ANTI-ASTHMATIC EFFECTS OF *Warburgia ugandensis* USING BALB/C MOUSE MODEL FOR ASTHMA AND ISOLATED RABBIT TRACHEA

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Anti-asthmatic effects of *Warburgia ugandensis* using BALB/c mouse model for asthma and isolated rabbit trachea

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DECLARATION

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

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DEDICATION

I dedicate this thesis to my family and friends. A special feeling of gratitude to my loving parents, Samuel and Ann whose words of encouragement and push for tenacity ring in my ears. My sister Silvia, brothers Eric and Dennis who have never left my side and are very special. I also dedicate this thesis to my friend and mentor Dr. Maingi for his support throughout the process. I will always appreciate all they have done for me. Above all I thank God for His grace throughout my Master's studies.

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LIST OF ABBREVIATIONS

Ach	Acetylcholine
ANOVA	One-way Analysis of Variance
BALF	Bronchial alveolar lavage fluid
CE	Catechin equivalent
CO_2	Carbon dioxide
DALYs	Disability Adjusted Life Years
DF	Degrees of freedom
DPPH	2-Diphenyl-1-picryl hydrazyl
DWB	Dry weight bases
EPO	Eosinophil peroxidase
eNOS	Endothelial Nitric oxide synthase
FENO	Fraction of exhaled nitric oxide
G-CSF	Granulocytes colony stimulating factors
GSNO	S- nitrosoglutathione
GSNOR	S- nitrosoglutathione reductase
HCL	Hydrochloric acid
iNOS	Inducible Nitric oxide synthase
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KO	Knock Out
KH	Krebs–Heinsleit solution

M_3	Muscarinic 3
MOP	Myeloperoxidase
NS	Normal saline
NO	Nitric oxide
NOS	Nitric oxide synthase
NO^{2-}	Nitrite
NO^{3-}	Nitrate
nNOS	Neuronal Nitric Oxide synthase
OVA	Ovalbumin
PCT	Pre Convulsion Time
RNS	Reactive nitrogen species
RSA	Radical scavenging activity
SPSS	Statistic Product for Service Solutions
TM	Traditional medicine
Th2	T helper cell
WHO	World Health Organization
WT	Wild type
YLD	Years of Life lived with Disability

DEFINITION OF TERMS

BALB/c mouse: an albino laboratory-bred stain of the house mouse.

EC50 (Half maximum effective concentration): concentration of a drug, antibody or toxicants which induces a response halfway between the baseline and maximum after a specified exposure time.

IC 50 (half maximum inhibitory concentration): a quantitative measure that indicates how much of a particular inhibitory substance is needed to inhibit in vitro a given biological process or biological component by 50%

L-NIL (N^6-(1-Iminoethyl)-lysine, hydrochloride): a potent and moderately selective inhibitor of inducible nitric oxide synthase (iNOS).

ABSTRACT

Asthma is a chronic respiratory disease characterized by recurrent airway inflammation, hyper-responsiveness and reversible airway obstruction. *Warburgia ugandensis* is widely used by herbalist due to its therapeutic properties with observational studies showing it can be used in management of asthma. This study through experimental study sought to find out the anti-asthmatic effects of W *ugandensis* using BALB/c mouse model for asthma and isolated rabbit trachea. Percent protection to developing airway resistance by measure of Pre Convulsion Time (PCT), efficacy in reduction of inflammatory cells count and Nitric Oxide levels in BALF, reduction in bronchiole wall thickness and muscarinic and beta receptor activity were determined. Animals were induced with asthma and challenged with methacholine one hour after final OVA inhalation to determine baseline PCT. Intervention was done with (500mg/kg, 250mg/kg, negative control (NS), positive control (budesonide) then challenged with methacholine 72 hours after final OVA inhalation, PCT taken was compared to the baseline PCT and percent protection calculated. Hemocytometer was used to determine total inflammatory cell count and four- part differential counts of 200 cells per slide performed for differential cell count. NO level was determined using Griess reagent protocol. Bronchiole wall thickness was assessed using image j v1.50i©, Java 1.6.0_20© image analysis open source software. Receptor activity was demonstrated by percent relaxation dose response curves drawn from contraction recordings of isolated rabbit trachea hanged on an organ bath. Pre-convulsion time was not significantly different across all groups before intervention ($p>0.05$); however, it was significantly higher in 500mg/kg percent inhibition (88.02±1.72b) though not significantly different from positive control percent inhibition (82.03±1.29b). Negative control recorded highest total cell count (619.67±15.50a) most being eosinophils (494.33±12.01) and decreased significantly following interventions $p<0.05$. NO was highest in negative control (494.33±12.01a) and decreased significantly with interventions, positive control (0.082±0.003d), 250mg/kg (0.114±0.00c) and 500mg/kg (-0.013±0.003d) $p<0.05$. Bronchiole wall thickness significantly decreased with *W. ugandensis* 500 mg/kg intervention (0.633±0.292) and was comparable to positive control (0.587±0.439), $p<0.05$. *W. ugandensis* had duo relaxation effects, Beta receptor percent relaxation was 55.74% and anti-muscarinic percent relaxation was 13.6%, $P < 0.05$. Results attested that *W. ugandensis* stem bark extract has anti-asthmatic effects though there is need for further validation of anti-asthmatic potent molecules to augment the findings.

CHAPTER ONE

INTRODUCTION

1.1 Background

Asthma is a chronic respiratory disease characterized by allergen- induced recurrent airway inflammation, hyper-responsiveness and reversible airway obstruction. It is associated with increase in IgE antibodies that stimulate mast cells, infiltration of neutrophils, eosinophils, T helper type 2 cells and lymphocytes in the airway. These cells secrete cytokines and inflammatory mediators that lead to the development of airway hyper-responsiveness (AHR) to a variety of stimuli, including allergens, chemical irritants, and pharmacological agents such as histamine and methacholine. Secreted cytokines include but not limited to leukotrienes, bradykinins, prostaglandins, Nitric Oxide, platelet activating factors, chemokines and endothelin (Rang, Ritter, Flower& Henderson (2014). As a result of the chronic inflammatory process, the airways may suffer profound structural changes that include epithelial metaplasia, fibrosis and increased airway smooth muscle mass, referred to as airway remodeling (Keller, Rodriguez & Russo 2005).

Various treatment options have been applied in the management of asthmatic conditions ranging from the use of conventional medicine to ethno-medicine like *W. ugandensis.* Observational studies by clinicians showed patients who were treated with *W. ugandensis* for asthma had great improvement (Aluoch *et al*. 1990). Though data exist to support the use of *W. ugandensis* in management of Asthma, data on its broncho-spasmolytic activity, its effectiveness in lowering inflammatory cells, Nitric oxide levels in BALF and its receptor activity on M3 receptors and beta 2 receptors in the respiratory system is limited.

Across the world, traditional medicine (TM) is either the mainstay of health care delivery or serves as a complement to it (WHO 2013). More than 60 % of the world population, of which 80% is contributed by the developing countries, depend on

traditional medicine (Bannerman & Burton 1983). Integration of traditional medicine has been recommended by WHO since 1978 (WHO 2002). Traditional medicine strategy 2014-2023 by World Health Organization aims to support member states in developing proactive policies and implementing action plans that will strengthen the role traditional medicine plays in keeping populations healthy (WHO 2013).

Warburgia ugandensis tree belongs to the family of Canellaceae, it's commonly known as East African green wood/greenheart/pepper-bark tree (Orwa *et al*., 2009). Local names in Kenya are Ol-msogoni (Maasai), Muthiga (Kikuyu). The tree is common in East African forests, in Kenya its more concentrated in the Mt. Kenya forest. The stem bark of W. *ugandensis* extract, is used as an expectorant, treatment of constipation, fever, asthma, malaria, stomach ache and diarrhea (Karani *et al*. 2013)**.**

Bronchial asthma is the most common chronic respiratory disorder affecting man. The disease burden is approximately 8-10% of the world's population, with an increase in the prevalence of morbidity and mortality in the past 20 years (Barik1, Kanungo,Panda 2014). Asthma is one of the major causes of disability, health resource utilization and poor quality of life worldwide (Centers for Disease Control & Prevention 2013). The symptoms of asthma are due to the production of endogenous and intrinsic mediators from mast cells in response to an allergen in the airway resulting into symptoms like breathlessness, wheezing, dyspnea, chest tightness, cyanosis, coughing (Patil & Ninave 2016).

Asthma can be triggered by various factors like dust, cold or warm air, exercise, emotions, perfumes, smoke tobacco, chemicals, genetics, and histamine (Barik1*et al*., 2014).

Different classes of anti-asthmatic drugs are currently being used for management of asthma such as Beta 2 agonist, corticosteroids, mast cell stabilizers leukotriene inhibitors and methylxanthines whoever they have been reported to have significant side effects among them immune suppression, cardiac abnormality hyperglycemia, hypokalemia and muscle tremors (Barik1 *et al*., 2014).

Therefore, there is a high need to identify more safe and effective drugs to manage asthma.

1.2 Statement of the problem

Asthma is a common respiratory condition with a burden of 8-10% in the world and 10% in Kenya (Barik1*et al*., 2014). There is an increase in the prevalence of morbidity and mortality of asthma in the past 20 years (Barik1*et al*., 2014). Studies showing the effectiveness, mode of action and immune modulation effects of *W. ugandensis* in management of asthma are lacking yet the plant is commonly being used by traditional herbalist (Kokwaro,2009). Asthma is one of the major causes of disability, health resource utilization and poor quality of life worldwide (Centers for Disease Control & Prevention 2013). The current health indices suggest that the prevalence and incidence of asthma is still high in the Western world however due to economic and humanitarian effects coupled with lack of proper sensitization of the disease, incidence in developing countries have increased in folds where the prevalence is on steady rise (Eder, Ege & von Mutius 2006).

There are about 300 million patients suffering from asthma worldwide half of those are found in Africa, with a high mortality mainly because of poor accessibility of the health facility, lack of proper understanding of the disease and high cost involved in purchasing of the available anti-asthmatic drugs (van Gemert *et al.,* 2011). Moreover, the bigger chunk of the affected population are usually children between 1-10years hence this is also a major contributor to childhood mortality and high cost involved in hospital bills and drug purchase leading to poor related quality of life (Oni, Erhabor, & Egbagbe 2010). Besides the poor related quality of life, some drugs have shown resistance, fatal side effects, and lack of efficacy in the management of asthma (Barik1*et al.,* 2014). Different classes of anti-asthmatic drugs are currently being used for management of asthma such as beta 2 agonist, corticosteroids, mast cell stabilizers leukotriene inhibitors and methylxanthines but they have been shown to have a lot of side effects among them immune suppression, cardiac abnormality hyperglycemia,

hypokalemia and muscle tremors (Barik1*et al*., 2014). Therefore, knowledge on *W. ugandensis* will provide an insight into alternative in the treatment of asthma.

1.3 Justification of the study

More than 60 % of the world population, 80% of which is in the developing countries depend on traditional medicine (Bannerman & Burton 1983). *Warburgia ugandensis* has gained a lot of popularity in the management of respiratory tract disease including asthma even without scientific evidence on its efficacy in Kenyan communities (Kokwaro,2009). Study on its bronchial spasmolytic effects, mode of action and its effectiveness in the management of asthma will help to generate data that could possibly authenticate its claimed efficacy. Results will also be disseminated to health workers to give appropriate advice to the community on the medicinal value of *W. ugandensis* in the management of asthma. Data from this study will also create an impetus for further studies especially isolation and identification of candidate bioactive molecule/s that may be helpful for future pharmacological intervention.

1.4 Research questions

1. What are the phytochemicals found in *W. ugandensis* in ethanol and water extraction?
2. What is the acute toxicity of *W. ugandensis* using BALB/c male mice?
3. What is the duration of time taken to develop airway resistance in OVA/alum hydroxide-asthma-induced BALB/c mice following treatment with *W. ugandensis* extract then after being challenged with methacholine?
4. What is the differential inflammatory cell counts in BALF in OVA /alum hydroxide-asthma-induced BALB/c mice after treatment with *W. ugandensis* extract then after being challenged with methacholine?
5. What is the NO level in BALF in OVA/alum hydroxide-asthma-induced BALB/c mice following treatment with *W. ugandensis* extract then after being challenge with methacholine?

6. What is the Bronchiole alveolar wall thickness in OVA /alum hydroxide-asthma-induced BALB/c mice following treatment with *W. ugandensis* extract then after being challenged with methacholine?
7. What is the effect of *W. ugandensis* on Muscarinic 3 and beta 2 receptor activity on isolated rabbit trachea?

1.5 Broad objective

To determine anti-asthmatic effects of *W. ugandensis* in BALB/c mouse model for asthma and isolated rabbit trachea.

1.6 Specific objectives

1. To determine phytochemicals in *W. ugandensis* in ethanol and water extraction
2. To determine acute toxicity of *W. ugandensis* in BALB/c male mice
3. To determine the duration of time taken to develop airway resistance in OVA/alum hydroxide-asthma-induced BALB/c mice following treatment with *W. ugandensis* extract then challenged with methacholine.
4. To determine the differential inflammatory cell counts in BALF in OVA/alum hydroxide-asthma-induced BALB/c mice following treatment with *W. ugandensis* extract then challenged with methacholine.
5. To determine Nitric Oxide level in BALF in OVA/alum hydroxide-asthma-induced BALB/c mice following treatment with *W. ugandensis* extract then challenged with methacholine.
6. To determine Bronchiole alveolar wall thickness in OVA/alum hydroxide-asthma-induced BALB/c mice following treatment with *W. ugandensis* extract then challenged with methacholine.
7. To determine *W. ugandensis* receptor activity on Muscarinic 3 and beta 2 receptors using isolated rabbit trachea.

1.7 Hypothesis

H0*: Warburgia ugandensis* does not have anti-asthmatic effects in BALB/c mouse model for asthma and on isolated rabbit trachea.

H1: *Warburgia ugandensis* does has anti-asthmatic effects in BALB/c mouse model for asthma and on isolated rabbit trachea.

CHAPTER TWO

LITERATURE REVIEW

2.1 What is asthma

Asthma is a chronic respiratory disease characterized by airway hyper responsiveness, airway inflammation and reversible airway obstruction (WHO 2011). Symptoms of asthma are due to intrinsic mediators like leukotrienes, bradykinins ,prostaglandins ,Nitric Oxide, platelet activating factors, chemokines and endothelin's from mast cells (Barik1*et al*., 2014). The inflammation of the airway results in swelling of airway, narrowing of airway, increased mucus production, shortness of breath, coughing and wheezing.

Airway hype responsiveness which results from antibody antigen interaction or non-specific stimuli like exercise, cold air, emotional stress, pharmacological agents like histamine, methacholine, causes airway resistance hence limited flow of air (breathlessness). It resolves spontaneously or with treatment (Barik1*et al.*, 2014).

2.2 Types of asthma

2.2.1 Allergic asthma (atopic asthma)

Asthma starts in childhood at age of 2-6 years. Its triggered by an allergen e.g. pollen, molds, fur. It's also known as extrinsic asthma. Its associated with positive history of allergic condition in the family as well as other allergic conditions like eczema, nasal allergies. Remission occurs in early adulthood but in some cases may persist for life (Spergel, 2010).

2.2.2 Intrinsic asthma (non-atopic asthma)

This type of asthma sets in adulthood mostly after 30 years of age. Its related to intrinsic factors and not allergens. Usually follows upper respiratory infection with a virus (Spergel, 2010).

2.2.3 Exercise induced asthma

Results from physical exertion or sport that causes chest tightness and difficulty in breathing which resolves when the person stops exercise (Koshak *et al*., 2007).

2.2.4 Aspirin induced asthma

Some asthmatic individuals have sensitivity to aspirin and other nonsteroidal anti-inflammatory drugs that inhibit cyclooxygenase 1 pathway. On taking these drugs ,they develop sneezing, stuffy nose, wheezing and difficulty in breathing (Szczeklik & Stevenson 2003). This distinct clinical syndrome, called aspirin-induced asthma (AIA), is characterized by an eosinophilic rhino sinusitis, nasal polyposis, aspirin sensitivity, and asthma. Aspirin desensitization, followed by daily aspirin treatment, is a valuable therapeutic option in most patients with AIA, particularly those with recurrent nasal polyposis or overdependence on systemic corticosteroids (Szczeklik & Stevenson 2003).

2.2.5 Occupational asthma

Asthma that results from irritants at work place. The irritants could be smoke, chemicals like chloride fumes, paints (Ober & Yao 2011).

2.3 Burden of asthma

The prevalence of asthma is estimated to be about 300 million worldwide (WHO 2011). In 2016, asthma, across all ages, contributed 23.7 million Disability Adjusted Life Years (DALYs) globally. In the same year, asthma was ranked 16th as the leading cause of Years of Life lived with Disability (YLD) globally (Global Asthma Network, 2018). Asthma is rated as the most common respiratory disease in the US (Centers for Disease Control & Prevention 2013). Asthma is a common respiratory condition with a burden of 8-10% in the world and 10% in Kenya (Barik1*et al*., 2014). Prevalence in Nigeria 6.6%, Sweden 9%, Australia 10.7% and Norway 9.3% (Oni, Erhabor & Egbagbe 2010). The current health indices suggest that the prevalence and incidence

of asthma is still high in the Western world. However due to economic and humanitarian effects coupled with lack of proper sensitization of the disease, incidence in developing countries has increased in folds where the prevalence is on steady rise (Eder, Ege & von Mutius 2006). Despite advance existing knowledge on pathophysiology of asthma, its morbidity and mortality has remained high. Asthma affects all age groups, but boys are more affected than girls by the third decade, prevalence then becomes equal and subsequently more women become affected than men (Oni, Erhabor & Egbagbe 2010).

2.4 Pathogenesis and pathophysiology of asthma

Asthma is a chronic airway inflammatory disease characterized by infiltration of the airway T cells. Pathogenesis of allergic asthma is mediated by Th2 cell. In both normal and asthmatic airway mucosa, the prominent cells are the T lymphocytes, which are activated in response to antigen stimulation, or during acute asthma exacerbations, and produce high levels of cytokines. They are subdivided into two broad subsets according to their surface cell markers and distinct functions: the CD4+ (T helper) and the CD8+ (T cytotoxic) cells. CD4+ cells are further subdivided into TH1 and TH2 cells, depending on the type of cytokines that they produce. Another subtype of CD4+ is the TH3 cells, which produce high levels of growth factor, IL-4 and IL-10. Other cells involved in the pathogenesis of asthma include mast cells, basophils, macrophages, and eosinophils. The interactions among all these cells and their products perpetuate the inflammatory response. In asthma there is an imbalance between Th1 and Th2 cells, with either overproduction of Th2 or underproduction of Th1.Th1 is considered to be protective of asthma and other allergic conditions as it inhibits Th2 response (Nakagome and Nagata 2011).

Allergic reaction stimulate production of thymic stromal lymphopoietin (IL-7 like cytokine) from mast cells airway epithelial cells and fibroblast cells. IL-7 causes differentiation and maturation of antigen presenting dendritic cells which in turn induce differentiation of naïve CD4 cells into Th2 cells. Th 2 cells induce release of cytokines IL4, IL5, IL-9, IL-13, TNF, mediate recruitment of airway mast cells and

eosinophils, switch of IgG to IgE and mucus secretion. IL-13 stimulates airway hyper reactivity and mucus production. IL-4 promotes differentiation and proliferation of more Th2 cells and switching of IgG to IgE (Nakagome & Nagata 2011).

Dendritic cells also secret IL-6 that cause differentiation of Th2 and Th17. Th17 produce IL-17 that mediate neutrophils stimulating production of chemotactic factors CXC chemokines and G-CSF from bronchial epithelial cells (Cosmi *et al.*, 2011).

IL-4 and IL-13 also produce chemoattractant for eosinophils, 15- Lipoxygenases that generate pro-inflammatory mediators from arachidonic acid and inducible Nitric Oxide synthase that produce exhaled Nitric Oxide (FeNO) (Wills-Karp, 2004).

IL-5 helps in differentiation, maturation and survival of eosinophil. TNF, a pro-inflammatory cytokine promotes airway inflammation, hyper reactivity and mucin production (Cosmi *et al.* 2011).

Mast cells have granules that are releases upon activation. The granules contain histamine, prostaglandins D2 and leukotrienes which are potent broncho constrictors. Mast cells have receptors for Ig E that bind on them and activate them. They are also activated by osmotic stimuli like in cases of exercise induced bronchoconstriction. Mast cells are associated with airway hyper-responsiveness by releasing a large number of cytokines even when exposure to allergen is limited (Saglani & Lloyd 2014).

The symptoms of asthma are due to production of endogenous and intrinsic mediators from mast cells in response to an allergen in the airway resulting in to symptoms like breathlessness, wheezing, dyspnea, chest tightness, cyanosis, coughing (Patil & Ninave 2016)

Eosinophils are granulated leukocytes. Asthmatic patients have a large number of eosinophils along the airway. The number increases more during exacerbation of an asthmatic attack and for asthmatics who smoke. The high number correlates with severity of the disease. Granules of eosinophil have pro-inflammatory mediators and

leukotrienes. It has been shown that steroid use lowers the levels of eosinophil in asthmatic patients (Saglani & Lloyd 2014).

Ig E antibodies are increased in allergic reactions. They attach to cell surface receptors of mast cells, basophils, dendritic cells and lymphocytes hence activating these cells. As a result, monoclonal antibodies against Ig E have been developed and are useful in treatment of asthma (Froidure *et al*. 2016).

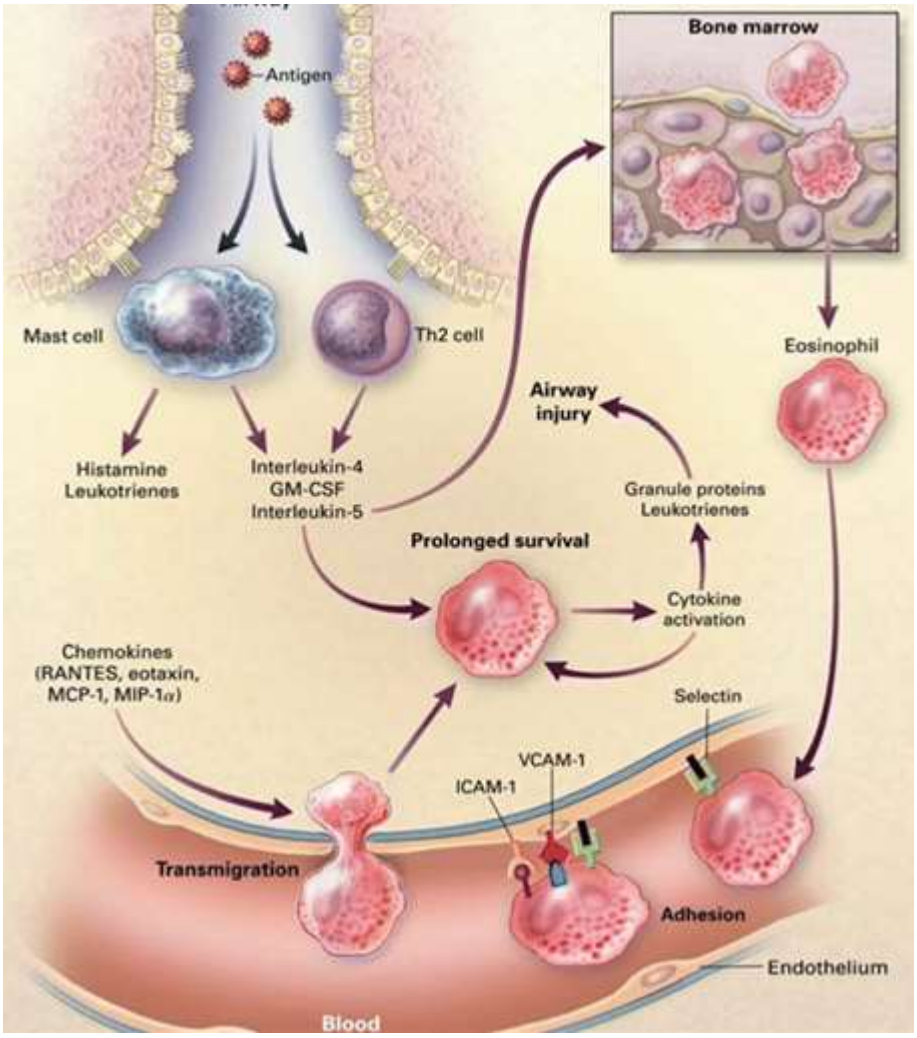

Figure 2.1Pathophysiology of asthma (Panel on the Management of Asthma. 1998)

2.4.1 Role of Nitric Oxide in the pathophysiology of asthma

The role of Nitric Oxide (NO) in asthma is of particular interest as fraction of exhaled Nitric Oide (FeNO) is clinically used as a marker of eosinophilic bronchial inflammation. High exhaled NO concentrations in asthmatic patients may reflect induction of NO synthase, which is known to be inhibited by steroids. Measurement of exhaled NO concentrations may be clinically useful in detection and management of cytokine mediated inflammatory lung disorders (Kharitonov , Yates & Robbins 1994).

Several mechanistic pathways have been postulated in the NO role in pathophysiology of asthma. In asthmatic airway, high levels of NO may lead to greater formation of detrimental reactive nitrogen species (RNS), which modify proteins that adversely mediate inflammation and injury. It's also noted that high levels of NO causes suppression of S-nitrosothiols, which causes bronchodilation in the airway. Nitric Oxide also causes recruitment of neutrophils, monocytes, eosinophils and mucus hypersecretion playing a significant role in pathophysiology of asthma (Prado, Martins & Tib 2011). Infiltrated eosinophils produce peroxidases like myeloperoxidase that use NO to generate nitrogen radicals that cause more inflammation (Erzurum, 2013). Exhaled NO levels are used as an indicator of good control in asthmatic individuals. NO is generated by three Nitric Oxide synthase; neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS), (i/n/eNOSs) isoforms (Akata, Yatera, & Keisuke 2016). The isoforms are found in many epithelial tissues, with Nitric oxide synthase induced (iNOS) being the most abundant isoform synthase enzyme in the lung (Kobzik *et al.*, 1993). NO derived from iNOS, seems to play a primary role as pro-inflammatory mediator.

The iNOS protein expression levels are increased in asthmatic airway epithelial cells. The iNOS expression is mostly regulated at the level of transcription and Janus kinase (JAK)/signal transducer, activator of transcription. An animal study using wild type (WT) mice sensitized and induced with Ovalbumin (OVA) to induce asthma with targeted deletions of the three isoforms of NOS, identified a role for iNOS in the

disease progression. Ovalbumin (OVA) sensitized and challenged wild-type (WT) mice lung showed significant up regulation of iNOS, whereas iNOS expression was undetectable in similarly treated iNOS knock out (KO) mice. However, airway hyper-responsiveness between these two groups was similar. Airway hyper-responsiveness in nNOS-deficient and n/eNOS deficient mice was significantly less than that observed in WT mice. On the other hand, iNOS KO mice sensitized and challenged with OVA had less inflammatory cell infiltration of the lung, particularly eosinophils, as compared to WT animals (Keller, Rodriguez, & Russo, 2005). In a similar study by Akata, Yatera, and Keisuke (2016) showed the significance of NO in pathophysiology of asthma, using a mouse model for asthma with knock out of Nitric Oxide synthase (i/n/eNOSs) and there was significant decrease in eosinophilic inflammatory cells, bronchial thickening and mucus secretion, Th2 cytokines like IL-4, 5 and -13, monocyte chemoattractant protein-1, eotaxin-1 and thymus and activation-regulated chemokine expressions.

2.4.2 BALF NO levels are used as a surrogate marker of inflammation in asthma

Concentration of Nitric Oxide (NO) in exhaled air is now recognized as a critical component in evaluating control of the asthmatic (Wechsler *et al.*, 2000). Measurement of exhaled nitric oxide levels provides a rapid, reproducible, and reliable test which may reflect airway inflammation in asthma (Stirling *et al*. 1998). Corticosteroids lower NO levels which causes exacerbation of inflammatory cell mediators and mucus producing airway epithelial cells hence their use as controllers (Permanente *et al.*, 2011). Persistent elevation of exhaled Nitric Oxide in treated asthmatics may suggest either more severe inflammation or inadequately treated inflammation. Elevated exhaled nitric oxide is a clinical indicator of uncontrolled asthma in asthmatic patients on inhaled corticosteroids. It is evident that corticosteroids lower the FENO levels and the asthmatic individuals are better controlled as compared asthmatics who are on short acting bronchodilator (SABA) (Permanente *et al.*, 2011.).

2.4.3 Metabolism of nitric oxide in relation to asthma pathophysiology

Nitric oxide synthases use L-Arginine as substrate to produce NO and L Citrulline.

L-Arginine is also consumed by Arginases I and II to generate urea and Lornithine.

L-ornithine serves as a precursor for Glutamine, Proline, Putrescine, Spermidine

and Spermine, which are important to cell proliferation hence causing airway remodeling in asthmatics.

NO can be rapidly oxidized to nitrite (NO^{2-}), which can be further oxidized to nitrate

(NO^{3-}). Under disease conditions with superoxide generation, Nitric Oxide can rapidly oxidize to peroxynitrite ($ONOO^{-}$) that readily Nitrate tyrosine residues of proteins. Myeloperoxidase (MPO) and eosinophil peroxidase (EPO) can also use nitrite as a substrate to nitrate protein tyrosine promoting production of radicals that cause inflammation in asthma. In the presence of thiols (RSH), nitrite undergoes nitrosylation reaction to generate S-nitrosothiols (RSNO) a molecule that promotes bronchodilatation.

In asthmatic airway, high levels of NO lead to greater formation of reactive nitrogen species (RNS), which modify proteins adversely affecting functional activities. In contrast, high levels of NO are associated with lower than normal levels of S-nitrosothiols, which serve a bronchodilator function in the airway (Ghosh *et al.*, 2013).

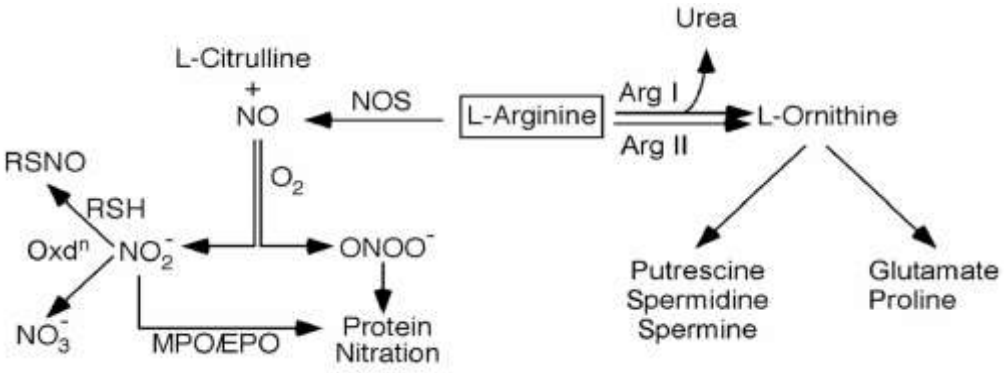

Figure 2.2 Metabolism of Nitric Oxide

Source; Keller, Rodriguez &Russo 2005

2.4.4 Nitric Oxide pathways in asthma pathogenesis and the interventions

i) Inhibition of iNOS

The NOS inhibitor effectively reduced eosinophilia and airway resistance in animal models of asthma. For example, L-NIL effectively caused iNOS inhibition, and reduced exhaled NO in asthmatics within 15 min of oral treatment. In a cigarette-smoke exposure mouse model selective iNOS inhibitors (GW274150 and BYK402750) was effective in decreasing airway inflammation. This may indicate that specific iNOS inhibition is important to achieve therapeutic outcome. Paradoxically, NO effects via the phosphodiesterase inhibitor sildenafil effectively relaxes carbachol-induced contractions in isolated tracheal rings prepared from a rodent model of allergic asthma (Keller, Rodriguez & Russo 2005).

ii) Arginase blockade as a strategy for asthma

The pharmacologic inhibitor of arginase, nor-NOHA [L-2-Amino-(4-(2'-hydroxyguanidino) butyric acid], increases NO production. On the other hand, BEC [(S)-(2- Boronoethyl)-L-cysteine], a highly potent arginase inhibitor, increased protein s-nitrosylation and nitration in a mouse asthma model (Bratt, *et al.*, 2008)

iii) Augmentation of S-nitrosothiol through blockade of GSNOR

Studies have tested the therapeutic role of S-nitrosothiols in the murine model of allergic inflammation and found that instillation of GSNO suppressed NF-kB activation and bronchial hyper-reactivity, but did not significantly alter airway inflammation. Since blockade of GSNOR is protective in murine asthma model Sarkar *et al* (2011) explored the possibility of using GSNOR inhibitors, GSNORi, to treat asthma. GSNORi raised levels of S-nitrosylated proteins in cytokine stimulated murine macrophage cells and interestingly lowered levels of other immune modulators, e.g. osteopontin, cyclooxygenase-2, and iNOS. Edwan *et al* (2019) identified a pyrrole based potent and novel GSNORi, N6022, that lowers GSNOR activity, and leads to bronchodilation in the OVA induced asthmatic mouse model.

2.5 Medicinal uses of *Warburgia ugandensis*

Plants are important source of medicine both in modern and traditional system (Maobe *et al.,* 2012) *Warburgia* is a genus that includes four species W.salutaris Bert of Chiov, W. elongate Verd and W. stuhlmannii Engl. found along East African coastline Africa and *W. ugandensis* Sprague found within East African highland and belongs to the family of Canellaceae (Muchugi *et al.* 2008). Its commonly known as East African green wood/greenheart/pepper bark tree (Orwa *et al.*, 2009). Local names in Kenya are Ol-msogoni (Maasai), Muthiga (Kikuyu). Warburgia *ugandensis* an endangered indigenous species and has been rated as the second highest medicinal plant in Kenya after Prunus africana. The tree is common in East African forests, in Kenya its more concentrated in the Mt. Kenya forest. It has been found to have antifungal effects against candida albicans antibiotic effects against E.coli, staphylococcal aureus, anti-inflammatory effects, microbial activity against Mycobacterium aurum, M fortuitum , and antiseptic effects (Kairu *et al.* 2013). It has also been shown to have phytochemicals that have antidiabetic, anti-hypertensive and anti-oxidant *(Maobe et al.* 2012). *W. ugandensis* has been used in treatment of diabetes, asthma, hypertension, diarrhea, pneumonia (Mwitari *et al*. 2013).

Stem bark of W.*ugandensis* extract, is used as an expectorant ,treatment of constipation, fever, asthma, malaria, stomach ache and diarrhea (Malik *et al*. 2006).

Figure 2.3 Photograph of *W. ugandensis* plant

2.6 Phytochemical analysis of Warburgia *ugandensis*

Phytochemicals are compounds found in plants, have no nutritive value but protect the plant from diseases (Maobe *et al.,* 2012). Phytochemicals are of different type and have been found to have pharmacological value like anti-microbial, anti-inflammatory, anti-diabetic, anti-cancer among many other values hence the use of plants as herbal medicine (Maobe *et al.,* 2012). Examples of phytochemicals are flavonoids that have protective value against ultraviolent radiation and pathogens in plants and hence used as antioxidants in human beings as they scavenge for free radicals (Maobe *et al.*, 2012). Phenolic compounds are another example of phytochemicals that have been shown to have pharmacological activity such as anti-microbial, anti-inflammatory, anti-diabetic, anti-cancer, cytotoxic and anti-mutagenic activity (Kutima *et al*., 2015). A study done by Maobe *et al*., (2012) showed that *W. ugandensis* contain Naringenin, flavanone, Monin, Kaempterol, Galangin as phytochemical though many are yet to be identified. *Warburgia*. *ugandensis* has been shown to have secondary metabolites such as alkaloids, terpenes, flavonoids, cardiac

glycosides, polyphenol, and terpenoids responsible for its anti-plasmodia activity hence its continued use in management of malaria (Kutima *et al*., 2015).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The present study was carried out at SAFARI Animal House in Jomo Kenyatta University of Agriculture and Technology and Medical Physiology laboratory, University of Nairobi.

3.2 Study design

Experimental, laboratory-based study.

3.3 Study population

BALB /c male mice age 6-7 weeks, weighing 25gms to 35gms were used. BALB/c mice have been frequently used in asthma model because of their well-characterized immune system, availability of many tools to manipulate their immunological process and low cost (Carey *et al.* 2007). Giant rabbits 4-6 months old weighing 2600-3000gms were used.

3.3.1 Sample size calculation

Resource equation method (Charan & Biswas, 2013)

The study was divided into 2 sets, one set used BALB/c mice and the other used rabbits each set had the same number of groups and animals.

E = Total number of animals - Total number of groups (The value of E should lie within 10 to 20 for optimum sample size)

Number of groups: 5

Total number of animals: 15

E value is: 10

Each group had 3 animals

20 percent was added to cater for non- response and mortality, totalling to 20 BALB/c mice and 20 Giant rabbits.

3.3.2 Acquisition of the Animals

Animals were purchased from SAFARI animal house at JKUAT and housed at the same place in standard cages that measured 330cm cubed and the floor was 60cm^2 for the BALB/C mice. Each cage accommodated six BALB/c mice. The rabbits were housed in hutches of 12 square feet. The animals were maintained under ambient conditions of temperature (26 ± 4 ºC), relative humidity (60 ± 10%) and normal light/dark cycle (Albus 2012)

3.3.3 Feeding of the animals

The mice were fed on commercial rodent pellets, 5gms /mice/day and the rabbits were fed on 60gms /rabbit/day sourced from Unga Feeds Limited, Kenya and water *ad libitum*.

3.4 Processing of medicinal plant part for extraction

3.4.1 Collection of medicinal plant part

Five kilograms of medicinal plant part (bark) of *W. ugandensis* was collected in August, 2018 from Mt. Kenya forest by the researcher with the help of a plant taxonomist from JKUAT. Authentication was achieved by comparison with Herbarium specimens by taxonomists and a voucher specimen was deposited at the JKUAT herbarium.

3.4.2 Processing of the extract

Stem bark was cleaned to remove physical impurities. Clean bark of the plant was air dried at room temperature for 30 days shred, ground into powder form using

laboratory millers. The powder was weighed and stored in air tight khaki bags. 1200gms was recovered from the 5 kg bark that was collected and was used for aqueous and ethanol extract.

3.4.2.1 Aqueous extract

Water extraction was done using distilled water. Eight hundred grams of ground powder was put in four separate freeze drier glass containers and was dissolved and soaked in 0.5 liters of distilled water per container for 3 hours, placed in warm water bath at 60ºC for 1 hour. The extract was filtered, freeze dried using a freeze drier. A total of 13.4 grams' powder was collected stored in sterile vials.

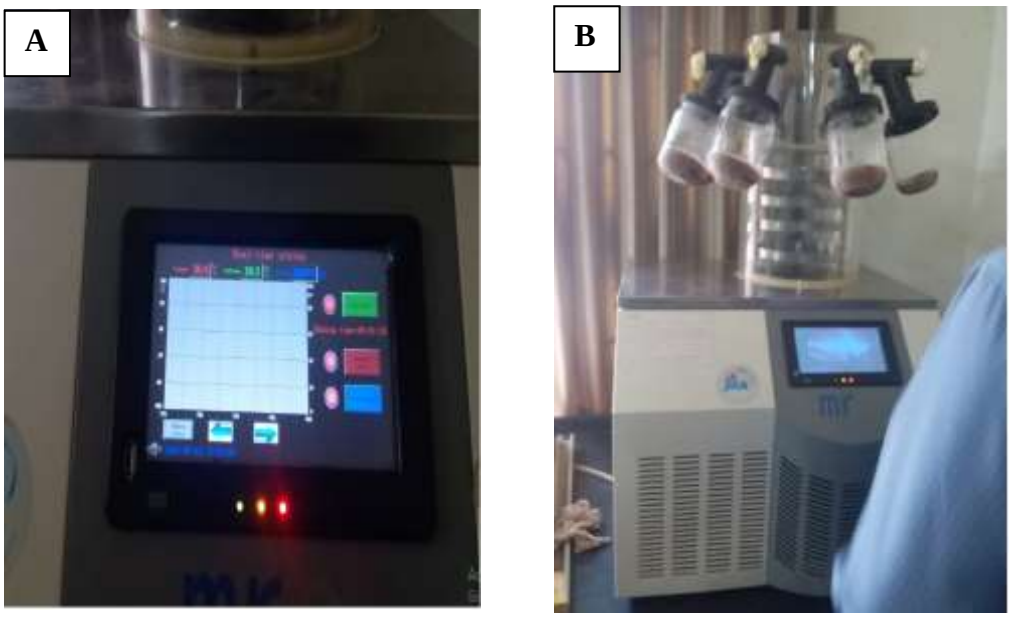

Figure 3.1 Freeze Drier monitor (A) and Freeze Drier with a sample of *W.ugandensis* (B)

Figure 3.2 Freeze dried *W. ugandensis* (13.2gms) in air tight sterile glass container

3.4.2.2 Ethanol extract

Ethanol extraction was done using distilled ethanol as organic solvent, 400gms of ground powder was soaked overnight and concentrated using rotor evaporator. 100mls (paste form) of extract was obtained from the solvent. Extracts was put in sterile vials and stored at -20^{o}C until required for reconstitution.

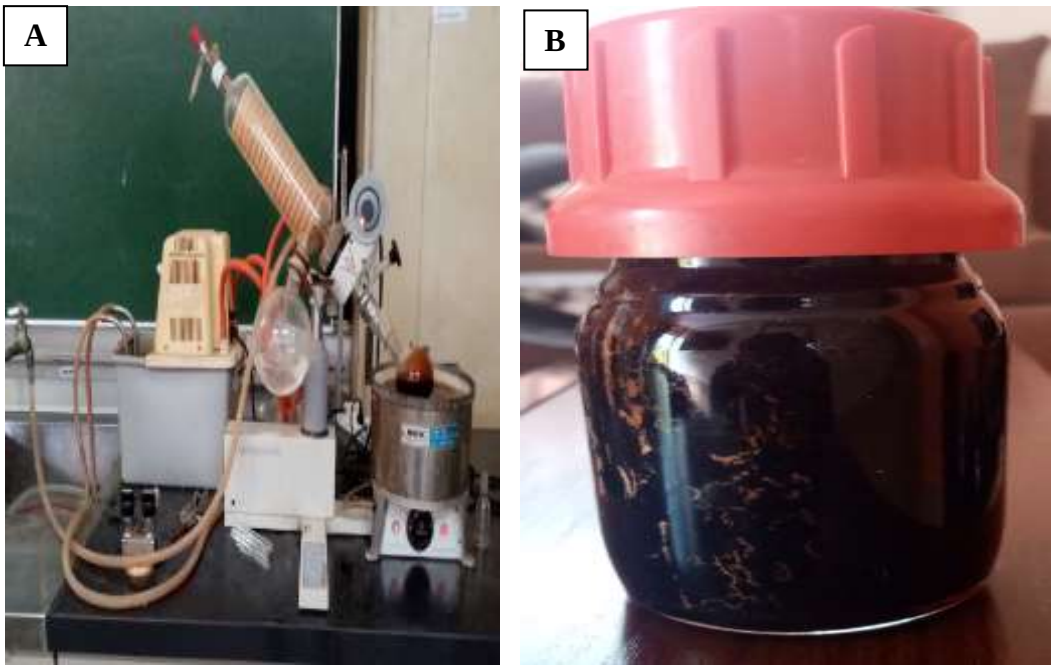

Figure 3.3 Rotter evaporator(A) with *W. ugandensis* ethanol extract in sterile glass container(B)

3.5 Protocol for phytochemical analysis

3.5.1 Determination of radical scavenging activity (RAS)

The radical scavenging activity of the aqueous and ethanol of *W. ugandensis* extracts was determined against 2-Diphenyl-1-picryl hydrazyl radical (DPPH) method using UV visible spectrophotometer at 517 nm. Radical scavenging activity was measured by a modified method described by (Cheung *et al*, 2003). The following concentrations of the extracts were prepared, 0.05, 0.1, 0.5, 1.0, 2.0 and 5 mg/ml ethanol. Ascorbic acid was used as the antioxidant standard at concentrations of 0.02, 0.05, 0.1, 0.2, 0.5 and 0.75 mg/ml. To I ml of the extract in a test tube, 3 ml ethanol was added followed by 0.5 ml. 1mM DPPH in ethanol. Incubation was done for 5 minutes after which the absorbance was read. A blank solution was prepared containing the same amount of ethanol and DPPH. Ascorbic acid dilutions were equally treated; absorbance read and a standard curve generated using the data

obtained. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation:

%RSA=[(ADPPHAS)/ADPPH]100

Where: AS is the absorbance of the solution when the sample extract has been added

ADPPH is the absorbance of the DPPH solution.

The extract concentration providing 50 % inhibition (EC_{50}) was calculated.

3.5.2Determination of flavonoids

Flavonoid content in the extracts was determined by a colorimetric method as described by Barros *et al*, (2007) with minor modifications. To 1ml of aqueous and ethanol *W. ugandensis* extract 0.3 ml 5% sodium nitrite and 4 ml distilled water were added and held for 5 minutes. To the mixture 0.3 ml of 10 % aluminum chloride was added and held for 6 minutes. Finally, 2ml of 1M sodium hydroxide was added and the content made to 10 ml with distilled water. Using UV Visible spectrophotometer, the intensity of pink color was measured at 415 nm. Pure quercetin was prepared in different concentrations and absorbance read at same wavelength. The readings were used to make standard curve, against which flavonoids in the sample were calculated and expressed as mg of quercetin equivalent (QE)/100 g dwb. The same procedure was repeated but with catechin as standard in place of quercetin. Flavonoids in the sample were similarly calculated and expressed as mg catechin equivalent (CE)/ 100g dwb.

3.5.3 Determination of polyphenols

Phenolic compounds in the *W. ugandensis* extracts were estimated by a colorimetric assay, based on procedures described by Barros *et al*, 2007 with minor modifications. To 5 ml distilled water was added 0.5 ml Folin Ciocalteu‟s reagent. After 3 min, 1 ml 7.5 % sodium carbonate solution, 1ml of both aqueous and

ethanol extract were added to the mixture and made to 10 ml with distilled water. The mixture was kept in water bath maintained at 50^0C for 16 minutes. UV-V machine was used to read the absorbance at 765 nm. Gallic acid was prepared in different concentrations and the absorbance equally read at 765 nm. The data obtained was used to generate the standard curve against which polyphenols in the *W. ugandensis* were calculated and expressed as Gallic Acid Equivalent (GAEs) / 100g dwb.

3.5.4 Determination of tannin content

Condensed tannins were assayed according to vanillin-hydrochloric acid method (Price, Van & Butler, 1978). Quarter a gram of ground sample was extracted with 10 ml of 4% HCl in Methanol by shaking for 20 min using a shaker (Labortechnik KS 250b, Germany) and separation done using a refrigerated centrifuge (Kokusan, Type H-2000C, Japan) at 4,500 rpm for 10 min at 25°C. The supernatant was put into a 25 ml volumetric flask and extraction from the residue was repeated with 5 ml of 1 % HCl in methanol. The second supernatant was combined with the first one and diluted to 25 ml. Standards were prepare using Catechin hydrate at 0, 10, 20, 40, 60, 80 and 100 µg/ml. Duplicate aliquots of 1 ml of sample extracts were put into test tubes where one served as sample blank. The samples and standard solutions were reacted with 5 ml vanillin-HCl reagent (prepared by mixing just before use, equal volumes of 8% HCl in methanol and 1% vanillin in methanol) and allowed to stand for 20 min. To the sample's blanks were added 5 ml of 4% HCl in methanol. Absorbance for all prepared solutions was read at 500 nm and tannin content calculated as percent catechin equivalent (CE) using the standard calibration curve.

3.5.5 Phytochemical screening

Ethanolic and aqueous extracts were subjected to preliminary phytochemical screening for the identification of various classes of active chemical constituents present as described by Harborne, (1998) with minor modifications.

3.5.6 Test for saponins

Foam test: To 1 ml of the extracts 5ml distilled water was added and shaken vigorously. Formation of foam that persisted for over 10 minutes indicated presence of saponins.

3.5.7 Test for anthraquinones

Weighed *W. ugandensis* 0.5 g, was boiled in 10 % hydrochloric acid and filtered while still hot. To the filtrate, 2 ml chloroform and 10 % ammonia solution each were added. Formation of pink color in the aqueous layer indicated presence of anthraquinones.

3.5.8 Test for terpinoids

Aqueous and ethanol extract 5 ml were separately mixed with 2ml chloroform followed by sulfuric acid along the tube wall. Formation of brown colored ring at interface was a positive indicator.

3.5.9 Test for alkaloids

On silicagel-coated plates, 10 µl extract was spotted equidistant from each other and eluted with methanol-sulfuric acid solution. The dried plates were sprayed with Dragendroff reagent. Formation of red-brown coloration was a positive indicator.

3.5.10 Detection of unsaturated sterols

The petroleum ether extracts were prepared for both aqueous and ethanol extract then evaporated to dryness. The residue was dissolved in 10 mL choroform, dried over anhydrous sodium sulfate and filtered. The filtrate was divided into three portions. The first portion was subjected to Liebermann-Burchard test; a blue green colour indicated a positive test for sterols. The second portion was subjected to Salkowski

test; a red colour indicates a positive test for sterol. The third portion was used as control for colour changes.

3.5.11 Detection of cardiac glycosides

Five microliters of the solution were placed in small porcelain evaporating dish, 5 mL of kedde's reagent, and 5 mL of 2 N sodium hydroxide solution were added. The appearance of purple color indicates a positive test for cardiac glycosides. Another 10 mL of the solution were evaporated to dryness, the residue was triturated with 3 mL of ferric chloride solution and filtered. The filtrate was transferred to a test tube and 1 mL of concentrated sulphuric acid was added slowly down the side of the test tube. The appearance of purple ring indicates presence of cardiac glycosides (desoxysugar). If the above two tests are positive, 5 mL of the solution were evaporated to dryness. The residue was dissolved in 2 mL chloroform and transferred to a small test tube. Acetic anhydride (0.3 mL) was added and mixed gently, then, a drop of concentrated sulphuric acid was added. The appearance of blue-green colour, observed during 60 min, indicates presence of cardiac glycosides (as steroids).

3.6 Acute Oral Toxicity Determination

The acute toxicity was determined by use of modified Lorke's method (Lorke 1984).

Fifteen male BALB/c mice weighing approximately 25-35 grams were obtained from SAFARI animal Biomedical department in Jomo Kenyatta University of Agriculture and Technology (JKUAT). They were housed in standard mice cages and exposed to 12hour light/dark cycles under humid tropical conditions. Litter papers in the cage was changed on alternate days. Each cage was labelled with a cage card showing experiment number, date of starting the experiment, dosage level, and age, number of animals, species and sex of the animal. The mice were allowed unrestricted access to standard feed Rodent pellets obtained from UNGA Mills and water ad libitum throughout the experimental period. The mice were handled in accordance with the guidelines for the care and use of laboratory animals.

The acute toxicity study was conducted in two phases. The animals were fasted overnight prior administration of the bark extract of *W. ugandensis*. Phase one; four groups each with 3 mice, group 1, 2 & 3 animals were administered with single oral dose of 10, 100 & 1000 mg/kg of the bark extract in 5% dimethyl sulfoxide (DMSO), respectively. Group 4; was the control group with three mice, which were given 5% DMSO in distilled water (5 ml/kg body weight).

Phase II; had three animals, and each received a single oral dose of 1600, 2900 and 5000 mg/kg of the bark extract in 5% DMSO respectively. The bark extract was administered orally using sterile gavage needles. All the animals were monitored closely for signs of toxicity which are mortality, changes in gross appearance of the skin and fur, mucous membrane of the eye, respiratory distress, somatomotor activity, behaviour, and special attention was given to observation of tremors, salivation, diarrhoea, coma and convulsions, changes during the first 48 hours post dosing. The observation schedule was as follows; immediately, ½ an hour, 1 hour, 4 hours, 24 and 48 hours, then monitoring for signs of toxicity continued daily for 14 days. The body weight was monitored as follows; day 0 (initial weight), day 7 and day14 (terminal weight)

Then the LD50 was calculated by the formula;

$$LD50 = \sqrt{D0 * D100}$$

D0 = Highest dose that gave no mortality

D100 = Lowest dose that produce mortality

3.7 Mouse model for asthma

Mouse model have become by far the most popular animals for modelling allergic responses in the airway because there is a detailed understanding of their genetics and it's easy to manipulate outcome using transgenic technology. Also they are relatively cheap and easily sensitized to some antigens like ovalbumin. Sensitization and

subsequent challenge with these antigens results in a Th-2 allergic response simulated in asthma pathogenesis (Zosky & Sly 2007).

3.7.1 Protocols for inducing asthma in BALB/c mice using ovalbumin (OVA)/ alum hydroxide then challenged with methacholine.

Protocol- 20 μg of ovalbumin (OVA; Sigma-Aldrich, St. Louis, MO) with 2 mg of alum hydroxide (Sigma-Aldrich) as an adjuvant dissolved in phosphate buffered saline (PBS), and a total of 200 μL was injected intraperitoneal on day 1 and 14. Inhalation challenge was performed with 1% OVA in a 5.9 L Pyrex glass box for 30 min with a nebulizer on day 28, 29, and 30 (Chang *et al.,* 2005). Ovalbumin is an antigen that acts as an allergen that evokes a hypersensitivity reaction and airway remodeling. Remodeling is thought to be as a result of repeated exposure to allergen which causes repeated or continuing inflammatory events in the airways. As a consequence, the airway wall structure is altered which has an impact on airflow and may be linked to the non-specific hyper-responsiveness that is typical of asthma. Alum is an adjuvant that helps to promote development of a Th2 phenotype by the immune system when BALB/c mice are exposed to an antigen (Zosky & Sly 2007).

3.7.2 Protocol for inducing airway resistance by challenging with methacholine.

Inhalation with methacholine will induce symptoms like asphyxia convulsions resembling bronchial asthma in BALB/c mice(Patil & Ninave 2016).
Bronchodilators and anti-inflammatory drugs can delay the occurrence of these symptoms (Okechukwu & Ekeuku 2012).
At 1 hour (to get a base line PCT for each group) and 97 hours after final ova inhalation BALB/c mice were put in an aerosol chamber, challenged with methacholine 2.5mg/ml, at a constant pressure of 40mm/Hg and constant flow rate of 0.5mls ml/minute from inbuilt nebulizer of methacholine chamber to cause airway resistance by aerosol method (Chang *et al.* 2005). Symptoms resembling bronchial asthma like dyspnea, asphyxia and convulsion were observed (Okechukwu & Ekeuku 2012)

3.7.3 Determination of time to developing airway resistance or Pre Convulsion Time (PCT)

Pre Convulsion Time (PCT) i.e. time from aerosol exposure to onset of dyspnea was measured and recorded. Group 2, 3, 4 and 5 were challenged with methacholine 1hours after last dose of OVA inhalation and PCT mean calculated and recorded.

T1-Negative control NS (group 2)

T2- Test Drug *W. ugandensis* 250mg/kg (group 3)

T3- Test Drug *W. ugandensis* 500mg/kg (group 4)

T4- Positive Control Budesonide 1mg/kg (group 5)

Animals were then moved to fresh air chambers.

Group 2 (negative control)-20 µg of ovalbumin with 2 mg of alum hydroxide dissolved in phosphate buffered saline (PBS), and a total of 200 µL was injected intraperitoneal on day 1 and 14, inhalation challenge was performed with 1% OVA in a 5.9 L Pyrex glass box for 30 min with a nebulizer on day 28, 29, and 30 *(Chang et al.* 2005). Saline was given 24 hours after last does of OVA inhalation as from day 32 at 0, 24 and 48, and 72 hours. 1 hour after last normal saline dose BALB/c mice were put in an aerosol chamber, challenged with methacholine 2.5mg/ml, at a constant pressure of 40mm/Hg and constant flow rate of 0.5mls/minute from inbuilt nebulizer of methacholine chamber to cause airway resistance by aerosol method. Pre convulsion time (PCT) in seconds i.e. time from aerosol exposure to onset of dyspnea was measured and recorded as T1^ value. A mean of the observed PCT was calculated.

Group 3- Extract of *W. ugandensis* was given 24 hours after last does of OVA inhalation at dose of 250mg/kg (Karani *et al*., 2013) as from day 32 at 0, 24, 48, and 72 hours. 1 hour after giving the last extract animals were put in an aerosol chamber, challenged with methacholine 2.5mg/ml, at a constant pressure of 40mm/Hg and constant flow rate of 0.5mls ml/minute from inbuilt nebulizer of methacholine chamber to cause airway resistance by aerosol method (Chang *et al.*, 2005). Symptoms resembling bronchial asthma like dyspnea, asphyxia and convulsion were

observed (Okechukwu & Ekeuku 2012). PCT was measured and recorded as T2^ value. Animals were moved to fresh air chambers immediately the PCT was measured.

Group 4 – Experiment was done as per the group 3 protocol but dose of *W. ugandensis* was increased to 500mg/kg. PCT was recorded as T3^.

Group 5- positive control. Animals were given budesonide 1mg/kg oral (Birrell *et al*., 2006) on day 32,33,34 at 0, 24, 48, and 72hours. 1 hour after giving the last dose of budesonide animals were put in an aerosol chamber, challenged with methacholine 2.5mg/ml, at a constant pressure of 40mm/Hg and constant flow rate of 0.5mls ml/minute from inbuilt nebulizer of methacholine chamber to cause airway resistance by aerosol method. PTC was measured and recorded as T4^ value. Animals were moved to fresh air chambers immediately the PCT was measured.

Percentage Protection = (T1^ - T1/T1) X 100; (T2^-T2/T2) X100; (T3^-T3/T3) X100; (T4^-T4/T4) X100

Where,

T1 = the mean of PCT for negative control using NS.

T2 = the mean of PCT after administration of *W. ugandensis* 250mg/kg at 0, 24, 48, and 72 hours.

T3 = the mean of PCT after administration of *W. ugandensis* 500mg/kg at 0, 24, 48 and 72 hours.

T4= the mean of PCT for positive control using budesonide.

Animals that with stood exposure to methacholine for 15 min were considered to be protected

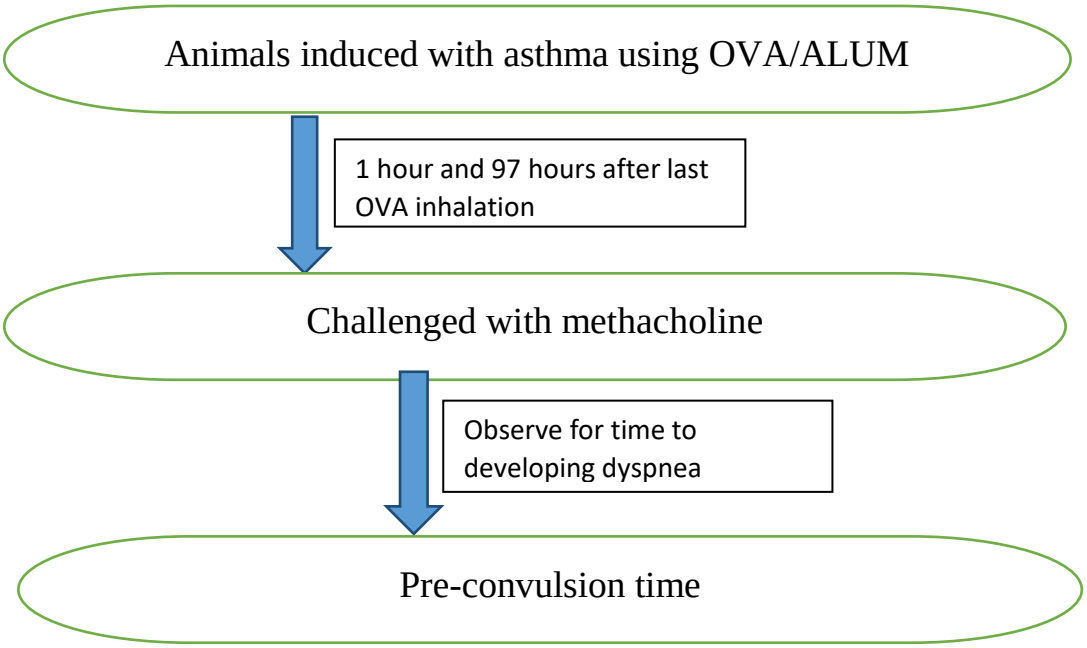

Figure 3.4 Flow chart for determination of airway resistance by measuring PCT for animals induced with asthma then challenged with methacholine.

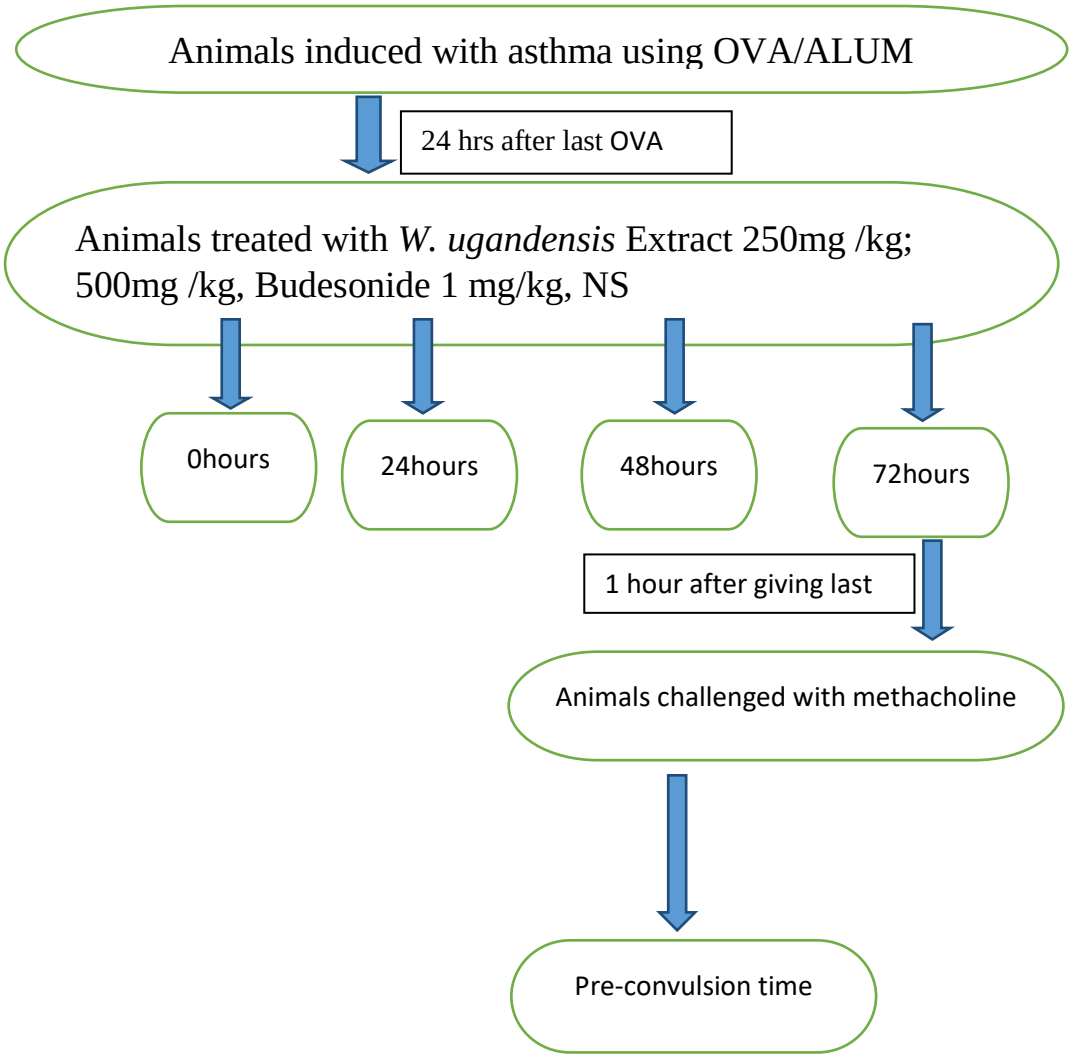

Figure 3.5 Flow chart for determination of time to developing airway resistance by measuring PCT for animals induced with asthma treated with *W. ugandensis* and Budesonide then challenged with methacholine.

3.8 Protocol for obtaining Broncho Alveolar Lavage Fluid

Animals were euthanized using CO_2 inhalation in a CO_2 chamber then put in the biosafety cabinet and were dampened with 70% ethanol. The animals were then placed front side up on styrofoam panel and fixed the arms and legs with needles. An incision was made on the skin from abdomen to neck and thoracic cage and neck was exposed. Muscles around the neck were removed to expose the trachea, the ribs were

cut to expose the lungs and the heart. A 22 G × 1" Exel Safelet Catheter was inserted into the trachea and tied firmly with a nylon string together with the trachea to avoid movement. 0.8ml of PBS was slowly injected and aspirated 4 times and recovered lavage fluid stored in 1.5 ml Eppendorf (EP) tubes on ice. BALF was then centrifuged at 800 × revolutions for 4 minutes. The supernatant was then put in a new 5ml tube at 4 °C s and processed for Nitric Oxide levels immediately. The residue was used for cell count in BALF. This procedure was repeated and samples were pooled for each animal.

3.8.1 Determination of inflammatory cell levels (Eosinophil, neutrophils, basophils and macrophages) in BALF for group 1, 2, 3, 4, and 5.

Protocol for slide preparation and cell count

BALF collected was put in centrifuge tubes and spun down for 4 minutes at 800 RPM, after which the supernatant was separated. Hemocytometer analysis was performed for total cell count. Using a WBC pipette BAL centrifuged fluid was sucked up to 0.5 mark. Diluted with Turk's fluid up to 11^{th} mark. Mixed the content in the WBC bulb for 3 minutes. Turk's fluid contains Glacial acetic acid 1.5mls for distribution and dissolution of red blood cells and fixes WBC, 1.5mls Gentian violet 1% for staining WBC diluted in 100mls of distilled water. Discarded the first two drops from the pipette and run the mixture under hemocytometer cover slip carefully. Allowed the corpuscles to settle for 4 minutes. Counted cell under light microscope at 100X objective. Four- part differential counts on 200 cells per slide was performed following standard morphological criteria and the percentage of eosinophils, lymphocytes, macrophages cells and neutrophils was determined.1-2 drops of the cell suspension was placed on a slide in the central area spread to form a thin and even film with a glass spreader. The slide was fixed with methanol and air dried on the bench for 3 minutes. The cells were stained with methylene blue/eosin; two drops of methylene blue (1%) were added to the slides and left on for 2 min, after which the excess stains were removed by placing a piece of paper towel on the stain briefly (1 second). Subsequently, two drops of 1% eosin were added to the slides and incubated

for 2min at room temperature. The slide was rinsed briefly with little amounts of tap water, after which one small drop of mounting medium was added to the slide and covered with a coverslip. The methylene blue stain makes nuclei more visible although it stains both nuclei and cytoplasm; the eosin mainly colors the cytoplasm and cell membranes (Hu *et al*., 2015) .

Total cells per micrometer volume = counted cells per surface area(mm^2)X depth of the chamber.

BALF smears were stained with May-Grünwald Giemsa stain. Cell count done under Nikon Optiphot light microscope at a total magnification of 100x.

3.8.2 Protocol for measuring Nitric Oxide levels (Griess Reagent protocol 1879) in BALF for group 1, 2, 3, 4, and 5.

Measurement of non-volatile Nitrite

NO level was determined using Griess reagent. Briefly 0.5 ml aliquots of each cell-free supernatant were added to an equal volume of Griess reagent (a freshly prepared 1:1 mixture of 1% sulfanilamide (Sigma Chemical Co., St Louis, MO) in 5%H3PO4 and 1% N(ÿ1-napthyl)-ethylenediamine dihydrochloride (NED)(Sigma) in distilled water).

Sulfanilamide Solution and NED Solution were allowed to equilibrate to room temperature for 15–30 minutes. 50µl of each experimental sample was added to wells in triplicate, 50µl of the1% sulfanilamide (Sigma Chemical Co., St Louis, MO) was then added to all experimental samples and wells containing the dilution series for the Nitrite Standard reference curve. Samples were incubated for 5–10 minutes at room temperature and protected from light. Using a multichannel pipette, dispensed 50µl of the NED solution to all wells and incubated at room temperature for 5–10 minutes, protected from light. A purple/magenta color was observed in approximately 5 minutes. Absorbance was measured within 30 minutes in a plate reader at 520nm Wavelength.

To determine Nitrite Concentrations in Experimental Sample a Nitrite Standard reference curve was generated using 10mM $NaNO_2$ as a standard. The average absorbance value of each concentration of the Nitrite Standard was plotted as a function of "Y" with nitrite concentration as a function of "X". Average absorbance value of each experimental group was determined and the concentration determined by comparison to the Nitrite Standard reference curve (Váradi *et al.*, 2019).

3.9 Histopathology of the lung tissue for determination of bronchiole inflammatory area for group 1, 2, 3, 4, and 5.

3.9.1 Harvesting of the lungs and staining

Animals were euthanized using CO_2 inhalation in a CO_2 chamber then put in the biosafety cabinet and were dampened with 70% ethanol. The animals were then placed front side up on styrofoam panel and fix the arms and legs with needles. An incision was made in the skin from abdomen to neck and thoracic cage and neck was exposed. The intrathoracic organs were inspected for gross changes and the lungs dissected out one after the other. Dissection of the lungs was done at the hila upon its identification. The harvested lungs were embedded in paraffin wax (Paraplast©) then placed on an electric cold plate for cooling. After 24hours, the tissue blocks were oriented along their long axis on the Medimeas© microtome with the microtome set at 5 micrometer thick sections.

The achieved ribbon upon sectioning was placed in a water bath at 37^0C and fishing done on glass slides.

Leitz sledge microtome was used to cut longitudinal thin sections of 5µm thick, floated in water at 37^0 then stuck onto glass slides using egg albumin, applied as thin film with a micro-dropper. Four slides in each subgroup selected with systematic random sampling was then dried in an oven at 37^0 for 24 hours then stained with Haematoxyline and eosin to demonstrate the bronchiole inflammation area.

3.9.3 Staining Method for Histology

The lung sections were stained using Haematoxyline and eosin (H.E) as described by (Cardiff *et al.* 2014)

Procedure

1. The glass slides that hold the paraffin sections of the lung was placed in staining racks. The paraffin from the samples was cleared in three dips of xylene for 2 minutes per change.

2. Hydration of the lungs samples was done as follows.

   i. Transferring the slides through three changes of 100% ethanol for 2 minute per change.
   ii. Transferring to 95% ethanol for 2 minutes.
   iii. Transferring to 70% ethanol for 2 minutes.
   iv. The slides were rinsed in running tap water at room temperature for at least 2 minutes.

3. The lung samples were dipped in haematoxyline solution for 3 minutes.

4. The slides were placed under running tap water at room temperature for at least 5 min.

5. The samples were stained in working eosin Y solution for 2 minutes.

6. Dehydration of the samples was done as follows.

   i. Dipping the slides in 70% ethanol about 20 times.
   ii. Transferring to 95% ethanol for 2 minutes.
   iii. Transfer through two changes of 100% ethanol for 2 minutes per change.

7. The samples were cleared in three changes of xylene for 2 minutes per change.

8. A drop of Permount was placed over the tissue on each slide and a coverslip was added.

9. The slides were examined under light microscope, in powered fields of x40, x100 and x400.

3.9.4 Determination of bronchiole wall thickness in BALB/c mice

The bronchial wall thickness was determined using Image J v1.50i©, Java 1.6.0_20© image analysis open source software (Schindelin *et al.,* 2015). The Heamatoxyline and Eosin stained slides were selected using systematic random sampling technique and the reviewer was blinded to the treatments. Tissue sections to be stained were picked based on the K^{th} value (skip) of 10 calculated as follows:

Number of sections made from each lung (N) =40

Number of desired sections from each lung (n) =4

K^{th} or skip value=N/n

=40/4

=10

Every 10^{th} section was picked beginning from section number 4 upon doing simple random sampling to determine the beginning section.

Upon staining with Haematoxylin and Eosin, photomicrographs were taken using the Labomed light binocular microscope Lx 400© with the assistance of the PixelPro© image acquisition and analysis software version 2.6.0.0 by Labo American Inc. Photomicrographs were taken at a total magnification of x40 with a field of view of 0.5mm (10x/20, total magnification of 40). Upon capturing, selected

photomicrographs were uploaded in Image J v1.50i© stereology analysis software for bronchiole wall measurements as follows:

From the main menu bar, the "file tab" was clicked and from the drop down menu the "open" tab was clicked so as to import the stored image to the software image analysis window then from the main menu bar the "analyze" tab was clicked and from the drop down menu in a sequential manner "select measurements" "select area" "area fraction" "OK" were clicked. From here, the scale was set by clicking on "analyze" from the main menu bar and set (distance in pixels=264.5833, known distance=1µm, pixel aspect ratio=1, unit of length=µm then click OK). To calculate the bronchial wall thickness, click on the line tool and drag it over the bronchiole wall. Click on analyze button then from the drop-down menu click on measure upon which a pop-up window will appear with the length measurements. Repeat the above steps to obtain 6 measurements and get the average bronchiole thickness.

3.10 Demonstration of Beta 2 and M3 receptor activity of *W. ugandensis*

Rabbits weighing 2600-3000gms were sacrificed and the trachea strips were prepared. The trachea strips were then mounted under a resting tension of 2 g in an organ bath containing 25 ml of Krebs–Heinsleit solution (KH, pH 7.4) at 37 °C and perfused with a gas mixture (O2 95%, CO2 5%). The trachea strips were left for 1.5 hours and KH solution was changed at 15 min intervals before drugs (propranolol and acetylcholine) and *W. ugandensis* extract was accumulatively added to the bath. The maximum effects of *W. ugandensis* produced in absence and in presence of each blocker was recorded and dose–response curves were made (Gan, Wang, Cheng, & Pan 2003). The Krebs solution used had the following composition *NaCl (6.92), KCl (0.35), $MgSO_4 \cdot 7H2O$ (0.15), $NaHCO_3$ (2.1), KH_2PO_4 (0.16), glucose (2.0) and $CaCl_2$ (0.24)*

3.10.1 Demonstration of *W. ugandensis* activity on isolated rabbit trachea strips

Rabbits weighing 2600-3000gms were sacrificed and the trachea strips were prepared. The trachea strips were mounted under a resting tension of 2 g in an organ bath containing 25 ml of Krebs–Heinsleit solution (KH, pH 7.4) at 37 °C and perfused

with a gas mixture (O2 95%, CO2 5%).The trachea strips were left for 1.5 h and KH solution was changed at 15 min intervals before *W. ugandensis* extract was accumulatively added to the bath. The maximum effects produced by *W. ugandensis* was taken as 100 % and dose–response curves was made. The experiments were repeated in triplicate.

3.10.2 Demonstration of Beta 2 receptor activity of *W. ugandensis* on isolated rabbit trachea strip using acetylcholine as a blocker

The isolated rabbit trachea strips were pre contracted with acetylcholine (3mM). The maximum contraction induced by acetylcholine was taken as 100%. *W. ugandensis* extract was added accumulatively and the cumulative concentration–response curves for *W. ugandensis* was established. The contact time was 20 min, enough to produce a steady level of contraction. EC50, the concentration needed to decrease the tone of trachea strips by 50%, was obtained from the dose–response curves to express the relaxant effects of *W. ugandensis.* The same was repeated using salbutamol as a positive control. The two dose response curves were compared. The experiments were repeated in triplicate.

3.10.3 Demonstration of the M3 receptor effect of *W. ugandensis* on isolated rabbit trachea using propranolol as a blocker

The isolated rabbit trachea strips were pre-contracted with 5mM of histamine. The maximum contraction induced by histamine was taken as 100%. Following the addition of beta 2 adrenoceptor antagonist, Propranolol 0.2 mM, *W. ugandensis* extract was added into the organ bath cumulatively, and the maximum relaxation produced by *W. ugandensis* was taken, the dose–response curve of *W. ugandensis* in the presence of propranolol was established. The same was repeated using atropine as a positive control, the two dose response curves were compared. The experiments were repeated in triplicate.

Figure 3.6 Organ bath with trachea strip hanged

3.11 Statistical analysis

The collected clinical data was recorded and cleaned using Microsoft Excel. The data was then keyed into Statistical Product for Service Solutions (SPSS) software version 25.0 for statistical analysis. Data analysis involved both descriptive and inferential

statistics whereby for descriptive statistics, means and standard error of mean were used. Frequencies were generated for categorical data. Inferential statistics were used to test for association and test for mean differences among variables under the study. An independent t-test and a One-way Analysis of Variance (ANOVA) were used to compare the means between and among the groups while Pearson's correlation analysis was used to test for association between variables that were continuous in nature. The research assumed a 95% level of confidence (α=0.05) and the analyzed data was presented in tables, charts and graphs.

3.12 Ethical consideration

Ethical approval was sought from the Animal Ethics Committee for Care and Use of laboratory animals of Jomo Kenyatta University of Agriculture and Technology. Animal experimentation was carried out in a Level 2 Biosafety laboratory. All activities during the studies conformed to accepted principles for laboratory animal use and care (EU directive of 1986: 86/609/EEC). All the technical team observed institutional biosafety guidelines for protection of personnel and laboratory. Animals were handled in a humane manner and were monitored twice daily for any ill health related to experimental interventions. Sacrificing of the animals was after euthanizing using an overdose of concentrated carbon dioxide.

Reduction: The resource equation method was employed to reach at the desired scientific sample size that led to reliable, valid and a robust research.

Refinement: paper shredding for enrichment, proper and standard housing was provided. A stand-by veterinary was available to identify and take care of sick animals. For humane end point, all animals were to be moved to fresh air chamber immediately they develop dyspnoea. Animals were euthanized on day 35 of the experiment. All animals having laboured breathing, acute weight loss of more than 20% of baseline body weight and inability to ambulate were to be removed from the experimental group and euthanized.

CHAPTER FOUR

RESULTS

4.1 Qualitative and quantitative phytochemical screening

4.1.1 Yields of *W. ugandensis* extract from collected bark

One thousand two hundred milligrams of ground power were recovered from five kilograms of the bark collected from the forest, this was 24% yield.

4.1.2 Quantitative phytochemical screening of aqueous and ethanol extracts

An independent t-test was performed to compare ethanol and water extractions on FLAg/100g. The results in Table 4.1 shows that FLAg/100g in the sample which received the ethanol intervention (M = 0.339, SE = 0.001) compared to the one which received water (M = 0.056, SE = 0.003) was significantly different, t (4) =88.041, p <0.001. Tannins were found to be significantly different in water (M=3.152, SE = 0.050) compared to ethanol (M= 0.892, SE = 0.060), t (4) = 37.337, p <0.001. Phenols were found to be significantly higher in water (M = 2.868, SE = 0.509) compared to ethanol (M = 0.406, SE = 0.020), t (4) = 4.835, p = 0.040. The anti-oxidant activity was better with ethanol extract IC 50 (0.66 mg/ml) compared to water extract at 0.8 mg/ml. Ascorbic acid was used as a standard, IC 50 (0.52mg/ml) Fig 4.1.

Table 4.1 Quantitative comparison of water and ethanol bark extract of the *W. ugandensis*

Parameter	FLA	Tannins	Phenols
Water	0.056±0.003	3.152±0.050	2.868±0.509
Ethanol	0.339±0.001	0.892±0.060	0.406±0.020
t-statistic (4)	-88.041	37.337	4.835
P-value	<0.001*	<0.001*	0.040*

*Key*denotes statistical significance at p<0.05*

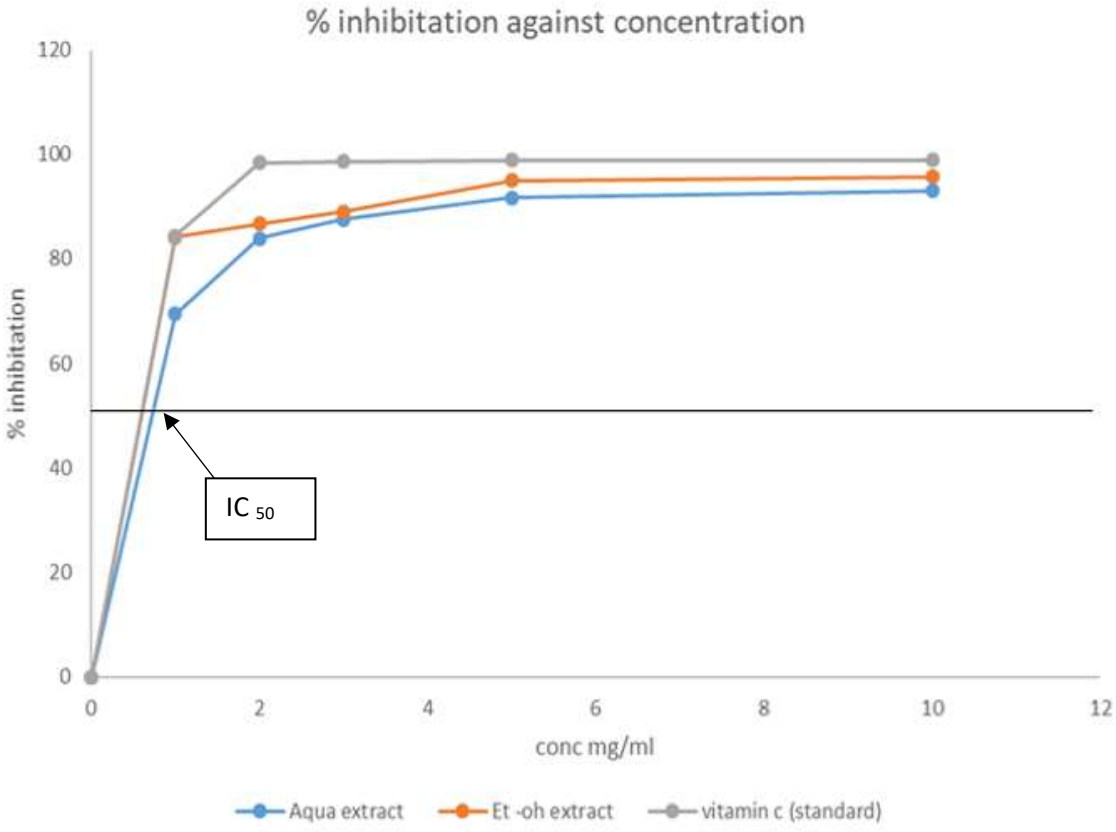

Figure 4.1 Antioxidant activity of *W. ugandensis.*

4.1.3 Qualitative phytochemical Screening of aqueous and ethanol extracts

Qualitative phytochemicals were determined by screening test as per the protocols. Saponins and terpenoids has similar results in aqueous and ethanol extract. Alkaloids, steroids and terpenoids were higher in aqueous extract as compared to ethanol extract. Cardiac glycosides had undetectable levels in both aqueous and ethanol extract.

Table 4.2 Qualitative comparison of water and ethanol extraction of *W. ugandensis* bark extract

Phytochemical	Aqua extract	EtOH extract
Saponins	+++	+++
Anthraquinones	+	++
Terpenoids	+++	+++
Alkaloids	++	+++
Steroids	+	Negative
Cardiac glycosides	Negative	Negative

Key +++ = Highly present, ++ = moderately present, += mildly present, Negative =not detected

4.2 Acute oral toxicity of *W. ugandensis*

4.2.1 Mortality rate and behavioral observations

The *W. ugandensis* aqueous bark extract was administered with the aim of establishing a standard dose that can cause toxicity and marked distress to the animals. There was no mortality observed within the first 48 hours and during the entire 14 days of observation in all groups administered with aqueous bark extract of *W. ugandensis.* General behaviour of each animal was observed for the first 30 minutes,1 hour, 4 hours, 24 hours, 48 hours and then daily for entire 14 days. The following general parameters were normal for the entire period of observation; fur appearance, alertness, muscle tone on hind limbs, pain, feeding, activity, respiratory rate apart from overcrowding and inactivity that was observed at the of dose of 5000mg/kg but resolved within 24 hours' (Table 4.3).

Table 4.3 Acute toxicity of *W. ugandensis* as per behavioral observation and mortality rate

Experiment	Doses (Mg)	Observation in hours							
		Immediate	½ hour	1 hour	4 hours	24 hours	48 hours	Mortality	Mortality rate (%)
Phase I (B.E in 5% DMSO)	10	Normal activity	Normal activity	Normal activity	Normal Activity	Normal activity	Normal activity	0/3	0
	100	Normal activity	Normal activity	Normal activity	Normal Activity	Normal activity	Normal activity	0/3	0
	1000	Normal activity	Normal activity	Normal activity	Normal Activity	Normal activity	Normal activity	0/3	0
Control [Distilled water + 5%DMSO]	2mls	Normal activity	Normal activity	Normal activity	Normal Activity	Normal activity	Normal activity	0/3	0
Phase II (B.E in 5% DMSO)	1600	Normal activity	Normal activity	Normal activity	Normal Activity	Normal activity	Normal activity	0/1	0
	2900	Normal activity	Normal activity	Normal activity	Normal activity	Normal activity	Normal activity	0/1	0
	5000	Over crowding	Over crowding	Over crowding	Over Crowding	Normal activity	Normal activity	0/1	0

Key: DMSO - Dimethyl Sulphoxide, B.E- bark extract of W. ugandensis

4.3 Pre Convulsion Time (PCT)

ANOVA and Tukey post hoc test was done to find out if PCT was significantly different between groups before intervention and between groups after intervention. Pre convulsion time was not significantly different across all groups before intervention ($p>0.05$). After intervention PCT was significantly lower in negative control (576.67±39.43a) in comparison to other groups Table 4.4. T- test was done to find out whether PCT was significantly different before and after the interventions in each group. There was a significant difference in each group before and after interventions ($p <0.05$) apart from negative control whose p value was >0.05 Table 4.5. Percent inhibition to developing airway resistance was significantly lower in 250mg/kg (53.52±7.30a) when compared to positive control (82.03±1.29) and

500mg/kg intervention. However, positive control was not significantly different from 500mg/kg intervention (88.02±1.72) Table 4.6.

Table 4.4 Comparison of PCT between groups before and after intervention

Parameter	Negative control (0.0)	Positive Control (1.0 mg/kg)	250 mg/kg	500 mg/kg	F value	P-value
PCT-Before (Base line)	599.67±26.12[a]	604.33±3.28[a]	611.67±6.01[a]	602.00±9.87[a]	0.986	0.447
PCT-After	576.67±39.43[a]	1100.0±2.31[c]	938.33±38.19[b]	1188.33±21.79[c]	102.76	0.000*

*Notes The means, followed by the same letter in a row are not statistically different at (P>0.05) using one-way ANOVA. with Tukey test on post-hoc t-tests. * indicates significance (p<0.05).*

Table 4.5 Comparison of PCT in each group before and after intervention

Conc.	Group	Mean	df	t-stat	p-value
250mg/kg	Before	611.67±6.00	4	-8.449	0.001
	After	938.33±38.19			
500mg/kg	Before	602.00±9.87	4	-23.259	0.000
	After	1188.33±21.79			
Positive	Before	604.33±3.28	4	-123.489	0.000
	After	1100.00±2.31			
Negative	Before	599.67±26.12	4	0.486	0.652
	After	576.67±39.43			

Key: df. degrees of freedom

Table 4.6 Percent inhibition to developing airway resistance

Parameter	Positive Control (1 mg/kg)	250 mg/kg	500 mg/kg	F (2,6) value	P-value
%Inhibition	82.03±1.29b	53.52±7.30a	88.02±1.72b	17.61	0.003*

*Notes The means, followed by the same letter in a row are not statistically different at (P>0.05) using one-way ANOVA. with Tukey test on post-hoc t-tests. * indicates significance (p<0.05).*

4.4 The differential inflammatory cell counts in BALF

Inflammatory cells were compared across the different interventions (-ve control, normal, +ve control, 250mg/kg and 500 mg/kg). This was achieved through Analysis of variance test (ANOVA) with Tukey post hoc test. The results showed that total cells were significantly high in the negative control (619.67±15.50) compared to the rest of the treatments which were significantly lower, $F = 998.39$, $p \leq 0.001$. Total cells under the normal (74.00±3.06), positive control (44.00±3.00), 250mg/kg (65.33±6.64) and 500 mg/kg (46.67±3.84) were not significantly different. The number of lymphocytes decreased significantly from 3.33±0.88 in the negative control to 0.67±0.33 when the positive control was applied and 0.33±0.33 when 500 mg/kg intervention was applied, $F = 6.23$, $p = 0.009$. However, the number of lymphocytes in the negative control did not vary significantly from normal and 250 mg/kg interventions.

Neutrophils were compared across the various interventions and found to drop significantly from 15.33±1.76 $x10^3$/µl under the negative control to 0.00±0.00 under the normal, 3.67±0.33 $x10^3$/µl in the 250 mg/kg and 1.67±0.33 $x10^3$/µl in the 500 mg/kg, $F = 54.02$, $p = <0.001$, but was insignificantly different from the positive control.

Eosinophils were also compared across the different interventions using one-way ANOVA and Tukey post hoc t-test. Eosinophils were found to be significantly high in the negative control 494.33±12.01 $x10^3$/µl and dropped significantly when a positive control 8.00±1.16 $x10^3$/µl was applied, base line eosinophils were 18.00±2.65 $x10^3$/µl 250 mg/kg 11.33±2.40 $x10^3$/µl and 500 mg/kg 7.33±2.40 $x10^3$/µl, $F = 1424.02$, $p = <0.001$. Lastly, Macrophages were compared across different interventions using one-way ANOVA with Tukey post hoc test. They were found to be significantly higher in the negative control 106.67±4.67 $x10^3$/µl compared to positive control 34.33±2.73 $x10^3$/µl, normal 53.67±1.20 $x10^3$/µl, 250 mg/kg 49.00±4.04 $x10^3$/µl and 500 mg/kg 37.33±4.10 $x10^3$/µl, $F = 67.41$, <0.001. Again,

macrophage in the positive control were found to be significantly different from the normal.

Total cells in 250 mg/kg (M=65.33, SE = 6.64) $x10^3$/µl were found to be higher though not significantly different from those in 500 mg/kg (M = 46.67, SE = 3.84) $x10^3$/µl, t (4) = 2.432, p = 0.072. Lymphocytes were also not significantly different in 250mg/kg (M = 1.33, SE = 0.33) $x10^3$/µl compared to the 500mg/kg, t (4) = 1.33, p = 0.33. However, Neutrophils in 250mg/kg were significantly higher (M = 3.67, SE = 1.67) $x10^3$/µl compared to those in 500 mg/kg (M = 1.67, SE = 0.33) $x10^3$/µl, t (4) = 4.243, p = 0.013. An independent t-test was used to compare eosinophils of the samples for 250mg/kg and 500mg/kg. Although there were more eosinophils in 250mg/kg (M=11.33, SD = 2.40) compared to 500mg/kg (M7.3, SD2.40), they were not statistically and significantly different, t (4) = 1.177, p = 0.305 Table 4.7. The results again show an insignificant difference between macrophages in 250mg/kg (M = 49.00, SE = 4.04) and 500 mg/kg (M = 37.33, SE 4.10), t (4) = 2.027, p = 0.113.

Table 4.7 Inflammatory cells in different groups

Parameter	Baseline	Negative control (0.0)	Positive Control (1mg/kg)	250 mg/kg	500 mg/kg	F value	P-Value
Total cells	74.00±3.06^{b}	619.67±15.50^{a}	44.00±3.00^{b}	65.33±6.64^{b}	46.67±3.84^{b}	998.39	<0.001*
Lymphocytes	2.33±0.33^{ab}	3.33±0.88^{a}	0.67±0.33^{b}	1.33±0.33^{ab}	0.33±0.33^{b}	6.23	0.009*
Neutrophils	0.00±0.00^{b}	15.33±1.76^{a}	16.00±0.58^{a}	3.67±0.33^{b}	1.67±0.33^{b}	54.02	>6.001
Eosinophils	18.00±2.65^{b}	494.33±12.01^{a}	8.00±1.16^{b}	11.33±2.40^{b}	7.33±2.40^{b}	1424.02	<0.001*
Macrophages	53.67±1.20^{b}	106.67±4.67^{a}	34.33±2.73^{c}	49.00±4.04^{bc}	37.33±4.10^{bc}	67.41	<0.001*

*Notes: The means, followed by the same letter in a row are not statistically different at (P<0.05) using one way ANOVA.with Tukey test on post-hoc t-tests. * indicates significance (p<0.05). Cell count done $X10^3$/µl*

4.5 Nitric Oxide levels in BALF

The results show that the Nitric oxide levels were significantly different in the Normal (0.301±0.011), negative control (0.513±0.005), Positive Control (0.082±0.003), 250 mg/kg (0.114±0.00) and 500 mg/kg (-0.013±0.003), F = 1209.17, p = <0.001. However, this was not significantly different in positive control and 500mg/kg interventions table 4.8. An independent t-test was used to compare Nitric Oxide levels

in 250 mg/kg and 500 mg/kg. The results show in 500mg/kg (-0.0127±0.00) it was significantly lower compared to 250 mg/kg, t (4) = 2.027, p = 0.113 table 4.9

Table 4.8 Comparison of NO levels across all groups

Parameter	Baseline	Negative control (0.0)	Positive Control (1 mg/kg)	250 mg/kg	500 mg/kg	F value	P-value
Nitric oxide	0.301±0.011[b]	0.513±0.005[a]	0.082±0.003[d]	0.114±0.00[c]	-0.013±0.003[d]	1209.17	<0.001*

*Notes The means, followed by the same letter in a row are not statistically different at (P>0.05) using one-way ANOVA. with Tukey test on post-hoc t-tests. * indicates significance (p<0.05).*

Table 4.9 T- test results comparing NO levels with different doses of *W. ugandensis*

Parameter	Nitric Oxide
250 mg/kg	0.114±0.00
500 mg/kg	-0.0127±0.00
t-statistic (4)	40.00
p-value	<0.001*

*Key: * indicates statistical significance (p<0.05).*

Table 4.10 Correlation of NO, PCT, eosinophils, and bronchiole wall thickness

		Nitric oxide	PCT	Eosinophils	Thickness
Nitric oxide	Pearson Correlation	1			
	Sig. (2-tailed)				
	N	15			
PCT	Pearson Correlation	-.988**	1		
	Sig. (2-tailed)	.000			
	N	12	12		
Eosinophils	Pearson Correlation	.847**	-.961**	1	
	Sig. (2-tailed)	.000	.000		
	N	15	12	15	
Bronchiole wall thickness	Pearson Correlation	.870**	-.904**	.819**	1
	Sig. (2-tailed)	.000	.000	.001	
	N	12	12	12	12

Notes: The results show a strong negative significant correlation between Nitric Oxide and PCT, r = -0.988, p=0.000. Again, there exist a strong negative significant correlation between Eosinophils and PCT, r = -0.961, p=0.000. There was a strong positive significant correlation between Nitric Oxide and Eosinophils, r = 0.847, p=0.000. Again, there exist a strong positive significant correlation between eosinophils and bronchiole wall thickness r = 0.819

4.6 Bronchiole wall thickness of BALB/c mice

Bronchiole wall thickness was significantly higher in the negative control (5.21±0.237) compared to 250 mg/kg (3.10±0.519), 500 mg/kg (0.633±0.292) and positive control (0.587±0.439), F (3,8) = 32.68, p=0.000. However, bronchiole wall

thickness was not significantly different in 500 mg/kg and positive control interventions.

Table 4.11 Bronchiole wall thickness per different groups

Parameter	Negative Control	250 mg/kg	500 mg/kg	Positive control	F (3,8) value	P-value
Bronchiole wall thickness	5.21±0.237[a]	3.10±0.519[b]	0.633±0.292[c]	0.587±0.439[c]	32.68	0.000*

*Notes: The means, followed by the same letter in a row are not statistically different at (P<0.05) using one way ANOVA. with Tukey test on post-hoc t-tests. * indicates significance (p<0.05).*

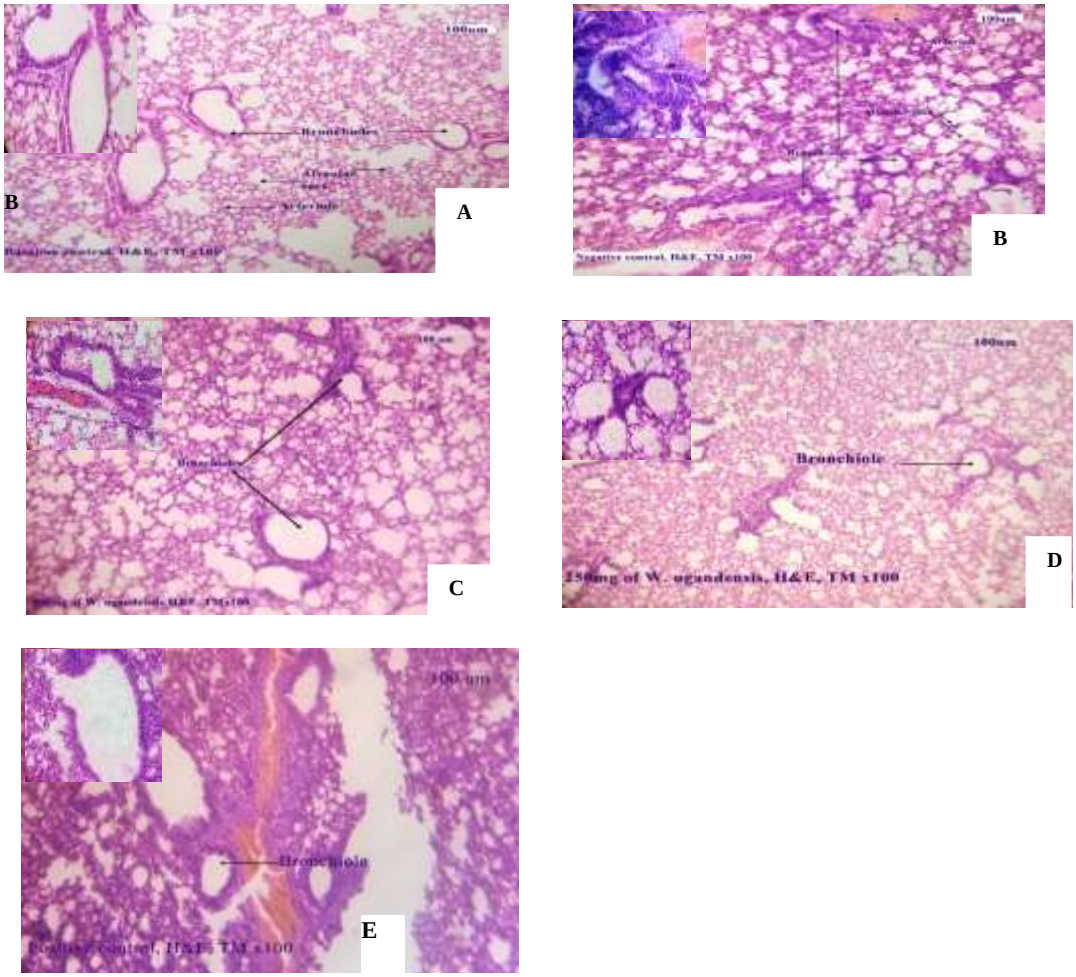

Figure 4.2 Photomicrographs of the lung tissue (A, B, C, D) for the different interventions

A: Baseline Control mouse lung tissue. There is normal cell density in the lamina propria of the bronchioles. Top left photomicrograph insert shows a normal bronchiole cell density in the lamina propria bronchiole as well as wall thickness, TM x400.

B: Negative control mouse lung tissue with increased inflammatory cells in the lamina propria of the terminal bronchioles. Top left photomicrograph insert shows a bronchiole with high number of inflammatory cells in the lamina propria as well as an increase in wall thickness, TM x400.

C: Lung tissue in the treatment group (500mg). There are few inflammatory cells in the lamina propria of the terminal bronchioles. Top left photomicrograph insert shows a bronchiole with high number of inflammatory cells in the lamina propria as well as an increase in lamina propria thickness, TM x400.

D: Lung tissue in the treat group (250mg). There are few inflammatory cells in the lamina propria of the terminal bronchioles. Top left photomicrograph insert shows a bronchiole with high number of inflammatory cells in the lamina propria as well as an increase in lamina propria thickness, TM x400.

E: Lung tissue in the treat group (500mg). There is mildly increased inflammatory cells in the lamina propria of the terminal bronchioles. Top left photomicrograph insert shows a bronchiole with high number of inflammatory cells in the lamina propria as well as an increase in lamina propria thickness, TM x400

4.6 Receptor activity of *W. ugandensis*

The coefficient of determination is $R^2 = 0.8557$ indicating that 85.57% of any change that occurs in the relaxation of the extract,

Equation will be:

$y = 20.486x + 18.272$

Therefore, at Y=50, the X value will be

$50 = 20.486x + 18.272$

$20.486x = 50 – 18.272$

$X = 1.549$

This is in log format so we find the antilog of 1.549 to get the value of the extract which will be 4.706.

Therefore, at EC50, the value of the extract will be **4.706**.

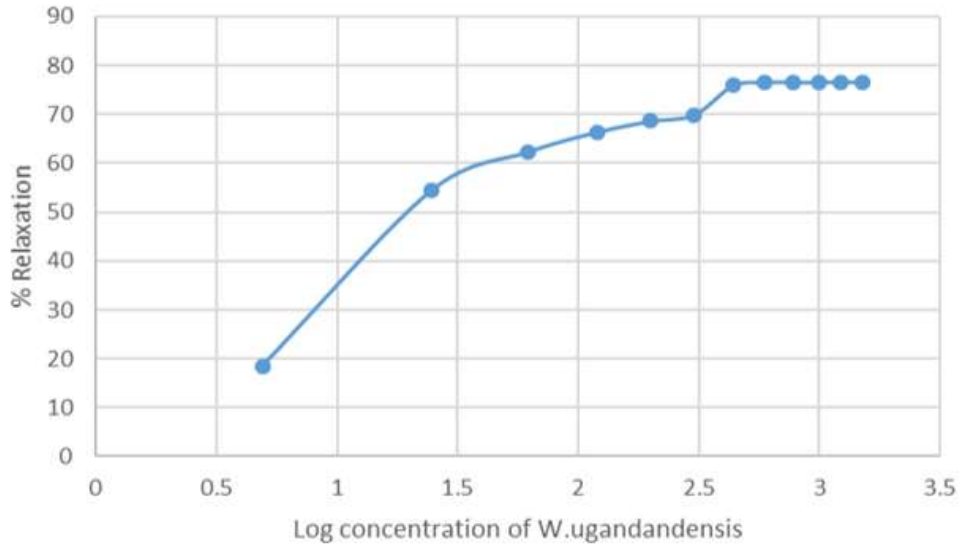

Figure 4.3 Dose response curve of relaxation effect of *W. ugandensis* on a resting isolated rabbit trachea strip.

To the right of fig 4.6 the coefficient of determination is $R^2 = 0.7578$ indicating that 75.78% of any change that occurs in the relaxation of *W. ugandensis* as a beta agonist, is explained by the extract.

Equation will be:

$y = 17.024x + 18.239$

Therefore, at Y=50, the X value will be

$50 = 17.024x + 18.239$

$17.024x = 50-18.239$

$X = 1.866.$

This is in log format so we find the antilog of 1.866 to get the value of the extract which will be 6.462.

Therefore, at EC_{50}, the value of the extract will be **6.462.**

Left of fig 4.6 the coefficient of determination is $R^2 = 0.9374$ indicating that 93.74% of any change that occurs in the Relaxation of Salbutamol, is explained by the extract.

Equation will be:

$y = 25.444x + 75.255$

Therefore, at Y=50, the X value will be

$50 = 25.444x + 75.255$

$25.444x = 50 -75.255$

$X = -0.9926$

This is in log format so we find the antilog of -0.9926 to get the value of salbutamol which will be 0.3706.

Therefore, at EC_{50}, the value of salbutamol will be **0.3706**

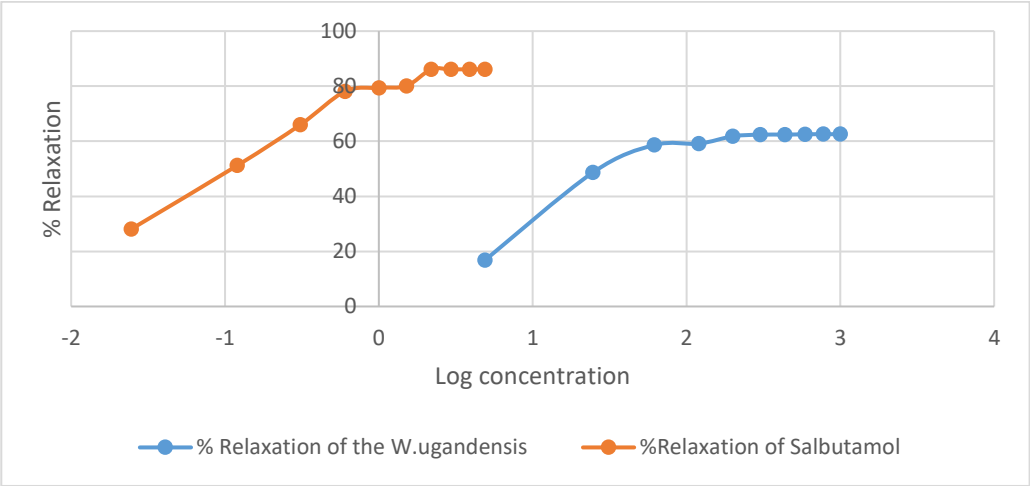

Figure 4.4 Dose response curve of relaxation effect of *W. ugandensis* on beta receptors compared to salbutamol as a positive control using isolated rabbit trachea

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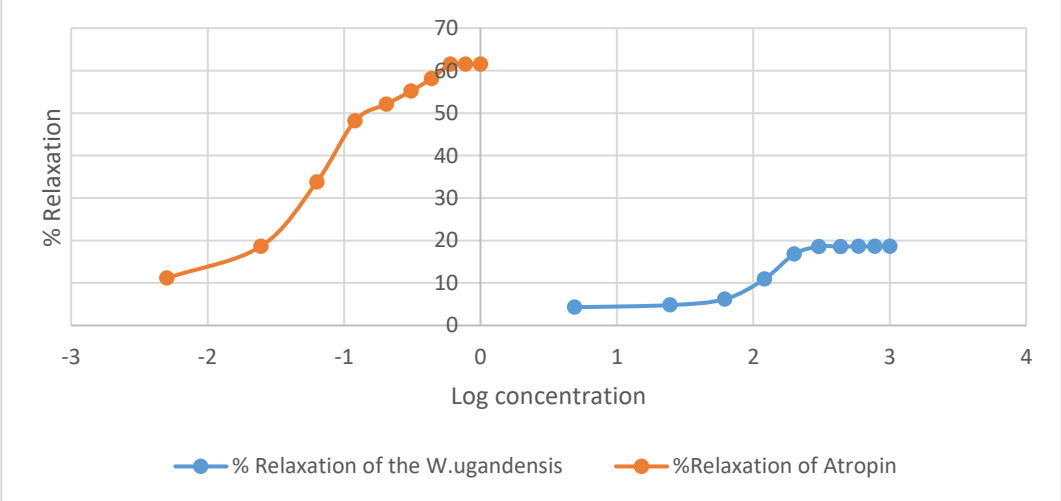

Figure 4.5 Dose response curve of relaxation effect of *W. ugandensis* on muscarinic receptors compared to Atropine as a positive control using isolated rabbit trachea

Fig 4.7 right, coefficient of determination is $R^2 = 0.859$ indicating that 85.9% of any change that occurs in the Relaxation of w. u as a M3 antagonist, is explained by the extract.

Equation will be:

$y = 8.0046x - 4.0352$

Therefore, at Y=50, the X value will be

$50 = 8.0046x - 4.0352$

$8.0046x = 50 + 4.0352$

$X = 6.75$

This is in log format so we find the antilog of 6.75 to get the value of the extract which will be 854.50.

Therefore, at EC_{50}, the value of the extract will be **854.50**.

Fig 4.7 left, the coefficient of determination is $R^2 = 0.9583$ indicating that 95.83% of any change that occurs in the Relaxation of Atropine, is explained by Atropine.

Equation will be:

$y = 24.849x + 65.834$

Therefore at Y=50, the X value will be

$50 = 24.849x + 65.834$

$24.849x = 50 - 65.834$

$X = - 0.6372$

This is in log format so we find the antilog of -0.6372 to get the value of Atropine which will be 0.5289.

Therefore, at EC_{50}, the value of Atropine will be **0.5289**.

4.6.1Testing significance difference of percent relation between the test drug and the positive controls.

Testing significance difference of the percent relaxation between the test drug *W. ugandensis* with Acetylcholine as a blocker and the positive control (salbutamol) was done using a student T test. The results showed there was a significant difference between relaxation of *W. ugandensis* as beta agonist (%) and %relaxation salbutamol since the P value (0.038) was less than 0.05. Salbutamol proved to have a better Beta agonist effects compared to *W. ugandensis* (Table 4.12)

Table 4.12 Comparison Beta receptors effects of *W. ugandensis* with salbutamol

	Relaxation of W. U as beta agonist (%)	**%relaxation salbutamol**
Mean	55.74	72.77
Variance	205.75	371.57
Observations	10	10
Df	18	
t-Stat	-2.23	
P-value	**0.038**	

Key: df. degrees of freedom

Testing significance difference of the percent relaxation between the test drug *W. ugandensis* with propranolol as a blocker and the positive controls (Atropine) was done using a student T test. The results showed there was a significant difference between relaxation of *W. ugandensis* as M3 antagonist and atropine since the P value (<0.001) was less than 0.05. Therefore, Atropine proved to have a better muscarinic (M3) antagonist effects compared to *W. ugandensis.*

Table 4.13 Comparison of muscarinic receptor effects of W. *ugandensis* with Atropine

	% Relaxation of w. u as a M 3 antagonist	**%Relaxation of Atropine**
Mean	13.60	46.15
Variance	40.12	345.25
Observations	10	10
Df	18	
test Statistic (t)	-5.24	
P-value	**<0.001**	

Key: df. degrees of freedom

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Water and ethanol extract phytochemicals of *W. ugandensis*.

Antioxidant activities of *W. ugandensis* ethanol and water bark extract was evaluated and ascorbic acid was used as a standard. Concentration of the extract that decrease the DPPH concentration by 50% (IC_{50}) was used to compare the antioxidant activity of the extract. Ethanol extract had an IC_{50} of 0.66mg/ml while water extract had IC_{50} of 0.8mg/ml. Comparing the anti-oxidant activity of ethanol and water extract to the standard whose IC_{50} was 0.52mg/ml, it was evident that ethanol extract had better antioxidant activity compared to water extract. Water proved to be a better solvent for extraction of phenols and tannins compared to ethanol solvent since the concentration for each of the phytochemicals was higher in water extract compared to ethanol. Ethanol performed better for flavonoids extraction by having 0.339 g/100g compared to water extract which was at 0.056 mg/100g. This agreed with a study done by Dharani, (2020) who concluded that *Warburgia ugandensis* has antioxidant phytochemicals such as flavonoids and phenols.

From the result it was evident that *W. ugandensis* bark extract of both water and ethanol is rich in saponin, antraquinone, alkaloids and short cardiac glycoside phytochemicals. This agreed with a research done by Abuto, Muchugi, & Machocho, (2018) whereby *W. ugandensis* bark was extracted using methanol and analyzed by gas chromatography-mass spectrometry (GC-MS) to determine the variations in the phytochemical profiles from different populations across the Kenyan Rift Valley, from this study they concluded that *W. ugandensis* has abundant levels of Sesquiterpenoids and low concentrations of other classes of compounds such as phenolics, phytosterols, tocopherols.

5.2 Acute toxicity

Upon administration of *W. ugandensis* to the BALB/c mice at different doses, no mortality was recorded across all doses though overcrowding and reduced activity was noted for the mice administered with 5000mg/kg body weight of the bark extract. Conversely; this behaviour was not observed in the other dose categories. From the current study the LD_{50} of *W. ugandesis* was found to be > 5000 mg/kg body weight *W. ugandensis* and thus classified as relatively harmless (Lorke, 1984). This agrees with a cytotoxicity study of *W. ugandensis* done by Karani *et.al* (2013) on Vero E6 cells using MTT assay which showed that it was safe for use as its CC_{50} was >250 ug/ml in reference to Rukunga and Simon classification of cytotoxicity and its LD_{50} was > 5000mg/kg. In another study done by Githinji, Edward, Irungu (2010) it showed insignificant toxicity levels of crude bark extract of *W. ugandensis* on BALB/c peritoneal macrophages whereby 3/100 macrophages died at a concentration of 1000ug/ml in comparison to the positive control (pentostam) that had a mean of 45/100 BALB/c peritoneal macrophages dead at the same concentration. Similarly; *W. ugandensis* was shown to be non-toxic to *Drosophila melanogaster* at acute exposure but toxic at chronic exposure (Ahmad *et al*., 2017). Bark extracts, leaves and young shoots of *W. ugandensis* have been used for ages with no adverse effects (Kokwaro, 2009).

5.3 Protective effects of *W. ugandensis* against development of airway resistance

Warbugia ugandensis was found to have a protective effect to developing airway resistance as evaluated by use of PCT and computed percent inhibition. The protective effect was dose dependent whereby animals that received 500mg/kg body weight of bark extract exhibited higher protection with percent inhibition of 88.02% as compared to 250mg/kg body weight intervention whose percent inhibition was 53.52%. Protective effect with 500mg/kg body weight of *W. ugandensis* intervention was comparable to positive control budesonide since the PCT and percent inhibition to developing airway resistance was not statistically significant. The protective role would be attributed to the many phytochemicals found in *W. ugandensis* that have

antioxidant and anti-inflammatory effects. The study finding concur with a study done by Karani, *et al.,* (2013) that showed *W. ugandensis* water extract was effective in treating asthma as it improved clinical symptoms of asthma in a murine asthma model and the effects of the extract were similar to orally administered dexamethasone.

5.4 Inflammatory cells in BALF

In this study it was observed that the murine asthma model applied caused a statistically significant increase in the total number of BALF inflammatory cell count when compared to the base line cell count. Intervention with *W. ugandensis* at different doses caused a statistically significant reduction in total inflammatory cell count in BALF when compared to the negative control and the count was not statistically significant when compared to the positive control. Considering the total inflammatory cell count in BALF, the test drug was as effective as the positive control (budesonide) whereas low dose (250mg/kg bwt) and high dose (500mg/kg bwt) had comparable effectiveness. Contrary to this, there was a considerable variation when the individual differential inflammatory cell count in BALF was considered whereby eosinophils registered the highest number of cell count following the murine asthma model. Upon administration of *W. ugandensis* at different doses, there was significant reduction in the number of eosinophils in BALF though this was not in a dose dependent manner and this was not statistically significant when compared to the positive control. These results agreed with a study by Karani, *et al.*, (2013) that employed the murine asthma model to assess the acclaimed efficacy of *W. ugandensis* in treatment of asthma. In their study, intervention with *W. ugandensis* was found to be effective as it lowered the eosinophil levels in BALF and this was considered to be the hallmark of asthma treatment response. In addition, Karani *et al*., (2013) noted that the reduction in eosinophil count with *W. ugandensis* intervention was not significantly different from the positive control (dexamethasone). The reduction in eosinophil count in the BALF in the current experiment could be attributed to possible presence of steroid-like anti-inflammatory molecules in *W. ugandensis*. Corticosteroids have been shown to reduce eosinophils in the BALF by subsequent

reduction of cytokines such as interleukin 5 (IL-5) and granulocyte-macrophage colony-stimulating factor (GM-CSF) that cause eosinophil infiltration and survival. Secondly, the postulated steroid-like molecules in *W. ugandesis* might have induced a reduction in exhaled NO levels hence a reduction in the eosinophils since NO causes eosinophil migration. This is evidenced by the strong positive correlation of BALF NO level with BALF eosinophil count in the current study. Furthermore; BALF eosinophil count in negative and positive control agreed with a study done by Roos, *et al.,* (2014) that elucidated significant elevation of airway eosinophils in asthma after allergen provocation with methacholine in murine asthma model that significantly decreased with use of corticosteroid. Roos, *et al.,* (2014) concluded that eosinophils can be used as a marker for monitoring anti-inflammatory therapy response in asthmatics.

Although lymphocytes are markers of inflammation in asthma, the murine asthma model applied did not show a significant rise in BALF lymphocyte count when the base line cell count was compared to the negative control. Interestingly, there was a statistically significant drop in lymphocyte count when negative control group was compared to interventional groups with *W. ugandensis* at 500mg/kg and positive control (budesonide). This was not the case with low dose of *W. ugandensis* at 250mg/kg intervention as it was not statistically significant when compared to the negative control. It was therefore evident that *W. ugandensis* water extract at high dose of 500mg/kg was effective in lowering the lymphocytes that promote inflammation in asthmatics and its' effectiveness was comparable to that of positive control budesonide. Of note is that the intervention caused a dropped in the lymphocyte count to a level that was not statistically significant when compared to the base line. Just like the lymphocytes the neutrophils are a key marker of inflammatory cells. Compelling evidence suggests that common triggers of asthma exacerbations are preferentially mediated by neutrophilic airway inflammation (Jr 2018). The murine asthma model caused a significant rise in neutrophils. Intervention with *W. ugandensis* at 250mg/kg and 500mg/kg was effective and none proved to have better results than the other in lowering the neutrophil.

Alveolar macrophages (AMs) are key orchestrators of pulmonary immune responses (Zaslona *et al,*. 2019). In this study macrophages registered the highest number of cell count amongst all the inflammatory cells in the baseline. This was in agreement with others studies that under steady-state condition macrophages account for majority of the leukocytes in the lower respiratory tract (Hamid, Tulic & Liu 2003). Although the study did not delineate between the resident and circulating alveolar macrophages since they have different roles in airway inflammation, it was evident that *W. ugandensis* water extract statistically decreased the macrophage count in comparison to the negative control though not in a dose dependent manner. There was no significant difference in macrophage count reduction between *W. ugandensis* and the positive control interventions. This proved that *W. ugandensis* is effective in asthma management with similar effects like budesonide.

5.5 Nitric oxide levels

Concentration of Nitric Oxide (NO) in exhaled air is now recognized as a critical component in evaluating control of the asthmatic (Wechsler *et al*., 2000). Measurement of exhaled Nitric Oxide (NO) levels provides a rapid, reproducible, and reliable test which may reflect airway inflammation in asthma (Stirling *et al*., 1998). Exhaled NO is used as a noninvasive biomarker of inflammation in respiratory conditions (Birrell *et al.,* 2006). Corticosteroids lower NO levels which causes exacerbation of inflammatory cell mediators and mucus producing airway epithelial cells hence their use as controllers (Permanente *et al.*, 2011). Levels of BALF NO was used as a surrogate marker of inflammation in this study hence able to predict the control ability. This Study demonstrated the effectiveness of *W. ugandensis* in the control of asthma using NO as a surrogate marker of inflammation. The observed steep increase of NO between the base line and the negative control demonstrated that an inflammatory process had occurred as animals had been induced with asthma using OVA/Alum model for asthma. This agreed with Roos, *et al* (2014) that elucidated Fractional Exhaled Nitric Oxide was significantly higher in asthmatics, compared to healthy controls. There was a significant difference between the negative control when compared to intervention groups with *W. ugandensis* at 250mg/kg and500mg/kg

with high dose recording a significantly lower value of NO than low dose group. This showed that *W. ugandensis* extract had an anti-inflammatory effect and can be used as a controller in asthma management and that a higher dose proved adequate treatment compared to a lower dose of 250mg/kg. This agreed with Permanente *et al.,* 2011 that persistent elevation of exhaled Nitric Oxide in treated asthmatics may suggest either more severe inflammation or inadequately treated inflammation. Reduction of Nitric Oxide levels in murine asthma model for the intervention group with *W. ugandensis* at 500mg/kg was not significantly different from the positive control but intervention with 250mg/kg of *W. ugandensis* recorded a significantly higher level when compared to positive control budesonide. This proved *W. ugandensis* has anti-inflammatory molecule/s whose effects at a high dose of 500mg/kg body weight are comparable to budesonide. This was in agreement with a study by Permanente *et al., (*2011) that elucidated that corticosteroids lower the FENO levels in a dose dependent manner. The therapeutic effects of *W. ugandensis* could be attributed to its wide variety of phytochemicals with high antioxidant and anti-inflammatory molecules (Odongo *et al.,* 2017).

There was a correlation between symptomatology PCT, inflammatory cells eosinophils, and exhaled NO levels. This was in agreement with Akata *et al.,* (2016) study that showed levels of eNO correlated with sputum eosinophilia, broncho alveolar lavage fluid eosinophilia, blood eosinophilia, airway hyperactivity and peak-flow variability in asthmatics.

5.6 Bronchiole wall thickness

Much effort in the pharmaceutical industry is geared towards developing therapies that inhibit inflammation in respiratory diseases in the belief that this will impact on disease pathology (Akata, Yatera & Keisuke 2016). In the study, the negative control showed significant increase in thickness of the epithelial layer compared to the baseline, proving that an inflammatory process had occurred. There was a significant bronchiole wall reduction following intervention with *Warburgia ugandensis* water extract when compared to the negative control group. The bronchiole wall thickness

in intervention group with *W. ugandensis* was dose dependent, with a high dose (500mg/kg bwt) proving to have better results when compared to low dose (250mg/kg bwt). Remarkably at high doses (500mg/kg bwt) *W. ugandensis* was as effective as positive control (budesonide). There was a significant strong positive correlation between bronchiole wall thickness, NO in BALF and eosinophils in BALF. The study postulated that the attributed effectiveness of *W. ugandensis* in reducing bronchiole wall thickness was due to phytochemicals that had direct effect on the epithelium or through reduction of NO levels hence inhibiting recruitment of inflammatory cells. The study agreed with other researchers that concluded inflammation in asthmatics is associated with increased cellular infiltration especially eosinophils which causes more inflammation by producing peroxidases like myeloperoxidase that use NO to generate nitrogen radicals (Ghosh *et al.*, 2012) and that high Nitric Oxide levels is associated with inflammation in asthmatics and causes recruitment of inflammatory cells especially eosinophils and to a lesser extent neutrophils, monocytes (Prado, Martins, & Tib 2011). The study also agreed with a similar study by Akata, Yatera, & Keisuke (2016) showed the significance of NO in pathophysiology of asthma, using a mouse model for asthma with knock out of Nitric Oxide synthase (i/n/eNOSs) and there was significant decrease in eosinophilic inflammatory cells, bronchial thickening and mucus secretion. Interventions done to curb airway inflammation by aiming at the reduction in NO levels and inflammatory cells achieve a good outcome Rensen *et al.*, 1999 like *W. ugandensis* did.

5.7 Beta and muscarinic activity of *W. ugandensis*

In healthy lungs, muscarinic and beta receptors control smooth muscle tone, mucus secretion, vasodilation, and inflammation. In asthma, cholinergic mechanisms contribute to increased bronchoconstriction and mucus secretion that limit airflow. Functional experiments demonstrate that contraction induced by muscarinic ligands in isolated trachea and bronchi is mediated by M3 and beta receptors in all species including humans (Buels & Fryer 2014; Wiegand, *et al.* 2006). Acetylcholine induces release of leukotriene B4 and other factors from alveolar macrophages that induce human peripheral blood monocyte, neutrophil, and eosinophil chemotaxis, and M3

receptor antagonists prevent acetylcholine-induced release of chemotactic activity from macrophages. Additionally, acetylcholine may also contribute to inflammation by inducing release of chemotactic factors from airway epithelial cells (Buels &Fryer 2012).

Beta and muscarinic receptor activity of *W. ugandensis* was evaluated using isolated rabbit trachea strips with or without the pre contraction with broncho contractor (acetylcholine & histamine). Potency values (EC_{50}) were determined from the cumulative concentration–response curves. It was interesting that *W. ugandensis* extract proved to have a duo relaxation effect in isolated trachea though the effects were more skewed to beta receptor activity compared to anti-muscarinic effects.

Maximum effects were predetermined using a normal trachea without a pre contracting chemical. *Warburgia ugandensis* relaxed the trachea by 76.6% at concentration of 1.39mg /ml of Kreb's solution and the EC 50 was 4.76mg. In pre contracted trachea strips with acetylcholine as a blocker, the maximum relaxant effect of *W. ugandensis* was compared to salbutamol as positive control. The results showed that *W. ugandensis* dose-dependently relaxed the isolated rabbit trachea strips and the EC_{50} was 6.462mg for *W. ugandensis* and 0.3706mg for salbutamol. The relaxing effects of *W. ugandensis* with acetylcholine as a pre contractor was comparable to that of normal trachea without any pre contractor. Moreover, it was confirmed that the bronchodilator effect of *W. ugandensis* was more due to the activation of beta 2 adrenoceptor because this effect was easily antagonized by propranolol a beta 2 adrenoceptor antagonist. A student T test was applied to compare the effectiveness in relaxation of salbutamol to that of the extract *W. ugandensis* as a beta agonist, there was a significant difference with P value of 0.038. It was therefore evident that salbutamol had better relaxation effects at 86.1% compared to *W. ugandensis at* 62.56%.

Muscarinic effect of *W. ugandensis* was evaluated with used to block the beta receptors and atropine was used as a positive control. At a concentration of 1.39mg/ml (maximum relaxing dose of the extract) in Krebs solution, *Warburgia ugandensis* had

an EC 50 of 845.5mg and maximum relaxation of 18.6 %. The dose response curve was compared to that of atropine used as a positive control and the EC 50 was 0.5289 with maximum relaxation effects at 61. 5 %. It was therefore evident that *W. ugandensis* extract had anti-muscarinic like relaxing effects though it was significantly low when compared to atropine. Although *W. ugandensis* extract proved to have muscarinic receptor antagonist effects, the effect/s were not as potent as those of beta activity since the EC_{50} was 845.50 and 6.462 respectively and maximum relaxation effect was 62.56 % for beta activity and 18.6% for anti-muscarinic activity.

5.8 Conclusion

Warburgia ugandensis water extract has many phytochemicals and is safe for use. *Warburgia ugandensis* water extract can be used as a controller in asthmatics. Its protective effect is dose dependent and a dose of 500mg/kg body weight is comparable to budesonide. Results in this study indicated that *W. ugandensis* was effective in management of asthma as it lowered the inflammatory cells in BALF. Also these inflammatory cells may be useful for monitoring response of asthmatic on *W. ugandensis* extract management. It was evident that *W. ugandensis* inhibited NO production and had good efficacy in control of asthma just like budesonide used as a positive control. A higher dose of 500mg/kg would have better control results compared to a lower dose. The therapeutic effects could be attributed to its wide variety of phytochemicals with high antioxidant levels. *W. ugandensis* water extract has anti-inflammatory effects. A higher dose of 500mg/kg has similar anti-inflammatory effects as a budesonide used as a positive control and has better anti-inflammatory effects compared to a lower dose at 250mg/kg. Comparison of the effect of budesonide and test drug *W. ugandensis* water extract on bronchiole wall thickness demonstrated a significant correlation between the impact on BALF NO and inflammatory cell burden in the airway and was does dependent. *W. ugandensis* proved to have a duo relaxing effects on isolated rabbit trachea with more beta activity compared to anti-muscarinic effects.

5.9 Recommendations

1. Though use of *W. ugandensis* as a medicinal plant has proved to be safe for use further pre-clinical and clinical studies need to be carried out to validate its safety furthermore its safety on chronic use should also be evaluated. It is evident that *W. ugandensis* extract has potent molecule/s that have anti-inflammatory activity, and therefore specific molecule need to be isolated. Large-scale clinical trials to elucidate the anti- inflammatory mechanism of action should be considered.
2. Further studies should be done to find out if *W. ugandensis* has a protective mechanism in preventing airway remodeling in chronic asthma since high levels of NO in asthmatic is associated with airway remodeling.
3. Corticosteroids play a central role in the treatment of asthma and have been reported to have detrimental effects on the gastric mucosa and also have systemic and local side effects after prolonged and high dosage use compared to herbal extract therefore *W. ugandensis* extracts should be evaluated for such side effects as would offer a safe alternative medicine if found to have no such side effects.
4. Potent molecules with beta and muscarinic receptor effects should be isolated and more in vivo studies should be carried out to ascertain the receptor activity.

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APPENDICES

Appendix I: Data collection form

	ID	ID	Conc.	spl Abs+added 0.1 Normal (NO)	conc NO 0.1 normal	diffrence	conc /Normal
control							
wu/500mg							
wu/500g							
control							
wu/250mg							
wu/500g							
Budesonide							

Appendix II: Ethical approval form

JOMO KENYATTA UNIVERSITY
OF
AGRICULTURE AND TECHNOLOGY
P. O. Box 62000-00200 Nairobi, Kenya Tel 0672520225 OR Extn 3209
Institutional Ethics Review Committee

February 23rd, 2018 REF: JKU/2/4/896B

Victoria Wanjiku Ngugi,
Department of Medical Physiology

Dear Ms. Ngugi,

RE: ANTI-ASTHMATIC EFFECTS OF WARBURGIA UGADENSIS USING BALB/C MOUSE MODEL FOR ASTHMA

The JKUAT Institutional Ethics Review Committee has reviewed your responses to issues raised regarding your application to conduct the above mentioned study with you as the Principal Investigator.

The is to inform you that the IERC has approved your protocol. The approval period is from February 23rd 2018 to February 23rd 2019 and is subject to compliance with the following requirements:

a) Only approved documents (informed consent, study instruments, study protocol, etc.) will be used.
b) All changes (amendments, deviations, violations, etc.) must be submitted for review and approval by the JKUAT IERC before implementation.
c) Death and life threatening problems and severe adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the IERC immediately.
d) Any changes, anticipated or otherwise that may increase the risks to or affect the welfare of study participants and others or affect the integrity of the study must be reported immediately.
e) Should you require an extension of the approval period, kindly submit a request for extension 60 days prior to the expiry of the current approval period and attach supporting documentation.
f) Clearance for export of data or specimens must be obtained from the JKUAT IERC as well as the relevant government agencies for each consignment for export.
g) The IERC requires a copy of the final report for record to reduce chances for duplication of similar studies.

Should you require clarification, kindly contact the JKUAT IERC Secretariat.

Yours Sincerely,

Dr. Patrick Mbindyo
SECRETARY, IERC

J.K.U.A.T
DIRECTOR,
RESEARCH DEPARTMENT (RPE)
P.O. Box 62000-00200
NAIROBI

Setting Trends in Higher Education, Research and Innovation

Appendix III: Publication

East African Medical Journal Vol. 96 No. 4 April 2019
ANTI-ASTHMATIC EFFECTS OF WARBURGIA *UGANDENSIS* USING BALB/C MOUSE MODEL FOR ASTHMA
Victoria Wanjiku Ngugi, BSc Clinical Medicine, Department of Clinical Medicine, School of Medicine, Jomo Kenyatta University of Agriculture & Technology, P.O Box 62000-00200, Nairobi, Kenya. Simon Karanja, BVM, MSc-TVE PhD, School of Public Health, Jomo Kenyatta University of Agriculture & Technology, P.O. Box 62000 - 00200 Nairobi, Kenya. Justus Maingi Simba, MBChB, MMed (Paeds) Department of Child Health and Paediatrics, School of Medicine, Jomo Kenyatta University of Agriculture and Technology P.O Box 8064-01000, Thika, Kenya. Samuel Kariuki Ngugi, MBChB, MMed (ENT), Department of Medical Physiology, School of Medicine, Jomo Kenyatta University of Agriculture and Technology P.O Box 59-01000 Thika, Kenya.

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ANTI-ASTHMATIC EFFECTS OF WARBURGIA *UGANDENSIS* USING BALB/C MOUSE MODEL FOR ASTHMA

V. W. Ngugi, S. Karanja, J. M. Simba and S. K. Ngugi

ABSTRACT

Background: **W. *ugandensis* is widely used by herbalist due to its therapeutic properties with observation studies suggesting it can manage asthma. This research sought to find out the anti-asthmatic effects of W *ugandensis* using BALB/c mouse model for asthma.**

Methodology: **Percent protection to developing airway resistance by measure of Pre-Convulsion Time (PCT), efficacy in reduction of inflammatory cells count and Nitric Oxide levels in BALF were assessed. Animals were induced with asthma and challenged with methacholine one hour after final OVA inhalation to determine baseline PCT. Intervention was done with (500mg/kg, 250mk/kg, negative control (NS), positive (budesonide) then challenged with methacholine 72 hours after final OVA inhalation, PCT taken was compared to the baseline PCT and percent protection calculated. Hemocytometer was used to determine total inflammatory cell count and four- part differential counts of 200 cells per slide performed for differential cell count. NO level was determined using Griess reagent protocol.**

Results: **PCT was not significantly different across all groups before intervention ($p>0.05$). Percent inhibition for 500mg/kg of W. *ugandensis* was 88.02±1.72b was not significantly different from positive control 82.03±1.29b. Negative control recorded highest total cell count (619.67±15.50a) most being eosinophils (494.33±12.01) and decreased significantly following interventions $p<0.05$. NO was highest in negative control (494.33±12.01a) and decreased significantly with**