CHAPTER ONE

General Introduction

1.1. General Introduction

The hallmark of biomedical research over the years has been the investigation of the causes and nature of diseases as well as development of medication for treatment of these diseases. Whereas research involving cell culture and animal diseases could be carried out with some ethical prescription, many invasive procedures employed for studying human diseases are often subjected to intense scrutiny and the use of human subjects and specimens usually requires a very high degree of thorough ethical clearance. Gene therapy (especially germ-line gene therapy) for various diseases can only be administered in line with arduous clinical protocols especially because of possibilities of serious trans-generational side effects.

Studies on diseases can be investigated at a molecular, sub-cellular, cellular, tissue, organ or even systemic level. There are difficulties in many drug development processes because of the limited understanding of the molecular pathogenesis of various diseases. Molecular and cell culture studies have thus far provided useful information and knowledge about the causes and progressions of many human diseases. The use of human cells in culture experiments has also provided insights into the various mechanisms involved in human diseases even though not all *in vitro* conditions have been successfully reproduced in *in vivo* models.

In spite of many reported shortcomings, cell culture experiments have remained useful means of studying biosystems since the researcher is able to alter experimental conditions to suite the desired research objectives. In general, the use of *in vitro* model systems makes it easy to conduct reproducible experiments

under controlled conditions such as measuring cellular damage, screening dosages and evaluating substance cytotoxicity. Another major advantage of *in vitro* studies is that only small amounts of the test compound are usually required to perform several statistically significant experiments. Furthermore, a wide range of bioassay systems is already available for use in cell culture experiments.

Cell viability assays are used to measure the proportion of viable cells affected by the treatment of cells in culture with different test agents. Most of the assays rely on the fact that there is a breakdown of cell membranes to allow the uptake of a dye to which cells are normally impermeable or the release of a dye that is usually retained by cells. The Neutral Red (NR) assay distinguishes viable from non-viable cells because only viable cells absorb the NR dye and sequester it in their lysosomes. The MTT assay is based on the principle that a yellow water-soluble dye MTT [3-(4, 5-dimethylthiazol-zyl) 2, 5-dimethyl tetrazolium bromide] is reduced by live cells in culture to a purple or blue product (MTT-formazan) which can be quantified using absorbance readings (Mosmann, 1988). Densitometric readings of Crystal Violet (CV) are directly related to the number of viable cells. It thus implies that cell culture assays could be used to effectively determine cell viability, membrane integrity as well as cell number, all of which find application in the determination of cytotoxicity as well as dose effects. In this study, cell number, cell viability and lysosomal membrane integrity were determined using a combined NR/MTT/CV assay.

In most cases, *in vitro* experiments are used for preliminary investigations prior to *in vivo* studies with animals. Test doses determined to be safe from *in vitro* studies are further used in animal experiments and then eventually in drug trials. The need

to effectively represent or reproduce human diseases in animals led to the development of animal models of human diseases. Understanding the molecular processes that lead to the manifestations of human-like disease symptoms in these animal models became even more of a possibility as researchers were permitted within limits to subject animals to more invasive techniques than could be used for humans.

Modelling human diseases in animals has greatly enhanced knowledge about human diseases in general. A guinea-pig system that was utilized for more than 90 years has contributed to the basic understanding of physiological and immunological processes involved in allergic respiratory sensitization (Karol, 1994). Currently, animal models exist for many human diseases like Alzheimer's disease (Richardson and Burns, 2002) and cancer in nude mice (Hunter and Williams, 2002); diabetes, obesity and asthma in Sprague-Dawley rats (Schaan and Machado, 2006; Speakman et al., 2007). Animal models of mice, guinea pigs, rats, dogs, cats, monkeys, sheep, and horses have been developed in order to study disease pathogenesis and to discover effective drug treatments (Epstein, 2004a, b). However, since the first demonstration of allergic mouse asthma was reported in 1994, mice have become one of the most extensively studied model systems (Epstein, 2006).

In the case of asthma, animal models have been developed over the years with the aim of understanding the exact mechanisms causing the disease and with a view to developing drugs that can cure the associated chronic inflammatory condition. Although all of the currently available animal models of asthma have their strengths and weaknesses, the mouse asthma model appears to be the most

commonly used and seemingly preferred model of the disease. Different strains of mice have been adequately modelled and a broad spectrum of molecular and immunological tools (including gene deletion technology) is now available particularly for studying the balance between Th1 and Th2 responses (Kips et al., 2003). Inbred strains of mice may also be useful for studies on genetic susceptibility and predisposition to asthma (Herman, 2002).

Th1 responses are proinflammatory in nature and tend to be responsible for killing intracellular parasites or pathogens like viruses and certain bacteria as well as for perpetuating autoimmune responses. Th1 cells secrete interleukin (IL)-2 and interferon (IFN)-γ which are responsible for these responses. On the other hand, Th2 cells secrete interleukins (IL)-4, IL-5 and IL-13 and Th2-responses are more anti-inflammatory or humoural in nature, causing allergic diseases and asthma. They are associated with the promotion of IgE and eosinophilic proliferation in atopy and are important in the inhibition of macrophage activation. Th-2 responses are thus essential for antibody-mediated immunity (Mosmann and Coffman, 1989; Tadao et al., 2004).

Excessive proinflammatory responses can lead to uncontrolled tissue damage and excessive Th2 responses will counteract the Th1-mediated microbicidal action. Because Th1 and Th2 cells cross-regulate each other, the Th1/Th2 theory predicts that allergic diseases like asthma develop when there are too many Th2 cells and not enough Th1 cells. Accordingly, allergy results from an imbalance in favour of a Th2 response, and is negatively regulated by Th1 cells (Gereda et al., 2000; Berger 2000).

Epstein (2006) reported that despite differences between the allergic mice models and the human disease, mice models develop features of the clinical disease (albeit with several notable caveats) and are therefore useful for testing novel therapeutic agents aimed at reducing lung inflammation, mucus hypersecretion, airway hyperresponsiveness (AHR) and immunoglobulin E (IgE) levels. Mouse models are especially beneficial because many different materials and methods can be used to study the disease pathology from numerous and complementary angles. For instance numerous models from different mice strains are available for laboratory experimentation, a large variety of different antigens and many different routes of administration exist for introducing experimental substances. In addition, even the non-allergic (intrinsic) form of asthma could be modelled using strains of mice (t-bet knockout or t-bet deficient transgenic mice) in which asthma-like symptoms occur spontaneously (Epstein, 2006).

All the above factors seem to allude to the possible preference of the mouse model. Moreover, the use of other animals like horses, dogs, sheep and primates is limited due to several factors such as size, difficulty to handle, availability of large sample sizes and general costs making the mouse a much better alternative. In general, the efficient use of animal models would surely advance the recognition, treatment and prevention of asthma (Karol, 1994; Epstein 2006).

Asthma is considered one of the most common respiratory complaints and affects the respiratory passages and indeed the whole lung. Triggers of an asthma attack may include among others, animal skin, hair and feathers, cockroaches, infections, dust and house mites, exercise, pollen and outdoor molds, smoke, strong odours and sprays, tobacco smoke, weather, occupational dust and vapours such as

plastic, wood, metals and grains; air pollutants such as cigarette smoke, auto exhaust and sulphur dioxide. Incidence of asthma appears to be on the increase worldwide despite improving therapeutic advances. The pathophysiology of asthma involves airway inflammation, hyperresponsiveness and bronchospasm, mucus hypersecretion and airway remodelling (Schieken, 2002). The need to develop effective treatment and management regimens for asthma still remains a high clinical priority.

Although corticosteroids are considered among the best medication for asthma, much of the world's population and the poor in particular, seem to rely on herbal remedies and other traditional means for the management and treatment of the disease. The use of herbal remedies for the treatment of many diseases has gained popularity globally as part of the complimentary and alternative medicine (CAM) revolution (Chevrier et al, 2005).

A number of herbal CAMs have reportedly been tested experimentally for their effects on allergy, asthma or other inflammatory conditions. These include the Echinacea family *(Echinacea augustifolia, Echinacea pallida,* and *Echinacea purpurea*); garlic (*Allium sativu*); angelica (*Angelica archangelica*); chamomile flower (*Chamomilla recutita*); ephedra (*Ephedra sinica*) and gingko (*Gingko biloba*). Others include red grape seed extract, licorice root (*Glycyrrhiza glabra*), peppermint oil and leaf (*Mentha piperitae*), stinging nettle root and leaf (*Urtica dioica*) and ginseng (*Panax ginseng)*, among others (Bielory, 2004).

Other known anti-asthma herbs include *Astragalus membraneous*, *Ammi visnaga*, *Brassica* spp, *Boswellia serrata (frankincence), Convallaria majalis*, *Commiphora myrrha*, *Datura stramonium*, *Euphorbia hirta (E. hirta), Grindelia robusta*, *Ephedra*

vulgaris, *Lobelia inflata*, *Marrubium vulgare*, *Petasites hybridus, Polygala senega*, *Sanguinaria canadensis*, *Seleneicerus grandifluros*, *Symplocarpus goetidus*, *Thymus vulgaris*, *Tylophora astmatica, Verbascum thapsus* and *Viburnum opulus*. Interestingly, most patients adopting CAM interventions use them to complement conventional care rather than as the sole form of treatment (Eisenberg et al., 1993; Astin, 1998 and Eisenberg et al., 1998)

In parts of Africa, Asia and Australia the herb *E. hirta* (known commonly as asthma weed or commercially as *Euphorbia pilulifera*) has been reportedly used for treatment of numerous diseases including allergies, bronchitis, asthma, hypertension, oedema, worm infestation, amoebic dysentery, conjunctivitis, and syphilis. Very little documented information is currently available on the biomedical properties and mechanism of action of this plant especially with respect to its use against asthma.

In this study therefore, the possible medicinal effects of *E. hirta* herb were explored. Extracts of this plant were used in cell culture assays to determine cytotoxicity and later administered to asthmatic BALB/c mice to determine possible improvements in a number of observed parameters in the treated animals compared to untreated animals. Changes in the blood coagulation system, total eosinophil count as well as lung ultrastructure were examined and compared.

Results from this study are expected to provide more information on some dose-related effects of the herb *E. hirta* in experimental asthmatic conditions.

CHAPTER TWO

Literature Review

2.1. Asthma: an introduction

The word "asthma" is derived from the Greek word "Panos," which means “panting” or “laboured breathing” possibly referring to the airway obstruction often associated with this condition. This term has been used by such ancient medical pioneers as Hippocrates, Galen and Bernardino Ramazziniin, in their description of the airway condition that causes wheezing, chest tightness and obstructs the airways. (Rosner, 1981; Marketos and Ballas, 1982). There has been continuous revision of the scientific description of asthma as more knowledge of asthma pathogenesis becomes available. A comprehensive description would consider asthma as a syndrome in which genetic predisposition and environmental factors interact to produce complex inflammatory reactions in respiratory passages such as airway hyper-responsiveness (AHR), mucus overproduction, proliferation and infiltration of inflammatory cells and airway wall remodelling, among others (Kon and Kay, 1999).

An operational description of asthma adopted by a team of experts is as follows: “asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment” (GINA, 2006).

The working definition of asthma, as proposed in the Expert Panel Report of the National Heart, Lung and Blood Institute is as follows:

"Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role, in particular, mast cells, eosinophils, T-lymphocytes, neutrophils and epithelial cells. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and cough, particularly at night and in the early morning. These episodes are usually associated with widespread, but variable airflow obstruction that is either often reversible spontaneously or with treatment. The inflammation also causes an associated increase in the existing bronchial hyperresponsiveness to a variety of stimuli" (NHLBI, 1997).

All the descriptions given above are based on current knowledge about asthma and are likely to change when more information becomes available on the pathogenesis of this airway condition.

2.2. Development and expression of asthma

A number of factors influence the development of asthma and others trigger asthma symptoms; although some factors do both. The factors which influence the development of asthma (referred to as host factors) are primarily genetic while those which trigger asthma symptoms are usually environmental factors (Busse and Lemanske 2001). However, the mechanisms by which these factors influence the development and expression of asthma are complex and interactive. For example, genes likely interact both with other genes and with environmental factors to determine asthma susceptibility (Holgate, 1999; Ober, 2005). In addition, certain characteristics have been linked to an increased risk for asthma, but are not themselves true causal factors. These include racial, ethnic differences and socioeconomic variation in asthma prevalence. A higher prevalence of asthma is

found in developed rather than in developing nations. In populations from developed countries, there is a higher prevalence of atopic asthma in affluent populations compared to non-atopic asthma in poor populations. This may reflect lifestyle differences such as exposure to allergens and access to health care (Aligne, 2000; McGeady, 2004).

2.2.1. Host factors

2.2.1.1. Genetics

Asthma can be inherited and research has shown that multiple genes may be involved in the pathogenesis of asthma (Holloway et al., 1999; Wiesch et al., 1999). It has also been suggested that different genes may be 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 IgE antibodies (atopy); expression of airway hyperresponsiveness; generation of inflammatory mediators, such as cytokines, chemokines, and growth factors; and determination of the ratio between Th1 and Th2 immune responses (as relevant to the hygiene hypothesis of asthma) (Strachan 1989). The tendency to produce an elevated level of total serum IgE is co-inherited with airway hyperresponsiveness and the gene governing airway hyperresponsiveness is located near a major locus that regulates serum IgE levels on chromosome 5q (Postma et al., 1995).

Some genes do not predispose individuals to asthma but are associated with the response to asthma treatments. For example, variations in the gene encoding the beta-adrenoreceptor have been linked to differences in subjects' responses to β2-agonists (Israel et al., 2004). Other genes of interest modify the responsiveness to glucocorticosteroids (Ito et al., 2006) and leukotriene modifiers (In et al., 1997).

These genetic markers will likely become important not only as risk factors in the pathogenesis of asthma but also as determinants of responsiveness to treatment (Lane et al., 1994; In et al., 1997; Drazen and Weiss, 2002; Israel et al., 2004; Tattersfield and Hall et al., 2004).

2.2.1.2. Obesity

Obesity has also been shown at the molecular level to be a risk factor for asthma. Certain mediators such as leptins may affect airway function and increase the likelihood of asthma development (Shore and Fredberg, 2005; Beuther et al., 2006).

2.2.1.3. Sex

Male sex is a risk factor for asthma in children and prior to the age of fourteen (14) the prevalence of asthma is nearly twice as great in boys as in girls (Horwood et al., 1985). As children get older, the differences narrows and by adulthood the prevalence of asthma is greater in women than in men. The reasons for this sex-related difference are not clear. However, lung size is smaller in males than in females at birth (Martinez et al., 1995) but larger in adulthood (Weiss, 1998).

2.2.2. Environmental factors

There is some overlap between environmental factors that influence the risk of developing asthma and factors that cause asthma symptoms (occupational sensitizers for example belong in both categories). However, there are some important causes of asthma symptoms such as air pollution and some allergens that have not been clearly linked to the development of asthma.

2.2.2.1. Allergens

Although allergens are known to cause asthma exacerbations, their specific role in the development of asthma is still not clear. The prevalence of sensitization with allergens derived from house dust mites and cockroaches appears to correlate directly with exposure (Wahn et al., 1997; Huss et al., 2001). Although some data suggest that exposure to house dust mite allergens may be a causal factor in the development of asthma (Sears et al., 2003), other studies tend to disagree (Charpin et al., 1991; Sporik, 1995). Cockroach infestation has been shown to be an important cause of allergic sensitization, particularly in inner-city homes (Rosenstreich et al., 1997).

In the case of animal-borne allergens, some epidemiologic studies have shown that early exposure to dogs and cats may protect a child against allergic sensitization or the development of asthma (Platts-Mills et al., 2001; Melen et al., 2001; Ownby et al., 2002; Almqvist et al., 2003; Gern et al., 2004). Other studies have however suggested that such exposure may increase the risk of allergic sensitization (Ownby et al., 2002; Celedon et al., 2002) and so this issue remains unresolved. Research has also shown that the prevalence of asthma is reduced in children raised in a rural setting, which may be linked to the presence of endotoxin in these environments (Braun-Fahrlander, 2003).

Findings from this study seem to indicate that prolonged environmental exposure to microbial products as assessed by the measurement of endotoxin levels in mattress dust is associated with the development of tolerance toward ubiquitous allergens found in natural environments. Mechanisms relating to the recognition of these microbial compounds by the innate immune system and the regulation of the

resulting inflammatory responses through adaptive immunity are likely to be of key importance for the development of atopic childhood asthma.

2.2.2.2. Infections

During infancy, a number of viruses have been associated with the inception of the asthmatic phenotype. Respiratory syncytial virus (RSV) and parainfluenza virus produce a pattern of symptoms including bronchiolitis that parallel many features of childhood asthma (Sigurs et al., 2000; Gern and Busse, 2002). A number of long-term prospective studies of children admitted to the hospital with documented RSV have shown that approximately 40% will continue to wheeze or have asthma into later childhood (Sigurs et al., 2000). On the other hand, evidence also indicates that certain respiratory infections early in life, including measles and sometimes, even RSV, may protect against the development of asthma (Shaheen et al., 1996; Stein et al., 1999). The data did not allow specific conclusions to be drawn.

The “hygiene hypothesis” of asthma suggests that exposure to infections early in life influences the development of a child’s immune system along a “nonallergic” pathway, leading to a reduced risk of asthma and other allergic diseases. Although the hygiene hypothesis continues to be investigated, this mechanism may explain observed associations between family size, birth order, day-care attendance, and the risk of asthma. For example, young children with older siblings and those who attend day care are at increased risk of infections, but enjoy protection against the development of allergic diseases, including asthma later in life (Ball et al., 2000; Illi et al., 2001; de Meer et al., 2005).

The interaction between atopy and viral infections appears to be a complex relationship (Zambrano et al., 2003) in which the atopic state can influence the lower airway response to viral infections. Viral infections in turn can then influence the development of allergic sensitization, and interactions can occur when individuals are exposed simultaneously to both allergens and viruses (Venables and Chan-Yeung, 1997; Zambrano et al., 2003; Malo et al., 2004).

2.2.2.3. Occupational sensitizers

Over 300 substances have been associated with occupational asthma (Newman, 1995; Fabbri et al., 1997; Venables and Chan-Yeung, 1997; Chan-Yeung and Malo, 1999; Malo et al., 2004), which is defined as asthma caused by exposure to an agent encountered in the work environment. These substances include highly reactive small molecules that may cause an alteration in airway responsiveness, and that stimulate the production of IgE. Occupational asthma occurs predominantly in adults (Chan-Yeung and Malo, 1994; Bernstein et al., 1999) and occupational sensitizers are estimated to cause about 1 in 10 cases of asthma among adults of working age (Nicholson et al., 2005). It is now known that asthma is the most common occupational respiratory disorder in industrialized countries (Blanc and Toren 1999) and occupations associated with a high risk for occupational asthma include farming and agricultural work, painting (including spray painting), cleaning work, and plastic manufacturing (Venables and Chan-Yeung, 1997).

Two types of occupational asthma can be distinguished according to the presence or absence of a latency period, the type with latency being the most common. This type develops after a period of exposure ranging from a few weeks to several

years. Occupational asthma with latency includes all instances of immunologic asthma, although the immunologic mechanism for some agents has yet to be identified. Occupational asthma without a latency period on the other hand follows exposure to high concentrations of irritant gases, fumes, or chemicals on one or several occasions (Brooks et al., 1985).

Most occupational asthma is immunologically mediated and has a latency period of months to years after the onset of exposure Sastre et al., (2003). IgE-mediated allergic reactions and cell-mediated allergic reactions are involved (Frew et al., 1998). Levels above which sensitization frequently occurs have been proposed for many occupational sensitizers. However, the factors that cause some people but not others to develop occupational asthma in response to the same exposures have not been identified. Very high exposures to inhaled irritants may cause "irritant induced asthma" (formerly called the reactive airways dysfunctional syndrome) even in non-atopic persons. Atopy and tobacco smoking may increase the risk of occupational sensitization, but screening individuals for atopy is of limited value in preventing occupational asthma (Bernstein, 1993). The most important method of preventing occupational asthma is elimination or reduction of exposure to occupational sensitizers.

2.2.2.4. Tobacco smoke

Tobacco smoking is associated with accelerated decline of lung function in people with asthma, increases asthma severity, may render patients less responsive to treatment with inhaled (Chalmers et al., 2002) and systemic (Chaudhuri et al., 2003) glucocorticosteroids, and reduces the likelihood of asthma being controlled (Bateman et al., 2004). Exposures to tobacco smoke both prenatally and after

birth are associated with measurable harmful effects including a greater risk of developing asthma-like symptoms in early childhood. However, evidence of increased risk of allergic diseases is uncertain (Strachan and Cook, 1998). Studies of lung function immediately after birth have shown that maternal smoking during pregnancy has an influence on lung development (Martinez et al., 1995) and other studies have shown that infants of smoking mothers are four times more likely to develop wheezing illnesses in the first year of life (Dezateux et al., 1999). In contrast to the latter, there is little evidence that maternal smoking during pregnancy has an effect on allergic sensitization in children (Strachan and Cook, 1998). Exposure to environmental tobacco smoke (passive smoking) was also found to increase the risk of lower respiratory tract illnesses in infancy (Nafstad et al., 1997) and childhood (AAPCEH, 1997).

2.2.2.5. Outdoor/indoor air pollution

The role of outdoor air pollution in causing asthma remains controversial (American Thoracic Society, 2000). Children raised in a polluted environment were found to have diminished lung function (Gauderman et al., 2004), but the relationship of this loss of function to the development of asthma is not known. Outbreaks of asthma exacerbations have been shown to occur in relation to increased levels of air pollution, and this may be related to a general increase in the level of pollutants or to specific allergens to which individuals are sensitized (Anto et al., 1999; Marks et al., 2001; Chen et al., 2004). Indoor pollutants, e.g., smoke and fumes from gas and biomass fuels used for heating and cooling, moulds and cockroach infestations have also been associated with different airway conditions.

2.2.2.6. Diet

The role of diet, particularly breast-feeding, in relation to the development of asthma has been extensively studied and, in general, the data reveal that infants fed formulas of intact cow's milk or soy protein have a higher incidence of wheezing illnesses in early childhood compared with those that have been fed breast milk (Friedman and Zeiger, 2005). Some data also suggests that certain characteristics of Western diets, such as increased use of processed foods and decreased intake of antioxidants (in the form of fruits and vegetables), increased n-6 polyunsaturated fatty acid (found in margarine and vegetable oil), and decreased n-3 polyunsaturated fatty acid (found in oily fish) intakes have contributed to the recent increases in asthma and atopic disease (Devereux and Seaton, 2005).

2.3. Classification of asthma

Asthma can be categorized into four: extrinsic (allergic or atopic), intrinsic (non-allergic), occupational and exercised-induced asthma.

2.3.1. Extrinsic (allergic or atopic) asthma

This form of asthma is IgE-mediated, atopy-associated and usually begins in childhood or early adolescence. Atopy is the genetic predisposition for the development of IgE-mediated response to common aeroallergens and has been described as the strongest predisposing factor for developing asthma (NHLBI 1997; Nadel and Busse, 1998). Allergic asthma is the most common form of asthma and is characterized by reversible obstruction of airway, bronchospasm, infiltration of inflammatory cells into lung tissues, airway hyper-responsiveness

(AHR), mucus overproduction and over-expression of Th2-mediated cytokines among others (Kon and Kay, 1999; Renauld, 2001).

2.3.2. Intrinsic (non-allergic or non-atopic) asthma

A number of factors could cause intrinsic asthma but its onset is usually during adulthood (NHLBI, 2003). There is little or no IgE-mediation and the observed bronchoconstriction and airway hyper-responsiveness could possibly be due to stimulation of airway postganglionic parasympathetic nerve endings by inhaled antigens, leading to the release of acetylcholine which then binds to M_3 muscarinic receptors to sustain the process (Jacoby et al., 2001). Neutrophils instead of eosinophils appear to be the most prominent cell type in this form of asthma (Sur et al., 1993; Amin et al., 2000) and therefore the mechanism of non-allergic asthma could be said to be associated more with smooth muscle constriction and less with inflammatory response.

2.3.3. Occupational asthma

Occupational asthma is often considered as a temporary form of asthma caused by occupational exposure to workplace materials (animal products, biological enzymes, plastic resins, wood dusts and metal particles). The airway inflammation, bronchial hyperresponsiveness and clinical signs of asthma observed after inhalation of these workplace materials can be reduced by removal of the causative agent (NHLBI 1997; Venables and Chan-Yeung 1997) but the asthmatic conditions could persist even after removal of the causative agent(s) (Venables and Chan-Yeung, 1997).

2.3.4. Exercise-induced asthma (EIA)

This form of asthma is characterized by narrowing of the airways when triggered by vigorous activity, often beginning 5-10 minutes after a brief exercise. Patients with EIA have airways that are overly sensitive to sudden changes in temperature and humidity, especially when breathing colder, drier air. One explanation for this is that during strenuous activity, people tend to breathe through their mouths, allowing the cold, dry air to reach the lower airways without passing through the warming, humidifying effect of the nose (Jeffery 1999).

A type of EIA called “ski asthma” has been modelled in dogs (Davis et al., 2002) and in horses (Davis et al., 2005) and results compare closely with findings from studies in human winter athletes. Macrophage, lymphocyte and eosinophil concentrations were raised (Karjalainen et al., 2000) and there was expression of airway cytokines (Davis et al., 2005). The inflammatory mechanisms involved in EIA have also been investigated in human subjects (Hallstrand et al., 2005) and it appears activation of mast cells by osmotic stimuli through high-affinity IgE receptors is one of the mechanisms for exercise-induced bronchoconstriction (Robinson, 2004).

Other identified forms or sub-types of asthma include aspirin sensitive asthma (Stevenson 1984; Nasser et al., 1996; Szczeklik et al., 2000; Szczeklik and Stevenson, 2003; Szczeklik et al., 2004), severe infant asthma (Balfour-Lynn, 1999) and ‘steroid resistant’ asthma (Woolcock, 1993). The exact mechanism underlying steroid resistance is uncertain, but abnormalities in glucocorticoid receptor number, defective glucocorticoid receptor binding, or abnormalities in the glucocorticoid-glucocorticoid receptor complex binding to DNA have been

implicated for the poor response to corticosteroid therapy in affected patients Spahn and Leung (1999).

2.4. Epidemiology of asthma

Asthma is considered one of the most common respiratory complaints in the world today. According to the World Health organization (WHO) estimates, approximately 300 million people worldwide currently have asthma and 255 000 people died of asthma in 2005. It is projected that by 2025, an additional 100 million people will suffer from asthma due, in part, to growing urbanization and pollution (Masoli et al., 2004). Researchers have not yet determined the cause of this increase in asthma prevalence. Worldwide, the rate of asthma is increasing significantly, rising by 50 percent every decade. It is estimated that asthma accounts for about one in every 250 deaths worldwide (Masoli et al., 2004) and deaths due to asthma are projected to increase by almost 20% in the next 10 years unless urgent action is taken (WHO, 2006). Among the many socio-economic costs of asthma in many countries is the loss of economic work hours as asthma sufferers stay away from work whenever their condition requires them to be hospitalized (Karr et al., 1978; Thompson, 1984; Barnes et al., 1996; Sculpher and Price, 2003).

2.5. Aetiology and pathophysiology of asthma

The aetiology of asthma is complex and multifactorial (Maddox and Schwartz, 2002); the exact mechanisms inducing and regulating this condition are poorly understood (Hamelmann and Gelfand, 2001). It is however known that the disease is elicited by allergic reactions to certain agents (Jarjour and Kelly, 2002) and that there is a strong correlation between increased serum immunoglobulin type E

(IgE) levels and the progression and severity of asthma (Anupama et al., 2005). The failure of current therapies to cure asthma stems from the poor understanding of its mechanism.

IgE is the initiator of the airway inflammatory cascade that produces the classic early and late phase airway response to an inhaled allergen. Airway inflammation is initiated when an inhaled allergen forms a crosslink with a mast cell or basophil-bound IgE. Linking of the allergen and receptor-bound IgE provokes mast cell/basophil degranulation and release of inflammatory mediators including histamine, prostaglandins, tryptase and leukotrienes as well as such cytokines as IL-4, IL-5 and IL-13 (Travis et al., 2002; Puxeddu and Levi-Schaffer, 2004). Together, these mediators are responsible for mucosal oedema and smooth muscle contraction that are characteristic of the early asthma response (Fahy 1997).

As more information became available on asthma over the years, perception of the asthmatic condition shifted from that of a disease primarily characterized by altered bronchial smooth muscle function, bronchoconstriction and airway hyperresponsiveness to that of a disease mainly characterized by acute, sub acute, and/or chronic inflammation driven by a variety of agents (Davies et al., 1997; Drazen, 1998). The chronic airway inflammation seen in asthma adversely affects normal lung function as a result of which many new treatments have focused on control of the underlying inflammation.

2.6. Asthma and genetics

Several studies on asthma seem to suggest that genetic predisposition and environmental factors interact to produce asthma even though more studies are

required to define the exact manner of the interactions between genes and the environment as well as provide information on how gene therapy can provide the much-needed cure for the disease. Understanding gene-environmental interaction would facilitate risk prognostication, improve preventive strategies and develop targeted interventions in people with asthma (Yang et al., 2007).

Using genetic linkage techniques, the human chromosome 5q31 has been identified as the region likely to contain the genes related to asthma and asthma-related phenotypes (Hoffjan and Ober, 2002; Hoffjan et al., 2003). Other studies have also suggested the possibility of asthma being an inheritable condition with about half of its causative factor considered to be due to genetic susceptibility and the other half due to environmental factors (Duffy et al., 1990; Palmar et al., 2000). Studies of twins have shown that concordance rates for asthma are significantly higher in monozygotic twins than in dizygotic twins, and that the heritability of asthma may be as high as 75% (Duffy et al., 1990).

Five asthma susceptibility genes have already been identified and include *ADAM33, PHF11, DPP10, GRPA and SPINK5* (Walley et al., 2001; Van Eerdewegh et al., 2002; Zhang et al., 2003; Allen et al., 2003; Laitinen et al., 2004). *ADAM33* seems to function in airway remodelling and hyperresponsiveness (Van Eerdewegh et al., 2002) while the expression of *DPP10, GRPA and SPINK5* in terminally differentiating epithelium seems to suggest that these genes deal with threat or damage from the external environment (Cookson, 2004). Other genes exert their effect within the cells that make up the mucosa like *IL13,* which modifies mucus production and *FCεRI-β*, which modifies the allergic trigger on mast cells

(Cookson, 2004). The chromosome 13q14 gene *PHF11* was identified as a locus for IgE levels in asthma (Wills-Karp and Ewart, 2004).

2.7. The inflammatory process of asthma

The inflammatory process of asthma involves a wide range of cell types and cellular mediators. Asthma was originally described as an inflammatory disease that predominantly involves the central airways. Pathological and physiological evidence suggests that the inflammatory process extends beyond the central airways to the peripheral airways and lung parenchyma (Tulic et al., 2001). The presence of airway inflammation appears to be a consistent feature in asthma and the pattern of inflammation in the airways appears to be similar in most clinical forms of asthma. The relationship between the severity of asthma and the intensity of inflammation not clearly understood (Cohn et al., 2004; Bousquet et al., 2004).

The process of inflammation in asthma is described by an inflammatory cascade which is divisible into seven phases viz: sensitization, stimulation, cell signalling, migration, cell activation, tissue stimulation or damage and resolution. The sensitization or antigen presentation phase occurs as a result of presentation of antigens to T-lymphocytes usually by dendritic cells, monocytes and even B-lymphocytes (Holt et al., 1999). There is increasing evidence that the underlying mechanism driving and maintaining the asthmatic inflammatory process is an abnormal or inadequately regulated CD4+ T-cell immune response to otherwise harmless environmental antigens (Miller, 2001). Over-expression of Th2-mediated cytokines including IL-4, IL-5, IL-13 and TNF-α, as well as chemokines such as eotaxin and RANTES (regulated upon activation, normal T cell expressed and

secreted) was observed in the airways of allergic asthmatics (Kon and Kay, 1999; Renauld, 2001; Zimmermann et al., 2003).

The T-lymphocytes respond by changing from naive lymphocytes to allergic type of cells (called T-Helper 2 or TH-2 cells) which produce cytokines interleukins IL-4, IL-5, IL-9 and IL-13 (Barnes et al., 1998). The released cytokines influence conversion of B-lymphocytes to plasma cells that produce IgE that are specific for the particular antigen (Maddox and Schwartz, 2002). The IgE then attach mostly to mast cells where it can bind allergens, thereby completing the first step in the inflammatory cascade.

A number of factors usually stimulate an exacerbation of asthma, including allergens and environmental agents, mostly through the triggering of mast cells. Studies show that early exposure of genetically predisposed individuals to indoor aeroallergens, occupational antigens and respiratory viral infections sensitizes them to certain allergens (Holt and Macaubas, 1997). Recent studies suggest that IgE and the triggered mast cells can cause long-term asthmatic inflammation. Mast cell activation causes degranulation and leads to the release of such mediators as histamine, tryptase, platelet-activating factor (PAF), leukotriene-C4, Prostaglandin B2 (Wenzel et al., 1988; Wenzel et al., 1990) and such cytokines as IL-4, IL-5 and IL-13 (Puxeddu and Levi-Schaffer, 2004).

Allergen stimulation activates a complex communication network in which signaling cells issue biological commands that lead to recruitment of inflammatory cells into the airways. Th2 cytokines such as IL-4/IL-13 are involved in cell signaling and signal transducers and activators of transcription-6 (STAT6) is a cytoplasmic factor which plays a vital role in Th2 cell differentiation (Mullings et al.,

2001). Increase in eosinophil numbers and T lymphocytes in the bronchial mucosa and bronchoalveolar lavage (BAL) fluid are distinctive features of the inflammatory response in patients with asthma and appear to correlate with the severity of the disease (Walker et al., 1991; Caramori et al., 2005; Tillie-Leblond et al., 2005). Inflammatory cells only function after they have been activated and this occurs at the site of inflammation when they are exposed to cytokines and other potential activators including IL-1, IL-5, tumour necrosis factor-alpha (TNF-α), and chemokines such as eotaxin and IL-8 (Fireman, 2003). The major cellular components in late-phase allergic asthma appear to be eosinophils known to contribute greatly to the initiation and maintenance of the allergic response (Gleich, 2000; Dombrowicz et al., 2001). Under the influence of IL-5, undifferentiated bone marrow eosinophils differentiate and migrate to the area of allergic inflammation in the airways via a variety of interactions with integrins and adhesion proteins, through the influence of chemoattractant substances (Busse and Lemanske 2001; Prescott 2003; Lampinen et al., 2004).

The inflammatory processes of asthma lead to tissue alterations (including stimulation and damage) at the level of the epithelium, basement membrane, smooth muscle and nerves (Laitinen and Laitinen, 1994). At the site of inflammation, eosinophils release cationic proteins, mainly the major basic protein (MBP) and the eosinophil cationic protein (ECP) besides several cytokines, eosinophil peroxidase, oxygen metabolites and proteolytic enzymes. MBP has a rapid and highly cytotoxic effect on airway epithelial cells, damaging the airway mucosa and its associated nerves, causing epithelial shedding, increased epithelial permeability to external agents, hypersecretion of mucus, smooth muscle

contraction and increased vascular permeability (Gleich, 2000; Dombrowicz et al., 2001; Kay et al., 2004).

Besides the roles of various migrant cells, the structural cells of the airways also produce inflammatory mediators that contribute to the persistence of airway inflammation in various ways including contributing to the release of the over 100 different mediators now recognized to be involved in asthma and that mediate complex inflammatory responses in the airways (Barnes et al., 1998). Various airway structural cells involved in the pathogenesis of asthma make useful contributions. Airway smooth muscle and epithelial cells are capable of expressing multiple inflammatory proteins in asthma and release cytokines, chemokines, and lipid mediators (Chung, 2000). Endothelial cells of the bronchial circulation play a role in recruiting inflammatory cells from the circulation into the airway while fibroblasts and myofibroblasts produce connective tissue components, such as collagens and proteoglycans that are involved in airway remodelling. Reflex triggers in the airways may activate airway cholinergic nerves and cause bronchoconstriction and mucus secretion, causing sensory nerves to possibly release inflammatory neuropeptides (Groneberg et al., 2004).

2.7.1. The main mediators of asthma

2.7.1.1. Chemokines

These are involved in the recruitment of inflammatory cells into the airways and are mainly expressed in airway epithelial cells (Miller, 2004).

2.7.1.2. Cysteinyl leukotrienes

These are potent bronchoconstrictors and proinflammatory mediators mainly derived from mast cells and eosinophils. They are the only mediators whose

inhibition has been associated with an improvement in lung function and asthma symptoms (Leff, 2001).

2.7.1.3. Cytokines

These are the main drivers of the inflammatory process in asthma and determine its severity (Barnes, 2002). Key cytokines include IL-1 and TNF-α which amplify the inflammatory response, and GM-CSF, which prolongs eosinophil survival in the airways. Th2-derived cytokines include IL-5, which is required for eosinophil differentiation and survival; IL-4, which is important for Th2 cell differentiation; and IL-13, needed for IgE formation.

2.7.1.4. Histamine

This substance is released from mast cells and contributes to bronchoconstriction and to the inflammatory response. Histamine can cause inflammation directly (Andriopoulou et al., 1999) and indirectly (Marone et al., 1999) as well as smooth muscle contraction (Schmidt *et al.,* 1999).

2.7.1.5. Nitric oxide (NO)

This is a potent vasodilator, is produced predominantly from the action of inducible nitric oxide (NO) synthase in airway epithelial cells (Ricciardolo et al., 2004). Exhaled NO is increasingly being used to monitor the effectiveness of asthma treatment, because of its reported association with the presence of inflammation in asthma (Smith and Taylor, 2005).

2.7.1.6. Prostaglandin D2

Prostaglandin D2 is a bronchoconstrictor derived predominantly from mast cells and is involved in Th2 cell recruitment to the airways.

2.7.2. Cellular influx during asthma

2.7.2.1. Mast cells

Activated mucosal mast cells release bronchoconstrictor mediators (histamine, cysteinyl leukotrienes, prostaglandin D2) (Galli et al., 2005). Increased mast cell numbers in airway smooth muscle may be linked to airway hyperresponsiveness (Robinson, 2004).

2.7.2.2. Eosinophils

Eosinophils numbers are always increased in the airways and these cells are known to release basic proteins and growth factors that may damage airway epithelial cells and cause airway remodelling (Kay et al., 2004).

2.7.2.3. T-lymphocytes

These cells are usually present in increased numbers in the airways and these cells release the cytokines IL-4, IL-5, IL-9, and IL-13 that orchestrate eosinophilic inflammation and IgE production by B lymphocytes (Larche et al., 2003; Akbari et al., 2006).

2.7.2.4. Dendritic cells

These cells sample allergens from the airway surface and migrate to regional lymph nodes, where they interact with regulatory T cells and ultimately stimulate production of Th2 cells from naïve T cells (Kuipers and Lambrecht, 2004).

2.7.2.5. Macrophages

These cells are increased in number in the airways and may be activated by allergens through low-affinity IgE receptors to release inflammatory mediators and cytokines that amplify the inflammatory response (Peters-Golden, 2004).

2.7.2.6. Neutrophils

Neutrophil population is usually found to be increased in the airways, in sputum of patients with severe asthma and in smoking asthmatics, but the pathophysiological role of these cells is uncertain and their increase could be due to glucocorticosteroid therapy (Wenzel, 2003).

2.8. Structural changes in asthmatic airways

In addition to the inflammatory response of asthma, there are characteristic structural changes, often described as airway remodelling, that may represent repair in response to chronic inflammation. The release of Th2-mediated cytokines is known to also cause persistent inflammatory cell recruitment and to induce structural changes in airway walls, such as increased basement membrane thickness, increased collagen deposition, smooth muscle hypertrophy and goblet cell hyperplasia (Chiapara et al., 2001). Some of these changes are related to the severity of the disease and may result in relatively irreversible narrowing of the airways (Vignola et al., 2003; James et al., 2005). Tschumperlin and Drazen (2001) studied the role of mechanical stimuli in airway remodelling. Airway hyperresponsiveness is linked to both inflammation and repair of the airways and is partially reversible with therapy; its mechanisms are poorly understood (Hargreave et al., 1986; Elias, 2000).

Increase in thickness of the airway walls may result from subepithelial fibrosis (deposition of collagen fibres and proteoglycans under the basement membrane) as well as from hypertrophy (increased size of individual cells) and hyperplasia (increased cell division) of the airway smooth muscle (Hirst et al., 2004). Subepithelial fibrosis is a cardinal feature of bronchial asthma. It is known that

deposition of collagens I, III, and V, fibronectin and tenascin-C in the basal lamina contributes to this process (Roche, 1989; Laitinen et al., 1997). Airway smooth muscle contraction in response to multiple bronchoconstrictor mediators and neurotransmitters is the predominant mechanism of airway narrowing (Black, 2004) and is largely reversed by bronchodilators. In contrast, airway thickening due to structural changes (often termed “remodelling) is not fully reversible by current therapy. Trifilieff et al., (2001) reported that data from their studies suggested that glucocorticoids, although potent anti-inflammatory agents, may not be potent in reducing the lung remodelling process associated with asthma.

Mucus hypersecretion results from increased numbers of goblet cells in the airway epithelium and increased size of submucosal glands. It may lead to luminal occlusion (“mucus plugging”) and is a product of increased mucus secretion and inflammatory exudates (Barnes, 2004). Airway oedema usually results from increased microvascular leakage in response to inflammatory mediators. Thickening of the airway wall by oedema and structural changes amplifies airway narrowing (Wang et al., 2003) and in severe asthma, it is not only the bronchial walls that are affected but also the bronchial epithelium, which is usually damaged and sloughed (Laitinen et al., 1985; Lozewicz et al., 1990; Benayoun et al., 2003).

Although the exact reason for epithelial shedding is not clear, it is possible that it occurs in response to abnormal changes in the structural integrity of both cell-to-cell and cell-to-basement membrane attachments.

2.9. Treatment of asthma

There are difficulties in the drug development process for the treatment of asthma because of the limited understanding of molecular pathogenesis of various

diseases including asthma (Umetsu and DeKruyff, 2004). For now, there is no known treatment for asthma (Sheffer et al., 1992) and available medications only control or relieve its progression. Controller medications are taken daily on a long-term basis to keep asthma under clinical control mainly through their anti-inflammatory effects. Reliever medications are quick-acting agents used to reverse bronchoconstriction and to relieve asthmatic symptoms. Great variability in patient responses to current asthma therapy has been reported and this is possibly due to genetic and genomic variability (Pahl et al., 2006). It is expected that an ideal medication for asthma should act to inhibit the activities of specific inflammatory mediators or to inhibit specific processes like cell differentiation, cell proliferation, cell migration and cytokine production, all of which are associated with different aspects of asthma.

2.9.1. Controller medications

Although asthma treatment can be inhaled, taken orally or injected, the inhaled therapy seems to be most effective. Controller medications are effective in decreasing airway hyperresponsiveness (The Childhood Asthma Management Program Research Group, 2000), controlling airway inflammation (Jeffery et al., 1992), reducing frequency and severity of exacerbations (Pauwels et al., 1997), and reducing asthma mortality (Suissa et al., 2000). However, when controller medications are discontinued, deterioration of clinical control follows within weeks to months in some patients (Waalkens et al., 1993; Jayasiri and Perera, 2005).

Controller medications include leukotriene modifiers like cysteinylleukotriene 1 (CysLT1) receptor antagonists (e.g. montelukast, pranlukast and zafirlukast) and a 5-lipoxygenase inhibitor (like zileuton). Clinical studies have demonstrated that

leukotriene modifiers have a small and variable bronchodilator effect but are capable of reducing symptoms like cough (Dicpinigaitis et al., 2002), improving lung function and reducing airway inflammation and asthma exacerbations (Lipworth 1999; Drazen et al., 1999; Barnes and Miller, 2000).

The long-acting inhaled β2-agonists (e.g. formoterol and salmeterol) only work effectively against airway inflammation in asthma when combined with inhaled glucocorticosteroids and inhaled short-acting β2-agonists (salbutamol, terbutaline, and bambuterol) (Lemanske et al., 2001; Lazarus et al., 2001). In this way, the two processes fundamental to asthma (bronchoconstriction and inflammation) are addressed via the additive or synergistic effects of these combined therapy (Pahl, 2006). The bronchodilator theophylline has modest anti-inflammatory properties when given in a lower dose (Sullivan et al., 1994; Kidney et al., 1995; Barnes, 2003) while treatment of mild-to-moderate allergic asthma usually involves the use of such medication as tranilast, repirinast, tazanolast, pemirolast, ozagrel, celatrodast, amlexanox, and ibudilast (Kurosawa, 1994). The association of intranasal and inhaled corticosteroids (ICS) with systemic side effects is still an important issue against their use and recent research focuses on development of soft corticosteroids that are capable of separating local activity from these side effects (Bodor and Buchwald, 2006).

The inadequacy of using ICS alone to control asthma has led to the development and introduction of combination products usually consisting of an ICS and a long-acting beta-2 agonist. The two combination products currently available are Symbicort (budesonide/formoterol in a single inhaler) and Seretide

(salmeterol/fluticasone) using fixed or adjustable dosing options (Lötvall, 2004; Miller-Larsson and Selroos, 2006).

For unknown reasons the inflammation and underlying mechanisms seen in asthma may not cease even after medication. A better understanding of the resolution process could help explain the differences among mild, moderate and severe asthma and lead to the development of more effective therapies (Woolley et al., 1996).

The biological product omalizumab is a recombinant, humanized, monoclonal anti-IgE that has been reported to be capable of enhancing asthma control in patients with difficult-to-treat asthma. Omalizumab binds to the portion of the circulating IgE recognized by the high-affinity IgE receptor on the surface of the mast cell or basophil. Formation of omalizumab-IgE complexes reduces (in a dose-dependent manner) the amount of free IgE available to crosslink with an allergen, minimizes effector cell activation and greatly attenuates release of inflammatory mediators (Boulet, 1997; Busse et al., 2001; Buhl et al., 2002).

2.9.1.1. Hydrocortisone (HC)

The *in vitro* effects of HC on cells in culture as well as the *in vivo* effects in experimental animals have been well established. This steroidal anti-inflammatory medication has long been reported to be highly effective in the spontaneous treatment of Trichinella and Trichuris in mice infected with *Trichinella spiralis* and *Trichuris muris* (Campbell, 1968). In a later study, the interaction of HC with the German homeopathic remedy *pseudomonas aeruginosa* in cultured peripheral mononuclear blood cells was investigated (Kunze and Hartmann, 1997). In another study, the effects of treatment of pulmonary fibrosis (PF) with HC in rats

were compared with those seen in treatment with some Chinese herbal medicines (Dai et al., 2004). Furthermore, a low-dose HC treatment was found to have the most remarkable effects of improving the biological indices of lung injury, inflammatory mediators and pathological changes in rats with early septic shock (Zhou et al., 2004).

Besides herbal products, functional foods and so-called nutraceuticals such as whey proteins have also been studied for their biomedical effects in comparison with HC. The gel-like Malleable Protein Matrix (MPM) is a novel fermented whey protein with potent anti-inflammatory activity. In a study by Beaulieu et al., (2007), MPM demonstrated anti-inflammatory activity in an atopic contact dermatitis mouse model (ACD) with no side-effects or neutrophil extravasation in tissue compared to HC.

In other studies, the anti-oxidant and anti-inflammatory activities of crude extracts of oregano (*origanum vulgare* L) were studied in a mouse model. The anti-inflammatory activities of the oregano extracts tended to be weaker than those of HC used as pharmaceutical control (Yoshino et al., 2006).

Effects of corticosteroids on cells in culture have been studied. Kraft et al., (2001) reported that IL-4, IL-13 and dexamethasone significantly increased fibroblast proliferation in subjects with mild asthma whereas IFN-γ did not significantly alter airway fibroblast proliferation.

In this study, HC was used as a pharmaceutical control for *E. hirta* in a BALB/c mouse asthma model.

2.9.2. Reliever medications

These are medications that act quickly to relieve bronchoconstriction and accompanying acute symptoms. Beta-2 agonists act on redundant receptors on the muscle of the bronchioles and cause them to relax. Rapid-acting inhaled β2-agonists are the medications of choice for relief of acute bronchoconstriction and for the pre-treatment of exercise-induced bronchoconstriction in both adults and children of all ages (Tattersfield et al., 2001). Examples include salbutamol and terbutaline. Anticholinergic bronchodilators (ipratropium bromide and oxitropium bromide), short-acting oral β2-agonists and theophylline are also being used as reliever medications. In addition to its bronchodilator effect, theophylline is known to be capable of attenuating inflammation in asthma (Sullivan et al., 1994). It is considered that continuous use of reliever medications is a sign of deteriorating asthma control and indicates the need to re-assess treatment.

2.9.3. Complementary and alternative medicine (CAM)

Over the years, complementary and alternative treatment modalities used for most inflammatory diseases have gained popularity because these procedures are purported to show clinical efficacy with minimal side effects compared to mainstream treatments (Chevrier et al., 2005). In the case of asthma, Ming-Chun et al., (2005) concluded that anti-asthma herbal medicine intervention appears to be a safe and effective alternative medicine for treating asthma.

Traditional medical practices especially in most developing countries remain an integral part of the primary healthcare system of these nations albeit informally. The failure of relevant authorities to recognise and integrate these traditional practices into the mainstream healthcare system led to many patients embracing

both allopathic with traditional practices. It thus seemed that only the patient integrated the two systems and this is known to have attendant consequences. It is only imaginable how many lives have been lost to the adverse complications of drug-plant extract interactions that may have occurred.

The WHO traditional medicine strategy 2002-2005 reported (in Fink 2002) some adverse effects of using prescribed drugs and herbal products like gingseng and Saint John's wort. Hodges and Kam (2002) also reported adverse effects such as increased bleeding tendencies and drug interactions associated with patient use of herbal products before surgery. Anaesthetists and surgeons are therefore required to specifically inquire about the use of herbal medicines during pre-operative assessment and ensure that an interval of at least two weeks is allowed between drug use and surgery.

It is however, commendable that many countries of the world have come to terms with the reality of extensive use of traditional medicine by their citizens leading to the formulation of regulatory policies on these practices (Hodges and Kam, 2002).

Corticosteroids, the most potent nonspecific anti-inflammatory agents, produce substantial improvement in objective lung functions of patients with asthma and are the cornerstone of asthma treatment. However, prolonged use of systemic corticosteroids also induces serious systemic adverse effects (Leonard and Sur, 2002) resulting in the demand for complementary and alternative therapies (Partridge et al., 2003).

Although most CAMs are known to be effective for asthma, their methodological shortcomings need to be addressed so that their scientific integrity can be

validated (Yu et al., 1991; Federspil and Vettor, 2000). Research shows that most patients adopting CAM interventions use them to complement conventional care rather than as the sole form of treatment (Eisenberg et al., 1993; Astin, 1998; Eisenberg et al., 1998).

Complementary and alternative therapies such as acupuncture, homeopathy, Ayurvedic medicine, ionizers, other massage therapy, hypnotherapy, yoga, osteopathy, chiropractic manipulation, speleotherapy, herbal medicine and dietary supplements continue to gain popularity as modalities for the treatment of asthma (Bielory et al., 2004). Apart from studies involving herbal medicine, homeopathy and dietary supplements, there are only very few studies in literature from which conclusions about the efficacy of other forms of CAMs can be drawn.

A single controlled trial of chiropractic spinal manipulation failed to show benefit of this therapy in asthma (Balon and Mior, 2004). One study of the Butyeko breathing method suggested minor benefit but a later study of two physiologically contrasting breathing techniques showed similar improvements in reliever and inhaled glucocorticosteroids use in both groups, suggesting that perceived improvement with these methods are the result of non-physiological factors (Slater et al., 1999). Studies on the use of acupuncture for treatment of asthma have also been reported (Jobst, 1996; Anmin, 1998). Hypnotherapy has been used for decades as an important tool in ameliorating asthma, improving ventilatory capacity and promoting relaxation with no recourse to pharmacologic agents and their side effects (Hanley, 1974; Aronoff et al., 1975).

2.9.3.1. Herbal remedies and medicines for asthma

Phytomedicine (the use of plants or plant parts for therapeutic purposes) is an ancient discipline and practice used worldwide. According to a 1985 World Health Organization report, an estimated 80% of the world's population relies on herbal remedies for primary health care (Farnsworth et al., 1985). Herbs have been widely used over the years for the treatment of asthma especially in rural communities of most poor nations. There are now claims worldwide that herbal remedies and other alternative medical practices could be used to combat a number of chronic diseases like diabetes, cancer, HIV/AIDS, arthritis and asthma.

The present study investigates the possible effects of extracts from the herb *E. hirta* (used for the traditional treatment of asthma) on the MRC-5 cell line and asthmatic BALB/c mice. Like the currently used pharmaceutical products, herbs are expected to function effectively in reversing the various symptomatic conditions that characterize the onset and progression of asthma without causing too many side effects. (Bielory and Lupoli, 1999) found that many medicinal plants provided relief for symptoms as much as, or better than the effects of the known allopathic medicines used in the study.

Herbal products in different preparations or packaged in homeopathic remedies have been reported in literature as part of CAM (Slader et al., 2006). Botanicals such as *Boswellia serrata, Petasites hybridus, Astragalus membranaceus*, *Echinacea angustifolia* and *Ananas comosus* (common pineapple) and specific extracts such as bromelain from pineapple are being investigated as therapeutic agents in inflammatory conditions such as ulcerative colitis, multiple sclerosis and asthma (Gupta et al., 1998; Miller, 2001; Mialovyts'ka, 2003; Shinto et al., 2004;

Patwardhan and Gautam, 2005; Guggi and Bernkop-Schnurch, 2005; Hale et al., 2005; Bellavite, 2006).

Eupatilin (a pharmacologically active flavonoid in the herb *Artemisia asiatica*) was found to block multi-signal pathways and Ca^{2+} influx in the mast cells activated by specific antigen/antibody reaction (Kim et al., 2005). Bromelain is an extract from the juice and stems of pineapples commonly used clinically as an anti-inflammatory agent in rheumatoid arthritis, soft tissue injuries, colonic inflammation and chronic pain. This botanical extract is normally delivered as a powder either encapsulated in gelatine or prepared in an enteric-coated tablet. Bromelain is available in combination with other natural products or as a single stand-alone product. Bromelin treatment was shown to inhibit and modulate critical components of the allergic airway disease response in a murine model of allergic airway disease (AAD), which include influx of lymphocytes and eosinophils into the lung, reduction of T-lymphocytes and bronchoalveolar lavage (BAL) IL-13 levels (Hale et al., 2002; Secor Jr., 2005). Bromelain thus appears to be capable of reducing the inflammation of cells associated with asthma.

Ephedra (*Ephedra sinica*; pharmacopeial name: Herba ephedrae) also known as *ma huang* is commonly used in the treatment of asthma, bronchitis, and nasal congestion due to its high content of ephedrine, ephedra (Goodman et al., 1993; Hutchins, 2001). Gingkolides are derived from the botanical Gingko (*Gingko biloba*; pharmacopeial name: Folium ginkgo) and have long been shown to inhibit the development of bronchial hyper-reactivity in a small study of asthmatic patients; and to inhibit eosinophil influx into animal airways induced by platelet-activating factor (PAF) or antigen exposure (Guinot et al., 1987; Coyle et al., 1988;

Braquet and Hosford, 1991). In an *in vitro* model of mast cell activation, an extract of ginseng (*Panax ginseng*; pharmacopeial name: Radix Ginseng) was found to inhibit mast cell activation in a dose-dependent fashion; and to generate and release TNF-α and IL-6 (Jeong et al., 2001).

Even the effects of plant extracts on possible airway remodelling following chronic asthma have been studied. DA-9201 (an ethanolic extract of black rice, *Oryza sativa*) was found to significantly reduce total serum and bronchoalveolar lavage fluid (BALF) IgE levels, eosinophilia, inflammatory cell infiltration, mucus hypersecretion and significantly reduced subepithelial collagen deposition (Lee, 2006). Studies by Chaabi et al., (2006) showed that extracts from *Euphorbia stenoclada* displayed anti-proliferative activity on cultured human airway smooth muscle (HASM) due to the presence of quercetin (Chaabi et al., 2007).

Huntley and Ernst (2000) reviewed seventeen randomized controlled trials on herbal remedies used for asthma over three decades including six using traditional Chinese medicines, eight using traditional Indian preparations and three using other preparations. A number of findings were made viz: a total of 775 asthmatics were involved and each received different herbal remedies and also, each of the studies used different herbal preparation. Nine of the trials were described as double blind. The overall methodological quality was poor; only three studies (all of which used traditional Indian remedies and lasted for four weeks or more) were of acceptable quality (Shivpuri et al., 1972; Mathew and Shivpuri, 1974; Gupta et al., 1979). Only one of these studies involving 123 asthmatics showed a benefit with respect to lung function (Mathew and Shivpuri, 1974).

Barak et al., (2002) studied the effect of five herbal remedies possessing immune-enhancing properties and sold as food additives on the production of cytokines. These remedies included *Sambucol Black Elderberry* extract, *Sambucol Active Defence Formula* and *Sambucol for Kids* (with known antiviral properties), *Protec* and *Chizukit N* (containing propolis and Echinacea, claimed to be immune enhancers). Only the three *Sambucol* formulations were found to be very effective in activating the healthy immune system by increasing inflammatory and anti-inflammatory cytokines production (Barak et al., 2002). An experiment with BALB/c mice treated with *Silymarin* (a complex mixture of flavonoids routinely isolated from the seed and fruits of the common milk thistle *Silybum marinum*) for 5 consecutive days showed that the $CD4^+$ and $CD8^+$ T-helper cell population was increased (Johnson et al., 2002).

In many low-income and developing nations of the world, herbal products are abundant and are freely available in the open market but their safety has always been of great concern especially because of poor regulatory standards in most of these poor countries. An example is a study by Obi et al., (2006) in which some randomly sampled Nigerian herbal products screened for their heavy metal content were found to contain elevated amounts of heavy metals. Generally, there is a lack of control of quantity and quality of the components in most herbal remedies although many have fewer side effects than current therapy (Bielory and Lupoli, 1999).

In spite of reported shortcomings, the use of herbal products seems to have been deeply entrenched in many health and medical cultures and traditions and hence there is need for them to be studied in order to elaborate their mechanisms of

action. It is expected that these herbal products will at least show potent anti-inflammatory and bronchodilator effects without any toxicity and side effects.

2.9.3.2. Effects of vitamins and other food supplements

Different food supplements and vitamins present in either fruits or vegetables or in isolated preparations have been reported to relieve different aspects of asthma progression. Some of the substances studied include vitamin C (Ting et al., 1983; Forastiere et al., 2000); lycopene in tomatoes (Neuman et al., 2000); vitamin B6 (Collipp et al., 1975; Reynolds and Natta, 1985), magnesium (Rylander et al., 1997; Hill et al., 1997) and fish oil supplements (Dry and Vincent, 1991; Broughton et al., 1997; Mickleborough et al., 2006). The use of intravenous treatment with multiple nutrients, including magnesium, for acute and chronic asthma has also been reported (Shrader, 2004). Findings showed that pulmonary function improved progressively with longer periods of treatment and thus this treatment with multiple nutrients may be of considerable benefit to asthma patients.

2.9.3.3. *Euphorbia hirta*

E. hirta (Euphorbiacea) is a plant with great anti-inflammatory potential (Dickshit, 1943; Hazleton and Hellerman, 1954; Watt and Breyer-Brandwijk, 1962; Le Strange, 1977; Wong, 1980; Lanhers, 1990, 1991; Skidmore-Roth, 2001; Lindsey, et al., 2002). Although the cytotoxic potential of the plant has been studied, little is known of the effects of the plant on cellular function and morphology.

E. hirta is found worldwide but it is also indigenous to Africa. In East and West Africa, extracts of the decoction of the flowering and fruiting plant have long been used (and are still being used) for the treatment of asthma and respiratory tract infections and are sometimes combined with bronchial sedatives like *Grindelia*

robusta in preparations for inhalation (Oliver, 1959; Kokwaro, 1976). *Euphorbia hirta* is also used for the treatment of coughs, chronic bronchitis and pulmonary disorders; for relieving hay fever and catarrh; as an anti-hypertensive agent, analgesic, anti-pyretic and sedative; and the diuretic properties of the plant have also been reported (Dickshit, 1943; Hazleton and Hellerman, 1954; Watt and Breyer-Brandwijk, 1962; Le Strange, 1977; Wong, 1980; Lanhers, 1990, 1991).

E. hirta contains a great number of active ingredients including alkaloids, flavonoids, glycosides, sterols, tannins and triterpenoids (Gupta and Garg, 1966; Atallah and Nicholas, 1972; Sofowora, 1984; Galvez et al., 1992). The exact mechanisms by which *E. hirta* relieves asthma are not clear, but significant and dose-dependent anti-inflammatory effects have been observed (Martinez-Vazquez et al., 1999). Research also showed that aqueous extracts of *E. hirta* strongly reduced the release of prostaglandins I_2, E_2, and D_2 in rats (Hiermann and Bucar, 1994).

Despite the array of chemical compounds identified in *E. hirta* and the diverse local medicinal uses of the plant, very little pharmacological evaluations have been carried out to ascertain the rationale behind most of the folkloric claims of its efficacy (Johnson et al., 1999). However, *E. hirta* contains a bioflavonoid, Quercitrin (3-rhamnosylquercetin), which is usually converted to Quercetin (3-O-alpha-L-rhamnopyranoside - Quercetrin) in the alimentary canal and it seems this is the compound that has great therapeutic potential. Quercitrin possesses antioxidant properties as well as anti-inflammatory activities and is the glycosylated form of Quercetin (Comalada et al., 2005). Another flavonoid in *E.*

hirta, Myricitrin also seems to be a powerful anti-oxidant, inhibiting Nitric Oxide Synthase (NOS).

The sterols 24-methylene-cycloartenol and β-sitosterol exert significant and dose-dependent anti-inflammatory activity (Martinez-Vazquez et al., 1999). The triterpene β-amyrin also seems to have anti-inflammatory effects. The combined effects of β-amyrin, 24-methylene-cycloartenol and β-sitosterol, may therefore account for the potent dose-dependent anti-inflammatory activity of *E. hirta*.

Unfortunately, many of the other components of *E. hirta* extracts have not been studied sufficiently to know if they too might have anti-inflammatory effects. However, its effectiveness in treating asthma probably lies in the synergistic relationships between particularly the flavonoids, sterols and triterpenoids. The current research therefore investigates the effect of *E. hirta* on the different parameters in a BALB/c murine model with HC used as a pharmaceutical control for treatment of asthma.

2.10. Animal asthma models

Animal disease models are pathological states or induced injuries that present with signs and symptoms similar to conditions seen in humans. The use of animal models enables researchers to investigate disease states and perform potentially harmful procedures that would otherwise be unethical to carry out on humans, living or dead. Virtually all medical advances over the last century have been made using animals despite many diverse limitations.

Effective animal models usually provide useful information for understanding the mechanism of action of the modelled disease and are normally used for *in vivo*

testing of treatment modalities. For instance, behavioral analogues of anxiety or pain in laboratory animals can be used to screen and test new drugs for the treatment of these conditions in humans.

Apart from diseases occurring naturally in animals (e.g. genetic diabetic mice), most modelled diseases are induced by physical, chemical or biological means. Examples include healing models of physically inflicted wounds (Bergman et al., 1983), radiation-induced tumours (Setlow et al., 1989), alloxan-induced diabetes mellitus (Soto et al., 1998), invasive models for ischemic stroke in rats (Gerriets et al., 2003) and pentylenetetrazol-induced animal epilepsy (Patsoukis et al., 2004).

In a review article, Kurucz and Szelenyi (2006) emphasized the usefulness of animal models in terms of how successfully they have helped biomedical science to recognize or introduce new, more effective pharmaceutical products for asthma. However, since no one animal model can adequately mimic the entire asthma phenotype, it is probably more suitable to develop an animal model for modelling a trait associated with asthma, rather than for modelling the entire asthma phenotype (Pabst, 2003; Kips et al., 2003).

Attempts at understanding the immunology of human asthma led to the development of severe combined immunodeficiency (SCID) mice. Reconstitution of SCID mice with human peripheral blood mononuclear cells (Hu-PBMC) resulted in a human-mouse chimera with a functional human antigen-reactive system, which enabled the *in vivo* study of human T-cell biology. Exposure of the Hu-PBMC SCID mice to an aerosolised allergen has been reported by different groups to induce airway hyperresponsiveness that would appear to be driven purely by human T-cells (Duez et al., 2000; Tournoy et al., 2001).

Studies on asthma modelling have been extended to new areas like exercise-induced asthma, occupational asthma as well as to less studied aspects of modelled asthma such as airway remodelling. For instance, the sheep asthma model was reported to have features that correlated with the structural changes observed in the remodelling of lung tissues seen in chronic asthmatics. In their study, Snibson et al., (2005) repeatedly challenged the lungs of sheep with the house dust mite (known to be the major trigger of asthma in up to 80% of cases) in order to induce many of the distinguishing features of airway remodelling often seen in asthmatics. These include increased collagen deposition and airway smooth muscle bulk, mast cell and goblet cell hyperplasia as well as epithelial cell hypertrophy.

One interesting study on modelling exercise-induced asthma involved athletic dogs whose airway conditions after exercise compared closely with the airways of susceptible humans. Athletes who play sports in cold weather, particularly skaters and cross-country skiers, are known to have an increased prevalence of lower airway disease that is hypothesized to result from repeated penetration of incompletely conditioned air into the lung periphery (Davis et al., 2002) usually with significantly more airway inflammation than their sedentary counterparts (Sue-Chu et al., 1999).

Racing sled dogs like humans also perform strenuous exercises under frigid conditions and studies by Davis et al., (2002) suggest that the racing sled dog could be a useful naturally occurring animal model of the analogous human “ski asthma”, a term used to describe a syndrome of non-atopic airway inflammation and hyper-reactivity in elite winter athletes (Sue-Chu et al., 1999). Results from a

later study with horses also suggest that cold weather exercise can lead to asthma-like airway disease through the local induction of cytokines typical of the TH2 phenotype (Davis et al., 2005).

Treatment of induced asthma in animal models with botanicals has been reported in literature. Johnson et al., (2002) exposed asthmatic BALB/c mice to the botanical *Silymarin* and found that it caused increased T-lymphocyte population. Similarly, treatment of BALB/c mice with DA-9601 (a formulated ethanol extract of *Artemisia asiatica Nakai or Asteraceae*) was found to cause reduced IgE level, recruitment of inflammatory cells into the BAL fluid and lung tissues, expression of CD40, CD40L and VCAM-1 molecules, goblet cell hyperplasia and production of various cytokines (Kim et al., 2006).

In general, the use of animal models of human diseases in scientific investigations will continue since they appear to be far more beneficial. In the case of asthma, animal models progressively remain useful through the elucidation of disease mechanisms and development of medications that are often more active than previously used ones. For studies in mice, a broad spectrum of molecular and immunological tools is available (Bice et al., 2000).

2.10.1. BALB/c mouse models

BALB/c asthma models are widely studied and findings are reported in the literature. Viana et al., (2002) studied the response of BALB/c mice to the fungus *stachybotrys chartarum* in causing asthma-like conditions. Concurrent exposure of BALB/c mice to ovalbumin and ozone appeared to cause increased goblet-cell hyperplasia in a study by Last et al., (2004). DA-9201 (an ethanolic extract of black rice *Oryza sativa* L.) appeared to play an important role in attenuating the

progression of airway inflammation and remodelling in BALB/c mice sensitized to ovalbumin (OVA) (Lee et al., 2006).

The asthmatic mouse models of C57/BL, BALB/c, 129J, DBA/2, C3H/HeJ and CBA/J strains have also been studied (Joachim et al., 2003; Shinigawa and Kojima, 2003). BALB/c mice are chosen for this study primarily because so much is already known about their immune responses and genetics from literature. Despite the few identified shortcomings, results from previous studies have shown that the BALB/c mouse model still manages to paint a good pattern of the human airway disease better than any other model (Gleich et al., 1988, Zhao et al., 2000, Blyth et al., 2000; Leigh et al., 2002; McMillan and Lloyd, 2004; Johnson et al., 2004; Jungsuwadee et al., 2004).

BALB/c mice have long been used for other studies in immunology (Alvarez et al., 2004; Chen et al., 2005), drug dose testing (Fayer and Fetterer, 1995), carcinogenesis (McIntire and Princler, 1969; Pawlowski et al., 1979; Gambotto et al., 2000) as well as microbiology and infection studies (Adjei et al., 1993; Sheu et al., 1999; Singh et al., 2000; Padigel and Farrell, 2005).

2.11. Cell cultures

Cell culture systems are useful means of evaluating different biological processes *in vivo* prior to undertaking further studies with animals or final studies in human subjects. Many drugs are screened for toxicity and dosage using cell cultures. Cell culture studies could be done either with primary cultures or with cell-lines depending on the objectives of the study and availability. In the present study, the MRC-5 cell-line was used for testing toxicity of the test substances in order to determine the appropriate doses.

The MRC-5 cell line is a normal finite cell line derived from human embryonic or foetal diploid lung fibroblasts (Jacobs et al., 1970; Freshney, 2000). The plethora of literature on the MRC-5 cell line could be indicative of its excellent biological properties. Diverse studies are carried out on these cells, ranging from their use as control cells in experiments involving other cells or cell lines, to their use for cytotoxicity testing, drug potency testing, medicinal plant testing and vaccine production. In one study, Yang et al., (1997) found that cadmium induced oxidative cellular damage in cultured MRC-5 cells. In another study, extracts of thirty - three plants commonly used for the treatment of malaria by traditional healers in west tropical Africa were tested for antiplasmodial activity and cytotoxicity using MRC-5 cells (Zirihi et al., 2005). In addition, extracts prepared from the leaves of seven Panamanian tropical forest plants were found to have direct virucidal as well as intracellular antiviral activity against both DNA and RNA viruses. Antiviral activity was achieved with extract concentrations significantly lower than those required to produce cytotoxic effects. In addition, extracts were less toxic to normal MRC-5 cells than to the tumour cells tested (Romin et al., 2006).

A study by Maes et al., (2004) showed that PX-6518 exhibits minimal toxicity for mammalian cells including MRC-5 cells. Wu et al., (2005) used MRC-5 cell culture systems for the production of a smallpox vaccine and their findings suggest that optimization of growth conditions for MRC-5 cells resulted in enhanced vaccinia virus production. In another study, extracts of *Commelina diffusa* and *Spathodea campanulata* used as wound-healing agents in Ghana also exhibited significant antioxidant activity by protecting MRC-5 cells from hydrogen peroxide-induced oxidant injury at concentrations between one and ten microgram per millilitre (1-10µg/mL) (Mensah et al., 2006). MRC-5 cells have also been used as control cells

in many studies (Chang et al., 2002; Merlin et al., 2002; Conforti et al., 2006), among others.

In conclusion, it appears from the reviewed literature that a large number of studies have been done on the mechanism and treatment of asthma using different experimental models, therapeutic modalities and substances. The present study is expected to contribute to the general body of knowledge on asthma treatment by examining the effects of a locally-used medicinal plant on a murine model. It is hoped that sooner than later, a cure for asthma will be developed from all the effort of past and current researchers.

2.12. Aims and objectives of study

The general aims and objectives of this study therefore include the following:

a. To test for possible cytotoxic effects of the aqueous, acetone, hexane and dichloromethane extracts of *Euphorbia hirta* on the MRC-5 cell line using the combined NR/MTT/CV assay for lysosomal membrane integrity, cell viability and cell number, with a view to determining safe doses for use in the animal experiments.

b. To evaluate the possible dose-dependent therapeutic effects of the aqueous extract of *E.hirta* in the BALB/c mouse model by studying its effects on white blood cell count, platelet aggregation, fibrin network pattern and fibre morphology using the scanning electron microscope (SEM).

c. To use light and transmission electron microscopic (TEM) techniques to investigate histological and ultrastructural changes in the airways and

lungs including inflammatory cell migration, collagen fibre distribution, alveolar wall thickness, smooth muscle hyperplasia as well as mucous gland distribution.

d. To determine body weight changes in asthmatic BALB/c mice treated with the aqueous extract of *E.hirta* and comparing results with the control mice.

e. To compare findings from all experiments involving treatment with the aqueous extract of *E.hirta* with findings from experiments involving treatment with hydrocortisone in both the cell culture and the animal studies.