POTENTIAL ANTIASTHMATIC, ANTIHISTAMINIC AND ANTIDIARRHOEAL EFFECTS OF CRYPTOLEPINE, THE MAJOR ALKALOID OF *CRYPTOLEPIS SANGUINOLENTA* (LINDL.) SCHLTR (PERIPLOCACEAE), IN EXPERIMENTAL ANIMALS.

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DECLARATION

The experimental work herein described was carried out at the Department of Pharmacology, KNUST. I declare, that this work has not been submitted for any other degree.

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DEDICATION

To my late uncle Robert Winful and my sisters, Maudina and Josephine.

ABSTRACT

Cryptolepine (CLP) is a known antimuscarinic at M_1, M_2 and M_3 receptors. Antimuscarinics tend to find use in asthma due to their bronchodilatory effect. Coupled with this, is its established antiinflammatory effect. As asthma medications are either bronchodilative or anti-inflammatory agents, the effect of CLP was studied in certain animal models of asthma. In addition some asthma patients tend to present with certain conditions such as diarrhoea due to release of certain mediators like histamine and general vagal stimulation. In that vein, the antidiarrhoeal effect of cryptolepine was also evaluated.

Ovalbumin-induced airway guinea pig model was used in the study to evaluate airway inflammation. Allergic inflammatory response was also evaluated using skin prick test.

Results revealed that CLP at a dose range of 10-100 mg/kg significantly and dose dependently inhibited inflammatory cells in the periphery. These effects corresponded with the histopathology in the airways. Also evident in the histopathology is protection of CLP against airway inflammation and remodeling viewed in parameters such as thickening of basement membrane and epithelial cells, smooth muscle hypertrophy and hyperplasia in goblet cells.

There was significant reduction in the oedema at various doses of CLP compared to the control in the skin prick test, the effect still greater after 24 hours suggesting that it might have an effect in both early and late phase of inflammation.

Cryptolepine showed dose-dependent protection in histamine-induced bronchoconstriction from a dose of 10- 100 mg /kg which was significant even after 24 hrs. At doses of 10, 30 and 100 mg/kg of CLP there was graded decrease mucus secretion in the tracheal phenol red model.

These effects could also be attributed to stablilizing mast cells apart from its known antimuscarinic and antihistaminic effect. This is evident in the protection showed by cryptolepine at a dose of 30 and 100 µg/ml in compound 48/80 induced rat mesentery mast cell degranulation.

CLP (10, 30 and 100 mg/kg) reduced diarrhoea in the castor oil induced diarrhoea model from 100% in control group to 46.5-78.89%. This effect could be partly due to inhibition of peristalsis of GIT by cryptolepine, evidenced by inhibition of transition of charcoal meal from 100% in control group to 17.7% and 37.07%in the charcoal meal test.

CLP therefore demonstrated antiasthmatic, antidiarrhoeal and antihistaminic in animal models.

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AHR	Airway hyper responsiveness
APC	Antigen presenting cells
ASM	Airway smooth muscle
BALF	Bronchoalveolar lavage fluid
COX	Cycloxygenase
CLP	Cryptolepine
DNA	Deoxyribonucleic acid
FcεRI	Fc epsilon receptor 1
FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
GIT	Gastrointestinal tract
GINA	Global initiative for asthma
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HPA	Hypothalamic-pituitary axis
ICAM-1	Intracellular adhesion molecule-1
IFN	Interferon
Ig	Immunoglobulin
IgE	Immunoglobulin E
IL	Interleukin
INOS	Inducible nitric oxide synthase
LO	Lipoxygenase
LPS	Lipopolysaccharide
LT	Leukotriene

MAPK	Mitogen-activated protein kinases
MCP	Monocyte chemotactic protein
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
NANC	Non- adrenergic non-cholinergic
NF-κB	Nuclear factor kappa B
NHLBI	National Heart Lung and Blood Institute
NKJV	New King James Version
NO	Nitric oxide
NOS	Nitric oxide synthase
PAF	Platelet activating factor
PDE	Phosphodiesterase
PDGF	Platelet-derived growth factor
PEF	Peak expiratory flow
PG	Prostaglandin
PGHS	Prostaglandin H synthase
RANTES	Regulated upon activation normal T-cell expressed
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
SLPI	Secretory leukoprotease inhibitor
Th	T helper
Th1	T helper type 1
Th2	T helper type 2
TNF	Tumour necrosis factor
Tx	Thromboxane
VCAM-1	Vascular cell adhesion molecule-1
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 OVERVIEW

Over the past centuries, plants have become important for the discovery of novel bioactive agents, which have in turn served as lead molecules for development of new drugs (Cragg *et al.,* 1997). According to Agosta, almost all medicines, centuries before the advent of modern medicine, synthetic chemistry and pharmaceutical industry, came from plants (Agosta, 1997). Interestingly, more than 25% of drugs in the modern pharmacopeia are derived from plants and many others, which are synthetic analogues or built on prototype compounds, were isolated from plants (Cox and ballick, 1994). Actually, the use of plants for treatments of ailments transcends that of scientific background to that of religious beliefs. The good book for instance talks about the use of leaf for medicine (Ezekiel 47:12b, NKJV). The scenario is not different from today, as natural products continue to dominate research in the academic and industrial field. This probably could be attributed to availability, acceptability and affordability of these natural products. More so, because of the limited efficacy, cost and untoward effects associated with current medications, the importance and need for search of alternative medicines cannot be overemphasized. Aspirin, atropine, scopolamine, theophylline, taxol are few examples of medicines derived from plant sources (Cox and Ballick, 1994).

Cryptolepine (CLP), the major alkaloid of *Cryptolepis sanguinolenta*, is another molecule that has been isolated quite recently. Its use as an antidiabetic (Luo *et al.,* 1998) and antimalarial agent (Noamesi *et al.* 1991) have been validated. Despite this, the demonstration of antimuscarinic,

adrenergic, anti-inflammatory and antimicrobial properties by CLP makes it a potential for the management of other conditions such as asthma and diarrhoea.

Both Asthma and diarrhoea present a global worry to the quality of life of individuals. One report estimates that the most common chronic disease of childhood is asthma causing significant morbidity and mortality in both adults and children (World Health Organization, 2012). According to that report about 235 million people worldwide suffer from asthma with a further 100 million expected to be affected by 2020 (WHO, 2012).

Diarrhoea on the other hand, though not a disease in itself but a symptom of underlying conditions is attributed to the death from most ailments such as malaria and cholera. In allergic conditions such as asthma, diarrhoea has also been implicated, probably due to general vagal stimulation and release of mediators such as histamine which increases peristalsis (Awortwe *et al.*, 2013).

This work thus seeks to evaluate the effect of Cryptolepine in parameters that characterize animal models of asthma such as airway inflammation and airway obstruction.

1.2 *CRYPTOLEPIS SANGUINOLENTA*

Fig 1. 1 A picture of the dried roots of *Cryptolepis sanguinolenta*

1.2.1 Description and distribution

Botanical source – aqueous *Cryptolepis sanguinolenta* is obtained from the roots of *Cryptolepis sanguinolenta* of family Periplocaceae or Asclepiadaceae. It is locally known as nibima (Twi), Kadze (Ewe), gangamau (Hausa) or Ghana quinine, likely because of its bitter taste and its substitution for quinine as an antimalarial. The plant is a thin-stemmed and scrambling shrub. It can be found scattered in open areas (Luo *et al.,* 1998). The leaves are petiolate, glabrous, elliptic or oblong-elliptic, and up to 7 cm long and 3 cm wide. The blades have an acute apex and symmetrical base. The inflorescence cymes, lateral on branch shoots, are few flowered, with a yellow corolla tube up to 5 mm long. Its fruits are paired in linear follicles and are horn-like. The seeds are oblong in shape, small (averaging 7.4 mm in length and 1.8 mm in the middle), and pinkish, embedded in long silky hairs (Irvine, 1961). *Cryptolepis sanguinolenta* has a sweet aroma when dry. The root has a light brown colour and bitter taste. The root varies from 0.4 - 6.6 cm long and 0.31-1.4 cm wide. It is hard and brittle.

1.2.2 Traditional uses

Decoction of the dried roots of the plant is used to treat various forms of fevers, including malaria, urinary and upper respiratory tract infections, rheumatism and venereal diseases in Ghana (Boakye –Yiadom, 1979a; Frederich *et al.,* 2008). Amongst the Fulani‘s in Guinea-Bissau, the aqueous extract of the plant is used to treat jaundice and hepatitis (Silva *et al.,* 1996). In Zaire and the Casamance district of Senegal, infusions of the roots are used in the treatment of stomach and intestinal disorders (Kerharo and Adam, 1974). In Congo the aqueous extract of the roots is used in amoebiasis (Tona *et al.,* 1998).

1.2.3 Previous work on cryptolepine

Cryptolepine is the major alkaloid in the aqueous extract (Dwuma_Badu *et al.,* 1978) of *Cryptolepis sanguinolenta*. Most of the major pharmacological activities exhibited by cryptolepis are due to cryptolepine. Cryptolepine is unique, as synthesis by Fichter and Boehringer in 1906 first preceded the isolation by Clinquart (1929), Gellert and Schlittler (1951) and later by Dwuma-Badu *et al.,* (1978)

1.2.3.1 Anti-malarial effect

Cryptolepine exhibits antimalarial (Noamesi *et al.,* 1991; Kirby *et al.,* 1995) and antiplasmodial activity (Paulo *et al.,* 2000).

1.2.3.2 Adrenergic and anticholinergic effect

Cryptolepine exhibits presynaptic alpha-adrenoceptor blocking actions (Noamesi and Bamgbose, 1980; Noamesi and Bamgbose, 1982) and antimuscarinic property (Rauwald *et al.,* 1992)

1.2.3.3 Cardiovascular effect

Antiplatelet, antithrombotic, and fibrinolytic activity (Oyekan *et al.,* 1988; Oyekan and Okafor, 1989; Oyekan and Ablordeppey, 1993a; Oyekan and Ablordeppey, 1993b) have been reported. It is also known to have hypotensive activity (Bamgbose and Noamesi, 1978).

1.2.3.4 Antidiabetic effect

Cryptolepis reduces plasma glucose (Luo *et al.,* 1998) and cryptolepine enhanced insulin-mediated glucose disposal in a mouse model of diabetes and in an in vitro system using the glucose transport assay (Luo *et al.,* 1998).

1.2.3.5 Antimicrobial effect

Cryptolepine also has antibacterial (Boakye-Yiadom and Heman-Ackah, 1979; Cimanga *et al.*, 1996), antimycobacterial (Gibbons *et al.*, 2003) and antifungal activities (Cimanga *et al.*, 1998).

1.2.3.6 Anti-inflammatory effect

It has potent anti-inflammatory activity without inducing gastric ulceration (Bamgbose and Noamesi, 1981; Olajide *et al.*, 2009) and it inhibits neuroinflammation in lipopolysaccharide (LPS) activated

microglial cells (Olajide *et al.*, 2013).

After cryptolepine had been shown to inhibit nitric oxide production, and DNA binding of Nuclear Factor-kappa B following inflammatory stimuli in vitro, in vivo anti-inflammatory property were validated by Olajide *et al.*, (2009) in a number of animal models of inflammation. Cryptolepine (1040 mg/kg i.p.) produced significant dose-dependent inhibition of the carrageenan-induced rat paw oedema, and carrageenan-induced pleurisy in rats. These effects were compared with those of the non-steroidal anti-inflammatory drug indomethacin (10 mg/kg). At doses of 10-40 mg/kg i.p., cryptolepine inhibited lipopolysaccharide (LPS)-induced microvascular permeability in mice in a dose-related fashion. Oral administration of up to 40 mg/kg of the compound for four consecutive days did not also induce gastric lesion formation in rats. All these models are an indication of its acute anti-inflammatory effect as acute anti-inflammatory has three major components: (1) alterations in vascular tone that lead to an increase in blood flow, (2) structural changes in the microvasculature that permit the plasma proteins and leukocytes to leave the circulation,

and (3) emigration of the leukocytes from the microcirculation and their accumulation at the focus of injury (Pulichino *et al.*, 2006;Schmid-Schönbein, 2006).

1.2.3.7 Cytotoxic and Genotoxic effect

Both cryptolepis and its main alkaloid, cryptolepine, are cytotoxic (Ansah and Gooderham, 2002) with low genotoxicity (Ansah *et al.*, 2005). The molecular mechanisms include deoxyribonucleic acid (DNA) intercalation and inhibition of topoisomerase II (Bonjean *et al.*, 1998; Lisgarten *et al.*, 2001).

1.3 ASTHMA

Asthma is a chronic inflammatory disorder of the airways with many cells and cellular elements such as eosinophils, T lymphocytes, mast cells, neutrophils, macrophages and epithelial cells playing different roles. It is usually characterized by reversible airway obstruction, airway hyperresponsiveness (AHR) and airway inflammation causing recurrent wheezing, chest tightness, breathlessness and coughing (Currie *et al.*, 2005). It is now recognized that airway remodeling may occur, resulting in a fixed or irreversible airway defect. Asthma is a common disease that is rising in prevalence worldwide, with the highest prevalence in industrialized countries. Asthma affect about 300 million people worldwide and it has been estimated that a further 100 million will be affected by 2025 (Masoli *et al.*, 2004). Interestingly, it is estimated that asthma accounts for death of about 1 in every 250 deaths worldwide. Many of the deaths are preventable. They occur as a result of suboptimal long-term medical care and delay in obtaining pharmaceutical care during an attack (Masoli *et al.*, 2004).

The situation is not different in West Africa. In 2004, for instance out of a population of 239.5 million West Africans, 13.7 million persons were living with asthma representing a mean clinical prevalence of 5.7%. The prevalence of asthma has increased over recent decades having previously been rare within the countries that make up this region. For example, in 1975, no cases of asthma could be found among over 1,000 children and adults in a rural Gambian community, whereas 3% of a rural Gambian population reported current asthma symptoms in 1997. With increasing urbanization and lifestyle changes it is likely that the prevalence of asthma will increase further in West Africa over the next decade (Beasley, 2004).

Social and economic factors are integral to understanding asthma and its care, whether viewed from the perspective of the individual sufferer, the health care professional or entities that pay for health care. Therefore in the analyses of economic burden of asthma, attention needs to be paid to both direct (hospital admissions and cost of medications) and indirect, non-medical cost (time lost from work and premature death). Although from the perspective of both the patient and society the cost to control asthma seems high, the cost of not treating asthma correctly is even higher. For instance asthma is a major cause of absence of from work in many countries (Yan *et al.*, 2005).

1.3.1 Factors influencing the development and expression of asthma

Factors that influence the risk of asthma can be divided into those that cause development of asthma and those that trigger asthma symptoms with some doing both. The former include host factors which are primarily genetic and the latter are usually environmental factors (Busse *et al.*, 2001). However, the mechanisms whereby they influence the development factors and expression of asthma are complex and interactive (Ober, 2005).

1.3.1.1 Host factors

Genetic

Current data reveals that several genes may be involved in the pathogenesis of asthma (Holloway *et al.,* 1999) with different genes probably being involved in different ethnic groups. The search for genes linked to the development of asthma has focused on four major areas: production of allergen-specific immunoglobulin E (IgE) antibodies (atopy): expression of airway hyperresponsiveness; generation of inflammatory mediators such as cytokines and determination of the ratio between T helper type 1 (Th1) and T helper type 2 (Th2) immune responses (Wiesch, 1999).

Obesity

Asthma is frequently observed more in obese subjects and is more difficult to control (Lavokie *et al.,* 2006; Pakhale *et al.,* 2010). How this occurs is still uncertain but is proposed that obesity could influence airway function due to its effect on the lung mechanics, development of a proinflammatory state in addition to genetic, development, hormonal or neurogenic influences. In this light, obese people have a reduced expiratory reserve volume, a pattern of breathing which may possibly alter airway smooth muscle plasticity and airway function (Beuther *et al.,* 2006).

Sex

Prior to age of 14, asthma is nearly twice as great in boys as in girls—an indication of male sex being a risk for asthma in children (Horwood *et al.,* 1985; Martinez *et al*., 1995). However by adulthood the prevalence is greater in women than in men, probably because lung size is smaller in males at birth but larger in adulthood (Martinez *et al.,* 1995).

1.3.1.2 Environmental factors

Allergens

Both indoor and outdoor allergens are known to cause asthma exacerbations. Examples of allergens are house mites, cat dander, dog dander, and aspergillus mold. The relationship between allergen exposure and sensitization is complex. It depends on the allergen, the dose, the time of exposure, age and genetics (Huss *et al.,* 2001). Epidemiological studies suggest that exposure to cats and dogs early in life may protect a child against sensitization or development of asthma (Platts-Mills *et al.,* 2001; Ownby *et al.,* 2002) but others suggest that such exposure may increase the risk of allergic sensitization (Melen *et al.,* 2001; Celedón *et al.,* 2002).

Infections

A number of viruses have been associated with the inception of the asthmatic phenotype. Respiratory syncytial virus (RSV) and parainfluenza produce a pattern of symptoms including bronchiolitis that parallel many features of childhood asthma (Sigurs *et al.,* 2000). On the other hand, evidence also indicates that certain respiratory infections early in life may protect against the development of asthma (Stein *et al.,* 1999). This seems to be in favour of the 'hygiene hypothesis' of asthma, which suggest that the exposure to infections early in life influences the development of a child's immune system along a 'non-allergic' pathway leading to a reduced risk of asthma and other allergic diseases (Ramsey and Celedon, 2005).

Occupational sensitizers

Occupational asthma is defined as asthma caused by an exposure to an agent encountered in the work environment. Over 300 substances have been implicated in occupational asthma including highly reactive small molecules such as isocyanates (Chan-Yeung and Malo, 1995).

Diet and tobacco smoking are also other environmental factors associated with development and expression of asthma (Strachan and Cook, 1998; Devereux *et al.*, 2005).

1.3.2 Mechanism of asthma

1.3.2.1 Airway inflammation in asthma

It has been assumed in the past that, the basic defect in asthma is the abnormal contractility of airway smooth muscle giving rise to variable airflow with associated symptoms of wheezing and shortness of breath. Even though this remains an important aspect in the dysfunction of asthma, current studies suggest that inflammation has a central role to play in the pathophysiology of asthma. Inflammation affects all airways including in most patients the upper respiratory tract and nose but its physiological effects are most pronounced in medium-sized bronchi. This is evident by inflammation and infiltration of inflammatory cells in the airway of patients who have died of asthma attacks (Dunnil, 1960; Barnes, 2010). Direct bronchoscopy also reveals that the airways of the asthmatic patient are often reddened and swollen indicating acute inflammation, even in patients with mild asthma (Djukanovic *et al.,* 1990). Airway inflammation involves an interaction between different cell types and multiple mediators (**Fig 1.2**) with the airways that result in the characteristic features of the disease such as hyperresponsiveness. Evidence also shows that certain transcription factors such as nuclear factor kappa B (NF-κB), play a critical role in the expression of inflammatory genes (Epstein *et al.*, 1997).

It is also evident that no single inflammatory cell is able to account for the complex pathophysiology of asthma. Some cells are however predominant.

Mast cells are definitely important in eliciting the acute response to allergen and also other indirect stimuli such as exercise and hyperventilation. Its role in asthma cannot be disputed. Activation of

mucosal mast cells release bronchoconstrictor mediators (histamine, prostaglandin, cysteinylleukotrienes) (Boyce 2003; Robinson 2004). Increased numbers of mast cells in airway smooth

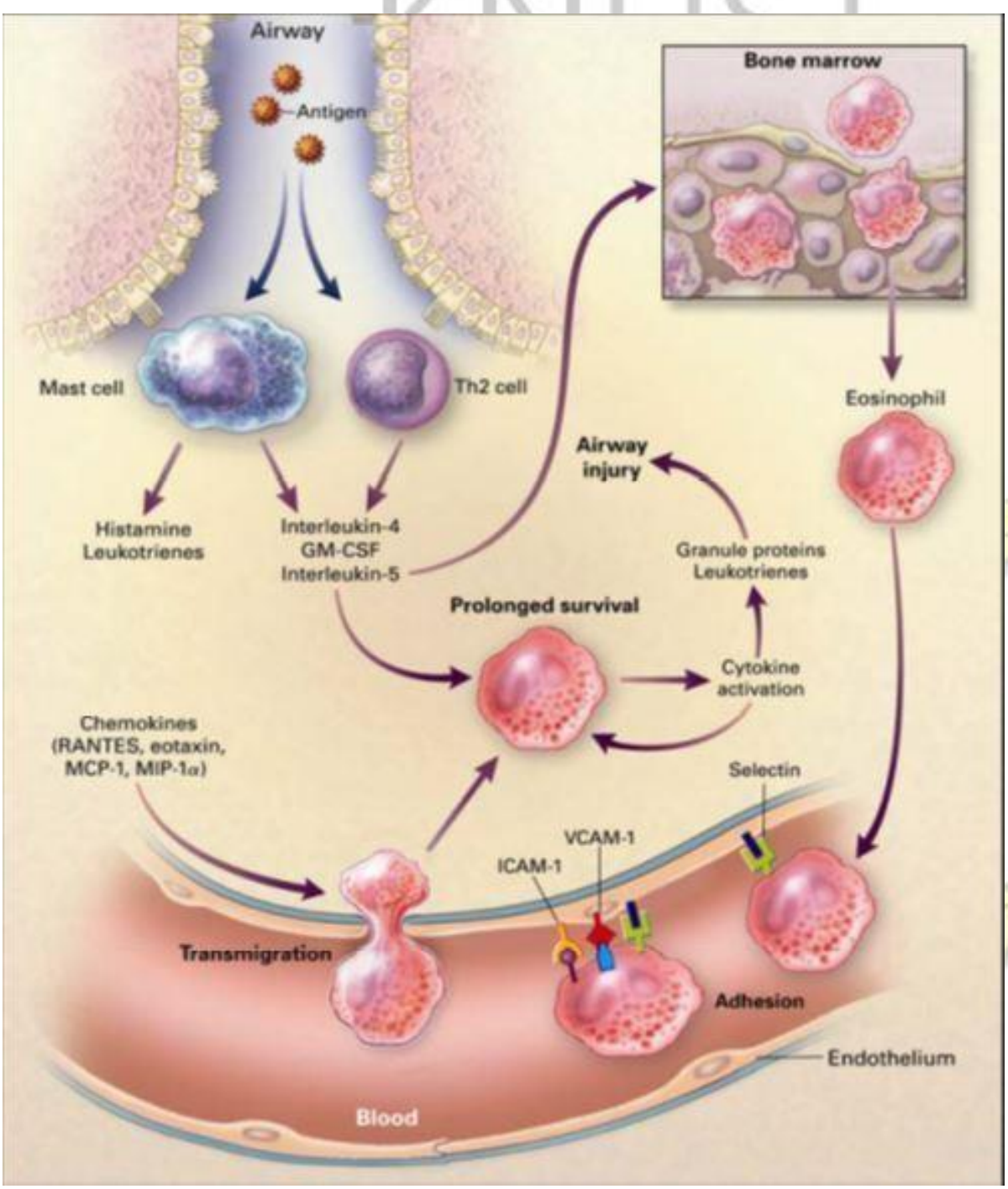

Fig 1. 2 A schematic representation of cells and mediators that mediate airway inflammation. Adapted from Busse and Lemanske (2001)

muscle may be connected to airway hyperresponsiveness (Brightling *et al.,* 2002). They can also release a large number of cytokine to change the environment of the airway and promote inflammation. However their role in chronic inflammation has been questioned with other cells such as eosinophils being implicated. It is however worth mentioning that certain agents that stabilize mast cells such as cromoglycate have found use in the management of asthma. These cells are activated by allergens through the high-affinity IgE receptors as well as by osmotic stimuli (accounting for exercise-induced asthma).

Basophils are similar to mast cells in expression of IgE receptor known as Fc epsilon receptor 1 (FcεRI) which degranulate in response to allergen challenge. They produce a wide range of mediators and are a dominant and rapid source of interleukin-4 (IL-4) in patients with atopic asthma (Perrigoue *et al.,* 2009).

Eosinophil infiltration is a characteristic feature of asthmatic airways and is known to differentiate asthma from other inflammatory condition of the airways. Asthma in 1916 was known as chronic eosinophilic bronchitis. Eosinophils play a significant role in the chronic phase of asthma which is linked to the development of airway hyperresponsiveness (AHR) through the release of basic proteins and oxygen derived free radicals. They may also have a role in the release of growth factors and airway remodeling (Gleich, 1990). Eosinophils were long regarded as the effector cells responsible for much of the pathology of asthma (Hogan *et al.*, 2008). However, this concept was challenged when anti-interleukin-5 (anti-IL-5) therapy had little or no impact on the clinical outcomes, although the number of eosinophils in blood and sputum were significantly reduced

(Leckie *et al.*, 2000). Eosinophils migrate to the airway by attaching to the endothelium of blood vessels through the interactions of integrins (glycoproteins on the surface of eosinophils) with VCAM-1 (vascular-cell adhesion molecule 1) and ICAM-1 (intercellular adhesion molecule 1) both members of the immunoglobulin superfamily of adhesion molecule (Kwon *et al.*, 1995).

T-lymphocytes play a very significant role in coordinating the inflammatory response in asthma. Peradventure nothing depicts more the immune response involvement of asthma than the imbalance in Th1 and Th2 lymphocyte expression (Anthony *et al.*, 2007). Th1 expressions are largely responsible for host defense against infection through production of interleukin-2 (IL-2) and interferon gamma (IFNγ) whiles Th2 produce a variety of cytokines (IL-4, IL-5, IL-9, and IL13) which drive allergic responses by varying mechanisms. In asthmatics there is a tilt towards Th2 arm. Interleukin-5 (IL-5) is responsible for eosinophil terminal differentiation and survival, IL-4 plays an important role in Th2 cell differentiation and interleukin-13 (IL-13), IgE formation. Lastly, interleukin-9 (IL-9) is associated with eosinophils and mast cells recruitment and activation (Kay, 1991).

Macrophages are the most numerous cells in the airways and also can be activated by allergens through low-affinity IgE receptors to release inflammatory mediators and cytokines that amplify the inflammatory response (Peters-Golden, 2004).

Neutrophils are increased in the airways and sputum of persons who have severe asthma, during acute exacerbations and in the presence of smoking. Their pathophysiological role remains uncertain; they may be a determinant of a lack of response to corticosteroid treatment (Kimb and Liub, 1995).

Dendritic cells function as key antigen-presenting cells that interact with allergens from the airway surface and then migrate to regional lymph nodes to interact with regulatory cells and ultimately to stimulate Th2 cell production from naïve T cells (Kuipers and Lambrecht, 2004).

Structural cells of the airway also produce inflammatory mediators and contribute to the persistence of inflammation in various ways. Structural cells that contribute to the inflammatory process are enumerated below.

Epithelial cells provide inflammatory mediators such as endothelins, pro-inflammatory cytokines, chemokines and growth factors (Devalia *et al.*, 1993). The may play an important role in translating inhaled environmental signals into an airway response and are probably the main target cell for inhaled glucocorticoids.

Airway smooth muscles express similar inflammatory proteins to epithelial cells. This tissue regulates the bronchomotor tone and also contributes to the inflammatory milieu of the disease through the expression of cell surface molecules and Toll like receptors (Chaudhuri *et al.*, 2007) that mediate the release of cytokines upon stimulation with environmental pathogens

Endothelial cells of the bronchial circulation play a role in recruiting inflammatory cells in from the circulation into the airway.

Fibroblasts and myofibroblasts produce connective tissue components such as collagens and proteoglycans, which are involved in airway remodeling.

Airway nerves are also involved. Cholinergic nerves may be activated by reflex triggers in the airways and cause bronchoconstriction and mucus secretion. Sensory nerves which may be sensitized by inflammatory stimuli including neurotrophins cause reflex changes and symptoms

such as cough and chest tightness and may release inflammatory neuropeptides (Groneberg *et al.*, 2004).

Apart from the cells (inflammatory and structural cells) mentioned above, which are involved in the pathophysiology of asthma, many different mediators have been implicated in asthma and they may have a diversity of effects on the airways which could account for the pathological features of asthma (Barnes *et al.*, 1998). Mediators such as histamine, prostaglandins and leukotrienes contract airway smooth muscles, increase microvascular leakage, mucus secretion and attract other inflammatory cells. The role of each inflammatory mediator in the pathophysiology of asthma still remains unclear because each mediator has many effects. Therefore antagonizing a single mediator may not result in significant impact in clinical asthma due to the multiplicity of mediators involved.

The cysteinyl-leukotrienes (LTC4, LTD4 and LTE4) may play an important role in asthma and are known potent constrictors of airways smooth muscle which are reported to increase airway hyper responsiveness (Arm and Lee, 1993). Development of their antagonist has made it possible for their role in asthma to be evaluated. LTD4 antagonists protect (by about 50%) against exercise and allergen-induced bronchoconstriction (Manning *et al.*, 1990; Taylor *et al.*, 1991). They are the only mediators whose inhibition has been specifically associated with an improvement in lung function and asthma symptoms (Busse 1996; Leff, 2001). This mediators which are mainly derived from mast cells however have modest improvement in lung function compared with corticosteroids in chronic treatment (Spector *et al.*, 1994).

Platelet activating factor (PAF) is another mediator which has attracted considerable attention since it mimics many of the features of asthma including airway hyper responsiveness (Barnes *et al.*, 1988). However initial results with potent PAF antagonist such as apafant in chronic asthma has been disappointing (Spence *et al.*, 1994).

Cytokines direct and modify inflammatory response in asthma and are likely to be responsible for severity. Th2-derived cytokines include IL-5, which is needed for eosinophil differentiation and survival, 1L-4 which is important for Th2 cell differentiation and with IL-13 important for IgE formation. Key cytokines include interleukin 1β (IL-1β) and tumor necrosis factor-α (TNF-α), which amplify the inflammatory response, and granulocyte colony-stimulating factor (GM-CSF), which prolongs eosinophil survival in airways (Barnes, 1994). Recent studies of treatment directed toward single cytokines (e.g., monoclonal antibodies against IL-5 or soluble IL-4 receptor) have not shown benefits in improving asthma outcomes. Many inflammatory cells (macrophages, mast cells, eosinophils, lymphocytes) together with structural cells such as epithelial and endothelial can synthesize and release these proteins. Unlike mediators such as histamine and leukotrienes that play a role in acute and subacute inflammatory process and exacerbations of asthma, cytokines have a dominant role in chronic inflammatory process (Barnes, 1994).

Chemokines are important in recruitment of inflammatory cells into the airways and are mainly expressed in airway epithelial cells (Zimmermann *et al.,* 2003).

Nitric oxide (NO) is produced by several cells in the airway by Nitric Oxide synthases (NOS) (Barnes, 1995). Inducible nitric oxide synthase (iNOS) is the inducible form of the enzyme expressed in the epithelial cells of asthmatic patients and can be induced by cytokines in airway epithelial cells (Hamid *et al.,* 1993). This may be the reason for increased concentration of nitric oxide in exhaled air of the asthmatic patient. Measurements of fractional exhaled NO (FeNO) may be useful for monitoring response to asthma treatment because of the purported association between FeNO and the presence of inflammation in asthma (Green *et al.,* 2002). NO may increase plasma exudation in airways because of its potent vasodilator property and may amplify the TH2lymphocyte mediated response (Strunk *et al.,* 2003).

1.3.2.2 Bronchoconstriction

The most dominant physiological event leading to clinical symptoms is airway narrowing and subsequent interfering with airflow. In acute exacerbations of asthma, bronchial smooth muscle contraction (bronchoconstriction) occurs quickly in response to exposure to a variety of stimuli including allergens or irritants bronchial. IgE-dependent release of mediators from mast cells including histamine, tryptase, leukotrienes, and prostaglandins that directly contract airway smooth muscle mediate allergen-induced acute bronchoconstriction (Busse and Lemanske 2001). Nonsteroidal anti-inflammatory drugs can also cause acute airflow obstruction in some patients, and evidence indicates that this non-IgE-dependent response also involves mediator release from airway cells (Stevenson and Szczeklik 2006). In addition, other stimuli (including exercise, cold air, and irritants) can cause acute airflow obstruction. The mechanisms controlling the airway response to these factors are less well defined, but the intensity of the response appears linked to underlying airway inflammation. Stress may also play a role in precipitating asthma exacerbations. The mechanisms involved have yet to be established and may include enhanced generation of pro-inflammatory cytokines

1.3.2.3 Hypersecretion, Hyperresponsiveness and Remodelling

As a result of persistence and progression of inflammation, airflow obstruction is compounded by mucus hypersecretion and oedema which combines with desquamated epithelial cells to form mucus plugs. There is also evidence of submucosal gland hyperplasia confined to larger airways with increased numbers of epithelial goblet cells. Goblet cells are the main source of mucus production in peripheral airways with recent study suggesting the control of goblet cells in guinea pigs are under the stimulating effect of cholinergic, adrenergic and sensory neuropeptide (Kuo *et al.,* 1990). An exaggerated response in the airway to a wide variety of stimuli is referred to as

hyperresponsiveness. The mechanisms influencing airway hyperresponsiveness are multiple and include inflammation, dysfunctional neuroregulation, and structural changes with inflammation appearing to be a major factor in determining the degree of airway hyperresponsiveness. Also permanent structural changes can occur in the airway resulting in progressive loss of function of the lungs. These structural changes which is known as remodeling can include thickening of the sub-basement membrane, subepithelial fibrosis, airway smooth muscle hypertrophy and hyperplasia, blood vessel proliferation and dilation, and mucous gland hyperplasia (Holgate and Polosa, 2006).

1.3.2.4 Neural effects

The airway in man is innervated by efferent and afferent autonomic nerves. In asthmatic patients, it is suggested that the neuronal control of the airway may be deficient and that neurogenic mechanisms may contribute to the pathogenesis and pathophysiology of asthma (Van der Velden and Hulsmann, 1999). Parasympathetic nervous system is the dominant neuronal pathway in the control of airway tone. When stimulated there is bronchoconstriction, mucus secretion and bronchial vasodilation. Recent data suggest that enhanced neural activity may play a key role in the symptomatology and pathophysiology of airway inflammatory diseases (Belvisi, 2002). It is not surprising therefore that anticholinergics such as ipratropium bromide (Atrovent (®)) and tiotropium bromide (Spiriva (®)) have been proven to be important when used as bronchodilators for the treatment of obstructive airway diseases. Though the human airway smooth muscle is not innervated by sympathetic nervous system, beta-adenergic receptors inundate the human airway smooth muscle and may control tracheobronchial blood vessels. Stimulation of these receptors also results in bronchodilation (Dinh *et al.*, 2011). It is noteworthy that this classical view of one excitatory (cholinergic) and one inhibitory (noradrenergic) component, of the innervation of

airway smooth muscle is incomplete, and at least two other , possibly peptidergic, types of innervation must be included when the innervation of airways is considered. Inhibitory nonadrenergic noncholinergic (NANC) nerves, containing vasoactive intestinal peptide, may be the only direct neural bronchodilator pathway in human airways. Stimulation of excitation NANC nerves causes bronchoconstriction, mucus secretion, vascular hyperpermeability, cough and vasodilation, a process called 'neurogenic inflammation' (Andersson and Grundstrom, 1987).

1.3.2.5 Diarrhoea in asthma

Recent studies have suggested that diarrhoea could be implicated in certain asthmatic patients. According to Awortwe *et al.,* (2013), inflammatory mediators, such as histamine and eicosanoids are related to the pathophysiology of asthma which include bronchospasm, vasodilation, acute functional changes in the lungs and diarrhoea due to increased intestinal motility. This increased intestinal motility could also be due to increased vagal stimulation (Awortwe *et al.,* 2013) Survey conducted by a group of scientists discovered that children with asthma had a greater frequency of gastrointestinal symptoms particularly diarrhoea (Caffareli *et al.,* 2000).

1.3.3 Diagnosis of asthma

The diagnosis of asthma requires the evaluation of the patient's medical and family history. Symptoms, such as episodic dyspnea, wheeziness, persistent cough and chest tightness, along with family history of allergies or asthma, strongly suggest an asthma diagnosis. Physical examination of the respiratory function could further support the presence of airflow limitation or other features such as hyperinflation if the examination is performed during symptomatic periods (NHLBI, 2007). Of all the different methods used in the assessment of airflow limitation, two have gained widespread acceptance. These are spirometry, particularly the measurement of forced expiratory

volume in 1 second (FEV1) and forced vital capacity (FVC), and peak expiratory flow measurement. (PEF).

Spirometry is the recommended method for measuring airflow limitation and reversibility to establish a diagnosis of asthma. During the spirometry test, air flow and volume are measured during a forced expiratory maneuver to determine the forced expiratory volume in one second (FEV1) and forced vital capacity (FVC). Reversibility is assessed by the improvement in FEV1 after inhalation of a short-acting bronchodilator, or following a more effective controlled treatment such as glucocorticoids; while variability which refers to an improvement or deterioration in symptoms and lung function occurring overtime is assessed by measuring the changes in lung function during the day, from month to month or seasonal (Pellegrino *et al.*, 2005). A 12% improvement (or > 200 ml) in FEV1 post bronchodilator therapy is accepted as reversible airflow obstruction and therefore indicative of asthma (Pellegrino *et al.*, 2005). In addition, spirometry is also useful to differentiate between restrictive and obstructive lung disorders. Asthma, for example, is an obstructive lung disease. Therefore, in contrast with restrictive lung diseases, FVC remains close to normal while FEV1 decreases and the ratio FEV1/FVC is decreased (Pellegrino *et al.*, 2005). The FEV1/FVC ratio of about 0.75-0.80 or higher in children (0.90) is considered normal, while any lower value indicates airflow obstruction.

Peak expiratory flow measurements (PEF) are made using a peak flow meter and can be an important aid in the diagnosis and monitoring of asthma. Measurements of PEF are not interchangeable with other measurements of lung function such as FEV1. PEF measurements can underestimate the degree of airflow limitation. Because values for PEF obtained with the different peak flow meters vary and the range of predicted values is too wide, measurements should be

compared to the patient's own previous best measurements using his or her own flow meter (Reddel *et al.,* 2004).

Measurements of airway responsiveness to direct airway challenges such as inhaled methacholine and histamine or indirect airway challenges such as inhaled mannitol or exercise challenge may help establish asthma in patients with symptoms consistent for asthma, but normal lung function (Cockcroft, 2010). These test are sensitive for diagnosis of asthma, but have limited specificity (Cockcroft *et al.,* 1992). A negative outcome can be useful to exclude a diagnosis of persistent asthma in a patient who is not taking glucocorticosteroids but a positive outcome does not always mean that a patient has asthma. This is because airway hyperresponsiveness has been described in patients with allergic rhinitis (Ramsdale *et al.,* 1985) and those with airflow limitation caused by conditions other than asthma, such as cystic fibrosis (Van Haren *et al.,* 1995).

Also, because of the strong association between asthma and allergic rhinitis, the presence of allergies, allergic diseases, increases the probability of a diagnosis of asthma in a patient with respiratory symptoms. Therefore the presence of allergies in asthma patients (identified by skin testing or measurement of specific IgE in serum) can help identify the risk factors that cause asthma in individual patients. Skin tests with allergens represent the primary diagnostic tool in determining allergic status. They are simple and rapid to perform, having a low cost and high sensitivity as well (Hoeppner *et al.,* 1985).

Non-invasive markers of inflammation such as levels of exhaled nitric oxide (FeNO) and carbon monoxide (FeCO) are known to be elevated in people with asthma (Kharitonov *et al.,* 1997).

1.3.4 Management of asthma

Therapy for chronic asthma is directed at suppressing the underlying inflammation and normalizing pulmonary function. Because of the varying presentation of asthma, treatment guidelines for asthma therapy should serve as a guide for therapy with the therapeutic plan individualized for each patient (GINA, 2011).

1.3.4.1 Non-pharmacological management

Non-pharmacological therapy should be incorporated into each step of therapy. Patients should be taught basic facts about asthma, including the difference between the asthmatic and normal lung, what happens to the lung during an asthma attack, how medications work, environmental control measures, and self-management of asthma, including skills for self-monitoring of pulmonary function, symptoms of asthma deterioration, and when and how to take rescue actions. The role of long-term medications and quick relief medications should be clearly understood in the treatment of their conditions. Proper use of medication device should also be clearly demonstrated and enforced with patient advised to be on the lookout for triggering factors.

1.3.4.2 Pharmacotherapy

Anti-inflammatory drugs and bronchodilators are the main stay of most common asthma therapies. The former suppresses the underlying inflammation; while the latter acts by reverting airway smooth muscle contraction (Barnes, 2011a). Glucocorticoids belong to the anti-inflammatory class and by far, are the most effective therapy for controlling asthma (Barnes, 2011b). Although glucocorticoids do not cure asthma, their effectiveness probably stems from their effect on multiple inflammatory pathways. They reduce airway inflammation by suppressing inflammatory cells, preventing microvascular leakage which leads to improve lung function, decrease AHR and reduce

the frequency of acute exacerbations. Mechanisms of action of glucocorticoids include activation of genes encoding anti-inflammatory secretory leukoprotease inhibitor (SLPI) and MAPK phosphatase-1 (MKP-1) that inhibits MAPK pathways. Glucocorticoids are also capable of switching off activating inflammatory genes that code for cytokines, chemokines, adhesion molecules, inflammatory enzymes and receptors via attenuation of NF-κB-associated co activator activity. Despite this, their use is limited because of serious side effects associated with them. These include Cushing's habitus, fragile skin, purple striae, hyperglycaemia, muscular weakness, susceptibility to infection, delayed healing of wounds and surgical incisions, peptic ulceration, osteoporosis, glaucoma, growth retardation, psychiatric disturbances, suppression of hypothalamicpituitary-adrenal (HPA) axis (Barnes, 2011b).

Beta 2 (β_2)-adrenergic agonist are the most effective bronchodilators. By activating adenylyl cyclase through the stimulatory G-protein, they result in relaxing the smooth muscle of the airway. β_2 – receptor agonists may also relieve bronchoconstriction indirectly by inhibiting the release of bronchoconstriction agents from inflammatory cells, and neurotransmitter from airway nerves. In spite of the effectiveness of β_2 –agonists as bronchodilator agents, they are unable to mitigate the underlying chronic inflammation. Combination with glucocorticoid is therefore necessitated (Gibson *et al.,* 2007). Side effects include cardiovascular stimulation, skeletal muscle tremor and hypokalemia. Regular use also leads to refractoriness.

By interfering with cysteinyl leukotrienes pathways, there is improvement in pulmonary function, symptoms, and exacerbation of asthma. Montelukast is an example of the several cysteinyl leukotrienes receptor antagonist that have been developed for the treatment of asthma. In addition, pharmacological blockers of cysteinyl leukotrienes pathways 5'-lipoxygenase, for example, the enzyme inhibitor zileuton, have also shown therapeutic benefits for asthma treatment (NHLBI,

2007). When used alone as asthma controller, leukotrienes modifiers exhibit less efficacy than low doses of glucocorticoids. However when given in combination of glucocorticoids the dose of the glucocorticoid is reduced and there is improvement in glucocorticoid treated patients with severe asthma (GINA, 2011).

Theophylline is an example of methylxanthine used in the management of asthma because of its bronchodilatory effect. It enhances the anti-inflammatory effect of corticosteroids when used in combination. Side effects such as convulsions, shock, arrhythmias, increased muscle tone, tachypnoea, (dose-dependent) flushing, hypotension, restlessness, tremors, vomiting, palpitation, diuresis, dyspepsia, insomnia as well as the small therapeutic window have limited their use.

Anticholinergics, such as ipratropium bromide, are antagonist of muscarinic receptors, which block the effect of endogenous acetylcholine. Although they are potent bronchodilators their potency is less than β_2-agonists. However its combined actions exert an additional bronchodilator effect, especially in patients with more severe disease.

Generally, current therapies allow adequate control of asthma especially the combination therapy of inhaled corticosteroids and β_2-agonists. However concerns for usage stems from its long term usage and side effects associated with it. Additionally, corticosteroids do not cure or modify the course of the disease and inflammation recurs as soon as the medication is interrupted (Barnes, 2010). Furthermore, patients with severe asthma and are resistant to corticosteroids, achieve poor levels of control of asthma with current therapies (Wenzel *et al.*, 2007). Obviously, efforts are still required to fill these gaps.

1.3.5 Animal models of asthma and diarrhoea.

As a result of the complex nature involving the pathophysiology of asthma, it is impossible to get a model that fully mimics that found in nature (Nials and Uddin, 2008). However, most of the features of the human syndrome have been successfully recapitulated in animal models (Bates *et al.*, 2009).

The focus in times past and present have been on species involved in the animal model of asthma. Mice, rats, monkeys, guinea pigs, sheep, monkeys and horses have been employed to study the inflammatory processes and alterations in airway function (Shin *et al.*, 2009). However, their use has been limited because of lack of availability of species, specific reagents, along with increased ethical considerations regarding their use for research purposes (Bates *et al.*, 2009). In the present study guinea pigs and mice were used with reasons for their use enumerated below. Sprague-Dawley rats were used in the diarrhoea models.

Guinea pig models of asthma

Guinea pigs have been utilized as animal model for many decades. Studies with guinea pigs showed increase in IgE and immunoglobulin G1 (IgG1) in response to allergen (Noelpp and NoelppEschenhagen, 1952). One benefit associated with this model is that the lungs serve as primary target organ of type 1 hypersensitivity reaction to allergen sensitization. Besides this, is the rich eosinophilic and neutrophilic pulmonary infiltration (Ricardolo *et al.*, 2008). Although there are various models of asthma, guinea pig airways react to histamine, leukotrienes, acetylcholine and other bronchoconstrictors in a way similar to that in man (Agrawal *et al.*, 1991). Guinea pig models of asthma have been used in recent studies to evaluate large number of different classes of compounds. Among them include the use of phosphodiesterase (PDE) inhibitors especially those specific for PDE4 (Kim *et al.*, 2003) and immunosuppressives (Xie *et al.*, 2002).

Histamine-induced bronchoconstriction in guinea pigs

The role of histamine in asthma is well established (Nelson, 2003). In the early stages of asthma, release of inflammatory mediators like histamine, acetylcholine, leukotrienes, and prostaglandins are triggered by exposure to allergens, irritants, cold air or exercise. Some of these mediators like histamine cause bronchoconstriction. The close resemblance of pulmonary responses to histamine challenge in both guinea pigs and humans, as well as the anaphylactic sensitization made this species the model of choice. In the present study, guinea pigs were used because of the extreme sensitivity of their airways to the primary mediators of bronchoconstriction, including histamine and leukotrienes, and their ability to be sensitized to foreign proteins. Histamine antagonists such as mepyramine can be conveniently recognized and assayed by their ability to protect guinea pigs against lethal effects of histamine-induced bronchospasm (Broadbent & Bain, 1964). When guinea pigs are exposed to histamine aerosols, it results in intense smooth muscle contraction, hypoxia leading to convulsion, asphyxia and death. Bronchodilators can delay the occurrence of these symptoms. Hence molecules that delay this occurrence are potential bronchodilators (Nayampalli *et al.,* 1986).

Mouse models of asthma

The growing use of mice for asthma models could be attributed to availability of molecular and immunological tools, lower maintenance cost, and shorter gestation period (Bates *et al.*, 2009). However, some limitations such as mast cells which are visibly present and involved in the pathogenesis of asthma in man, are not noted in the respiratory mucosa of mice. Another limitation is that there is transient airway hyperreactivity and weak Late Phase Response (LPR); Airway hyperresponsiveness requires high doses of agonist; unresponsive to many of the mediators

associated with pathogenesis of asthma. In this study, mice were used to evaluate the effect of cryptolepine in mucus secretion using the phenol red mucus secretion method.

Tracheal phenol red secretion in mice (Engler and Szelenyi, 1984)

This is a simple method for screening drugs that influence tracheobronchial secretion. When phenol red is applied intraperitoneally, part of the dye is secreted into the tracheal lumen. This basal amount is increased by both parasympathomimetics and sympathomimetics, in addition to expectorants. The reverse is true for parasympatholytics. As such, drugs that have low output have higher proclivity to inhibit mucus secretion.

Castor oil-induced diarrhoea

This model is based on the principle that the active agent of castor oil, that is, ricinoleic acid, acts primarily in the small intestines, where it reduces permeability changes in mucosal fluid and electrocyte transport resulting in increased intestinal transit (Ammon *et al.*, 1974). This is so, because, it causes irritation and inflammation of the intestinal mucosa which leads to production of prostaglandins in the process. Prostaglandins are known to cause secretion and also increase the motility of the gastrointestinal tract (GIT) (Pierce *et al.*, 1971).

Charcoal meal test

This model is based on the fact that charcoal is not absorbed in the GIT. Therefore the distance travelled by the charcoal, gives an idea of the effect of the pre-administered agent on the propulsive effect of the GIT.

1.4 JUSTIFICATION OF STUDY

The purpose of asthma therapies is to optimize lung function, prevent acute exacerbations and maintain the quality of life of asthma patients while minimizing the side effect of asthma medications. However, current therapies are beset with challenges. As stated above a combination of bronchodilators such as, beta-2 adrenergic agonists, methylxanthines (theophylline), antimuscarinics and anti-inflammatory agents with corticosteroids being the main class, are used in the management of asthma. Each class of drugs have side effects associated with it. This is further compounded by recent recommendations, which suggest the combination of drugs, a bronchodilator and an anti-inflammatory agent. As a consequence, the search for effective lowrisk drug molecules that have both bronchodilator and anti-inflammatory effect on the airway will provide a valuable adjunctive or alternative treatment in asthma management making it clinically attractive and relevant.

Cryptolepine is the major alkaloid of *Cryptolepis sanguinolenta*, the West African antimalarial plant. Both cryptolepine and other alkaloidal fractions of *Cryptolepine sanguinolenta* have been reported to have in vitro antimuscarinic action at muscarinic subtype 1, 2, 3 (M_1, M_2, M_3) receptors (Rauwald *et al.*, 1992). The role of antimuscarinics in asthma management cannot be overemphasized. In addition to this, recent findings indicate that cryptolepine is an antiinflammatory agent in both *in vitro* and *in vivo* animal models (Olajide *et al.*, 2009). Cryptolepine also inhibits neuro-inflammation in LPS mediated microglia (Olajide *et al.*, 2013). As to whether this anti-inflammatory effect will translate to the airways and ultimately be of benefit in asthma is yet to be established.

Also, recently diarrhoea has been implicated in some asthmatic patients. This cause is attributed to release of mediators such as histamine and general vagal stimulation which lead to increased intestinal motility (Awortwe *et al.,* 2013). Cryptolepine is reported to possess both antihistaminic and antimuscarinic properties by Noamesi and Bamgbose (1982) and Rauwald *et al*., (1982) respectively. These properties could be of benefit in asthma-induced diarrhoea.

This work evaluates the antiasthmatic and antidiarrhoea effects of Cryptolepine.

1.5 AIMS AND OBJECTIVES

1.5.1 Aims

This study investigates the possible anti-asthmatic and anti-diarrhoeal effects of cryptolepine.

1.5.2 Specific objectives

Specific objectives include:

- Isolation of cryptolepine from the roots of *Cryptolepine sanguinolenta*
- Assessment of the effect of cryptolepine on airway obstruction
    - Determining the effect of cryptolepine on histamine-induced bronchoconstriction
    - Estimation of the effect of cryptolepine on tracheal phenol red mucus secretion
    - Evaluation of the effect of cryptolepine on mast cell stabilizing action
- Assessment of the effect of cryptolepine on airway inflammation
    - Estimation of the effect of cryptolepine on extent of inflammatory response to antigens-skin prick test
    - Estimation of the effect of cryptolepine on inflammatory cells (eosinophils, basophils, lymphocytes)

- Evaluation of the effect of cryptolepine on smooth muscles, epithelial cells, basement membrane of the airway (hypertrophy)

- Assessment of the effect of cryptolepine in diarrhoea
  - Determination of the effect of cryptolepine in castor oil-induced diarrhoea
  - Evaluation of the effect of cryptolepine on charcoal meal test

2 CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 PLANT COLLECTION

The root of *Cryptolepis sanguinolenta* used in this study was collected at the Centre for Scientific Research into Plant medicine (CSRPM), Mampong-Akwapim, Ghana in November 2012 and was

confirmed by Dr. Ameyaw, a Botanist of the CSRPM. Its authenticity was confirmed by Dr Kofi Annan of the Department of Pharmacognosy, KNUST and afterwards compared to a voucher specimen KNUST/HM1/2008/L056 at the herbarium of the Department of Pharmacognosy/ Herbal Medicine, College of Health Sciences.

2.2 ISOLATION AND PURIFICATION OF CRYPTOLEPINE

The approach used by Cimanga and co-workers in the isolation of cryptolepine hydrochloride and the determination of its content in the root (Cimanga *et al.*, 1996; Cimanga *et al.*, 1997) was used in the extraction. The roots were pulverized with a ball miller after it had been cut and sun-dried. Powdered root (600 g) was exhaustively extracted with methanol (2.5 L) by Soxhlet extraction at 50 ºC for 48 hours to afford a dark crude alkaloid mixture. The crude extract was concentrated in vacuo to about 300 mL using a rotary evaporator, (rotavapor® R-215, Buchi, Switzerland). The mixture was rendered alkaline (pH >11) with aqueous ammonium hydroxide and extracted with 5 portions of 100 ml chloroform in a 1 L separation funnel. The combined organic layer was again concentrated in vacuo to about 50 mL, adsorbed onto aluminium oxide, and air-dried. The dried aluminium oxidealkaloid mixture was dry-loaded onto a 500 mL aluminium oxide packed column and eluted with dichloromethane followed by chloroform and chloroform containing 10 % methanol. Fractions (10 ml) were collected and identical fractions were combined following TLC on silica gel plates (5×8 cm) using a mixture of dichloromethane, chloroform, and methanol (4:4:1) as the mobile phase. Identical fractions containing cryptolepine (deep purple solution) (Cimanga *et al*., 1997) was concentrated and acidified with aqueous HCl (200 mL, pH 4). The aqueous phase was separated and basified with aqueous ammonium hydroxide to precipitate cryptolepine, which was then extracted with 200 ml of chloroform. Finally, the chloroform layer was treated with

acidified ether to precipitate cryptolepine as a yellow hydrochloride salt. The solvent was removed in vacuo and the material dried into a free-flowing yellow powder. The melting point was determined using an electrothermal melting point apparatus. Purity was confirmed by UV and melting point determination.

The yield of the isolated cryptolepine as the hydrochloride salt was 5.67 g. Melting point was determined with an electrothermal apparatus.

2.3 DRUGS AND CHEMICALS

Histamine, mepyramine, atropine, ammonium chloride, phenol red, sodium bicarbonate, compound 48/80, ketotifen fumarate, formaldehde, toluidine blue, acetone, xylene, castor oil, methanol, chloroform, ammonium hydroxide, aluminium oxide, dichloromethane and activated charcoal were purchased from Sigma Aldrich Inc., St Louis, MO, USA and Phyto-Riker Pharmaceuticals Accra Ghana.

2.4 ANIMALS

Male ICR mice (20-25 g), guinea pigs (350-500 g) and Sprague-Dawley rats were all obtained from Noguchi Memorial Institute for Medical Research, Accra, Ghana and kept at the vivarium of the

Department of Pharmacology, KNUST, Ghana. They were grouped in stainless steel cages (34 × 47

× 18 cm^3) with soft wood shavings as bedding and fed *ad libitum* with commercial pellet diet (Agricare limited, Tanoso-kumasi) and water. All animals used were naïve and used only once. All procedures employed were in accordance with the National Institute of Health Guidelines for Care and Use of laboratory animals and were approved by the Departmental Ethics Committee.

2.5 EFFECT ON AIRWAY OBSTRUCTION

2.5.1 Histamine-induced bronchoconstriction

Overnight fasted guinea pigs were exposed to histamine aerosol (0.2%) produced by an ultrasound nebulizer in an aerosol chamber (30 x 15 x15cm) made of perspex glass to induce bronchospasm. The pre-convulsion time (PCT), that is, the time of aerosol exposure to the onset of dyspnea leading to the appearance of convulsion was noted and recorded as the basal value. The animals were immediately removed from the chamber and placed in fresh air to recover from the dyspnea. After 24 hours, the animals were allotted to five different groups of 4 animals per group. Animals in Group 1 served as control and received distilled water. Groups 2, 3 and 4 were given 10, 30, and 100 mg/kg of the Cryptolepine intraperitoneally (*i.p*) respectively, while group 5 received the standard drug – mepyramine 8 mg/kg *i.p*. All the animals were again exposed to histamine aerosol in the chamber, 1 hour, 6 hours and 24 hours after receiving the drugs, to determine pre-convulsive time (PCT). The protection offered by the treatment was calculated using the formula

Percentage protection = (1-T1/T2) X 100 where T1 = mean of PCT before administration of test drugs, and T2 = mean of PCT after administration at 1, 6 and 24 hours.

2.5.2 Tracheal phenol red secretion

The mucus secretion assay was based on the method of Engler and Szelenyi (1980). Forty (40) mice were assigned to 6 different treatment groups. Group I served as control and received water. Group II-IV received cryptolepine 10, 30 and 100 mg/kg respectively. Group V and VI were treated with atropine (100 mg/kg) and ammonium chloride (1500 mg/kg) respectively. Each mouse was treated by oral administration of the test drug. Thirty minutes later, each mouse was injected

with phenol red (5% in saline solution, 0.2/20g body weight *i.p*). All the animals were sacrificed by cervical dislocation thirty minutes after the injection of the phenol red. The whole trachea was excised, cleared of adhering tissues and washed in 2 ml physiological saline. Ultrasonification was carried out for 15 minutes with sodium bicarbonate (2 ml, 5%w/v) added to stabilize the pH of the lavage fluid. The absorbance of the phenol red was read at 542 nm using a spectrophotometer (T-70 UV/visible). A graph of absorbance against concentration was plotted from which concentrations of phenol red were extrapolated.

2.5.3 Mast cell stabilizing effect of cryptolepine

Studies on Compound 48/80 Induced Rat Mesenteric Mast cell degranulation

Rats were sacrificed and the pieces of mesentery were collected in petri dish containing Ringer Locke solution and then subjected to following conditions

Petri dish no. 1 - Ringer Locke solution (Positive control)

Petri dish no. 2 - 0.1ml of Ketotifen fumarate (10 µg/ml)

Petri dish no. 3 - 0.1ml of test agent in distilled water (CLP-10 µg/ml)

Petri dish no. 4 - 0.1ml of test agent in distilled water (CLP-30 µg/ml)

Petri dish no. 5 - 0.1ml of test agent in distilled water (CLP-100 µg/ml)

Each petri dish was incubated for 15 min at 37°C. Later Compound 48/80 (0.1 ml, 10 µg/ml) was added to each petri dish and again incubated for 10 min. at 37°C. Following that, all pieces of the mesenteric cells were transferred to 4% formaldehyde solution containing 0.1% toluidine blue and kept aside for 20 to 25 min for fixation and staining of cells. After staining and fixation of mast

cells, mesentery pieces were transferred through acetone and xylene two times and mounted on slides. All the pieces were examined under the high power of light microscope. Percent protection of the mast cells in the control group and the treated groups were calculated by counting number of degranulated mast cells from a total of 100 mast cells counted. Percent mast cell degranulation (MCD) for each treatment was calculated by following formula:

% MCD= [[number of degranulated cells]/ (total number of mast cells)]*100

2.6 EFFECT OF CRYPTOLEPINE ON AIRWAY INFLAMMATION.

2.6.1 Sensitization and treatment of guinea pigs

Chronic model of asthma used in this study was developed as described by Awortwe *et al.,* 2013 with slight modifications. Guinea-pigs used in the study were randomly put into six different groups of five animals each: group A (non-sensitized and unchallenged controls); group B (ovalbumin (OA)-sensitized controls treated orally with normal saline); group C (OA-sensitized treated orally with 1 mg/kg of dexamethasone as reference standard) and group D to group F (OA-sensitized treated with cryptolepine 10, 30 and 100 mg/kg orally respectively)

All animals (except group A) were sensitized with two different doses of 10 mg OA and 30 mg aluminium hydroxide intraperitoneally and subcutaneously respectively, each on day zero. Immune response boosting of antigen was done using 0.1 ml solution containing 1 mg OA dissolved in 0.9% saline intraperitoneally on day-14.

2.6.2 Ovalbumin challenge

On the 21st day through to 60th day, sensitized guinea-pigs were challenged with 1% aerosolized OA (0.1 g OA dissolved in 10 ml saline) for 7 min after 1 h of drug treatment. Challenge was done

every other day till the end of the period. Group A animals were not challenged. The challenge was conducted in Perspex chamber (dimensions = 20 × 30 cm) connected to a jet nebulizer. Figure 2.1 illustrates the schematic diagram of the experimental protocol indicating the events and durations.

Fig 2. 1 Schematic diagram of the experimental protocol indicating the events and durations in days

2.6.3 Skin prick test

On day-50, the back of each guinea-pig was shaven and injected intradermally with 1 % OA dissolved in 0.1 ml saline. The diameter of wheals formed in the skin of each animal was monitored and measured until complete disappearance of the oedema. The average skin oedema was calculated for each group.

2.6.4 Histopathological study

The lungs were swiftly excised from the thoracic cavity. They were immediately washed 4 times with 0.9% saline. The tissues were fixed with 10% neutral buffered formaldehyde (pH = 7.4), embedded in paraffin wax (56 to 60°C) and sectioned at 4 μm for histopathological examination. The sectioned tissues were stained with hematoxylin and eosin (H&E) and Periodic Acid Schiff

(PAS) for light microscopy of airway inflammation, epithelium,subepithelial smooth muscle thickness and mucin secreting goblet cells. Lung sections were evaluated microscopically using Olympus BX 51TF (Olympus Corporation, Tokyo, Japan) light microscope connected to a digital camera for (Leica ICC50 HD Camera) morphology in the bronchiolar structure captured at 40× magnification. The degree of airway inflammatory cell infiltration was scored according to Myou *et al*., (2003). The degree of peribronchiole and perivascular inflammation was evaluated by a subjective scale of 0–4. The scoring system for cell infiltration was: 0, no cells; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2–4 cells deep; 4, a ring of cells>4 cells deep. Goblet cell hyperplasia in the airway epithelium was quantified based on a five-point system: 0, no goblet cells; 1, <25% of the epithelium; 2, 25–50% of the epithelium; 3, 50–75% of the epithelium; 4, > 75% of the epithelium. For each mouse, 5 airway sections that were randomly distributed throughout the lung were analyzed, and their average scores were calculated.

2.7 ANTI-DIARRHOEAL EFFECT

2.7.1 Castor oil induced diarrhoea

A modification of the method of Awouters *et al*. (1978) by Nwodo and Alumanah (1991) was adopted. The rats were fasted for 24 h but allowed free access to water. They were randomized and placed in cages of five rats per cage. Group I served as the control and received normal saline. Group II-IV were treated with cryptolepine 10, 30 and 100 mg/kg (*i.p*) respectively with group V receiving atropine at a dose of 3 mg/kg (*i.p*)

After 1 h, each rat received 2 ml castor oil (p.o) and was observed for consistency of faecal matter and the frequency of defaecation for 4 h. Faeces were allowed to collect beneath the slit wire

gauzed cages. The wet faecal matters were easily weighed at the end of the experiment by lifting off the upper part of the cage containing the slit wire gauze and the animals.

2.7.2 Charcoal meal test

The effect of extract on intestinal propulsion in unanaesthetized rats was tested using the charcoal method by Capasso et al. (1976). They were fasted for 24 h but allowed free access to water. They were randomized and placed in five cages of five animals per cage.

Group 1 received normal saline (p.o) using an orogastric cannula. Groups II–IV were pretreated with cryptolepine 10-100 mg/kg (p.o), respectively. Group V was pretreated with 100 mg/kg atropine (p.o). After 1 h, each rat received 1 ml charcoal meal (5% activated charcoal suspended in 10% aqueous tragacant) orally. The rats were sacrificed 30 min later by cervical dislocation and bled, and the small intestine rapidly dissected out and placed on a clean surface. The small intestine was carefully inspected and the distance traversed by the charcoal meal from the pylorus was measured. The length of the whole small intestine was also measured. The distance traversed by the charcoal meal from the pylorus was expressed as a percentage of the distance from the pylorus to the ileocaecal junction.

$$\text{Intestinal propulsion \%} = \frac{\text{Distance moved by the suspended charcoal}}{\text{Whole length of small intestine}} \times 100$$

2.7.3 Statistics

All results are presented as mean ± SEM. Data was analyzed using one-way analysis of variance (ANOVA). Where ANOVA was significant, multiple comparisons between treatments was done

using Turkey multiple comparison or Neuman keuls post hoc test. GraphPad Prism for Windows Version 6 (GraphPad Software, San Diego, USA) was used for all statistical analyses. A sognificance level of 0.05 was used.

CHAPTER THREE

3.0 RESULTS

3.1 CONFIRMATION OF PURITY OF CRYPTOLEPINE

The UV/Vis spectrum of the methanolic solution of isolated cryptolepine yielded a data (fig 3.1) with maximum wavelength (λmax) at 275, 281 and 369 which was also comparable to that described by Dwuma-Badu *et al.*, (1978). Melting point was determined to be 264-268 °C.

Ultraviolet/visible absorption confirmation of isolated cryptolepine

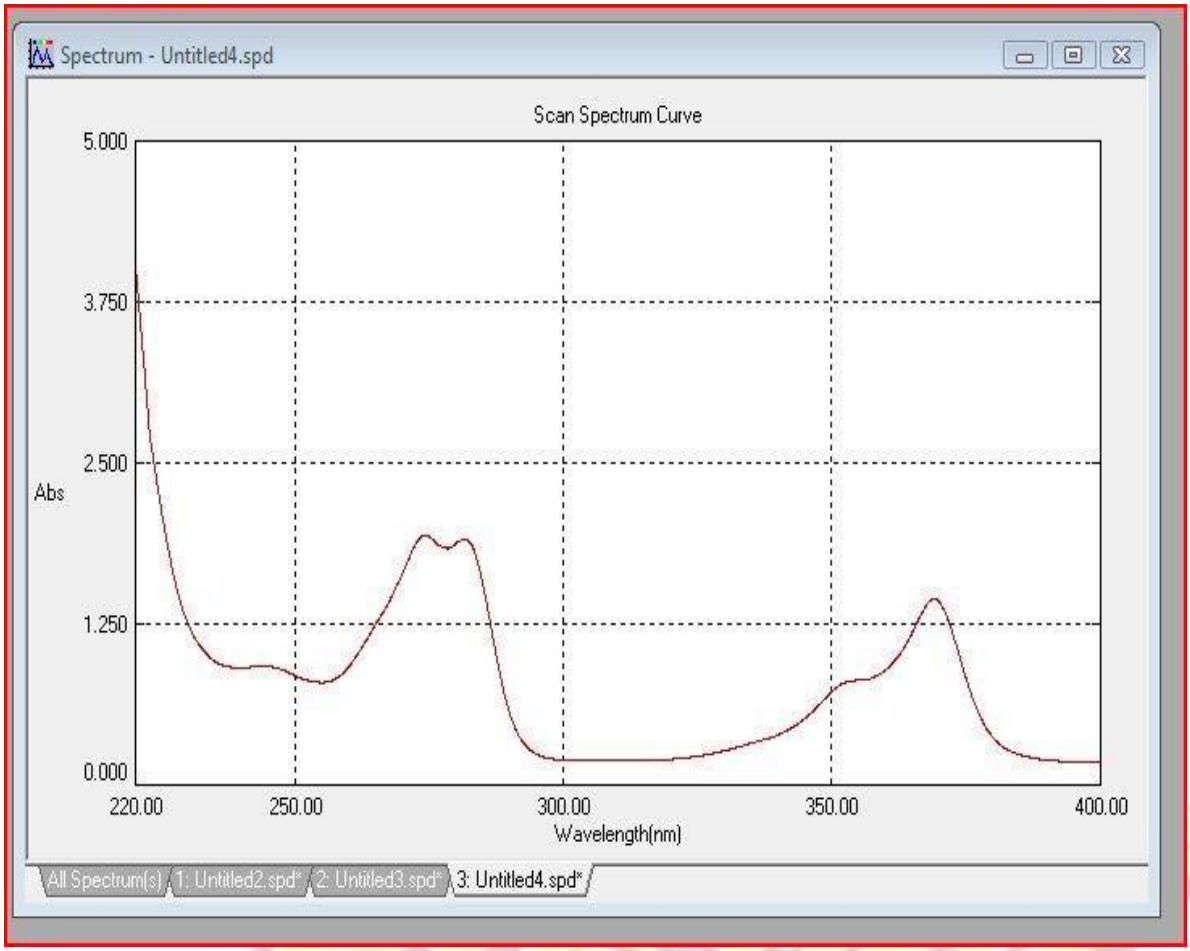

Fig 3. 1 UV/Vis spectrum of methanolic solution of isolated cryptolepine

3.2 EFFECT ON AIRWAY OBSTRUCTION

3.2.1 Histamine-induced bronchoconstriction

Cryptolepine (CLP) showed a delay in pre-convulsive time in gunea pigs in a dose-dependent fashion ($p<0.05$). The effect was prominent after 24 hours. Mepyramine 8 mg/kg showed the highest effect at 1 and 6 hours but no effect after 24 hours (Fig 3.2).

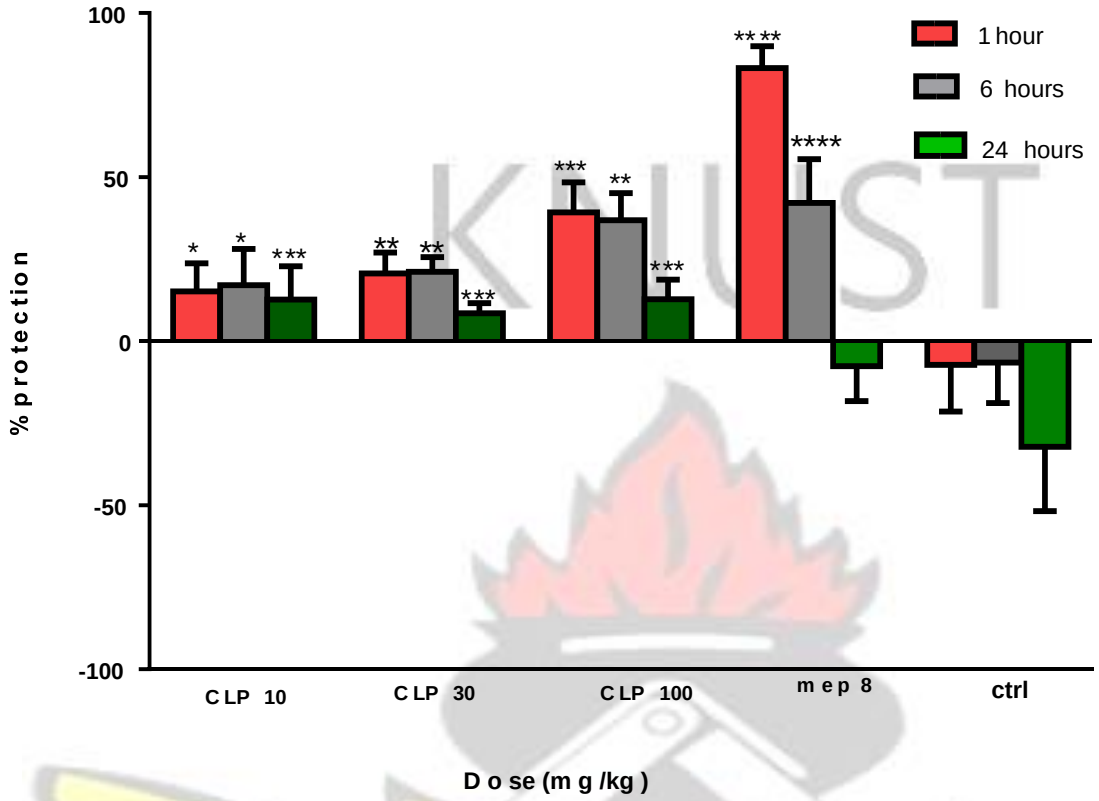

Fig 3. 2 Effect of Cryptolepine on histamine induced bronchoconstriction in guinea pigs. Data are presented as group means (±SEM). Significantly different from control: *p<0.05 **p<0.01, ***p<0.001, by one –way ANOVA followed by Turkey's multiple comparison test.

3.2.2 Tracheal phenol red secretion

Tracheal phenol-red mucus secretion is for the evaluation of drugs that influence tracheobronchial secretion. Cryptolepine showed dose-dependent decrease in tracheal phenol-red mucus secretion ($p<0.05$). The effect have a levelling effect from a dose of 30 to 100 mg/kg (Table 3.1).

Table 3. 1 Effect of cryptolepine on tracheal phenol red mucus secretion.

GROUP	TREATMENT	DOSE (mg/kg)	OPTICAL DENSITY	CONCENTRATION OF PHENOL RED (µg/ml)	%INCREASING OR DECREASING
1	Control(distilled water)		0.261±0.058	8.18±2.17	----------
2	Cryptolepine	10	0.181±0.011*	5.17±0.40*	36.8↓
3	Cryptolepine	30	0.160±0.011**	4.39±0.41**	46.3↓
4	Cryptolepine	100	0.151±0.028***	4.03±1.06**	50.7↓
5	Atropine	100	0.140±0.030***	3.62±1.12***	55.7↓
6	NH_4Cl	1500	0.513±0.039***	17.71±1.48***	116↑

Data are presented as group means (±SEM). Significantly different from control: *p<0.05, **p<0.01, ***p<0.001, by one –way ANOVA followed by Turkey's multiple comparison test

3.2.3 Mast cell stabilizing effect-compound 48/80 induced degranulation of rat mesentery mast cells

Cryptolepine showed protection of mast cell viewed under higher power microscope of ×40. Intact mast cells which have well defined boundaries were counted in relation to degranulated mast cells (Fig 3.3).

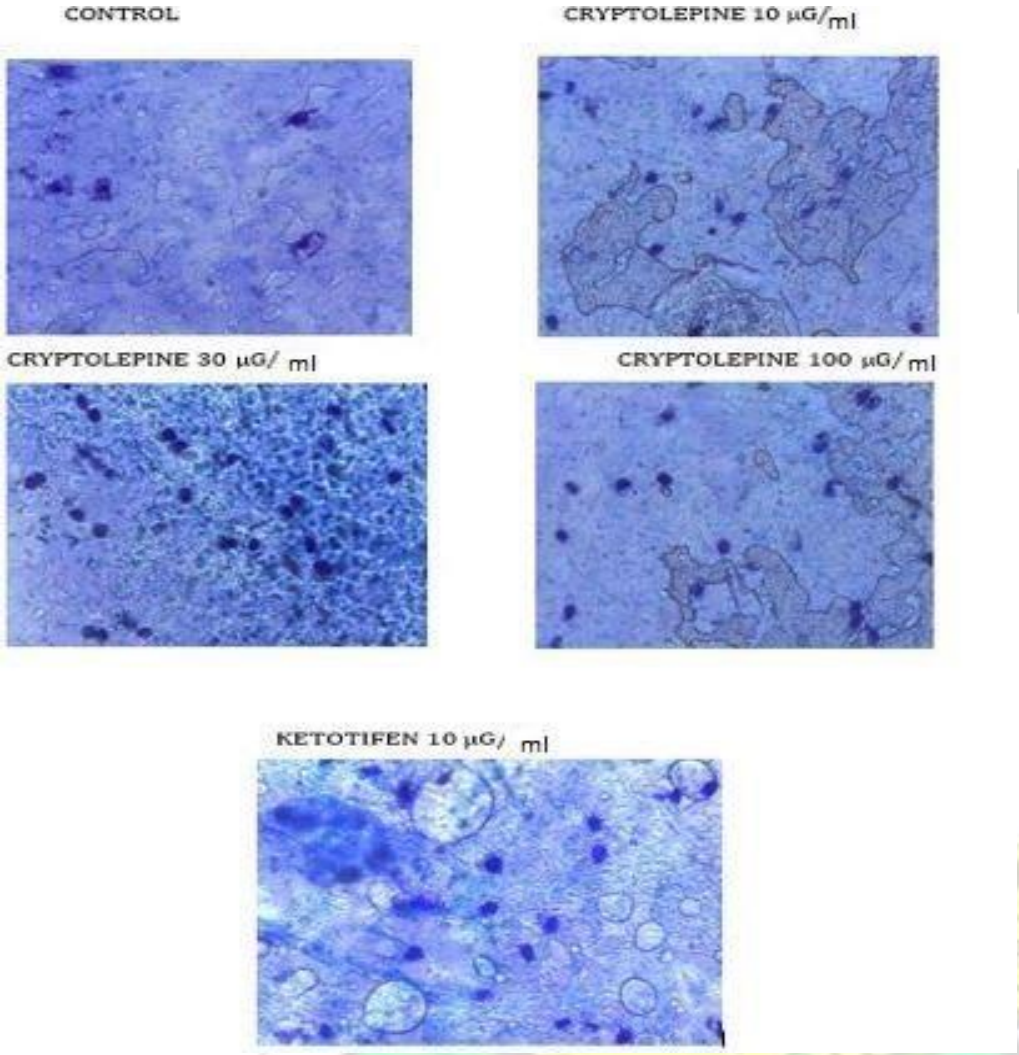

Fig 3. 3 View of stained mast cells under high power light microscope at a magnification of ×40 CLP (30-100 µg/ml) showed significant and dose dependent protection of rat mesentery mast cell induced by compound 48/80 ($p<0.01$) (Fig 3.4). 10 µg/ml showed no significant effect. Ketotifen (10 µg/ml), a known mast cell stabilizing antihistamine showed the highest effect.

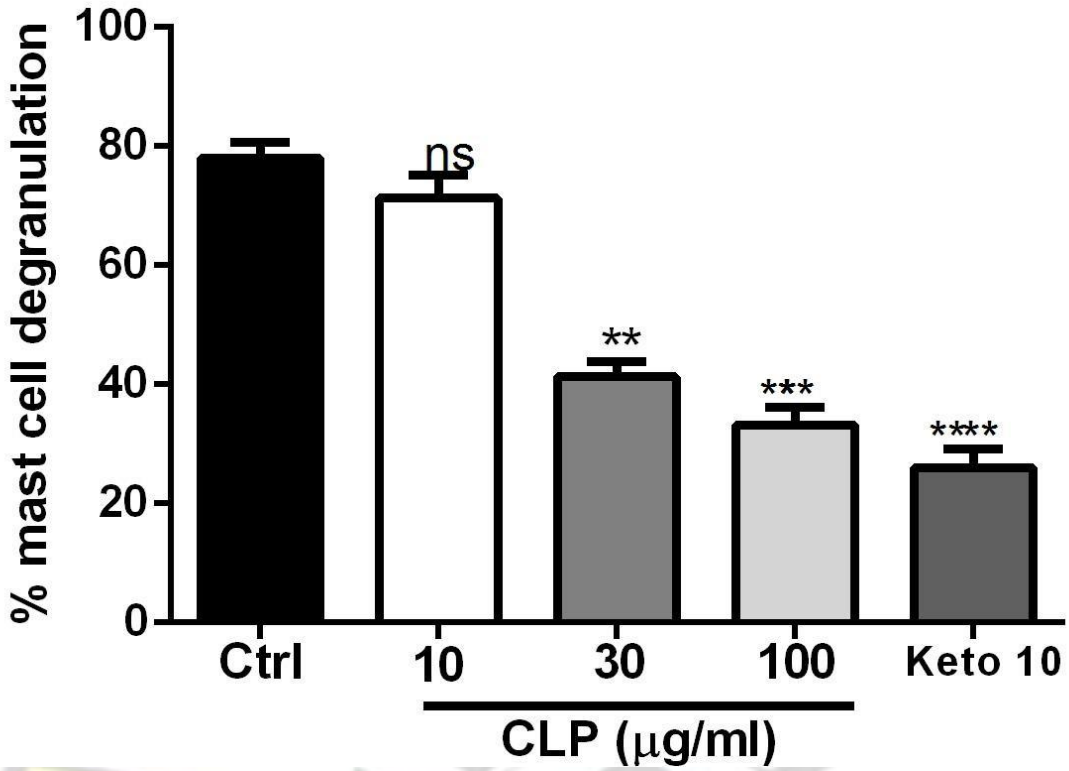

Fig 3. 4 Effect of cryptolepine on compound 48/80 induced degranulation of rat mesentery mast cells, Data are presented as group means (±SEM). Significantly different from control: **p<0.01, ***p<0.001, by one –way ANOVA followed by Turkey's multiple comparison test**.**

3.3 EFFECT ON AIRWAY INFLAMMATION

3.3.1 Skin prick test

The skin test was used to assess the extent of inflammatory response in OA-sensitized guinea-pigs. Oedema was observed in all the sensitized groups except the non-sensitized controls (Figure 3.5). CLP showed inhibitory effect of oedema formation compared to saline group at 1 h and 24 h ($p<0.01$)

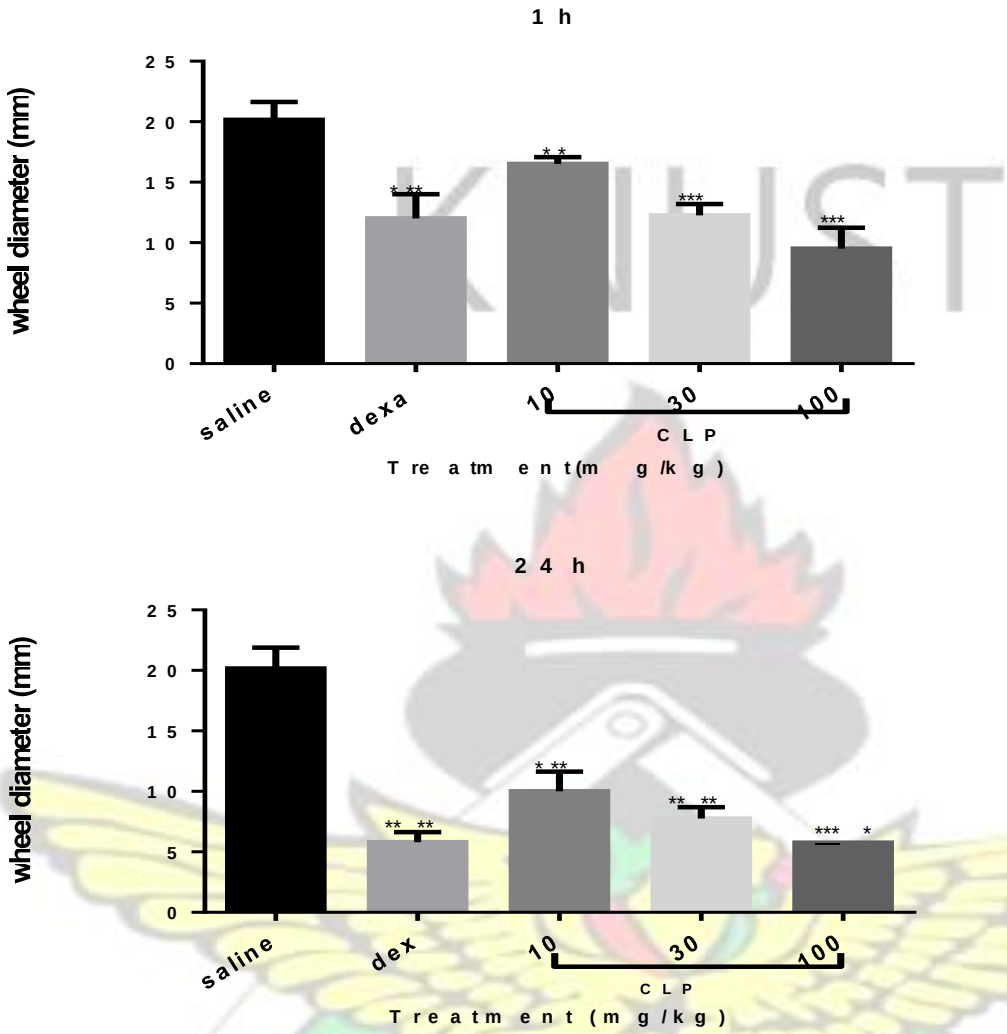

Fig 3. 5 Effect of CLP on skin oedema with respect to time showing at 1 h and 24 h. Data is expressed as mean descent time ± SEM observed at various time points within the period ofobservation. Significantly different from control: **p<0.01, ***p<0.001, ****p<0.0001, by one –way ANOVA followed by Neman-Keuls' *post hoc* test.

3.3.2 Effect on CLP on inflammatory cells in the periphery

CLP showed general inhibition of inflammatory cells in the blood. There was dose-dependent inhibition of basophils and eosinophils at all doses. Inhibition of monocytes and lymphocytes was not significant at lowest dose. Only highest dose showed significant inhibition against neutrophils in comparison with ova sensitized control (p<0.01).

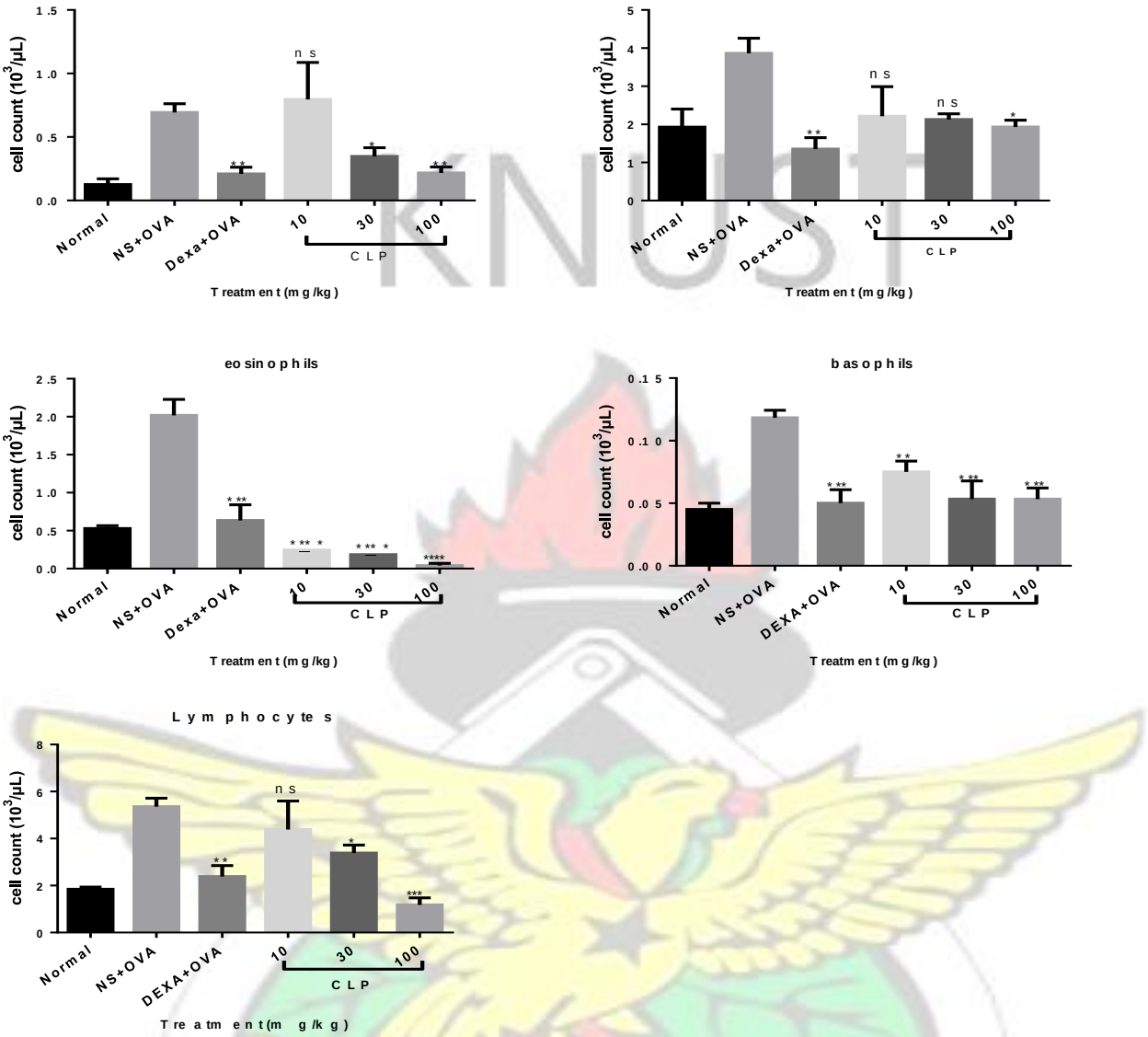

Fig 3. 6 Effect of CLP on inflammatory cells in the blood. Data is expressed as mean cell count ± SEM. Significantly different from ovalbumin sensitized control: **p<0.01, ***p<0.001, ****p<0.0001, by one –way ANOVA followed by Neuman-Keuls' *post hoc* test

3.3.3 Histopathological analysis

3.3.3.1 Hematoxylin and eosin stain (H&E stain)

CLP at all doses showed protection against airway inflammation. Basement and smooth muscle thickening together with epithelial lining was improved in cryptolepine treated groups.

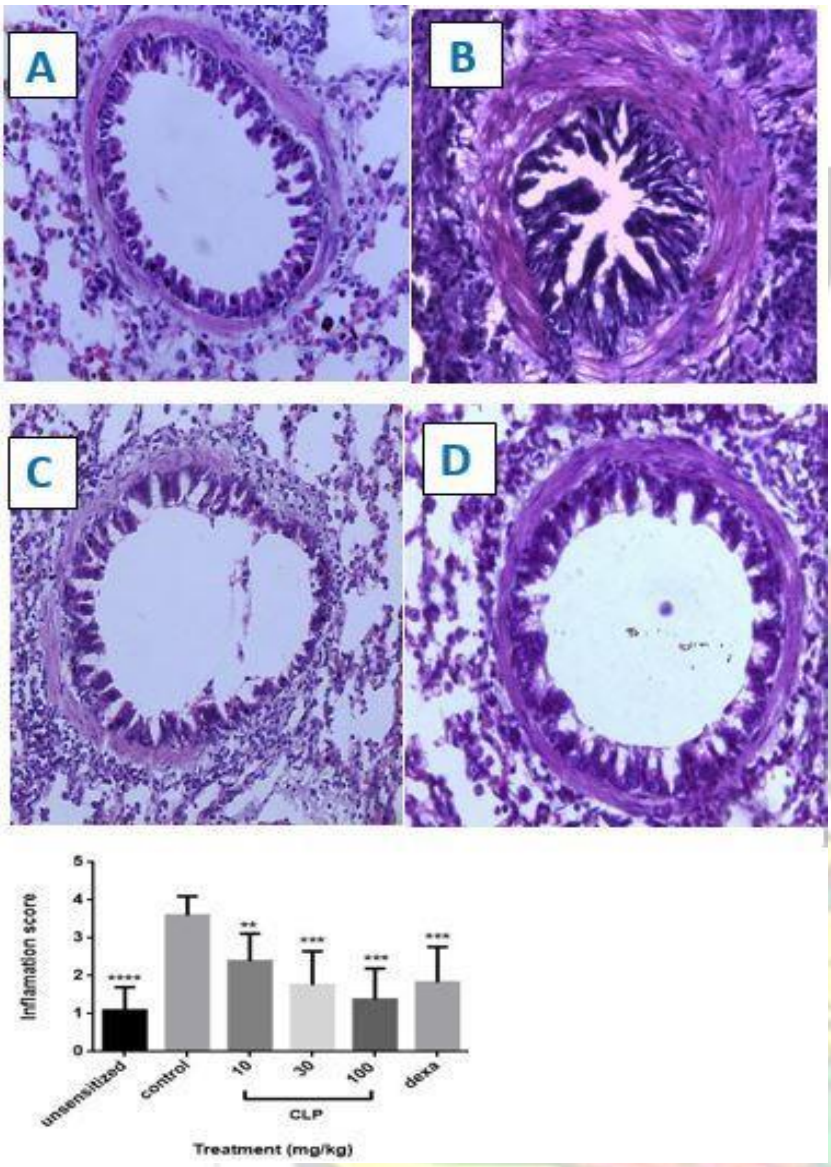

Fig 3. 7 Representative micrographs of the effects of CLP on lung tissue eosinophilia and airway remodeling. The lung tissues were fixed, embedded, cut into slices, and stained with H&E solution, which enables one to distinguish inflammatory cells infiltrated into peribronchiole and perivascular connective tissue: (A) Normal airway of guinea pig;(B) OVA-challenged guinea-pig; (C) asthmatic guinea pig treated with dexamethasone (1 mg/kg); (D) asthmatic guinea-pig treated with CLP (30 mg/kg) . Beneath is graphical representation of inflammation score.

2.7.3.1 Periodic Acid Schiff (PAS) staining

PAS is a specific stain that stains for goblet cells and mucin. CLP at all doses prevented goblet cells hyperplasia. Dexamethasone showed no significant effect.

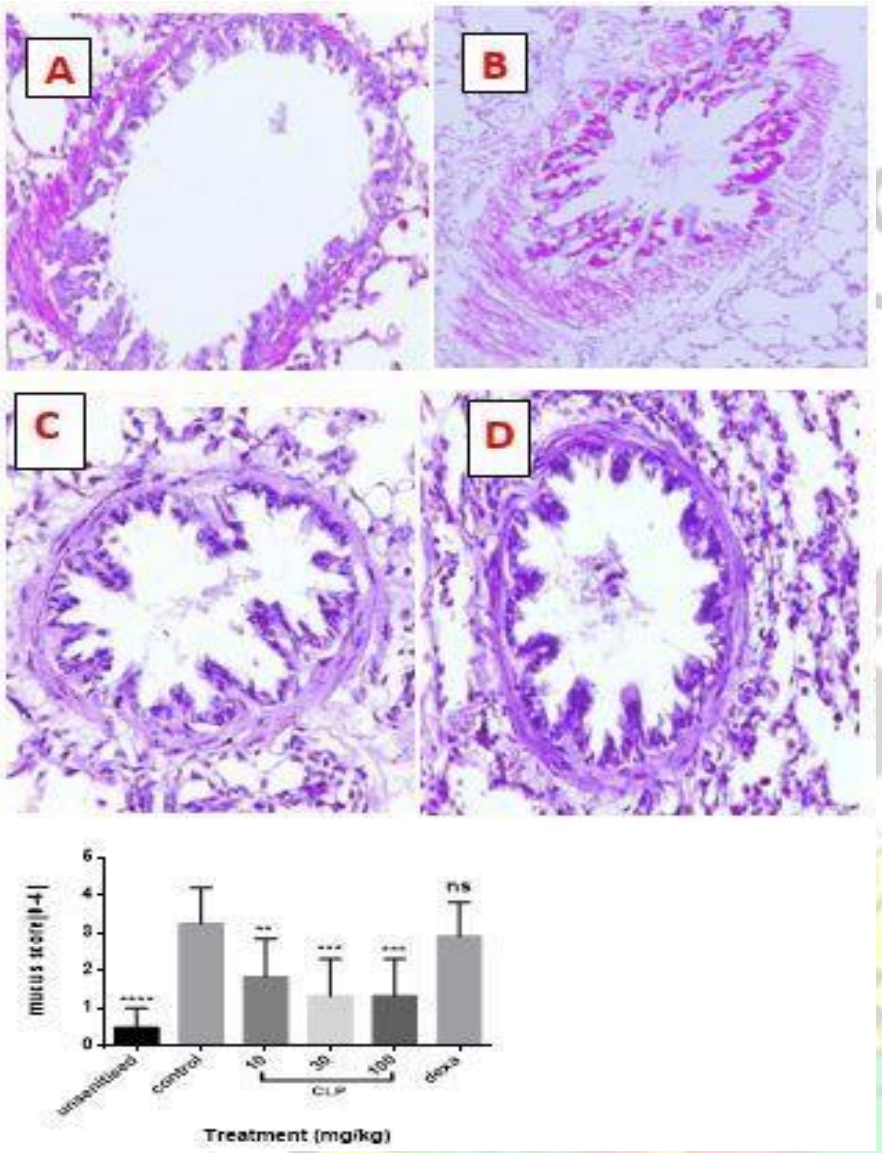

Fig 3. 8 Representative micrographs of the effects of CLP on lung tissue goblet cells and airway mucus. The lung tissues were fixed, embedded, cut into slices, and stained with PAS solution, which enables one to see hyperplasia of goblet cells, the mucus producing cells and mucin within the airway or bronchioles: (A) Normal airway of guinea pig;(B) OVA-challenged guinea-pig; (C) asthmatic guinea pig treated with dexamethasone (1 mg/kg); (D) asthmatic guinea-pig treated with CLP (30 mg/kg). Beneath is graphical representation of mucus score.

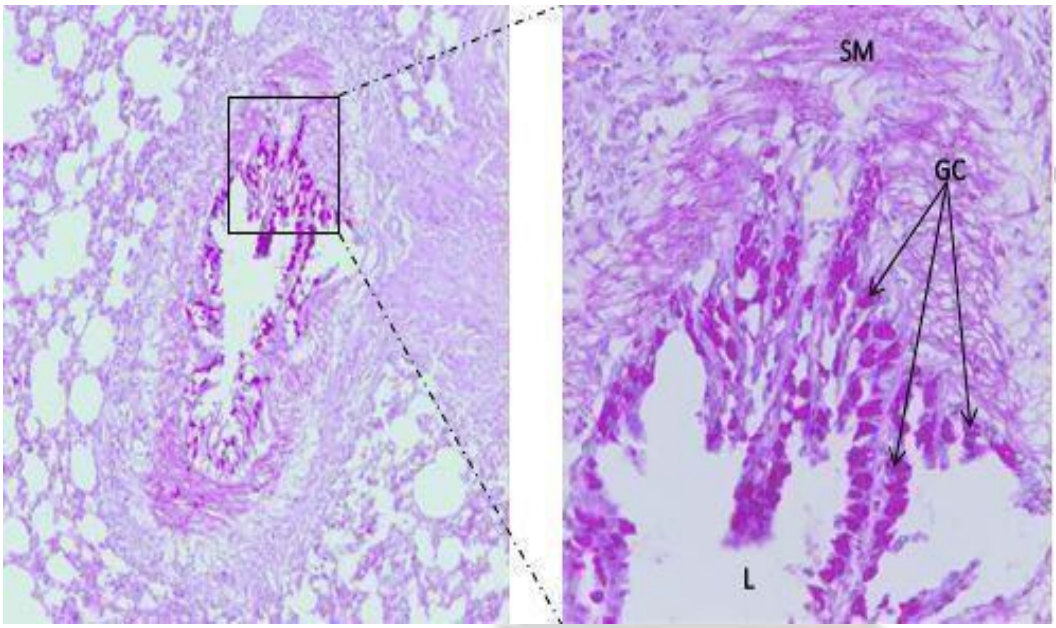

Fig 3. 9 Enlarged representative micrographs of histopathological specimens from ovalbumin sensitized controls treated with normal saline stained with PAS solution. PAS. SM: smooth muscle, GC: goblet cell

3.4 ANTI-DIARRHOEAL EFFECT

3.4.1 Castor oil- induced diarrhoea

Cryptolepine showed dose dependent inhibition of castor oil-induced diarrhoea (Fig 3.10). The incidence and severity of diarrhoea as well as the frequency of defecation and wetness of faecal droppings were reduced compared to control ($p<0.01$)

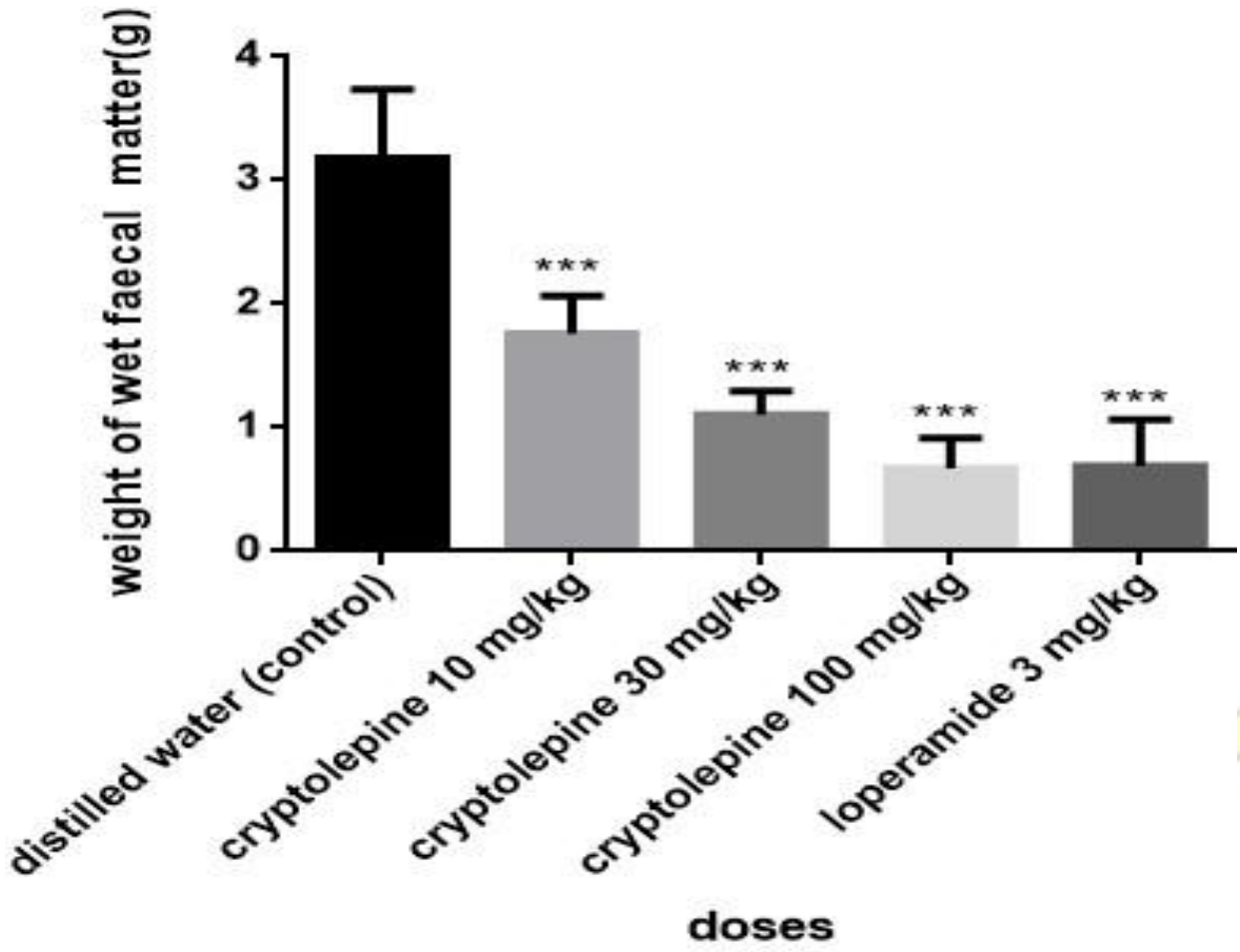

Fig 3. 10 Effect of cryptoolepine on castor oil induced diarrhoea. Data are presented as group means (±SEM). Significantly different from control: ***P<0.001, by one –way ANOVA followed by Turkey's multiple comparison test

3.4.2 Charcoal meal test

Cryptolepine dose dependently increased transit time in the GIT with a graded effect from 30-100 mg/kg ($p<0.01$). Atropine which was used as the standard also increased the transit time of the charcoal in the GIT.

Table 3.2 Effect of cryptolepine on movement of charcoal in the gastrointestinal tract of rats.

GROUP	TREATMENT	DOSE(mg/kg)	% MOVEMENT	% of inhibition
1	Control		80.54±4.73	
2	Cryptolepine	10	66.28±4.87**	17.7
3	Cryptolepine	30	52.06±1.70***	35.36
4	Cryptolepine	100	50.68±2.57***	37.07
5	Atropine	100	50.06±3.82***	37.84

Data are presented as group means **(**±SEM**).** Significantly different from control: **P<0.01, ***P<0.001, by one –way ANOVA followed by Turkey's multiple comparison test

CHAPTER FOUR

4.0 DISCUSSION

Cryptolepine was successfully isolated from the dry roots of *C. sanguinolenta*. The melting point of the isolated cryptolepine was found to be 264-268 °C and its authenticity was confirmed by determination of UV which were in agreement with those reported by Grellier *et al.,* (1996) and

Dwuma-Badu, *et al.*, (1978). Cryptolepine is reported to have a melting point between 263-265°C. Melting point is used in the determination of purity. The isolated cryptolepine falling within the range suggest purity of cryptolepine isolated. Also in the UV/Vis spectrum the methanolic solution of isolated cryptolepine yielded a data with maximum wavelength (λmax) at 275, 281 and 369 which was also comparable to that described by Dwuma-Badu, *et al.,* (1978).

Cryptolepine (CLP) 10-100 mg/kg showed dose-dependent protection against histamine-induced bronchoconstriction after 1, 6 and 24 hours. The standard drug mepyramine 8 mg/kg however showed the highest effect at 1 and 6 hours with no effect after 24 hours. In the histamine-induced bronchoconstriction, exposing the guinea pigs constantly to histamine at different times reduces the time taken for the guinea pigs to respond. This is evident in the control group as the time for convulsion decreases in comparison with prior exposure. This probably could be as a result of type II hypersensitivity reaction after first histamine exposure. Inhalation of histamine is a classical model of inducing bronchoconstriction. The role of histamine in asthma is well established (Nelson, 2003). In the early stage of asthma, release of inflammatory mediators like histamine, acetylcholine, leukotrienes, and prostaglandins are triggered by exposure to allergens, irritants, cold air or exercise (Bosquet *et al.,* 2000). This mediators induce bronchoconstriction. The close resemblance of pulmonary responses to histamine challenge in both guinea pigs and humans, as well as the anaphylactic sensitization and extreme sensitivity of the airways to primary mediators made this species the model of choice. In the guinea pig, exposure to histamine aerosols results in intense smooth muscle contractions, hypoxia leading to convulsion, asphyxia and death. Bronchodilators can delay the occurrence of these symptoms (Nayampalli *et al.,* 1986). The results of the study suggest that cryptolepine may be acting via dilatation of the bronchial smooth muscles. Though the result is interesting, it is not surprising. This is because cryptolepine is a known

antimuscarinic agent. Parasympathetic nervous system is the dominant neuronal pathway in the control of airway tone. When stimulated there is bronchoconstriction, mucus secretion and bronchodilation. . By blocking those receptors the sympathetic arm dominates leading to relaxation of the airway. Also Noamesi *et al.,* (1982) reports of antihistaminic effect of cryptolepine on isolated guinea pig ileum. It is also interesting that this effect of cryptolepine persisted after 24 hours.

Pathogenesis of asthma has been associated with infiltration of inflammatory cells in the bronchial airway such as eosinophils, mast cells, lymphocytes and macrophages (Busse *et al.,* 2002). There has been further association with increased peripheral blood eosinophils and other inflammatory cells directly correlating with the severity of asthma in man (Koh and Choi, 2002) and other animal models (Wolyniec *et al.,* 1998). In this study cryptolepine (CLP) at the doses of 10, 30 and 100 mg/kg showed dose-dependent reduction of eosinophils, monocytes and basophils in the periphery. Eosinophils present in increased numbers in the airway leads to release of basic protein that may damage epithelial cells of the airway. They may also have a role in the release of growth factors and in the process evoke airway remodelling (Kay *et al.,* 2004). Monocytes on the other hand differentiate into macrophages that release inflammatory mediators and cytokines that amplify the inflammatory response. CLP at a dose of 30 and 100 mg/kg significantly inhibited T-lymphocytes proliferation in the blood with no significant effect at the dose of 10 mg/kg, whereas CLP only inhibited neutrophils at the highest dose of 100 mg/kg. Although the pathophysiological role of neutrophil is uncertain, their numbers are increased in the airways, sputum and blood of severe asthma and smoking asthmatics (Wen, 2003). The role of T-lymphocytes in asthma however cannot be overemphasized. Through the release of specific patterns of cytokines (IL-4, 5, 9,1 3) they result in the recruitment and survival of eosinophils, IgE production by B-lymphocytes and

maintenance of mast cells. Type 2 helper (Th_2) cells are known to drive the asthmatic pathogenesis with an imbalance between TH_2 and TH_1 being the brain behind the hygiene hypothesis of asthma. In this study however alumium hydroxide was used as an adjuvant in the sensitization to promote the development of the Th_2 immune response when exposed to antigen.

Generally CLP at a dose of 10-100 mg/kg inhibited inflammatory cells and the possible cascade of responses associated with it. It is not surprising that the response corresponded with that of the airway histology. The cluster of inflammatory cells within and around the bronchiolar airway was prominent and extensive in the saline treated group compared with that of CLP treated group. Clearly there was improvement in all aspects of remodeling including epithelial and basement membrane thickening and goblet cells numbers was seen at all doses of cryptolepine compared to normal saline group exposed to ovalbumin (OV).

Basement membrane and smooth muscles thickening are important aspect of airway remodeling in asthma which are associated with airway obstruction and hyperreactivity. It has therefore been the subject of research for therapeutic agents. Despite contradictory reports, inhaled and systemic steroid treatment is usually accepted to decrease basement membrane thickness and smooth muscle hypertrophy, therefore forming the mainstay of asthma treatment (Baraket *et al.,* 2012; Ward *et al.,* 2002; Hoshino *et al.,* 1998). In agreement with this, asthmatic guinea pigs treated with dexamethasone in our study had significantly thinner basement membrane compared to the normal saline asthma group. However, basement membrane and smooth muscle thickness was not significantly different between dexamethasone and cryptolepine group of 30 and 100 mg/kg.

Epithelial lining of the airway has a role in secretion of cytokine and this is related to the exposure of epithelium to allergens and other cytokines. They are usually the target of products of mediators

leading to destruction of the cells. CLP at all doses showed improvement in the epithelial airway lining compared to that of the saline treated group. In certain instances there was subepithelial fibrosis developing in the saline treated group which was visibly absent in the CLP treated groups.

Increased mucous production is a component of asthma pathophysiology and is associated with increased goblet cell mass (Durrani *et al.,* 2011). Periodic acid Schiff staining is a special kind of staining that stains glycoprotein and mucin, hence it is specific for goblet cells. The results suggest a decrease in goblet cell number with CLP treatment compared to guinea-pigs treated with normal saline. Empirical comparison with the dexamethasone group shows that this effect was also greater than dexamethasone treated group. Decrease in goblet cell numbers might be an important advantage of CLP over dexamethasone treatment.

In asthma an increase mucus secretion is not just as a result of goblet hyperplasia but also under the influence of parasympathetic arm. Therefore to determine the effect of cryptolepine on secretion of mucus by the parasympathetic arm, the tracheal phenol red mucus secretion by Engler and Szelenyi

(1984) was employed. It is a method for screening drugs that influence tracheobronchial secretion. When phenol red is applied intraperitoneally, part of the dye is secreted into the tracheal lumen. This basal amount is increased by both parasympathomimetics and sympathomimetics, in addition to expectorants. The reverse is true for parasympatholytics. As such, drugs that have low output of dye in trachea have higher proclivity to inhibit mucus secretion. Cryptolepine (CLP) 10-100 mg/kg showed dose dependent inhibition of mucus secretion. However it seems to have a levelling effect from a dose of 30 to 100 mg/kg.

Asthma is usually classified as an allergic condition as a result of the immunological response associated with it. The guinea-pig skin prick test is a basic test to check the extent of inflammatory response to antigens in sensitized animals. In the current study, skin prick test was used to assess the effect of CLP to allergic stimuli in ovalbumin-sensitized guinea-pigs. The response manifested as oedema in the skin of OA-sensitized guinea-pigs. The ability of CLP to reduce oedema formation in the skin of OA-sensitized guinea-pigs after intradermal injection of ovalbumin confirms the antiinflammatory activity of CLP. The development of oedema in the skin is biphasic process with first phase occurring within an hour and the second phase beyond an hour. Preformed mediators such as cytoplasmic enzymes, histamine, and serotonin are released from mast cells during the first phase (Vinegar *et al.*, 1969). These preformed mediators are capable of enhancing vascular permeability, contraction of non-vascular smooth muscles, dilating precapillary sphincters and postcapillary venules (Kim and Camilleri, 2002). The second phase is mediated by arachidonic acid metabolites including prostaglandins, leukotrienes and thromboxanes. The effects of these mediators are 10-fold higher than that of the preformed. Additionally, the test serves as an indicator for T-cell response in ovalbumin sensitized animal models. The effect of CLP in decreasing the oedema within the first hour and the sustenance effect after 24 hours could suggest effect on both phases of the allergic reaction. This could be either an effect on mast cell release or antagonism of mediators released or a combination of both. To ascertain this, compound 48/80 induced mast cell degranulation on rat mesenteric cells were used.

Activation and degranulation of mast cells result in an early phase response that involves acute bronchoconstriction. This is because activation of mucosal mast cells release bronchoconstrictor mediators (histamine, prostaglandin, cysteinyl-leukotrienes) (Boyce 2003; Robinson 2004). Increased numbers of mast cells in airway smooth muscle may be connected to airway

hyperresponsiveness (Brightling *et al.*, 2002). CLP 30-100 µg/ml exhibited significant and dose dependent protection of mesentery mast cell from degranulation induced by compound 48/80 *in vitro* but had no significant effect at a concentration of 10 µg/ml. This protection showed by CLP is comparable at higher doses to ketotifen—an antihistamine with a potent mast cell stabilizing effect. This finding suggest that CLP exhibits the anti-asthmatic like effect partly could be by stabilizing mast cell and that prevention of bronchoconstriction and airway inflammation may not just be as a result of its' reported antimuscarinic and antihistaminic effect but also because it stabilizers mast cell. Actually there may be a link between the antihistaminic effect and mast cell stabilizing effect as mast cells tend to have H_1 and H_2 receptors and stimulation of it results in mast cell activation. Therefore, by antagonizing those receptors it could lead to stability of the mast cells. Notably, some antihistamines like ketotifen are mast cell stabilizers (Molderings, 2010).

Recent studies have suggested that diarrhoea could be implicated in certain asthmatic patients. According to Awortwe *et al.*, (2013), inflammatory mediators, such as histamine and eicosanoids are related to the pathophysiology of asthma which include bronchospasm, vasodilation, acute functional changes in the lungs and diarrhoea due to increased intestinal motility. This increased intestinal motility could also be due to increased vagal stimulation. Survey conducted by a group of scientists discovered that children with asthma had a greater frequency of gastrointestinal symptoms particularly diarrhoea (Caffareli *et al.*, 2000). In such instances, medications for asthma that tend to have constipative effect tend to be useful by reducing side effects associated with combining effects of anti-asthma and antidiarrhoeal medications, enhancing compliance and reducing the cost of medicines. In line with this, the effect of cryptolepine was evaluated in animal models of diarrhoea.

In the castor oil-induced diarrhoea model CLP at doses of 10, 30 and 100 mg/kg exhibited significant dose dependent reduction of cumulative wet faecal mass. The incidence and severity of diarrhoea as well as the frequency of defecation and wetness of faecal droppings were reduced compared to the control and the highest dose showed effects comparable to the standard drug, loperamide (3 mg/kg). This model is based on the principle that the active agent of castor oil, that is ricinoleic acid, acts primarily in the small intestines where it induces permeability changes in mucosal fluid and electrocyte transport, speeding transit (Ammon *et al.*, 1974). This is so because it causes irritation and inflammation of the intestinal mucosa which leads to production of prostaglandins.

Prostaglandins are known to cause secretion as well as increase motility of the GIT (Pierce *et al.*, 1971). It is therefore likely that the antidiarrhoeal effect of cryptolepine could be as a result of prostaglandin biosynthesis inhibition or by decreasing the peristaltic movement of the mucosa. Cryptolepine has been suggested to inhibit COX-2 (Olajide *et al.*, 2009) and COX-2 is responsible for the production of prostanoid mediators of inflammation (Vane and Botting, 2001). Therefore by inhibiting COX-2, there is no production of prostanoid mediators leading to a decrease in secretion and motility of the GIT.

To confirm whether cryptolepine could have had an effect on the motility of the GIT, the charcoal meal test was performed. The charcoal meal test is based on the time taken for administered charcoal to travel from the pylorus to the caecum. As charcoal is not absorbed in the GIT, the distance travelled by the charcoal, gives an indication of the effect of the agents administered on the propulsive effect of the GIT.

CLP 10- 30 mg/kg significantly decreased the propulsion of charcoal meal through the GIT as compared with the control. This result confirms that cryptolepine reduces the propulsive action of

agents in the GIT. This findings again could be attributed to the its antimuscarinic at M_1, M_2 and M_3 receptors. In the GIT, the type of receptors mainly found are M_3 receptors and by activating those receptors it produces excitatory effect resulting in contraction of the smooth muscles. Therefore cryptolepine by possibly antagonizing at M_3 receptors could have led to decrease in contraction of the smooth muscle leading to increase in transit time.

CHAPTER FIVE

5.0 CONCLUSIONS

- From the results obtained, it is derived that oral administration of CLP in a guinea-pig asthma model significantly inhibited the increase in the total inflammatory cell count induced by ovalbumin, in blood.
- Histological studies demonstrate that CLP substantially inhibited ovalbumin-induced inflammatory cells in lung tissue, smooth muscle and basement membrane thickening and increase in the number of goblet cells in the airway. These findings suggest that CLP may effectively delay the progression of airway inflammation and remodeling.
- CLP also showed significant protection in histamine induced bronchoconstriction as well as reducing mucus secretion in tracheal phenol red model. This effect of cryptolepine could be attributed to the ability to stabilize mast cells.
- Its antidiarrhoeal effect was demonstrated by the castor oil-induced diarrhoea model and the effect could be partly due to inhibition of peristalsis seen in the charcoal meal test.

5.1 RECOMMENDATION AND FUTURE WORK

Though the results from animal studies cannot be directly extrapolated to man, the results of the present study suggest that cryptolepine has the potential to be used in asthma. This potential of cryptolepine should be further studied. Investigating the potential mechanism involved in mast cell protective properties by exploring CLP effect on intracellular Ca^{2+} accumulation in mast cells

could be paramount. Effect of CLP on arachidonic acid pathway which is a major pathway in asthma could be also investigated.

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