College of Medicine

PHYTOCHEMICAL, ANTIOXIDANT ACTIVITY AND TOXICITY EVALUATION OF MEDICINAL PLANTS COMMONLY USED FOR ASTHMA MANAGEMENT IN MALAWI

BY

TUNTUFYE MWAMBYALE

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DECLARATION

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where states otherwise by reference or acknowledgment, the work presented is entirely my own.

Name: Tuntufye Mwambyale

Signature:

Date:

CERTIFICATE OF APPROVAL

The Dissertation of Tuntufye Mwambyale is approved by the Dissertation Examination Committee:

_____

Professor Genesis Chorwe

(Chairman, Post Graduate Committee)

_____

Assoc. Prof. Mwapatsa Mipando

(Supervisor)

_____

Mr. Ibrahim Chikowe

(Supervisor)

_____

Mr. Joseph Fulakeza

(Head of Biomedical Sciences Department)

DEDICATION

I dedicate my dissertation work to my family and many friends. A special feeling of gratitude to my loving parents, Charles and Lyazzat Mwambyale whose constant words of encouragement and push for tenacity ring in my ears. My brother Kalyoto who brought smiles in times of sadness.

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I dedicate this work and give special thanks to my wonderful daughter Imaan for giving me the courage and strength to go on. For you I will be excellent so that you become excellent.

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ABSTRACT

Asthma is a chronic disease characterized by recurrent attacks of breathlessness and wheezing, which vary in severity and frequency from person to person. There is minimal scientific research on the efficacy, doses, toxicity and safety of herbal remedies used in the management of asthma in many countries including Malawi. Therefore, this study determined the brief chemical composition and toxicity and/or safety of herbal extracts from the medicinal plants that are commonly used to manage asthma in Malawi. This was achieved through qualitative and quantitative assessment of flavonoids and phenolics, screening for antioxidant activity using 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) assay and Ferric reducing antioxidant power assay (FRAP) as well as validated *in vitro* and *in vivo* acute toxicity test on Wistar rats of ethanolic herbal extracts of *Erythrina abbysinica Lam. ex DC., Paederia bojeriana (A.Rich. ex DC.) Drake* and *Trichodesma zeylanicum (Burm.f.) R. Br*. The results showed that *Trichodesma zeylanicum (Burm.f.) R. Br*, *Paederia bojeriana (A.Rich. ex DC.) Drake* and *Erythrina abbysinica* (*Lam. ex DC)* contained flavonoids and phenols. Highest total flavonoid content was found in *E. abyssinica (Lam. ex DC.)*, (806.12±0.01 QE/mg). Highest total phenolic content was also found in *E. abyssinica (Lam. ex DC.)* (98.48±0.08 mg GAE/g). Antioxidant activity using DPPH % inhibition was highest in *E. abyssinica (Lam. ex DC.) (*86.59%) while FRAP results were highest in *T. zeylanicum (Burm.f.) R. Br* (21.85mg TAEC/g). *In vitro* toxicity showed that ethanolic root extract of *P. bojeriana (A.Rich. ex DC.) Drake* had the lowest LC_{50} values (LC_{50} = 11.09µg/ml) however, *in vivo* toxicity studies showed LC_{50} of 2000mg/kg in all extracts. This study has revealed that the ethanolic extracts of all extracts are rich in phytochemicals and antioxidant activities that are indicative of asthmatic activity in human beings according to the literature. The study has also shown that the plants are toxic *in vitro* but non-toxic *in vitro*.

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LIST OF ABBREVIATIONS AND ACRONYMS

ACEPHEM	Africa Center of Excellence in Public Health and Herbal Medicine
ANOVA	Analysis of Variance
BALF	Bronchoalveolar fluid
cAMP	Cyclic Adenosine Monophosphate
COMREC	College of Medicine Research and Ethics Committee
DALY	Disability Adjusted Life Years
DPPH	2,2-diphenyl-1-picrylhydrazyl
ELISA	Enzyme-Linked Immunosorbent Assay
FeNO	Exhaled Nitric Oxide
FEV	Forced Expiratory Volume
FEV1	Forced Expiratory Volume in 1 second
FRAP	Ferric reducing antioxidant power
FVC	Forced Vital Capacity
H&E	Hematoxylin and Eosin
IgE	Immunoglobin E
ISAAC	International Study of Asthma and Allergies in Childhood
LPS	Lipopolysaccharides
NCD	Non-communicable disease

NHBG	National Herbarium and Botanical Gardens
OVA	Ovalbumin
PAF	Platelet Activating Factor
PAS	Periodic acid-Schiff
PDE	Phosphodiesterase
PEF	Peak Expiratory Flow
PEFR	Peak Expiratory Flow Rate
Th	T-Helper
TM	Traditional Medicine
Treg	T – Regulator
TPTZ	2,4,6-Tripyridyl-S-triazine
WHO	World Health Organisation

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1 Definition of Asthma

Asthma is a chronic non-communicable disease (NCD) in the respiratory system that affects both children and adults. It is characterized by recurrent and reversible attacks of wheezing, breathlessness, spasms, shortness or difficulty of breathing and coughing as a result of allergic reactions that trigger inflammation and narrowing of narrowing of airways, which vary in severity and frequency from person to person (2,11,12). These usually arise from the interaction between persons inherited genotype and the environment and its risk factors include allergen exposure such as pets, dust, mites, cockroaches, mold, pollen and viral infections (13).

Asthma is generally classified into allergic (extrinsic or non-atopic) and non-allergic (intrinsic or atopic depending on the causative agents) (14). Allergic asthma is the most common type of asthma in childhood and it is also highly associated with allergies such as nasal allergies and eczema. However, this subsides as the child grows older and may reappear in adulthood. Non-allergic asthma accounts for small proportion of asthma cases, mostly occurs in adulthood, and highly affects women and its risk factors include respiratory tract infections and obesity (over 30 years). This is the most challenging kind of asthma in disease management and its symptoms persist throughout the year. Atopic asthma is usually characterized by eosinophil infiltration and increased Immunoglobin E (IgE) concentration compared to non-atopic asthma (15).

1.2 Prevalence of Asthma

The Global Asthma Report estimated in 2018 that 339 million people have asthma, with 14 % of the population being children, 4.5% being young adults aged 18 to 45(1). Within the same young adult population 8.6% experience asthma symptoms such as wheezing, breathlessness, tightening of the chest and coughing (1). The World Health Organization (WHO) estimates that 383,000 deaths were due to asthma in 2015 (11).

According to the International Study of Asthma and Allergies in Childhood (ISAAC), the prevalence of asthma in children aged 13-14 years in Africa was 10.4% with the highest being Oceania with 25.9% and the lowest being eastern Europe with 4.4% (16). Prevalence rates within African countries are restricted to the International Study of Asthma and Allergies in Childhood in which seven countries participated of which the highest prevalence was South Africa (20.3 %) followed by Kenya (15.8%), Nigeria (13.0%), Tunisia (11.9%), Morocco (10.4%), Ethiopia (9.1%) and finally Algeria (8.7%) (17).

In Malawi, around 40% of Malawian adults in urban areas have abnormal lung function (18) and it is estimated that the prevalence of asthma is 5.1% (2). Banda et al, assessed chronic respiratory symptoms in the population of two rural Malawian communities and found that 55.3% of the population reported cough only symptom whilst 44.7% reported a combination of wheeze and shortness of breath symptom (18). The results also showed that 4.6% of the participants had the diagnosis of chronic obstructive pulmonary disease, asthma, bronchitis or tuberculosis within their medical records (18). Asthma, however is not a common presentation in government hospitals in adults and children (19) .

It is anticipated that there is a higher prevalence of asthma in developed countries as compared to developing countries however true prevalence in developing countries is not known due to insufficient data available on asthma cases (19). The ISAAC figures show that developing countries have already high prevalence even with the minimal data collected (20).

1.3 Pathophysiology of Asthma

Asthma is characterized by airway inflammatory cells, including eosinophils, macrophages, mast cells, epithelial cells and activated lymphocytes that release various cytokines, adhesion molecules and other mediators. Inflammation results in an acute, sub-acute or chronic process that alters airway tone, modulates vascular permeability, activates neurons, increases secretion of mucus, and alters airway structure reversibly or permanently (21).

During an initial reaction to an allergen, dendritic cells interact and engulf the allergens on the mucosal surface of the bronchi, and make their way to the lymph nodes. The presence of the allergens stimulates the production of antigens, these antigens when in contact with Th2 cells, cause the Th2 to proliferate and enhance the allergic reaction. The initial reaction is the sensitization of the mucosal surface with the coding and storage of the allergen information through memory T-cells (15,22–24).

The subsequent reactions involve the entry of the allergens into the airway where the sensitized Th2 and mast cells are stimulated and mobilized to produce pro-inflammatory mediators and cytokines against the allergen and pro-inflammatory mediators such as leukotrienes, histamines,

bradykinin, prostaglandins, thromboxane A_2 and platelet-activating factor (PAF) cause bronchoconstriction, increase mucus secretion and edema. Cytokines such as leukotriene B_4 attract eosinophils, neutrophils and platelets thus increasing the production of more pro-inflammatory mediators, cytokines and enzymes further exacerbating the reaction to the allergen and the cycle repeats itself every time there is an interaction between the mucosal surface and the allergen (15,24,25).

1.4 Diagnosis and Treatment

There is no single adequate test for diagnosing asthma. Pulmonary function tests measure ventilation using parameters such as airflow and lung volume. The most common procedure for these parameters is spirometry and peak expiratory flow rate.

Spirometry is an effective procedure evaluating airflow and lung volumes; it is carried out over days and weeks to determine the diagnosis of asthma. Airflow limitation is measure by the forced expiratory volume in 1 second over forced vital capacity, FEV1/FVC ratio; asthma is usually diagnosed if there is a greater than 15% change in forced expiratory volume (FEV) after use of bronchodilators as it determines the reversibility in airflow. Spirometry has standardized volume charts for various lung parameters in order to specify and adequately analyze the volumes measured (26,27).

Peak expiratory flow rate (PEFR) measures airflow exiting the lungs after forced inhalation and measured using a peak flow meter. PEFR is used to monitor changes in flow rates in order to determine the trend in a patient's asthma control and determines the reversibility in airflow if a

bronchodilator is used. PEFR has standardized charts for the peak expiratory flow measurement (27,28).

Exhaled nitric oxide test (FeNO test) is a simple procedure that measures the amount of nitric oxide that is exhaled. Nitric oxide is an endogenous mediator that relaxes smooth muscle within the airways, high levels of nitric oxide in exhaled air is an indicator of eosinophilic inflammation in the lung airways (29). The American Thoracic Society highly recommends the use of FeNO test in diagnosing eosinophilic inflammation and monitoring its responsiveness to steroid treatment for chronic respiratory symptoms (30).

In conjunction to the respiratory function tests mentioned above, other assessments are available to further aid in the diagnosis of asthma such as exercise tests, skin tests, allergy provocation tests, blood and sputum tests and radiology that include measurement in different lung volumes and flow volumes such as the peak expiratory flow (PEF) (15).

1.5 Oxidative Stress in Asthma

Oxidative stress occurs when there is an imbalance in the production and removal of reactive oxygen species (ROS) in cells and tissues (31) . ROS are residues from oxygen metabolism, however can increase due to various internal and external processes such as drugs (xenobiotics), environmental factors (radiation, UV rays, pollutants), aging, stress, that results in damage to the cell and tissues (32). ROS have several functions, commonly known for cell signaling. inflammation factor production and modulation of cell survival (32,33). ROS cause damage to various biomolecules such as DNA, proteins and lipids through removal of nucleotides,

modification of bases and strand breakage (32), enzyme inactivation, protein strand breakage, altered electric charges and change in cellular membrane permeability (34,35). There is now strong evidence that oxidative stress is involved in the etiology of a variety of lung diseases, including asthma, COPD, acute lung injury, pulmonary fibrosis, and lung cancer (36).

Oxidative stress in asthma is linked to chronic inflammation and disease severity due to the increased production of reactive oxygen and nitrogen species (37,38). Concurrently there is also a reduction in the activity of the antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase responsible for the inactivation of the reactive oxygen species (33). Many of the pathophysiologic characteristics associated with asthma are exacerbated by reactive oxygen species (ROS). ROS cause mast cells to produce histamine and airway epithelial cells to secrete mucus (39). Increased ROS production can harm epithelial cells directly and promote cell shedding (39,40). ROS have been shown in studies to cause endothelial barrier failure, which leads to an increase in cell permeability to fluid, macromolecules and inflammatory cells (33). Bronchial hyperreactivity, which is characteristic of asthma, is caused by an excess of reactive oxygen species (ROS) or a depression of the protective mechanism in cells (41,42). The effect of ROS on airway smooth muscle contraction is amplified when the epithelium is damaged or eliminated (40). There are external ways that counteract the effects of ROS through antioxidant found in dietary supplements and food. ROS stimulate pro-inflammatory gene transcription factors such as NFB, AP-1, and hypoxia-inducible factor (HIF)-1 through multiple signaling pathways involving mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/Akt, and protein kinase C [PKC] (43). By activating phospholipase A_2 (PLA2), ROS are also implicated in the synthesis of a number of inflammatory mediators, most notably

eicosanoids (31). ROS are also involved in the acquisition of immunological responses such as antigen interactions with IgE or IgG antibodies (35,44). Antioxidative compounds such as Vitamin A, C and D have shown to reduce inflammation when taken in moderation (32), various phenols and flavonoids have potent free radical scavenging ability and improve antioxidant defenses by acting directly on DNA, protein and lipid structures (45,46). However, excessive intake of the Vitamin C and Vitamin D can also cause proinflammatory and prooxidative damage to cells (33).

1.6 Management of Asthma

There is no medical cure for asthma and all the available treatment options so far are aimed at managing asthma by abating the symptoms, reducing the risk of attacks and improving lung function (47).

The mainstream drugs used to treat asthma are usually bronchodilators (Salbutamol), steroids (Prednisone), leukotriene antagonists (Montelukast) and recently anti-Immunoglobin E (IgE) antibody drugs (Omalizumab). Treatment is usually tapered to the severity of asthma and stepwise management is taken up by medical professionals (Table 1) (48,49).

Table 1: Stepwise Management of Asthma

Step		**PEFR**	**Treatment**
1	Occasional symptoms, less frequent than daily	100% predicted	As-required bronchodilators If used more than once daily, move to step 2
2	Daily Symptoms	<80% predicted	Anti-inflammatory drugs Sodium cromoglicate or low-dose inhaled corticosteroids up to 800 ug If not controlled, move to step 3
3	Severe Symptoms	50-80% predicted	High-dose inhaled corticosteroids up to 2000ug daily
4	Severe symptoms uncontrolled with high-dose inhaled corticosteroids	50-80% predicted	Add regular long-acting β2 agonists (e.g salmeterol)
5	Severe symptoms deteriorating	<50% predicted	Add prednisolone 40mg daily
6	Severe symptoms deteriorating in spite of prednisolone	<30% predicted	Hospital Admission

1.6.1 The Pharmacology of Anti-asthmatic Drugs

Anti - asthmatic drugs are classified into two major categories and these are bronchodilators and anti-inflammatory agents. Bronchodilators commonly known as relievers work on the early response in asthma which presents as bronchospasm; whilst the anti-inflammatory agents known

as controllers work on the late response in asthma in which the underlying cause is the continuous inflammatory process caused the recruitment of inflammatory cells such as prostaglandins, leukotrienes and other enzymes (50).

Bronchodilators come in various chemical forms such as sympathomimetics, methylxanthines and anticholinergics. Sympathomimetics such as salbutamol are usually β_2-adrenoceptor agonists, which work on the bronchi that are rich with β_2 receptors to reduce bronchial smooth muscle tension by increasing the production of cyclic adenosine monophosphate (cAMP) and works towards reducing overall bronchospasm with the airways (15,22,51). Sympathomimetics can inadvertently consumed in large doses and can induced adverse effects such as tremor, tachycardia, agitation, metabolic acidosis, hyperglycemia and hypokalemia (52).

Methylxanthines such as theophylline, although the method of action is not clearly known, acts by inhibiting the enzyme phosphodiesterase, which reduces the breakdown of cAMP thus giving a bronchodilating effect on the airways; Phosphodiesterase (PDE) also reduces the release of inflammatory mediators, which reduce immune cell activation and migration into the airway tissues (15,53). Adverse effects of methylxanthine can manifest as any of the following signs and symptoms intractable nausea, cardiac arrhythmias, rhabdomyolysis, seizures and cardiac arrest (54,55).

Anticholinergics for example, Ipratropium, block the release of acetylcholine into the airways that causes bronchoconstriction by activation of muscarinic receptors in bronchial smooth muscle. By blocking the receptors with atropine-like structures, bronchodilation can be elicited

thereby opening the airways (15,53,56). The negative consequences of anticholinergics are angina, myocardial infraction, bladder distention, urinary retention, constipation, impaired concentration, confusion and memory impairment (57,58).

The mode of action of bronchodilators is summarized in Figure 1, the various forms act on different enzymes to affect the overall bronchial tone.

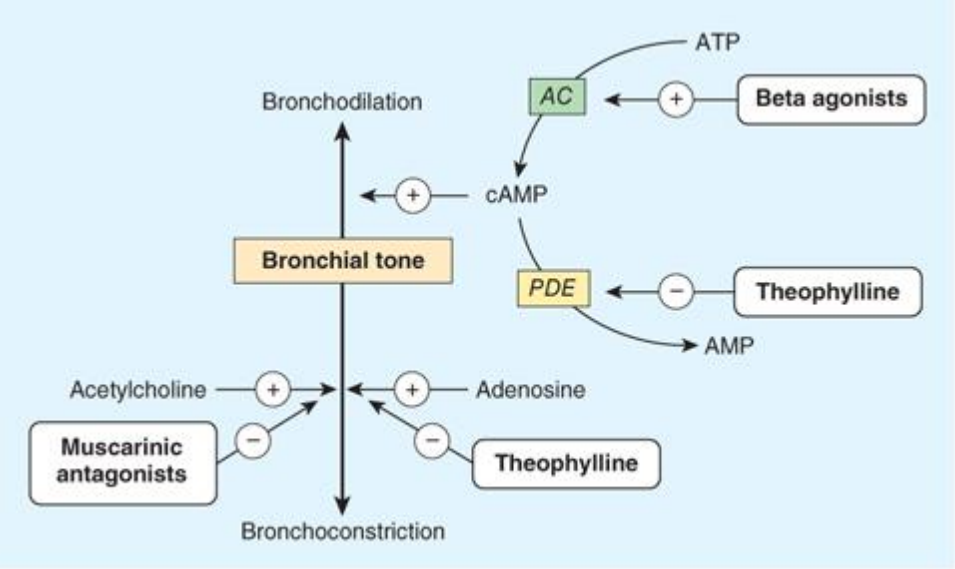


Figure 1: Drugs Used in Asthma (59)

Anti-inflammatory agents are used for long-term control in asthma and act against mucosal inflammation. Examples of anti-inflammatory agents are leukotriene pathway inhibitors and corticosteroids (60).

Leukotriene pathway inhibitors are a class of non-steroidal drugs that work on various stages of the lipoxygenase pathway to inhibit the production of the cysteinyl-leukotrienes that provoke bronchoconstriction in asthma. Leukotrienes are by-products of arachidonic acid using the lipoxygenase pathway by facilitating the migration and accumulation of inflammatory cells such

eosinophils and neutrophils. Leukotriene pathway inhibitors interfere in the production of the enzyme 5-lipoxygenase responsible for turning fatty acids into leukotrienes, therefore interfering in the production of the enzyme reduces the production of leukotrienes. The action of cysteinyl leukotrienes on the CysLT1 receptor are responsible for anaphylactic reactions, which induce inflammation, leukotriene receptor antagonists work to block cysteinyl leukotrienes from acting on the receptor and reducing bronchoconstriction and alleviating asthma symptoms. (61–64). Common adverse effects that occur with the intake of leukotriene pathway inhibitors are headaches, nausea, viral infection, abdominal pain, cough, dyspepsia and neuropsychiatric disorders (65).

Corticosteroids such as hydrocortisone interrupt the inflammation process by acting directly on genes responsible for inflammation, thus reducing the number of cytokines, enzymes, adhesion molecules and the number of inflammatory cells in the circulation and epithelial mucosa. Inhaled corticosteroids are considered the backbone of asthma management due to their efficacious mechanism of reducing airway response to an allergen, by directly reducing the number of inflammatory cells and mediators by manipulating gene transcription for various pro-inflammatory mediators (56,66,67).

The currently available treatment for asthma work by relaxing bronchospasm (bronchodilators) or reducing inflammation (corticosteroids). Unfortunately these available treatment options are not efficient for treating asthma completely as they have many adverse effects (12,68). The *Materia Medica* of India suggests that the herbal drug *Leucas aspera* have anti-inflammatory and antihistaminic activity (69,70). It had effects on histamine induced bronchospasm and

inflammation, mast cell degranulation and inflammatory cells like leukocytes and eosinophils. These parameters were helpful in evaluating antiasthmatic activity of Leucas aspera using various experimental animals like guinea pig, Wistar rat and Swiss mice (71). Ayurveda suggests that the herbal plants have comparatively fewer toxic values and are more efficacious. They also have fewer chances of side effects and complications to patients as compared to available synthetic drug treatments (36,72). Several studies have documented the use of medicinal plants in many parts of the world for asthma management and several studies have also been conducted to evaluate these plants for efficacy, quality doses and toxicity (30,73,74).

1.7 Traditional Medicine and Medicinal Plants Usage in Asthma

1.7.1 Traditional Medicine and Medicinal Plants

According to the WHO, traditional medicine (TM) refers to *"health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being*" (75).

Traditional medicine differs within the continent of Africa, between countries, regions, states and towns but the widely used materials are medicinal plants. However, there are streams of commonality and differences in certain aspect of the practice such as the parts of the plants used, routes of administration and preparation methods. Studies within different regions of Nigeria (76), Zimbabwe (77), Ethiopia (78), Cameroon (4), Ghana (79) and Kenya (80) have shown similarities and differences in proportions of the frequency of plant part used, routes of

administration and preparation methods. Table 2 below summarizes these similarities and differences

Table 2: Common ways TM is used in African countries

	Nigeria	**Zimbabwe**	**Ethiopia**	**Kenya**	**Cameroon**	**Ghana**
Parts of plant used	Leaves (51%) Roots (12.7%) Stem/bark (9%)	Roots (61.3%) Leaves (32.3%) Bark (12.9%)	Roots (49%) Leaves (43%)	Leaves (43%) Roots (30%) Stem/Bark (10%)	Leaves (45%) Seeds (11%) Whole plant (9%)	Leaves (57%) Combination of various plant parts (18%), Whole plant (9%)
Routes of administration	Oral (83%)	Oral (69.9%)	Oral (72%)	Oral (98%)	Oral*	Oral (77%)
Preparation	-	In the form of extracts (76.3%)	Juice/paste (no dilutions) (61%)	Hot decoctions (48%) Cold decoctions (19.4%)	Concoction* Decoction*	Decoctions (67%) Infusions (33%)
Type of extraction	Water* Alcohol*	-	Water processed (40%)	-	Water* Alcohol (palm wine)*	Water * Alcohol

						(local gin)*
Dosage	-	Based on type of disease	Based on age, type of ailment and seriousness of ailment	Based on herbalist's judgement, age, gender and type of ailment.	-	-
Key: " - " no indication for particular item in the study " * " no figures mentioned in the study						

African TM is extremely biodiverse with most (90%) of the medicines being plant-based and it is thought that 25% of the current conventional medicines might have originated from traditional use (79). Traditional medicine is the most predominantly used health care system in developing countries with an overwhelming 80% of the African population relying on it for its health care needs and use in developed countries is also on the rise as demonstrated in Table 1 (20,79).

Table 3: Percentage of TM use in some developing and developed countries.

Developing Country	**Percentage (%)**	**Developed Country**	**Percentage (%)**
Ethiopia	90	**Canada**	70
Benin	70	**Australia**	48
India	70	**France**	49
Rwanda	70	**USA**	42
Tanzania	60	**Belgium**	31
Uganda	60		

1.8 Traditional Medicine and Medicinal Plant Usage in Malawi

In Malawi, there were 45,000 registered members of the Traditional Healers Association and an estimated 90,000 traditional healers scattered throughout the country (81). A survey by Harries et al., showed that traditional healers saw an average 28 patients a week (82). There have been many studies of herbal medicine usage for different ailments in Malawi, 30 medicinal plant species were found to treat general ailments in pregnant women based in Mulanje, with

pneumonia and cough being one of the most prevalent diseases in the area (83). In Karonga, a survey showed that 71 out of the 102 woody species were used as herbal medicines with respiratory diseases being the second most prevalent ailment in the area (84).

1.8.1 T**raditional Medicine and Medicinal Plant Use in Asthma**

Several plants have been reported to be used for asthma management in many countries (85,86). Several studies have also been conducted to test the many plants reported in traditional medicine (16) focusing on their plant metabolites (primary and secondary), phytochemistry (study of chemical compounds in plants), bioactivity and toxicity (87–89). In Malawi, medicinal plants have also been widely reported to be used in traditional medicine (6,84,90). Out of the many plants that are used in Malawi, *Trichodesma zeylanicum (Burm.f.) R. Br*, *Erythrina abyssinica(Lam. ex DC.)* and *Paederia bojeriana (A.Rich. ex DC.) Drake* were found to be widely used. These plants were selected on the basis of indigenous usage specifically on asthmatic symptoms or asthma management, availability and distribution in the Southern region and subjective quantitative index reported in a compendium (Table 4). The subjective quantitative index was used to grade the yield of traditional medicine usage by herbalists and determine plants worthy of further research of which the highest 10% yield figures were of 9.10 and 11.60 and lowest figure was 1.45 (6). The *Trichodesma zeylanicum (Burm.f.) R. Br*, *Erythrina abyssinica (Lam. ex DC.)* and *Paederia bojeriana (A.Rich. ex DC.) Drake* are commonly used in different parts of Malawi to treat bronchitis, cough and asthma (6).

Table 4: Factsheet of the medicinal plants selected for the study

	Trichodesma zeylanicum (Burm.f.) R. Br	*Erythrina abyssinica (Lam. ex DC.)*	*Paederia bojeriana (A.Rich. ex DC.) Drake*
Family	Boraginaceae	Fabaceae - Papilionoideae	Rubiaceae
Common Name	Camel Bush; Cattle Bush; Jilarga; Rough Bluebell;	Red hot poker tree; Lucky bean tree; Mulunguti;	Skunkvine
Local Name	Chilingummwamba	Lindimira; Muwale	Mnunkhamanyi, Mtuvituvi,
Subjective Quantitative Index	6.49	5.31	6.39
Botanical Description	Annual, short-lived perennial, which grows up to 1.5m tall. Has a general and dense distribution bristly hairs. Leaves are narrowly elliptic with flowers that become nodding, the sepals are bristly hairy, enlarging in	Medium-sized deciduous tree that grows to the height of 5-15m, it is well branched, rounded and thickset with a spreading crown and a short trunk. The bark is a yellow buff when fresh, otherwise grey-brown to creamy	A foul-smelling climber with leaves opposite or in coils of 3, that are elliptic to ovate in shape and grey-green colour that are velvety in texture on the bottom aspect. The stalk can grow up to 15 cm long and is velvety in texture. Flowers are

	fruit. Corolla (7-9mm) scarcely exserted from the sepals and the lobes are pale blue to lilac or pinkish. Fruits split into four brown nutlets that are smooth and ovoid (91).	brown. The outer layer is deeply grooved, thickly corky and spiny and when damaged the exudate is gummy brown sap. Leaves have a trifoliate arrangement and area broad and long with scattered prickles on the underside of the veins. Fruits are a woody pod with constrictions in between the seeds (92).	whitish to pale creamy yellow with flowered heads on the peduncles. Fruits are oval-shaped and are yellowish to brownish in colour, crowned by persistent calyx lobes (93).

Uses	Roots and green leaves are used as an analgesic (94), applied to wounds, boils and snakebites to enhance healing (95) The plant has diuretic properties and is used to treat dysentery and fevers. The ash of the plant is used to treat coughs and scabies (94,95). In Malawi, the leaves and roots are used to treat wounds, asthma and diarrhea (6,96,97) .	The bark is commonly used to treat snakebites, malaria (98), sexually transmitted diseases, cough, liver inflammation (99), stomach-aches, measles, anthelmintic (100) and conjunctivitis. In Malawi, the bark is used to treat bronchitis and chest sores (6). The bark sap is used as an anthelmintic. Flowers are used as abortifacient and treat dysentery. Roots are taken to treat peptic ulcers and diarrhea. Leaf poultice is applied to wounds and joints. Fruit extracts are used in the treatment of asthma and meningitis (101).	Based on available literature, in Malawi, the root is taken to treat cough (6)

1.8.2 Plant Metabolites

Metabolism refers to the collection of biochemical events that occur in living organisms (102). Metabolites are the end products of these processes as well as their intermediates, which are usually tiny molecules (102). They are categorized as primary and secondary metabolites based on their inherent roles inside the plant body.

Primary metabolites (termed essential nutrients) are organic substances released by plants that are involved in physiological functions such as growth and development, hormone and protein production, photosynthesis, reproduction and respiration (103,104). Primary metabolites can be found in almost every component of a plant species and are generated using the same or nearly identical metabolic pathways (105). Examples of primary metabolites are carbohydrates, proteins, lipids, minerals and vitamins.

Secondary metabolites (also called phytochemicals) also play a significant role in the plants (102). They are organic compounds that are not directly involved in plant survival, but they do produce a limited number of products that facilitate normal growth and development but are not essential for survival (102,103). Secondary metabolites are substances that are biosynthetically produced from primary metabolites but are restricted to a specific taxonomic group (species, genus, family, or closely related set of families) in the plant kingdom (105).

Plants' primary metabolism is aided by the secondary metabolism. Secondary metabolites are critical for the health of plants and ensures that all of the plant's systems are functioning effectively (103,104). Secondary metabolites offer protection from herbivores, pests, and

diseases through anti-feeding activity, toxicity, and acting as precursors to physical defense mechanisms (106). Examples of secondary metabolites are alkaloids, terpenoids, polyphenolics, flavonoids.

1.8.3 Phytochemistry and Phytochemicals

Phytochemistry is a discipline that investigates the chemical elements of plants called phytochemicals. Chemical structures, metabolism (biosynthesis and degradation), natural distribution, roles, and modes of action in biological systems, extraction, and qualitative-quantitative evaluation are all part of the study of such compounds (107,108). Phytochemistry is important in the search for new medications and repurposing of current ones, characterizing and standardizing crude extracts, and determining the toxicity of the herbal plant extracts (103,104).

Phytochemicals are crucial part of plant metabolites that help plants survive momentary or long-term dangers in their environment such as insects, pests, pathogens, herbivores, UV radiation, and environmental threats while also controlling vital development and reproduction functions (103,106). All of these functions are generally beneficial to the species that produce them. Nonetheless, such properties have been exploited for human use in disease management as well as food and such effects may be an important indicator of desirable qualities, such as therapeutic potential, particularly if the mechanism of bioactivity can be identified (103).

1.8.4 Classification of Phytochemicals

The major classes of phytochemicals are grouped into four major categories: phenolics, terpenes, nitrogen (N), and sulphur (S) containing compounds. The compounds are summarized in Table 5 with the information extracted from Egbuna et al. (107) & Adetunji et al. (104).

Table 5: Classes of phytochemicals

Major classes	**Sub-classes**	**Representatives**	**Medicinal Uses**	**Available toxicity**	**References**
Phenolics	Polyphenols	Flavonoids, isoflavonoids, chalconoids, lignans, stilbenoids (e.g., resveratrol), curcuminoids, tannins (e.g., protocatechuic and chlorogenic acids)	Hyperlipidemia, hyperglycemia, anticancerous, antioxidative, antiartherosclerotic, Antiasthmatic, Antidiabetic, Anti-inflammatory, hepatoprotective, antithrombotic,	Compounds can be toxic in high concentrations due to the pro-oxidant estrogenic and mutagenic potential	(32,109–113)
	Aromatic acids	Phenolic acids (e.g., gallic acid, tannic acid, vanillin, ellagic acid), hydroxycinnamic acids (e.g., coumarin)			
Terpenes	Monoterpenes (C_{10})	Geraniol, limonene, pyrethroids, myrcene	Antimicrobial, antifungal, antiviral, antihyperglycemic, antiinflammatory,	Majority of the compounds a mildly toxic and dermal irritants however some compounds such as pulegone	(114,115)
	Sesquiterpenes (C_{15})	Costunolides			

	Diterpenes(C_{20})	Abietic acid, cafestol, gibberellins	antioxidants, antiparasitic, immune modulatory, skin permeation enhancer Nutraceuticals, functional foods, natural food, preservatives	(liver toxicity and seizures) and thujone (neurotoxic)	
	Triterpenes(C_{30})	Azadirachtin, phytoecdysones			
	Polyterpenes $(C_5)_n$	Tetraterpenes, for example, carotenoids, rubber			
Terpenoids	Carotenoids (tetraterpenoids)	β-carotene, lycopene, phytoene			
	Xanthophylls	Lutein, zeaxanthin			(116–118)
	Triterpenoid	Saponins, ursolic acid			
	Steroids	Tocopherols (vitamin E), phytosterols (β-sitosterol, campesterol)			
Nitrogen containing compounds (organonitrides)	Alkaloids	Nicotine, morphine, codeine, caffeine, theobromine, Theophylline, quinine,	Diuretic, Local anesthetic, anticancerous, antiasthmatics,	Most common toxic groups are: glycolalkoids, pyrrolizidine alkaloids, tropane alkaloids	(119–122)

		artemisinin, vincristine, vinblastine	antimalarials antihypertensive		
	Cyanogenic glycosides			Toxic due to release of hydrogen cyanide some clinical signs are stomach ache, vomiting, diarrhea, convulsion and death	(123–125)
	Nonprotein amino acids	Canavanine, azetidine-2-carboxylic acid		Known to be neurotoxic	(126,127)
Sulphur containing compounds (organosulphides)	Allicin, alliin, piperine Glutathione, phytoalexins		Antioxidants, anticancerous, Antimicrobial, antifungal, antiviral Anti-inflammatory Antidiabetic cardioprotective	Toxic in high concentrations due to hydrogen sulphide particles	(128,129)

1.9 Phytochemistry and Toxicity Testing of Herbal Plants

Several studies have attributed the medicinal plants activity to the presence of a number of compounds specifically known as phytochemicals or metabolites (primary and secondary) that differ in numbers, composition, concentration and combinations from species to species (107,117,130,131). The same phytochemicals are also responsible for the toxicity of these plants (122,132).

1.9.1 Phytochemicals and Asthma

Literature has shown a wide variety of diverse substances have moderate to strong inhibitory efficacy against the molecular targets/ indicators of asthma reported in *in vitro*, *ex vivo*, and mouse models (Table 6) (133,134). The majority are alkaloids, coumarins, flavonoids, polyphenols, and terpenoids. Many polyphenols that include phenolic acids, polyphenolic amides and flavonoids have been found to have a wide range of actions against various enzymes linked to asthma (85). They also have antioxidant properties that keep human bodies healthy as well as protecting them from diseases by neutralizing harmful free radicals that can cause oxidative stress (38,73,111). The oxidative stress has been found to be involved in the etiology of a variety of lung diseases, including asthma, COPD, acute lung injury, pulmonary fibrosis, and lung cancer (36).

Table 6: Phytochemicals and their Asthma-related activities

Phytochemical		**Mechanism of action**	**References**
Curcumin	Polyphenolic	Downgrading nitric oxide formation, inhibits histamine release, reduces expression of cytokines (IL-2, IL-5, and GM-CSF), reduces nitric oxide synthase levels by acting on IFN-γ in lung tissue.	(135–137)
Resveratrol	Polyphenolic	Stabilizes antioxidant enzymes Inhibits production of prostaglandins, inflammatory mediators (IFN-γ, TNF, COX-2, iNOS), angiogenesis pathway and activity of transcription factors such as NF-κB.	(138,139)
Kaempferol	Polyphenolic	Reduces eosinophil deposition and degranulation in lung tissue and downregulates the NF-κB pathway.	(140–142)
Ursolic acid	Terpenoid	Reduces the level of Th2 cytokines and IgE in bronchoalveolar fluid and reduces the infiltration of inflammatory cells in murine model	(143–145)

		of asthma.	
α-Lipoic acid	Organosulphide	Regulates transcription factors like as NF-κB, reduces intracellular ROS levels reduces serum IgE levels, suppresses Th2 cytokines, IL-4, IL-5, IL-13, and IL-18.	(146–148)

1.9.2 Qualitative and Quantitative Phytochemical Tests

Bioactivity and toxicity of compounds as well as medicinal plants components such as phytochemicals sometimes depends on the presence and concentration or dose that reaches the target. Studies have therefore been often been conducted to find out or confirm the presence or absence of particular phytochemicals. In some cases, some phytochemicals have also been quantified (149,150) as well as related to bioactivities (85,134,151–154). Since medicinal plants contain many phytochemicals, it might me one or a combination of them that would lead to bioactivity. However, when in plants, it is known which ones are responsible for the activity, so different solvent systems are used to extract the compounds based on any factors and the extracts are tested for bioactivity or toxicity and based on results, they are taken further, abandoned or tested in a different way or for different purpose (108,149,150). When using solvents to extract and purify organic molecules, certain guidelines are normally followed based on structural similarities between the material to be extracted and the solvent to be employed for that purpose (149). When examining the solubility of a solute in a certain solvent, another factor to consider is the polarity of the molecules. As a result, extremely polar solvents dissolve ionic or highly polar

solutes, whereas low-polar solvents dissolve low-polarity solutes but not ionic solutes. The extraction of the plant material is carried out in a series of solvents, starting with the least polar and progressing to the most polar, water (125,149,151). An opposite approach can be used depending on circumstances. In order to investigate the presence and quantify the total amount of secondary metabolites, standardized tests were used (Table 10). This study focused on the quantitative testing of flavonoids and phenols since there are validated methods for them and they are also related to the study disease. The study also evaluated the antioxidant activity of the plants since these polyphenols have also been found to have the effect of reducing oxidative stress as described above.

1.9.2.1 Phytochemical Testing of Flavonoids and Phenols

The commonly used assays that quantify flavonoid and phenolic content are the Aluminum chloride assay and Folin-Ciocalteu assay. Aluminum Chloride assay makes use of aluminum chloride and is based on the creation of a complex between the aluminum ion, Al (III), and the carbonyl and hydroxyl groups of flavones and flavonols, which results in a yellow color which is measured spectrophotometrically (155–157). Folin-Ciocalteu assay is based on the transfer of electrons from phenolic compounds to a blue chromophore composed of a phosphotungstic/phosphomolybdenum complex (Folin-Ciocalteu reagent) in an alkaline solution, with the maximum absorption (750nm) determined by the concentration of phenolic compounds spectrophotometrically (158–160).

1.9.2.2 Antioxidant Activity and Assays

An antioxidant is described as "any chemical that considerably slows or prevents oxidation of an oxidizable substrate (proteins, lipids, carbohydrates, and DNA) when presented at low concentrations compared to those of that substrate" (161). Antioxidants' primary role is to protect the body from the damaging effects of free radicals which results in oxidative damage (33). Internal sources such as inflammation, illnesses, or metabolism and external sources such as irradiation, pollution, food and medications are all causes of free radicals within cells and tissues, similarly a reduction in protective capacity can also cause an increase in free radicals (33,162,163).

Total antioxidant capacity (TAC) measures the number of free radicals scavenged by a test solution (162) and it is used to assess the antioxidant ability of biological samples (164). There are various tests that measure total antioxidant capacity and some of them are described in Table 7.

Table 7: Examples of types of antioxidant assays

Antioxidant Assay (Electron transfer-based)	**Principle of the method**	**End-product determination**	**Strengths**	**Limitations**	**References**
DPPH	Antioxidant reaction with an organic radical	Colorimetry	- Simple cheap and fast - Quantifiable antioxidants - Highly sensitive - Reproducible and comparable results - Efficient for	- Radical chromogens dissolve only organic solvents - Poor model for radical quenching in vivo and food samples - Absorbance decreases with exposure to light,	(163,165–167)
ABTS	Antioxidant reaction with an organic radical	Colorimetry	- Radical is stable in both organic and aqueous media as compared to the DPPH radical	- Does not resemble physiological processes - Lengthy process to generate ABTS•+ radical	(158,164,167)

			- Reproducible results - Radical is more stable than DPPH and can be kept for two days in the dark - Good correlation with bioactive compounds (phenolics and flavonoids)		
FRAP	Antioxidant reaction with Fe(III) complex	Colorimetry	- Simple and cheap - Reproducible and sensitive - Screens diverse	- Non-specific	(164,168,169)

			biological samples		
CUPRAC	Cu (II) reduction to Cu (I) by antioxidants	Colorimetry	- Cheap, stable and easily available reagents - Assay performed at neutral pH (pH 7)	- Lengthy and cumbersome process in order for some compounds to develop colour - Does not measure antioxidant enzymes	(158,163,164)

ABTS = 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), DPPH = 1,1-diphenyl-2-picrylhydrazyl, FRAP = Ferric Ion Reducing Antioxidant Power, CUPRAC = Cupric Reducing Antioxidant Capacity

1.9.3 Toxicity of Medicinal Plants and Testing

Toxicity refers to the amount or degree of a substance that must be present in order for it to be poisonous (170). Toxicity is determined by the amount and concentration of the material used, the frequency of usage (acute, subacute or chronic), the interactions of the person receiving the chemical, and the person's particular reaction (171). Toxicity can manifest itself as local, which is limited to the organ that absorbs or eliminate the toxic compound such as liver, kidney, stomach, skin, or lungs as well as systemic, whereby there is organ failure and the potential death of the organism is due to the toxicity as a cellular level (172). Several medicinal plants have been tested for toxicity since some of the have been found to be toxic and several poisoning cases from plants have widely reported (122,132,173,174). Therefore, it has also been recommended that medicinal plants should be subjected to toxicity tests (10). A literature study of the plants in showed that there were no studies that had evaluated these plants for toxicity in Malawi, hence this was recommended as one of the areas for study before others are done since the plants are being used by many people. In addition, toxicity testing is one of the important factors when conducting other studies as a safe dose is needed for the test models such as cells and animals if the efficacy studies are to be successful (175,176). Several approaches have been widely used for studying toxicity of plants and these are generally categorized into *in vitro* and *in vivo* studies (177,178). Various models have also been developed to assist with the interpretation of toxicity data and these include Global Harmonized System of Classification and Labelling of Chemicals (GHS).

1.9.3.1 *In vitro* and *In vivo* Testing

A study that is conducted outside of a living creature is known as an *in vitro* test. Typically, isolated tissues, organs, or cells are used in the test and are carried out in glass containers, petri dishes. An *in vivo* test, on the other hand, is a study that is carried out on living organisms such as rats, Guinea pigs, monkeys as models (179,180). Chemical compounds' intrinsic hazardous qualities can be predicted using both *in vitro* and *in vivo* approaches. *In vitro* data and methods, on the other hand, have limits and are rarely directly applicable to predicting biological reactions of organisms to chemical exposure *in vivo* (177). Screening compounds for *in vivo* testing should be obtained more frequently through *in vitro* procedures as consideration must be given to animal welfare and ethics (181). In this study, the Brine Shrimp Lethality assay was used to screen toxicity. This was followed by an *in vivo* toxicity testing in Wistar rats following the standardized protocols widely reported in literature (153,182–185).

The brine shrimp lethality assay is a single species toxicity test, that is simple, cheap and fast for screening bioactive compounds (186). It is based on the capacity of test compounds to kill brine shrimp (*Artemia salina*) (187). Many studies have used the assay to pre-screen herbal plant extracts for toxicity due to its high degree of repeatability (125,186,188–191). However, the assay has some downfalls such as any change in the standard operating procedure of the assay such as salinity, pH, light, temperature and aeration could affect the outcome of the assay and delay the overall experiment (9,186,192).

1.9.3.2 Global Harmonized System of Classification and Labelling of Chemicals (GHS)

The Globally Harmonized System of Classification and Labelling of Chemicals (GHS) was created to standardize the various hazardous material classification and labeling schemes previously in use by different countries around the world. The GHS focuses mainly on regulating hazard testing standards, universal warning pictograms, and harmonized safety data sheets (193). The main objectives of the GHS are to enhance the protection of human health and the environment by providing an internationally comprehensible system for hazard communication; provide a recognized framework for those countries without an existing system; reduce the need for testing and evaluation of chemicals; and facilitate international trade in chemicals whose hazards have be properly assessed and identifies on an international basis (193).

1.9.3.2.1 Acute Toxicity Based on GHS

The GHS document has four parts: Introduction, Physical hazards, Health hazards and Environmental Hazards (194). This study focused more on Health hazards mainly Acute Toxicity.

Acute toxicity is a health hazard classification that describes the adverse effects of a chemical substance or drug as a result of a single or many exposures during a short period of time (usually within a 24-hour time period) (195–197). It is commonly used as a guiding reference to repeated dose and bioactivity studies (10). Oral, cutaneous, or inhalation exposure are all modes of administration (Table 8) and the consequences of chemical exposure must appear within 14 days to be classified as acute toxicity (194–196). Acute toxicity is measured using LD_{50} value which

is the estimated medium lethal dose of a compounds which results in the death of half of the population which is categorized in different modes of administration (Table 8) (194).

Table 8: GHS dose dependent descriptor for Acute Toxicity - Modes of administration

Method of administration	**Category 1**	**Category 2**	**Category 3**	**Category 4**	**Category 5**
Oral: LD_{50} measured in mg/kg of bodyweight	≤5	>5 ≤50	>50 ≤300	>300 ≤2 000	Criteria • Anticipated oral LD_{50} between 2000 and 5000 mg/kg (>2000 ≤ 5000); • Indication of significant effect in humans; • Any mortality at category 4; • Significant clinical signs at category 4; • Indication from
Dermal: LD_{50} measured in mg/kg of bodyweight	≤50	>50 ≤200	>200 ≤1 000	> 1 000 ≤2 000	
Gas Inhalation: LC_{50} measured in ppmV	≤100	> 100 ≤500	>500 ≤2 500	>2 500 ≤20 000	
Vapour Inhalation: LC_{50} measured in mg/L	≤0.5	>0.5 ≤2.0	>2.0 ≤10	>10 ≤20	
Dust and Mist Inhalation: LC_{50}	≤0.05	>0.05 ≤0.5	>0.5 ≤1.0	>1.0 ≤5.0	

measured in mg/L					other studies; • If assignment to a more hazardous category is not warranted

1.10 Statement of Problem

Traditional medicine usage in sub-Saharan Africa is high and is the most commonly utilized form of medicine (83,84). With an increase in the prevalence of non-communicable diseases (NCDs) in developing countries such as asthma and the perceived failure of modern medicine in curing the diseases (84), majority of the population have preferred to use traditional medicine in managing diseases (85). Whilst the prevalence of asthma is ever increasing and its management is still costly and time consuming due to various reasons, such as inaccessibility to asthma medication, culture, and cost of asthma medication (198–200), it is most likely that the demand for herbal medicines will also increase. However there is minimal scientific research to prove the safety of herbal remedies in the management of asthma (3,4). Furthermore, there is limited data on the toxicity and safety of many medicinal plants used, yet several studies have shown that herbal medicines can also be toxic (119,201–203), sometimes at any concentration or at certain concentrations only (149,174). Furthermore, studies have also shown that herbal medicines toxicity is influenced by the presence of some phytochemicals at certain concentrations (132,203). It is further reported that the presence and concentrations of these phytochemicals can vary from place to place due to differences in weather and soil properties. Hence it is recommended that studies need to be conducted in each locality (204). However, literature search about toxicity of plants used in Malawi is sparse, let alone for the plants studied have been widely used for asthma management. These limitations have led to inadequate specific regulations that target efficacy, quality and safety of traditional medicines in Malawi and Africa (85–87). Hence, there is need to assess their efficacy and toxicity. In such a case, we need to take the idea that safety should be the first to be evaluated than efficacy based on the *'primum non nocere'* ('first, not do harm') (205) since the people are already using it so toxicity and safety

should be prioritized. In addition, safe concentrations are needed when performing efficacy studies. This study is therefore aimed at determining the toxicity and safety evaluation of the herbal extracts of the locally available medicinal plants that are used for the management of asthma.

1.11 Objectives of the study

1.11.1 General objective

To investigate the toxicity and safety of herbal extracts of *Trichodesma zeylanicum (Burm.f.) R. Br, Erythrina abyssinica (Lam. ex DC.)* and *Paederia bojeriana (A.Rich. ex DC.) Drake* on wistar rats after single oral dose delivery

1.11.2 Specific objectives

a. To conduct the phytochemical screening of the herbal extracts of *Trichodesma zeylanicum (Burm.f.) R. Br, Erythrina abbysinica* and *Paederia bojeriana (A.Rich. ex DC.) Drake*
b. To quantify flavonoids and phenolics in the herbal extracts of *Trichodesma zeylanicum (Burm.f.) R. Br, Erythrina abbysinica* and *Paederia bojeriana (A.Rich. ex DC.) Drake*
c. To determine antioxidant activity of the herbal extracts of *Trichodesma zeylanicum (Burm.f.) R. Br, Erythrina abbysinica* and *Paederia bojeriana (A.Rich. ex DC.) Drake*
d. To evaluate the *in vitro* toxicity for *Trichodesma zeylanicum (Burm.f.) R. Br, Erythrina abbysinica* and *Paederia bojeriana (A.Rich. ex DC.) Drake* after single dose delivery in *Artemia salina*

e. To evaluate the *in vivo* toxicity for *Trichodesma zeylanicum (Burm.f.) R. Br*, *Erythrina abbysinica* and *Paederia bojeriana (A.Rich. ex DC.) Drake* after single dose delivery in wistar rats

1.12 Significance of the study

This study will generate data that will be used to suggest whether people are using medicinal plants that are safe for their lives. The study will also assist with the data that will give a preliminary knowledge about the phytochemicals available in the plants present in Malawi, which would assist in further studies on many parameters such as compounds isolation and efficacy studies. It is recommended that during efficacy studies, safe concentrations should be used (206), and this study will also generate data on concentrations that are safe and toxic for the animals and might provide baseline data for further toxicity tests for the same or using the same data for the efficacy studies. Additionally, this study will assist regulatory authorities and other researchers to see if the Malawian plants are comparable to those found in other places out of Malawi or within Malawi where they have also been studied. This would help to know whether the data from those places can be relevant for decision making in Malawi about the plants or not.

CHAPTER TWO: METHODOLOGY

2.1 Study Place

The selected medicinal plants were collected from the fields and forest reserves in Zomba, and Chikwawa districts (Table 5) during the dry cold season; where they were found abundantly. The experiments took place in various laboratories of the Biomedical Science (BMS) department at the Kamuzu University of Health Sciences (KUHeS) formerly College of Medicine in Blantyre. Table 5 below shows the sites where the samples were collected.

Table 9: GPS data and Google map links of places where the study plants were collected

Erythrina abbysinica	***Trichodesma zeylanicum (Burm.f.) R. Br***	***Paederia bojeriana (A.Rich. ex DC.) Drake***
Lat Long: -15.522536 | 35.209941 DMS: 15° 31' 21.13" S | 35° 12' 35.79" E UTM: 737049.10E 8282653.97N 36L MGRS: 36LYH 37049 82654 URL: https://www.google.com/maps/place/-15.522536247113443,35.20994111895561	Lat Long: -15.521955 | 35.209869 DMS: 15° 31' 19.04" S | 35° 12' 35.53" E UTM: 737041.99E 8282718.37N 36L MGRS: 36LYH 37042 82718 URL: https://www.google.com/maps/place/-15.521955084707455,35.2098686993122	Lat Long: -16.000535 | 34.789332 DMS: 16° 0' 1.92" S | 34° 47' 21.59" E UTM: 691469.46E 8230181.17N 36K MGRS: 36KXH 91469 30181 URL: https://www.google.com/maps/place/-16.000534654667433,34.78933151811361

Figure 2 : Images of *Erythrina abbysinica*, *Trichodesma zeylanicum (Burm.f.) R. Br* and *Paederia bojeriana (A.Rich. ex DC.) Drake* respectively

2.2 Ethical Clearance, Data Collection and Methods Used

The study was approved by the College of Medicine Research and Ethics Committee (COMREC) with a clearance certificate number P.11/18/2523.

Data collection occurred in 4 phases:

- Phase 1: Plant Identification and Collection
    - Meeting with National Herbarium and Botanical gardens to identify the places in Malawi where the plants are abundantly found.
    - Visit to the places and GPS and satellite map locations for the various collection points of the plants with on-site photographs and the physical collection of the plants.

- Phase 2: Phytochemical Analysis and Quantification of Some Phytochemicals of the Plant samples
    - Various validated and published tests that measure the qualitative and quantitative properties of the plant samples were done and the tests were repeated three times and all the outcomes recorded (Table 10).
- Phase 3 Antioxidant Activity
    - Various validated methods published in literature were used for antioxidant activity.
- Phase 4: Brine Shrimp Lethality Assay (*In vitro* toxicity assay)
    - Various validated methods published in literature were used for Brine Shrimp Lethality Assay and were repeated three times and all the outcomes recorded (Table 17).
- Phase 5: Acute Oral Toxicity Test (*In vivo* toxicity assay)
    - Validated protocol from OECD was used (Test 423 ((Acute Toxic Class Method)) (Figure 3) (74,207–209)

2.2.1 Materials

Machines: Rotavapor R-100, Buchi, Switzerland; TopMix FB15024, Fisher Scientific, USA; BK-UV1800, Spectrophotometer, China; Axioscope A1 Microscope, Zeiss, Germany; Zeiss Axiocam 105, Zeiss, Germany; Multimode Plate reader, Victor X3, PerkinElmer, USA; PW 254 Balance, AE Adam, South Africa Leica RM; 2245 Biocut microtome, Leica, Nussloch, Germany; Shandon Citadel 1000, Labotek, South Africa; BS 230 Biochemistry Analyzer, Mindray, China

Reagents and Solvents: Potassium mercuric iodide, Dragendoff's reagents, Picric Acid, Benzene, Ammonia 25%, Sulphuric Acid, Chloroform, Sodium Hydroxide, Ferric Chloride, Lead Acetate, Gelatin powder, Sodium Chloride, Paraffin wax, Formaldehyde (37-40%) / formalin – Glassworld, South Africa

Tin powder, Thionyl Chloride, Magnesium powder, Ether, Acetic anhydride, Hexane, Ethyl Acetate, Acetone, Methanol, Ethanol, Hydrochloric acid, DMSO, Acid fuchsin, Eosin Y solution, Acetic acid (glacial), Hematoxylin, Phosphomolybdic acid solution, Phosphotungstic solution, Aniline blue, Mayer's Hematoxylin solution, Xylene – Associated Chemical Enterprises (ACE), South Africa

MN 615, 125mm filter paper, Macherey-Nagel, Germany

2.2.2 Medicinal Plant Sample Collection and Preparation

The study was approved by the College of Medicine Research and Ethics Committee (COMREC) with a clearance certificate number P.11/18/2523. Collection procedure was according to the National Herbarium regulations and comprised of healthy roots of *Trichodesma zeylanicum (Burm.f.) R. Br* and *Paederia bojeriana (A.Rich. ex DC.) Drake* and stem bark of *Erythrina abyssinica (Lam. ex DC.).* Collection of the plants was ecologically safe and without the harm of continual plant growth within the surrounding area. The chosen plants were identified and located with the help of a botany technician from the National Herbarium and Botanical Gardens Office in Zomba, Malawi. Only healthy and clean parts of the species were collected for analysis.

Plants were taken to the laboratory at the KUHeS, cleaned using distilled water and air-dried under shade at room temperature. The dried plant parts were processed into powder using a blender and stored in airtight containers in a cool, dry place, away from direct sunlight. The powder was macerated with 99.9% Ethanol for 48 hours, filtered using MN 615, 125mm filter paper. Filtrate was evaporated using a rotary evaporator to obtain the crude extract. (7,8,210,211).

2.2.3 Preliminary Phytochemical Analysis of Plant Extracts

Plants contain biologically active compounds that affect the physiology of living organisms. In humans, these compounds can have of therapeutic importance in treatment of diseases and nutrition purposes (212). Phytochemical analysis of plants is important to determine the bioactive constituents that occur within different species of plants and aids in understanding the pharmacological basis of the plants (212,213). The tests used in determining the qualitative phytochemical properties of the plants are found Table 10.

Table 10: Qualitative Phytochemical test for Secondary metabolites

Constituents	**Test**	**Expected Observations**
Alkaloids	Mayer's Test Filtrate was treated with Mayer's reagent(Potassium Mercuric Iodide).	Yellow precipitate
	Dragendroff's Test Filtrate was treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide)	Red precipitate
	Wagner's Test Filtrates was treated with Wagner's reagent (Iodine in Potassium Iodide)	Brownish/reddish precipitate
Flavonoids	Alkaline Reagent Test 2ml extracts added 2ml aqueous NaOH solution	Yellow colour appears
Glycosides	Bontrager's Test	Rose-pink/violet colour

	1. 3ml extract + 3ml benzene + 5ml NH_3(10%)	appears in ammonical layer indicates presence Anthraquinone glycosides
	Keller-Killani Test 0.5g extract + 5ml H_2O + 2ml acetic acid with drops of ferric chloride + 1ml H_2SO_4	
Saponins	Foam Test a) 5ml extract + 5ml H_2O + Heat	Froth appears
Phytosterols (steroids)	Salkowski's Test 2ml $CHCl_3$ + 2ml H_2SO_4 (conc.)	Brown ring at the junction appears
	Libermann-Burchard's Test 2ml extract + 2ml $CHCl_3$ + 2 drops of H_2SO_4 added from the side of test tube	Red, then blue and green colouration

Phenols	Ferric Chloride Test 2ml extract + 2ml H_2O + 2-3 drops $FeCl_2$ (5%)	Green precipitate
Tannins	Gelatin Test 2ml extract + 2ml H_2O + 1% gelatin solution with 10% NaCl	White precipitate

2.2.4 Quantitative Phytochemical Analysis

Quantitative tests measure the concentration of flavonoids and phenolics in *Trichodesma zeylanicum (Burm.f.) R. Br, Erythrina abyssinica (Lam. ex DC.)* and *Paederia bojeriana (A.Rich. ex DC.) Drake*. In this study the total flavonoid content and phenolic content were measured using the assays below.

2.2.4.1 Aluminum Chloride Assay for Total Flavonoid Content in crude extracts

The Aluminum chloride assay is a common technique used to measure the total amount of flavonoids in a given sample. The aluminum reacts with the flavonoids to create a complex which can be detected spectrophotometrically (155).

This experiment used 100 grams of the extracts, which were weighed using an analytical balance and dissolved in 2ml of analytical grade methanol (80% v/v). The mixtures were made up to 20ml with distilled water and vortexed to mix well. The extracts (1ml) were transferred into fresh falcon tubes and further diluted 10-fold with distilled water. Then 1ml of the diluted extracts were added into fresh falcon tubes in which 1ml of 2% aluminum chloride was added and vortexed. The mixtures were then incubated at room temperature for 30 minutes. Then 300uL was added into generic 8x12 size plate and absorbance was measured using UV-Vis Spectrophotometer at 415 nm with all results being performed in triplicate. The standard calibration curve was prepared by using different concentrations of quercetin as a standard (155,214,215). The standard calibration curve was used to interpolate and determine the concentrations of the test compounds.

2.2.4.2 Folin – Ciocalteu assay for Total Phenolic Content in crude extracts

Folin - Ciocalteu assay is a standardized method of approximating the total amount of phenolics present in a given sample (216). The method involves the formation of a blue chromophore from the mixture of phenols, reagent and alkaline solution which is measured spectrophotometrically (160,217).

This experiment used 100 grams of the samples weighed and dissolved in methanol (80% v/v, 2ml). The mixtures were made up to 20ml with distilled water vortexed to mix well. 1 ml of the extracts was transferred into another falcon tube and diluted 10-fold with distilled water. Then 1ml of the diluted extracts were added into another falcon tubes in which 10-fold diluted Folin - Ciocalteu reagent (5ml) was added followed by addition of sodium carbonate (1M, 4ml) to make 20-fold dilution of the extracts. This was done within 3 - 8 minutes and the mixtures were vortexed and left for 2 hours. Then 300uL was added into generic 8x12 size plate and absorbance was measured using UV-Vis Spectrophotometer at 760nm, with all results being performed in triplicate. The standard calibration curve was prepared by using different concentrations of gallic acid as a standard (97,157,218). The standard calibration curve was used to interpolate and determine the concentrations of the test compounds.

2.2.5 Anti -oxidant activity Assays

2.2.5.1 DPPH Free Radical Scavenging Assay

DPPH (2,2-diphenyl-1-picryl-hydrezyl) Free Radical Scavenging Assay was one of the methods used in order to measure the reducing ability of DPPH when coupled together with antioxidants

through the transfer of a hydrogen atom. Antioxidants found in the herbal extracts cause a colour change from purple to yellow (167,215).

The solution of DPPH radical is prepared by dissolving 39.432mg DPPH into 100ml methanol. Then 0.1 mL of the extract solution was then added to 3.9ml DPPH solution and vigorously shaken and kept in darkness for 30 minutes at room temperature. The absorbance was measured at 517 nm against methanol using the spectrophotometer with all results being performed in triplicate. Percentage inhibition was used to determine scavenging capability of DPPH using the following formula; DPPH Scavenged (%) = ((AB–AA)/AB) ×100 where, AB is absorbance of the control; AA is absorbance of the sample (211,219).

2.2.5.2 Ferric Reducing Antioxidant Power Assay

Ferric reducing antioxidant power (FRAP) is a method that measures the reducing ability of antioxidants that are present in the sample through the reduction of the Fe^{3+} TPTZ (2,4,6-Tripyridyl-S-triazine) complex which is colourless to Fe^{2+} TPTZ which is blue in color caused by antioxidants found in the extracts (166,214).

Preparation of FRAP reagent is done by in three steps, the first step is making the acetate buffer by dissolving 455.30mg of anhydrous sodium acetate in 50mL of distilled water and then adding 3.97mL of glacial acetic acid with a final dilution into 250mL. The second step is making the TPTZ solution by dissolving 156.20mg of TPTZ in distilled water, adding 0.17mL of concentrate hydrochloric acid and further diluting it into 100mL of distilled water. The third step is making the iron chloride solution by diluting 270.03mg iron chloride hexahydrate into 50 mL of water.

Finally, a mixture of 300mM acetate buffer, 10mM of TPTZ in 40mM of HCl and 20mM of Iron chloride hexahydrate in a ratio of 10:1:1 at room temperature is done as the final preparation of the FRAP reagent (167,169,219).

Then 0.2mL of the diluted extracts to 6mL of FRAP reagent and then vigorously shaken and incubated at room temperature for 10 minutes. The absorbance was measured at 593nm against a reagent blank using a spectrophotometer with all the results being performed in triplicate. The standard calibration curve was prepared by using different concentrations of Trolox as a standard and FRAP values were expressed as mg of Trolox equivalent per gram of sample (158). The standard calibration curve was used to interpolate and determine the concentrations of the test compounds.

2.2.5 Toxicity testing of Herbal Extracts

This test involved the determination of the tolerable concentration(s) of the herbal extracts on the test animals. This is so since we cannot rely on other studies concentrations entirely because toxicity of a chemical depends on many factors, one of which is intrinsic properties that may vary from chemical to chemical. In addition, composition of herbal products can vary place to place or plant to plant as already started earlier (68). Hence the tests were conducted as follows:

2.2.5.1 *In vitro* Toxicity and Safety of the Herbal Extracts Using Brine Shrimp Lethality Assay

One of the validated assay used to test *in vitro* toxicity of the herbal extracts is the Brine Shrimp Lethality assay (BSLA). The assay provides broad spectrum preliminary toxicity screening and

is an effective predictor of bioactivity and commonly used to test cytotoxicity and pesticide activity of medicinal plants (9,220).

2.2.5.1.1 Procedure

Firstly, a stock solution of the herbal extracts was prepared whereby 4 milligrams of crude extract was diluted in 4 mL of 0.5% Dimethyl sulfoxide (DMSO), which gives a concentration of 1 milligram per mL (1000µg/mL). Serial dilution was done by taking 1 mL from the stock solution and adding 9mL of 0.5% DMSO. In order to obtain the lower concentrations, 1 mL was taken from the previous concentration and 9 ml of 0.5% DMSO was added, the process was repeated until the to prepare other test concentrations.

The process started by hatching the shrimp eggs. This was done by incubating the eggs under a fluorescent light (60-watt bulb) for 48 hours in saline solution prepared from table salt (38grams in 1-liter of distilled water) in a 2-liter rectangular container. The eggs hatched into larvae (nauplii) and were ready for tests. Ten (10) larvae were placed into test tubes using pipettes and three test tubes were used for each plant making three replicates (30 larvae per herbal extract). The herbal extracts were added to the test tubes in concentrations of 1, 10, 100 and 1000 µg/mL. Positive control (potassium dichromate) and negative control (saline solution) were prepared as per standard protocol (9) The test tubes were checked after 24 hours to check the number of live nauplii in each test tube, and the figures recorded. The results were plotted on a chart and lethality concentration (LC_{50}) was calculated; the mortality of the nauplii was calculated in percentages (7–9,220). For each of the concentrations, a positive and negative control (saline solution) was prepared. For each test and control, there were at least 2 replicates.

The results of the BSLA were used in the evaluation of the toxicity and safety of the herbal extracts using Clarkson toxicity criteria and Meyer's toxicity index (125). Meyer's toxicity index classifies extracts of LC_{50} <1000µg/ml as toxic whilst extracts of LC_{50} > 1000µg/ml as non-toxic. Clarkson toxicity criteria further groups the extracts into detailed classification such as LC_{50} > 1000µg/ml as non - toxic, 500 – 1000 µg/ml as of low toxicity, 100 - 500µg/ml as of moderate toxicity and 0 - 100µg/ml as having high toxicity (186,221) .

2.2.5.2 *In-vivo* Toxicity and Safety of the Herbal Extracts Using Wistar rats; Acute Oral Toxicity Test

The Acute oral toxicity test is a testing procedure that consecutively evaluates the toxicity of chemicals using the lowest number of animals per group at pre-defined doses in order to assess the test substance (196).

This is a stepwise method with the use of minimal numbers of animals in each step, being orally subjected to herbal extracts at single set doses of 300mg/kg and 2000mg/kg (222,223). The test substances can be administered through many routes, but in this study, the oral route was used at a defined dose to a group of animals, the animals were monitored over a period of 14 days (196,224). The addition of consequent doses is determined by the mortality in the previously dosed group (225,226). In this experiment, general observations particularly behavior, changes in skin, fur and eyes, biochemical, histopathological and weight analysis were carried out and compared between the control group and test groups.

2.2.5.2.1 Procedure

The animals used were female Wistar rats that were nulliparous, non-pregnant and 8 to 12 weeks old. The animal room was 22^{o}C with relative humidity at 30%; the lighting was artificial with alternating 12-hour light and 12-hour dark durations. In terms of food, rat chow and access to water was available *ad libitum.* Rats were randomly selected, grouped marked for individual identification and caged as per dose.

Herbal extracts were administered in a constant volume over two fixed dose levels, 300 and 2000mg/kg body weight and negative control of 0.5% DMSO (196,227,228) and three animals were used for each dose level as shown in Figure 3. The rats were fasted overnight however water was *ad libitum*. After the fast, the rats were weighed and extract was administered in a single dose by gavage using an intubation cannula after which food was withheld for a further 3-4 hours (196,228). After dosing the rats were observed once at the first 30 minutes then at 4th hour and daily over the span of 14 days.

Observations were made for signs of toxicity, which include changes in behavior, changes in skin and fur and eyes and mucosal membranes. All observations were recorded for each individual rat. Any animals found in severe distress and pain and morbid condition, were euthanized (196,226,229).

After the span of 14 days, the rats were euthanized. Gross necroscopy and microscopic examination was done, and pathological changes recorded for each animal.

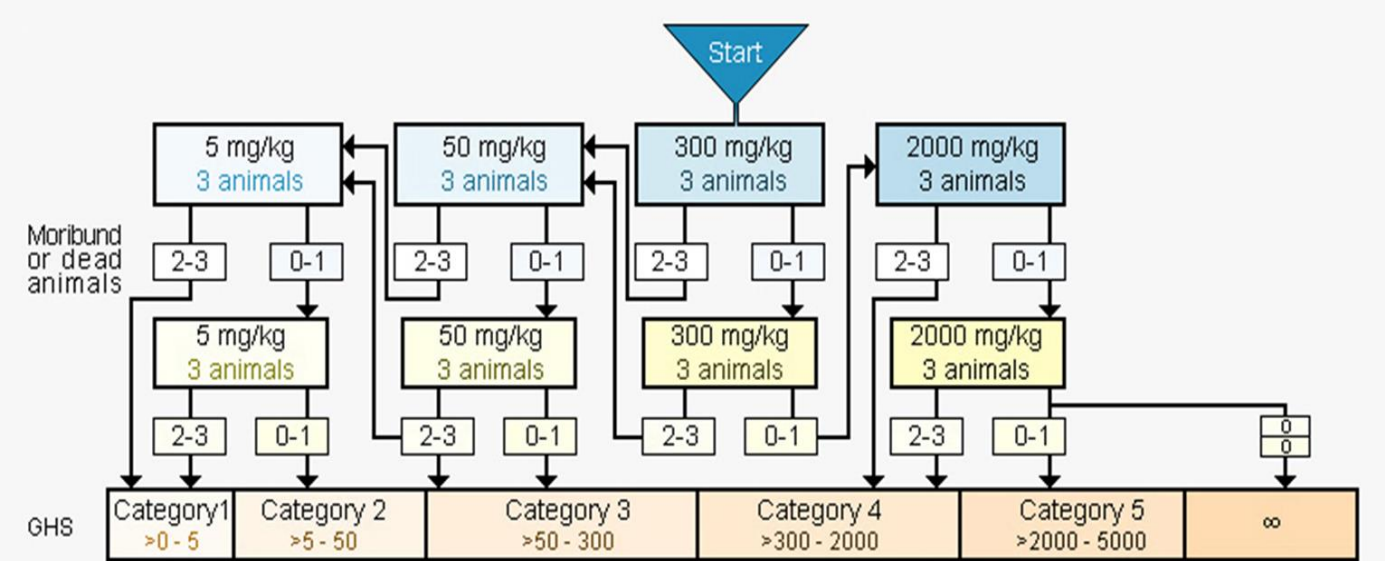


Figure 3: Organization for Economic Co-operation and Development (OECD), Acute Toxicity Study (423) Chart (230)

The results of the acute toxicity test were evaluated using the Global Harmonized Classification System summarized in Figure 2. Substances with relatively low toxicity were grouped under Category 5 (> 2000 – 5000mg/kg body weight whilst substances with relatively high toxicity were grouped under Category 1 (> 0 – 5 mg/kg body weight) (193). The crude plant extracts were assigned a category based on the number of rats that died in the fixed dosed group (group of 3). If 2 to 3 rats died in the group then the dosage was reduced and tested on a new group of rats. If 1 or no rat died then the plant was assigned to that category (193,231).

2.2.5.3 Tissue Preparation and Scoring

2.2.5.3.1 Dehydration, Embedding and Sectioning of tissues

Tissues were cut at 3 mm thickness, then placed in cassettes, which were then immersed in 10% buffered formalin for forty - two hours and then dehydrated through a graded concentration of ethanol series. The tissues were immersed in 70% ethanol for one hour in 2 consecutive baths, 95% ethanol for two hours in 2 consecutive baths and 100% ethanol for two and a half hours for 2 consecutive baths and absolute alcohol for thirty minutes for 2 final consecutive baths. The tissues were cleared by being soaked in xylene for two hours for 2 consecutive baths all of which was done at room temperature (232). Finally, the tissues were embedded in paraffin wax for two and a half hours in 2 consecutive baths at 51 degrees Celsius. After embedding the tissue in paraffin wax, thin sections/ribbons (5µm thick) were cut using a microtome. Slides were wiped and labelled prior to mounting. The wax section was dropped into the water bath (temp. at 40 degrees Celsius filled with distilled water) and picked up by the slide with the aid of brush mounting the tissue on the slide (233).

2.2.5.3.2 Dewaxing and Hydrating the paraffin wax sections

The slides were placed in the staining rack and the reversal series of dewaxing and rehydrating began. They were immersed in xylene for five minutes for 2 consecutive baths then transferred into absolute alcohol for thirty seconds whilst agitating the rack for 2 consecutive baths, further immersed in 95% ethanol for thirty seconds for 2 consecutive baths and finally rinsing the slide in running water for two minutes (233).

2.2.5.3.3 Hematoxylin and Eosin staining of slides

After the slides were deparaffinized and rehydrated, they were dipped into hematoxylin for five minutes, and washed in tap water till blue stain stops coming off, then dipped in 1% acid alcohol 3 times, the slides were washed under running water for five minutes and counter stained with eosin for one minute and briefly washed in running water. They were dehydrated through graded series of alcohol (90%, 95% and absolute alcohol for 1 minute for 2 consecutive baths) and cleared with xylene for one minute for 3 consecutive baths. The cover slips were mounted on the slides and left to dry at room temperature (232).

A total of 20 images per animal were assessed to generate the results. The observed fields were randomly selected and assessed by three independent histologists. Light microscopic examinations were done on the liver and kidneys in order to identify structural changes caused by the herbal plant extracts. The effect of the herbal extracts was measured using semiquantitative histologic scoring systems (234) adapted from previous studies on the liver (235) and kidneys (236) (Table 11).

Statistical Analysis

Data were expressed as means ± standard deviations and differences were considered statistically significant when $p<0.05$. Data were analyzed using Students' T-test and one-way ANOVA using Microsoft Excel and GraphPad Prism.

Table 11: Histological grading parameters of the rat liver and kidney

Liver	**Kidney**
Parameters	Parameters:
(1) Hepatocellular necrosis or degeneration	(1) Tubule interstitial leukocytic infiltration or nephritis in cortex and medulla
(2) Sinusoidal congestion	(2) Widening of the urinary space
(3) Portal triad and lobular inflammation	(3) Tubular epithelial degeneration (necrosis with dark acidophilic cytoplasm; acellular sections of tubules; loss of tubular epithelial cells into tubular lumen)
(4) Cytoplasmic vacuolization	(4) Congestion of glomeruli
	(5) Podocyte hyperplasia
	(6) Diffuse cortical and medullary regions

Histological grading: 0 = normal histological tissue; 1 = mild (1-10%); 2 = moderate (11-25%); 3 = severe (26-50%); 4 = very severe (51-100%)

CHAPTER THREE: RESULTS AND DISCUSSIONS

In this section, results for the analyses conducted on the widely used medicinal plants in Malawi for asthma management namely *Trichodesma zeylanicum (Burm.f.) R. Br , Erythrina abyssinica (Lam. ex DC.)* and *Paederia bojeriana (A.Rich. ex DC.)* are presented. The first section presents the results and discussion of phytochemical analysis of the medicinal plants, dwelling on how the presence or absence of some phytochemicals relates to the potential uses of the medicinal plants for asthma as well as toxicity and safety of the medicinal plants. The second part focusses on the quantification of the phytochemicals and how they relate to the toxicity of the studied medicinal plants. The third part focuses on the antioxidant activity of the plants and how they relate to the potential efficacy against the asthma disease as well as toxicity and safety of the plants. The fourth section deals with the *in-vitro* and *in-vivo* toxicity of the medicinal plants. The fifth part relates the phytochemical presence and the toxicity results found. The sixth part of the report summarizes the results of the whole study**.**

3.1 Qualitative and Quantitative Phytochemical Analysis of Plant Extracts

In this section, the results and discussion of the presence of secondary metabolites and quantity of phenolics and flavonoids was analyzed, in order to determine the anti-oxidant activity of *Trichodesma zeylanicum (Burm.f.) R. Br , Erythrina abyssinica (Lam. ex DC.)* and *Paederia bojeriana (A.Rich. ex DC.).*

3.1.1 Results on the Qualitative and Quantitative Phytochemical Analysis

3.1.1.1 Qualitative Phytochemical Analysis

The results showed that *Erythrina abyssinica (Lam. ex DC.)* (stem bark) and *Trichodesma zeylanicum (Burm.f.) R. Br* (root) had flavonoids, phenols, tannins, saponins and phytosterols while *Paederia bojeriana (A.Rich. ex DC.)* (root) had phenols, tannins, saponins, glycosides and phytosterols. None of the test plant crude extracts had alkaloids. Table 12 below summarizes the results of the tests.

Table 12: Qualitative phytochemical analysis for the ethanolic extracts of *Trichodesma zeylanicum (Burm.f.) R. Br, Erythrina abyssinica (Lam. ex DC.) and Paederia bojeriana (A.Rich. ex DC.)*

Secondary Metabolites	***Trichodesma zeylanicum (Burm.f.) R. Br* (root)**	***Erythrina abyssinica (Lam. ex DC.)* (stem bark)**	***Paederia bojeriana (A.Rich. ex DC.)* (root)**
Alkaloids	-	-	-
Flavonoids	+	+	-
Phenols	+	+	+
Tannins	+	+	+
Saponins	+	+	+
Glycosides	-	-	+
Phytosterols	+	+	+

+ = presence; - = absence

3.1.2 Quantitative Phytochemical Analysis

3.1.2.1 Total Flavonoid Content of the crude extracts

The total flavonoid content was quantified spectrophotometrically by the using Aluminum Chloride assay in a Victor X3, PerkinElmer, USA. Firstly, a calibration curve was produced from different known concentrations of quercetin reference standard and their corresponding absorbance values were used to calculate the concentrations. The results were used to derive a standard curve that showed a good linear relationship with the regression equation of absorbance versus concentration of quercetin (0 - 300µM) solution (Figure 4) which was expressed in quercetin equivalents in mg per gram of extract. Then the concentrations of the test samples were derived from the calibration curve after extrapolating their absorbance from the calibration curve.

The results showed that there was a significant difference between the total flavonoid content in all the extracts dissolved in 99% Ethanol and those dissolved in 0.5% DMSO with p values <0.05. Table 13 provides the summary of the total flavonoid content found in both groups for the three crude plant extracts.

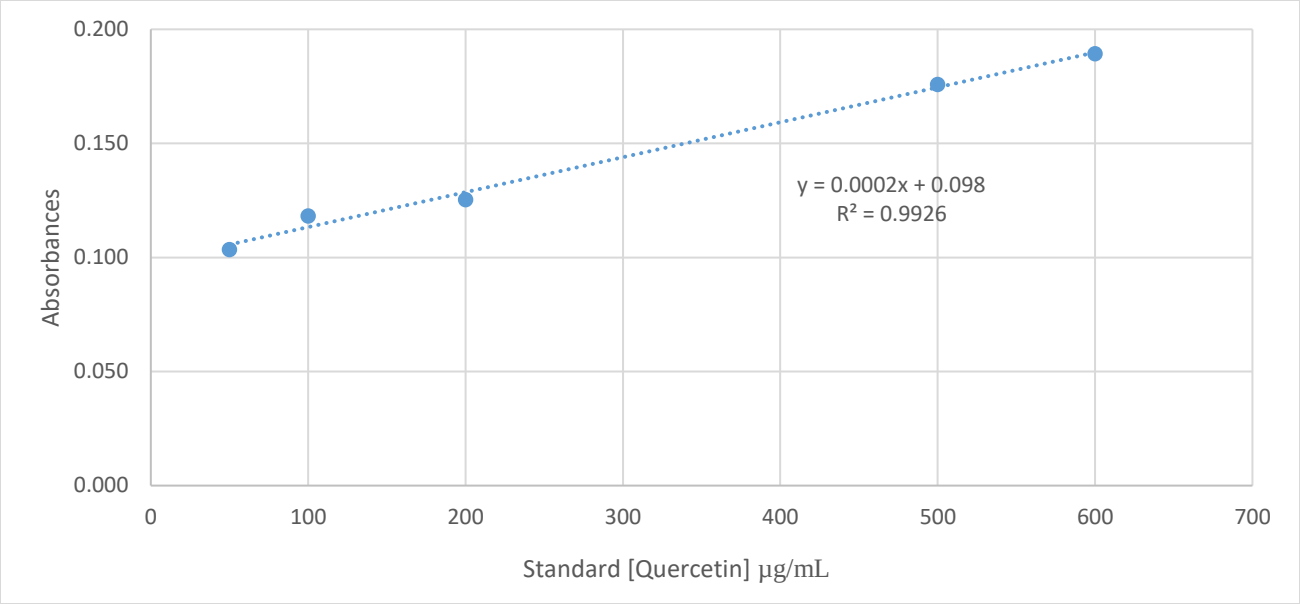


Figure 4: Calibration curve for determination of Flavonoid content using Quercetin as a standard

Table 13: Total flavonoid content for ethanolic extracts of *Trichodesma zeylanicum (Burm.f.) R. Br*, *Erythrina abyssinica (Lam. ex DC.)* and *Paederia bojeriana (A.Rich. ex DC.)*

Plant	**Total Flavonoid content (QE/mg)**		
	Dissolved in 99% Ethanol	**Dissolved in 0.5% DMSO**	**P -value**
Erythrina abyssinica (Lam. ex DC.)	806.12±0.01	436.52±0.01	1.16×10^{-27}
Trichodesma zeylanicum (Burm.f.) R. Br	114.96±0.02	163.83±0.01	1.06548×10^{-13}
Paederia bojeriana (A.Rich. ex DC.)	64.37±0.06	13.49±0.01	5.52085×10^{-28}

3.1.2.2 Total Phenolic Content of the crude extracts

The total phenolic content was quantified spectrophotometrically by combining Folin-Ciocalteau reagent, Gallic acid and Sodium Carbonate respectively, then incubate the solution for two hours at room temperature followed by spectrophotometric analysis at an absorbance of 760nm. The results derived from the standard curve showed a good linear relationship with the regression equation of absorbance versus concentration of Gallic acid (0 - 300µM) solution (Figure 5) which was expressed Gallic acid equivalents (GAE) in milligrams per gram dry weight. There was no significant difference between the percentage inhibition of DPPH radical in the *Trichodesma zeylanicum (Burm.f.) R. Br* and *Paederia bojeriana (A.Rich. ex DC.)* extracts dissolved in 99% ethanol and those dissolved in 0.5% DMSO however, there was significant difference in *Erythrina abyssinica (Lam. ex DC.)* extracts dissolved in 99% ethanol and those dissolved in 0.5% DMSO with p values shown in Table 14. Table 14 provides the summary of the total phenolic content found in both groups for the three crude plant extracts.

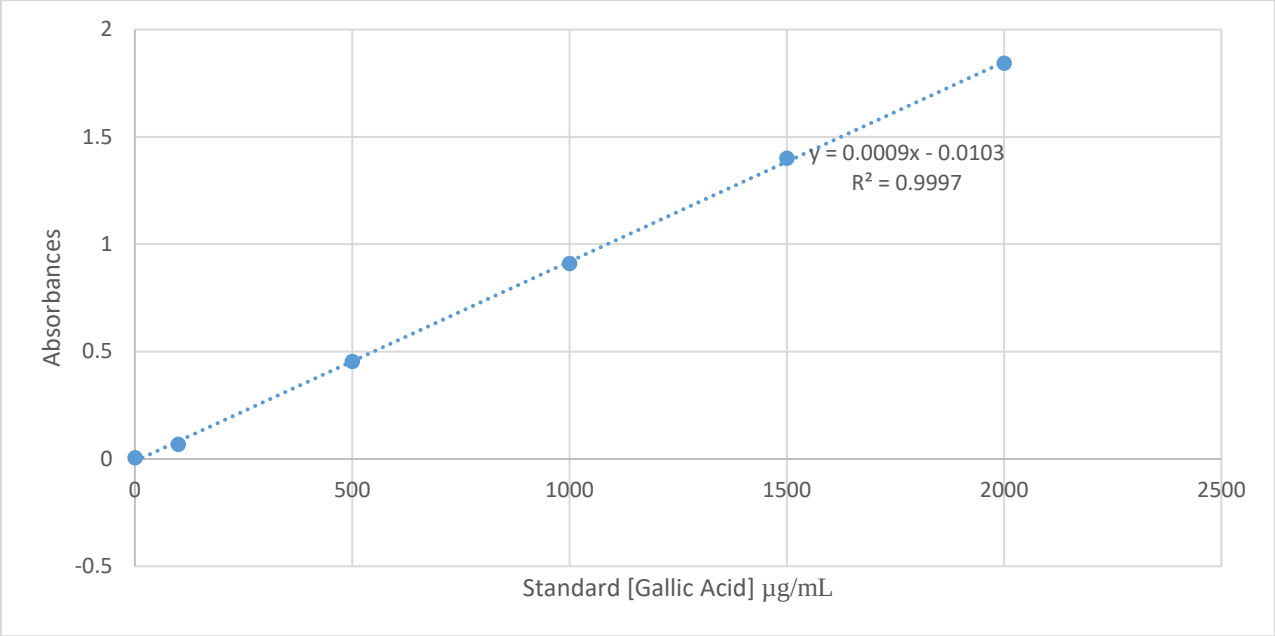


Figure 5: Calibration curve for determination of Phenolic content using Gallic acid as a standard

Table 14: Total phenolic content for ethanolic extracts of *Trichodesma zeylanicum (Burm.f.) R. Br*, *Erythrina abyssinica (Lam. ex DC.)* and *Paederia bojeriana (A.Rich. ex DC.)*

Plant	**Total Phenolic content (mg GAE/g)**		
	Dissolved in 99% Ethanol	**Dissolved in 0.5% DMSO**	**P-value**
Erythrina abyssinica (Lam. ex DC.)	98.48±0.08	109.63±0.02	0.30
Trichodesma zeylanicum (Burm.f.) R. Br	43.37±0.02	41.55±0.03	0.58
Paederia bojeriana (A.Rich. ex DC.)	-1.67±0.01	-22.60±0.2	0.33

3.3 Anti-oxidant Activity Assays

3.3.1 Percentage inhibition of DPPH Radical of the crude extracts

The DPPH Free Radical Scavenging Assay quantified the reducing ability of DPPH when coupled together with antioxidants through the transfer of hydrogen. There was no significant difference between the percentage inhibition of DPPH radical in the all the plant extracts dissolved in 99% ethanol and those dissolved in 0.5% DMSO with p-values shown in Table 11.

Table 15: Percentage inhibition of DPPH radical (%) of ethanolic extracts of *Trichodesma zeylanicum (Burm.f.) R. Br , Erythrina abyssinica (Lam. Ex DC.)* and *Paederia bojeriana (A.Rich. ex DC.)*

Plant	**Percentage inhibition of DPPH radical (%)**		
	Dissolved in 99% Ethanol	**Dissolved in 0.5% DMSO**	**P-value**
Erythrina abyssinica (Lam. Ex DC.)	86.59 ± 0.008%	74.09 ±0.002%	0.03
Trichodesma zeylanicum (Burm.f.) R. Br	66.18±0.03%	70.33±0.02%	0.40
Paederia bojeriana (A.Rich. ex DC.)	82.85±0.07%	80.68±0.04%	0.80

3.3.2 Ferric Reducing Antioxidant Power of the crude extracts

Ferric reducing antioxidant power quantified the reducing ability of antioxidants that are present in the crude extracts through the reduction of the Fe^{3+} TPTZ complex to Fe^{2+} TPTZ caused by antioxidants. The results derived from the standard curve showed a good linear relationship with the regression equation of absorbance versus concentration of Trolox (0 – 300µM) solution (Figure 6) which was expressed Trolox equivalent antioxidant capacity (TEAC) mg/g dry weight. There is no significant difference between the antioxidant potential in all the plant extracts dissolved in 99% ethanol and those dissolved in 0.5% DMSO with p values shown in

Table 16 and the antioxidant potential in between the plant extracts dissolved 99% ethanol and 0.5% DMSO is significantly different with the p value <0.05.

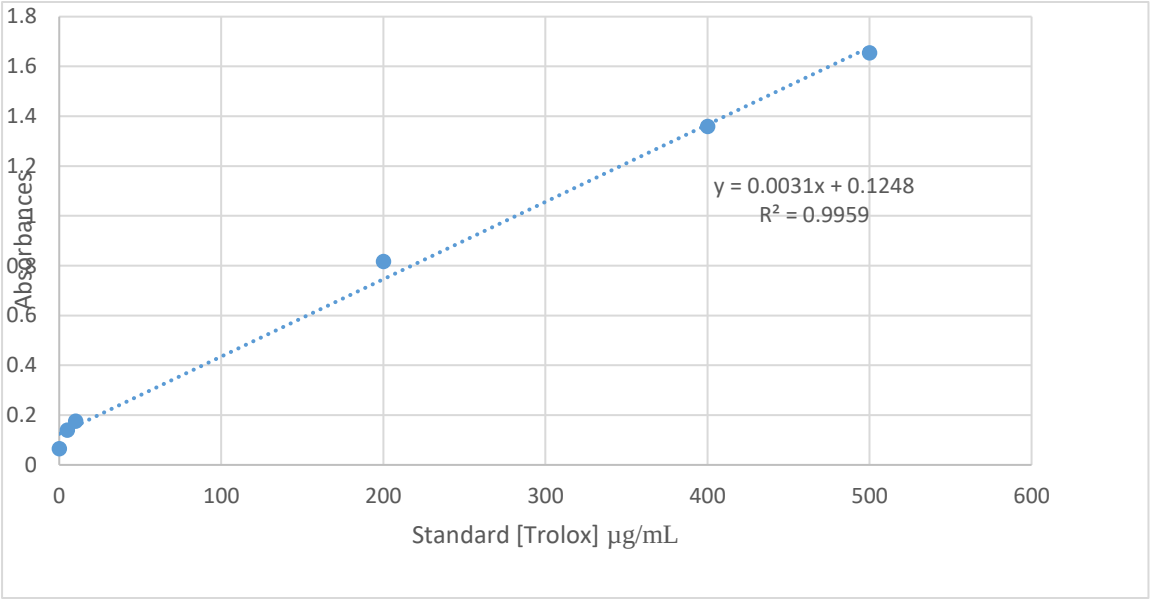


Figure 6: Calibration curve for determination of Ferric reducing antioxidant power using Trolox as a standard

Table 16: Ferric reducing antioxidant power of ethanolic extracts of *Trichodesma zeylanicum (Burm.f.) R. Br , Erythrina abyssinica (Lam. Ex DC.) and Paederia bojeriana (A.Rich. ex DC.)*

Plant	**FRAP (mg TAEC/g)**		
	Dissolved in 99% Ethanol	**Dissolved in 0.5% DMSO**	**P-value**
Erythrina abyssinica (Lam. Ex DC.)	21.09±0.03	14.02±0.11	0.06
Trichodesma zeylanicum (Burm.f.) R. Br	21.85±0.08	8.07±0.01	0.09
Paederia bojeriana (A.Rich. ex DC.)	9.42±1.00	6.00±0.08	0.23

3.2.4 Discussion on the Qualitative and Quantitative Phytochemical Analysis

The basis for tackling the presence of the phytochemicals is that some studies have shown that the presence of phytochemicals could be an indicator of the potential for a particular bioactivity or toxicity (117).

Erythrina abyssinica (Lam. Ex DC.) contained a wide range of phytochemicals which included flavonoids, phenols, tannins, saponins, phytosterols (Table 12) which were similar results found in studies by Musyoka et al. (152), Marume et al. (214) and Orwa et al. (101). However, these studies recorded methanolic extracts of *Erythrina abyssinica (Lam. Ex DC.)* that showed the presence of alkaloids of which was not present in this study, this could be due to the type of

solvent (ethanol) used to extract the secondary metabolites (237). *Erythrina abyssinica (Lam. Ex DC.)* showed to have a high flavonoid and phenolic content as compared with the other crude extracts as well as notable antioxidant capacity with immense ability to scavenge free radicals and reduce oxygen species (Table 9; Table 10). These findings were also reported by Kamadyaapa et al. (238), whereby the ethanolic leaf extract of *Erythrina abbysinica* had a notable free radical scavenging activity; Marume et al. (214) showed that the bark and leaves of *Erythrina abbysinica* had moderate amounts of flavonoid and phenolic content and potent DPPH scavenging activity. A further analysis in the study showed that the extract contributed to accelerated wound healing (EC_{50} values of 41.30 µg/ml and 75.57 µg/ml) which was also in part due to the anti-inflammatory properties found in the plant. The aqueous extract of *Erythrina abbysinica* has shown to have anti-inflammatory properties against meningoencephalitis in chronic *Trypanosoma brucei brucei* mouse model by the reduction of lymphocytic infiltration, perivascular cuffing and astrocytosis (239). Ethanolic and ethyl acetate extracts of *E. caffa, E. lysistemon, E. 73driamyci* had high cyclooxygenase inhibiting activity that reduced synthesis of prostaglandins(240), *E. variegate* had phospholipase A_2 inhibiting activity (241), and *E. sigmoidea* reportedly reduced 5-lipoxygenase and which reduced oedema formation in the induced mouse paw oedema assay (156). *In vitro* activity of erycristagallin from *Erythrina mildbraedii* inhibited 5-lipoxygenase pathway in rat polymorphonuclear leukocytes and reduced *in vivo* phospholipase A(2)-induced mouse paw oedema and mouse ear edema induced by tetradecanoylphorbol 13-acetate (110). These compounds have also been isolated from *Erythrina abyssinica (Lam. Ex DC.)* and could contribute to the extracts anti-oxidant properties and traditional use for anti-inflammatory and anti-asthmatic properties through the reduction of edema, reduction in the influx of inflammatory cells and potent inflammatory mediators which

promote bronchoconstriction and airway hyperresponsiveness. The *Erythrina* genus is reported to be rich in alkaloids and flavonoids that have potent anti-inflammatory and anti-asthmatic properties (242) with 106 flavonoids isolated from *Erythrina abbysinica* (45,131,243).

Trichodesma zeylanicum (Burm.f.) R. Br contained a similar secondary metabolite composition to *Erythrina abyssinica (Lam. Ex DC.)* which came in second with a moderate amount of flavonoid and phenolic content and exhibits high antioxidant and free radical scavenging activity (Table 9; Table 10). The findings are similar to a study done by Ngonda (97), in which the methanolic extract of *Trichodesma zeylanicum (Burm.f.) R. Br* had flavonoid content of 6.28mg/gram of dried extract equivalent to phloroglucinol, the hydrogen peroxide scavenging activity of 74.82% , showing that the plant had significant free radical scavenging and antioxidant activity which improved wound healing. Ethanolic leaf extracts of *Trichodesma khasianum,* a plant of the same genus also showed high flavonoid and phenolic content with potent antioxidant activity which reduced inflammatory parameters such as nitric oxide and prostaglandin E_2 (PGE_2) in lipopolysaccharide (LPS)-induced RAW264.7 cells (111). Different extracts of *Trichodesma indicum* from multiple solvent systems (n-hexane, ethyl acetate, ethanol, and water) had notable amounts of flavonoids (ethylacetate extract, 52.48 μg of GAE/mg) and phenolics (ethylacetate extract, 52.48 μg of RUE/mg) with potent antioxidant activity(159). Chloroform extract of *Trichodesma indicum* showed significant anti-inflammatory activity in acute and chronic inflammatory models against carrageen, dextran, histamine and serotonin induced edema (244). The methanol extract of *Trichodesma indicum* also showed significant inhibition of cough in the cough reflex induced by sulphur dioxide in mice which persisted for 90 minutes after ingestion of the extract (245). Extracts of *Trichodesma indicum* showed

antispasmodic activity on rabbit jejunum by reducing intestinal contractions and moderately inhibited *in vitro* lipoxygenase activity (246). The *Trichodesma* genus shows high number of alkaloids, flavonoids, steroids and phenolics and significant anti-oxidant and free radical scavenging activity using various solvent extracts (111,247). These results support its traditional use in the management of asthma through the reduction of edema and potent inflammatory mediators which promote bronchoconstriction and airway hyperresponsiveness.

Paederia bojeriana (A.Rich. ex DC.) Drake contained the negligible amount of phenols and low amount of flavonoids but had a high DPPH scavenging activity compared to the other crude extracts in this study, which could be explained by the solubility of the compounds in solvents. The soluble phenolics are easily extracted by a solvent compared to bound phenolics, which are covalently bound within the plant structure (248). Sricharoen et al. (168) and Eroglu & Girgin (249) found that DMSO provided a good environment for phenolic activity, which could explain the low amounts of phenolics and high antioxidant activity in this study.

There were no phytochemical studies found for *Paederia bojeriana (A.Rich. ex DC.) Drake* in the literature at the time of this study report. However, these results reflect those of Osman et al (250) on the methanolic extract of leaves of *Paederia foetida* (same genus) contained high levels of antioxidant activity (ABTS scavenging activity of dry leaves 67.74% and fresh leaves 75.38%) but it had a high amount of phenols and flavonoids. This was also similar to investigations by Upadhyaya (112) on the ethanolic extracts of the leaves of *Paederia foetida* which showed 74.7% – 88.2% DPPH scavenging activity with high levels of phenols and flavonoids. An *in vitro* and *in vivo* study showed that treatment with *Paederia foetida* reduced

paw edema, arthritic index induced by Complete Fruend's Adjuvant(CFA) in the knee joints and suppressed prostaglandin E_2 and COX – 2 expression (251). A poly herbal formulation containing *Paederia foetida* showed significant reduction in carrageenan induced paw edema and potent analgesic activity (252). Similarly, extracts of *Paederia scandens* have been found to suppress pro-inflammatory mediators(TNF-α and IL-1β) in MSU crystals-induced gouty arthritis rats in synovial tissue (253). Iridoid glycosides of *Paederia scandens* have shown suppressed NF-κBp65, MCP-1, α-SMA, TNF-α and TGF-β1 activity and expression in renal tissue in uric acid nephropathy induced rats (202). The *Paederia* genus has shown to have impressive anti - inflammatory and immunomodulatory effects which corresponds to their traditional use in the management of asthma.

3.4 *In vitro* and *In vivo* Toxicity Testing

3.4.1 Results on *In vitro* and *In vivo* Toxicity Testing

3.4.1.1 *In vitro* Brine Shrimp Lethality Assay

The BSLA was used to determine the toxic potential of *Paederia bojeriana (A.Rich. ex DC.) Drake, Erythrina abyssinica (Lam. Ex DC.)* and *Trichodesma zeylanicum (Burm.f.) R. Br*.

Table 17: Brine shrimp mortality rates after 24hrs

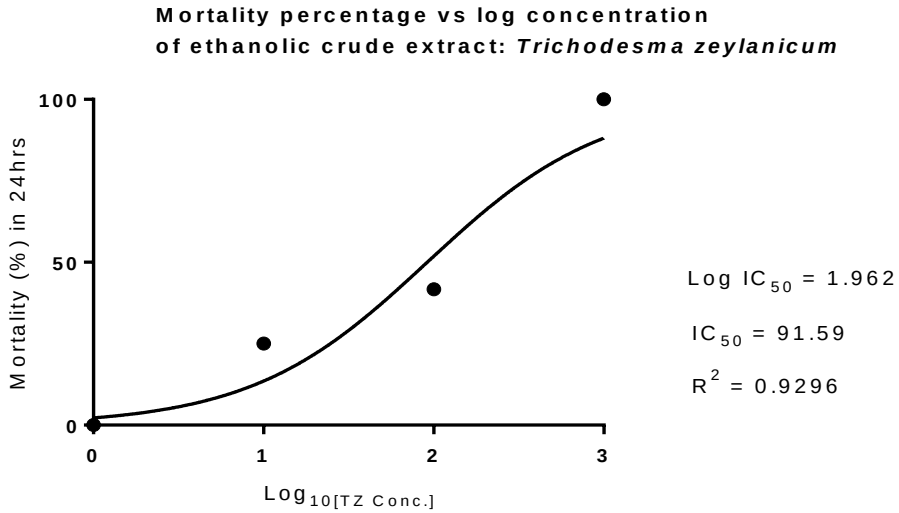
Crude extract	**Concentrations and Mortality rates after 24hrs (n=20)**				**LC_{50}**
	1µg/ml	**10µg/ml**	**100µg/ml**	**1000µg/ml**	
Trichodesma zeylanicum (Burm.f.) R. Br	8	11	13	20	91.59
Erythrina abyssinica (Lam. Ex DC.)	10	11	18	20	41.05
Paederia bojeriana (A.Rich. ex DC.)	12	14	20	20	11.09
Potassium dichromate	14	16	18	20	31.62

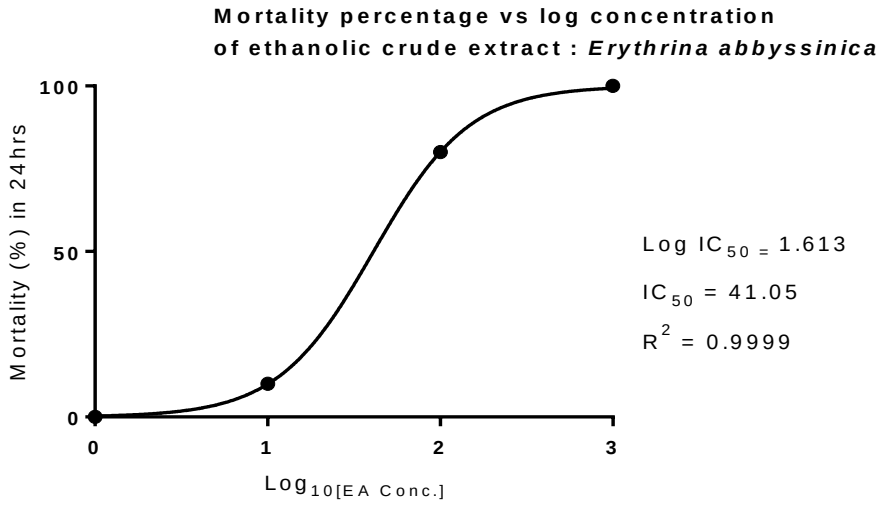
The results shown in Table 17 above and Figure 7 below show that the lowest and most toxic LC_{50} values were of ethanolic root extract of *Paederia bojeriana (A.Rich. ex DC.) Drake* (LC_{50} = 11.09µg/ml), followed by the bark extract of *Erythrina abyssinica* (LC_{50} = 41.05µg/ml) and root extract of *Trichodesma zeylanicum (Burm.f.) R. Br* (LC_{50} = 91.59µg/ml). the extracts were also evaluated to determine whether they were safe or toxic using the Clarkson toxicity criteria and Meyer's toxicity index (189), as shown in Table 18 below.

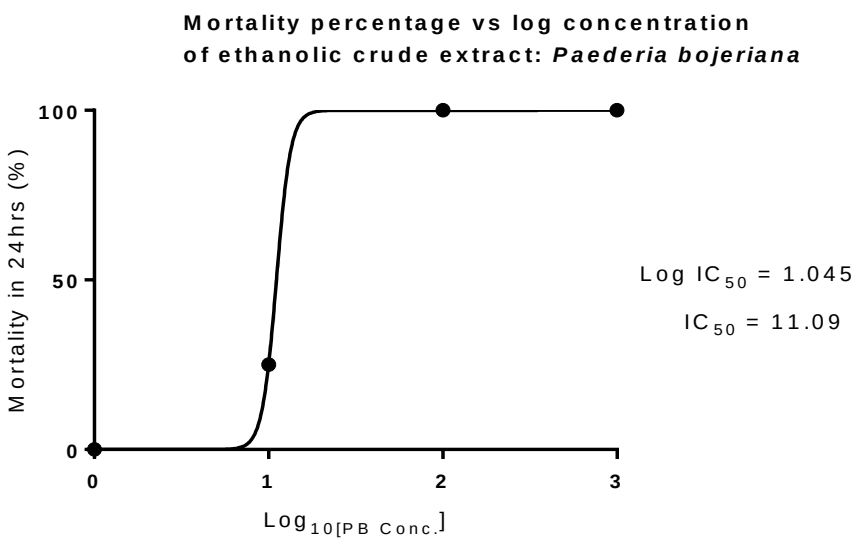
Table 18: Clarkson and Meyer's Toxicity Ratings

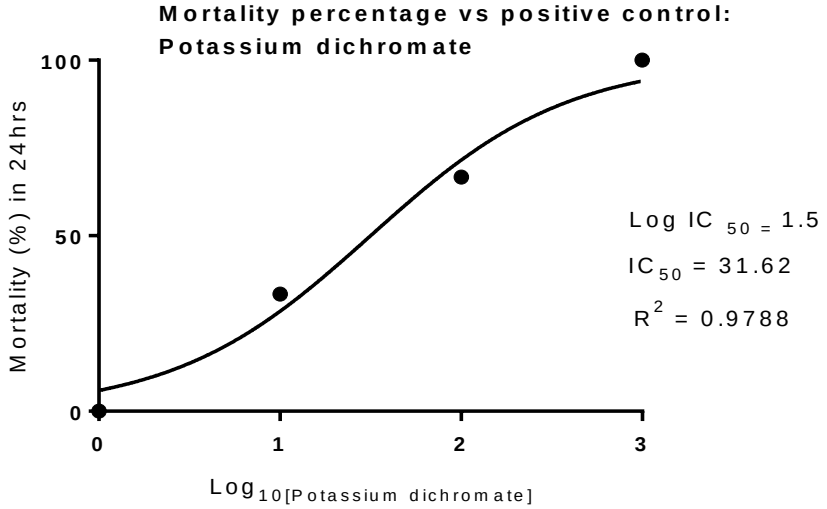
Toxicity model	**Toxicity category**	**Plant extracts in the category**
Clarkson toxicity criteria	High toxicity = 0 – 100µg/ml	• *Paederia bojeriana (A.Rich. ex DC.) Drake* • *Erythrina abyssinica (Lam. Ex DC.)* • *Trichodesma zeylanicum (Burm.f.) R. Br*
	Moderate toxicity = 100 – 500µg/ml	-
	Low toxicity = 500 – 1000 µg/ml	-
	Non – toxic = LC_{50} > 1000µg/ml	-
Meyer's toxicity criteria	Toxic = LC_{50} <1000µg/ml as	• *Paederia bojeriana (A.Rich. ex DC.) Drake* • *Erythrina abyssinica (Lam. Ex DC.)* • *Trichodesma zeylanicum (Burm.f.) R. Br*
	Non-toxic = LC_{50} > 1000µg/ml	-

Figure 7: Brine Shrimp Mortality (24hrs) for *Trichodesma zeylanicum (Burm.f.) R. Br*, *Erythrina abyssinica (Lam. Ex DC.)*, *Paederia bojeriana (A.Rich. ex DC.) Drake* and positive control









3.4.1.2 Discussion on *In vitro* Toxicity Testing

Based on the results, the extracts of the three plants show a concentration-response relationship of lethality. The lower toxicity of *Erythrina abbysinica* and *Trichodesma zeylanicum (Burm.f.) R. Br* could be due to the solvent used (ethanol) in the extraction of the compounds specifically not extracting alkaloids from the samples, which are known to be toxic (237). The toxicity of *Paederia bojeriana (A.Rich. ex DC.) Drake* could be due to the presence of cardiac glycosides, which are known to be poisonous (201,254), and their moderate toxicity could be attributed to their lower concentration since toxicity also depends on the concentration of a toxicant.

Erythrina abbysinica is typically known to have moderate toxicity due to the presence of curare-like alkaloids (45), alkaloids were not extracted in this experiment. However, based on the results alone, the toxicity could be attributed to the high level of flavonoids and anti-oxidant activity, which are known to enhance proinflammatory and prooxidative damage (33). Furthermore, the toxicity results are in agreement with those obtained by Nguyen et al. (255), whereby the ethanolic extract of *Erythrina abbysinica* showed strong cytotoxic activity against MCF7, tamoxifen-resistant MCF7, 80driamycin-resistant MCF7 and MDA-MB-231 breast cancer cell lines. In addition, ethanolic leaf, stem and root extracts of *Erythrina abbysinica* have shown to immobilize and subsequently kill *Ascaridia galli* and have shown to be a potent anthelmintic substances, which was additionally attributed to tannins found in the extracts (100). Other studies have shown the presence of alkaloids in crude extracts from the bark and leaves of *Erythrina abbysinica* with the use of solvents such as methanol and water with LC_{50} values of which show weak *in vitro* levels of toxicity(45,238), anticancer properties (118,255) and antiparasitic properties (98,100).

Trichodesma zeylanicum (Burm.f.) R. Br, which belongs to the Boraginaceae family known for containing hepatotoxic pyrrolizidine alkaloids (256), alkaloids were not extracted from this plant as well. Similarly with *Erythrina abbysinica*, the mild toxicity could be attributed to the high level of present flavonoids and anti-oxidant activity which enhances proinflammatory and prooxidative damage (33) which can exacerbate the disease process of asthma. However, this study showed that *Trichodesma zeylanicum (Burm.f.) R. Br* had low toxicity, which is similar to studies done on the methanolic extracts of *Trichodesma zeylanicum (Burm.f.) R. Br* by Matata et al. (257) that showed through Brine Shrimp assay a LC_{50} of 2045µg/ml. Another study on this plant by Moustafa et al. (258) also showed low cytotoxicity to A-549, BJ-1, MCF-7, HCT-116 and HepG2 cell lines.

Paederia bojeriana (A.Rich. ex DC.) Drake was the plant with the most toxic crude extracts, which is similar to results of a study by Ahmed et al. (259) and Morshed et al. (260) whereby the methanolic leaf extract and whole parts of the plant of *Paederia foetida* showed Brine Shrimp assay LC_{50} of 65.31 µg/ml and 1.5625 µg/ml respectively showing cytotoxicity of the leaves and plants.

Overall, the crude plant extracts of all the plants showed mild to moderate toxicity at the cellular level which could manifest adverse effects when being used as traditional medicines. However, since toxicity depends on many factors, further studies are needed in order to have conclusive decisions which should include exploring other toxicity testing models such as human cells.

3.4.2 *In vivo* Acute Oral Toxicity Study

The results for the *in vivo* tests included general observations, biochemical and histopathological parameters.

3.4.2.1 General Observations

The overall results in Figure 7 showed that the herbal extracts of *Erythrina abyssinica (Lam. Ex DC.), Trichodesma zeylanicum (Burm.f.) R. Br* and *Paederia bojeriana (A.Rich. ex DC.) Drake* are of Category 5 (>2000mg/kg – 5000mg/kg) with the LC_{50} cut-off mg/kg b.w = ∞ mg/kg. The rats showed no abnormal behavioral changes during the course of the study with no death occurring in all the 4 groups inclusive of the control group. Table 19 shows a summary of the general observation results.

Table 19: General Observations for *Erythrina abyssinica (Lam. Ex DC.), Paederia bojeriana (A.Rich. ex DC.) Drake and Trichodesma zeylanicum (Burm.f.) R. Br*

Observations	**30 min**	**4hrs**	**24hrs**	**48hrs**	**1 week**	**2 weeks**
Eyes	Normal	Normal	Normal	Normal	Normal	Normal
Skin and Fur	Normal	Normal	Normal	Normal	Normal	Normal
Vomiting	N.O	N.O	N.O	N.O	N.O	N.O
Diarrhea	N.O	N.O	N.O	N.O	N.O	N.O
Salivation	Normal	Normal	Normal	Normal	Normal	Normal
Convulsion	N.O	N.O	N.O	N.O	N.O	N.O
Lethargy	N.O	N.O	N.O	N.O	N.O	N.O
Coma	N.O	N.O	N.O	N.O	N.O	N.O

N.O = not observed

3.4.2.2 Effect of the herbal extracts on rat body weight in the acute oral toxicity test

Although the body weights were different amongst the test animals, their body changes before and the administration of the crude extracts were not significant visually. Statistical calculations of the differences were also not significant as the p-value were greater than 0.05 ($p > 0.05$). The body weight changes are shown in Table 20 below.

Table 20: Body weight of Female rats in acute oral toxicity test for *Erythrina abyssinica (Lam. Ex DC.)* (EA), *Trichodesma zeylanicum (Burm.f.) R. Br* (TZ) and *Paederia bojeriana (A.Rich. ex DC.) Drake* (PB)

	Control	**0.5% DMSO**	**PB 300mg/kg**	**PB 2000mg/kg**	**EA 300mg/kg**	**EA 2000mg/kg**	**TZ 300mg/kg**	**TZ 2000mg/kg**
Day 0	175.00±15.13	168.00 ± 14	159.33±12.66	134.33± 11.93	138.33±13.01	152.67±25.54	168.00±16.00	160.00±13.11
Day 14	175.00±15.13	167.00± 10.39	161.33± 13.58	141.33± 17.21	144.00 ± 11.13	165.00 ± 25.87	176.00 ± 22.27	163.33±14.01
P-Value	1	0.93	0.86	0.59	0.60	0.57	0.64	0.78

3.4.3.3 Effect of the herbal extracts biochemical parameters in the acute oral toxicity test

Biochemical parameters are summarized in Figure 9. The results in the figure show that there is no significant association between all the biochemical parameters and dosage groups for all the three plants as the p ≥0.05. Indicating that the administration of the crude extracts did not have any significant effect on the biochemical parameters of the rats.

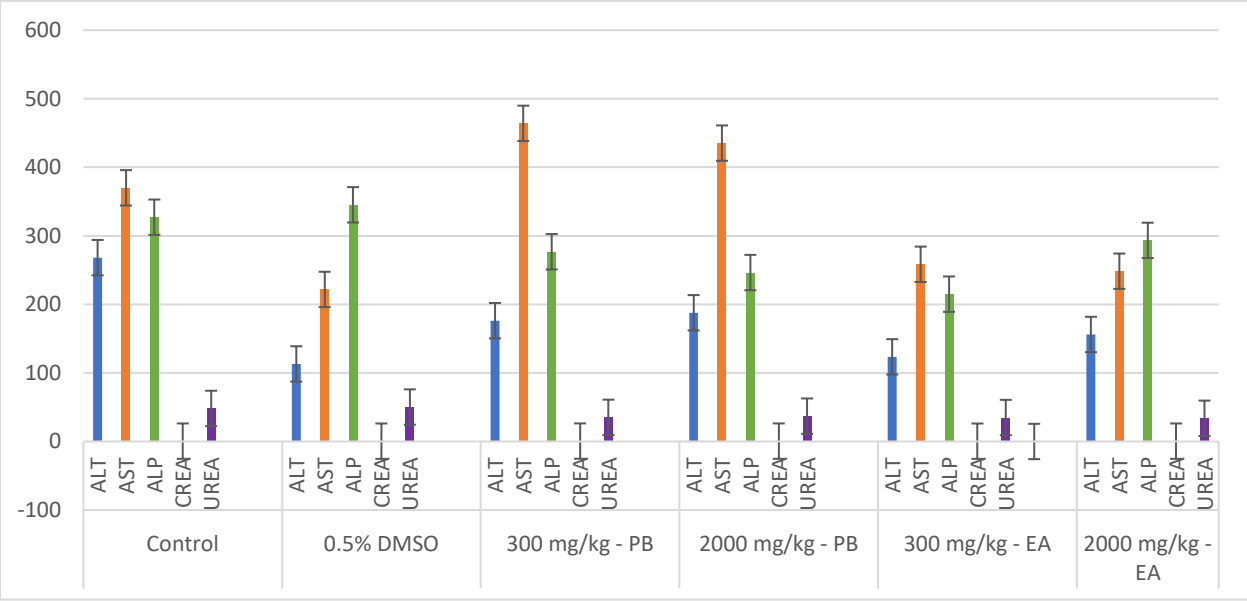


Figure 8: Biochemical biomarker parameters for *Paederia bojeriana (A.Rich. ex DC.)* and *E. abbysinica*, a null difference between the crude extracts, control and 0.5% DMSO; AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkaline phosphatase, CREA: creatinine

Paederia bojeriana (A.Rich. ex DC.) Drake

Figure 9 illustrates biochemical parameters found in the rats treated with 300mg/kg and 2000mg/kg dosages of the crude extract of *Paederia bojeriana (A.Rich. ex DC.) Drake*. The

parameters showed a lowered ALT, and ALP, elevated AST and unchanged amounts for creatinine and urea in rats treated with 300mg/kg of crude extract as compared with the control. The parameters showed a lowered ALT, and ALP, elevated AST and unchanged amounts for creatinine and urea in rats treated with 300mg/kg of crude extract as compared with the control.

Erythrina abyssinica (Lam. Ex DC.)

Figure 9 illustrates biochemical parameters found in the rats treated with 300mg/kg and 2000mg/kg dosages of the crude extract of *Erythrina abyssinica (Lam. Ex DC.).* The parameters showed a lowered ALT, ALP and AST amount and unchanged amounts for creatinine and urea in rats treated with 300mg/kg of crude extract as compared with the control. The parameters showed a lowered ALT, ALP, and AST amount and unchanged amounts for creatinine and urea in rats treated with 300mg/kg of crude extract as compared with the control.

3.4.3.4 Effect of herbal extracts on the histopathological parameters in the acute oral toxicity test

3.4.3.4.1 Histopathological analysis of the Liver

Of the four histological parameters, there were significant differences between the control groups and *Paederia bojeriana (A.Rich. ex DC.) Drake* 300mg/kg and 2000 mg/kg treated groups ($p \leq 0.05$) in three histological parameters (cytoplasmic vacuolization, hepatocellular necrosis and degeneration, portal triad and lobular inflammation) except sinusoidal dilation and congestion (Figure 10 and Figure 11); indicating that the administration of the crude extracts generally affected the histological architecture of the liver. There were no significant differences in the liver architecture between the control group and 0.5% DMSO treated rats ($p \geq 0.05$).

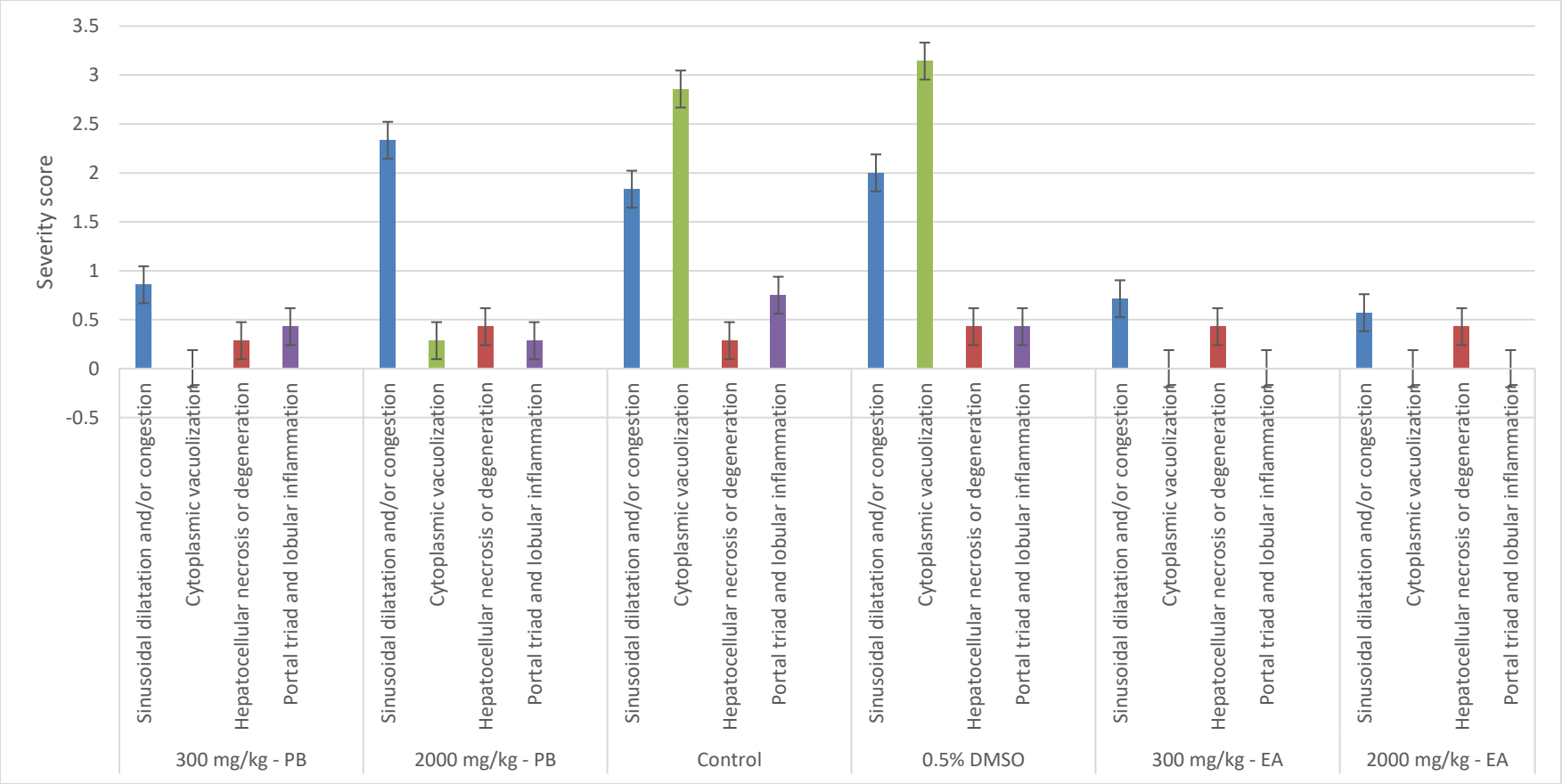


Figure 9: Histological grading for rat liver

Control and 0.5% DMSO treatment

Figures 9 & 10 illustrates some of the main histopathological characteristics and severities found in the rats treated that were not treated with any dosage of the crude extract and those treated with 0.5% DMSO. The liver tissues showed moderate sinusoidal dilatation and moderate cytoplasmic vacuolization.

Paederia bojeriana (A.Rich. ex DC.) Drake

Figure 10 & 11 illustrate some of the main histopathological characteristics and severities found in the rats treated with 300mg/kg and 2000mg/kg dosages of the crude extract of *Paederia bojeriana (A.Rich. ex DC.) Drake.* The liver tissues showed mild sinusoidal dilatation in the rats treated with 300mg/kg of crude extract whilst in the rats treated with 2000 mg/kg, there was moderate sinusoidal dilatation.

Erythrina abyssinica (Lam. Ex DC.)

Figure 10 & 11 illustrates some of the main histopathological characteristics and severities found in the rats treated with 300mg/kg and 2000mg/kg dosages of the crude extract of *Erythrina abyssinica (Lam. Ex DC.).* The liver tissues showed mild sinusoidal dilatation in the rats treated with 300mg/kg and 2000 mg/kg of crude extract.

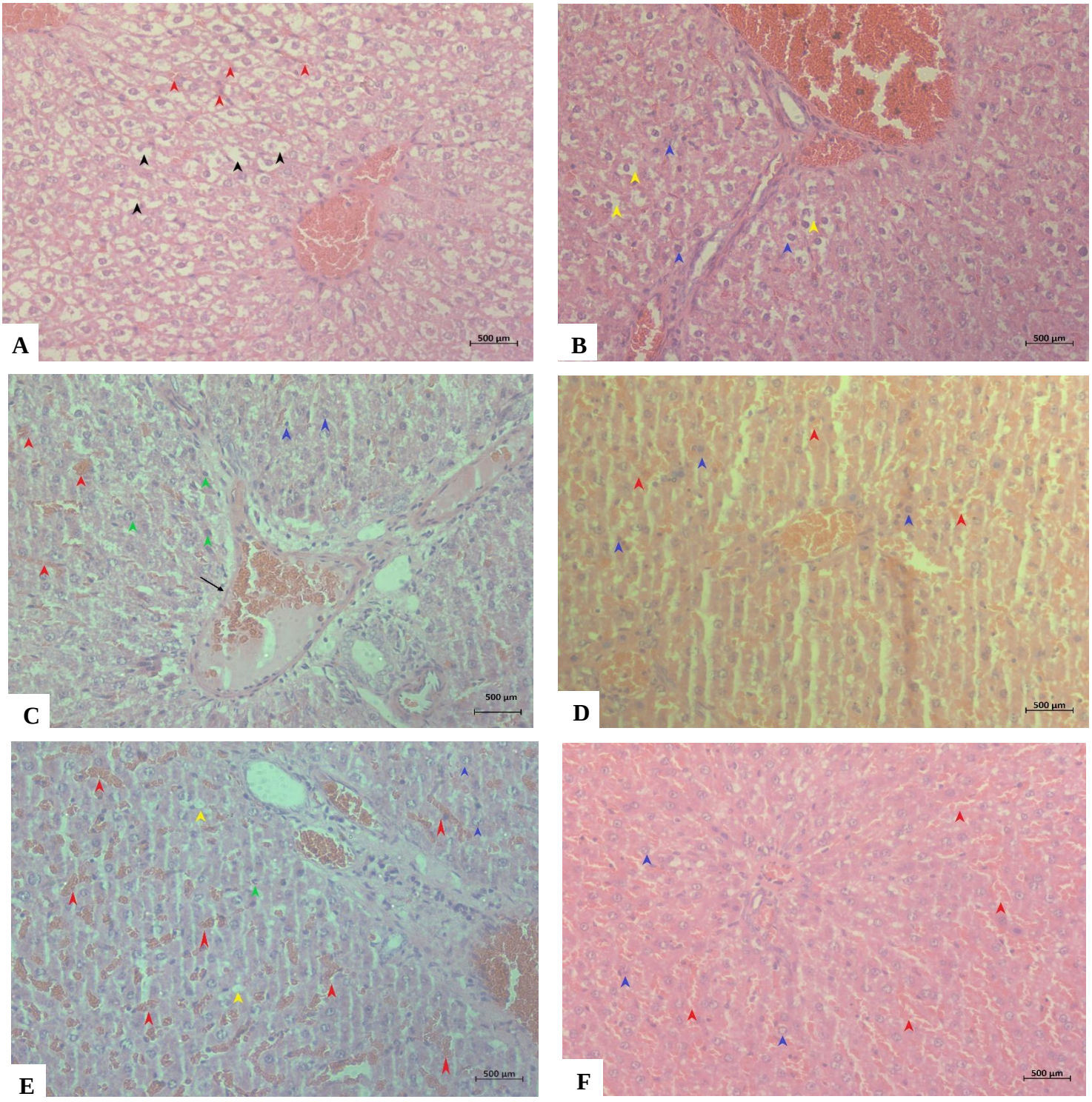


Figure 10: Histological slides for rat liver: A– Control(x10), B – 0.5% DMSO(x10), C – 300mg/kg *Paederia bojeriana (A.Rich. ex DC.)* (x10), D- 300mg/kg *Erythrina abyssinica (Lam. Ex DC.)*(x10), E – 2000mg/kg *Paederia bojeriana (A.Rich. ex DC.)* (x10) F – 2000mg/kg *Erythrina abyssinica (Lam. Ex DC.)*(x10)*;* Red arrow heads – sinusoidal congestion Blue arrow heads – hepatocellular necrosis Green arrow heads- lobular inflammation Yellow and black arrow heads cytoplasmic vacuolization

3.4.3.4.2 Histopathological analysis of the Kidney

There were no significant differences between the control and 0.5% DMSO treated, *Paederia bojeriana (A.Rich. ex DC.) Drake* dosages 300mg/kg and 2000 mg/kg in all parameters as the p-value < 0.05, however there was an extensive amount of diffuse cortical and medullary regions in *Erythrina abyssinica (Lam. Ex DC.)* 2000mg/kg. There was a significant difference between the control and *Erythrina abyssinica (Lam. Ex DC.)* 300mg/kg in all the histological parameters.

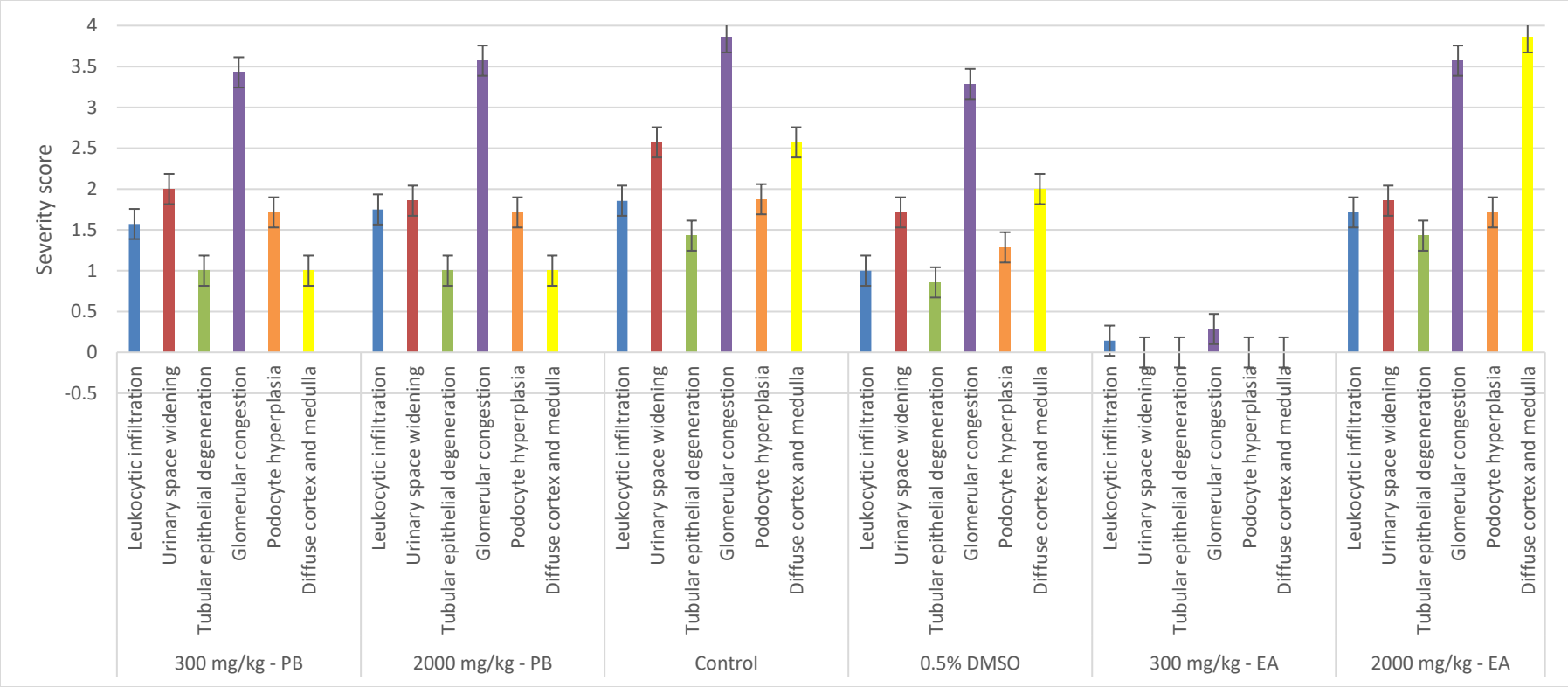


Figure 11: Histological grading for rat kidney

Control and 0.5% DMSO treatment

Figure 12 & 13 illustrates some of the main histopathological characteristics and severities found in the rats that were not treated with any dosage of the crude extract and those treated with 0.5% DMSO. The kidney tissues showed moderate leukocytic infiltration, moderate urinary space widening, mild tubular epithelial degeneration, severe glomerular congestion, moderate podocyte hyperplasia and mild diffuse cortical and medullary regions for the rats that had not treatment given whilst the 0.5% DMSO treated rats showed mild leukocytic infiltration, moderate urinary space widening, mild tubular epithelial degeneration, severe glomerular congestion, mild podocyte hyperplasia and mild diffuse cortical and medullary regions for the rats that had not treatment, the difference was not statistically different.

Paederia bojeriana (A.Rich. ex DC.) Drake

Figure 12 & 13 illustrates some of the main histopathological characteristics and severities found in the rats treated with 300mg/kg and 2000mg/kg dosages of the crude extract of *Paederia bojeriana (A.Rich. ex DC.) Drake.* The kidney tissues showed mild leukocytic infiltration, moderate urinary space widening, mild tubular epithelial degeneration, severe glomerular congestion, mild podocyte hyperplasia and mild diffuse cortical and medullary regions in the rats treated with 300mg/kg of crude extract whilst in the rats treated with 2000 mg/kg of the crude extract, there was moderate leukocytic infiltration, moderate urinary space widening, mild tubular epithelial degeneration, severe glomerular congestion, mild podocyte hyperplasia and mild diffuse cortical and medullary regions.

Erythrina abyssinica (Lam. Ex DC.)

Figure 12 & 13 illustrates some of the main histopathological characteristics and severities found in the rats treated with 300mg/kg and 2000mg/kg dosages of the crude extract of *Erythrina abyssinica (Lam. Ex DC.).* The kidney tissues showed normal kidney architecture with no leukocytic infiltration, urinary space widening, tubular epithelial degeneration, glomerular congestion, podocyte hyperplasia and diffuse cortical and medullary regions in the rats treated with 300mg/kg of crude extract whilst in the rats treated with 2000 mg/kg of the crude extract, there was overall general cellular degeneration with moderate leukocytic infiltration, moderate urinary space widening, mild tubular epithelial degeneration, severe glomerular congestion, moderate podocyte hyperplasia and severe diffuse cortical and medullary regions .

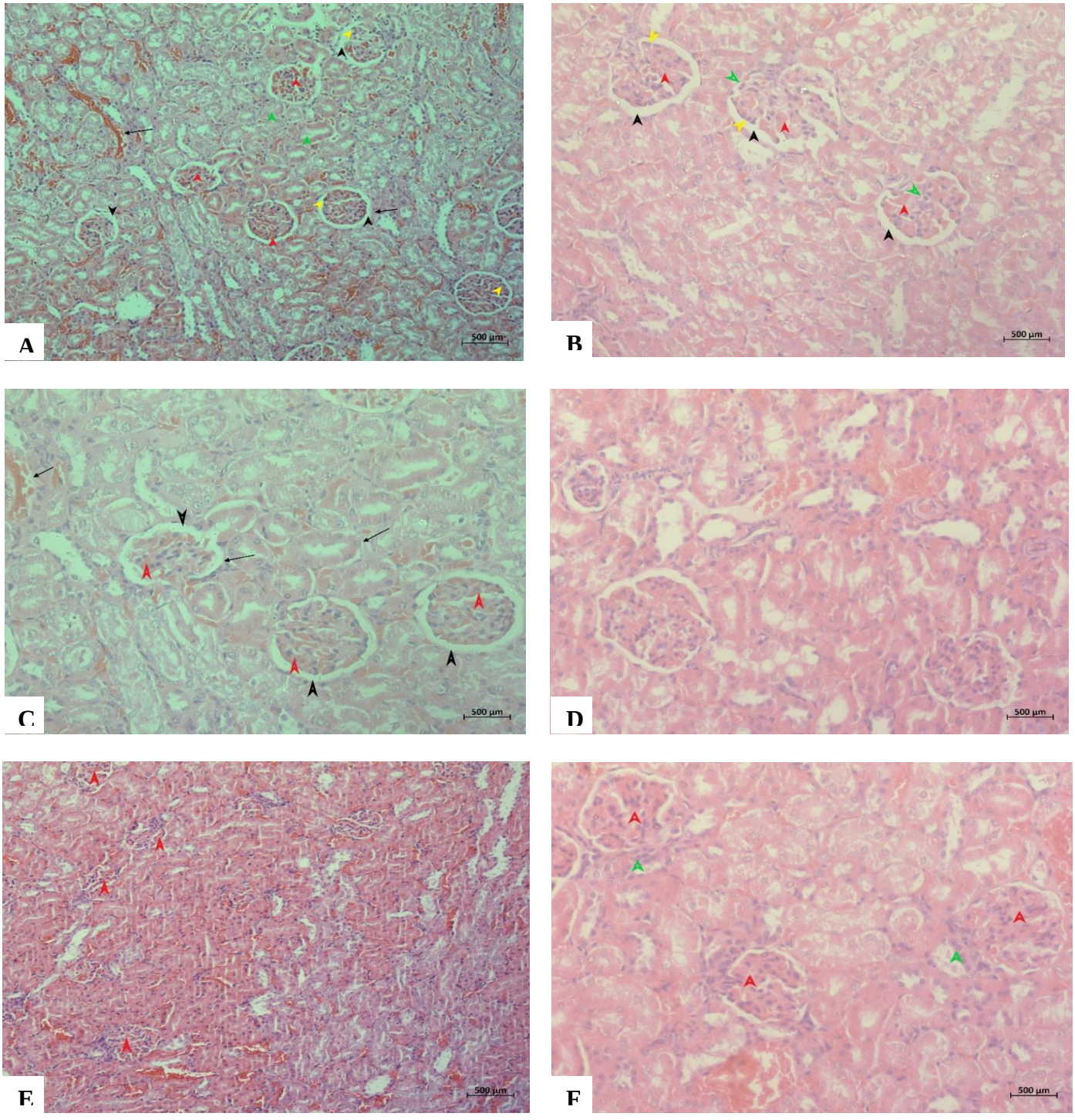


Figure 12: Histological slides for rat kidney A– Control(x10), B – 0.5% DMSO(x20), C – 300mg/kg *Paederia bojeriana (A.Rich. ex DC.)* (x20), D- 300mg/kg *Erythrina abyssinica (Lam. Ex DC.)*(x20), E – 2000mg/kg *Paederia bojeriana (A.Rich. ex DC.)* (x10) F – 2000mg/kg *Erythrina abyssinica (Lam. Ex DC.)* (x20)*;* Red arrow heads – glomerular congestion; Yellow arrow heads – podocyte hyperplasia;

Green arrow arrowheads- leukocyte infiltration ; Black arrow heads – urinary space widening ; Black arrows are showing the glomerulus, blood vessel and collecting duct found in the kidney cortex

Table 21: Overview of Brine Shrimp Assay and Acute Oral Toxicity (423) Study

Assay		**Duration**	***Erythrina abyssinica (Lam. Ex DC.)* extract**	***Trichodesma zeylanicum (Burm.f.) R. Br* extract**	***Paederia borjeriana* extract**
Brine Shrimp Lethality Assay (LC_{50})		24 hours	41.05 µg/ml	91.59 µg/ml	11.09 µg/ml
	Clarkson's Toxicity Criteria		Highly toxic	Highly toxic	Highly toxic
	Meyer's Toxicity Index		Toxic	Toxic	Toxic
Acute Oral Toxicity Study (423) (LC_{50})		14 days	>2000 mg/kg Non-toxic	>2000 mg/kg Non – toxic	>2000 mg/kg Non-toxic

3.4.4 Discussion on *In vivo* Acute Oral Toxicity Study

The three extracts showed no overall induced mortality, change in behavioural pattern, clinical signs, or body weight of the rats and were grouped in Category 5 (>2000mg/kg – 5000mg/kg) (Figure 8).

The results from *Erythrina abyssinica (Lam. Ex DC.)* concurred with a study done by Atsamo et al. (261); whereby there were no significant findings in rat weight and biochemical parameters against the controls. However, Bunalema et al. (262) found an LC_{50} = 776.2mg/kg and an increase in sedation and reduced motor activity and a presence of alkaloids in the methanolic extracts of root bark of *Erythrina abyssinica (Lam. ex DC.).* The decreased toxicity observed in this study could be attributed to the absence of alkaloids found in this experiment (237). There was an absence of gross pathological lesions in the treated rats in this study, which infers the low toxicity of crude extracts (Figure 11 & 13). The *Erythrina abyssinica (Lam. ex DC.)* extract showed hepatoprotective effects, which could be attributed to the presence of flavonoids and the high antioxidant activity found in the extract (263). Musyoka et al. (152) noted that any dosage above 1000mg/kg increased renal, liver and cardiac impairment, which explains the histopathological lesions found in the 2000mg/kg *Erythrina abyssinica (Lam. ex DC.)* group as compared to the 300mg/kg *Erythrina abyssinica (Lam. ex DC.)* group (Figure 11 & 13).

There is scanty literature on *Paederia bojeriana (A.Rich. ex DC.) Drake* and no acute toxicity studies were found in regard to this plant. However, studies done by Kumar et al. (197), Wang et al. (264) and Das et al. (265) on the family Rubiaceae (*P. foetida and P. scandens*) concur that

the LC_{50} = 2000mg/kg and there was similarly no sign of mortality and adverse effects to the LC_{50} dosage.

This study showed elevated AST levels in the liver, which could be due to the presence of cardiac glycosides (87) found in *Paederia bojeriana (A.Rich. ex DC.) Drake,* in both 300mg/kg and 2000mg/kg groups, however, the extract did not show hepatoprotective or renoprotective effects as *P. foetida* and *P. scandens* (264) since there were no significant differences between the control and 300mg/kg and 2000mg/kg groups in the kidney tissues and sinusoidal dilatation trait in the liver tissue.

Regarding *Trichodesma zeylanicum (Burm.f.) R. Br*, the extract showed no induced mortality, significant change in behavioural pattern, clinical signs, or body weight of the rats. This correlates with studies done by Omar et al. (266) and Preveen et al. (267) on *Trichodesma africanum* and *Trichodesma indicum* showing similar outcomes with dose dependent hepatoxicity and an LC_{50} above 2000 mg/kg.

Despite showing mild to moderate toxicity of the crude extracts the *in vitro* Brine Shrimp assay results, the three extracts were found to be non-toxic and safe to use in animals (Table 21). The disparity between the *in vitro* and *in vivo* model results in this investigation could be due to the extracts being metabolized and compounds detoxified by liver enzymes (268). The organic solvent used to extract the active compounds could have increased cytotoxicity by extracting more active compounds as compared to if an aqueous counterpart was used (268).

3.4.5 Limitations

The results in this study are limited to the cold dry season this would affect the antioxidant activity and toxicity as secondary metabolites are affected by various environmental factors such as season, location, weather and so thus affects amount in which the plants produce them (204,238). Animal studies are complex as dietary, environmental, and genetic influences can affect the biological framework of the animal (269,270). Similarly, parasitic (271), bacterial, viral (272) and fungal (273) infections could alter the physiological functions of the animal which could have affected the outcome of the study. The protocol-based sample size in this study was small (3 rats per group) and could have affected the accuracy of the statistical tests that were used. Increasing the same sample size (5 – 6 rats) could help generate more accurate results.

Trichodesma zeylanicum (Burm.f.) R. Br tissues and bloods samples were damaged due to machine malfunction thus affecting data analysis and in depth histological and biochemical review. However, the overall observations, clinical signs (Table 17) and mortality (Figure 8) of the rats shown in the results was enough to conclude the GHS grade. If there were enough funds other experiments would have taken place. However, measures were put in place throughout the experiments to minimize these limitations which included among others repeating the results as well as using controls.

CHAPTER FOUR: CONCLUSION AND RECOMMENDATIONS

The study has only established baseline data for flavonoid and phenolic content, antioxidant activity and acute toxicity for the crude extracts of *Erythrina abyssinica (Lam. ex DC.), Paederia bojeriana (A.Rich. ex DC.) Drake* and *Trichodesma zeylanicum (Burm.f.) R. Br*. Active components found in *Erythrina abyssinica (Lam. ex DC.), Paederia bojeriana (A.Rich. ex DC.) Drake* and *Trichodesma zeylanicum (Burm.f.) R. Br* should be identified, isolated and screened for anti-asthmatic activity both *in vitro* and *in vivo*. Further investigation is warranted to explore the *in vivo* bioavailability of antioxidants, sub- chronic and chronic toxicity of the crude extracts so as to enhance the knowledge of the implications in the use of traditional medicines without adequate safety data and to standardize the use of animal model protocols in the Malawian setting in Biomedical research.

Herbal medicine is usually seen as a preferential choice especially in developing countries such as Malawi. Asthma medication can be costly, inaccessible and expensive due to the nature of the disease. There is limited literature on the effects of acute toxicity in plants such as *Erythrina abyssinica (Lam. ex DC.), Paederia bojeriana (A.Rich. ex DC.) Drake* and *Trichodesma zeylanicum (Burm.f.) R. Br* in Malawi and yet these are regularly used in the management of asthma. This study has revealed that the ethanolic extracts of *Erythrina abyssinica (Lam. ex DC.),* and *Trichodesma zeylanicum (Burm.f.) R. Br* are rich in flavonoids and phenolics as compared to *Paederia bojeriana (A.Rich. ex DC.) Drake.* However, all the three plants have the ability to scavenge free radicals and reduce reactive oxygen species which have shown potential anti-oxidant properties which could have anti-inflammatory and immunomodulatory effects which needs to be investigated further. The extracts exhibited toxicity *in vitro* but were deemed

safe *in vivo* up to 2000mg/kg with mild to moderate hepatic and renal lesions which affirms its traditional use in the management of asthma at adequate dosages. In light of the results, there should be further studies done to explore the sub-chronic, chronic and anti-asthmatic effects of these plant extracts.

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