CHAPTER FIVE

Analysis of Inflammatory Leukocytes

5.1. Introduction

The formed elements of blood (particularly white blood cells) play key roles in the pathogenesis and progression of asthma especially the inflammation aspect of the disease. The process of asthmatic inflammation is described by an inflammatory cascade of seven phases beginning with sensitization (Wenzel et al., 1994). Sensitization involves presentation of antigens to T-lymphocytes usually by dendritic cells, monocytes and even B-lymphocytes (Holt et al., 1999) and is usually followed by other phases including stimulation, cell signaling, migration, cell activation, tissue damage and resolution. It is known that one of the predominant inflammatory cells recruited into asthmatic lung tissues is eosinophils, but neutrophils and macrophages have also been found to be elevated in the bronchoalveolar lavage fluid (BALF) and lung tissues (McKay et al., 2004).

Eosinophils are the predominant cells in the late phase of allergic inflammation and some authors have shown that neutrophils are also observed in bronchial biopsies and BALF from asthmatics although in relatively low numbers (Bradley et al., 1991; Lacoste et al., 1993; Persson et al., 1993). Studies by Lommatzsch et al., (2006) show that monocyte and neutrophil counts increased significantly 18 hours after challenge and returned to control levels 42 hours after challenge. Neutrophils (but not monocytes) were significantly decreased 3 days after challenge, before returning to control levels after 7 days. Lymphocyte counts did not change significantly at all time points. In contrast, eosinophils were significantly elevated at all time points, with a maximum 42 hours after challenge.

A study using the wild mouse model presented many of the characteristic features of allergic asthma, including eosinophil and lymphocyte infiltration, mucus

production and airway hyper reactivity (Gama Landgraf et al., 2003; Gama Landgraf et al., 2004). In another study, total white blood count (WBC) showed a significant increase following treatment of male albino rats with extracts of garlic. Neutrophil, lymphocyte and monocyte counts were significantly higher than in the control group whereas basophil count did not differ significantly from the controls. Eosinophil count on the other hand showed no significant change when compared with the control (Iranloye, 2002).

5.1.1. Eosinophils

The association of airway eosinophilic inflammation and asthma has been known for more than 100 years and the number of eosinophils in bronchial tissues, in bronchoalveolar lavage and in sputum correlates with symptoms, bronchial hyperresponsiveness and airflow obstruction (Bousquet et al., 1990). A significant positive correlation was found to exist between the clinical severity of bronchial asthma and total peripheral eosinophil count (TPEC) (Koshak and Alamoudi, 1999). One previous study documents a significant positive correlation between the clinical severity of bronchial asthma and eosinophil counts (Kamafar et al., 1999). Another study demonstrated a relationship between reduced sputum eosinophil apoptosis and increased clinical severity of chronic stable asthma, providing additional evidence that eosinophil apoptosis may be important in the resolution of eosinophilic airway inflammation in asthma (Duncan et al., 2003). Eosinophils in bone marrow are undifferentiated bone cells. These cells usually differentiate and migrate to the area of allergic inflammation in the airways via a variety of interactions with integrins and adhesion proteins under influence of chemo-attractant substances and interleukin-5 (IL-5) (Busse and Lemanske 2001; Prescott 2003; Lampinen *et al.,* 2004).

Eosinophils appear to be the major cellular components in late-phase allergic asthma and contribute greatly to the initiation and maintenance of the allergic response (Dombrowicz and Capron, 2001 and Gleich, 2000). Increase in eosinophil numbers and T-lymphocytes in the bronchial mucosa and BALF 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 and Tillie-Leblond et al., 2005). Eosinophilia in the BALF of asthmatic patients is associated with production of IL-5, which plays a critical role in the differentiation, infiltration, and activation of pulmonary eosinophils (Wills-Karp and Karp, 2004).

There is convincing evidence that early phase bronchoconstriction in asthma is attributable to IgE-mediated mast cell degranulation (Bingham and Austen, 2000). In contrast, the underlying mechanisms of the late asthmatic response are still in dispute. Eosinophils, the most characteristic leukocyte subpopulation within allergen-challenged airways (Virchow et al., 1995) are one example for this debate (Williams, 2004). Animal studies have suggested a role for eosinophils in the development of late phase bronchoconstriction (Cieslewicz et al., 1999). However, a specific reduction of endobronchial and peripheral eosinophils did not affect the development of a late asthmatic response in human asthma (Leckie et al., 2000). In humans, eosinophil numbers are always increased in the airways of asthmatics 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).

Data from one previous study suggests that in both human and experimental asthma, the recruitment of eosinophils peaks approximately 2 days after allergen

exposure (Lommatzsch et al., 2006). Other human studies have shown that eosinophils and IL-5 levels in BALF increase between 4 and 24 hours after allergen challenge (Teran et al., 1999). Notably, hypereosinophilia can even prevent airway dysfunction, possibly because of the release of anti-inflammatory mediators by eosinophils (Kobayashi et al., 2003). Repeated exposure of sensitized mice to ovalbumin aerosol induced airway inflammation characterized by eosinophil infiltration in lung tissue, trachea, and BALF and development of airway hyperresponsiveness of methacholine and serotonin (Hessel et al., 1994; Hessel et al., 1997; De Bie et al., 1996).

5.1.2. Lymphocytes

The role of lymphocytes in the asthmatic process has been well documented. 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). Inflammatory cells only function after they have been activated and this occurs at the site of inflammation when the cells are exposed to cytokines and other potential activators including interleukins IL-1, IL-5, tumour necrosis factor-alpha (TNF-α) and such chemokines as eotaxin and IL-8 (Fireman, 2003).

T-lymphocytes respond to inflammation by changing from naïve lymphocytes to allergic type of cells (called T-Helper 2 or TH-2 cells) which produce IL-4, IL-5, IL-9 and IL-13 (Barnes et al., 1998). The released cytokines influence conversion of B-lymphocytes to plasma cells known to produce antigen specific IgE as well as orchestrate eosinophilic inflammation (Maddox and Schwartz 2002; Larche et al.,

2003 and Akbari et al., 2006). The IgE molecules then attach mostly to mast cells where they bind allergens to complete the first step in the inflammatory cascade.

Over-expression of the Th2-mediated cytokines like IL-4, IL-5, IL-13, and TNF-α as well as such chemokines as eotaxin and RANTES was observed in the airways of allergic asthmatics (Kon and Kay, 1999; Renauld, 2001). T-lymphocyte numbers in the bronchial mucosa and BALF are also usually high in airways of asthmatics (Walker et al., 1991, Caramori et al., 2005 and Tillie-Leblond et al., 2005).

Current hypotheses hold that lymphocytes are recruited into the lung after allergen challenge to orchestrate the activity and differentiation of various effector cells (Hamid and Cameron 2000). Migration of lymphocytes from peripheral blood may contribute to the 15-fold increase of assorted types found in the BALF and lung parenchyma of sensitized and challenged Brown Norway rats (Schuster et al., 2000).

5.1.3. Neutrophil

Neutrophils are considered the dominant leukocyte subpopulation within airways during status asthmaticus (Lamblin et al., 1998) and are known to be more closely related to airway obstruction and asthma severity than eosinophils (Jatakanon et al., 1999; Ordonez et al., 2000). Neutrophils are usually found to be higher in count only in non-atopic asthmatic patients (Tobin 2001) and increased neutrophil numbers in airway mucosa or recovered from within the lumen has also been described in sudden-onset fatal asthma (Sur et al., 1993; Carroll et al., 1996).

Results from one study showed that neutrophils predominated more frequently than eosinophils as the major inflammatory cell in sputum from patients with acute

exacerbation asthma (Fahy et al., 1995). Another study showed that neutrophils are the dominant inflammatory leukocyte characterizing airway inflammation in acute severe asthma that requires mechanical ventilation, and that IL-8 is an important mediator of the observed neutrophilia (Ordoñez et al., 2000).

5.1.4 Aim of study

Since the number of inflammatory cells is known to increase in most forms of asthma, the aim of this study was to determine the presence of this effect in the BALB/c mice as a way of establishing their asthmatic status. In addition the effects of treatment of the "asthmatic" mice with the high and low doses of both HC and *E. hirta* were studied to determine which of the doses of these treatment agents used in this study had potential beneficiary effects in the asthmatic mouse model of asthma.

5.2. Materials and methods

Blood samples of all mice from each group were collected by cardiac puncture on day 46 of the study, pooled and used to prepare histological blood smears with Giemsa Wright staining technique. BALF was not used in this study. Five fields in each blood smear slide were evaluated and hundred (100) leukocytes were counted. Under X 100 magnification, cells were identified by standard morphology and counted manually. The cells are categorized into the respective leukocyte sub-population (lymphocytes, neutrophils, eosinophils, basophils and monocytes).

5.3. Results

Results from this study (*table 5.1* and *figure 5.1a*), show that most values for cell counts in all the treated groups are relatively lower than the values for the control

group (except neutrophil count in the HHC group). The percentage values were not however the lowest in the CT group. In addition, the AS group had the highest total leukocyte count while the lowest total count was observed in the LHC group and not the control group as would have been expected.

Table 5.1: Summary of the mean values obtained from white blood cell counts for all experimental groups

Group/ Cell type	CT	AS	LHC	HHC	LEH	HEH	Total
Neutrophil	39	336	36	91	24	31	557
Eosinophil	13	104	10	3	8	4	142
Basophil	7	58	3	2	3	1	74
Lymphocyte	144	1203	41	81	70	82	1621
Monocyte	203	737	32	63	89	113	1237
Total	406	2438	122	240	194	231	3631

The highest mean cell count recorded (1203 lymphocytes) was in the asthma group while the lowest cell count recorded (one basophil) was in the HEH group. In all the groups, the mean basophil counts were consistently the least followed by eosinophils (except in the AS group).

Generally, lymphocyte and monocyte counts were higher when compared to other cell types, followed by neutrophils. Interestingly, monocyte counts were the highest in three groups viz: CT, LEH, HEH groups and represented 50%, 45.88% and 48.92% respectively. Comparison according to dose shows that apart from eosinophil and basophil counts which were slightly higher than observed in all other treatment groups, the low dose treatment groups (LHC and LEH) generally had fewer cells than their corresponding high dose groups (HHC and HEH).

In order to understand the relationship between the mean cell counts per group and the total cell counts, the percentage values for the cell count were determined as given in *Table 5.2*.

Table 5.2: Summary of the percentage values obtained from white blood cell counts for every exposure group.

Cell type/Group	CT	AS	LHC	HHC	LEH	HEH
Neutrophil	9.61	13.78	29.50	37.92	12.37	13.42
Eosinophil	3.20	4.27	8.20	1.25	4.12	1.73
Basophil	1.72	2.38	2.50	0.83	1.55	0.43
Lymphocyte	35.47	49.34	33.60	33.75	36.08	35.50
Monocyte	50.00	30.23	26.20	26.25	45.88	48.92
Total	100	100	100	100	100	100

Analysis of the percentage values in table 5.2 shows the following:

i) Most of the percentage values in the HHC group were lower than values for both the control and asthma.

ii) HC appears to raise neutrophil levels in this study.

iii) The high doses of HC and *E. hirta* appear to reduce eosinophil levels when compared with other groups

iv) Basophils had the smallest percentage values in all groups and their numbers appear to increase slightly in asthma, as well as following treatment with low dose HC.

v) In the AS group, percentage lymphocyte levels are raised, whereas all other groups (LHC, HHC, LEH and HEH) had values that are similar to the percentage value in the control.

vi) The percentage monocyte values are reduced in the AS, LHC, HHC but are very close to normal levels in LEH and HEH.

vii) Monocytes had the highest percentage of all the cells in the LEH and HEH groups.

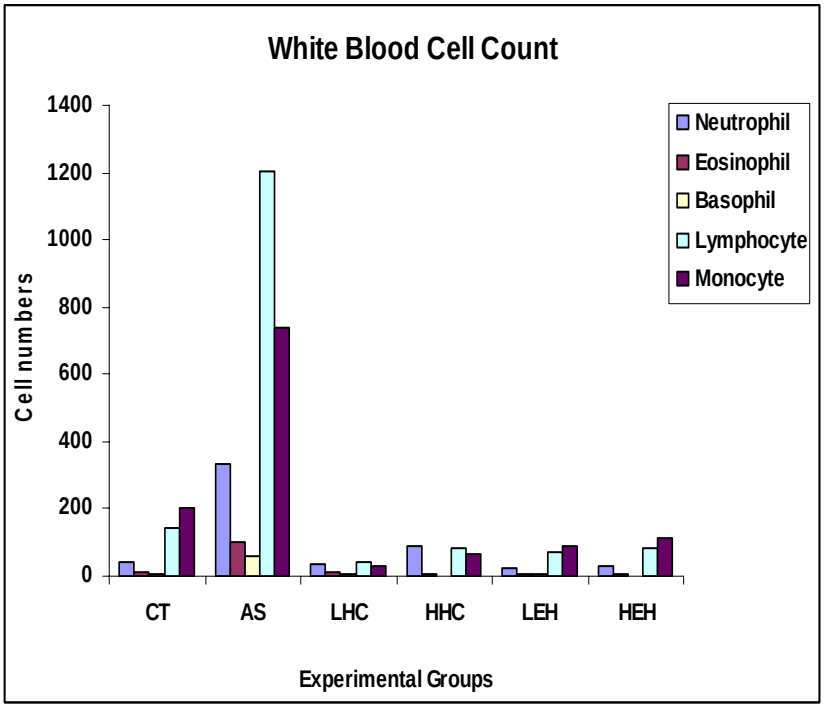

Figure 5.1a: Histogram showing the mean values obtained from white blood cell counts per group

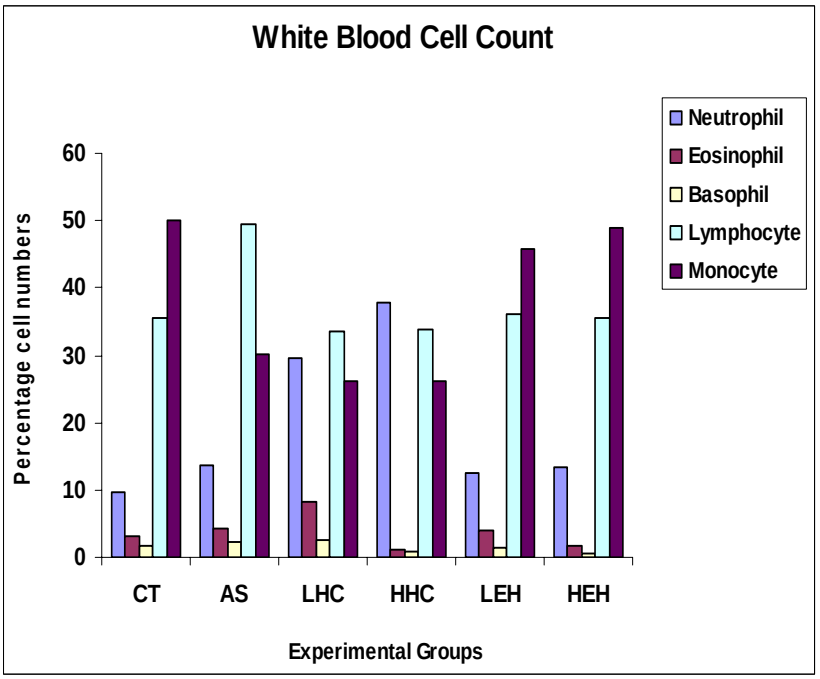

Figure 5.1b: Histogram showing the percentage white blood cell counts per group

5.4. Discussion

In this study, most values for cell counts in all the treated groups were relatively lower than the values for the control group. Since higher total cell counts could indicate the likely presence of inflammation, in this case experimental asthmatic inflammation, it could be concluded that mice in the “asthma” group (with the highest total leukocyte count) were actually asthmatic via inflammation.

Values for mean cell count in all other groups (except the control) were generally lower than in the asthma group but this is not necessarily an indication that all the treatment agents had a potential to relieve asthmatic effects. However the percentage values *(table 5.2)* show that only treatment with HHC could considerably reduce the numbers of most of the cells (except neutrophils). The LHC and HEH (in order) treatments also appeared to have limited positive effects at reducing asthma-induced leukocyte surge.

Basophils are said to be absent in healthy airways but present in asthmatic airways under a variety of circumstances (Hamid et al., 2003). In this study, basophil numbers were seen to be consistently the lowest of all cell types evaluated in all the treatment groups. This finding is therefore in line with findings from previous studies.

The respective effects of HC and *E. hirta* aqueous extract on the different subpopulations of leukocytes are discussed in the following sections.

5.4.1. Effects of *E. hirta* aqueous extract

The potential use of natural products to inhibit eosinophilic inflammation in both experimental models (Lopes-Martins et al., 2002; Rogerio et al., 2003) and human

diseases (Gupta et al., 1998; Takano et al., 2004) has been demonstrated. Many natural products have been considered more useful because they are purported to exert their positive effects with little or no side effects like some known pharmaceutical products.

A study by (Lee et al., 2006) revealed a significantly increased percentage of eosinophils in BALF of OVA-exposed animals. However, treatment of the animals with DA-9201, an extract from rice, resulted in a dose-dependent reduction in eosinophil count. Another study by Secor et al., (2005) utilized a well-established OVA-induced model of allergic airway disease (AAD) to demonstrate the effects of extract of *bromelain* in reducing CD4+ and CD8+ T cell as well as eosinophil counts in BALF of mice.

A different study showed that the initial eosinophilia observed in OVA-exposed mice was reduced following treatment with *Vimang*, an aqueous extract of the stem bark of *Mangifera indica* (Sá-Nunes et al., 2006). In this case, the airway inflammation was induced by *Toxocara canis* and resulted in an IL-5-dependent systemic eosinophilia that mimicked the features observed in asthma (Faccioli et al., 1996; Sá-Nunes et al., 2006).

In this present study, both the high and low doses of *E. hirta* extract were effective in lowering the cell counts of specifically lymphocytes, eosinophils and basophils known to be active in inflammation, in line with previous studies which showed that the predominant inflammatory cells in asthmatic inflammation include eosinophil, T-lymphocytes, mast cells and basophils (Denburg 1991; Kepley et al., 2001).

Increase in the number of neutrophils is usually transient in murine asthma (Tomkinson et al., 2001; Taube et al., 2003). Although there was an increase in neutrophils numbers following asthma, (AS group), treatment with LEH and HEH appeared to have lowered the numbers appreciably.

5.4.2. Effects of hydrocortisone

Corticosteroid therapy, which is the mainstay of treatment of patients with severe asthma, is capable of influencing neutrophilic trafficking in the airways via decreased programmed cell death (apoptosis) of neutrophils (Meagher et al., 1996). Lowered neutrophilic apoptosis usually results in increased neutrophil trafficking into the peripheral blood and possibly into the airways.

In this study, the high dose of HC appeared to effectively lower blood cell counts in all other cellular subpopulation except the neutrophils. Neutrophil counts were the highest (37.92%) in the HHC group (*table 5.1* and *5.2*) compared to all the other groups except the AS group (*table 5.1*). This high neutrophil count is in line with previous studies in which the increased neutrophil count observed in the blood of dexamethasone-treated *T. canis*-infected mice was attributed to inhibition of neutrophil apoptosis by dexamethasone (Meagher et al., 1996; Sá-Nunes et al., 2006). In another study, neutrophil counts were found to have increased significantly after treatment with the systemic glucocorticosteroid 6-methyl-prednisolone while eosinophil count significantly reduced (Paggiaro et al., 1995).

5.4.3. Effects of treatment on monocyte count

Monocytes are not a common feature in the progression of asthma but in this study, the high monocyte count called for a detailed analysis. Monocytes develop in the bone marrow and migrate to peripheral blood. After a few hours in the

bloodstream, they migrate to tissues such as spleen, liver, lung, and even bone marrow tissue, where they mature into macrophages (Van Furth et al, 1973).

Monocytes are the main 'effector' cells of the immune system and play a crucial role in defence mechanisms. These cells help other white blood cells to remove dead or damaged tissues, destroy tumour cells and regulate immunity against foreign substances. Therefore, a relatively high monocyte count in peripheral blood may not necessarily be an indication of pathology but usually occurs only in response to chronic infections. However, a very low monocyte count could increase the risk especially of bacterial infections.

A previous study by Rinehart et al., (1974) showed that a 16µg/ml concentration of hydrocortisone succinate (cortisone) could significantly impair random migration of monocytes and so treatment with glucocorticoids is capable of causing a marked reduction in monocyte count in peripheral blood. In the present study, monocyte counts were highest in the "asthma" group followed by the control group but values were remarkably lower in both HC groups.

In this study, a high monocyte count was observed in the asthma group compared to other groups but this value represented only 30.23% of the total cell count in the AS group and was much lower than the 50% value in the CT group. It is not too clear why the control group had the highest percentage value of monocytes. In the case of the 30.23% monocytes in the AS group, an automatic immune response to the prolonged presence of foreign agents (e.g. OVA used in the airway challenge procedure) in the body system of challenged mice can implicated. The remarkably lower counts (with their relatively low percentage values) observed in both the HHC and LHC groups could be due to the effects of cortisone treatment as

previously reported (Rinehart et al., 1974) and this could possibly be due to reduced cellular participation in host defence, which is then taken over by the administered cortisone.

The effects of medicinal plants on monocyte counts have also been reported. Extracts of the Mediterranean desert shrub *Teucrium polium* L. (Labiatae) for an example, were found to increase monocyte count in a dose-dependent manner while lowering lymphocyte counts (Khleifat et al., 2002). Garlic extracts administered to albino rats also caused significant increase in monocyte counts (Iranloye, 2002). Results from this study showed that monocyte counts in the HEH and LEH groups were lower than counts in the control and 'asthma' groups but their respective percentage values were only slightly lower than in the control group but higher than values of the asthma group .

This disparity in the monocyte numbers is not clearly understood but lower cell counts could be indicative of the involvement of the *E. hirta* extract in reduction of cellular involvement in immune processes.

5.5. Conclusion

Results from the white blood cell counts indicate that asthma was indeed induced in the BALB/c mice. *E. hirta* extract was effective in lowering the number of active inflammatory cells remarkably lymphocytes, eosinophils and basophils. Both the high and low doses of *E. hirta* also caused as much percentage increase in neutrophil numbers as in the asthmatic mice while mean monocyte counts were also lowered by treatment with the *E. hirta* extract.

In summary, *E. hirta* extract caused reduction of raised leukocyte numbers observed in asthmatic mice but in this study such effects were more pronounced following treatment with HC.

CHAPTER SIX

Ultrastructural Studies of the Blood Coagulating System

6.1. Introduction

In vivo animal models have been used successfully during the past few years to study diseases like asthma (Epstein, 2004a, b). The murine model in particular, is used successfully because mice allow for a variety of *in vivo* immunological applications (Bice et al., 2000). According to Epstein (2006), the allergic asthma as observed in an experimental mouse model is a reliable, clinically relevant facsimile of the human disease. Furthermore, antigen-induced mouse allergic asthma is a useful model for testing novel therapeutics (Epstein, 2006) and has been used for testing many novel agents aimed at reducing lung inflammation, mucus hypersecretion, airway hyperresponsiveness and IgE profiles.

It appears however, that only few non-immunologic studies with the BALB/c mouse model are available in literature. Many studies on the involvement of a wide range of cells in the typical immunological processes of asthma have been done but in the present study, the possible involvement of blood platelets (thrombocytes) in asthma was explored. The ultrastructure of the components of the coagulation process (haemostasis) especially the cellular components involved (e.g. platelets, fibrin networks) was examined for any significant treatment effects. Comparing the effects of HC and phytomedicines on platelet and fibrin formation and morphology using the murine model might give researchers insight into how these products affect the coagulation system.

Platelets have traditionally been associated with disorders of the cardiovascular system, where they are known to be involved in the maintenance of haemostasis as well as in the initiation of the repair process following tissue injury (Herd and Page, 1994). Many cardiovascular diseases could be attributed to excessive

platelet aggregation, which in turn has a critical role in thrombus formation (Lee et al., 1998).

A number of stimuli activate platelets resulting in the expression and/or activation of surface receptors, secretion of vaso-active substances, their adhesion, aggregation and thrombus formation (Lazerus et al., 2003). In conditions like allergic asthma, platelets participate by acting as inflammatory cells, releasing mediators, spasmogens and/or by interacting with other inflammatory cell types. Some of these mediators are enzymes active in the coagulation cascade. Platelet activation may be due to damage of the vessel wall or activation of the endothelium by chemicals, cytokines and by the inflammatory processes (Camera et al., 1999; Butenas and Mann, 2002) typically involved in conditions like allergic asthma.

Interest in the use of phytomedicines for the treatment of diseases like asthma has greatly increased over the past years. One plant extract used for asthma is *E. hirta* (Euphorbiacea), 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 cytotoxicity potential of *E. hirta* has been studied, only few pharmacological evaluations have been carried out to ascertain the rationale behind most of the folkloric claims of its efficacy (Johnson 1999) and not much is known about the effects of extracts of the plant on cellular function and morphology.

The effects of some medicinal plants or natural products on platelets and the blood coagulation system have been documented in literature. Plant products like garlic

(Rahman and Billington, 2000) and tomato (Dutta-Roy et al., 2001) have been shown to be potentially beneficial in protecting against cardiovascular diseases by inhibiting platelet aggregation. In addition, Mekhfi et al., (2004) examined the *in vitro* effects of aqueous extracts of five medicinal plants (*Arbutus unedo*, *Urtica dio¨ıca*, *Petroselinum crispum*, *Cistus ladaniferus* and *Equisetum arvense*) used for cardiovascular diseases. In their study on rat platelet aggregation induced separately by thrombin and ADP, they found that extracts derived from all five tested plants elicited a dose-dependent inhibitory activity on platelet aggregation.

Effects of different traditional herbal medicines have been reported. The anti-thrombic Korean herbal medicine, Dae-Jo-Whan (DJW), consists of 11 herbs of *Rehmanniae Radix*, *Hominis Placenta*, *Testudinis Carapax*, *Eucommiae Cortex*, *Asparagi Radix*, *Phellodendri Cortex*, *Achyranthis Radix*, *Liriopis Tuber*, *Angelicae Sinensis Radix*, *Ginseng Radix*, and *Schizandrae Fructus*. DJW was reported to have inhibitory effects on collagen-and ADP-induced blood platelet aggregation, thrombin-induced conversion of fibrinogen and fibrinolysis in *in vitro* experiments (Min, 1997). The effects observed with total DJW extract were stronger than the additive effects since the sum of the single contributions was lower than the effects of the total.

In other studies, extracts of *Andrographis paniculata* (Acanthaceae) used as a traditional medicine in India, China, Thailand, and Scandinavia, remarkably decreased and inhibited platelet aggregation induced by thrombin in a concentration-and time-dependent manner (Thisoda et al., 2006). Tetramethylpyrazine, one of the active ingredients of the Chinese herbal medicine *Chuanxiong* was reported to demonstrate unique antiplatelet characteristics -

selective inhibition of platelet activation, aggregation and thrombus formation under high shear rate conditions (Li et al., 2001).

6.1.1. Aim of study

The aim of this study was to describe the possible contribution of the blood coagulation system to the general pathology of asthma as well as investigate the effects of treatment with HC and *E. hirta* on this aspect of the asthma pathology. The effects of the plant extracts were compared with the effects of HC, a known anti-inflammatory pharmaceutical used for the treatment of asthma. Presently, not much is reported on the effects of *E. hirta* extracts on the morphology of the coagulation system. Since the plant is known to be used for asthma, the BALB/c mouse asthma model thus provides a medium for investigating the effects of phytomedicines (in this case *E. hirta*) on the blood coagulation process in the presence of asthma, and particularly the effects on the ultrastructure of platelets and fibrin networks.

6.2. Materials and methods

6.2.1. Inducing and treating asthma in the BALB/c mice

Ethical clearance was obtained for the animal studies. Mice were divided into the following groups: control mice, asthmatic mice, mice exposed to low dose (100mg/kg) HC, mice exposed to higher dose (125mg/kg) HC, mice exposed to physiologically comparable levels of *E. hirta* (0.01 ml of 62.5mg/kg plant material).

The BALB/c mice were sensitized and challenged before treatment with either *E.hirta* or cortisone. Sensitization (on day 0 and day 5) of mice was via intraperitoneal injection of a mixture of 25mg OVA (grade V; Sigma-Aldrich) and 2mg of Aluminium hydroxide [Al $(OH)_3$] dissolved in 0.5ml of 0.9% saline solution.

All mice except those in the control group were sensitized. Nebulization (or challenge) was done twice daily on days 13, 14 and 15, each for 1 hour using 1% OVA in PBS (i.e. 1mg OVA in 100 ml PBS).

The plant material was prepared as described (in section 4.2.1.3). The stock solution of 50mg/ml was prepared into the dose solutions of 62,5mg/kg and 25mg/kg respectively. Administration of low and high doses of the treatment agents (*E. hirta* and HC**)** was done on days 15 -18, and again on days 22, 25, 29 and 32. Nebulization was repeated on days 34-36 before the treatment resumed daily on days 39 - 45. Animals were terminated on day 45.

6.2.2. Preparation of fibrin clots

On day 45 during termination, 100 - 500 µl of blood per group was drawn from the hearts of the mice in each of the groups and 11µl citrate for every 100 µl of blood, was added. The blood samples were then centrifuged at 1000 rpm for 2 minutes to obtain platelet rich plasma (PRP). Human thrombin (provided by The South African National Blood Services) was used to prepare fibrin clots (Pretorius et al., 2006). The thrombin is 20 U/ml and is made up in biological buffer containing 0.2% human serum albumin. When thrombin is added to PRP, fibrinogen is converted to fibrin and intracellular platelet components e.g. transforming growth factor (TGF), platelet derived growth factor (PDGF) and fibroblastic growth factor (FGF) are released into the coagulum.

Ten micro litres (10µl) of mouse PRP was mixed with 10 µl of human thrombin. The PRP and thrombin mix was transferred immediately with a pipette tip to a 0.2 µm millipore membrane to form the coagulum (fibrin clot) on the membrane. This millipore membrane was then placed in a Petri dish on filter paper dampened with

PBS to create a humid environment at 37 ºC for 10 minutes. The millipore membranes with the coagula were later placed in PBS and magnetically stirred for 120 minutes to remove any blood proteins trapped within the fibrin network (Pretorius et al., 2006).

6.2.3. Preparation of washed fibrin clot for scanning electron microscopy (SEM)

Washed fibrin clots were fixed in 2.5% glutaraldehyde in the buffer DPBS (Dulbecco's Phosphate buffered saline) at a pH of 7.4 for 1 hour. Each fibrin clot was rinsed thrice in phosphate buffer for 5 minutes before being fixed for 1 hour with 1% Osmium tetra oxide (OsO_4). The samples were rinsed thrice with distilled water for 5 minutes and later dehydrated serially in 30%, 50%, 70%, 90% ethanol as well as three times with 100% ethanol.

The Scanning Electron Microscopy (SEM) procedures were completed by critical point drying of the material, mounting and examining the tissue with a JEOL 6000F FEGSEM.

6.3. Results

Platelet and fibrin structure morphology of each of the mice in each group were investigated. Morphology was found to be constant for a particular group and homogenous for each sample and for each test field. SEM stubs were analyzed systematically to cover the whole fibrin network area for each animal. *Figures 6.1(a)* and *(b)* show fibrin networks and platelet aggregates from control mice. Both thick, major fibres (label A) and a thin network of minor fibres (label B) are present in the controls.

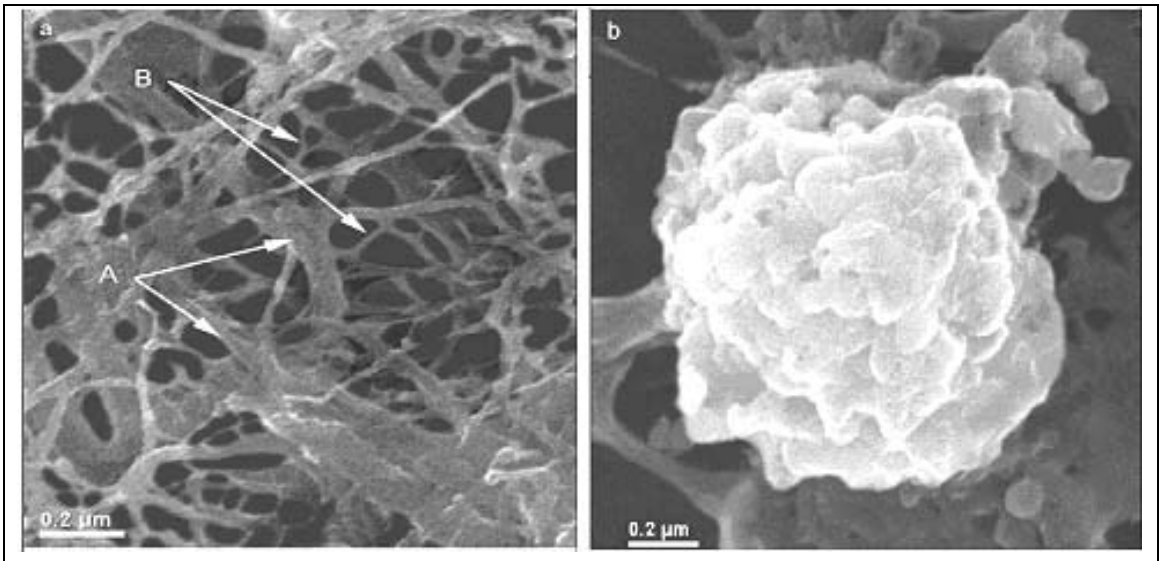

Figure 6.1. (a) Control fibrin network with thick, major fibres as well as thin, minor fibres. Label A ¼ thick, major fibres; Label B: ¼ thin, minor fibres. (b) Control platelet aggregate forming dense, round aggregate with pseudopodia.

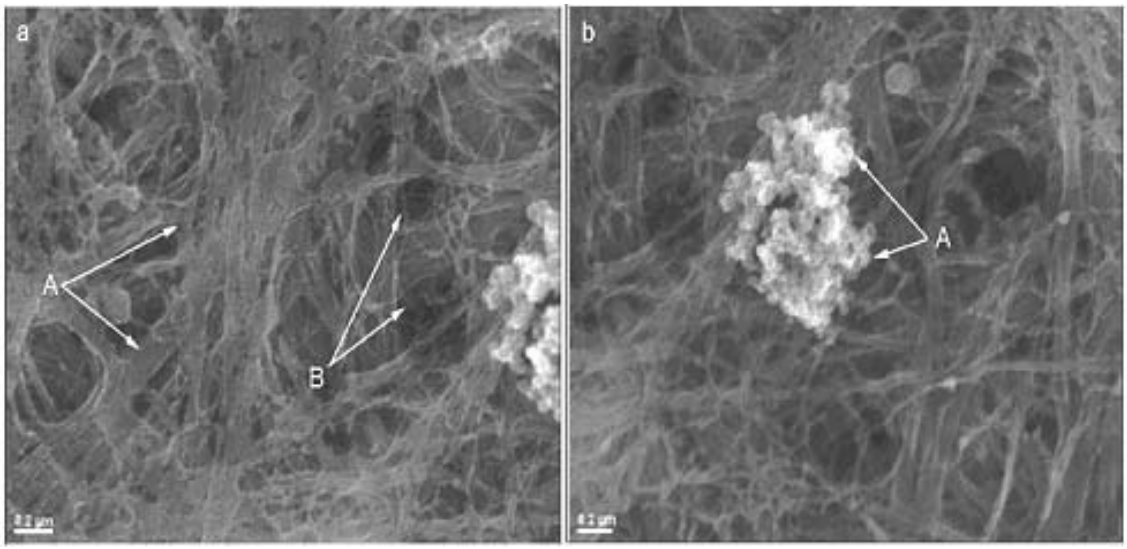

Figure 6.2. (a) Fibrin network from asthmatic BALB/c mice showing thick, major fibres as well as thin, minor fibres. Label A ¼ thick, matted, major fibres; Label B ¼ thin, minor fibre forming a dense network. (b) Platelet aggregate from asthmatic BALB/c mice forming course, granular aggregate

Platelet aggregates form round, dense groupings with pseudopodia extending from the aggregates. *Figure*s *6.2 (a)* and *(b)* are fibrin and platelet aggregates from asthmatic mice that were not treated with *E. hirta* or HC. Fibrin fibres in the asthmatic mice also consist of thick, major fibres and thin minor fibre networks;

however, major fibres seem to have a matted appearance [label A of *Figure 6.2 (a)*] and seem to be fused longitudinally.

Minor fibre networks are more prominent [label B of *Figure 6.2 (a)*] and cover the major fibre network. *Figure 6.2 (b)* shows an aggregation of platelets; however, they differ from the control aggregates in that they look more granular and do not clump together closely. Furthermore, although pseudopodia are visible [label A of *Figure 6.2 (b)*], they are much smaller and less bulbous than pseudopodia of the control animals.

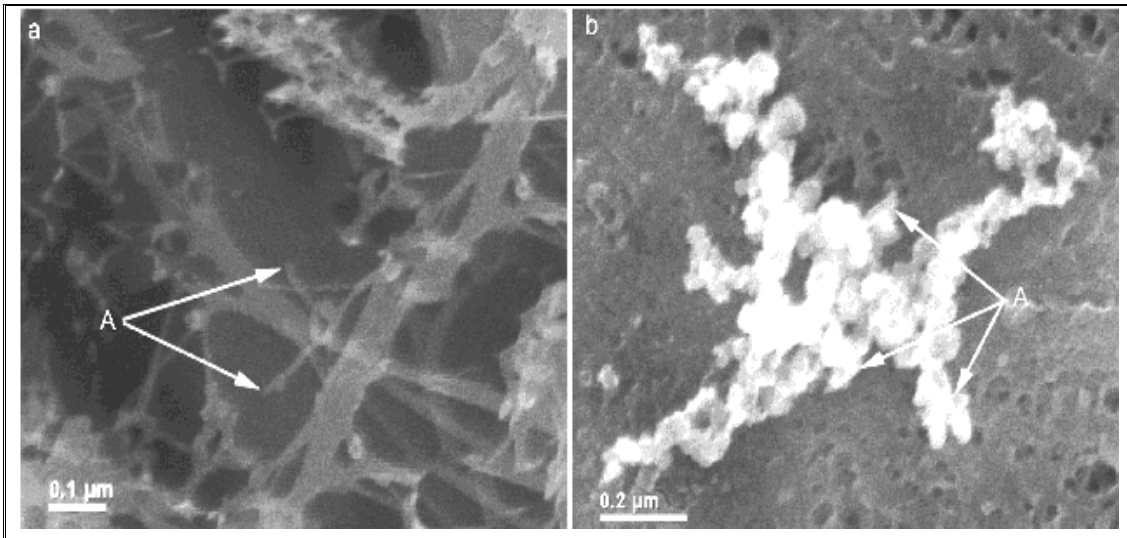

Figure 6.3. (a) Fibrin network from LHC (100 mg/kg) asthmatic BALB/c mice, forming flimsy fibrin network. Label A ¼ flimsy fibres with breakages. (b) Platelet aggregate from LHC (100 mg/kg) asthmatic BALB/c mice. Label A ¼ granular platelet aggregate.

Figures 6.3 (a) and *(b)* show fibrin and platelet aggregates of the mice treated with LHC. Fibrin fibres appeared flimsy and breakages are present and can be seen [*Figure 6.3 (a)* label B]. When viewing the networks using the SEM, the fibres tend to break just as the electron bundle moving over a region. This suggests that these fibres are much more prone to breakages than those of the controls or even those found in the asthmatic mice. Platelet aggregates were also granular (similar to the

untreated asthmatic mice) and the aggregates did not form the tight round, dense aggregates that were seen in the controls. Rather, a loosely associated aggregate with small pseudopodia is seen [*Figure 6.3 (b)* label B].

The HHC samples [*Figure 6.4(a)* and *(b)*] also showed a flimsy fibre network easily prone to breakages and both major and minor fibres are seen. These fibre networks have a similar appearance as the untreated asthmatic mice, where the fine minor fibres form a much thicker covering than is found in the controls. Platelet aggregates also did not have a tight round appearance and appeared granular and not attached closely to each other as seen in the controls [*Figure 6.4(b)*]. *Figure 6.5(a)* and *(b)* shows a fibrin network and platelet aggregate from mice that were treated with *E. hirta*.

Both major and minor fibres are present (*Figure 6.5a* labels A and B); however, fibres were much more stable and did not break as easily as the HC groups and the minor fibres are much less prominent than those of the asthmatic mice group are. *Figure 6.5b* shows a platelet aggregate. Aggregates from *E. hirta* look similar to that of the controls. The aggregate is much more condensed and round while the pseudopodia are more bulbous and more similar to that of the controls.

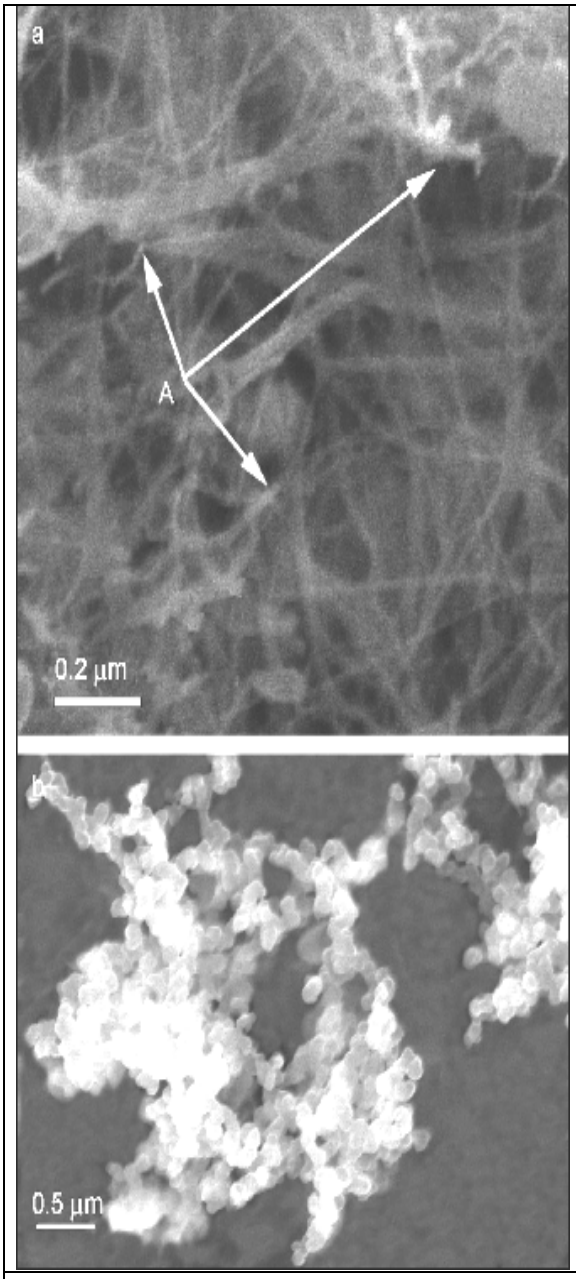

Figure 6.4. (a) Fibrin network from HHC treated (125 mg/kg) asthmatic BALB/c mice network. Label A ¼ Flimsy fibre network. (b) Platelet aggregate from high dose HC-treated (125 mg/kg) asthmatic BALB/c mice forming granular aggregate.

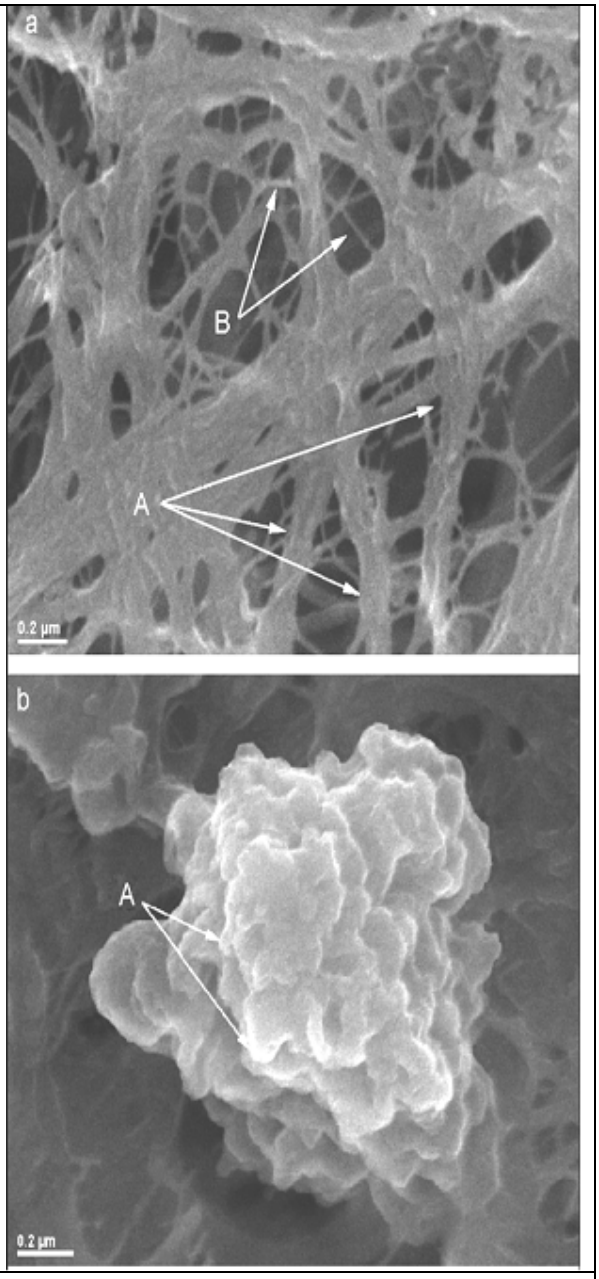

Figure 6.5. (a) Fibrin network from asthmatic BALB/c mice treated with *E. hirta* (0.01 ml of 62.5mg/kg plant material) showing fibrin network with thick, major fibres as well as thin, minor fibres. Label A ¼ thick, major fibres; Label B ¼ thin, minor fibres. (b) Round aggregate with pseudopodia. Label A ¼ pseudopodia.

6.4. Discussion

Although platelets have traditionally been associated with disorders of the cardiovascular system, they also play an important physiological role in allergic processes and immunological mechanisms. Platelets play an important and fundamental part in asthma, as inflammatory processes, typically involved in asthma, activate them. Furthermore, platelet-activating factor (PAF) as well as platelet factor 4 (PF_4) and also thrombin itself, fibrinogen, fibrin, are all known to be involved in asthma. Importantly, platelets contribute to the adhesion of eosinophils to inflamed endothelium of patients with allergic asthma. Platelet depletion is also known to reduce PAF, suggesting that PAF plays a central role in the processes by which platelets facilitate the induction of eosinophil accumulation, which is central in asthma.

In addition, PAF is involved in increased vascular permeability and platelets have been shown to be in contact with the vasculature of the bronchi of patients with asthma and in this way, platelets evoke contraction of smooth muscles of the respiratory passages that directly leads to an asthma attack. Fibrin and platelets are therefore two important factors in the disease. Fibrinogen itself is also widely recognised as a marker for systemic inflammation as it is considered an acute phase protein. Pithcford et al., (2004) mentioned that there is evidence of platelet recruitment to the lungs of asthmatics after allergen exposure, suggesting that platelets participate in various aspects of asthma. Morley et al., (1984) has earlier suggested that platelet activation may contribute to airway remodelling in asthma.

Furthermore, the association between thrombin, fibrin and asthma seems to be the following:

- Thrombin is known to increase airway smooth muscle contraction *ex vivo* (Panettieri et al., 1995).
- Increased thrombin generation occurs in the airway of patients with asthma (Gabazza et al., 1999). Thrombin may play a role in the pathogenesis of airway remodelling.
- Human platelets can produce PAF upon thrombin stimulation in the lungs (Touqui et al., 1985).
- Fibrinogen can be produced by lung epithelia because of inflammatory stimulus (Lawrence and Simpson-Haidaris, 2004).
- Fibrin degradation products have also been found to increase pulmonary vascular smooth muscle contraction (Kern et al., 1986).
- Airway fibrin deposition occurs in inflammatory disorders of the lung, and it is known that fibrin inhibits surfactant function (Wagers et al., 2003).
- Fibrin is typically formed at sites of vascular damage (Touqui et al., 1985).
- Extra-vascular thrombin, fibrinogen, and fibrin have been found in the sputum of patients with asthma (Banach-Wawrzenczyk et al., 2000; Pizzichini et al., 1996; Wagers et al., 2004).
- Wagner and co-workers in 2004 hypothesized that airway hyperresponsiveness seen in asthma is largely the result of decreased stability of airways and subsequent airway closure secondary to the formation of fibrin on the distal airway surface. According to the authors the coagulation system and fibrinolytic system proteins is associated with the pathogenesis of airway hyperresponsiveness in asthma.

- There is decrease in plasminogen activator (PA) activity in asthma (Wagers et al., 2004).

- Activity of PAI_{act} (plasminogen activator inhibitor) is increased in homogenates of lung tissue of mice with allergic airway inflammation, thereby potentially promoting the accumulation of fibrin by suppressing fibrinolysis (Wagers et al., 2004).

In control BALB/c mice we find major and minor fibrin networks (*Figure 6.1a*); this is similar to previous findings in humans (Pretorius et al., 2006). In addition, the morphology of platelet aggregates appears as a collection of platelets that has a dense, round shape. This is also similar to human platelet aggregates (Pretorius et al., 2006). However, in the asthmatic mice the minor fibres seem to be more dense and covering the major fibres (as seen in *Figure 6.2a*). This might be the reason why in asthma, an accumulation of fibrin is seen in airways (Wagers et al., 2004); perhaps because the fibrin mass is more dense and that the fibrin networks are not disaggregated so quickly (fibrinolysis) and stay in the airways for longer periods, forming fibrin plugs. It is known that surfactant function is inhibited in asthma (Wagers et al., 2003), possibly because of the denser fibrin network. In addition, degradation products of fibrin have been found to increase smooth muscle contraction. This might be because the fine minor fibrin network inhibits the surfactant to function optimally and interferes with smooth muscle function.

It is known that glucocorticosteroids are the most useful class of drugs employed in the treatment of patients with allergic asthma (Lantero et al., 1997; Pretorius, 2005). There is also increasing evidence that fibrin(ogen) physiology is affected by glucocorticoids. After fibrin is produced via the thrombin (intrinsic and extrinsic) pathway, it undergoes fibrinolysis. This process is under the control of plasminogen activators (PAs), which are serine proteases that convert the

proenzyme plasminogen to active plasmin, a broad-spectrum proteolytic enzyme that readily degrades fibrin as well as extracellular matrix glycoproteins including laminin, vitronectin, fibronectin and proteoglycans (Bator et al., 1998).

Platelets also seem to be affected by glucocorticoid treatment (Tutluoglu et al., 2005). Platelets have the capacity to release mediators with potent inflammatory or anaphylactic properties; these mediators include PF_4 and beta-thromboglobulin (BTG) (Tutluoglu et al., 2005). BTGs are also chemokines that play an important role in mediating cell recruitment and activation necessary for inflammation and the repair of tissue damage. Plasma levels of PF_4 and BTG also show changes in chronic inflammatory diseases such as asthma; and Tutluoglu and co-workers (2005) found that plasma levels of PF_4 among patients with an asthma attack were significantly higher than those of controls and a further increase in plasma PF_4 levels was detected after steroid therapy. From these results it appears that both the low and high HC dosages produce flimsy fibrin networks that break easily and that are not as stable as was found in the control fibrin networks (*Figure 6.3a* and *6.4a*). In addition, the platelet aggregates do not have the typical round, compact shape; rather the platelet aggregates are widely spread, granular, and not tightly associated with each other (*Figure 6.3b* and *6.4b*); also, aggregates appear more like those found in the asthmatic mouse.

An interesting observation was that the extract of *E. hirta*, did not cause the fibrin networks to be as flimsy and fragile (*Figure 6.5a*). However, the fine minor fibres seem to be more prominent than in the controls and more similar to those found in the asthmatic mice. Platelet aggregates showed the same morphology as those of the control mice, without the widely spread granular appearance found in HC

treated mice (*Figure 6.3b* and *6.4b*). However, further studies need to be done to determine the effect of the plant on lymphocytic lung infiltrates; Ag-specific production of IL-4 and IL-5 from spleen and lung cells *in vitro*, elevated levels of IgG1 as well as expression of Th2 cytokine RNA in lungs.

6.5. Conclusion

Asthma is a very complex condition, with many physiological factors playing a role in its presentation. However, it appears that platelets and products of the coagulation cascade form an intricate and important part of asthma and not only affect the presentation of the condition itself but also interact with the typical choice of treatment, namely glucocorticoid therapy. The question that arises is how the platelet activation process and the coagulation cascade contribute to asthma in the presence of other pharmaceutical products or phytomedicine.

It is known from previous research that the *E. hirta* is widely used for the treatment of asthma probably because it contains chemical ingredients that have anti-inflammatory properties. However, very little information is available of the effect of the plant on cellular systems of the body and the exact mechanism through which it plays a role in the treatment of asthma. The current research seems to indicate that treatment with *E. hirta* does not make fibrin fibres of mice as fragile as seen in treatment with HC; also, treatment with *E. hirta* did not change the integrity and morphology of the platelets as observed for treatment with HC.

This work focused on a small aspect of the coagulation process and now leaves more questions regarding the effect of HC on the coagulation process as well as the effects and the exact mechanisms of *E. hirta* action on asthma in general and on other cellular systems of the body. Further ultrastructural studies are

suggested especially involving the use of transmission electron microscopy to examine particularly platelet morphology.

We conclude that *E. hirta* does not affect the fragility of mouse fibrin and that it prevents the minor fibres from forming a dense netlike layer over the major fibres, as is seen in untreated asthmatic mice. This ultrastructural morphology might give a better insight into asthma and possible new treatment regimes for it.