agricultural and collections practices, non-adherence to good manufacturing practices, and poor storage of products (Ekor, 2014; Pan et al., 2013; Sharma, 2015). Quality problems can often be mitigated by observing good manufacturing practices which include proper authentication and identification of raw materials to prevent the use of adulterated or misidentified ingredients. In this regard, HPTLC based fingerprints have emerged as a cost effective tool for identifying and authenticating plant based raw materials by a simple comparison with a standard (Eloff et al., 2011).

In this study, an HPTLC method was used to create *Warburgia ugandensis* stembark and leaf fingerprints that can aid in identification of the highly valued medicinal plant. In the second part of the study, *W. ugandensis* aqueous leaf, aqueous stembark, 70% ethanolic leaf and stem bark extracts were administered to Wistar rats and observed for acute and sub-acute toxicities, to provide safety information to facilitate the safe and effective use of the medicinal plant.

5.2 Discussion of HPTLC results.

Herbal Fingerprinting is a technique used for the qualitative and quantitative analysis of herbal components in drugs (Custers et al., 2017; Lazarowych & Pekos, 1998). The United States Pharmacopoeia defines an HPTLC Fingerprint as an electronic image of the visual HPTLC chromatogram. The HPTLC fingerprint is mainly evaluated based on colour and retardation factor (rf) values of the bands or spots which represent compounds in the plant extract (Attimarad et al., 2011). In this study, the acetone extract of the leaf showed the highest number of bands in both white light and derivatized modes of visualization with 9 and 12 bands respectively corresponding to an RF range of 0.06 to 0.90. On the other hand, the acetyl acetate extract of the stembark produced the highest number of bands in the derivatized chromatogram with 7 corresponding to an RF range of 0.04-0.87.

The number of compounds resolved in the fingerprint is affected by the solvent of extraction and mobile phase system used (Rashmin et al., 2012; Sonia & Lakshmi, 2017; Srivastava, 2010). In this study, the intermediate polarity solvents of acetone and methanol were able to extract a wider variety of compounds from the plant matter than the typically non-polar solvents like hexane. The relatively non-polar mobile solvent systems used in the study, i.e. hexane: acetone (70: 30) for the leaf extracts, and ethyl acetate: acetone: hexane (10:5:3) for the bark extracts, similar in polarity to heptane: chloroform: acetone (30:20:10) used by (ONDORA, 2016) promoted the separating out of non-polar components in the extract against the polar stationary phase of silica. The compounds of interest in *warburgia* species have been previously characterized as non-polar sesquiterpenes for instance muzigadial, ugandensolide, cinnamolide among others (Dharani; Maroyi, 2014). Owing to the polar stationary used, these non-polar compounds are expected to correspond to the bands with the highest retardation factors in the HPTLC fingerprints.

The observation of compounds in any chromatogram visualization method is dependent on the presence of chromophores for that particular wavelength of light (Rashmin et al., 2012; Sonia & Lakshmi, 2017). Chromophores are regions in the molecules where the energy difference between two separate molecular orbitals falls within the range of the visible spectrum

(Shcherbakova & Verkhusha, 2014). Chromophores are characterized by highly unsaturated and conjugated structures (Shcherbakova & Verkhusha, 2014). In this study, 9 coloured bands, ranging from grey to yellow were only observed in the leaf fingerprint while the stem bark fingerprint did not show any bands in white light. The yellow coloured bands observed in white light in the leaf extract might include xanthophylls and carotenes, classes of pigmented (chromophore possessing) compounds that are ubiquitous in the leaves of all photosynthesizing species of the plant kingdom (Thomas & Johnson, 2018).

Compounds without chromophores on the other hand cannot be visualized in white light. The visualization of such compounds can be achieved by spraying with a staining agent. In this study, Vanillin sulphuric acid was used as a staining agent. Vanillin is a phenolic aldehyde similar in structure to ***p***-anisaldehyde, the staining agent used by (ONDORA, 2016). The sprayed fingerprints of the leaf and stembark displayed additional compounds with higher retardation factors of up to 0.9, seen as blue to purple coloured bands. The visualized compounds likely include the *W. ugandensis* sesquiterpenes whose structures are non-polar and also characterized by lower unsaturation and conjugation compared to typical coloured compounds like carotenes or xanthophyll (Brooks & Draffan, 1969a, 1969b). Vanillin introduced a highly unsaturated and conjugated structure to the hitherto non-light absorbing sesquiterpenes causing them to be visualized in white light. These findings are similar to the results obtained by Ondora et al (2016) who observed 8 spots using the naked eye in the TLC chromatogram of ethyl acetate leaf extract and additional compounds that were coloured purple, red to blue after spraying with a derivatizing agent of 0.5% anisaldhyde in sulphuric acid.

The findings from this study support the use of a relatively nonpolar mobile phase system, intermediate polarity extraction solvent and vanillin-sulfuric as a staining agent in developing analytical methods to fingerprint *W. ugandensis* extracts based on presence of sesquiterpenes. However, because of limited resources, this study did not undertake the validation of the method by using a set of known marker compounds in *W. ugandensis* as standards. This is essential in ensuring that the method is specific for the identification of the *W. ugandensis* extracts. Furthermore, because plant matter was collected from a single locality at one point in time, the fingerprints may not reflect the variability of phytochemical constitution of the plant due to climate, seasonality and geographical location.

5.3 Discussion of Acute toxicity results

Acute toxicity refers to adverse effects occurring following oral administration of a single dose of a substance given within 24 hours, and subsequently during a 14-day observation period (Colerangle, 2013). In this study, all animals exhibited symptoms of raised fur and abdominal spasms that resolved within 24 hours. General body weakness, polyuria and increased defaecation were observed up to one week after dosing but resolved completely by the 14^{th} day. Furthermore, all animals that received the different extracts of *W. ugandensis* gained weight at a similar rate compared to the control group. The mildness and self-limiting nature of the symptoms coupled with weight gain pointed to the non-noxious nature of the plant.

Acute toxicity tests are often used to determine the dose of a substance that causes death in 50% of the test population (LD_{50}) during short-term exposure (Akhila et al., 2007). In this study, there were no mortalities observed for *W. ugandensis* 70% ethanolic and aqueous extracts of the leaf and stembark. Based on this, the *W. ugandensis* extracts can be said to belong to category 5 of the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) with an estimated LD_{50} of greater than 2000 to 5000 mg/Kg of body weight for oral route (Toxicity–Up, 2001).

The finding on the aqueous bark extract (WUBA) is in agreement with (Karani et al., 2013) who determined an LD_{50} of 5000mg/Kg in mice for *W. ugandensis* aqueous extract. The findings in respect to the three other extracts investigated in this study suggest that the aqueous leaf (WULA), 70% ethanolic leaf (WULE) and stem bark (WUBE) extracts have a similar level of acute oral safety as the aqueous stembark extract (WUBA). The LD_{50} of 5000mg/Kg determined for *W. ugandensis* extracts is similar to the LD_{50} reported for *Vernonia amygdalina* (*mululuza*) by (Zakaria et al., 2016) and twice the LD_{50} of 2200mg/Kg reported for *Prunus africana* by (Karani et al., 2013). The safety of *W.ugandensis* extracts in acute use is therefore comparable to *V. amygdalina* and better than *P. africana* extracts. Information from this study supports the safety of 70% ethanolic extracts of *W. ugandensis* in addition to aqueous extracts of both leaf and stembark for use by pharmaceutical industry in developing herbal remedies for acute use.

5.4 Discussion of Subacute toxicity results.

The determination of oral subacute toxicity using repeated doses often proceeds initial acute toxicity testing (Gelbke et al., 2004). The test provides information on possible health hazards likely to arise from repeated exposure over a limited period of time, including effects on the

animal weight and target organ toxicity i.e. liver, kidneys, bone marrow (Bitsch et al., 2006; Wilhelm & Maibach, 2012). In this study, there were only two mortalities of one male rat each from the WULA and WUBA 500mg/Kg groups during the first week of dosing. The fact that the dead animals were observed to have physical body injuries consistent with bites suggested that those deaths were as a result of territorial fights and were unrelated to the extracts.

All treatment groups of the sub-acute tests reported a gain in average weight. However, at the dose of 1000mg/kg, the groups that received extracts of WUBA, WUBE and WULE reported less average weight gained compared to the control group. The fact that at the same dose, animals that received extracts of WUBE, WULA and WULE reported greater average daily food consumption compared to the control group suggests that the observed reduction in the rate of weight gain is not due to decreased food consumption.

According to (Chapman et al., 2013), body weight loss is one of the few objective measures assessed in short term toxicity studies, and is often the primary endpoint, and an indicator of the extreme toxic nature of the substance being tested. The findings of this study show that repeated doses of *W. ugandensis* extracts had a negative effect on the rate of weight again, however, this effect was not enough to cause weight loss. The reduced rate of weight again compared to control group can be attributed to increased energy requirements for the metabolism of a high quantity of phytochemicals from the *W. ugandensis* extracts which were constantly given to the animals.

Organ weight changes are accepted as a sensitive indicator of chemically-induced effects on organs (Lazic et al., 2020). The organ weight index is computed by dividing the weight of the organ by its corresponding animal body weight and multiplying by a factor. This value represents the relative mass of the organ and is more representative of target organ toxicity than the actual organ weight which might reflect chemically-induced changes in overall body weight (Lazic et al., 2020). Reduced organ weight indices might indicate target organ damage.

At the dose of 1000 mg/Kg, compared to the control group, the weight indices of the liver and left kidneys were increased in both WUBE and WULE animals but decreased in the corresponding satellite of groups of 1000 mg/Kg. The liver and the kidneys are the primary organs responsible for eliminating xenobiotics from the body (Shabbira et al., 2022). The liver is tasked with metabolizing the phytochemicals from the extracts, converting them to a form that can be easily eliminated by the Kidneys. The increase in the weight indices of the liver and left kidney in animals that received *W. ugandensis* 70% ethanolic leaf and stembark extracts can be attributed to a form of physiological adaptation arising from increased metabolic needs

due to the phytochemical load from the extracts. The liver and kidney tissue tend to proliferate to handle increased chemical load (Williams & Iatropoulos, 2002). The decrease of liver and kidney weight indices in the corresponding satellite groups is probably due to the reverse effect.

The weight indices of the stomach were reduced in animals that received *W. ugandensis* aqueous stem bark and ethanolic leaf extracts at the top doses of 500mg/Kg and 1000mg/Kg. The effects were maintained in the satellite group of 1000mg/Kg for the stem bark extract. This observation can be attributed to a possible eroding action of the extracts. This action has also been observed in histopathology analysis were extensive epithelial erosion was seen in some animals in the acute test. The offending chemicals are currently unknown but it appears they are prevalent in the WUBA and WULE extracts.

The functionality of the liver is often monitored using parameters like albumin (ALB), aspartate aminotransferase (AST) and alanine aminotransferase (ALT). In this study, the mean plasma levels of ALB, AST and, ALT in animals that received the various extracts of *W. ugandensis* at all doses studied fell outside the normal historical reference upper limits in Wistar rats of 122µg/L and 45 µg/L for Aspartate Transferase and Alanine aminotransferase respectively and the lower limit for Albumin (37g/L) as reported by (Giknis & Clifford, 2008).

Furthermore, the serum levels of AST were raised nearly two-fold in animals that received the extracts of *W. ugandensis* at all doses studied and in the corresponding the satellite groups of 1000 mg/Kg. The serum levels of ALT were also raised in animals that received WUBA and WULA extracts at all doses studied. This shows that all *W. ugandensis* extracts no matter the dose increased serum levels of liver functional enzymes with the AST levels not reversing to normal range even after 14 days without extract. Elevated levels of ALT and AST indicate on going liver injury (Lee et al., 2012) . The findings from study therefore suggest that repeated doses of *W. ugandensis* might have caused some degree of liver injury. However, (Lee et al., 2012) argues that mildly elevated liver enzymes (e.g., <2–3 times of the upper limit of normal) without a symptom may be considered benign. These results should therefore be taken in the context of the whole study. Furthermore, product information leaflets of *W. ugandensis* products may be updated with this information to warn patients of the possible raise in liver enzymes.

Creatinine and urea levels were not raised in animals that received extracts of *W. ugandensis* compared to the control group. Excessively high creatinine and urea levels are an indication of kidney function impairment or failure (Sharkey, 2017). The results from this study suggest that repeated doses of *W.ugandensis* extracts did not impair animal kidney function.

The mean levels of haematological parameters in the different treatment groups of *W ugandensis* extracts fell within the normal historical reference ranges reported for Wistar rats for red blood cells (RBC); 6.5 to 9.8 $9.17x10^6/\mu L$, white blood cells (WBC); 3.2 to 10.5$x10^3/\mu L$ and haematocrit (HCT);37% to 51% according to (Giknis & Clifford, 2008) and haemoglobin (HGB) of 10.7 to 17.7g/dL according to (Vigneshwar et al., 2021). Furthermore, there were no differences reported between the control group and the groups that received *W. ugandensis* extracts in the plasma counts of WBC and RBC at all doses of the extracts studied.

Precursors of blood cells are produced in the bone marrow, therefore any observed anaemia in test animals would indicate impaired bone marrow function (Leach, 2014). Interestingly, the RBC counts in the satellite groups of the aqueous stembark, 70% ethanolic stembark and 70% ethanolic leaf extracts were slightly increased compared to the control group suggesting that there was a delayed enhancement of red blood cell count 14 days after dosing with those extracts. These results therefore suggest that *W. ugandensis* extracts are not only nontoxic to the bone marrow of rodents after repeated subacute administration, but also might enhance the red blood cell count. The mechanism of enhancement of red blood cells is currently unknown.

Although this study found favourable data for the safety of *W. ugandensis* leaf and stem bark extracts in acute use, data from subacute studies suggests some caution should be taken in repeated use lasting 28 days. Repeated subacute toxicity tests are often designed to determine the no observable adverse effect level (NOAEL) , the NOAEL is the highest dose of a substance that produces no noticeable toxic effect on tested animals (Dorato & Engelhardt, 2005). This study was unable to determine a NOEL for *W. ugandensis* extracts since the lowest dose of extracts tested i.e. 250mg/Kg produced marked changes in Liver enzymes. Furthermore, caution must be taken in the extrapolation of safety data established by this study to different types of extracts. This study investigated the effects of aqueous and 70% ethanolic extracts and there was no fractional concentration of any particular phytochemical or close class of phytochemicals. The findings, might not apply to fractionated extracts or those where an attempt is made to concentrate particular active phytochemicals.

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

Conclusion

This study has demonstrated that a simple HPTLC method using vanillin-sulphuric acid as a staining agent can be used to develop fingerprints for *W. ugandensis* leaf and stem bark extracts.

The study has also demonstrated the safety of *W.ugandensis* leaf and stem bark, aqueous and ethanolic for acute use. However, there should be caution in the development of remedies that involve repeated dosing exceeding two weeks due to possible liver toxicity.

Recommendations

The use of intermediate polarity solvents of extraction and non-polar mobile solvent systems in the development of HPTLC methods for fingerprinting *W ugandensis* extracts should be considered by method developers for improved resolution. Furthermore, in order for any subsequent method to be adopted for routine identification of *W. ugandensis* extracts, there needs to be further studies to validate the specificity of the method and the fingerprints using known marker compounds of *W. ugandensis* as reference standards. Additional research should also be undertaken to determine the variability of the fingerprints due to seasonality and geographical location.

The inclusion of the raise of liver functional enzymes as a caution in the patient information leaflets (PIL) of *W. ugandensis* containing products may be considered by drug regulatory bodies to warn prospect users. Additional research might be undertaken to determine the NOEL of *W. ugandensis* extracts by considering repeated doses lower than 250mg/Kg which was the lowest dose considered in this study. Eventually the safety of any *W. ugandensis* based remedies should be validated by a clinical trial in humans.

Finally, further research is warranted to investigate the possible link between *W. ugandensis* repeated dosing and enhancement of red blood cell count. There is potential for this to become another therapeutic area for *W. ugandensis* based herbal remedies.

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APPENDICES

Appendix 1: Chromatograms from Mobile Phase Optimization

Stembark Chromatograms

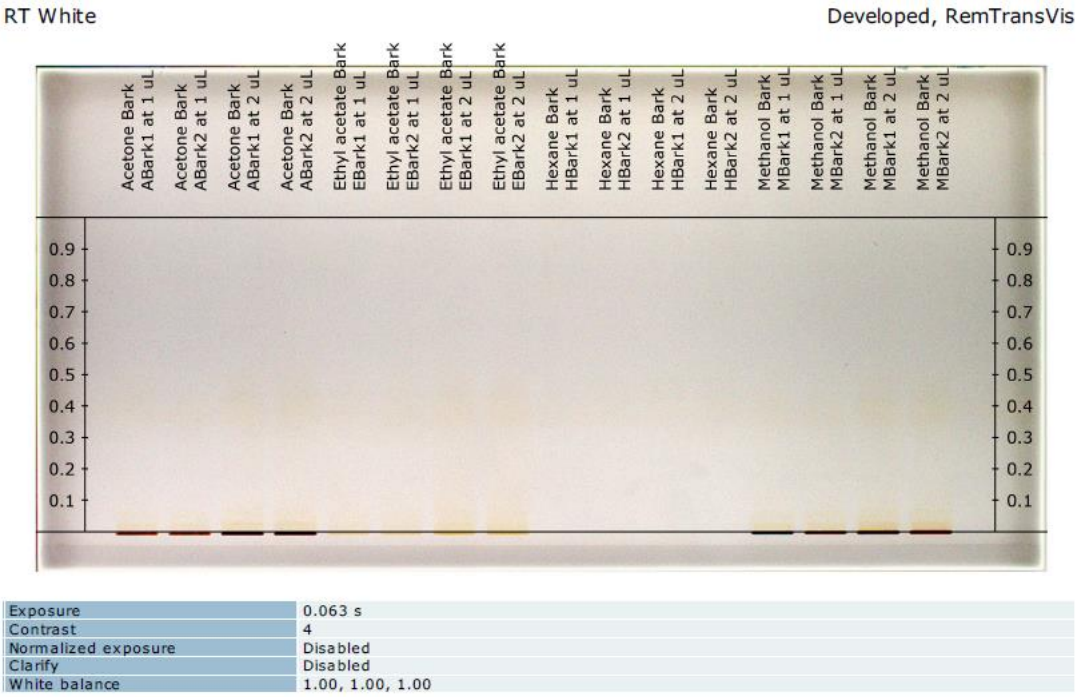

Figure 17: Stem bark Chromatogram obtained with Hexane: Acetone (60:40) Mobile phase in white light.

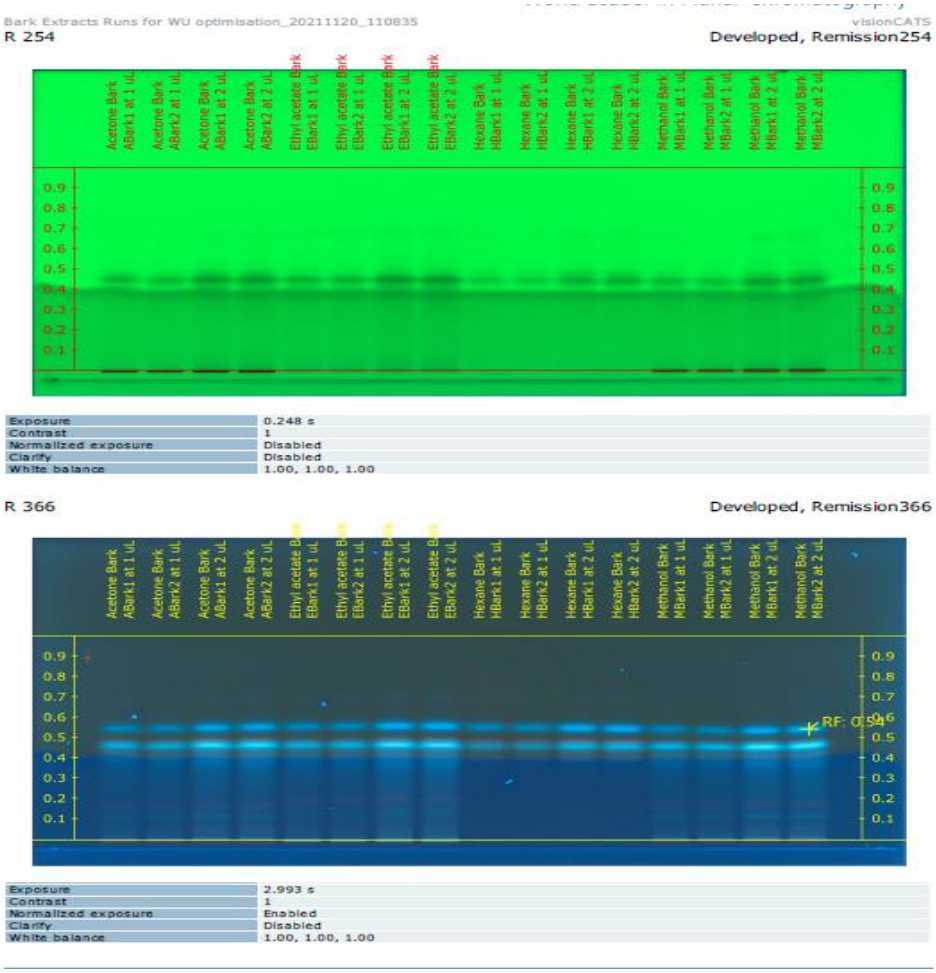

Figure 18: Stem bark Chromatogram obtained with *Hexane*: Acetone (60:40) Mobile phase in UV light (254nm top, 366nm bottom)

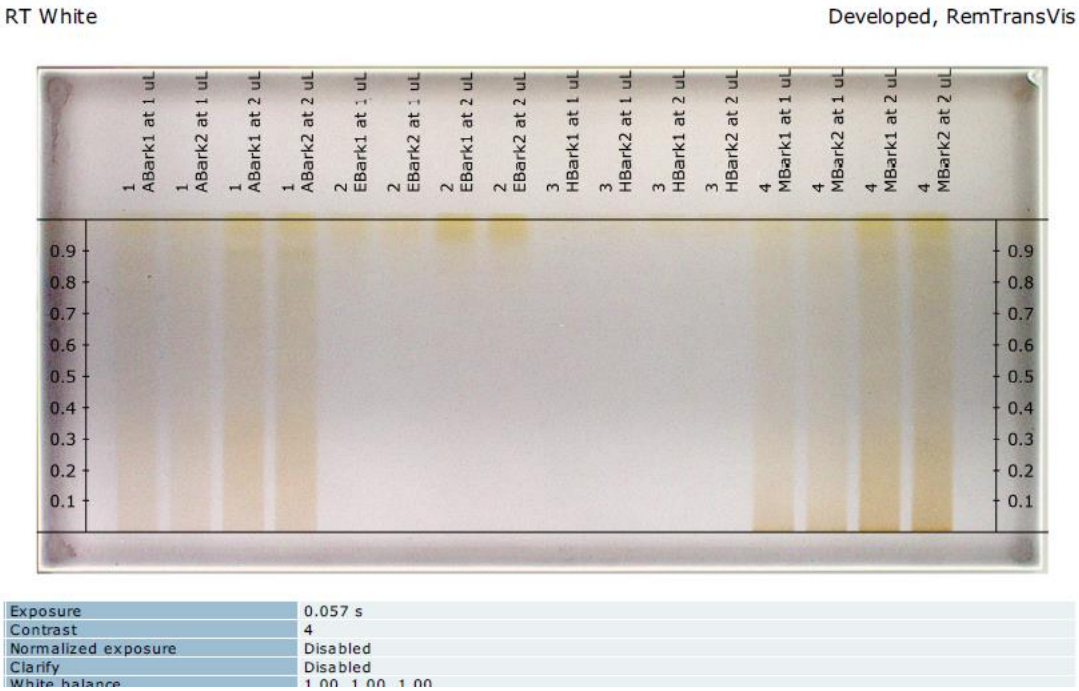

Figure 19: Stem bark Chromatogram obtained with Acetic Acid: Methanol: Water (1:8:3) Mobile phase in white light

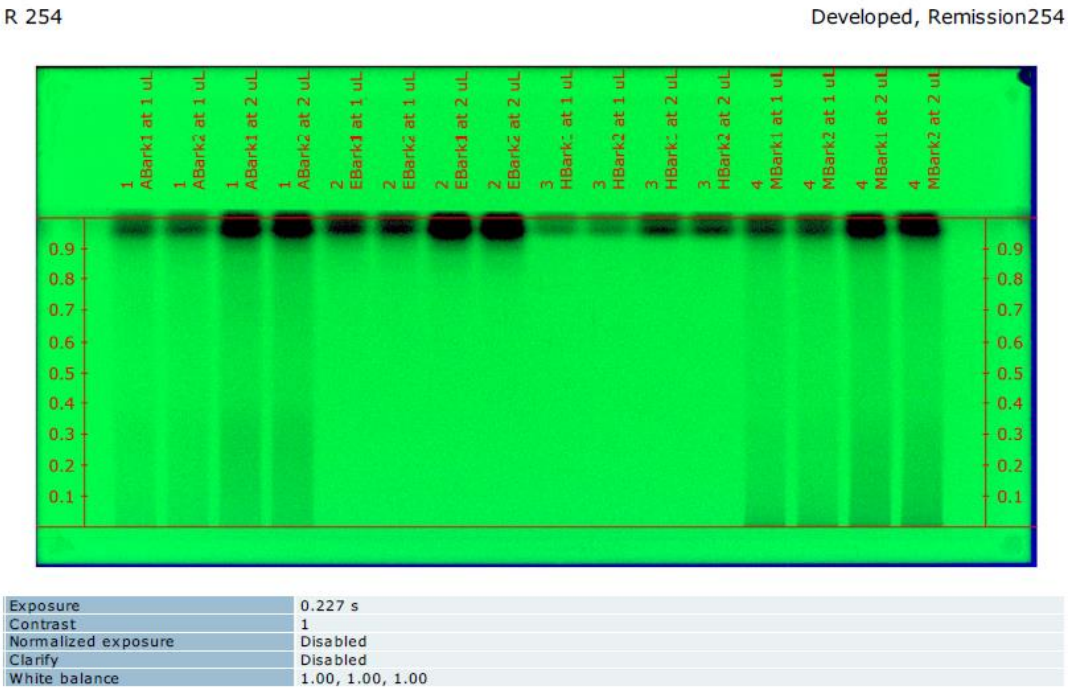

Figure 20: Stem bark Chromatogram obtained with Acetic Acid: Methanol: Water (1:8:3) Mobile phase in UV light in 254nm

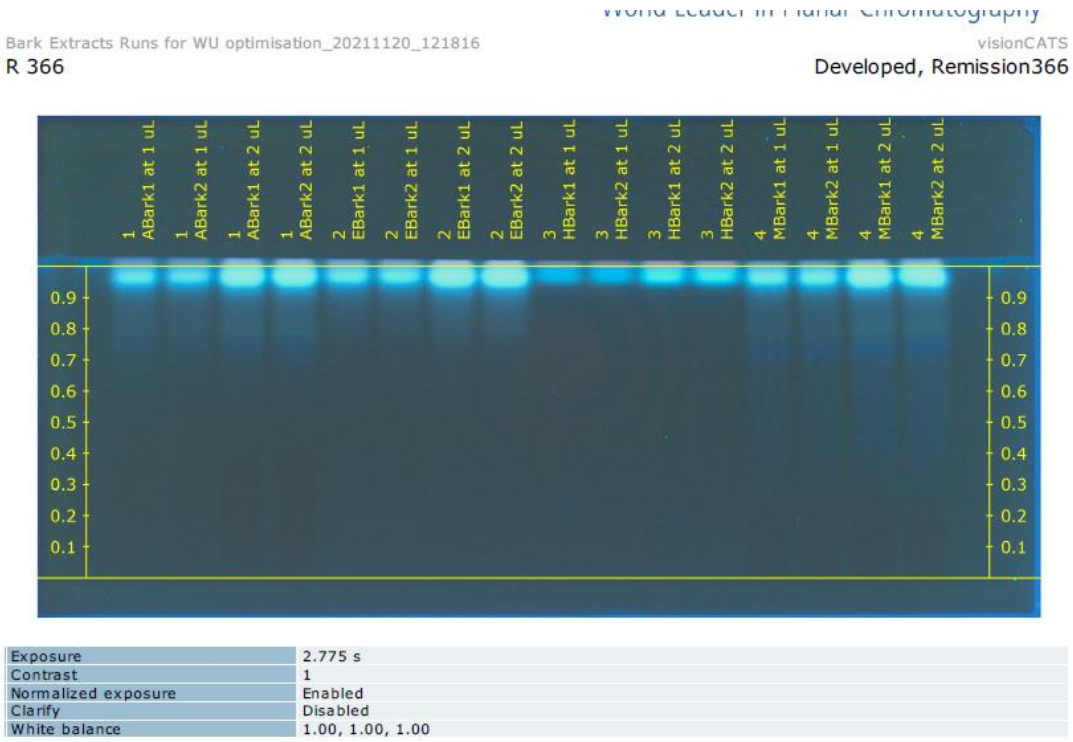

Figure 21: Stem bark Chromatogram obtained with Acetic Acid: Methanol: Water (1:8:3) Mobile phase in UV light 366nm.

Figure 22: Stem ba**rk Chromatogram obtained with Ethyl acetate: Methanol: Water (5:10:2) Mobile phase in white light**

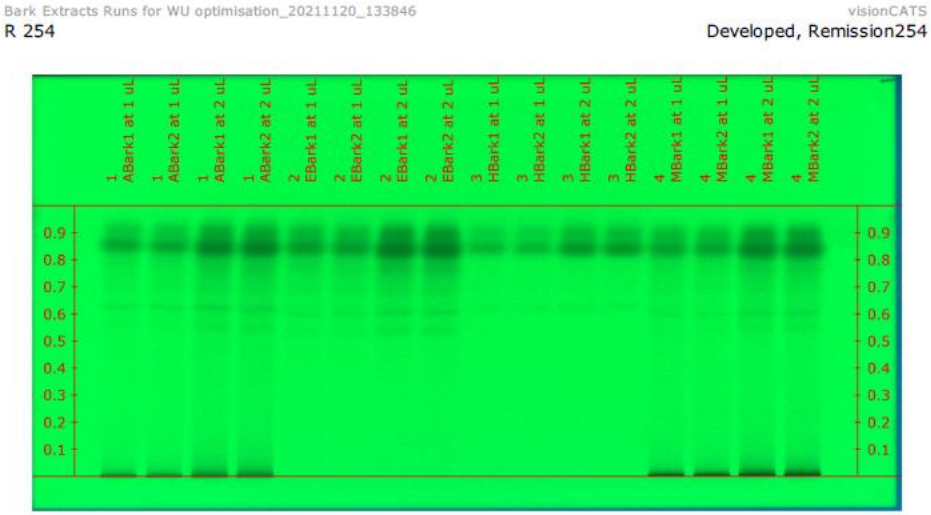

Figure 23: Stem bark Chromatogram obtained with Ethyl acetate: Methanol: Water (5:10:2) Mobile phase in UV 254nm.

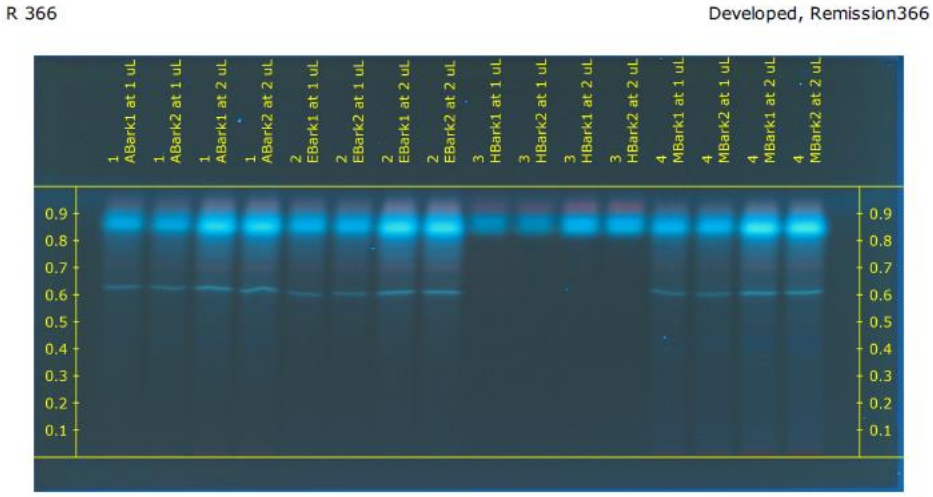

Figure 24: Stem bark Chromatogram obtained with Ethyl acetate: Methanol: Water (5:10:2) Mobile phase in UV 366nm

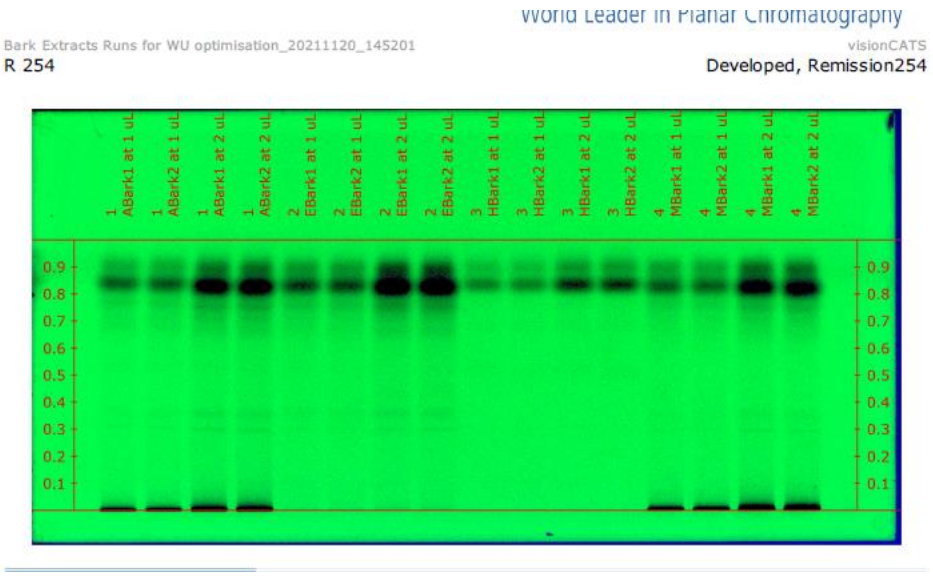

Figure 25: Stem bark Chromatogram obtained with Ethyl acetate: Methanol: Hexane (2:5:10) Mobile phase in UV 254nm

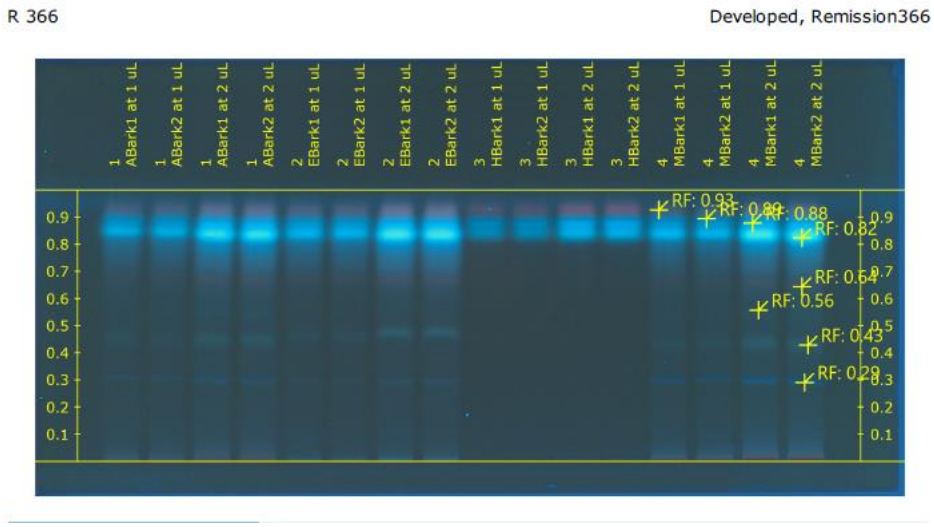

Figure 26: Stem bark Chromatogram obtained with Ethyl acetate: Methanol: Hexane (2:5:10) Mobile phase in UV 366nm

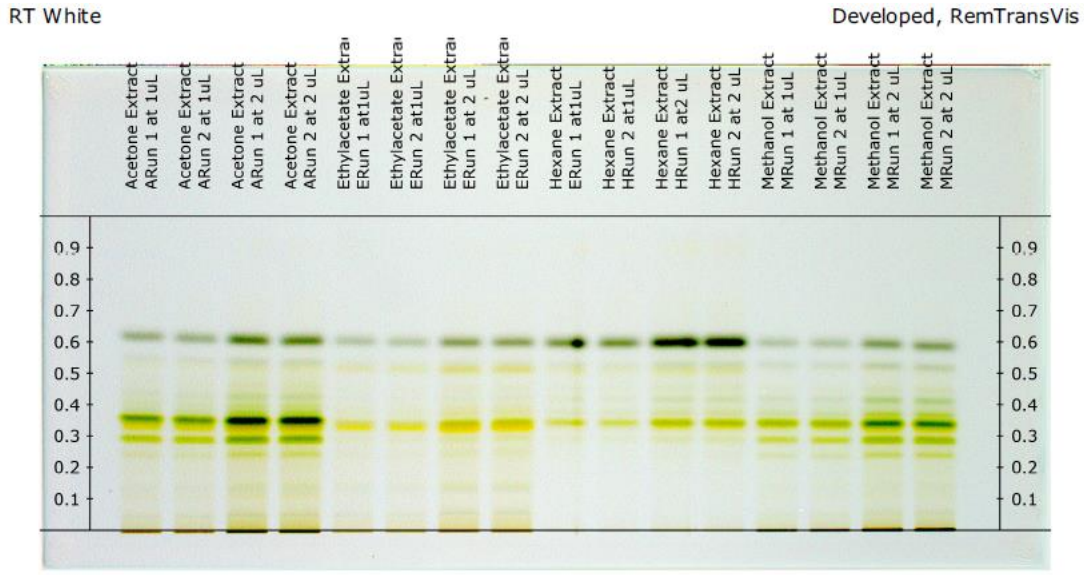

Figure 27: Leaf Chromatogram obtained with Hexane: Acetone (60:40) Mobile phase in white light.

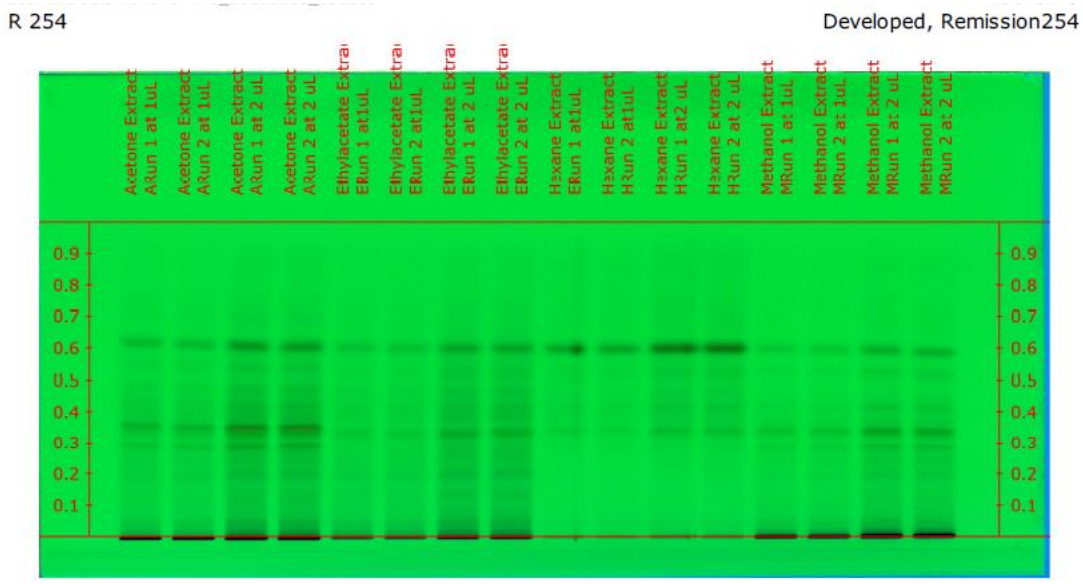

Figure 28: Leaf Chromatogram obtained with Hexane: Acetone (60:40) Mobile phase in white light.

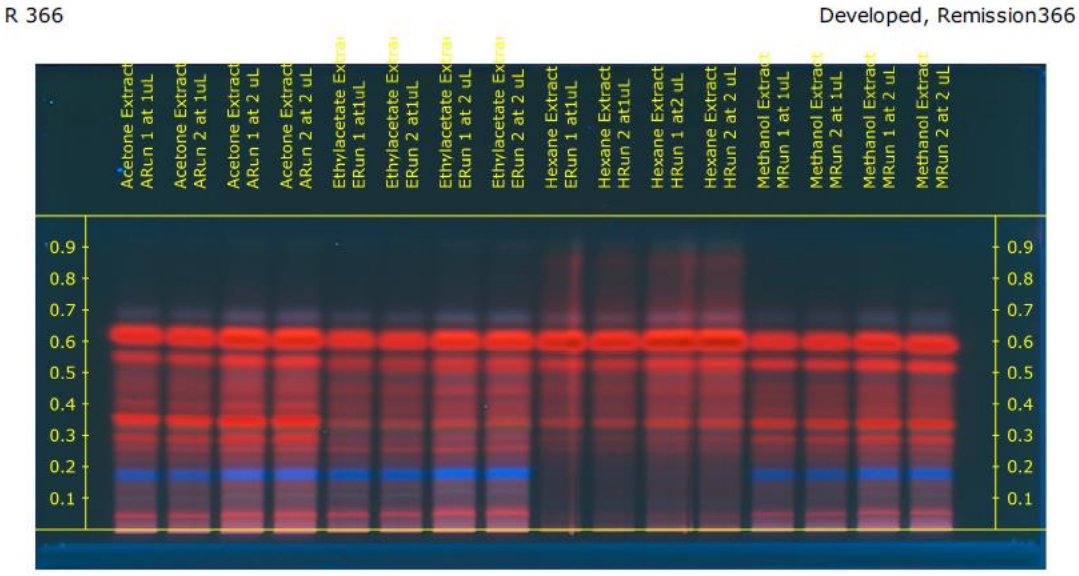

Figure 29 :Leaf Chromatogram obtained with Hexane: Acetone (60:40) Mobile phase in white light.

Appendix 2: IRB Appoval Letter

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COLLEGE OF HEALTH SCIENCES
SCHOOL OF HEALTH SCIENCES
OFFICE OF THE DEAN

17/05/2021

To: James Ochan

Type: Initial Review

Re: MAKSHSREC-2021-107: HPTLC fingerprinting and Toxicity profile of Warburgia ugandensis (Fam. Canellacaea, 01, 2021-05-11

I am pleased to inform you that at the **83rd** convened meeting on **27/04/2021**, the Makerere University School of Health Sciences REC, committee meeting, etc voted to approve the above referenced application.
Approval of the research is for the period of **17/05/2021** to **17/05/2022.**

As Principal Investigator of the research, you are responsible for fulfilling the following requirements of approval:

1. All co-investigators must be kept informed of the status of the research.
2. Changes, amendments, and addenda to the protocol or the consent form must be submitted to the REC for re-review and approval **prior** to the activation of the changes.
3. Reports of unanticipated problems involving risks to participants or any new information which could change the risk benefit: ratio must be submitted to the REC.
4. Only approved consent forms are to be used in the enrollment of participants. All consent forms signed by participants and/or witnesses should be retained on file. The REC may conduct audits of all study records, and consent documentation may be part of such audits.
5. Continuing review application must be submitted to the REC **eight weeks** prior to the expiration date of **17/05/2022** in order to continue the study beyond the approved period. Failure to submit a continuing review application in a timely fashion may result in suspension or termination of the study.
6. The REC application number assigned to the research should be cited in any correspondence with the REC of record.
7. You are required to register the research protocol with the Uganda National Council for Science and Technology (UNCST) for final clearance to undertake the study in Uganda.

The following is the list of all documents approved in this application by Makerere University School of Health Sciences REC:

No.	Document Title	Language	Version Number	Version Date
1	Protocol	English	01	2021-05-11
2	Corvid-19 Risk Assessment Plan	English	00	2021-05-08
3	Data collection tools	English	00	2021-05-08
4	Data collection tools	English	00	2021-05-08
5	Data collection tools	English	00	2021-05-08
6	Data collection tools	English	00	2021--08
7	Data collection tools	English	00	2021-05-08
8	Data collection tools	English	00	2021-05-08
9	Data collection tools	English	00	2021-05-08

Yours Sincerely

Kalidi Rajab
For: Makerere University School of Health Sciences, REC

Appendix 3: Post Hoc Dunnett's Test Results

Table 13: : Summary of the Results of post hoc Dunnett's Test versus control groups on significant ANOVA findings for average weight gained, food and water consumption

Parameter	Dose (mg/Kg)	P-Values			
		Extracts			
		WUBA	WUBE	WULA	WULE
Average Weight gain	250	<.001	<.001	<.001	<.001
	500	<.001	<.001	<.001	<.001
	1000	<.001	<.001		<.001
	SAT 1000	<.001		<.001	<.001
Water Consumption	250	<.001	<.001	<.001	<.001
	500	<.001	<.001	<.001	<.001
	1000			<.001	0.028
	SAT 1000	.017	<.001		<.001
Food Consumption	250	<.001	<.001		<.001
	500	<.001	<.001		<.001
	1000		<.001	.031	<.001
	SAT 1000	<.001		<.001	

Note: Only statistically significant results are reported, SAT- satellite

Table 14: Results of post hoc Dunnett test versus control group showing statistically significant results for weight Indices.

Organ	Dose mg/Kg	P-Values			
		Extracts			
		WUBA	WUBE	WULA	WULE
Heart	1000				.049
	SAT 1000		.004		<.001
Stomach	250				.003
	500	.013			<.001
	1000	.009			.046
	SAT 1000	.019			
Liver	250	<.001		.006	
	500			.002	
	1000		.015		.007
	SAT1000	.003	.008	<.001	.006
Left Kidney	1000		.009		.027
	SAT 1000	<0.001			
Right Kidney	1000		.005		<0.001

Note: Only statistically significant valued reported , SAT- satellite

Table 15; Post Hoc Dunnett's Test Results versus Control Group showing only statistically significant observations for biochemical parameters

Parameter	Dose (mg/kg)	P-Values Extracts WUBA	WUBE	WULA	WULE
ALB	250	<.001			
	500	.002			
	SAT1000	<.001		<.001	
AST	250	<.001	<.001	<.001	<.001
	500	<.001	<.001	<.001	<.001
	1000	<.001	.006	<.001	<.001
	SAT 1000	<.001	<.001	<.001	<.001
	250	<.001		.012	<.001
ALT	500	<.001		<.001	<.001
	1000	<.001		<.001	
CRET	250			,042	
	1000			.007	
NA	250	.049	<.001	.005	
	500		<.001	<.001	
	1000			.002	
	SAT 1000	.011	.010	<.001	<0.001
K	250		<.001		
	500		<.001		
	1000	.030			
	SAT 1000	.018			

Note: Only statistically significant valued reported, SAT- satellite

Table 16: Post Hoc Dunnett test results vs control group for Haematological parameters

Parameter	Dose (mg/Kg)	P-Values			
		Extracts			
		WUBA	WUBE	WULA	WULE
RBC	SAT 1000	.003	.021		<.001
WBC	SAT 1000				.038
HCT	250				.007
	500				<.001
	1000		<.001		.001
HGB	250	.034			
	500				.009
	1000		.008		
	SAT 1000			.022	.005
PLT	250		.006		
	500	.004	.001		
	1000		.027		

Note: **Only P-values <0.05 are reported**, SAT- satellite