CHAPTER EIGHT

Concluding Discussion

8.1. Concluding discussion

The search for permanent treatment medications and modalities for many disease conditions with no known cure has been the focus of most current scientific investigations in many parts of the world and these usually employ different experimental probes, methodologies and models. For asthma with a complex aetiology and pathogenesis, developing a cure will involve not only understanding how genetic predisposition, environmental and other factors interact but also detailed investigation of how the different components of the disorder especially the chemical and cellular inflammatory processes as well as airway remodelling could be prevented or controlled. It is clear over the years that the best way to study the progression of asthma is via the use of *in vivo* animal models, which have also helped with drug development efforts.

A wide spectrum of herbal and homeopathic products is now being widely investigated in many parts of the world for their biomedical efficacies using *in vivo* and *in vitro* systems and many pharmacological and biochemical studies investigate the mechanisms of action of these products.

The focus of this study was the investigation of *in vitro* and *in vivo* anti-inflammatory properties and cytotoxicity effects of *Euphorbia hirta* extracts. Hydrocortisone (HC) was used as a pharmaceutical control for the plant extract because of its known anti-asthma effects.

The rationales for this study included following:

- Knowledge of the high chances (about 75%) of heritability (genetic predisposition) of asthma (Duffy et al., 1990).

- The knowledge that the prevalence of asthma is on the increase even in many poor communities of the world due to increasing environmental pollution especially from industrialization (Masoli et al., 2004).

- The knowledge that approximately 80% of the people in the world's developing countries rely on traditional medicines (including plant remedies) for their primary health care needs (Vieira and Skorupa, 1993).

- The knowledge that about 85% of traditional medicine involves the use of plant extracts (Vieira and Skorupa, 1993) and in most cases without expert prescriptions.

- The possibility that cases of asthma in these poor rural communities could be treated with herbal medicines with no prescriptions as has been the practice.

- The need for *in vitro* and *in vivo* evaluation of a known anti-asthma herbal medicine (*E. hirta*) for scientific validation of its claimed potency in folk medicine.

The *in vitro* effects were evaluated by way of cytotoxicity testing of the aqueous, acetone, dichloromethane and hexane extracts of the plant on the MRC-5 cell line as previously reported (Zirihi et al., 2005). The Neutral Red (NR), MTT [1- (4, 5-Dimethylthiazol-2-yl) -3, 5- diphenylformazan] and Crystal Violet (CV) assays were used for the studies. Screening the plant extracts for cytotoxicity was necessary to establish the safe dose range for use in the animal studies. The aqueous, acetone, dichloromethane and hexane extracts of the whole plant material were prepared and tested on the MRC-5 cell line.

Different solvents used for extraction of plant materials isolate different compounds. The aqueous extracts will normally contain most of the polar compounds (e.g. polyphenols, triterpenes and flavonoids) while the solvent extracts will isolate compounds based on their polarities (e.g. glycerides, carotenoids, sterol compounds, lipid soluble vitamins, β-Sitosterol). Separate experiments were carried out with the organic solvents alone to determine their individual effects and compare them with the effects of their corresponding plant extracts.

At all concentrations for water, acetone, hexane and dichloromethane, no significant differences were observed compared to the control (no solvent or no solvent plant extract). This is possibly an indication that extracts of *E. hirta* contain few if any, toxic compounds and thus their biological activity could be due to the presence of anti-oxidant compounds. Futher studies on the use of plasmids in hydrogen peroxide protection systems could provide further information in this regard.

Further studies are also recommended with different assay systems at other concentrations of the plant extracts or with the isolated active compounds. In addition, apoptosis and morphology of the MRC-5 could be studied by flow cytometry and with fluorescence microscopy. The MRC-5 cell line has a few limitations: it is susceptible to a wide range of human viruses and only 42-46 population doublings are possible before the onset of decline in proliferation and eventually senesce. Other cell lines that could be used to test for *E. hirta* toxicity include the BEAS-2B cell line was derived from normal human bronchial epithelium especially because the lung epithelium is not simply a passive barrier

but plays an active role in immune and inflammatory responses to toxic stress through the release of inflammatory cytokines. The WI-38 is another cell line that could also be used since it is also derived from lung tissue and has a fibroblast-like morphology.

The *in vivo* studies involved the use of the BALB/c asthma mouse model to investigate the effects of only the aqueous extract of *E. hirta* treatment on inflammatory and structural changes in the airways after asthma was induced. The processes of inducing asthma included sensitization followed by airway challenge. Although the entire procedure used in this study has been previously used, it was necessary to evaluate the possible effects of these initial experimental procedures on animal weights. This investigation was informed by the reported obscurity in the relationship between airway inflammation (including that seen in asthma) with excessive body weight, anomalous body mass index, or obesity (Camargo et al., 1999; Hakala et al., 2000; Aaron et al., 2004; Weiss and Shore 2004; Beuther et al., 2006).

Both immunization and nebulization caused weight gain effects in the mice but the effects were more pronounced following immunization and only minimal following nebulization. These effects were however modulated variously by treatment with the test agents (HC and *E. hirta* extracts). Prolonged treatment with HC remarkably reduced the cumulative weight gained following prior experimental procedures (immunization and nebulization), followed by a slow and sustained increase in the rate of weight gain. On the other hand, prolonged administration of *E. hirta* causes only a minimal reduction in weight gained due to induced asthmatic

conditions. In addition, the lower doses were found to be more effective in lowering weights than the high doses.

Any weight loss associated with *E. hirta* treatment could be due to the effects of their constituent antioxidants in eliminating free radicals associated with weight gain since many weight dynamics in especially asthmatics are related to oxidative stress (Fenster et al., 2004; Johnson et al., 2007). Further studies with specific defined weight-related experimental goals would be required to clarify some of the effects reported above.

In chapter 5, the role of inflammatory cells in the BALB/c asthma model was investigated. Most of these cells generate free radicals which when in excessive amounts can cause a wide range of diseases. Asthma has long been associated with an overall increase in reactive groups and oxidative stress (Barnes, 1990; Kharitinov et al., 1994; Nadeem et al., 2003). It is possible that one way by which *E. hirta* functions for the treatment of asthma is through synergistic anti-inflammatory and antioxidant activities of especially the flavonoids, sterols and triterpenoids (Park and Lee, 2006).

Blood smears were prepared and a white blood cell count was undertaken. Results showed that In general, treatment with both the high and low doses of the *E. hirta* extracts effectively reduced the number of active inflammatory cells (neutrophils, eosinophils and basophils) and the high dose of HC appeared to effectively lower counts in all other cellular subpopulations except in the neutrophil smears.

Although a large number of blood smears was used in the determination of cell counts and results were pooled, the use of other techniques e.g. flow cytometry in addition to the methods used in this study could have been explored. In addition, bronchoalveolar lavage fluid (BALF) analysis could provide additional cell count data relating to the presence of inflammatory cells in the airway passages. Finally, assay systems like the Cellular Allergen Stimulation Test (CAST), Eosinophil Cationic Protein (ECP) Assay and the ECP fluoroenzyme immunoassay could provide information on whether or not high numbers of particular cells in the blood translates into the release of cytokines into the bloodstream following induced asthma.

In chapter 6, the effects of both HC and *E. hirta* on the blood coagulation system were reported. This aspect of the study was motivated by the lack of previous studies in literature on this aspect of asthma pathogenesis. 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. The ultrastructural outlook of the coagulation process (haemostasis) especially the cellular components involved (e.g. platelets, fibrin networks) was examined for any significant treatment effects and these were compared to the effects of HC and phytomedicines on platelet and fibrin formation and morphology using the murine model to give researchers insight into how these products affect the coagulation system.

Findings from this study showed that treatment with *E. hirta* did not cause fragility of blood fibrin fibres in the mice and did not change the integrity and morphology

of the platelets as seen in treatment with hydrocortisone. In addition, *E. hirta* prevented the minor fibres from forming a dense netlike layer over the major fibres, as is seen in untreated asthmatic mice. Knowledge of the ultrastructural morphology might give new insights into asthma pathology and possible new treatment regimes for it. Only a small aspect of the coagulation process was explored and this did not address the question of the mechanisms of action of both *E. hirta* and HC on the coagulation pathway. The morphological observations from this study however provide a clue on other possible effects. Further ultrastructural studies are suggested especially involving the use of transmission electron microscopy techniques to process and examine particularly platelet morphology. Measurement of other coagulation parameters in the animals e.g. coagulation times may also be necessary.

A major limitation of this study was however the size of the BALB/c mice even though the BALB/c asthma model has been widely acclaimed to be a reliable clinical facsimile of the human asthma (Epstein, 2006). Mice do not have enough blood to allow for long-term studies of individual mice because they have to be terminated and blood samples from many mice pooled to obtain about 900µ of blood required for a single coagulation study. The development of a rabbit asthma model appears to be the way forward especially because the coagulation factors, platelets and fibrin networks of rabbits have been reported to be similar to those of humans than are those of the mice (Humphries et al., 2007; Pretorius et al., 2007a).

In chapter 7, the effects of *E. hirta* and HC on possible inflammatory cell migration into the respiratory airway walls and lung parenchyma were reported. The effects

of these treatment agents on the general airway wall ultrastructure were also reported. Parameters studied included lymphocyte and plasma cell migration into the lung parenchyma, fibroblast and mucous cell proliferation, alveolar pneumocyte numbers, lamellar body formation, filopodia formation as well as migration of macrophages into the airway parenchyma were examined.

The light microscopic studies showed very thick and discontinuous alveolar walls in the asthma group, with smooth muscle masses seen in the walls of distal airways. These findings appeared to confirm the presence of asthma induced in the asthmatic mice. Treatment with HC did not however appear to reverse the asthmatic effects whereas the low *E. hirta* appeared to be effective in ameliorating the histological alterations observed in the respiratory structures studied. The histological findings were complemented with ultrastructural studies and results showed a variety of effects of treatment by both the high and low doses of *E. hirta* and hydrocortisone on different inflammatory cells, collagen fibre deposition, filopodia formation by fibroblasts, lamellar body formation, mitochondria population and smooth muscle hypertrophy. One strange finding in this study was the presence of abnormal muscle striations only in the 'asthmatic' specimens.

The summary of the remarkable ultrastructural findings in this study included the following (also see *table 7.1*):

- Both low doses of HC and *E. hirta* appeared to lower fibroblast proliferation whereas the high doses increased fibroblast proliferation
- HC doses above the low dose used in this study possibly inhibit cytokine-induced lymphocyte migration, resulting in less lymphocytes being attracted to the airways

- Treatment with either *E. hirta* or HC did not prevent the rapid elimination of neutrophils from the airways nor promote their retention in the airway and lung parenchyma.
- Monocytes appear to have differentiated extensively into the macrophage pool that usually addresses inflammatory conditions in the airways and even down to the alveoli (Landsman and Jung, 2007).
- Treatment with both HC and *E. hirta* extract appear to inhibit migration of the macrophages into the airway parenchyma.
- Treatment with both HC and *E. hirta* appeared to promote plasma cell migration hence the fewness or absence of these cells in treatment specimens.
- The numerical composition of alveolar pneumocytes was not distorted by the different experimental interventions.
- Treatment with HC and *E.hirta* extract did not generally cause significant reduction in collagen fibre deposition.
- Only treatment with the low HC and *E.hirta* extract doses appeared to increase lamellar body formation in type II cells.
- Only the high doses of HC and *E.hirta* extract appeared to be effective in reducing mucous cell proliferation and possibly mucus secretion.
- Striations were seen only present in the AS specimens group and were much similar to those observed in a typical striated muscle.
- Treatment with both HC and *E.hirta* extract appeared to be effective in preventing airway smooth muscle cell proliferation.
- Filopodia were absent in the fibroblasts of all other specimens but those of the low HC dose.

Most of the findings above were made from observations with the TEM and the use of additional methods like special staining techniques could have revealed more information. For an example, immunohistochemical staining techniques could show the specific types of collagen and smooth muscle actin proteins present in the specimens. In addition, quantitative analyses involving histomorphometric techniques to measure micro-distances or ultra-distances in specimens could provide numerical data that support the morphological observations described above. Such quantitative analyses could also be applied to investigate the effects of *E. hirta* extracts on the ultrastructure and function of the liver and kidney.

Although most of the goals set for this research were accomplished, there remain areas, which still need to be investigated. For example, there is need to explore the effects of the isolated active ingredients present in *E. hirta* on the different parameters examined in this study. It is also necessary to study the effects of the plant extracts on free radical scavenging systems (e.g. the Horse-radish system) since the pathogenesis of asthma appears to involve the activities of free radicals (Chanez et al., 1990; Andreadis et al., 2003). In addition, the effects of varying the durations of especially the sensitization and nebulization stages probably need to be investigated to possibly establish the exact time span needed to produce complete asthmatic effects in the chosen animal model. These findings would help optimize these animal models.

Finally, since the *E. hirta* extracts produced many positive effects in the ultrastructure of many cells and tissues of treated asthmatic mice, it may be necessary to investigate the possible effects of these extracts on asthma-induced

damage to the bronchiolar epithelium. These cells are the first cells of contact and many studies have shown that ploughing of this epithelium is a common feature of asthma and potentially harmful to the airway.

The conclusion therefore is that the aqueous *E. hirta* extract is non-toxic and could be used for the treatment of asthma in the BALB/c mice. Further studies are however required in the different areas recommended above.