ANTI-ASTHMATIC AND IMMUNOMODULATORY PROPERTIES OF EXTRACTS OF *ACACIA XANTHOPHLOEA, STRYCHNOS HENNINGSII* AND *MICROGLOSSA PYRIFOLIA* IN ASTHMA-INDUCED MICE

ODONGO GRACE AKINYI

MASTER OF SCIENCE

(Molecular medicine)

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Anti-asthmatic and Immunomodulatory properties of extracts of *Acacia xanthophloea, Strychnos henningsii* and *Microglossa pyrifolia* in asthma-induced mice

Grace Akinyi Odongo

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

Signature …………………………………. Date ………………………

Grace Akinyi Odongo

This thesis has been submitted for examination with our approval as university supervisors.

Signature………………………………… Date ………………………

Dr. Festus Tolo

KEMRI, Kenya

Signature ………………………………….. Date ……………………

Prof. Fred Wamunyokoli

JKUAT, Kenya

Signature………………………………… Date ………………………

Dr. Joseph Mwatha

KEMRI, Kenya

DEDICATION

I dedicate this work to my father Michael Odongo.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS/ACRONYMS

ACUC: Animal Care and Use Committee

AHR: Airway Hyper responsiveness

AIDS: Acquired Immune Deficiency Syndrome

ANOVA: Analysis of Variance

BAL: Bronchoalveolar lavage

BALF: Bronchoalveolar lavage fluid

BC: Baseline control

BW: Body weight

CBRD: Center for Biotechnology Research and Development

CC$_{50}$:   Cytotoxic Concentration 50

CO$_2$:   Carbon dioxide

COPD: Chronic Obstructive Pulmonary Diseases

CTMDR: Center for Traditional Medicine and Drug Research

DMEM: Dulbecco Modified Essential Medium

DMSO: Dimethyl sulfoxide

EDTA: Ethyl diamine tetra acetate

ELISA: Enzyme Linked Immunosorbent Assay

ERC: Ethical Review committee

FCS: Fetal calf's serum

GINA: Global Initiative on Asthma

HBSS: Hanks' balance salt solution

HIV: Human Immunodeficiency syndrome

IgE: Immunoglobin E

IL: Interleukin

IP: Intraperitoneal

JKUAT: Jomo Kenyatta University of Agriculture and Technology

KEMRI: Kenya Medical Research Institute

LABA: Long-Acting Beta 2 Adrenoceptor

MTT: (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide)

NC: Negative control

NSAIDs: Non-Steroidal Anti-Inflammatory Drugs

O.D: Optical density

OVA: Ovalbumin

PBS: Phosphate buffered saline

PC: Positive control

SABA: short-Acting Beta 2 Adrenoceptor

SD: Standard Deviation

SSC: Scientific Steering Committee

TLC: Thin Layer Chromatography

WHO: World Health Organization

DEFINITION OF OPERATIONAL TERMS

Allergen: An antigen that produces an abnormally vigorous immune response in which the immune system fights off a perceived threat that would otherwise be harmless to the body. Such reactions are called allergies. It is also described as an antigen capable of stimulating a type-1 hypersensitivity reaction in atopic individuals through immunoglobulin E (IgE) responses.

Allergy: is a hypersensitivity disorder of the immune system.

Asthma: refers to a chronic inflammatory disorder of the airways in which many cells and

Cellular elements play a role. Asthma is a chronic lung disease where inhalation and exhalation are obstructed by the production of excess mucus and the swelling of the airway membranes, giving rise to coughing and wheezing. Asthma is a chronic inflammatory disease of the airways characterised by early and late asthmatic responses (EAR & LAR) to allergen, airways hyper responsiveness (AHR) to inhaled spasmogens, airway inflammation and airway oedema.

Cytotoxic Concentration 50 (CC_{50}): it is dosage that kills or destroys 50% of the cells.

Cytotoxicity: destruction or damage or killing or limitation of growth of cells.

Decade: Period of ten years

Diff-Quick staining: is a modified Giemsa staining technique. It faster and more efficient in staining different types of smears and gives better results for differential white blood cells staining. It is a Romanowsky stain and hence operate in the same principle

Eosinophils: are white blood cells and one of the immune system components responsible for combating multicellular parasites and certain infections in vertebrates. Along with mast cells, they also control mechanisms associated with allergy and asthma.

Ethno-botanical: study of relationships that exist between people and plants e.g. use of plants for food, medicine, dye, clothing, rituals, cosmetics and construction.

Extracts: is a substance made by extracting a part of a raw material, often by using a solvent such as ethanol or water.

IgE: Immunoglobulin E is a class of antibody or immunoglobulin ("isotype") that has been found only in mammals.

Immunomodulatory: refers to an immunological change, adjustment or regulation and capable of modifying or regulating one or more immune functions.

MTT dye: Is a Tetrazolium salt that is metabolized by viable cells to form a purple colored formazan compound that is visible and can be quantified by spectrophotometry. Non-viable cells cannot metabolize the MTT dye thus serves as a marker for cell viability.

Ovalbumin: is a key reference protein constituent of chicken egg whites for immunization and biochemical studies.

Prednisolone: is a synthetic glucocorticoid, a derivative of cortisol which is used to treat a variety of inflammatory and autoimmune conditions such as Asthma, and other allergic conditions.

Vero E6 cells: are mammalian cells derived from kidney tissue of *Cercopithecus aethiops,* also known as green.

ABSTRACT

The increasing prevalence of asthma in developing countries during the last decade continues to represent a significant public health problem, causing both economic and social burdens. It remains an area of considerable unmet medical need which affects 235–330 million and kills about 300,000 people worldwide. Low and middle income countries make up more than 80% of the mortality and the prevalence of Asthma in Kenya is 15.8%. Treatment of asthma is hindered by severe side effects and high cost of the treatment. Methanol and aqueous extracts of *Acacia xanthophloea, Strychnos henningsii* and *Microglossa pyrifolia* have shown efficacy on antimicrobial and antioxidant properties but have not been investigated for anti-asthmatic activities. This study was aimed at evaluating the anti-asthmatic activities of extracts of *Acacia xanthophloea, Strychnos henningsii* and *Microglossa pyrifolia* on asthma-induced mice. A total of 81 female Swiss Albino mice grouped into 9 major groups with 9 mice each, aged 8weeks old and weighing 20 +/- 2g, were asthma-induced by Intraperitoneal injection of 1% Ovalbumin (grade VI; Sigma, Steinheim, Germany) followed by treatment using methanol and water extracts of *A xanthophloea, S henningsii* and *M pyrifolia* in concentrations of 50, 100 and 200mg/kg body weight except for positive control group of mice which was induced and not treated. Standard reference drug control group was given 10mg/kg Prednisolone. After treatment, serum total Immunoglobin E (IgE) levels were determine using mouse OVA specific IgE Enzyme Linked Immunosorbent Assay (ELISA) (Legend Max^{TM}). Bronchoalveolar lavage fluid eosinophils level was also determined using Diff-Quick staining Technique. Cytotoxicity determination of the plant extracts was tested on Vero E6 cells using MTT viability assay. Phytochemical Screening of the plant extracts was done using thin layer chromatography. Data were analyzed and expressed as Means and Standard Deviation and the parametric data was statistically analyzed using one way Analysis of Variance (ANOVA) followed with unpaired student's t-test at p-value < 0.001, GraphPad software was used. Cytotoxicity results were analyzed by Almar blue assay software to determine the Cytotoxic concentration 50 (CC$_{50}$). The key results showed that the extracts were able to reduce the serum total IgE levels and Balf eosinophils level by upto100% in reference to the positive control. The standard drug Prednisolone also demonstrated the same effects. Based on this study the extracts tested therefore have the ability to reduce IgE and eosinophils levels in an asthmatic attack. TLC revealed presence of Phenols, Alkaloids, and Flavonoids in the methanol and aqueous extracts of the medicinal plants. These results can be used as a guideline in setting other experiments using human cells or human subjects since this was a pilot study.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Asthma is a chronic inflammatory disease of the airways characterized by episodic attacks of airflow obstruction, wheezing, coughing and shortness of breath. Many risk factors contribute to the severity and expression of asthma and these include both environmental and occupational exposure as well as genetic associations. It is estimated that over 300 million people around the world suffer from asthma globally and the number of people affected with asthma in the current rising trends will grow by more than 100 million by 2025 (WHO, 2007; Holgate *et al*., 2011).

In 2005 there were more than three million deaths, from chronic obstructive pulmonary diseases (COPD) and out of this, 250,000 deaths were from Asthma. Ninety percent of all COPD deaths were in low- and middle- income countries. Globally, COPD is now the fourth leading cause of death, and by 2025 it is predicted to become the third and will surpass HIV/AIDS in Africa (WHO, 2008). Rates vary between countries with prevalence between 1 and 18% (GINA, 2011). For example the reported prevalence of wheezing illness in Western countries varies from as low as 5% in Sweden to as high as 25– 30% in Australia and New Zealand. Data from developing countries also show wide variation: Kenya 15.8%, Ethiopia 9.1%, Nigeria 13.0%, Mozambique 13.3%, and South Africa 20.3% (Hailu *et al*., 2003; Frederik van Gemert *et al*., 2011). Asthma appears to be more severe in sub-Saharan Africa than in affluent countries, although the latter have the highest symptom prevalence (Frederik van Gemert *et al*., 2011).

Non-steroidal anti-inflammatory drugs (NSAIDs) have been successfully used for the alleviation of pain, fever, and inflammation for many years and continue to be used daily by millions of patients worldwide (Rao *et al*., 2010; Vonkeman & van de Laar, 2010). They are well tolerated for short periods, but long-term administration may result in persistent adverse events (Quan *et al*., 2008). However, their use is limited by serious side effects, most common of which is gastro duodenal toxicity (Vonkeman & van de Laar, 2010). Other common adverse effect is related to the

kidneys which is acute renal insufficiency (Ziltener *et al*., 2010). Neither the standard drugs nor the newer generations are without their shortcomings. Given the limitations of these agents, there remains a clear need for identification and validation of new anti-inflammatory drug targets (Ward, 2008). The expensive cost of the current drugs used for asthma treatment is as a result of persistent continual usage and also to the elimination of the side effects caused.

Traditional medicine is currently the fastest growing medical field with herbal therapies becoming increasingly popular. This mode of treatment is preferred because it is considered more holistic, acceptable, accessible, low cost and proven to be safe (Gessler, 1995). Moreover, as far as asthma is concerned, the anti-inflammatory activities of plants are due to the secondary metabolites. Medicinal plants such as *Acacia xanthophloea, Strychnos henningsii,* and *Microglossa pyrifolia* have shown anti-bacterial, anti-cancer, anti- oxidant and anti-plasmodial activity (Katerere & Eloff, 2004; Kalaivani *et al*., 2010), but have not been investigated against anti-inflammatory activity even though they contain bioactive compounds that are known to show anti-inflammatory activity. These bioactive compounds consist of polyphenols, flavonoids, alkaloids, terpenoids, steroids, carotenoids, coumarins, curcumines and etc. (Saeed *et al*., 2010). Among these secondary metabolites responsible for the anti-inflammatory activity is isoretuline from *Strychnos henningsii* Gilg (Calixto *et al*., 2000), *Acacia xanthophloea*, and *Microglossa pyrifolia* are also believed to play role in anti-inflammatory activities but are not yet proven scientifically. Moreover, from the ethnobotanical survey it was realized that *Strychnos henningsii* is the most popular plants in management of respiratory tract infections in Kenya and Kibwezi in particular (Kariuki & Njoroge, 2011).

1.2 Problem statement

Worldwide Asthma cases are increasing at the rate of 50% every decade and according to WHO by the year 2025, Asthma along with COPD will become third leading cause of death. As of 2011, 235–330 million people worldwide were affected with asthma and approximately 250,000 people die per year from the disease. The

burden of asthma has been growing over the past 30 years, particularly in the low and middle income countries. Low and middle income countries make up more than 80% of the mortality. The economic cost of asthma is considerable both in terms of direct medical costs (such as hospital admissions and cost of pharmaceuticals) and indirect medical costs (such as time lost from work and premature death). The employment of anti-inflammatory agents may be helpful in the therapeutic treatment of those pathologies associated with inflammatory reactions. Non-steroidal or steroidal anti-inflammatory drugs are commonly used to treat different inflammatory diseases. The most commonly used drug is Prednisolone, available in pharmacy at a range price of $6.38-$89.00. In haled glucocorticoids pose a risk of developing glaucoma and cataract. The side effects of the currently available anti-inflammatory drugs pose a major problem in their clinical use. Attention is being given to the investigation of the efficacy of traditionally used plants as they are affordable and have fewer adverse effects.

Therefore, the aim this study is to evaluate the anti-asthmatic activities of extracts produced from the *Acacia xanthophloea, Strychnos henningsii* and *Microglossa pyrifolia* on asthma-induced mice as they have antimicrobial, anticancer, antimalarial and antioxidant properties.

1.3 Justification

Non-steroidal anti-inflammatory drugs (NSAIDs), steroidal drugs, and immuno-suppressant drugs, which have been usually used in the relief of inflammatory diseases worldwide for a long time, are often associated with severe adverse side effects, such as gastrointestinal bleeding and peptic ulcer (Valiollah *et al.*, 2009). Recently, many natural medicines derived from plants, marine organisms, etc. were considered effective and safer for the treatment of various diseases including inflammation and pain (Su *et al.*, 2011).

From the previous ethno-botanical survey there is empirical evidence that *Strychnos henningsii* is the most popular plants in management of respiratory tract infections in Kenya (Kariuki & Njoroge, 2011). *Acacia xanthophloea, Strychnos henningsii* and *Microglossa pyrifolia* have shown antimicrobial, anticancer, antimalarial, and

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antioxidant activity. However, they have not been investigated against anti-asthmatic activity.

Therefore, this study investigated the effects of *Acacia xanthophloea, Strychnos henningsii* and *Microglossa pyrifolia* extracts on the level of IgE and anti-inflammatory activities. Moreover, it is expected to lay a platform ground for development of new anti-asthmatic drugs that are cheaper, effective and with no or less side effects.

1.4 Research Questions

1) What are the effects of *Acacia xanthophloea, Strychnos henningsii* and *Microglossa pyrifolia* methanol and aqueous extracts on the level IgE in asthma induced mice model?

2) What are the anti-inflammatory activities of methanol and aqueous extracts of *Acacia xanthophloea, Strychnos heninningsii* and *Microglossa pyrifolia* in asthma induced mice model?

3) What are the cytotoxic concentrations (CC_{50}) of methanol and aqueous extracts of *Acacia xanthophloea, Strychnos heninningsii* and *Microglossa pyrifolia* on mammalian Vero E6 cells?

3) What are phytochemical constituents in the extracts of *Acacia xanthophloea, Strychnos henningsii* and *Microglossa pyrifolia*?

1.5 Null Hypothesis

The extracts of *Acacia xanthophloea, Strychnos henningsii* and *Microglossa pyrifolia* have no anti-asthmatic and immunomodulatory properties in ovalbumin induced asthma mice model.

1.6 Objectives

1.6.1 General Objective

To determine the anti-asthmatic and immunomodulatory properties of extracts of *Acacia xanthophloea, Strychnos henningsii* and *Microglossa pyrifolia* in ovalbumin induced-asthma mice model

1.6.2 Specific Objectives

1) To determine the effects of methanol and aqueous extracts of *Acacia xanthophloea, Strychnos heninningsii* and *Microglossa pyrifolia* on the level IgE using asthma induced mice model.

2) To determine the reduction in eosinophils level by aqueous and methanol extracts of *Acacia xanthophloea, Strychnos henningsii* and *Microglossa pyrifolia* in asthma induced mice model.

3) To determine the cytotoxic concentrations (CC_{50}) of methanol and aqueous extracts of *Acacia xanthophloea, Strychnos henningsii* and *Microglossa pyrifolia* on mammalian Vero E6 cells.

4) To determine the phytochemical constituents in the extracts of *Acacia xanthophloea, Strychnos henningsii* and *Microglossa pyrifolia.*

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Asthma

The prevalence of asthma has increased during the last two decades in both the developed and developing countries (Eder *et al*., 2006). According to the World Health Organization (2007), over 300 million individuals are affected with asthma worldwide, and there are approximately 250,000 deaths attributed to the disease each year. The current rising trend it is estimated that the number of people with asthma will grow by a further 100 million by 2025 (Holgate *et al*., 2011).

Asthma is a chronic inflammatory disorder of the lungs that is characterized by recurrent episodes of airflow obstruction, bronchial inflammation, mucus hyper secretion and airway hyper responsiveness (AHR) (Busse & Lemanske, 2001). Patients with asthma typically experience shortness of breath, wheezing, coughing and difficulty in breathing particularly after exposure to an allergen or non-specific irritants with symptoms often worsening at night. The most general form of asthma is allergic asthma which is attributable to airway inflammation triggered by an environmental allergen. Allergic asthma can be defined by the positivity to skin prick test or via the presence of IgE antibodies to common environmental allergens. Other forms of asthma are non-allergic asthma where the cause of airway inflammation is unclear and mixed-type asthma where there is a combination of allergic and non-allergic factors. It is estimated that allergic asthma patients account for slightly more than half of all asthmatics (Handoyo & Rosenwasser, 2009).

Asthma is associated to T-helper (Th) type 2 cells response, immunoglobulin (Ig) E-mediated mast cell activation, and other inflammatory factors, including eosinophils, B cells, cytokines and chemokines (Herrick & Bottomly, 2003).

Currently used effective pharmacological treatments of asthma include the following groups of drugs (Barnes, 2004):

- Adrenoceptor stimulants (selective ß 2- agonists) which have bronchodilator effect
- Glucocorticoids which inhibit inflammatory changes in airways
- Phosphodiestrase inhibitors which help to open constricted airways
- Cromones which have mast cells stabilizing activity
- Anticholinergic, which decrease airway mucus secretion
- Leukotriene modifiers which have anti-inflammatory effects

These medications are used alone or in combination to control the asthma symptoms experienced by many asthmatics. Immunotherapy, on the other hand, is a causal treatment for allergic disorder since it is able to modify the immunological background of the disease and thus to provide long-lasting remission of allergic symptoms. However, there is a small but real risk of IgE-mediated reactions, including anaphylaxis when using immunotherapeutic strategies (mainly subcutaneous injections). At the moment, glucocorticoids are the most effective and widely used therapy available for the control of asthma and are considered as the golden standard by which novel anti-inflammatory medications are measured.

Despite the availability of a wide range of anti-asthmatic drugs, the relief offered by them is mainly symptomatic and short lived. Moreover their side effects are also quite disturbing. Hence a continuous search is needed to identify effective and safe remedies to treat bronchial asthma (Barnes, 2004).

2.2 Medicinal plants

During the past decade, traditional systems of medicine have become a topic of global importance. Current estimate suggest that, in many developing countries a large proportion of the population relies heavily on traditional practitioners and medicinal plants to meet primary health care needs. Plants represent still a large untapped source of structurally novel compounds that might serve as lead for the development of novel drugs to mitigate unpleasant sensation occurring in varying

degrees of severity as a consequence of injury, tissue-damage, diseases-comprehensively termed pain (Shashank *et al*., 2013).

Nowadays, focus on plant research has increased all over the world. Alternate healthcare system which has a growing potential as export earner because plant based medicines are affordable, offer diversity and have freedom from side effects of synthetic drugs and resistance emergence to pathogenic microbes. The use of plants for healing purposes is very common in developing countries, especially in the rural areas. This is probably due to the perceived beneficial and lower side-effect profile of natural products that are extracted from plants (Leonardo *et al*., 2000). However, most medicinal plants are used indiscriminately without knowing their possible adverse effect. Over the past decades, several reports in both developed and developing countries have indicated adverse effects allegedly arising from the use of medicinal plants (Elvin-Lewis, 2001).

It has been reported that in Kenya for example, about 90% of the population has consented to have used traditional medicines at least once for various health conditions (Chirchir *et al*., 2006). In Kenya, the number of patients being treated in traditional health facilities is on the increase, sometimes reaching well over 500 patients per month attended to by just one herbalist (Njoroge, 2006). From the ethnobotanical survey it was realized that *Strychnos heninningsii* and other *Acacia species* and *Microglossa species* are the most popular plants in management of respiratory tract infections in Kibwezi, Kenya (Kariuki & Njoroge., 2011). All these plants are tree and occur in semi-arid areas.

2.2.1 *Acacia xanthophloea*

The genus name Acacia is derived from the Greek word acantha meaning spine, thorn or prickle and the species name xanthophloea is derived from the Greek words xanthos meaning yellow and phloios meaning bark (Orwa *et al.,* 2009). *Acacia xanthophloea* belongs to the pod bearing family *Fabaceae*. A special feature of this family is the pulvinus which is a conspicuous thickening at each petiole and petiolule base which allows the leaves to close at night and also during extreme heat. Stipules (or a stipular scar) are always present and are often modified into thorns or spines as is the case with the fever tree (Orwa *et al*., 2009).

Acacia xanthophloea is a large tree, 15-25 m tall, with a crown that is somewhat spreading, branching fairly up the trunk. Bark smooth, slightly flaking, yellow to greenish-yellow. New twigs purple tinged but flaking later to reveal the yellow under layer. Leaves are 4-10 cm long with a hairy midrib. Pinnae has 4-7 pairs, about 10-17 pairs of small leaflets. Stipules spinescent, spines white, straight, up to 7-10 cm in length, paired, often slender and conical at the base. Buds pink; flowers fragrant, in round golden balls on slender stalks; several borne together with a tuft of leaves, in the axils of the thorns. Pods 5-19 cm long, pale brown, straight, flat, rather papery, moniliform with segments mostly longer than wide, usually breaking into segments containing individual seeds borne in small clusters. Pods turn from green to pale greyish-brown when mature. Each pod contains 5-10 elliptic, flattened seeds, pale to dark green (Orwa *et al*., 2009)

This species occurs in groups in low-lying swampy areas in the Northern Province, KwaZulu-Natal and Mpumalanga in South Africa and as far North as Kenya in tropical Africa. The tree is spread from Kenya south to South Africa. It is often found in clusters where the water table is high, beside lakes or rivers, 600 - 2, 300 m, often on black cotton soils.

Traditionally used as a medicine to treat high cholesterol, diabetes, cancer, gingivitis, mouth sores and pharyngitis. The bark is used for treating fevers and eye complaints. In Kenya a bark decoction is used in traditional medicine to treat indigestion and in Tanzania it is used to treat sickle-cell anemia. In South Africa Zulu people use powdered bark as an emetic to treat malaria. The wood is used in building, being a hard, heavy and a useful general purpose timber; it should be thoroughly seasoned first to avoid it from cracking. (Exotichealingherbs, 2012)

A. xanthophloea is also reported to have antibacterial activity (Katerere & Eloff, 2004). Scientific studies reveal even more about A. xanthophloea and its health Benefits. There are three active compounds in Acacia; these are **Catechin, Catechol** and **Catecholamine** that have been scientifically researched to determine their medicinal uses.

Catechin is an antioxidant and an anticarcinogenic. Antioxidants are substances that protect cells against the effects of free radicals. Free radicals are molecules produced when the body breaks down food, or by harmful environmental exposure. Free radicals can damage cells, and may play a role in heart disease, cancer and other diseases.

Regarding the antioxidant activity, catechin has been found to be the most powerful scavenger between members of the different classes of flavonoids. Catechin exists in the form of a glycoside which is also found in tea. Antioxidants are well-known for their anti-aging properties and Age-related Neurodegeneration.

Small amounts of **Catechol** occur naturally in fruits and vegetables, along with the enzyme polyphenol oxidase also known as catecholase, or catechol oxidase. Upon mixing the enzyme out in open air, the colourless catechol oxidizes to reddish-brown melanoid pigment, a derivative of benzoquinone. Benzoquinone is said to be antimicrobial, which slows the spoilage of wounded fruits and other plant parts.

Catechol has been identified in the brain and various endocrine tissues. There are high concentration of catechol estrogens in the hypothalamus and pituitary. Catechols have potent endocrine effects and, because of their normal occurrence in the hypothalamic-pituitary axis, they have an important role in neuroendocrine regulation.

Catecholamines are significant hormones and neurotransmitters that are phenethylamines in which the phenyl group has a catechol skeleton structure. Catecholamines are molecules that include dopamine, as well as the "fight-or-flight" hormones adrenaline and nor adrenaline released in response to stress. In the human body catecholamines are epinephrine (adrenaline), nor epinephrine (nor adrenaline) and dopamine, which also constitute brain chemistry. The brain communicates with itself by sending out chemical information from one neuron or nerve cell to another. Brain chemistry is the sum of all the chemical messaging that takes place in the brain, which allows it to carry out its daily functions, such as generating movement,

speaking, thinking, listening, regulating the systems of the body, and countless others.

So considering its wide ethnomedical uses and broad spectrum activity against microbes, it became very compelling that its anti-asthmatic activity be investigated in the search for drugs for safe and effective treatment of Asthma.

2.2.2 *Strychnos henningsii*

Strychnos henningsii Gilg (Loganiaceae) is commonly known as umnono in Zulu language and mostly cultivated in South Africa, Tanzania, Uganda and Kenya (Oyedemi *et al.*, 2009). It is a small evergreen tree or shrub with leathery leaves and clean green-reddish stem. It is a tree that grown up to 2-12 months in a dry or moist forest, wooded hillsides, coastal forest and stream banks. The fruit is oblong and brown or orange when ripe. The bark is crown compact with dark green and glossy foliage. In local medicine of South Africa, the decoction or infusions of the stem bark is widely used for the management of DM (Oyedemi *et al.,* 2009). It is also used in the eastern part of Africa for the treatment of various ailments including abdominal pain, syphilis, snake bite, gastrointestinal pain, rheumatism, diabetes, malaria and to hasten wound healing in animal (Oyedemi *et al*., 2009). About five compounds have been isolated including indolinic alkaloids, strychnine, brucine, curarine and bitter glycoside (Penelle *et al.*, 2000). The plant has also been reported as potential agent in the development of new antinociceptive and antispasmodic drugs due to the presence of retuline-like alkaloids (Penelle *et al.*, 2000). Previous studies on the phytochemical constituents of *S. heninningsii* revealed the presence of flavonoids, tannins, saponins, proanthocyanidins, phenols and glycosides (Oyedemi *et al*., 2010). A strong antioxidant and free radical scavenging activity has also been reported, which was attributed to the presence of phenolic compounds indicating the ethno-therapeutic usage of this plant for the management of oxidative stress induced diseases. *Strychnos henningsii Gilg* (Loganiaceae) is widely used in South African traditional medicine for the treatment of various ailments. These include gynecological complaints, abdominal pain, snake bite, gastrointestinal pain, rheumatism, malaria and diabetes mellitus (Oyedemi *et al.,* 2009). Based on the fact

that *S. heninningsii* has strong antioxidant activities both in vitro and in vivo (Oyedemi et al., 2010), hence the determination of its anti-asthmatic properties in vivo is needful since the phytochemicals involved in antioxidant activity might have anti-inflammatory and anti-asthmatic properties.

2.2.3 *Microglossa pyrifolia*

M. pyrifolia (Lam) Kuntze (Astereceae) is a climbing shrub of up to 6 m, and it's widespread in tropical Asia and Africa. In Kenya it's commonly found in the Lake Vitoria regions and is known as *Nyabungu odide* by the locals (Kokwaro, 1993). It has finely ribbed branches and alternating petiolate leaves (Wild, 1975).

Traditional uses of *M. pyrifolia* include anti-malarial (Onegi *et al*., 2002), abdominal pain relief, rheumatism, diarrhea (Neuwinger, 1994), headache and cold relief, and treatment of limb fractures (Kokwaro, 1993), treatment of dermal infections, eye infections, cough, elephantiasis, mastitis, yellow fever and black fever.

M. pyrifolia has been proven scientifically to have anti-malarial properties (Köhler *et al*., 2002; Omollo C. O., 2011). It has also been shown to have anticonvulsant property (Bum N. E. *et al*., 2009). Moreover, antimicrobial, antioxidant, and free radical scavenging properties of *M. pyrifolia* have also been reported (Dickson R. A. *et al*, 2006). Other properties exhibited by *M. pyrifolia* include; complement modulating activity (Cos P. *et al*., 2002), and sedative activity (Bum N. E. *et al*., 2009). These findings create the need for investigating it anti-asthmatic property.

2.3. Mouse models of asthma

Experimental animal models provide important information on the outcome of a variety of diseases. Different animal models of asthma have been developed in recent years to study the pathophysiology of the disease, the activity and function of different genes and cellular pathways and to evaluate the safety of new drugs or chemicals before entering clinical studies. Allergic asthma models have been described in rodents (mice, rats and guinea-pigs) and dogs (Out et al., 2002). The mouse is being increasingly used as a model animal since it offers many advantages over larger species (Kips et al., 2003). For example, there is worldwide availability of genetically well-characterized inbred mouse strains at relatively low cost as well

as open access to the genetic map of the murine genome. The availability of genetically engineered transgenic and knock-out mice as well as a range immunological reagents available for analysis of cellular and mediator response further enhance the advantages of mice as valuable experimental animal, allowing the detailed investigation of the different mechanisms of allergic reactions (Shin et al., 2009; Taube et al., 2004).

Although experimental mouse models display many of the features of human asthma, including elevated levels of IgE, airway inflammation, goblet cell hyperplasia and airway hyperactivity to specific stimuli, a single animal model may not be able to reproduce all of the morphological and functional features of the disease. A large number of studies with asthma have been done by using models of acute allergic response, but chronic asthma models with clinical aspects related to human asthma have also been investigated by several research groups (Kumar *et al.,* 2008; Lloyd, 2007).

Mice are usually sensitized by intraperitoneal (i.p.) injection of chicken egg ovalbumin (OVA), frequently given together with an immune-boosting adjuvant such as aluminium hydroxide (alumn). Other natural allergens or purified proteins derived from potent allergens including house dust mite (HDM), ragweed or fungi have also been increasingly used as sensitizing allergens in mice instead of ovalbumin (Johnson *et al*., 2007; Leino *et al*., 2006). Sensitization may also be achieved by repeated administration of allergen via the skin (intradermally, subcutaneously or epicutaneously) or via the airways (Intranasal, intratracheally).

CHAPTER THREE

3. 0 MATERIALS AND METHODS

3.1 Study sites

The study was conducted at Kenya Medical Research Institute (KEMRI) Nairobi, Kenya at the Center for Traditional Medicine and Drug Research (CTMDR) which has advanced laboratories equipped with facilities that were mandatory for these experiments such the 4°c, -20°c, and -80°c freezers, CO_2 incubator, freeze drier, rotary evaporator, Ultraviolet chamber, multiplate spectrophotometer, clean-bench, centrifuge, etc. The laboratories are level 2. CTMDR focuses its research on traditional medicine and drug development; it is under KEMRI program named; Traditional Medicine and Drug Development Program (TMDDP). In vivo work with mice was done at KEMRI animal house based at the Center for Biotechnology Research and Development (CBRD). The mice were bred and reared from the KEMRI animal house.

3.2 Study Design

Laboratory based experimental study design was used.

3.3 Experimental animals

A total of 81 female healthy Swiss Albino mice aged 8 weeks old and weighing 20+/-2g were obtained from the KEMRI animal house. The animals were moved into the experimental room for acclimatization one week before onset of experiments. The experimental room was maintained at standard conditions of temperature 25ºc, and 12hrs light/dark cycle in environmentally controlled room in KEMRI animal house. Three mice were housed in 15cm $\times$ 21cm $\times$ 29cm transparent plastic cages bedded with wood shavings and equipped with continuous-flow nipple watering devices. They were fed with standard pellet diet (Mice pellets UNGA® feeds) and water *ad libitum*. The dressings in the cages were changed on a daily basis.

The mice were monitored daily and periodically after completion of a process. The care and handling of mice were in accordance with the internationally accepted standard guidelines for care and use of animals.

3.4 Medicinal plants collection and extraction

3.4.1 Collection of the medicinal plants

The stem bark of the *Acacia xanthophloea* was obtained from Nairobi county, while the leaves of *Strychnos henningsii* was obtained from Makueni county, and *Microglossa pyrifolia* were collected from Kisumu. The plants were botanically authenticated by a botanical taxonomist from the University of Nairobi Herbarium; Mr. Mutiso while in the field prior to collection. Upon transportation to the screening center, they were assigned voucher specimens numbers and voucher specimens deposited at the University of Nairobi Herbarium, Nairobi (Appendix I).

3.4.2 Plant material preparation and extraction

3.4.2.1 Plant material preparation

The plant materials: (stem barks of *Acacia xanthophloea* and leaves of *Strychnos henningsii* and *Microglossa pyrifolia*) were chopped into small pieces then carefully washed under running tap water to remove dust and any other foreign materials and left to drain off, this was followed by air-drying at room temperature under shade for 14 days and grinding using a laboratory mill (Christy & Norris Ltd., Chelmsford, England) (Appendix XII) at the Center for Traditional Medicine and Drug research, KEMRI. The resultant plant powders were packed in air tight polythene bags.

3.4.2.2 Aqueous and methanol extraction methods

Water extraction: A 150g of the powdered plant material were extracted with 1500 ml of distilled water in a water bath at 60°C for 1 hour. The extract were then decanted into a clean dry 3000ml conical flask and filtered through 2 layers of sterile gauze. The filtered extract were freeze dried in 200ml portions using a Freeze Dryer (Edwards freeze dryer Modulyo) (Appendix II). The freeze dried powder was weighed, labeled and stored in an air tight bijou bottle at 4 °C until used. For *in vitro*

cytotoxicity assay sterilization was done using 0.22µm Millex^R syringe driven filter unit after diluting the freeze dried powder with PBS.

Methanol extraction: A 150g of each plant powder were weighed and put in a flat-bottomed conical flask, methanol was then added to cover the plant material completely and left to stand for 24 hours. Filtration was done as stated above. The acquired filtrate was concentrated using a rotary evaporator (Buchi Rotavapor R-114) (Appendix III) and percentage yield noted. Storage was done in a cool dry place until use. Further sterilization was done as stated above but with first diluting in 0.1% DMSO before final dilution with PBS

3.5 Preparation of the extracted plant material for in vivo assays

Water or Methanol extracts were diluted in Phosphate buffered saline (PBS) to prepare concentrations of 50, 100, and 200mg/Kg body weight. The average weight of mice was determined to be 20g. A 0.2ml of the extracts solution was to be injected intraperitoneally. The principle C1V1=C2V2 was used. To prepare 50mg/Kg body weight; 1mg of the extract was diluted in 200µl of PBS, for the concentration of 100mg/Kg b.w; 2mg/200ul was used and for 200mg/Kg b.w; 4mg of extract per 200ul of PBS was used.

3.5.1 Asthma induction in Swiss Albino mice

3.5.2 Grouping of mice for asthma induction and treatment

Female healthy Swiss Albino mice aged 8 weeks old and weighing 20+/-2g were placed in 9groups with 9mice per group and each group had 3 different concentration to be administered hence a total of 3 mice were used per concentration (Appendix VIII). The groups were composed of the methanol and aqueous extracts of *Acacia xanthophloea, Strychnos heninningsii, Microglossa pyrifolia* and mix extract. The 9th group was composed of 3 subgroups; Positive control, Baseline control and standard reference subgroup. A total of 81 mice were used.

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Table 3.1 Grouping of mice for in vivo assay

Acacia xanthophloea group					
Methanol extracts groups			**Water extract groups**		
50mg/Kg b.w	100mg/Kg b.w	200mg/Kg b.w	50mg/Kg b.w	100mg/Kg b.w	200mg/Kg b.w
Strychnos henningsii group					
Methanol extracts groups			**Water extract groups**		
50mg/Kg b.w	100mg/Kg b.w	200mg/Kg b.w	50mg/Kg b.w	100mg/Kg b.w	200mg/Kg b.w
Microglossa pyrifolia group					
Methanol extracts groups			**Water extract groups**		
50mg/Kg b.w	100mg/Kg b.w	200mg/Kg b.w	50mg/Kg b.w	100mg/Kg b.w	200mg/Kg b.w
Mixed extract group					
Methanol extracts groups			**Water extract groups**		
50mg/Kg b.w	100mg/Kg b.w	200mg/Kg b.w	50mg/Kg b.w	100mg/Kg b.w	200mg/Kg b.w
Control Mice groups					
Positive control		**Standard Reference drug group**	**Baseline group**		
Induced with Asthma and not treated		Treated with 10mg/Kg Prednisolone	Non-induced and non-treated		
3mice used		3mice used	3miced used		

Note: All mice were induced with asthma except the baseline control group.

3.5.3 Sensitization and Challenge with Allergen

All mice except for the baseline control subgroup were sensitized by intraperitoneal (IP) injection of each mouse with 100μl of 20μg ovalbumin (grade VI; Sigma, Steinheim, Germany) emulsified in 2mg Aluminum hydroxide (Alum) (Pierce, Rockford, II., USA) in 200μl phosphate-buffered saline (PBS) (Gonzalo *et al.*, 1996;

Yoon-Seok *et al*., 2005) using a 29 gauge needle and syringe on day 0, 7 and 14 for induction of asthma (Appendix X). This was followed by ovalbumin challenge on day 21 and 22, where exposures to nebulizer 1% OVA in PBS (in a 5.9 L Pyrex glass box) for 30 minutes each day (Gonzalo *et al*., 1996; Yoon-Seok *et al*., 2005). A once per day treatment with 0.2ml of the desired drug (the plant extracts or standard reference drug; Prednisolone) was administered intraperitoneally on day 22, 23, 24 and 25 for all mice except those in Positive control and Baseline control subgroups as shown below. The plant extracts (methanol or water extracts) were administered in the concentrations of 50, 100, and 200mg/Kg body weight with 3 mice receiving each of the concentration. The standard reference subgroup received 10mg/kg Prednisolone. All treated mice were also observed for basic asthmatic symptoms including reduced physical activity, general discomfort, wheezing and difficulty in breathing 10 minutes before and after nebulization as well as one hour after each treatment exposure (Gonzalo *et al*., 1996; Yoon-Seok et al., 2005)
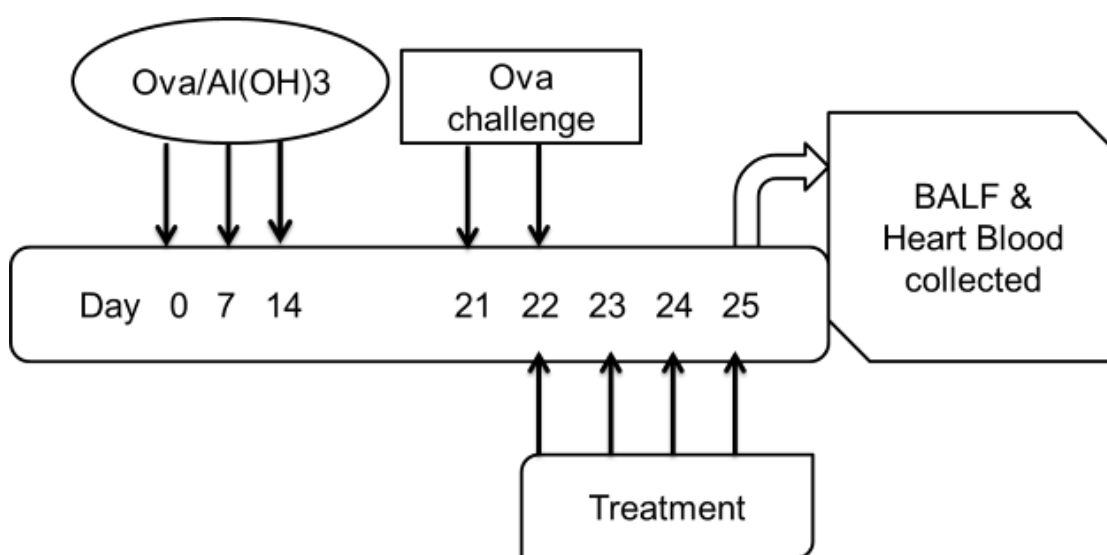

Figure 3.1 Sensitization and Challenge with Allergen

3.6 Mouse Ovalbumin specific IgE Enzyme linked immunosorbent Assay

3.6.1 Blood collection and Serum preparation

Blood was obtained from mice (anesthetized by intraperitoneal injection of 50mg/kg body weight of sodium pentobarbital - sagatal - conc. 0.5v/v distilled water) by

Cardiac puncture (Appendix XI) and allowed to stand at room temperature for 45minutes to clot and thereafter serum was obtained after centrifugation at 1000 xg for 10minutes at 20°c. The serum was kept at -80°c until analysis using ELISA kit (LEGEND MAX^{TM}).

3.6.2 Mouse OVA specific IgE ELISA protocol

All reagents were prepared prior to use and brought to room temperature. A 500µl of the 20ng/ml top standard was prepared by diluting 50 µl of standard stock solution in 450 µl of Assay Buffer A. Six two-fold serial dilutions of the 20ng/ml top standard was performed in separate tubes using Assay Buffer A as diluent. The final mouse OVA specific IgE standard concentrations in the tubes were 20ng/ml, 10ng/ml, 5ng/ml, 2.5ng/ml, 1.25ng/ml, 0.625ng/ml and 0.313ng/ml, respectively. Assay Buffer A served as the zero standard (0ng/ml). Washing of the plate was done 4 times with 300 µl of 1x Wash Buffer per well and any residual buffer was blot by firmly tapping the plate upside down on absorbent paper. All subsequent washes were performed similarly. A 50 µl of Matrix A was added to each well that was to contain the standard dilutions while Assay Buffer A of the same amount was added to wells that were to contain samples. This was followed by addition of 50 µl of standard dilutions or samples to the appropriate wells. The plates were then sealed with a Plate Sealer and incubated at room temperature for 2 hours while shaking at 200rpm. After incubation the plate content was discarded and the plate washed 4 times as described above. A 100 µl of Avidin-HRP D solution was added to each well, plate sealed and incubated at room temperature for 30 minutes while shaking. After incubation the content of the plate was discarded and washing was done 5 times as described above with soaking of wells in 1x Wash Buffer for 45 seconds for each wash. A 100 µl of substrate solution F was added to each well and incubation done for 15 minutes in the dark. The reaction was then stopped by adding 100 µl of stop solution to each well. Absorbance was read at 450 nm within 10 minutes.

3.7 Bronchoalveolar lavage fluid (Balf) collection and eosinophil evaluation

3.7.1 Balf collection

This was performed on one mouse from each group at different time points; for baseline control group it was performed immediately, while for positive control group it was done 48 hours after the last OVA challenge and to the experimental group 48hours after the last treatment.

The mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (50mg/kg) and then the trachea were cannulated for slowly delivery of 0.6ml warm

(37°c) Hanks' balanced salt solution ((HBSS) without calcium and magnesium but supplemented with 0.05mM EDTA) through the tracheal tube. This was followed by immediate withdrawal of the fluid (Haslam & Baughman, 1999; Klech *et al.,* 1989). The fluid is referred to as Bronchoaveolar lavage fluid (BALF).

The mice were allowed to recover from anaesthesia on a warm pad and then returned in their cages.

3.7.2 Balf eosinophil determination

The BALF was centrifuged at 300g for 8 minutes and the supernatant was decanted. The cell pellet was resuspended in 0.5ml of 10% foetal bovine serum (FBS). Total cells were counted with a haemocytometer after staining with Diff Quick stain (Appendix XIII) and viewing under light microscope (Gonzalo *et al.*, 1979). Percentage eosinophil level was determined against total white blood cells and recorded.

3.8 Preparation of the extracted plant material for in vitro assay

The extracted plant material was weighed 10mg in a 15ml centrifuge tube. A 1ml of PBS or DMSO (for water or methanol extract respectively) was added to the extract to dissolve it. This was then vortex using a vibrating mixer to dissolve completely. The volume was topped up to 10ml mark with PBS. The extract was sterilized by filtration using 0.22μM Millipore Millex^{TM} syringe driven filter. The final concentration of the sterilized plant extract was 1000μg/ml (1mg/ml), this was kept at -20°c freezer until use.

3.8.1 Cytotoxicity assay

On day one, Vero E6 cells in T-25 flask were trypsinized and 5 ml of complete Dulbecco´s Modified Eagle Medium (Gibco^{R} DMEM) was added to trypsinized cells to stop reaction, followed by centrifugation in a sterile 15 ml falcon tube at 300 xg for 5 min at 21°c. Media was removed and the cells resuspended with 1.0 ml complete media (DMEM supplemented with 15% fetal calf serum and 1% penicillin/streptomycin). Cells were then counted and recorded per ml. Dilution of cells was done using complete DMEM medium as per the following principle ($C_1V_1=C_2V_2$) to 200,000cells per ml. A 100 μl of cells (20,000 total cells) were

added into each well except for column 3, 6, 9 and 12 which served as controls then the plates were incubated for 24hrs at 37°c, 5% CO_2 and 95% humidity. On the second day cells were treated cells with the medicinal plant extracts after removal of the media. The highest concentration of the extract was placed at row H at a concentration of 1000µg/ml then serially diluted upwards until row A. (Serial dilution was done by placing 100µl of complete DMEM media in all the rows except H, then 50µl of the resuspended extract was transferred to the next row from H till A). The final concentration of the extract in each row is shown in the table 3.1 below. A single 96 well plate was enough to analyze four extracts. Since the first extract takes column 1,2,3, the second 4,5,6, the third 7,8,9 and the fourth extract 10,11,12 (Mosmann, 1983).

Table 3.2: Concentration of plant extract in each row

1	2	3	4	5	6	7	8	9	10	11	12
A	0.46µg/ml										
B	1.37µg/ml										
C	4.12µg/ml										
D	12.35µg/ml										
E	37.04µg/ml										
F	111.11µg/ml										
G	333.33µg/ml										
H	1000µg/ml										

The final volume in each well was 100µl. The plates were then incubated at 37°c/95% humidity and 5% CO_2 for 24hrs. On the third day, a 20 µl of 5 mg/ml MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) was added to each well. Including the wells without cells (control wells: 3, 6, 9 and 12) (Appendix XIV). This was incubated for 4 hours at 37°C in culture hood. Media was then removed carefully and 100µl of DMSO was added into each well without disturbing or rinsing the cells with PBS. Then cells were covered with tinfoil and agitated on orbital shaker for 15min. Absorbance was read at 562nm with a reference filter of 690nm (Mosmann, 1983). The results were then analyzed using Almar Blue assay software.

3.9 Preliminary phytochemical screening

The methanol and aqueous extract of *Acacia xanthophloea, Strychnos henningsii* and *Microglossa pyrifolia* were screened for the presence of selected phytochemical constituents such as phenols, alkaloids, and flavonoids, using thin layer chromatography (Appendix IV)

3.9.1 Thin layer chromatography procedure

The stationary phase was composed of pre-coated plates of aluminium foil coated with G/UV$_{254}$ silica gel. A 6 x 3 cm pre-coated plate was used for three samples. The line of origin was marked 0.5cm from the bottom and the spots for samples marked (A, S, M) for *Acacia xanthophloea, Strychnos heninningsii* and *Microglossa pyrifolia* respectively. Methanol and aqueous extracts were were run separately. A 1% concentration of the extract in methylene chloride or distilled water for methanol and aqueous extracts respectively was prepared and spotted onto the already marked spots using a microcapillary (10ul microcaps). The mobile phase was composed of two different types solvent systems (solvents with increasing polarity see Appendix V) for methanol and aqueous extract. Methanol extracts solvent system was that of Dichloromethane and Trichloromethane in a ratio of 1:1 respectively while Water extracts solvent system was composed of Butanol, Water, and Acetic Acid in a ratio of 4:1:1 respectively. The solvents were transferred to specific beakers and the pre-coated plate containing the extracts' spots dipped into the beaker with the marked line not touching the solvent. The beakers were then covered with a glass petri dish and allowed to run undisturbed (Appendix IV). After the run, the spots were visualized under UV light in the UV chamber (Appendix VI). The plates were allowed to dry then sprayed with specific spraying reagents for different phytochemical identification; phenols, alkaloids or flavonoids (see Appendix VII for spraying reagent preparation). The presence of specific phytochemical was determined by the spot color along the solvent run (Tolo *et al.,* 2006).

3.10 Disposal of mice after the experiments

At the end of the study the surviving mice were euthanized using 270mg/kg sodium pentobarbital (conc. 0.5v/v distilled water) and then incinerated**.**

3.11 Data Management and Analysis

A code book was used to explain variable names, meaning and codes. This served as a link between laboratory reports and data to be entered in the computer. Generated information was stored in a computer Microsoft office documents, and secured with passwords known only to the PI and colleagues. Soft copies of the data were stored on flash disks. Laboratory data sheets were duly completed.

Data was analyzed and expressed as Means ± Standard Deviation of replicates and the parametric data was statistically analyzed using one way Analysis of Variance (ANOVA) and unpaired student's t-test. A P-value of < 0.05 was considered statistically significant. All analyses were performed using Graphpad prism 6 software except for Cytotoxicity results which were analyzed by Almar Blue Assay software.

3.12 Ethical Clearance

Approval of this study was sought from KEMRI Scientific Steering Committee (SSC), KEMRI Animal Care and Use Committee (ACUC) and KEMRI Ethical Review Committee (ERC) with SSC No. 2845, see approval letters attached (Appendix XV, XVI, and XVII).

CHAPTER FOUR

4.0 RESULTS

4.1 Plant extraction percentage yield

The water and methanol extraction of dried bark of *Acacia xanthophloea* and the dried leaves of *Strychnos henningsii* and Microglossa *pyrifolia* was carried out successfully and percentage yield was calculated as shown below and the results were summarized in Table 4.1 and 4.2 below.

Percentage yield = Total weight of the extract obtained   X   100

Total weight of the dried plant material

Table 4.1: Water extracts percentage yield

Medicinal plant	Dry weight (g)	Freeze dried weight (g)	Percentage yield
Acacia xanthophloea	150	17.7	11.8
Strychnos heninningsii	150	18.9	12.6
Microglossa pyrifolia	150	20.1	13.4

Table 4.2: Methanol extracts percentage yield

Medicinal plant	Dry weight (g)	Rotary-evaporated weight (g)	Percent yield
Acacia xanthophloea	150	15.3	10.2
Strychnos heninningsii	150	11.9	7.9
Microglossa pyrifolia	150	13.1	8.7

Table 4.3: Asthmatic symptoms observed in Swiss albino mice after OVA challenge

First day of ovalbumin allergen challenge	Second day of ovalbumin allergen challenge
Reduced physical activity	Wheezing
General discomfort	Difficulty in breathing

4.4 Serum total IgE levels after treatments

The serum total IgE levels were calculated from the standard curve using the known standard concentrations and their specific optical density read at 450nm. The optical densities of the samples read at the same absorbance were used to calculate their specific serum levels using the following equation obtained from the standard curve in figure 4.1 below: $y = 0.2408x + 0.0233$ ($R^2 = 0.9985$), then multiplied by 2 which is the dilution factor for the serum. The positive control group (mice induced with asthma and not treated) exhibited the highest serum total IgE level of 7.25ng/ml. Standard drug reference group (treated with10mg/kg b.w Prednisolone), base line control group (non-induced and non-treated) and most extracts had their serum total IgE at 0ng/ml. The extracts that were able to reduce IgE level to zero include 50mg/Kg b.w of *A.xanthophloea* water and methanol extract, *S.heninningsii* methanol extract, *M.pyrifolia* methanol extract, Mix water extract, 100mg/Kg b.w of *A.xanthophloea* methanol extract, *M.pyrifolia* water and methanol extract, Mix water and methanol extract, 200mg/Kg b.w of *A.xanthophloea* water, *S.heninningsii* water extract, *M.pyrifolia* methanol extract, and Mix water extract. The results for serum total IgE levels are shown in figure 4.2 and 4.3 below. The percentage reduction of serum total IgE levels was calculated in reference to positive control, see figure 4.4 below. As high as 100% reduction in serum total IgE was seen in extracts mentioned above with the least reduction being 60.95%.

The final mean optical densities as read at 450nm were compared for all samples (figure 4.5). The optical densities for separate extracts type (methanol or water were also compared and analyzed using one way Anova and individual O.D for each concentration of extract compared with positive control value using unpaired student's t-test (figure 4.6 and 4.7) The Base line control (non-induced and non-

treated) and Standard reference drug control group were also compared to the positive control group (figure 4.8). Graph pad prism 6 software was used for analysis. There was a significant difference in the extracts optical densities and controls in reference to the positive control (P value <0.001). Figure 4.6, 4.7, and 4.8 below show the mean optical density and standard deviation.
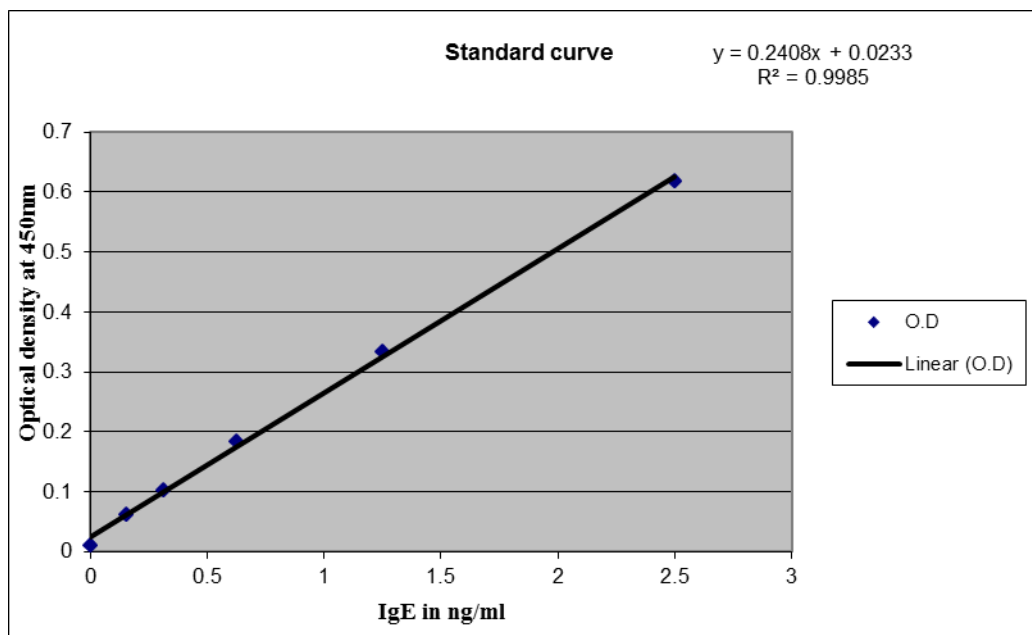
Figure 4.1: IgE concentrations standard curve.

Standard curve was obtained from standards Immunoglobin E (IgE) concentrations against their optical densities. The y-axis represents the optical density (O.D) read at 450nm while the x-axis represents the standards IgE concentration in ng/ml.
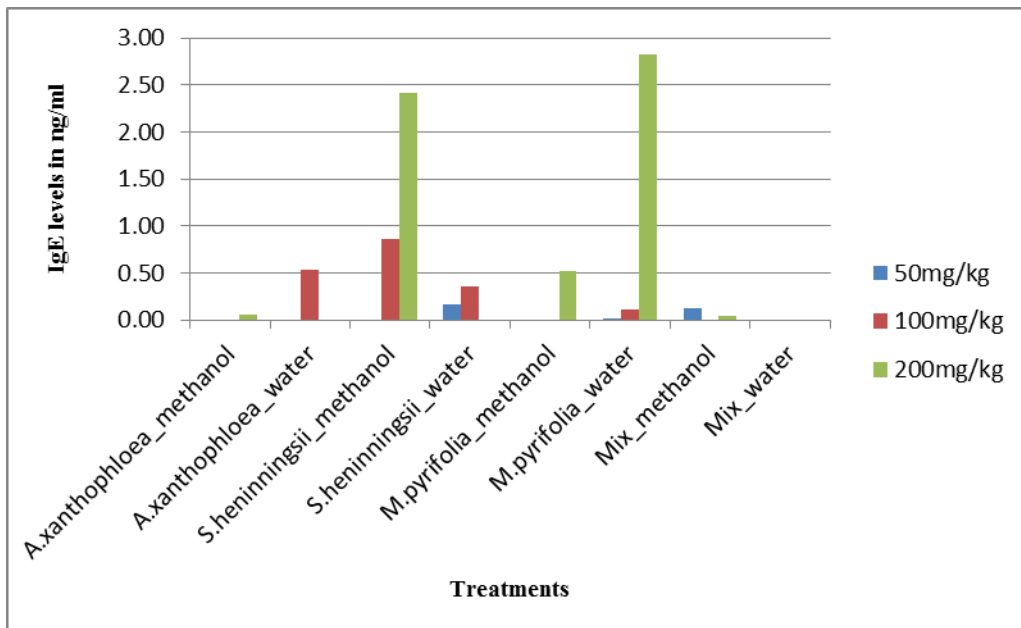
Figure 4.2: Serum total IgE levels in experimental mice groups.

Serum total IgE levels in mice treated with the plant extracts in concentrations of 50, 100, or 200mg/ml, the IgE levels were calculated using the equation y= 0.2408x + 0.0233 derived from standard curve in figure 1. The unknown 'x' which is the IgE concentration in ng/ml was determined. The y value is the optical densities obtained from the absorbance readings. The resultant IgE concentration was multiplied by two since the serum was double diluted in the experiment.
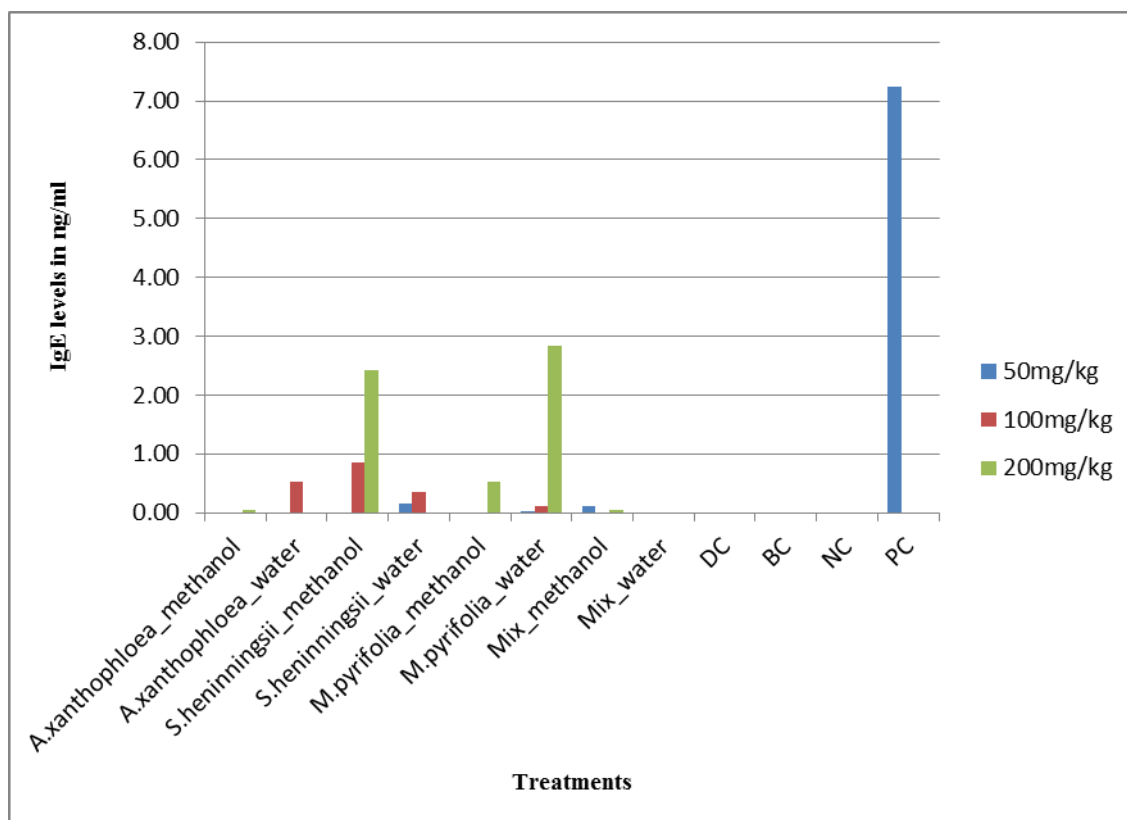
Figure 4.3: Serum total IgE levels in experimental mice groups and control mice groups. Serum total IgE levels in mice treated with the plant extracts in concentrations of 50, 100, or 200mg/ml and the controls including the standard reference drug control (DC) which is prednisolone, the Baseline control (BC) which are mice which were non-induced and non-treated, Negative control (NC) which is blank and the Positive control (PC) which are mice induced with Asthma and not treated. The IgE levels were calculated using the equation $y= 0.2408x + 0.0233$ derived from standard curve in figure 1. The unknown 'x' which is the IgE concentration in ng/ml was determined. The y value is the optical densities obtained from the absorbance readings at 450nm. The resultant IgE concentration was multiplied by two since the serum was double diluted in the experiment.
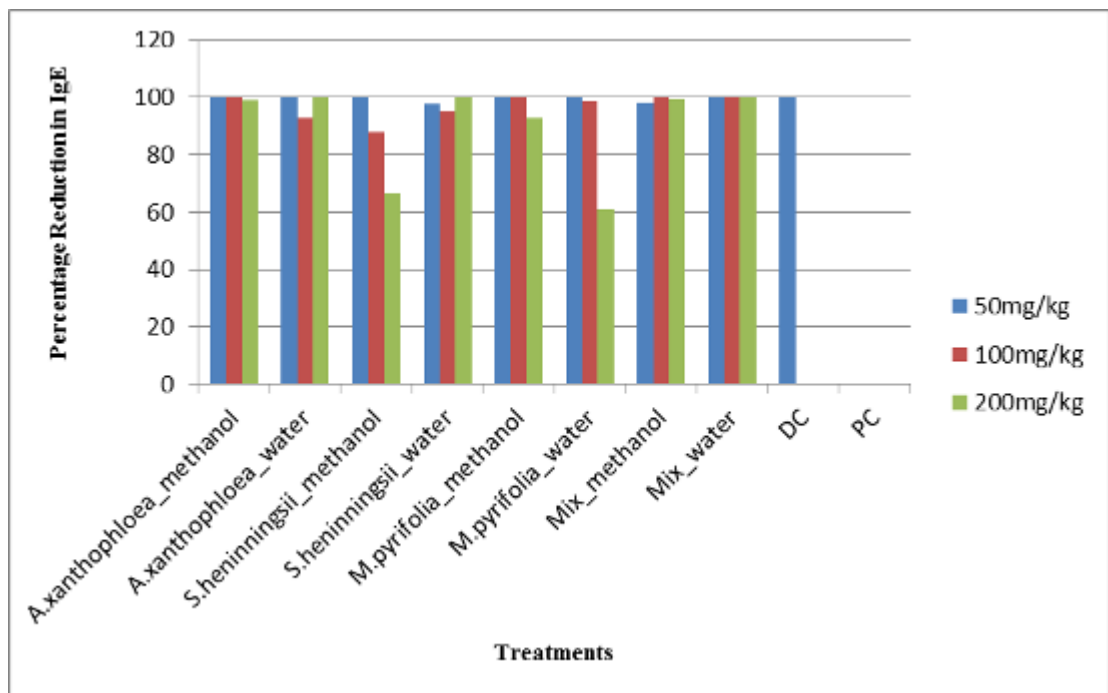
Figure 4.4: **Percentage reduction in serum total IgE levels in reference to Positive control**.

The percentage reduction was calculated by determining the difference between the serum IgE levels of extracts group mice or drug control group mice and the positive control group then dividing by the serum IgE level of the positive control group and finally multiplying by 100 to get the percentage. The x-axis represents treatments while the y-axis represents the percentages.
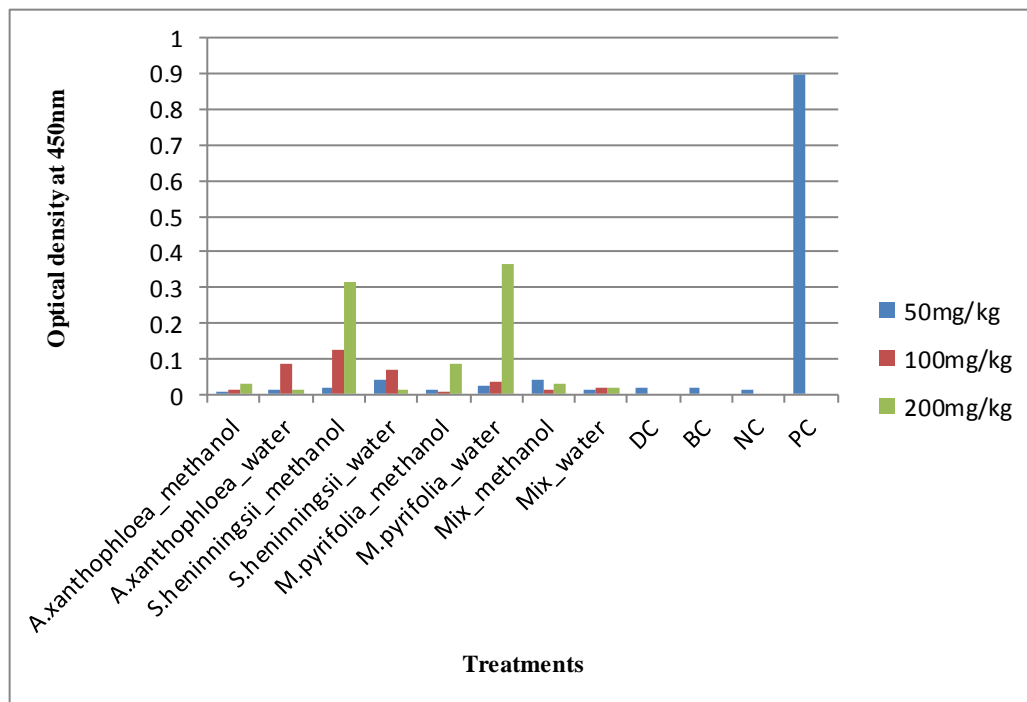
Figure 4.5: **Mean optical densities as read at 450nm in experimental and control groups of mice.**

These were obtained from serum samples of mice exposed to the above stated plant extracts at concentrations of 50, 100, and 200 mg/Kg body weight. The x-axis represents treatments while the y-axis represents the optical densities.
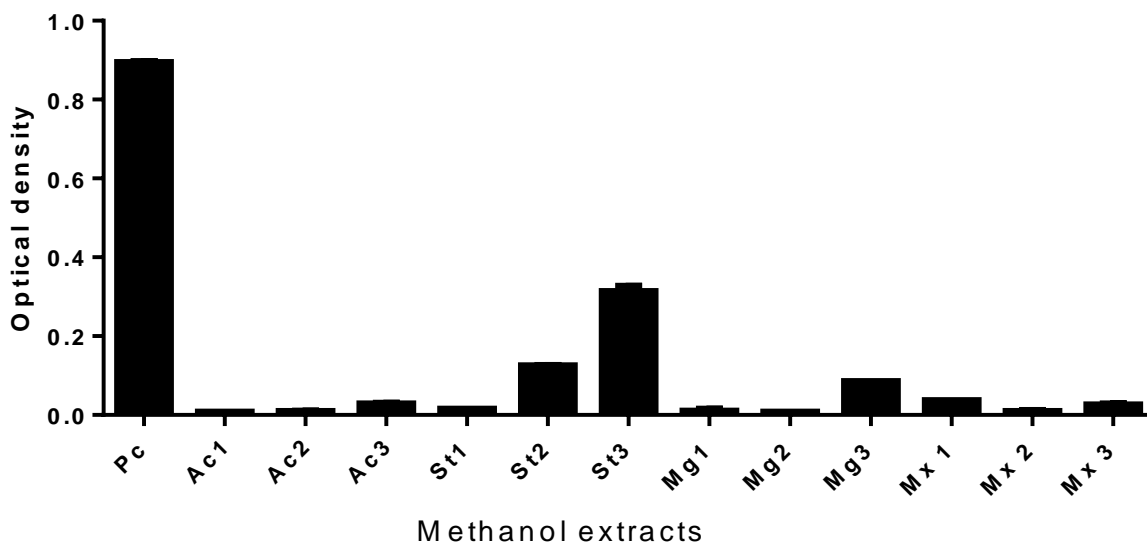
Figure 4.6: Comparison of optical densities of serum obtained from methanol extracts experimental mice group and positive control mice group.

Methanol extracts; *Acacia xanthophloea* (Ac), *Strychnos heninningsii* (St), *Microglossa pyrifolia* (Mg) and Mixed extract (Mx) in concentrations 1, 2, and 3 representing 50, 100 and 200mg/Kg b.w respectively against their optical densities at 450nm. Pc represents positive control. There is a significant difference (P<0.001) between the serum O.D in methanol extracts mice group and the positive control mice group analyzed using one way Anova, F is 6881. The individual extracts groups are also significantly different from the positive control group (P<0.001) as analyzed by unpaired student's t-test
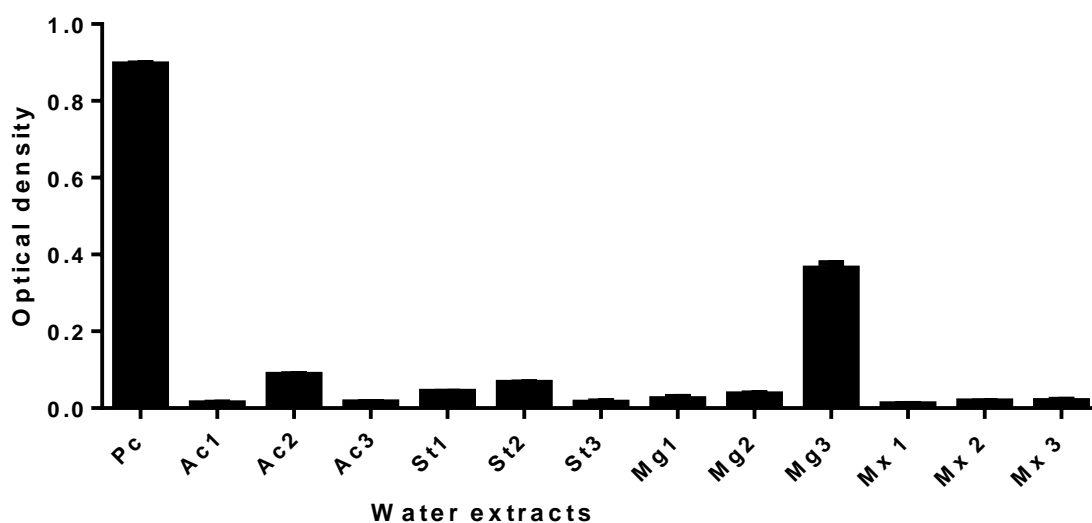
Figure 4.7: **Comparison of optical densities of serum obtained from water extracts experimental mice group and positive control mice group**.

Water extracts; *Acacia xanthophloea* (Ac), *Strychnos heninningsii* (St), *Microglossa pyrifolia* (Mg) and Mixed extract (Mx) in concentrations 1, 2, and 3 representing 50, 100 and 200mg/Kg b.w respectively against their optical densities at 450nm. Pc represents positive control. There is a significant difference (P<0.001) in serum optical densities from mice treated with water extracts and the positive control analyzed using one way Anova, F is 5979. The individual extracts are also significantly different from the positive control (P<0.001) as analyzed by unpaired student's t-test

31
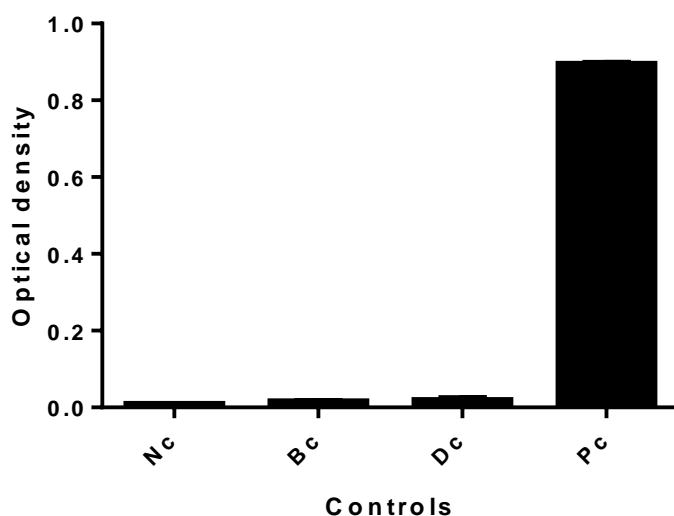
Figure 4.8: Comparison of optical densities of serum from control groups of mice.

Controls; Negative control (Nc), Baseline control (Bc) and Drug control (Dc) and Positive control (Pc). There is a significant difference between the individual controls and the positive control (P<0.001) analyzed with unpaired student's t-test.

4.5 Bronchoalveolar lavage fluid (Balf) eosinophil levels

The Balf eosinophils level in most extracts treatments and Prednisolone standard drug (Dc) was reduced from 28.67% (Positive control value; mice induced and not treated) to 0.33% (Figure 4.9 and 4.10). Some of the extracts that were not able to reduce the Balf eosinophils level to 0.33%, these include; methanol extracts of *S. heninningsii* 100mg/Kg b.w (6.67%) and 200mg/kg b.w (10.67%), *M. pyrifolia* 200mg/Kg b.w (4%) and Mixed extracts 50mg/Kg (2.33%). The water extracts include *A. xanthophloea* 100mg/Kg b.w (4.33%), *S. heninningsii* 100mg/Kg b.w (2.67%), and *M. pyrifolia* 100 and 200 mg/kg b.w (2.33% and 17.67% respectively), see figure 4.9, 4.10 and 4.11 below.
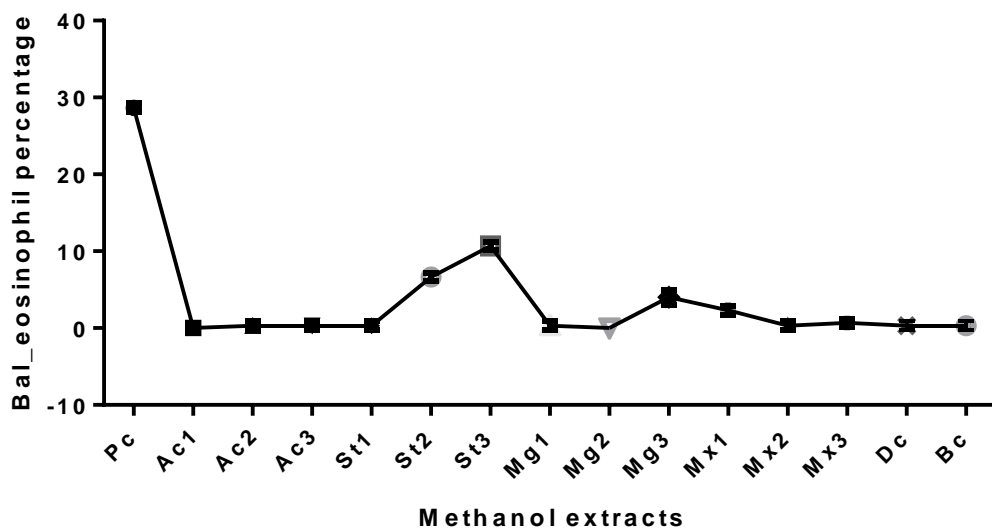
Figure 4.9: Percentage Balf eosinophils level in mice treated with methanol extracts compared with the Positive control (Pc), Standard drug control (Dc) and Baseline control (Bc).

Methanol extracts; *Acacia xanthophloea* in 50, 100, and 200mg/Kg is represented by Ac 1, 2, and 3 respectively, *Strychnos heninningsii* in 50, 100, and 200mg/Kg is represented by St 1, 2, and 3 respectively while *Microglossa pyrifolia* in 50, 100, and 200mg/Kg is represented by Mg 1, 2, and 3 respectively and Mixed extracts in the same concentrations are represented by Mx 1, 2, and 3 respectively. There was a significance difference (P < 0.001) between Balf eosinophils levels in methanol extract experimental mice group and Positive control mice group as analyzed by Anova (F value 513.4) using GraphPad prism 6 software. The individual methanol extracts concentrations mice groups are also significantly different (P < 0.001) from the positive control group as analyzed by unpaired student's t test in GraphPad software.

33
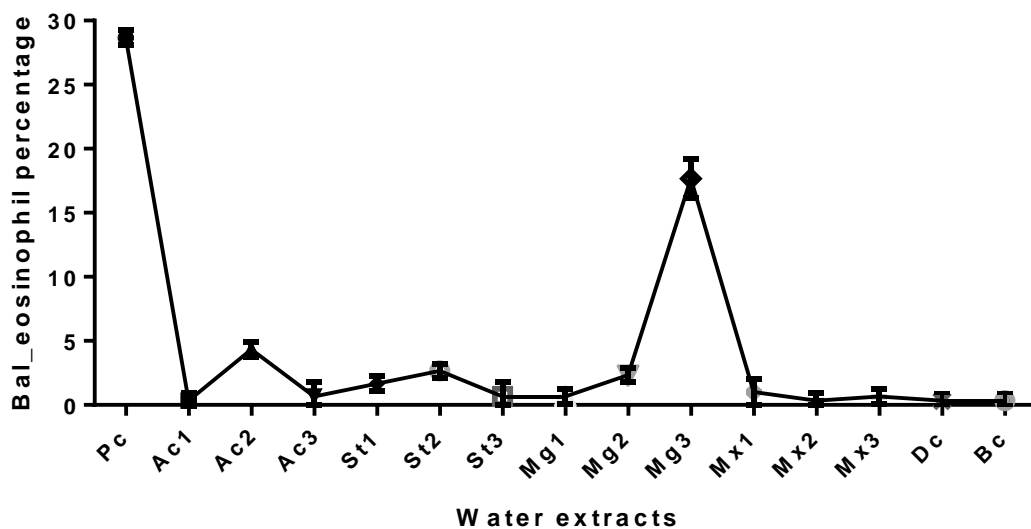
Figure 4.10: Percentage Balf eosinophils level in mice treated with water extracts compared with the Positive control (Pc), Standard drug control (Dc) and Baseline control (Bc).

Water extracts; *Acacia xanthophloea* in 50, 100, and 200mg/Kg is represented by Ac 1, 2, and 3 respectively, *Strychnos heninningsii* in 50, 100, and 200mg/Kg is represented by St 1, 2, and 3 respectively while *Microglossa pyrifolia* in 50, 100, and 200mg/Kg is represented by Mg 1, 2, and 3 respectively and Mixed extracts in the same concentrations are represented by Mx 1, 2, and 3 respectively. There was a significance difference ($P < 0.001$) between Balf eosinophils levels in water extract experimental mice group and Positive control mice group as analyzed by Anova (F value 303.3) using GraphPad prism 6 software. The individual water extracts concentrations mice groups are also significantly different ($P < 0.001$) from the positive control group as analyzed by unpaired student's t test in GraphPad software.
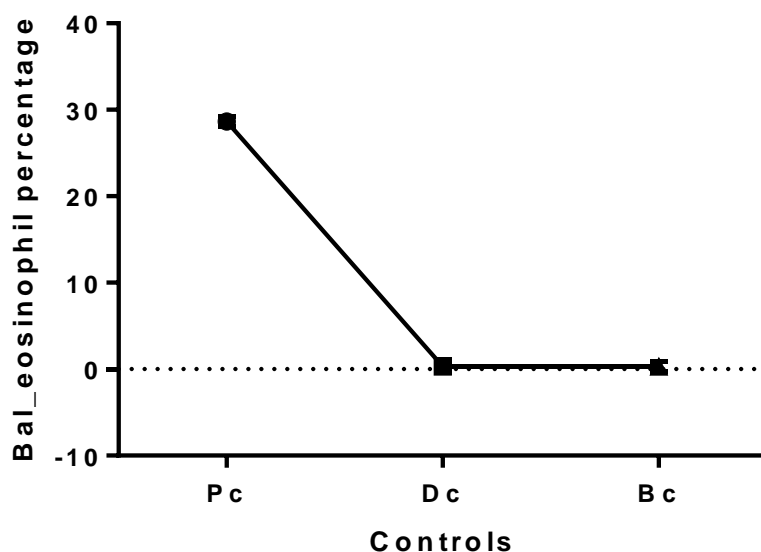
Figure 4.11: Comparison of percentage Balf eosinophils in control mice groups.
The Baseline control-Bc (un-induced and untreated mice), and Standard reference drug control-Dc (Prednisolone) was compared with Positive control-Pc (induced and untreated mice). There is a significant difference (P < 0.001) between the Baseline control and the Positive control as tested using unpaired student's t test in GraphPad prism 6 software. The same applies to Drug control. The three controls are also significantly different as test with Anova (P < 0.001 and F value is 2408) using GraphPad software.

4.6 Cytotoxic Concentration 50 (CC_{50}) of the plant extracts

The Cytotoxic concentration 50 (concentration with 50% viable cells – also known as Effective dilution; ED) measured with MTT dye and analyzed with Almar Blue assay software (figure 4.12, 4.13, 4.14, 4.15,4.16, 4.17 and 4.18) indicated that Methanol extracts of *Acacia xanthophloea*, *Microglossa pyrifolia* and *Strychnos heninningsii* had a CC_{50} of 83.79, 278.72, and 540.19µg/ml respectively (figure 4.12, 4.13 and 4.14), while the water extracts of the same plants indicated a CC $_{50}$ of 56.05, 198.60 and 389.08µg/ml respectively (Figure 4.16, 4.17, and 4.18). Mixed methanol extract had no significant loss of viability (Figure 4.15).
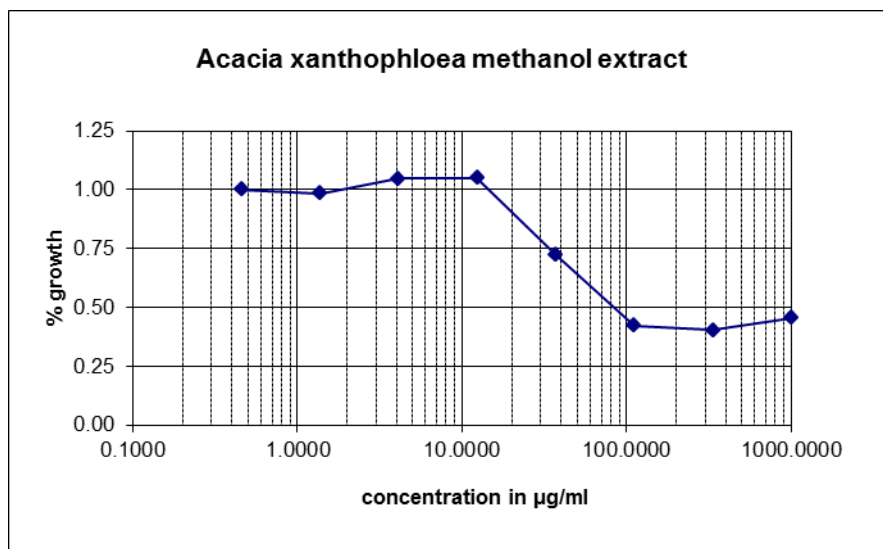
Figure 4.12: Cytotoxicity results for *Acacia xanthophloea* methanol extract as tested on Vero E6 cells and viability determined using MTT dye.

Absorbances were read at 562nm and a reference wavelength of 690nm was used. The optical densities were analyzed by Almar Blue assay software. Cytotoxic concentration 50 (CC_{50}) is 87.79µg/ml.
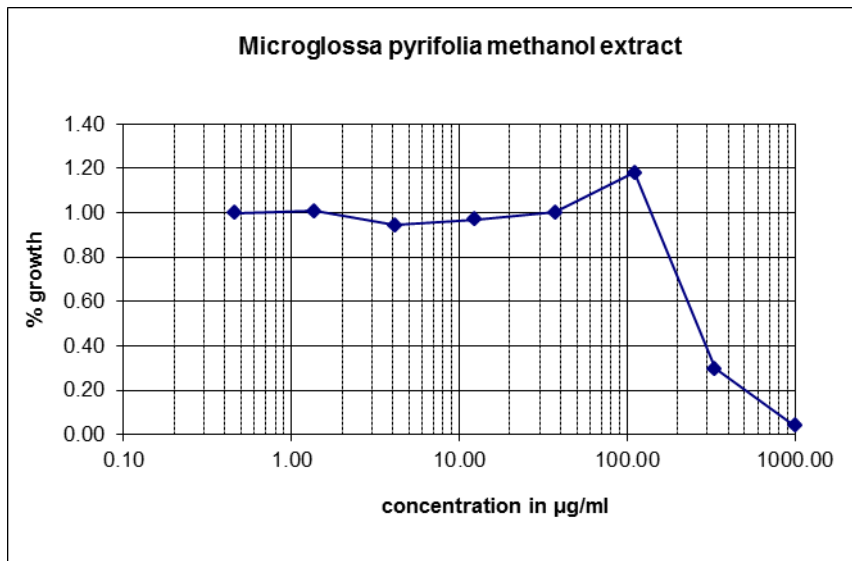
Figure 4.13: Cytotoxicity results for *Microglossa pyrifolia* methanol extract as tested on Vero E6 cells and viability determined using MTT dye.

Absorbances were read at 562nm and a reference wavelength of 690nm was used. The optical densities were analyzed by Almar Blue assay software. Cytotoxic concentration 50 (CC_{50}) is 258.72μg/ml.
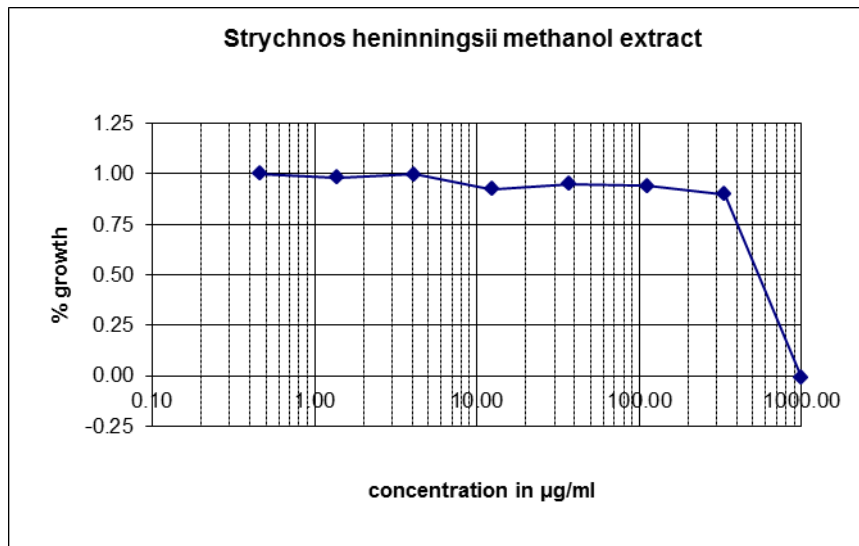
Figure 4.14: Cytotoxicity results for *Strychnos heninningsii* methanol extract as tested on Vero E6 cells and viability determined using MTT dye.
Absorbances were read at 562nm and a reference wavelength of 690nm was used. The optical densities were analyzed by Almar Blue assay software. Cytotoxic concentration 50 (CC_{50}) is 540.19μg/ml.
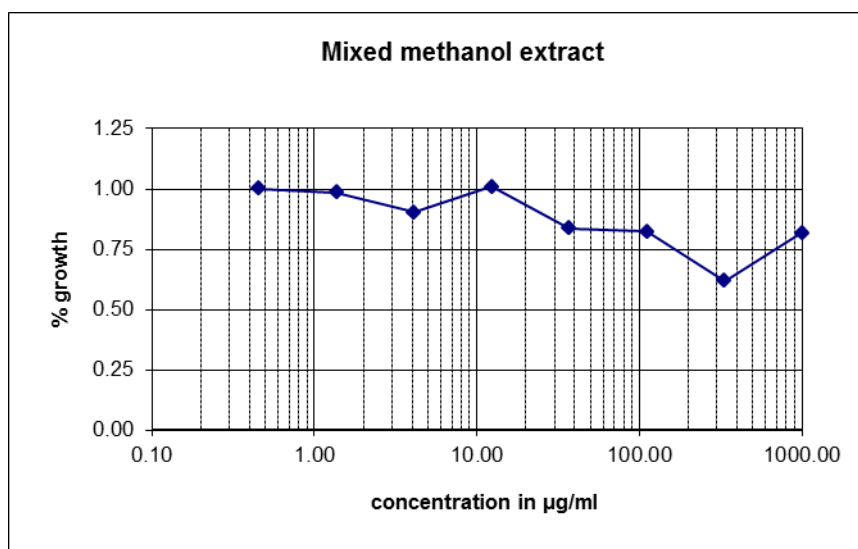
Figure 4.15: Cytotoxicity results for mixed methanol extract as tested on Vero E6 cells and viability determined using MTT dye.

Absorbances were read at 562nm and a reference wavelength of 690nm was used. The optical densities were analyzed by Almar Blue assay software Cytotoxic concentration 50 (CC_{50}) is not applicable for mixed extract since there was no significant loss of viability.
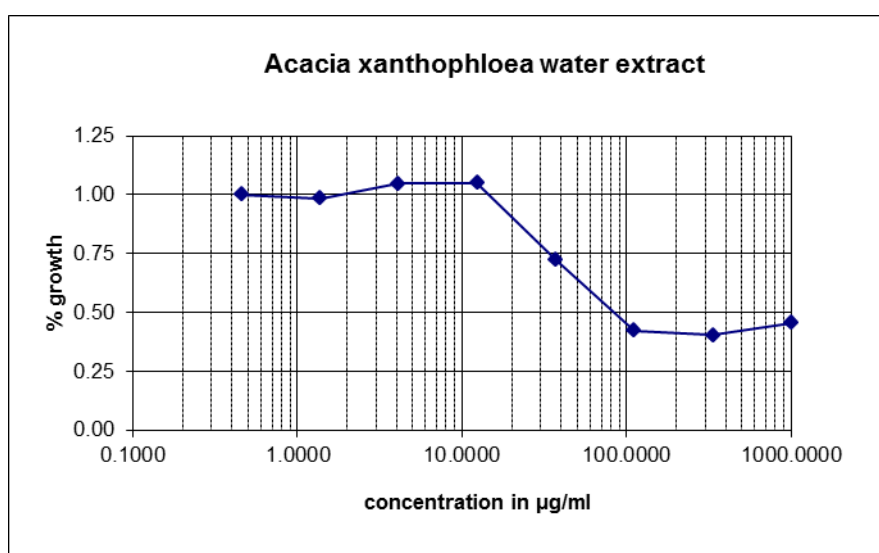
Figure 4.16: Cytotoxicity results for *Acacia xanthophloea* water extract as tested on Vero E6 cells and viability determined using MTT dye.

Absorbances were read at 562nm and a reference wavelength of 690nm was used. The optical densities were analyzed by Almar Blue assay software. Cytotoxic concentration 50 (CC_{50}) is 56.05µg/ml.
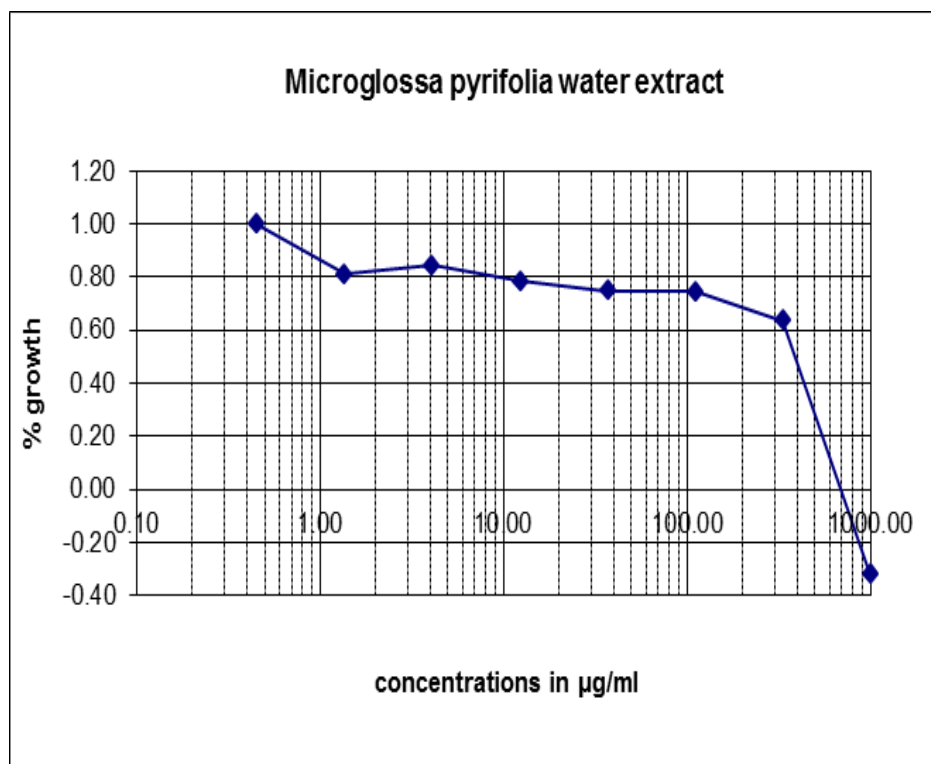
Figure 4.17: Cytotoxicity results for *Microglossa pyrifolia* water extract as tested on Vero E6 cells and viability determined using MTT dye.

Absorbances were read at 562nm and a reference wavelength of 690nm was used. The optical densities were analyzed by Almar Blue assay software. Cytotoxic concentration 50 (CC_{50}) is 389.08µg/ml.
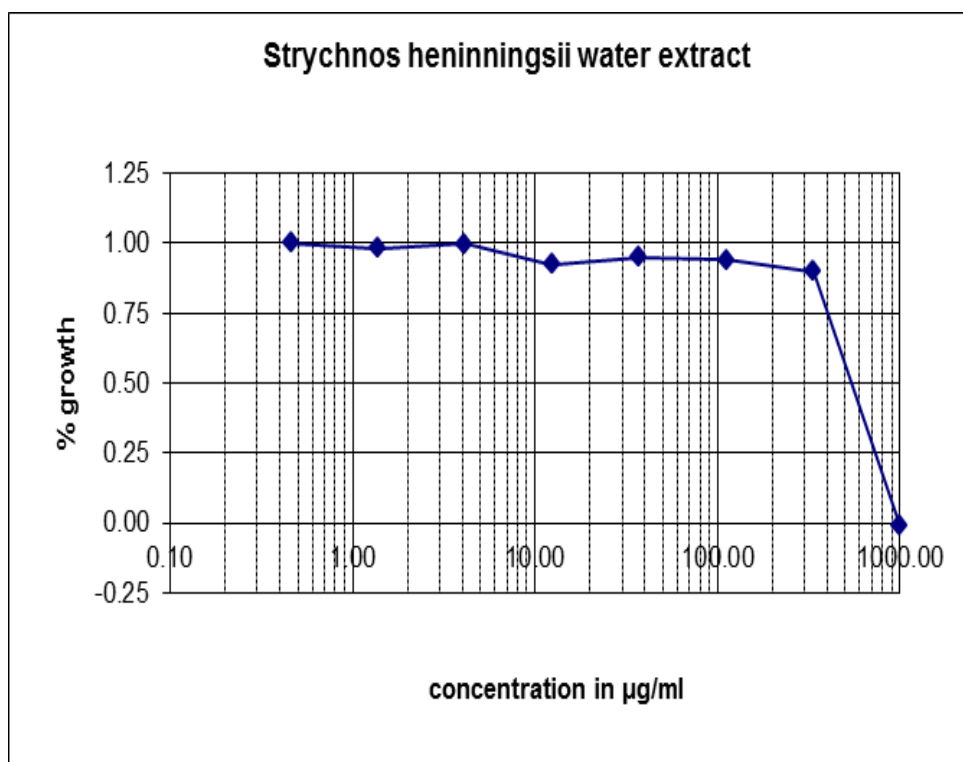
Figure 4.18: Cytotoxicity results for *Strychnos heninningsii* water extract as tested on Vero E6 cells and viability determined using MTT dye.

Absorbances were read at 562nm and a reference wavelength of 690nm was used. The optical densities were analyzed by Almar Blue assay software. Cytotoxic concentration 50 (CC_{50}) is 198.60μg/ml. 4.2 Phytochemical constituents of the medicinal plants identified by TLC

Phytochemical screening of water and methanol extracts of *Acacia xanthophloea*, *Strychnos heninningsii* and *Microglossa pyrifolia* done by thin layer chromatography (TLC), revealed the presence of phenols, alkaloids and flavonoids (Table 4.3)

40

Table 4.4: Phytochemical constituents of the medicinal plants

Acacia xanthophloea bark extracts		
Test	Water extracts	Methanol extracts
Phenols	+	+
Alkaloids	+	+
Flavonoids	+	+
Strychnos heninningsii leaves extracts		
Test	Water extracts	Methanol extracts
Phenols	+	+
Alkaloids	+	+
Flavonoids	+	+
Microglossa pyrifolia leaves extracts		
Test	Water extracts	Methanol extracts
Phenols	+	+
Alkaloids	+	+
Flavonoids	+	+

Key + present

  - Absent

4.3 Asthmatic symptoms observed in Swiss albino mice after Ovalbumin challenge

The mice induced with asthma exhibited basic asthmatic symptoms including reduced physical activity and general discomfort on the first day of nebulization, followed by wheezing and difficulty in breathing on the second day of nebulization. The symptoms reduced as treatment commenced in most groups with only two deaths recorded (in mice receiving 200mg/kg body weight of *S.heninningsii* methanol extract and *M.pyrifolia* water extract respectively) out of a total of 81mice (Table 4.4)

CHAPTER FIVE

5.0 DICUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

5.1.1 Extraction methods used in relation to yield

Generally the percentage yield of the water extracts was higher than that of methanol extracts. This could be attributed to the difference in polarity of the extraction solvents. Water has a polarity of 1.85 D while methanol has a polarity of 1.70 D, which is lower than that of water.

5.1.2 Asthma and Immunoglobin E (IgE) levels

A number of extracts and Prednisolone showed upto 100% reduction of the Immunoglobin E produced during the asthmatic attack. The Immunoglobin E (IgE) has long been implicated in asthmatic attack (Sunyer J.M., *et al*., 1996) with several cascade developed for the asthmatic process generally termed as allergic cascade. Asthmatic attack is an allergic reaction regarded as type 1 hypersensitivity reaction and IgE has evidently been known to play a key role in this reaction. The allergic cascade has been summarized into three steps, namely; a) Sensitization phase, b) Early allergen response phase- upon re-exposure to allergen and c) Late allergen response phase. The asthmatic induction process in this study was based on this approach. The sensitization phase is characterized by initial exposure to an allergen in minute doses, the allergen is then presented to T-helper cells by Antigen presenting cells (APC) such as macrophages and dendritic cells, and this triggers the T-helper cells to stimulate B cells to develop into plasma cells which produce IgE antibodies. In this study this process was successfully achieved evident by the detection of as high as 7.25ng/ml of serum total IgE levels in the positive control group (mice induced with Asthma and not treated). The IgE produced is normally specific to a particular allergen used. In our case Ovalbumin was used as an allergen and therefore the IgE antibodies produced were Ovalbumin specific. Mouse Ovalbumin specific IgE Enzyme Linked Immunosorbent Assay (Elisa) was used to detect the serum total IgE levels. The IgE antibodies produced in the sensitization phase have special receptors on mast cells, the bind to mast cells completing the sensitization phase. In the early phase response to allergen, this occurs when there is

a re-exposure to the specific allergen. IgE on mast cells binds to the allergen leading to crosslinking of multiple IgE antibodies. As a result the mast cells explode and release histamine and other inflammatory mediators leading to allergic/asthmatic symptoms such as wheezing, sneezing, shortness of breath, coughing, itchy eyes and running nose. Wheezing and difficulty in breathing are some of the symptoms that were evident in the mice used. The late allergen response phase involves the eosinophils. IgE antibodies which have receptors on eosinophils, binds to them leading to more adverse symptoms such as congestion, airway hyper responsiveness and tissue damage in chronic exposures. This phase normally occurs 10 to 24hours after the first allergic reaction.

5.1.3 Asthma and eosinophils level

The eosinophils percentage was reduced by a number of extracts and Prednisolone from 28.67% to 0.33%. Asthmatic attack is an inflammatory process that involves increase in the numbers of inflammatory cells such as Eosinophils, Neutrophils and Mast cells. The key cells that play a role in asthmatic attack are mast cells and eosinophils which have receptors for IgE antibodies involved in allergic reactions. Mast cells are involved in early stages of asthmatic attack while eosinophils are involved in the late stages of asthmatic process (10 to 24 hours after the first allergic reaction). Thus the Bronchoalveolar lavage fluid was collected in positive control group of mice 48 hours after the last ovalbumin challenge. The Bal-fluid for treatment groups was collected 48 hours after the last treatment while for baseline control group it was collected immediately after mice grouping. Diff-Quick staining technique was used for determination of the eosinophils level in relation to the total white blood cells in Balf. As high as 28.67% Balf eosinophils were recorded in positive control group of mice. Asthma has been long associated with increased eosinophils levels (Mehlhop P. D., *et al*., 1997) and this has also been proved in *in vivo* studies (Wardlaw A. J., *et al*., 2015). The normal level for Balf eosinophils is usually less than 1%. The most of the concentrations medicinal plants tested were able to reduce Balf eosinophils level to less than 1% with only few being in cable of this. These include methanol extracts of *S. heninningsii* 100mg/Kg b.w (6.67%) and 200mg/kg b.w (10.67%), *M. pyrifolia* 200mg/Kg b.w (4%) and mixed extracts

50mg/Kg (2.33%). The water extracts included *A. xanthophloea* 100mg/Kg b.w (4.33%), *S. heninningsii* 100mg/Kg b.w (2.67%), and *M. pyrifolia* 100 and 200 mg/kg b.w (2.33% and 17.67% respectively), see figure 4.9, 4.10 and 4.11 above. Eosinophils not only have receptors for IgE but are also involved in actively secreting mediators such as histamines and leukotriene (Wardlaw A. J. *et al,* 2015) evidence by increased eosinophils granules when view under microscope after Diff-Quick staining procedure. Diff- Quick staining technique is a modified Giemsa staining procedure that is very fast (short staining process) and efficient in differential staining of white blood cells in addition to a variety of cells hence the name Diff-Quick.

5.1.4 Cytotoxicity of the medicinal plants

The extracts showed varied levels of cytotoxicity against Vero E6 mammalian cells. Cytotoxicity test was done to determine the dosage of the plant extracts that does not kill or damage the mammalian cells after establishing that the plant have the ability to reduce serum total IgE levels and Balf eosinophil levels in mice. Vero E6 cells derived from kidney tissue of *Cercopithecus aethiops* (Vero ATCC$^{@}$ CCL81^{TM}) were used. The cells were obtained from the Kenya Medical Research Institute (KEMRI) tissue culture lab. The medicinal plants were tested to be cytotoxic to mammalian cells (Vero E6 cells) when used in higher concentrations. The concentrations differ from one extract to another. However mixed methanol extract were not highly cytotoxic showing less than 50% cytotoxicity even at high concentration. The cytotoxic properties of these plants can be attributed to the presence of some amounts of alkaloids and other compounds. Base on this study, the plants should be used at concentrations lower than mention cytotoxic concentration 50 (CC $_{50}$). Methanol extracts of *Acacia xanthophloea*, *Microglossa pyrifolia* and *Strychnos heninningsii* had a CC $_{50}$ of 83.79, 278.72, and 540.19μg/ml respectively (figure 4.12, 4.13, and 4.14), while the water extracts of the same plants indicated a CC $_{50}$ of 56.05, 198.60 and 389.08μg/ml respectively (figure 4.16, 4.17 and 4.18). Mixed methanol extract had no significant loss of viability (figure 4.15).

5.1.5 Phytochemical constituents of the medicinal plants

The thin layer chromatography revealed the presence of phenols, alkaloids and flavonoids. These plants have more than just the tested phytochemicals. The three phytochemicals were the only ones tested because polyphenols (phenols and flavonoids) have been reported to have anti-inflammatory activity in addition to their antioxidant and antibacterial properties (Smith & Eyzayuirre , 2007). Alkaloids were tested since it is normally poisonous when it's present in high amounts in medicinal plants; however it has antimicrobial activity (Chinedum E.k and Ogbonnaya C.E., 2013). *Acacia xanthophloea, Strychnos heninningsii* and *Microglossa pyrifolia* have been reported to have anticancer activity which is normally attributed to presence of glucosinolates (Block *et al*., 1992; Hu, 2003).

The use of methanol and water extraction methods led to obtaining optimal amounts of the bioactive compounds. Some of the chemical classes of the phytochemicals extracted from medicinal plants using the methanol extraction include saponins, tannins, phenols, flavonoids, sugars, amino acids, anthocyanins, terpenoids, xanthoxyllines,totarol, quassinoids, lactones, and polyphenols (Kumar *et al*., 2008)**.** Water exctraction achieves to obtain bioactive compounds such as saponins, tannins lectins, terpenoids, sugar, anthocyanins, starches, and polypeptides (Kumar *et al*., 2008).

5.2 CONCLUSION

The medicinal plants used in this study; *Acacia xanthophloea, Strychnos henningsii* and *Microglossa pyrifolia* showed the ability to reduce IgE levels and Bal-eosinophils levels by up to 100% evident by reduction of serum total IgE levels from 7.25ng/ml in positive control to 0ng/ml in most extract concentrations and the reduction of Balf eosinophils percentage from 28.67% to 0.33%. The extracts that showed these activities include: 50mg/Kg b.w of *A. xanthophloea* water and methanol extract, *S. henningsii* methanol extract, *M. pyrifolia* methanol extract, Mixed water extract, 100mg/Kg b.w of *A.xanthophloea* methanol extract*, M. pyrifolia* water and methanol extract, Mixed water and methanol extract, 200mg/Kg

b.w of *A. xanthophloea* water, *S. henningsii* water extract, *M. pyrifolia* methanol extract, and Mixed water extract. The ability of these medicinal plants to restore the eosinophils to normal levels is a reversion of immunological process and function. This shows that the plants have Immunomodulatory properties. The anti-asthmatic and Immunomodulatory activities of these extracts can be attributed to presence of anti-inflammatory phytochemical constituents that has not been investigated. Recently, many natural medicines derived from plants, marine organisms, etc. were considered effective and safer for the treatment of various diseases including inflammation and pain (Su S., *et al*, 2011)**.** Anti-inflammatory activity in medicinal plants has been accounted to presence of phytochemical bioactive compounds such as flavonoids, saponins, steroids, carbohydrates, phenols, and glycosides (Narendhirakannan, Kandaswamy & Subramanian, 2008).

Based on this study, *Acacia xanthophloea*, *Microglossa pyrifolia* and *Strychnos heninningsii* have the ability to reduce serum total IgE levels and eosinophils levels in an Asthmatic attack. The effective and safe dosage of the medicinal plants should be determined before usage.

5.3 RECOMMENDATIONS

Acacia xanthophloea, Strychnos henningsii and *Microglossa pyrifolia* having shown anti-asthmatic and Immunomodulatory properties should be further investigated using pure isolates from their plant material to determine the exact compounds/principle responsible for the activities. The use of pure isolates for further studies could also reduce the cytotoxic effects of these plants. The principle responsible for the cytotoxic effect should also be determined and eliminated in the course of use of the plants in drug development. Other plant parts not used in this study such as the roots, and seeds can also be investigated too in an effort to obtain better results. Other extraction methods can also be employed either singularly or multiply in a serial way to obtain more phytochemicals which could increase the observed activities.

Drug interaction studies should also be done to determine the right proportions of mixing the plant extracts and establish their interactions since mixed methanol

extract showed no loss of cell viability even when used in high concentrations. This would lead to development of a more safer and efficient drug. The results in this study can to be used for future possible large scale implementation in an effort to solve the burden of asthma as well as the current anti-asthmatic drug side effects.

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APPENDICES

Appendix I: Plant material voucher specimen numbers

Medicinal plant	Plant part collected	Voucher specimen number
Acacia xanthophloea	Stem bark	TFm 41
Strychnos heninningsii	Leaves	TFm 43
Microglossa pyrifolia	Leaves	TFm 45

Appendix II: Freeze drying photograph of water extracts

Appendix III: Rotary evaporation photograph of methanol extract

Appendix IV: Thin layer chromatography photograph of processing chamber

Appendix V: Polarity of solvents used for TLC of methanol and aqueous extracts

Methanol extracts' solvents system used in thin layer chromatography	
Solvents	**Polarity**
Dichloromethane	1.60 D
Trichloromethane	1.08 D
Water extracts' solvents system used in thin layer chromatography	
Solvents	**Polarity**
n-Butanol	1.63 D
Water	1.85 D
Acetic Acid	1.74 D

Appendix VI: Pre-coated plate after solvent run as viewed under UV light chamber
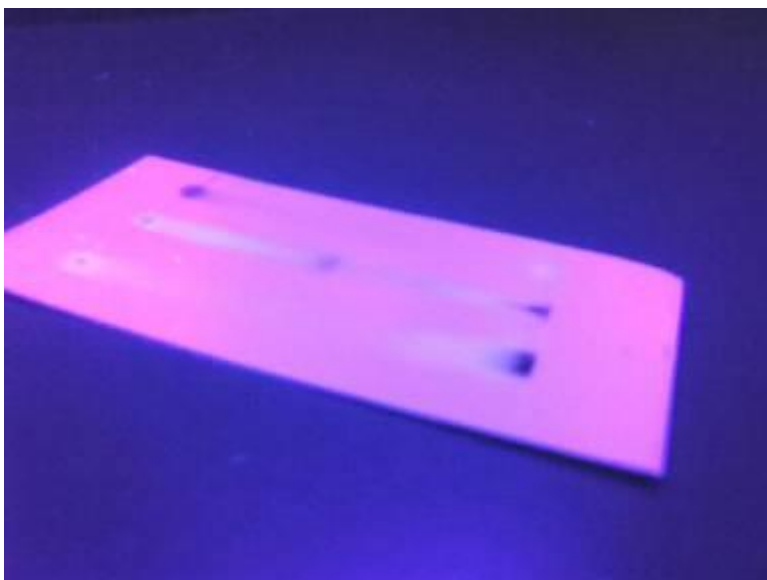
Appendix VII: Procedure for preparation of TLC spraying reagents

VII. a. Preparation of spraying reagent for phenols detection

A 0.1g of Ferric ferrichloride was dissolved in 10ml distilled water, and then mixed with Potassium ferricyanide solution prepared by dissolving 0.1g of potassium ferricyanide in 10ml distilled water. The presence of phenols was determined by blue colour formation along the spot solvent run.

VII. b. Preparation of spraying reagent for alkaloids detection

A 0.85g of bismuth suonitrate was dissolved in a solution of 10ml acetic acid and 40ml of distilled water. A second reagent was prepared by dissolving 8g of potassium iodite (KI) in 20ml distilled water. A 1ml each of reagent one and reagent two above was mixed with 2ml of fresh acetic acid and distilled 10ml water. The presence of alkaloid was determined by a yellow, orange and brown background spots.

VII. c. Preparation of spraying reagent for flavonoids detection

A 0.5g of vanillin was dissolved in 50ml of concentrated sulphuric acid. The presence of flavonoids was determined by the formation of purple coloured spot.

Appendix VIII: Grouping of mice for in vivo assay

Acacia xanthophloea group					
Methanol extracts groups			**Water extract groups**		
50mg/Kg b.w	100mg/Kg b.w	200mg/Kg b.w	50mg/Kg b.w	100mg/Kg b.w	200mg/Kg b.w
Strychnos henningsii group					
Methanol extracts groups			**Water extract groups**		
50mg/Kg b.w	100mg/Kg b.w	200mg/Kg b.w	50mg/Kg b.w	100mg/Kg b.w	200mg/Kg b.w
Microglossa pyrifolia group					
Methanol extracts groups			**Water extract groups**		
50mg/Kg b.w	100mg/Kg b.w	200mg/Kg b.w	50mg/Kg b.w	100mg/Kg b.w	200mg/Kg b.w
Mixed extract group					
Methanol extracts groups			**Water extract groups**		
50mg/Kg b.w	100mg/Kg b.w	200mg/Kg b.w	50mg/Kg b.w	100mg/Kg b.w	200mg/Kg b.w
Control Mice groups					
Positive control		**Standard Reference drug group**		**Baseline group**	
Induced with Asthma and not treated		Treated with 10mg/Kg Prednisolone		Non-induced and non-treated	
3mice used		3mice used		3miced used	

Note: All mice were induced with asthma except the baseline control group.

Appendix IX: Photograph of Swiss albino mice in cages

Appendix X: Intraperitoneal injection of mice photogragh

Appendix XI: Cardiac puncture photograph of mice during blood collection

Appendix XII: Laboratory grinding mill photograph

Appendix XIII: Diff-Quick staining procedure

A smear was made from Bal-fluid then air dried and fixed with Diff-Quick fixative for 30 seconds and drained. Solution II from Diff-Quick kit was then added on to the smear to stain for 30 seconds and drained, counter staining was done using Diff-Quick solution I for 30 seconds then drained. The smear was rinsed with tap water to remove excess stain and then dehydrated rapidly in absolute ethanol. Clearing was done using xylene followed by mounting using Dpx.

Appendix XIV: Vero E6 cells stained with MTT dye photograph
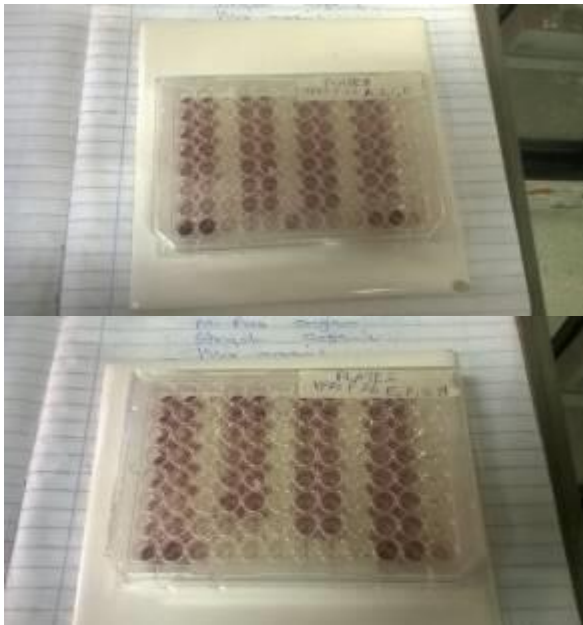
Appendix XV: Approval letter from KEMRI Scientific Steering Committee (SSC)

KENYA MEDICAL RESEARCH INSTITUTE

KEMRI/SSC/102927   25th June, 2014

Grace Akinyi

Thro'

    Acting Director, CTMDR

    NAIROBI

Forwarded 27/6/2014

REF: SSC No. 2845 (Revised) – Anti-asthmatic and Immunomodulatory Properties of Extracts of *Acacia nilotica, Strychnos heninningsii* and *Microglossa densiflora* in Asthma Induced Mice

Thank you for your letter dated 23rd June, 2014 responding to the comments raised by the KEMRI SSC.

I am pleased to inform you that your protocol now has formal scientific approval from SSC.

The SSC however, advises that work on the proposed study can only start after ERC approval.

Sammy Njenga, PhD
SECRETARY, SSC

Encl(s)

In Search of Better Health

Appendix XVI: Approval letter from KEMRI Animal Care and Use Committee (ACUC)

KENYA MEDICAL RESEARCH INSTITUTE

Centre for Virus Research, P.O.Box 54628 - 00200 NAIROBI - Kenya
Tel: (254) (020) 2722541, 254 02 2713349, 0722-205901, 0733-400003 Fax (254) (020) 2726115
Email: cvr@kemri.org

KEMRI/ACUC/ 02.07.14   24th July, 2014

Odongo Grace Akinyi
TM 305-2816/2013
ITROMID/KEMRI

Odongo Grace,

RE: Animal use approval for SSC 2845(revised) - "Anti-Asthmatic and
Immunomodulatory Properties of Extracts of *Acacia Nilotica, Strychnos
Heninningsii* and *Microglossa Densiflora* in Asthma Induced Mice" protocol

The KEMRI animal care and use committee acknowledges the resubmission of the above
mentioned protocol addressing the issues raised earlier.

The committee grants you the approval to use laboratory mice in your study but
recommends that you proceed after obtaining all the other necessary approvals that may
be required.

Approval is granted for a period of one year starting from when the final ethical approval
will be obtained. The committee expects you to adhere to all the laboratory animal
handling procedures as described in the protocol.

The committee wishes you all the best in your work.

Yours sincerely,

Dr. Konongoi Limbaso
Chairperson KEMRI ACUC

KENYA MEDICAL
RESEARCH INSTITUTE
★ 2 4 JUL 2014 ★
ANIMAL CARE AND USE COMMITTEE
Signature: ....

Appendix XVII: Approval letter from KEMRI Ethical Review Committee (ERC)

KEMRI/RES/7/3/1   **September 25, 2014**

TO: **GRACE AKINYI ODONGO,**
           PRINCIPAL INVESTIGATOR

THROUGH: **DR. PETER MWITARI,**
           THE DIRECTOR, CTMDR,
           NAIROBI

Dear Madam,

RE: SSC PROTOCOL NO. 2845 (*RESUBMISSION*): ANTI-ASTHMATIC AND
      IMMUNOMODULATORY PROPERTIES OF EXTRACTS OF *ACACIA NILOTICA,*
      STRYCHNOS HENINNINGSII AND *MICROGLOSSA DENSIFLORA* IN ASTHMA
      INDUCED MICE.

Reference is made to your letter dated 11th September, 2014. The ERC Secretariat
acknowledges receipt of the revised document on 22nd September, 2014.

This is to inform you that the Ethics Review Committee (ERC) reviewed the document
submitted, and is satisfied that the issues raised at the 229th meeting, have been adequately
addressed.

This study is granted approval for implementation effective this **September 25, 2014.**
Please note that authorization to conduct this study will automatically expire on **September**
25, 2015. If you plan to continue with data collection or analysis beyond this date please
submit an application for continuing approval to the ERC secretariat by **August 13, 2015.**

You are required to submit any amendments to this protocol and other information pertinent
to human participation in this study to the SSC and ERC for review prior to initiation.

You may embark on the study.

Yours faithfully,

PROF. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI/ETHICS REVIEW COMMITTEE

In Search of Better Health

Appendix XVIII: Working with mice in research course completion report

COLLABORATIVE INSTITUTIONAL TRAINING INITIATIVE (CITI)
WORKING WITH MICE IN RESEARCH SETTINGS CURRICULUM COMPLETION REPORT
Printed on 03/19/2014

LEARNER	GRACE AKINYI (ID: 4080000) 22585 - 00400 NAIROBI KENYA
DEPARTMENT	ITROMID
PHONE	+254 720 422 616
EMAIL	itromid@kemri.org
INSTITUTION	Kenya Medical Research Institute
EXPIRATION DATE	03/18/2017

WORKING WITH MICE IN RESEARCH

COURSE/STAGE:	Lab Animal Research/1
PASSED ON:	03/19/2014
REFERENCE ID:	12626658

ELECTIVE MODULES	DATE COMPLETED	SCORE
Introduction to Working with Mice in Research Settings	03/19/14	No Quiz
Research Mandates and Occupational Health Issues	03/19/14	3/3 (100%)
Alternatives Searches, Humane Standards, Housing, and Acclimation and Quarantine	03/19/14	2/2 (100%)
Detecting Pain and Distress, Genetics, and Biological Features	03/19/14	3/3 (100%)
Injections, Blood Collection, and Antibody Production	03/19/14	2/2 (100%)
Surgery, Supportive Care and Monitoring, Euthanasia, and References	03/19/14	4/5 (80%)

For this Completion Report to be valid, the learner listed above must be affiliated with a CITI Program participating institution or be a paid Independent Learner. Falsified information and unauthorized use of the CITI Progam course site is unethical, and may be considered research misconduct by your institution.

Paul Braunschweiger Ph.D.
Professor, University of Miami
Director Office of Research Education
CITI Program Course Coordinator

Appendix XIX: Biomedical responsible conduct of research course report

COLLABORATIVE INSTITUTIONAL TRAINING INITIATIVE (CITI)
BIOMEDICAL RESPONSIBLE CONDUCT OF RESEARCH CURRICULUM COMPLETION REPORT
Printed on 05/09/2014

LEARNER	GRACE AKINYI (ID: 4080000) 22585 - 00400 NAIROBI KENYA
DEPARTMENT	ITROMID
PHONE	+254 720 422 616
EMAIL	itromid@kemri.org
INSTITUTION	Kenya Medical Research Institute
EXPIRATION DATE	

BIOMEDICAL RESPONSIBLE CONDUCT OF RESEARCH : This course is for investigators, staff and students with an interest or focus in **Biomedical Research**. This course contains text, embedded case studies AND quizzes.

COURSE/STAGE:	RCR/1
PASSED ON:	05/09/2014
REFERENCE ID:	12953671

ELECTIVE MODULES	DATE COMPLETED	SCORE
Introduction to the Responsible Conduct of Research	05/09/14	No Quiz
Research Misconduct (RCR-Biomed)	05/09/14	5/5 (100%)
Data Management (RCR-Biomed)	05/09/14	5/5 (100%)
Authorship (RCR-Biomed)	05/09/14	5/5 (100%)
Peer Review (RCR-Biomed)	05/09/14	8/8 (100%)
Mentoring (RCR-Interdisciplinary)	05/09/14	5/5 (100%)
Using Animal Subjects in Research (RCR-Interdisciplinary)	05/09/14	5/5 (100%)
Conflicts of Interest (RCR-Biomed)	05/09/14	5/6 (83%)
Collaborative Research (RCR-Biomed)	05/09/14	5/5 (100%)
Research Involving Human Subjects (RCR-Interdisciplinary)	05/09/14	5/5 (100%)
Responsible Conduct of Research (RCR) Course Conclusion	05/09/14	No Quiz

For this Completion Report to be valid, the learner listed above must be affiliated with a CITI Program participating institution or be a paid Independent Learner. Falsified information and unauthorized use of the CITI Progam course site is unethical, and may be considered research misconduct by your institution.

Paul Braunschweiger Ph.D.
Professor, University of Miami
Director Office of Research Education
CITI Program Course Coordinator

Appendix XX: Biomedical research basic refresher course report

COLLABORATIVE INSTITUTIONAL TRAINING INITIATIVE (CITI)
BIOMEDICAL RESEARCH - BASIC/REFRESHER CURRICULUM COMPLETION REPORT
Printed on 05/09/2014

LEARNER	GRACE AKINYI (ID: 4080000) 22585 - 00400 NAIROBI KENYA
DEPARTMENT	ITROMID
PHONE	+254 720 422 616
EMAIL	itromid@kemri.org
INSTITUTION	Kenya Medical Research Institute
EXPIRATION DATE	05/09/2015

BIOMEDICAL RESEARCH - BASIC/REFRESHER: Choose this group to satisfy CITI training requirements for Investigators and staff involved primarily in biomedical research with human subjects.

COURSE/STAGE:	Basic Course/1
PASSED ON:	05/09/2014
REFERENCE ID:	12953669

REQUIRED MODULES	DATE COMPLETED	SCORE
Belmont Report and CITI Course Introduction	05/09/14	3/3 (100%)
History and Ethics of Human Subjects Research	05/09/14	7/7 (100%)
Basic Institutional Review Board (IRB) Regulations and Review Process	05/09/14	5/5 (100%)
Informed Consent	05/09/14	4/4 (100%)
Social and Behavioral Research (SBR) for Biomedical Researchers	05/09/14	4/4 (100%)
Records-Based Research	05/09/14	2/2 (100%)
Genetic Research in Human Populations	05/09/14	2/2 (100%)
Research With Protected Populations - Vulnerable Subjects: An Overview	05/09/14	4/4 (100%)
Vulnerable Subjects - Research Involving Prisoners	05/09/14	4/4 (100%)
Vulnerable Subjects - Research Involving Children	05/09/14	3/3 (100%)
Vulnerable Subjects - Research Involving Pregnant Women, Human Fetuses, and Neonates	05/09/14	3/3 (100%)
International Studies	05/09/14	3/3 (100%)
FDA-Regulated Research	05/09/14	5/5 (100%)
Research and HIPAA Privacy Protections	05/09/14	4/5 (80%)
Vulnerable Subjects - Research Involving Workers/Employees	05/09/14	4/4 (100%)
Conflicts of Interest in Research Involving Human Subjects	05/09/14	5/5 (100%)
Avoiding Group Harms - U.S. Research Perspectives	05/09/14	3/3 (100%)
Avoiding Group Harms - International Research Perspectives	05/09/14	3/3 (100%)
I Have Agreed to be an IRB Community Member. Now What?	05/09/14	5/5 (100%)
Stem Cell Research Oversight (Part I)	05/09/14	5/5 (100%)
Kenya Medical Research Institute	05/09/14	No Quiz

For this Completion Report to be valid, the learner listed above must be affiliated with a CITI Program participating institution or be a paid Independent Learner. Falsified information and unauthorized use of the CITI Progam course site is unethical, and may be considered research misconduct by your institution.

Paul Braunschweiger Ph.D.
Professor, University of Miami
Director Office of Research Education
CITI Program Course Coordinator

Appendix XXI: Working with International Animal Care and Use Committee (IACUC) course report

COLLABORATIVE INSTITUTIONAL TRAINING INITIATIVE (CITI)
WORKING WITH THE IACUC CURRICULUM COMPLETION REPORT
Printed on 06/10/2014

LEARNER

GRACE AKINYI (ID: 4080000)
22585 - 00400
NAIROBI
KENYA

DEPARTMENT   ITROMID
PHONE   +254 720 422 616
EMAIL   itromid@kemri.org
INSTITUTION   Kenya Medical Research Institute
EXPIRATION DATE   06/09/2017

INVESTIGATORS, STAFF AND STUDENTS : The CITI Basic Course in Laboratory Animal Welfare for Investigators, Staff and Students.

COURSE/STAGE: Lab Animal Research/1
PASSED ON: 06/10/2014
REFERENCE ID: 13177604

ELECTIVE MODULES	DATE COMPLETED	SCORE
Introduction to Working with the IACUC	06/06/14	No Quiz
Working with the IACUC	06/06/14	3/3 (100%)
Federal Mandates	06/06/14	5/5 (100%)
The Veterinary Consultation	06/06/14	1/1 (100%)
Getting Started	06/06/14	5/5 (100%)
Alternatives	06/06/14	8/8 (100%)
Avoiding Unnecessary Duplication	06/06/14	1/1 (100%)
USDA Pain/Distress Categories	06/06/14	8/8 (100%)
Endpoint Criteria	06/06/14	2/2 (100%)
Surgery	06/06/14	11/11 (100%)
Antibody Production	06/06/14	No Quiz
Collecting Blood Samples	06/06/14	3/3 (100%)
Personnel Training and Experience	06/06/14	3/3 (100%)
Occupational Health and Safety	06/06/14	1/1 (100%)
Using Hazardous and Toxic Agents in Animals	06/06/14	5/5 (100%)
Housing Social Animals	06/10/14	1/1 (100%)
Housing Rodents on Wire Floors	06/10/14	1/1 (100%)
Dog Exercise	06/10/14	1/1 (100%)
Primate Psychological Enrichment	06/10/14	1/1 (100%)
Prolonged Restraint	06/10/14	2/2 (100%)
Euthanasia	06/10/14	5/5 (100%)
Using Human Patient Care Areas for Animal Research	06/10/14	1/1 (100%)
Using Explosive Agents in the Animal Facility	06/10/14	1/1 (100%)
Making Changes after You Receive Approval	06/10/14	1/1 (100%)
Reporting Misuse, Mistreatment, or Non-Compliance	06/10/14	No Quiz
Final Comments	06/10/14	No Quiz

Paul Braunschweiger Ph.D.
Professor, University of Miami
Director Office of Research Education
CITI Program Course Coordinator