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The *in vitro* and *in vivo* anti-inflammatory properties and cytotoxicity of extracts of *Euphorbia hirta*

By

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ABSTRACT

Asthma is considered one of the most common respiratory complaints in the world today but a medical cure for this condition is currently not available. The use of herbal medicines to treat asthma has however been reported and *Euphorbia hirta* is one such herb. The alkaloids, flavonoids, glycosides, sterols, tannins and triterpenoids in *E. hirta* appear to exert the anti-asthma effects reported.

In the first part of this study, the aqueous, acetone, dichloromethane and hexane extracts of *E. hirta* were evaluated for their effects on the lysosomal membrane integrity, cell viability and cell number of MRC-5 cell-line using the NR/MTT/CV assay. Hydrocortisone was used as a pharmaceutical control. The differences between the effects of the different extracts were investigated and the effects of the extracts were compared with hydrocortisone. Results obtained showed that hydrocortisone was relatively toxic to the MRC-5 cells whereas all four extracts studied showed very limited cytotoxic effects, with the aqueous extracts generally exhibiting the least effects.

In the second part of this study, the effects of the aqueous *E. hirta* extract on the blood coagulation system and general airway wall microstructure and ultrastructure were investigated using the BALB/c mouse asthma model. Hydrocortisone was also used as a pharmaceutical control. Parameters studied included inflammatory cell population in peripheral blood and their migration into the lung parenchyma; platelet aggregation and fibrin fibre morphology; fibroblast and mucous cell proliferation; alveolar cell numbers, lamellar body formation as

well as filopodia formation. The animal weights were continuously being monitored throughout the study.

Results from the animal studies showed that the aqueous extract of *E. hirta* had limited effects on changes in the animal weights and did not cause fragility of blood fibrin fibres nor change the integrity and morphology of the platelets in the mice as seen in those treated with hydrocortisone. *E. hirta* extracts also significantly reduced the number of active inflammatory cells (especially neutrophils, eosinophils and basophils); restored the histological alterations observed in respiratory structures studied and had diverse, dose-dependent beneficial ultrastructural effects like reduction of smooth muscle hypertrophy, inhibition of macrophages into the airway parenchyma, among others.

The final judgment and conclusion of this study was that the aqueous *E. hirta* extract did not show cytotoxic effects and could be used for the treatment of asthma in the BALB/c mice at doses ranging 25-62.5mg/kg. Further research leading to clinical trials is recommended after testing the potency of equivalent doses of this extract in other animal asthma models.

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DECLARATION

I, Okobi Ekpo hereby declare that this thesis entitled:

"The *in vitro* and *in vivo* anti-inflammatory properties and cytotoxicity of extracts of *Euphorbia Hirta*"

which I herewith submit to the University of Pretoria for the Degree of Doctor of Philosophy in Anatomy, is my own original work and has never been submitted for any academic award to any other tertiary institution for any degree.

Date	**Okobi Ekpo**

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DEDICATION

To the memory of my former life coach and loving father, Chief Eko Ekpo Offem who laid for me, a solid foundation for morality, character, discipline and hard work but did not live long enough to see how these have helped to shape me.

LIST OF PUBLICATIONS

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TABLE OF CONTENTS

TITLE PAGE	i
ABSTRACT	ii
DECLARATION	iv
ACKNOWLEDGEMENTS	v
DEDICATION	viii
TABLE OF CONTENTS	x
LIST OF TABLES	xviii
LIST OF FIGURES	xix
LIST OF ABBREVIATIONS AND SYMBOLS	xxv
CHAPTER ONE: General Introduction	1
1.1 General Introduction	2
CHAPTER TWO: Literature Review	9
2.1. Asthma: an introduction	10
2.2. Development and expression of asthma	11
2.2.1. Host factors	12
2.2.1.1. Genetics	12
2.2.1.2. Obesity	13
2.2.1.3. Sex	13
2.2.2. Environmental factors	13
2.2.2.1. Allergens	14
2.2.2.2. Infections	15
2.2.2.3. Occupational sensitizers	16

2.2.2.4. Tobacco smoke 17
2.2.2.5. Outdoor/indoor air pollution 18
2.2.2.6. Diet 19
2.3. Classification of asthma 19
2.3.1. Extrinsic (allergic or atopic) asthma 19
2.3.2. Intrinsic (non-allergic or non-atopic) asthma 20
2.3.3. Occupational asthma 20
2.3.4. Exercised-induced asthma (EIA) 21
2.4. Epidemiology of asthma 22
2.5. Aetiology and pathophysiology of asthma 22
2.6. Asthma and genetics 23
2.7. The inflammatory process of asthma 25
2.7.1. The main mediators of asthma 28
2.7.1.1. Chemokines 28
2.7.1.2. Cysteinyl leukotrienes 28
2.7.1.3. Cytokines 29
2.7.1.4. Histamine 29
2.7.1.5. Nitric oxide (NO) 29
2.7.1.6. Prostaglandin D2 29
2.7.2. Cellular influx during asthma 30
2.7.2.1. Mast cells 30
2.7.2.2. Eosinophils 30
2.7.2.3. T-lymphocytes 30
2.7.2.4. Dendritic cells: 30
2.7.2.5. Macrophages 30
2.7.2.6. Neutrophils 31
2.8. Structural changes in asthmatic airways 31
2.9. Treatment of asthma 32
2.9.1. Controller medications 33
2.9.1.1. Hydrocortisone (HC) 35
2.9.2. Reliever medications 37
2.9.3. Complementary and Alternative Medicine (CAM) 37

2.9.3.1.	Herbal remedies and medicines for asthma	40
2.9.3.2.	Effects of vitamins and other food supplements	44
2.9.3.3.	*Euphorbia hirta*	44
2.10.	Animal asthma models	46
2.10.1.	BALB/c mouse models	49
2.11.	Cell cultures	50
2.12.	Aims and objectives of study.	52

CHAPTER THREE: Effects of HC and extracts of *E. hirta* on the fibroblast MRC-5 cell line 54

3.1.	Introduction	55
3.2.	Hypothesis	57
3.3.	Aims of study	57
3.4.	Materials	58
3.4.1.	MRC-5 cell line	58
3.4.2.	HC and *E. hirta*	58
3.4.3.	Media, supplements, reagents and plastic ware	59
3.5.	Methods	62
3.5.1.	Cultivation, maintenance and preservation of the MRC-5 fibroblast cell line	60
3.5.2.	Exposure of cells to the treatment agents	64
3.5.3.	The combined NR, MTT and CV (NR/MTT/CV) bioassay	63
3.6.	Data management and statistical analysis	64
3.6.1.	Pilot study with HC	65
3.6.2.	Treatment with the aqueous extract of *E. hirta*	67
3.6.3.	Effects of acetone and acetone extracts of *E hirta* on MRC-5 cells	68
3.6.4.	Effects of DCM and DCM extracts of *E hirta* on MRC-5 cells	72
3.6.5.	Effects of hexane and hexane extracts of *E hirta* on MRC-5 cells	75
3.6.6.	Analysis of the effects of the different solvents, compared with their *E. hirta* extracts and solvent extracts compared with water	81

extract.
3.6.6.1. Comparative effects in the NR Assays 80
3.6.6.2. Comparative effects in the MTT Assays 80
3.6.6.3. Comparative effects in the CV Assays 80
3.6.7. Comparison between all organic solvent extracts and the aqueous extract of *E. hirta*. 82
3.6.8. Comparison between treatment with HC and treatments with all three organic solvent extracts of *E. hirta*. 83
3.6.8.1. Comparative effects in the NR Assay 83
3.6.8.2. Comparative effects in the MTT Assay 84
3.6.8.3. Comparative effects in the CV Assay 84
3.6.9. Comparative effects between the three organic solvent extracts 85
3.7. Discussion 87
3.8. Conclusion 90

CHAPTER FOUR: Animal Experiments and Weight Studies 92
4.1. Introduction 93
4.1.1. Aim Of Study 93
4.2. Materials and methods 93
4.2.1. Materials 93
4.2.1.1. BALB/c Mice 93
4.2.1.2. Hydrocortisone (HC) 93
4.2.1.3. *Euphorbia hirta* 94
4.2.1.4. Reagents and equipment 95
4.2.2. Methods 95
4.2.2.1. Animal care and grouping 95
4.2.2.2. Experimental procedure 96
4.2.2.2.1. Sensitization 97
4.2.2.2.2. Nebulization 97
4.2.2.3. Administration of the test agents 98
4.3. Results and discussion 101
4.3.1. General effects of HC and *E. hirta* on asthmatic mice 101
4.3.2. Effects of HC and *E. hirta* on body weights of asthmatic mice 101

4.3.2.1.	Analysis of intra-group weight changes on selected days	105
4.3.2.1.1.	Early and late effects of immunization	107
4.3.2.1.2.	Effects of first time treatment on nebulization	111
4.3.2.1.3.	Midterm and late effects of continuous treatment	112
4.3.2.1.4.	Effects of repeated nebulization on weight changes	112
4.3.2.1.5.	Early and terminal post-nebulization treatment effects after nebulization on weight changes	113
4.3.2.2.	Comparison of progressive inter-group weight changes	113
4.3.2.2.1.	Control versus asthma group	113
4.3.2.2.2.	Control versus treatment groups	114
4.3.2.2.3.	Asthma versus other groups	114
4.3.2.2.4.	Low dose versus high dose groups	116
4.3.2.2.5.	Cortisone versus *E. hirta* groups	116
4.4.	Conclusion	118

CHAPTER FIVE: Analysis of Inflammatory Leukocytes 120

5.1.	Introduction	121
5.1.1.	Eosinophils	122
5.1.2.	Lymphocytes	124
5.1.3.	Neutrophils	125
5.1.4	Aim of study	126
5.2.	Materials and Methods	126
5.3.	Results	126
5.4.	Discussion	130
5.4.1.	Effects of *E. hirta* aqueous extract	130
5.4.2.	Effects of hydrocortisone	132
5.4.3.	Effects of treatment on monocyte count	132
5.5.	Conclusion	134

CHAPTER SIX: Ultrastructural studies of the Blood coagulating system 136

6.1.	Introduction	137
6.1.1.	Aim of study	140
6.2.	Materials and methods	140

6.2.1.	Inducing and treating asthma in the BALB/c mice	140
6.2.2.	Preparation of fibrin clots	141
6.2.3.	Preparation of washed fibrin clot for Scanning Electron Microscopy	142
6.3.	Results	142
6.4.	Discussion	147
6.5.	Conclusion	151

CHAPTER SEVEN: Inflammatory Cell Infiltration and Structural Changes in the Airways 153

7.1.	Introduction	154
7.2.	Mixed inflammatory infiltrate in the lung parenchyma	155
7.2.1.	Eosinophils	156
7.2.2.	Lymphocytes	156
7.2.3.	Mast cells	157
7.2.4.	Basophils	157
7.2.5.	Macrophages	158
7.2.6.	Polymorphonuclear neutrophils	158
7.2.7.	Fibroblasts	159
7.2.8.	Myofibroblasts	159
7.2.9.	Dendritic cells	160
7.2.10.	Aim of study	161
7.3.	Materials and methods	161
7.3.1.	Materials	161
7.3.1.1.	BALB/c mice	161
7.3.1.2.	Hydrocortisone (HC)	161
7.3.1.3.	*Euphorbia hirta*	162
7.3.1.4.	Reagents and equipment	162
7.3.1.5.	Light microscopy	163
7.3.2.	Transmission electron microscopy (TEM)	163
7.4.	Results	164
7.4.1.	Light microscopy	164
7.4.2.	Transmission electron microscopy (TEM) analysis	172

7.4.2.1.	Cell types	172
	Fibroblasts	172
	Lymphocytes	172
	Monocytes	172
	Neutrophils	172
	Macrophages	173
	Plasma cells	173
7.4.2.2.	Other structures	173
	Collagen fibres	173
	Lamellar bodies	173
	Thick alveolar walls	173
	Mitochondria	173
	Striations	173
	Mucous-secreting structures	173
	Smooth muscles	173
	Filopodia	174
7.5.	Discussion	181
7.5.1.	Cellular structures	181
	Fibroblasts	181
	Lymphocytes	182
	Neutrophils	183
	Monocytes	184
	Macrophages	184
	Plasma cells	185
	Pneumocytes	186
7.5.2.	Other structures	187
	Collagen deposition	187
	Lamellar bodies	188
	Alveolar wall thickness	190
	Mitochondria	190
	Striations	191
	Mucus-secreting structures	191
	Smooth muscle cells	192

Filopodia 193

7.6. Conclusion 196

CHAPTER EIGHT: Concluding Discussion 199

8.1. Concluding discussion 210

REFERENCES 210

APPENDICES 287

LIST OF TABLES

Table 3.1:	The effects of all solvent and extracts of *E hirta* as determined by the NR/MTT/CV assays.	79
Table 3.2:	Summary of the comparative effects of different E hirta solvent extracts as well as HC, as determined by the NR/MTT/CV assays	86
Table 4.1:	Groups for the short-term and long-term studies	96
Table 4.2:	Day of weighing and expected effects on weight changes	99
Table 4.3a:	Intra-group mean weights on selected reference days (ST study)	106
Table 4.3b:	Intra-group mean weights on selected reference days (LT study)	106
Table 4.4:	Percentage weights relative to baseline weights	107
Table 5.1:	Summary of the mean values obtained from white blood cell counts for all experimental groups	127
Table 5.2:	Summary of the percentage values obtained from white blood cell counts for every exposure group	128
Table 7.1:	Summary of the comparative effects of HC and E hirta extract treatments on inflammatory cell infiltration and structural changes in the airways as determined by TEM analysis	195

LIST OF FIGURES

Figure 3.1: The effect of 0-5.56 μM HC on lysosomal membrane integrity, cell viability and number measured using the NR/MTT/CV assay. Data expressed as mean (n=4) ± SD. *Differences are significant p ≤ 0.05 66

Figure 3.2: The effect of 0-11.11mg/ml aqueous extract of *E. hirta* on lysosomal membrane integrity, cell viability and number measured using the NR/MTT/CV assay. Data expressed as mean (n=4) ± SD. *Differences are significant p≤0.05 68

Figure 3.3: The effect of (a) 0-11.11% of acetone solvent, and (b): 0-11.11mg/ml acetone extract of *E. hirta*, on lysosomal membrane integrity, cell viability and number measured using the NR/MTT/CV assay. Data expressed as mean (n=2) ± SD. * Differences are significant at p ≤ 0.05. 70

Figure 3.4: Comparison of the effects of 24-hour exposure to the carrier acetone and *E. hirta* acetone extracts on the MRC-5 cell line; a) NR, b) MTT and c) CV assay. * Differences are significant for the NR and CV assays, p ≤ 0.05. 71

Figure 3.5: The effect of (a) 0-11.11% DCM and (b) 0-11.11mg/ml DCM extract of *E. hirta* on lysosomal membrane integrity, cell viability and number measured using the NR/MTT/CV assay. Data expressed as mean (n=2) ± SD. *No significant differences at p ≤ 0.05 73

Figure 3.6: Comparison of the effects of 24-hour exposure to the carrier DCM and DCM extracts of *E. hirta* on the MRC-5 cell line; a) NR, b) MTT and c) CV assay. * Differences significant at p ≤ 0.05 74

Figure 3.7:	The effect of (a) 0-11.11% carrier hexane and (b) 0-11.11mg/ml hexane extract of *E. hirta* on lysosomal membrane integrity, cell viability and number measured using the NR/MTT/CV assay. Data expressed as mean (n=2) ± SD. *Significant differences were seen at p ≤ 0.05	76
Figure 3.8:	Comparison of the effects of 24-hour exposure to hexane and hexane extracts of E. hirta on the MRC-5 cell line; a) NR, b) MTT and c) CV assay.* Differences are significant for the NR, MTT and CV assays respectively as indicated; p ≤ 0.05	78
Figure 3.9a-c:	Comparison of the effects of 24-hour exposure to HC; aqueous, acetone, DCM and hexane extracts of E. hirta on lysosomal membrane integrity, cell viability and number measured using the NR/MTT/CV assay. Data expressed as mean (n=2) ± SD. * Differences are significant at p ≤ 0.05	81
Figure 4.1:	Histogram illustrating weight changes during the ST study for the control, asthma, high and low hydrocortisone, high and low *E. hirta* treatment groups	109
Figure 4.2:	Histogram illustrating weight changes during the LT study for the control, asthma, high and low hydrocortisone, high and low *E. hirta* treatment groups	109
Figure 4.3:	Line graph illustrating the trend of changes in weight during the ST study for the control, asthma, high and low hydrocortisone, high and low E. hirta treatment groups	110
Figure 4.4:	Line graph illustrating the trend of changes in weight during the LT study for the control, asthma, high and low hydrocortisone, high and low *E. hirta* treatment groups	110

Figure 5.1a:	Histogram showing the mean values obtained from white blood cell counts per group	129
Figure 5.1b:	Histogram showing the percentage white blood cell counts per group	129
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.	143
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.	143
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.	144
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	146
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	146

Figure 7.1: Histological cross sections of control mice lungs showing A) and B) thin and continuous alveolar walls; regular shaped alveoli. H and E staining. Magnification X 100 166

Figure 7.2: Histological cross sections of asthma mice lungs showing A) thick and discontinuous alveolar walls and B) thick smooth muscle mass in walls of distal airways indicated with arrows. H and E staining. Magnification X 100 167

Figure 7.3: Histological cross sections of the lungs of mice treated with HHC showing A) and B) alveoli that are relatively small but numerous; alveolar walls moderately thick, alveoli compact and irregular in shape; alveolar walls discontinuous; smooth muscle thickenings of terminal airways seen, shown with arrows. H and E staining. Magnification X 100 168

Figure 7.4: Histological cross sections of the lungs of mice treated with low HC showing A) and B) alveolar walls discontinuous and irregular; smooth muscle, masses seen in walls of distal airways) H and E staining. Magnification X 100 169

Figure 7.5: Histological cross sections of the lungs of mice treated with high EH showing A) and B) thick alveolar walls; alveoli very compact and irregular in shape; alveolar walls discontinuous (shown with arrows in B). H and E staining. Magnification X 100 170

Figure 7.6: Histological cross sections of the lungs of mice treated with low EH showing A) thin alveolar walls; less compact and more patent alveoli; alveolar walls are only partially discontinuous (shown with arrows) and B) thinner alveolar walls; alveoli less compact and irregular in shape; alveolar walls only partially 171

discontinuous (arrowed). H and E staining. Magnification X 100

Figure 7.7: TEM micrograph of lung tissue from control mice showing A = Fibroblast (F), scanty collagen fibres [x7500]; B = Macrophage and lymphocytes in interstitium [x9800]; C = Collagen fibres [x13000]; D = Neutrophil, macrophage [x7500]; E = Fibroblast, neutrophils scanty fibres x5900; F = Lymphocytes and macrophage in lung interstitium [x4300] 175

Figure 7.8: TEM micrograph of lung tissue from asthma mice showing A = Types 1 and 2 (T1; T2); thick walls [x3600]; B = Fibroblasts (F), many collagen fibres, smooth muscle [x5900]; C = Thick walls (TW), Type 1 cells (T1); macrophage (Ma) [x4300]; D = Macrophage (Ma), collagen fibres, smooth muscle [x9800]; E = Large Lymphocytes (LL); fibres (F), mitochondria (Mi) [x18000]; F = Many collagen fibres (CF), fibroblast, striations [x5900] 176

Figure 7.9: TEM micrograph of lung tissue from asthma mice treated with LHC showing A = Monocyte (Mo), lymphocytes (L) [X5900]; B = Fibroblast, collagen fibres (CF) [X7500]; C = Mucous secreting structures [X4300]; D = Thick walls, lamellar bodies LB, Types 1, 2 cells [X3600]; E = Lymphocyte with vesicles (LV), Types 1, 2 cells [X2800]; F = Thick walls, macrophages (M), T2 cells [x3600] 177

Figure 7.10: TEM micrograph of lung tissue from asthma mice treated with HHC showing A = Fibroblast and scanty collagen fibres [x9800], B = Fibroblast and scanty collagen fibres [x9800], C = Fibroblasts, few fibres, many mitochondria [x9800], D = Fibroblast (F), relatively more fibres (CF) [x9800], E = Unmyelinated nerves and collagen fibres [x13000], F = Very scanty collagen fibres, red blood cells [x4300] 178

Figure 7.11: TEM micrograph of lung tissue from asthma mice treated with HEH showing A = Highly fibrous lung parenchyma, fibroblast [x5900]; B = Highly fibrous lung parenchyma, fibroblast [X5900]; C = Fibroblasts (F) and fibres [x7500]; D = Smooth muscle (SM) cell, many fibres [x9800] 179

Figure 7.12: TEM micrograph of lung tissue from asthma mice treated with LEH showing A = Mucus gland (Mu) and many fibres [x3600]; B = Fibroblast (F), many fibres [x13000]; C = Alveolar walls, Type 1 (T1) cells, lamellar bodies [x4300]; D = Bronchioles: Plasma cell (P), lymphocyte (L), Rough ER [x4300]; E = Bronchial seromucous gland and cell [x7500]; F = Bronchial artery containing red blood cells [x5900] 180

LIST OF ABBREVIATIONS AND SYMBOLS

AHR	Airway hyperresponsiveness
ASM	Airway smooth muscle
$Al(OH)_3$	Aluminium trioxide
ANOVA	Analysis of Variance
AS	Asthma
ACD	Atopic contact dermatitis
PWDs	Percentage weight differences
BTG	Beta-thromboglobulin
BALF	Bronchoalveolar lavage fluid
Ca^{2+}	Calcium ion
CD4+	Cluster of differentiation 4
cm^2	Centimetre squared
CAM	Complimentary and Alternative Medicine
Conc.	Concentration
CT	Control
CV	Crystal Violet
DCM	Dichloromethane
DMSO	DImethyl sulphoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate Buffered Saline
ddH_2O	Double distilled and deionized water
EMEM	Eagles Minimum Essential Medium
EIA	Exercise induced asthma
ECP	Eosinophil cationic protein

EDTA	Ethylene diamine tetra acetate
ECM	Extracellular matrix
FCS	Foetal Calf Serum
FER	Food efficiency ratio
GM-CSF	Granulocyte monocyte colony stimulating factor
HBSS	Hanks Balanced salt solution
HC	Hydrocortisone
HASMC	Human airway smooth muscle cells
Hu-PBMC	Human peripheral blood Mononuclear cells
IFN-γ	Interferon-gamma
IL	Interleukin
IgE	Immunoglobulin E
ICS	Inhaled corticosteroids
IFN-a	Interferon-alpha
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
Kg	Kilogram
LT	Long-term
LEH	Low EH
LHC	Low HC
M	Molar
MIP	Macrophage inflammatory protein
MBP	Major basic protein
MPM	Malleable Protein Matrix
Mg/ml	Milligram/Millilitre

MTT	[3-(4, 5-dimethylthiazol-zyl) 2, 5-dimethyl tetrazolium bromide]
NR	Neutral Red
NA	Not available
NOS	Nitric Oxide Synthase
NSD	No significant difference
Na_2HPO_4	Disodium hydrogen phosphate
$NaHCO_3$	Sodium hydrogen carbonate
OsO_4	Osmium tetraoxide
OVA	Ovalbumin
PAI_{act}	Plasminogen activator inhibitor
pH	Measure of the acidity or basicity
PBS	Phosphate Buffered Saline
PHA	Phytohemagglutinin
PA	Plasminogen activator
PRP	Platelet rich plasma
PAF	Platelet-activating factor
PF	Pulmonary fibrosis
SEM	Scanning Electron Microscope
ST	Short-term
SD	Standard deviation
SD	Significant difference
SO	Superoxide
Th1	T helper type 1 lymphocytes
TNF-α	Tumour Necrosis Factor-α
TEM	Transmission Electron Microscope

TGF	Transforming Growth Factor
µ	Micro
β	Beta
µl	Micro litre
g	Gram
%	Percent
≤	Equal or less than
µg/mL	Microgram per millilitre
^{0}C	Degree Celsius

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.

CHAPTER THREE

Effects of HC and extracts of *E. hirta* on the fibroblast MRC-5 cell line

3.1. Introduction

Asthma is one of the most common respiratory complaints in the world as it affects an estimated 300 million people worldwide. The marked increase in the prevalence of asthma has made it a worldwide health concern and global figures are expected to rise to 400 million by the year 2025 (Masoli et al., 2004).

Over the years, corticosteroids have been very effective in the treatment of asthma but serious systemic adverse effects have been associated with prolonged use of these medications (Leonard and Sur, 2002). With increasing popularity of complementary and alternative medicine, research geared towards developing more effective alternative anti-asthma agents appears to be gaining much attention.

Herbal medicines are purported to show clinical efficacy with minimal side effects compared to mainstream treatments (Chevrier et al., 2005). A number of studies support the use of herbal medicines in asthma and as reported by Ming-Chun et al., (2005), herbal medicine intervention appears to be safe and effective alternative methods for treating asthma. In addition, many pharmaceutical products have been developed from traditionally used plant products (McRae et al., 2007). Continuous investigation of how herbal medicines work could lead to the development of additional effective medication for the treatment of asthma, allergies and many other health conditions (Bielory and Lupoli, 1999). The present study aims at investigating the possible toxic effects of *E. hirta* on the MRC-5 cell line.

The MRC-5 cell line is one of the most common cell lines used for cytotoxicity studies. This cell line is even more suitable for modelling *in vitro* conditions of a

respiratory tract disease like asthma because as it is derived from normal human diploid foetal lung fibroblasts. The advantages of using *in vitro* model systems are that cellular damage is easy to measure, the system is simple and easy to reproduce, only small amounts of the compound are required and several statistically significant experiments under controlled conditions can be undertaken.

In one previous study involving the MRC-5 cell line, the biocompatibility of the Erlanger silver intravenous catheter was investigated. The possible acute cytotoxicity of extracts from the catheter was studied using MRC-5 cells and lymphocytes. The extracts were not cytotoxic to MRC-5 human fibroblasts or to sensitized phytohemagglutinin (PHA)-stimulated human lymphocytes (Greil, 1999). In another study, the physiological concentrations of the dipeptide L-carnosine were found to prolong the lifespan of MRC-5 cells and strongly reduce the normal features of senescence (Holliday and McFarland, 2000).

MRC-5 cells have also been used in many other studies including screening of medicinal plants for cytotoxicity (Zirihi et al., 2005), antimicrobial and antioxidant activities (Mensah et al., 2006); in vaccine production (Wu et al., 2006); in virology studies (McCarter and Robinson, 1997; Maes et al., 2004); as well as in inflammation studies (Hase et al., (2003). Others include carcinogenicity studies (Merlin et al., 2002; Tomatsu et al., 2003); studies on the damaging effects of heavy metals (Yang et al., 1997), stem cell research (Rieske et al., 2005) as well as studies on radiation-induced damage effects (Jones et al., 2006).

In this study, different types of bioassay systems were used to measure cytotoxicity in the MRC-5 cell line. Cell number, cell viability and lysosomal membrane integrity were determined using the Crystal Violet (CV); 3-(4, 5-

dimethylthiazol-zyl) 2, 5-dimethyl tetrazolium bromide (MTT); and Neutral Red (NR) assays. HC was used as a pharmaceutical control for the plant extract because of its known anti-asthma effects. A study by McLendon et al., (2006) showed that different cortisol concentrations did not induce structural damage in MRC-5 cells throughout the experiment.

A pilot cell culture study was carried out to determine the appropriate concentration of HC to be used as control and later, the final cell culture tests were done using the aqueous and organic (hexane, dichloromethane, acetone) extracts of *E. hirta.* The individual effects of each solvent were also evaluated.

3.2. Hypothesis

This study was carried out to test the hypothesis that extracts of *E. hirta* have limited cytotoxic effects on cultured MRC-5 cells and such effects are not only dose-dependent but vary between the different solvent extracts used.

3.3. Aims of study

- To evaluate the possible cytotoxic effects of *E. hirta* extracts using *in vitro* assays (NR, MTT and CV) to analyze the viability and lysosomal membrane integrity of MRC-5 cells as well as MRC-5 cell numbers.
- To compare the effects of the aqueous and organic solvent (acetone, dichloromethane and hexane) extracts of *E. hirta* on the MRC-5 cell line.
- To compare the effects of HC with the effects of the different extracts of *E. hirta* extract on the MRC-5 cell line.
- To provide information on the concentrations of *E. hirta* and HC that are safe to use in the animal studies

3.4. Materials

3.4.1. MRC-5 cell line

The MRC-5 lung permanent cell line is a normal, finite cell line derived from human embryonic diploid lung fibroblasts (Jacobs et al., 1970; Freshney, 2000). MRC-5 cells are common and commercially available and were obtained from Highveld Biological Company, Johannesburg, South Africa.

3.4.2. HC and *E. hirta*

HC was used as a pharmacologic control for the extracts of *E. hirta* in this study. The drug was purchased from Elwierda Apteek/Pharmacy Wirdapark, Pretoria, South Africa. One hundred milligrams (100mg) of the powdered HC were dissolved in 2ml of injection water giving a concentration of 50mg/ml. This solution has a molarity of 0.138M and the MRC-5 cells were exposed to five times progressive dilutions of HC in the following molar concentrations: (0.00 μM, 22.8μM, 3.8μM, 0.63μM, 0.16μM and 0.0263μM). The pilot study showed that the significant cytotoxic effects of HC on MRC-5 cells mostly affected the CV and NR assays.

E. hirta was obtained from fields in the Gezina area of Pretoria, South Africa and was identified and verified by staff in the Department of Botany, University of Pretoria, South Africa by comparison with a voucher specimen of the plant available at the H.G.W.J. Schweikerdt Herbarium of the University. Aqueous and organic solvent (acetone, hexane and dichloromethane) extracts of the plant used for the various experiments were prepared in the Cell Biology Laboratory of the Department of Anatomy, University of Pretoria. The aboveground parts of the plant were dried at room temperature for one week and a fine powder was then

prepared and was subdivided into four 50 g samples. Each 50 g sample was extracted with distilled water and the three different organic compounds (acetone, dichloromethane and hexane), filtered and then dried on a rotary evaporator at 40°C.

A concentration of 0.1g/ml (each 50g sample dissolved in 500ml distilled water or 500ml solvent) was prepared for each solvent and the samples were stored frozen as the stock solutions. Later the stored samples were thawed and fifty microlitres (50µl) of the working solution added to the culture wells in five times progressive dilutions to determine the effects of the different extracts on the MRC-5 cell line.

3.4.3. Media, supplements, reagents and plastic ware

Eagles Minimum Essential Medium (EMEM) powder, Hanks Balanced salt solution (HBSS) and Foetal Calf Serum (FCS) were from Highveld Biological Company, Johannesburg, South Africa. Sartorius cellulose acetate membrane filters 0.22µm were from National Separations, Johannesburg, South Africa. Fixatives, acids and organic solvents, such as glutaraldehyde, hydrochloric acid (HCl), acetic acid, isopropanol, and formic acid were analytical grade and were purchased from Merck, Johannesburg, South Africa. Streptomycin sulphate, penicillin G (sodium salt), Amphotericim B and Trypsin were obtained from Life Technologies Laboratory supplied by Gibco BRL Products, Johannesburg, South Africa. Ethylene diamine tetra acetate (EDTA), Dimethyl sulphoxide (DMSO), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), sodium chloride (NaCl) and sodium hydrogen carbonate ($NaHCO_3$) were from Merck, Johannesburg, South Africa.

MTT [1- (4, 5-Dimethylthiazol-2-yl) -3, 5- diphenylformazan] and Crystal Violet

(CV) powder were obtained from Sigma-Aldrich, Atlasville, South Africa. Bovine serum albumin (BSA) was from Boehringer Mannheim, Randburg South Africa. Water was double distilled and deionised (ddH_2O) with a Continental Water System and sterilized by filtration through a Millex 0.2μm filter. Glassware was sterilized at –140^{0}C in a Prestige Medical Autoclave (Series 2100). Twenty four (24-well) and 96-well 25cm^2 and 75cm^2 cell culture flasks, 10ml and 5ml pipettes, 15ml and 50ml centrifuge tube, micro centrifuge tubes were obtained from NUNC™ and supplied by AEC- Amersham, Johannesburg, South Africa.

3.5. Methods

3.5.1. Cultivation, maintenance and preservation of the MRC-5 fibroblast cell line

The MRC-5 fibroblast cell line was maintained in EMEM supplemented with 5% FCS and 1% antibiotic solution (EMEM/ 5% FCS). An antibiotic stock solution was prepared by mixing 10,000U/ml Penicillin G (sodium salt), 10,000μg/ml Streptomycin sulphate and 25μg/ml Amphotericim B in 0.85% saline. A volume of 10 ml of the working solutions was added to 1 litre of the prepared medium. The antibiotic solution was kept at -10 ^{o}C and thawed when needed. The media was sterilized by filtration through a 0.22μm membrane filters under aseptic conditions in a laminar flow cabinet. Aliquots of 100 ml were prepared and the medium was stored at 4°C and warmed to 37°C before use.

The MRC-5 cells were plated at a concentration of 5X10^4 cells per ml in 25cm^2 and 75cm^2 cell culture flasks and were maintained at 37°C and 5% CO_2 in a CO_2 - water-jacketed incubator from Forma Scientific. The cell culture medium was changed every three days or when the medium had become acidic. The cells were

later passaged once confluent with a 0.05% trypsin solution. A 10X Trypsin/EDTA stock solution of 5g/l Trypsin, 2g/l EDTA.4Na was prepared by mixing 0.25g Trypsin, 0.1g EDTA and 0.425g NaCl in 50ml Hanks buffer. Hanks buffer was prepared by dissolving 9.86g/ l Hanks salt and 0.35g/l $NaHCO_3$ in ddH_2O. A working solution of 1X was prepared by diluting the stock solution with DPBS that was stored at -10 ^oC and warmed up at 37^0C before use. The solutions were filtered through a 0.22µm membrane filter under aseptic conditions.

The cells were passaged by firstly removing the medium from the confluent monolayer. A volume of 0.08 ml/cm^2 trypsin working solution was added and the flask was placed at 37^0C for 5-10 minutes. The medium containing the detached cells was added to 10 ml medium. The cells were collected by centrifugation in a BTL Bench centrifuge from Baird and Tatlock at 6000xg (2000 rpm) for 5 minutes. The medium was removed and the cells were suspended in 10 ml fresh medium. The centrifugation step was repeated and the number of cells was determined by counting a 10µl aliquot of cells using a haemocytometer from Brand supplied by Merck, Johannesburg, South Africa.

The MRC-5 fibroblasts were either used for experiments described below or stored at –70°C. For storage, the cells were suspended in cell culture freezing medium at a concentration of $5x10^6$ cells per ml. The freezing medium was prepared by adding 10% DMSO and EMEM/5%FCS. One millilitre of the cell suspension was transferred to the 1.5ml freezing vials and stored by slow freezing (the vials were wrapped with tissue paper and placed into a large Styrofoam box) in a –70°C freezer.

The cells were stored for an indefinite period of time, with minimum loss of viability. The vials containing the MRC-5 fibroblasts were thawed rapidly by stirring the vial in warm water at 37^0C. The cells were suspended in EMEM containing 5% FCS to a volume of 15 ml. The cells were collected by centrifugation, the supernatant removed and the cells suspended in fresh medium. This step was repeated twice before the cells were suspended in a final volume of 3ml culture medium and plated in $25cm^2$ cell culture flasks. For each experiment, cells were plated at a cell concentration of $2x10^4$ cells per ml in 24 well plates with the culture area of $1.9cm^2$/well and were kept for 24 hours at 37°C and 5% CO_2 before conducting each experiment.

3.5.2. Exposure of cells to the treatment agents

Different experiments were done using MRC-5 cells. Some cells were exposed to HC and others were treated with a 0.1g/ml concentration of each of the stock solutions (aqueous, acetone, dichloromethane and hexane). In all experiments, cells in the first set of wells represented the controls and were left untreated. A five times dilution was done for the remaining wells by first adding 50µl of the treatment agents to the set of wells next to the control wells, mixing properly before transferring 100µl by pipette from these set of wells to the next, and again removing another 100µl to the next set of wells. The process was repeated in series for all the wells and the 100µl removed from the last set of wells was discarded.

After 24 hours of exposure cell number, cell viability and lysosomal membrane integrity were determined using the combined NR/MTT/CV assay. All experiments conformed to the ethical standards of the University of Pretoria.

3.5.3. The combined NR, MTT and CV (NR/MTT/CV) bioassay

In this study, cell viability, lysosomal membrane integrity and cell number were determined almost simultaneously using the combined NR/MTT/CV bioassay of Chiba and others (Chiba et al., 1998) with only a slight modification.

Briefly, 50µl of a 50µg/ml NR dye solution was first added to each well for uptake into lysosomes of viable cells. After an incubation period of 1-2 hours, 50µl of a 2.5mg/ml MTT solution was added. The yellow water-soluble MTT dye is converted by mitochondrial dehydrogenase in viable cells to insoluble purple/blue formazan (Mosmann, 1983).

After a further 1-2 hours, the culture medium was carefully removed, the plate carefully blotted dry and the cells rapidly fixed in 1% each of acetic acid and formalin in water for a few seconds. The fixative was decanted and the plate blotted dry before 100µl of the NR solvent (1% acetic acid in 50% ethanol) was added for extraction of NR. The extracted dye was then transferred to a 96-well plate. Likewise the formazan crystals were extracted by adding 100µl dimethyl sulphoxide (DMSO) and the extracted dye transferred to the 96-well plate.

The plates were later washed with water and dried before the attached cells were stained with 1% CV for 20 minutes. The CV dye was thereafter removed, the plates washed thoroughly with tap water, dried and the dye extracted with 100µl of a 10% acetic acid solution and transferred to the 96-well plate as well. Absorbency was measured at 570 nm, 450 nm and 590 nm using an EL900 plate reader for the NR, MTT and CV assays respectively (Borenfreund et al., 1990).

3.6. Data management and statistical analysis

The average of four independent experiments was determined and the data was expressed as percentage of the unexposed control wells. Graphs were plotted to show the effects of HC and the different plant extracts on lysosomal membrane integrity, cell viability and number. Tables were combined to obtain one final graph that is a summary of all experiments. All data was expressed as mean ± standard deviation (SD) and analysed for statistical significance using the Analysis of Variance (ANOVA) statistical package. A confidence level of 95% (i.e. $p < 0.05$) was considered statistically significant.

For the CV, MTT and NR assays, a one-way Analysis of Variance (ANOVA) was used to determine the presence or otherwise of statistically significant differences between unexposed control cells and the different dosage concentrations. For the solvent only experiments, a one-way ANOVA was used to compare between control and solvent extract dosage while a two-way ANOVA was used to compare each concentration of solvent with the solvent and plant extract.

In order to determine the differences in effects between the solvent only assays and the solvent and plant extract assays, comparisons were made between group-matched concentrations. The concentration range was designated '0-5' where '0' represents the control concentration, '1' the lowest concentration and '5' the highest concentration respectively. The 2-way ANOVA gives the results of statistically significant differences between corresponding concentrations in the solvent assays and the solvent with plant extract assays respectively.

3.6.1. Pilot study with HC

MRC-5 cells were exposed to a concentration range of 0-5.56 µM HC for 24 hours after which lysosomal membrane integrity, cell viability and number was determined using the NR/MTT/CV assay. From the data presented in *figure 3.3,* it is evident that HC had no effect on cell viability (MTT assay) but did cause a decrease in lysosomal membrane integrity and cell number. At concentrations of 1.11µM and 5.56µM, a significant decrease in NR ($p \leq 0.05$) was observed while at 5.56µM, a significant decrease in CV ($p \leq 0.05$) was observed.

Analysis of the mean results obtained showed that only the two highest concentrations of HC had statistically significant effects compared with the controls for the NR assay. There was no significant difference between the different dose concentrations for the MTT assay but a statistically significant difference was seen in the CV assay between control and the highest concentration.

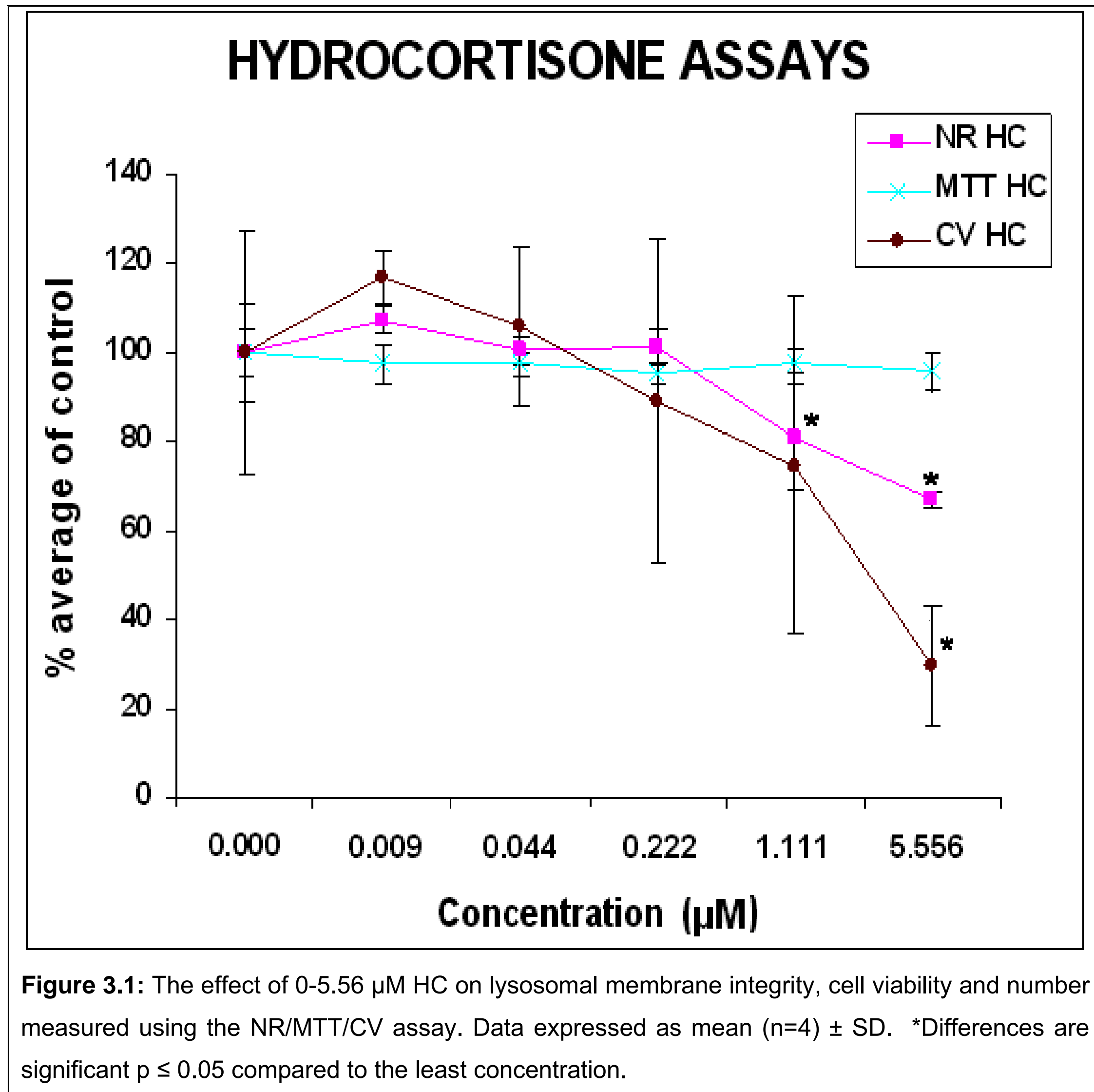


Figure 3.1: The effect of 0-5.56 µM HC on lysosomal membrane integrity, cell viability and number measured using the NR/MTT/CV assay. Data expressed as mean (n=4) ± SD. *Differences are significant $p \leq 0.05$ compared to the least concentration.

In addition, there was no statistically significant difference between the three lowest concentrations but differences were observed between the highest NR concentration and the three lowest concentrations as well as between the second highest NR concentration and the three lowest concentrations respectively. In the case of the CV assay, statistically significant differences were seen only when the three lowest concentrations were compared with the highest concentration.

3.6.2. Treatment with the aqueous extract of *E. hirta.*

The aqueous extract of most plants generally contain lipophobic compounds such as polyphenolic compounds, triterpenes and flavonoids, and most of these compounds have potent anti-inflammatory and antioxidant properties (Lanhers et al., 1991; Sharma et al., 2007; Fecka and Turek, 2007). MRC-5 cells were exposed to 0-11.11 mg/ml of the aqueous extract of *E. hirta* for 24 hours after which lysosomal membrane integrity, cell viability and number were determined using the NR/MTT/CV assay.

The aqueous fraction of *E. hirta* had no significant effect on lysosomal membrane integrity. For the MTT assay that measures cell viability. There was a significant decrease in cell viability when the 0.02mg/ml and 0.09mg/ml concentrations were compared to the 2.22mg/ml and 11.11mg/ml ($p \leq 0.05$ and $p \leq 0.05$) respectively. However, from 0.44 - 11.11mg/ml concentrations, the aqueous extract of *E. hirta* caused an increase in value for the CV assay from 100% to 300% compared to the control ($p \leq 0.05$). This finding appeared to imply that the aqueous extract of *E. hirta* could cause a three fold increase in cell number. This is very unlikely an increase in cell number, since such an increase is usually associated with a corresponding increase in the number of mitochondria and lysosomes and therefore an increase in the values for the NR and MTT assays. It is possible that the plant proteins bind either the tissue culture surface or to the surface of the MRC-5 cells resulting in false high values.

However, the MTT assay mean showed statistically significant differences between the control and least concentration, as well as between the third highest and the highest concentration.

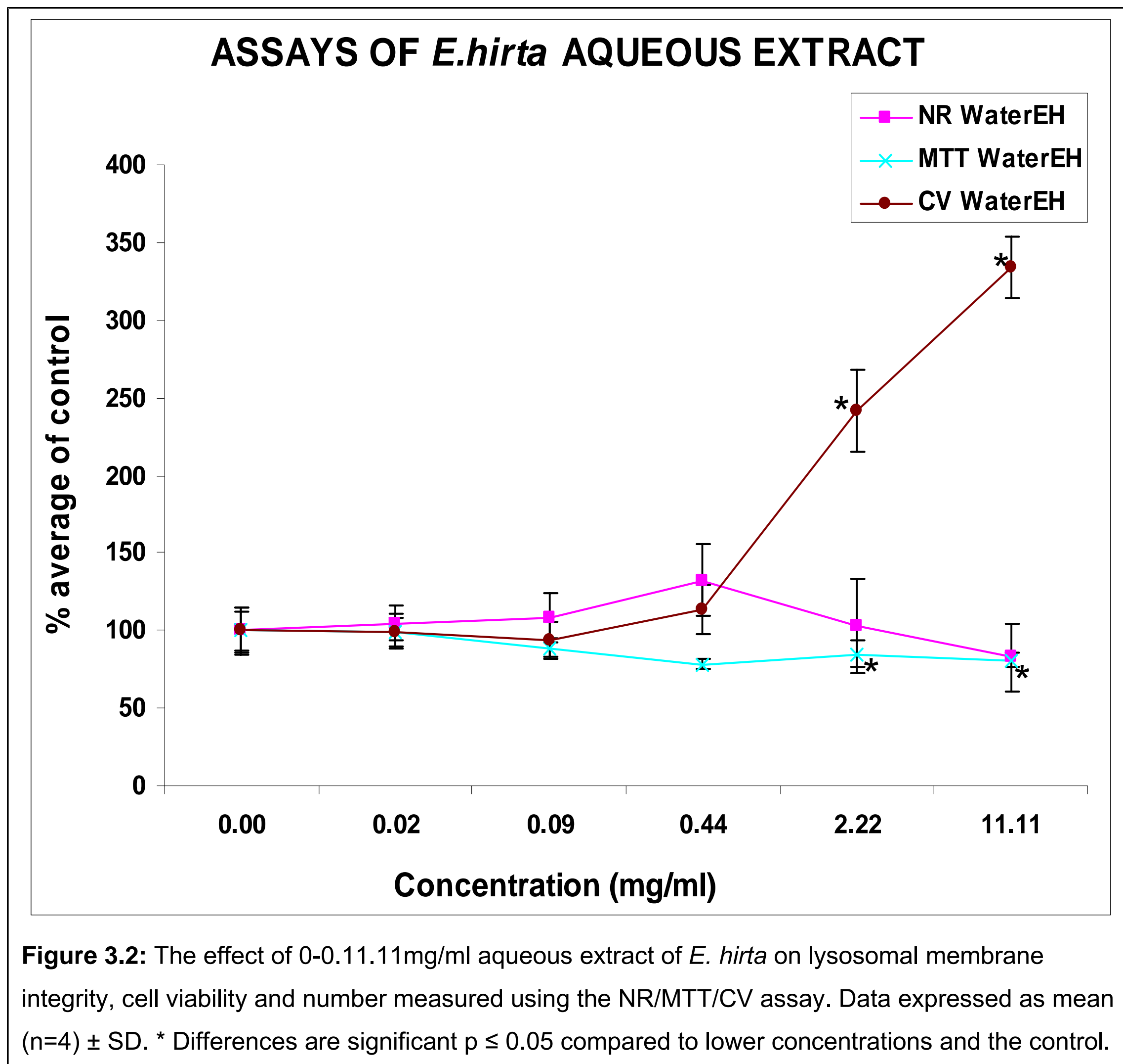


Figure 3.2: The effect of 0-0.11.11mg/ml aqueous extract of *E. hirta* on lysosomal membrane integrity, cell viability and number measured using the NR/MTT/CV assay. Data expressed as mean (n=4) ± SD. * Differences are significant $p \leq 0.05$ compared to lower concentrations and the control.

3.6.3. Effects of acetone and acetone extracts of *E hirta* on MRC-5 cells

Acetone extracts of plants generally contain glycerides, sterols, sterol esters, carotenoids, lipid soluble vitamins, monogalactoside diglycerides and sterol glycosides (Gouveia and Empis, 2003; Nyström et al, 2007). Acetone used as carrier itself can be cytotoxic to cells; therefore, the cytotoxicity of acetone in MRC-5 cells was determined. MRC cells were exposed to 0-11.11% acetone for 24 hours before lysosomal membrane integrity, cell viability and number was determined.

For acetone only assays (*Figure 3.3a*), following exposure to 11.11%, there was no significant difference in lysosomal membrane integrity and cell viability. However, the cell number appeared to rise initially to 125.4% before decreasing steadily until percentage concentration 2.22%; increasing again to 108.9% at the highest concentration. The differences found in cell number were significantly different from the controls as well as from 0.09% and 0.44% concentrations.

The MRC-5 cells in culture were also exposed to 0 - 11.11 mg/ml acetone extract of *E. hirta* (*Figure 3b*) and results show a steady progression between the different concentrations. Again, no statistical differences were observed between the control (without extract) and the different volumes/concentrations of the plant extract added to the cell culture medium of MRC-5 cells ($p \leq 0.05$ for both NR and MTT assays) (*Figure 3.3b*). Except at the 0.02 mg/ml concentration with 47% significant increase in cell number, all other differences in volumes/concentration were not significant ($p \leq 0.05$).

Two way ANOVA analysis showed significant differences in the NR and CV assay when corresponding concentrations of the acetone only and the acetone extract treatments were compared ($p \leq 0.05$ and $p \leq 0.05$)(*Figure 3.4b* and *c*). No statistical differences were observed for the MTT assay (*Figure 3.4c*).

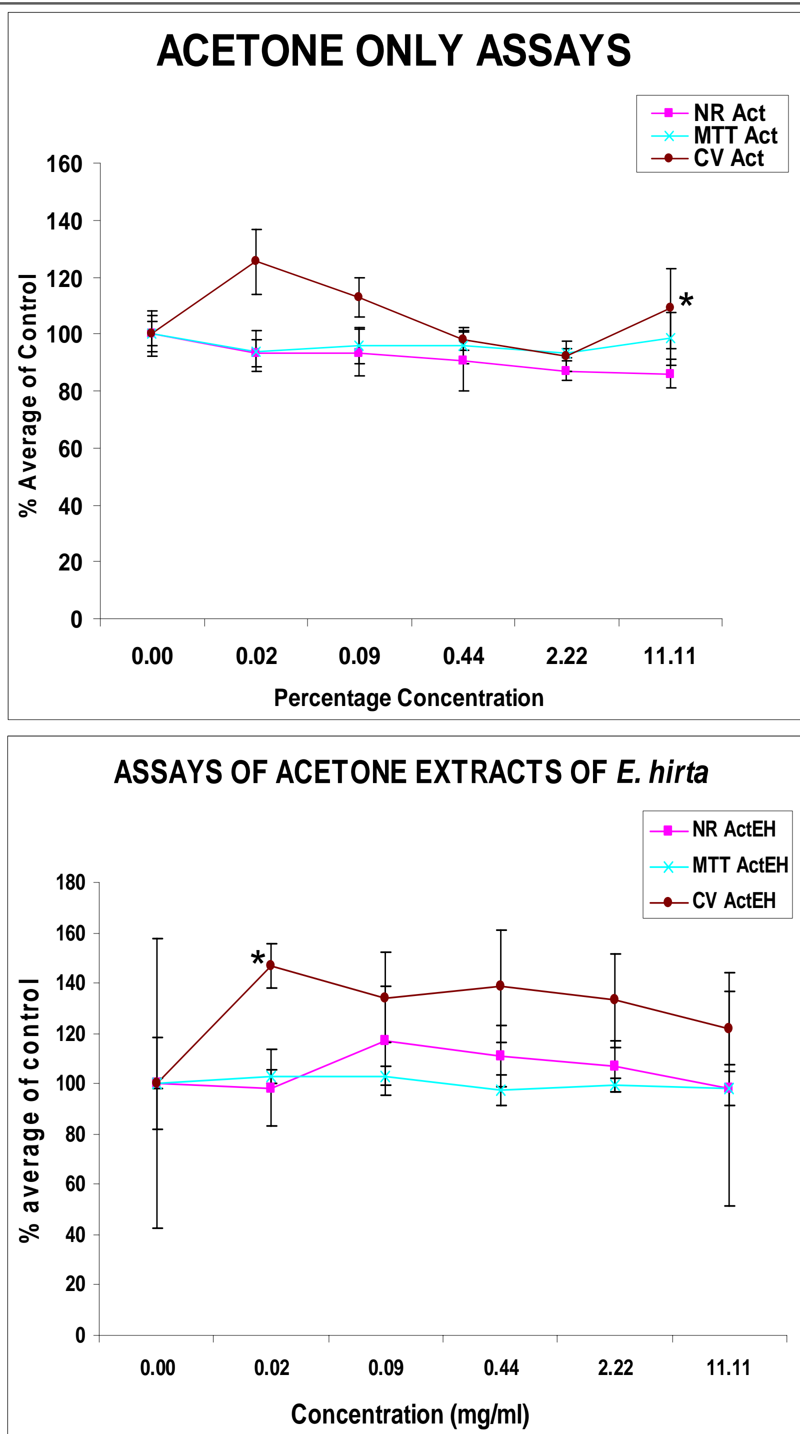


Figure 3.3: The effect of **(a)** 0-11.11% of acetone solvent, and **(b):** 0-11.11 mg/ml acetone extract of *E. hirta*, on lysosomal membrane integrity, cell viability and number measured using the NR/MTT/CV assay. Data expressed as mean (n=2) ± SD. * Differences are significant at p ≤ 0.05 compared to lower concentrations and the control.

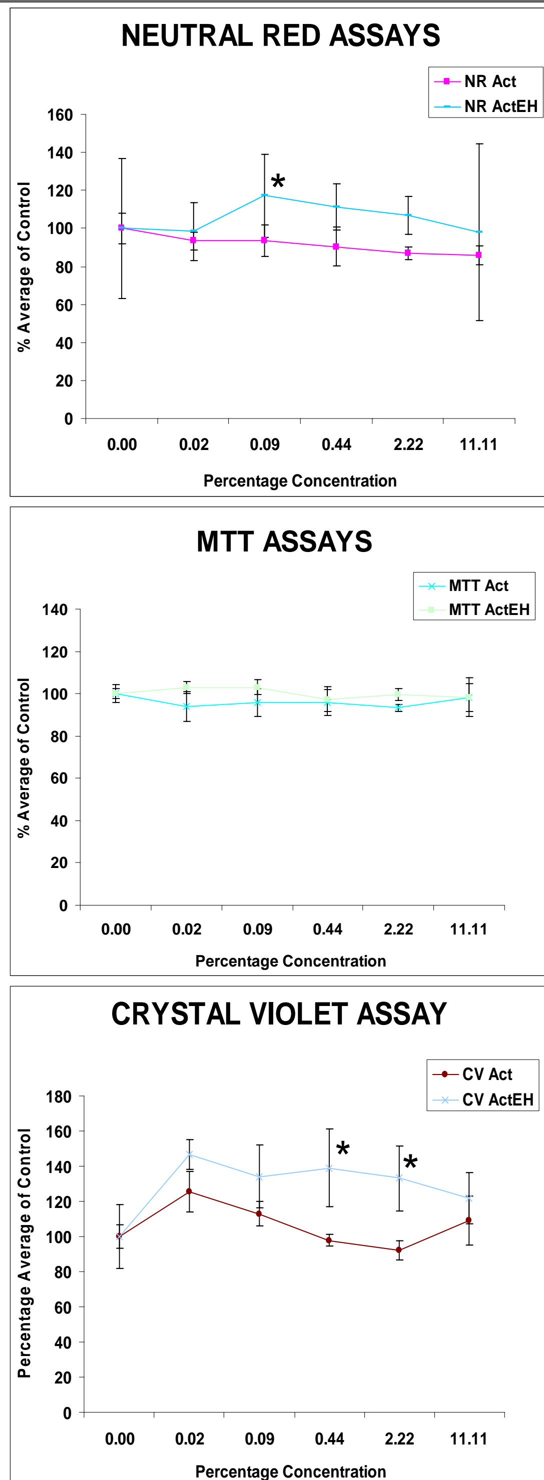


Figure 3.4: Comparison of the effects of 24-hour exposure to the carrier acetone and *E. hirta* acetone extracts on the MRC-5 cell line; **a)** NR, **b)** MTT and **c)** CV assay. *Differences are significant ($p \leq 0.05$) for NR and CV assays, each compared to ActEH.

3.6.4. Effects of dichloromethane (DCM) and DCM extracts of *E hirta* on MRC-5 cells

Most DCM plant extracts usually contain sesquiterpene lactones lychnopholide, centratherin, goyazensolide and 15-desoxygoyazensolide (Grael et al., 2005), terpene and naphthalene derivatives (Jing et al., 2007) as well as β-Sitosterol, 5, 6-Trihydroxy-3′, 7-dimethoxyflavone, Daucosterin and Ursolic acid (Moghaddam et al., 2007). The MRC-5 cells were exposed to dichloromethane (DCM) alone as well as to DCM of *E. hirta.*

After 24 hours exposure to 0 - 11.11% DCM (*Figure 3.5a*) and DCM containing 0-11.11 mg/ml plant extract (*Figure 3.5b*), lysosomal membrane integrity, cell viability and number was determined by the NR, MTT and CV assays respectively.

For the DCM treatment (*Figure 3.5a*), a one-way ANOVA showed that there was no significant difference ($p \leq 0.05$) between the control (no DCM) and other concentrations for all three assays. Likewise, no significant differences were observed (*Figure 3.5b*) for all three assays of the DCM extracts of *E. hirta*.

When treatment with DCM was compared to the DCM extracts of *E. hirta* using two-way ANOVA analysis (*Figure 3.6*), no significant differences were seen in the NR and MTT assays. However, the two-way ANOVA showed that only the difference between the second highest concentrations (2.22%) of the CV assays in the two treatments was statistically significant. Comparisons between the two treatments at other concentrations were not statistically significant.

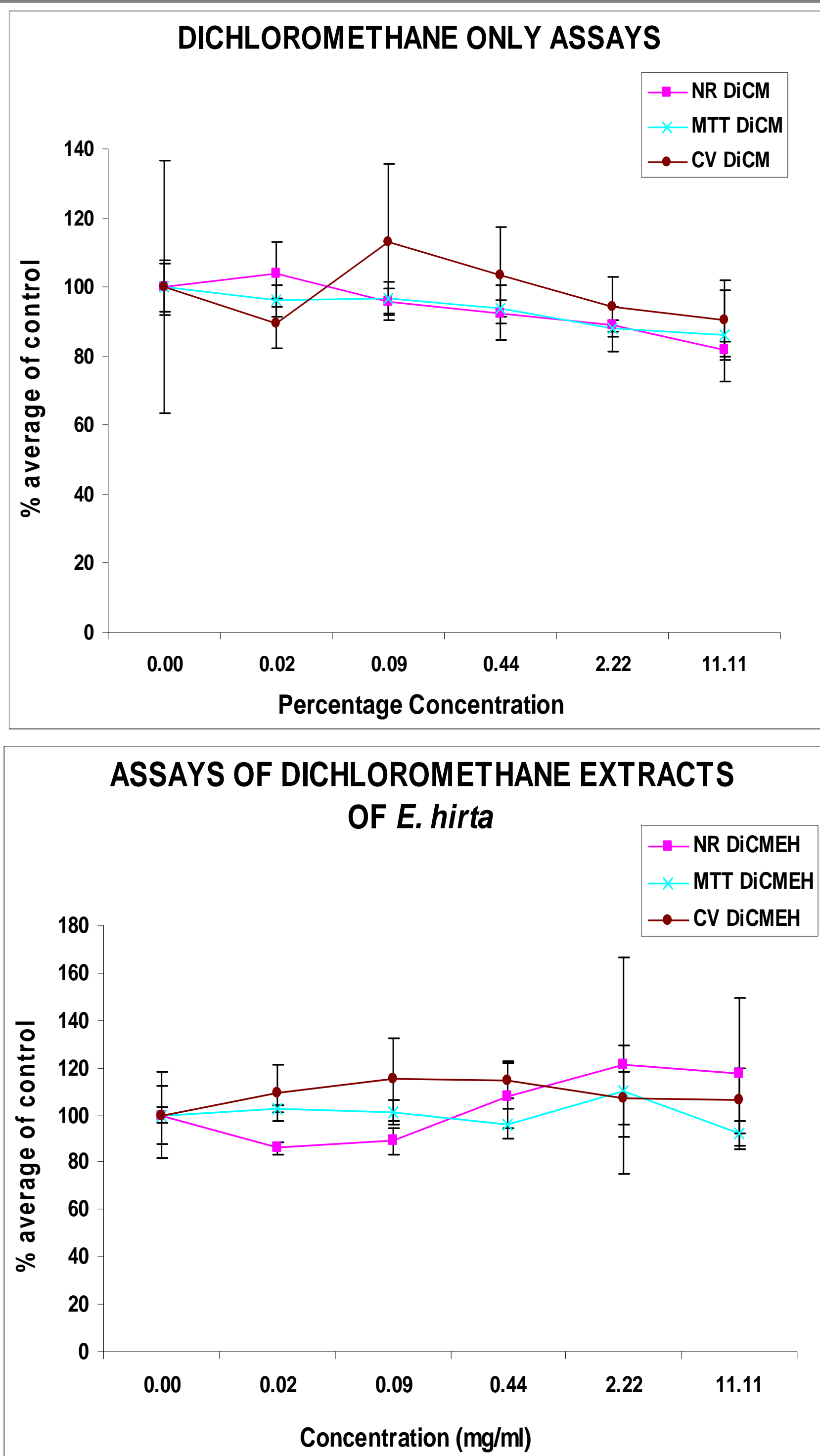


Figure 3.5: The effect of **(a)** 0-11.11% DCM and **(b)** 0-11.11 mg/ml DCM extract of *E. hirta*, on lysosomal membrane integrity, cell viability and number measured using the NR/MTT/CV assay. Data expressed as mean (n=2) ± SD. *No significant differences at all concentrations.

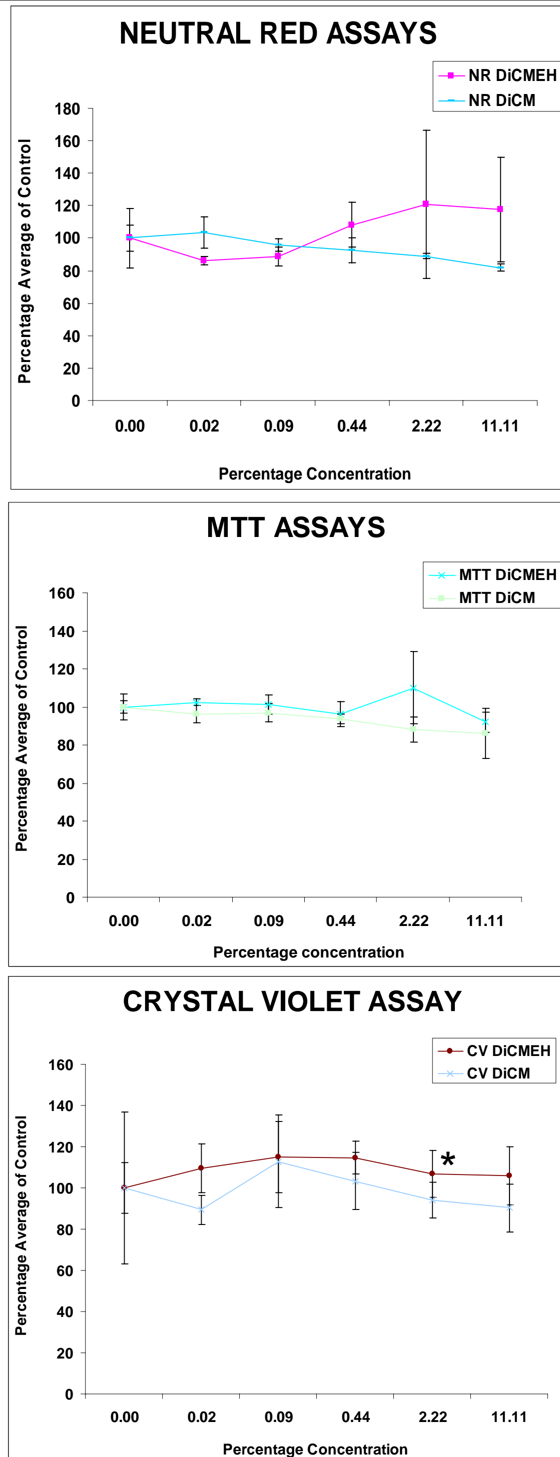


Figure 3.6: Comparison of the effects of 24-hour exposure to the carrier DCM and DCM extracts of *E. hirta* on the MRC-5 cell line; **a)** NR, **b)** MTT and **c)** CV assay. * Differences significant at $p \leq 0.05$ compared to the highest concentration.

3.6.5. Effects of hexane and hexane extracts of *E hirta* on MRC-5 cells

In most plant extracts, hexane extracts mainly contain terpenes, terpenic alcohols, terpenic aldehydes and ketones, xylene, dimethylformamide, and hexadecanoic acid (Quinn et al., 2007). The hot hexane extract of *E. hirta* was found to contain alpha and beta amyrin, sitosterol and taraxerone (Atallah and Nichola, 1972).

MRC-5 cells were exposed *in vitro* to 0-11.11% hexane alone as well as 0 - 11.11mg/ml hexane extracts of *E. hirta* for 24 hours. The effects of the hexane solvent alone on lysosomal membrane integrity, cell viability and number were determined using the combined NR/MTT/CV assays. Each assay in the two different experiments was tested for possible statistically significant differences between the control (no hexane or hexane extract of *E hirta*) and the different concentrations added. A two way ANOVA was used to determine differences between hexane alone and hexane *E. hirta* extract.

The one-way ANOVA test showed that treatment of MRC-5 cells with the hexane solvent alone did not show statistically significant differences between the different concentrations for the NR, MTT and CV assays (*Figure 3.7a*). Similarly, treatment with the hexane *E hirta* extract showed no significant differences at all concentrations for all three assays following (*Figure 3.7b*).

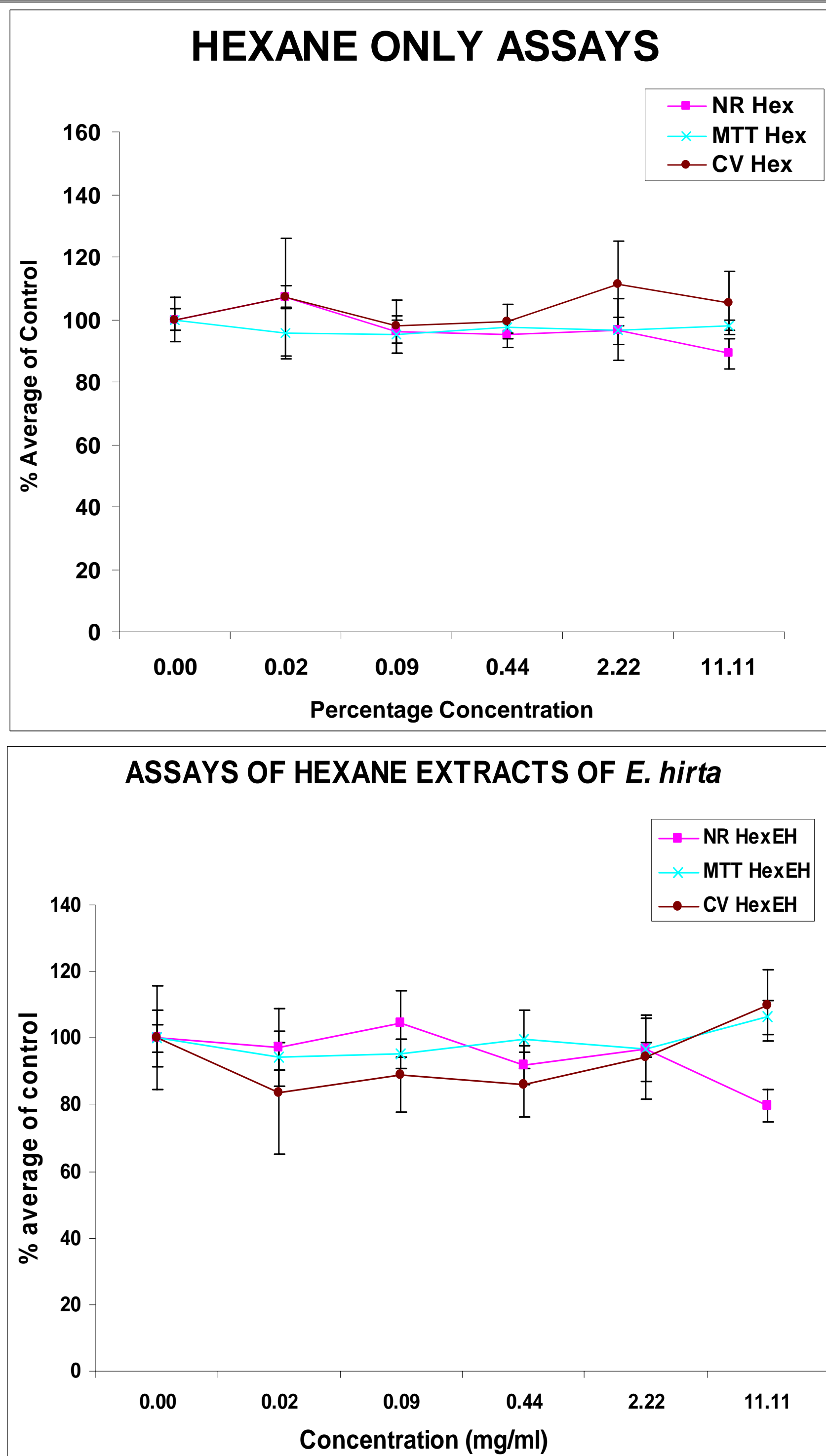


Figure 3.7: The effect of **(a)** 0-11.11% carrier hexane and **(b)** 0-11.11 mg/ml hexane extract of *E. hirta* on lysosomal membrane integrity, cell viability and number measured using the NR/MTT/CV assay. Data expressed as mean (n=2) ± SD. No significant differences at all concentrations.

Comparison between the hexane-treated MRC-5 cells and those treated with the hexane extract of *E. hirta* was done for the different assay types (*Figure 3.8a-c*) using the 2-way ANOVA analysis. Corresponding NR values showed statistically significant differences ($p \leq 0.05$) at all the concentrations compared. On the other hand, no statistically significant differences were observed between the hexane only and the hexane extract treatments in the MTT assays except at 2.22% concentration. This appears to indicate that the effects of MTT were relatively similar in both treatments.

For the CV assays, all percentage average of control values in the hexane only treatment were higher than corresponding values of the hexane extract treatment at the same concentration range (except the highest concentration), as shown in *Figure 3.8c*. The differences between these values were also statistically significant ($p \leq 0.05$) except at the highest percentage concentration (11.11%). At this concentration, the value of the percentage average of control for the 'hexane only' treatment decreased while that for the hexane extracts treatment increased.

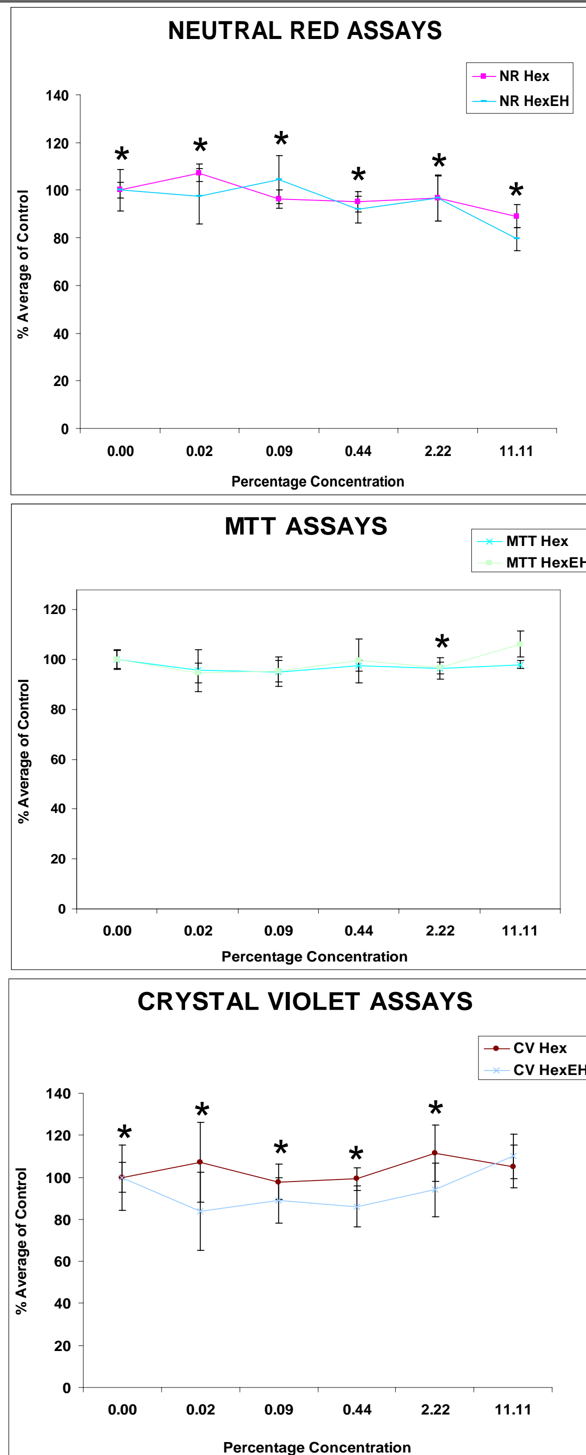


Figure 3.8: Comparison of the effects of 24-hour exposure to hexane and hexane extracts of *E. hirta* on the MRC-5 cell line; **a)** NR, **b)** MTT and **c)** CV assay.* Differences are significant ($p \leq 0.05$) as indicated for hexane only, compared to the hexane extract.

3.6.6. Analysis of the effects of the different solvents, compared with their *E. hirta* extracts and solvent extracts compared with water extract.

A summary of all the effects of treatment of MRC-5 cells with different solvents and extracts of *E hirta* as determined using the NR/MTT/CV assays is given in *table 3.1:*

Table 3.1: Effects of all solvent and *E hirta* extractas determined by NR/MTT/CV assays.				
Solvent only				
Assay	**Water**	**Acetone**	**DCM**	**Hexane**
NR	NA	NSD	NSD	NSD
MTT	NA	NSD	NSD	NSD
CV	NA	SD (⇑ highest conc only)	NSD	NSD
***E hirta* Extract**				
Assay	**Water**	**Acetone**	**DCM**	**Hexane**
NR	NSD	NSD	NSD	NSD
MTT	SD (⇓ highest conc only)	NSD	NSD	NSD
CV	SD (⇑ highest two concs. only)	SD (⇑ lowest conc only)	NSD	NSD
Solvent versus solvent *E. hirta* extract				
Assay	**Water**	**Acetone**	**DCM**	**Hexane**
NR	NA	SD (⇑ in the 2nd lowest conc only, compared to ActEH)	NSD	SD in all (⇑ for hexane in all except 2nd lowest conc)
MTT	NA	NSD	NSD	SD (⇓ for hexane; 2nd highest conc. only)
CV	NA	SD (⇑ 2nd and 3rd highest concs only, compared to ActEH)	SD (⇑ in the 2nd highest conc only, compared to DCM)	All SD except highest conc (⇑ for hexane)

Key: ⇓ **= decrease** ⇑ **= increase**

3.6.6.1. Comparative effects in the NR assays

Only the differences between the effects of acetone and the acetone extract at the second highest concentration (2.22mg/ml) as well as all comparisons between hexane and hexane extracts were statistically significant ($p \leq 0.05$). Comparisons between hexane and hexane extracts all showed statistically significant differences.

3.6.6.2. Comparative effects in the MTT assays

Only the highest two concentrations (0.118 mg/ml and 0.588 mg/ml) of the aqueous extracts of *E. hirta* were significantly different ($p \leq 0.05$) when compared to the control. In addition, the second highest concentrations (2.22 mg/ml) of the hexane and hexane extract of *E. hirta* showed statistically significant difference ($p \leq 0.05$).

3.6.6.3. Comparative effects in the CV assays

Most of the comparisons in the CV assays showed statistically significant differences. Statistically significant difference ($p \leq 0.05$) was observed in the acetone only treatment when the highest concentration was compared with the control as well as in the acetone extracts treatment when the lowest concentration was compared with the control. The highest two concentrations in the aqueous extract treatment also showed significant differences ($p \leq 0.05$) compared with the controls. In the hexane only treatment, all comparisons except at the highest concentration showed statistically significant differences ($p \leq 0.05$). In addition, only the second and third highest concentrations in the acetone versus acetone extract as well as the second highest concentration in the dichloromethane versus

dichloromethane extract comparisons showed statistically significant differences ($p \leq 0.05$).

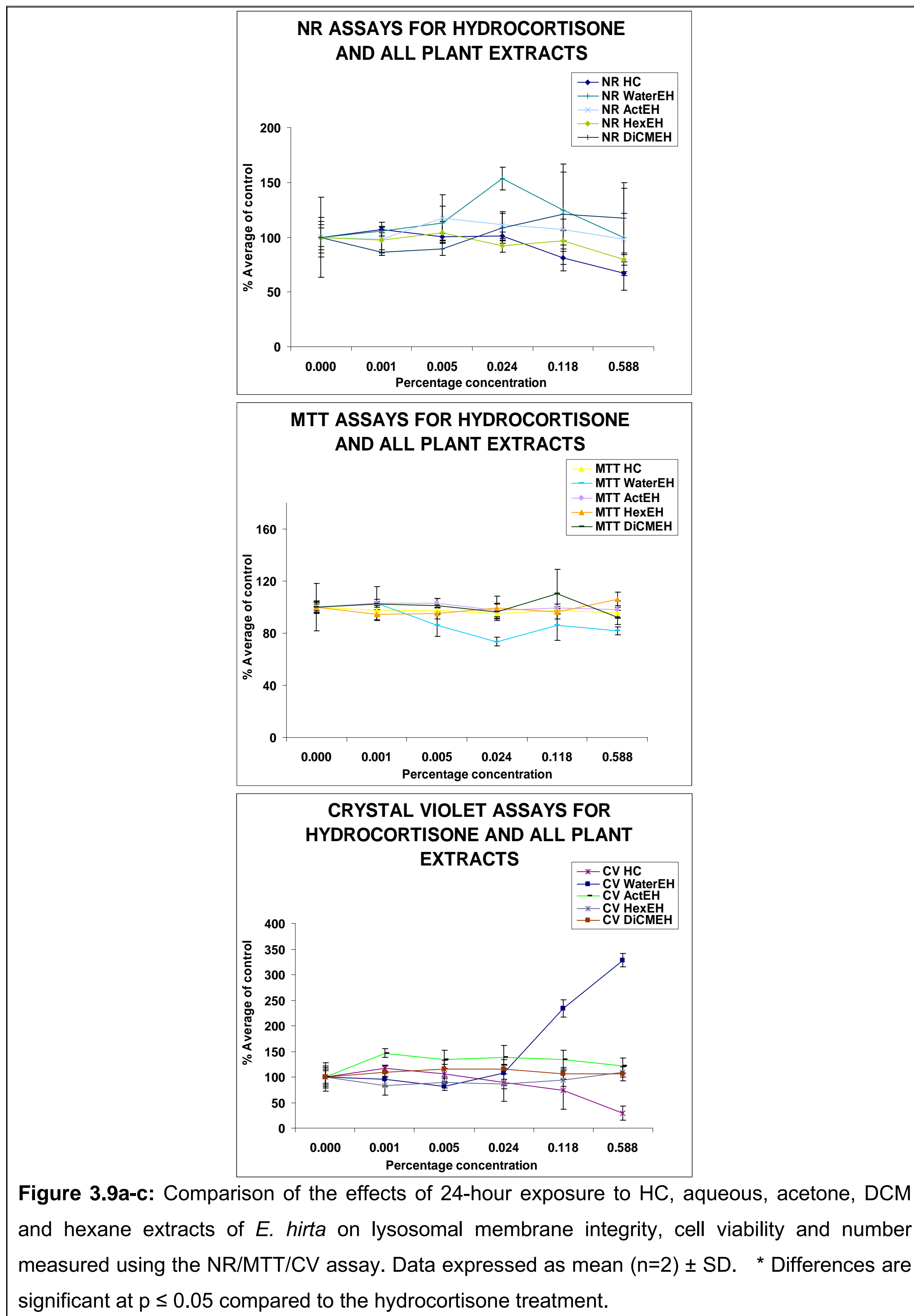


Figure 3.9a-c: Comparison of the effects of 24-hour exposure to HC, aqueous, acetone, DCM and hexane extracts of *E. hirta* on lysosomal membrane integrity, cell viability and number measured using the NR/MTT/CV assay. Data expressed as mean (n=2) ± SD. * Differences are significant at $p \leq 0.05$ compared to the hydrocortisone treatment.

3.6.7. Comparison between all organic solvent extracts and the aqueous extract of *E. hirta*.

At all concentrations and in all three assay systems used in this study, all the organic solvent (acetone, DCM and hexane) extracts used in this study generally showed statistically significant differences ($p \leq 0.05$) when compared with the aqueous *E. hirta* extract (*Figure 3.9a-c*). Some differences in the NR and CV assays in the acetone and water extract comparisons were however not significant at some concentrations (*table 3.2*).

NR values were relatively higher at most concentrations of all solvent extracts, indicating less toxic effects on lysosomal membrane integrity. However, most of the values for the MTT were much lower at all concentrations, indicating lower cell viability with the aqueous *E. hirta* extract treatment.

In the CV assays, there appeared to be a spike in cell numbers (that significantly differed from results obtained in the CV assays with organic solvent extracts) following addition of 2.22 mg/ml and 11.11 mg/ml aqueous *E. hirta* extract. The observed spike in cell numbers could not possibly be due to cell number increase and so the aqueous *E. hirta* extract could be said to have similar effects on cell number as other extracts.

3.6.8. Comparison between treatment with HC and treatments with all three organic solvent extracts of *E. hirta*.

Most herbal preparations of *E. hirta* used by traditional healers for the treatment of asthma are in aqueous solution, hence the need to investigate the effects of the aqueous extract. The organic solvent extracts were used in this study to determine their suitability for use. Different organic solvents may have effects that differ greatly as observed in previous studies (Jung et al., 2006), from the effects of the water extract or known anti-inflammatory medications like HC.

The effects of each of the different *E. hirta* extracts used in this study were compared with the effects of HC on the MRC-5 cells for every concentration of each assay type. The two-way ANOVA test was used to determine statistically significant differences (at $p \leq 0.05$) while graphs of the percentage averages of control plotted against concentrations (*figure 9a-c*) were used to compare the effects of the treatments.

3.6.8.1. Comparative effects in the NR assay

Figure 3.9a shows that for the NR assays, the hexane extract followed by the acetone extract, had effects that were similar in pattern to the effects of HC at all concentrations. The water extract showed a wide variation in pattern while the dichloromethane extract showed a sigmoid graphical pattern.

The observed graphical differences were tested statistically and results showed that only the differences between the HC and the aqueous extract treatments at all concentrations were statistically significant. The acetone extract showed significant differences only in the second lowest concentration; the hexane extract

showed significant differences between in the two lowest concentrations while the dichloromethane extract showed significant differences only in the two highest concentrations (*table 3.2*).

3.6.8.2. Comparative effects in the MTT assay

The graphs for the MTT assays (*figure 3.9b*) show similar graph patterns for both the acetone and hexane extracts as HC for all concentrations. The water extract showed a marked variation in pattern while the dichloromethane extract only showed variation in pattern at concentration 0.118mg/ml that later normalized at the highest concentration. In spite of the observed similarities in graphical patterns to the HC treatment, statistical analysis showed significant differences between the HC treatment and treatment with all the plant extracts at the different concentrations (*table 3.2*).

3.6.8.3. Comparative effects in the CV assay

The graphs of the acetone and the dichloromethane extract treatments at all concentrations show relative similarity in the patterns for the CV assays (*figure 3.9c*). In addition, only the graphs of these two treatments appear to resemble the HC graph. The water extract had a pattern that widely varied from the HC graph like in the other two assays while the hexane extracts had a graph pattern that differed from all other treatments.

Statistical analysis showed significant differences mostly in the two highest concentrations for all the three plant extract treatments relative to the HC treatment (*table 3.2*). In case of the aqueous extract, the lowest concentrations also showed significant differences when compared. Similarly, the differences

between HC and the hexane and DCM extracts treatment were also significant at the third lowest concentrations.

3.6.9. Comparative effects between the three organic solvent extracts

Comparisons between the different organic solvent extracts of *E. hirta* showed differences that were not statistically significant at $p \leq 0.05$ in most cases. Such differences were observed mostly in comparisons between the other two extract solvents and the DCM extract. Comparison between acetone and hexane showed only one significant difference at the lowest concentration. Other comparisons are given in *table 3.2*.

Table 3.2: Summary of the comparative effects of different *E hirta* solvent extracts as well as HC, as determined by the NR/MTT/CV assays.

Aqueous versus Solvent Extracts of *E hirta*				
Assay	**Water**	**Acetone vs. Water extract**	**DCM vs. Water extract**	**Hexane vs. Water extract**
NR	NA	SD (⇑ highest 3 concs. only)	SD (All)	SD (All)
MTT	NA	SD (All)	SD (All)	SD (All)
CV	NA	SD (⇑ lowest 2 concs. only)	SD (All)	SD (All)
***E hirta* Solvent Extracts compared**				
Assay	**Water**	**Acetone vs. DCM**	**Acetone vs. Hexane**	**Hexane vs. DCM**
NR	NA	SD (highest 3 concs only; ⇑ for DCM)	NSD	SD (highest 3 concs only;⇑ for DCM)
MTT	NA	SD (2nd highest conc only; ⇑ for DCM)	SD (lowest conc. only;⇑ for ACT)	SD (highest 2 concs; lowest conc. only;⇑ for DCM)
CV	NA	SD (highest conc. only;⇓ for DCM)	NSD	SD (lowest conc. only;⇑ for DCM)
Aqueous and Solvent *E. hirta* extracts versus HC				
Assay	**Water vs. HC**	**Acetone vs. HC**	**DCM vs. HC**	**Hexane vs. HC**
NR	SD (All)	SD (2nd lowest conc. only; ⇑ for ACT)	SD (highest 2 concs. only;;⇑ for DCM)	SD (lowest 2 concs. only;⇑ for HEX)
MTT	SD (All)	SD (All)	SD (All except 2nd lowest conc.;⇑ for DCM)	SD (All)
CV	SD (All except the 2nd and 3rd lowest concentrations;⇑ for water)	SD (highest 2 concs. only;⇑ for ACT)	SD (highest 3 concs. only;⇑ for DCM)	SD (highest 3 concs. only;⇑ for HEX)

Key: ⇓ **= decrease** ⇑ **= increase**

3.7. Discussion

Approximately 80% of the people in the world's developing countries rely on traditional medicines for their primary health care needs, and about 85% of traditional medicine involves the use of plant extracts (Vieira and Skorupa, 1993). Since most of these traditional plant remedies are used without expert prescriptions, there is need for scientific validation as well as *in vitro* and/or *in vivo* evaluation to understand their claimed effectiveness. Over the years, cell culture systems have provided the means for such scientific validation, commonly by evaluating medicinal plants for cytotoxicity (Zirihi et al., 2005).

The MTT assay is based on the reduction of the yellow coloured 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) by the enzyme mitochondrial dehydrogenase of metabolically active cells to a blue formazan which can be spectrophotometrically measured. Using the MTT colorimetric assay, the percentage of survived cells after treatment with the plant extract could be determined. Thus, the MTT assay measures cell viability.

The neutral red (NR) assay depends on the uptake of the neutral red dye solution into the lysosomes of viable cells and this uptake is proportional to the number of viable cells within the well and is usually expressed as percentage uptake by control cells. Thus, the NR assays measure lysosomal membrane integrity. The CV assay is a simple assay useful for obtaining quantitative information about the relative density of cells adhering to multi-well cluster dishes. The crystal violet dye stains DNA and upon solubilization, the amount of dye taken up by the monolayer can be quantitated in a spectrophotometer or plate reader. Thus, the CV assay measures protein levels and is an indirect measure of the cell number.

This study was carried out to test for the cytotoxic effects of the different extracts of *E. hirta* on the MRC-5 cell line using the NR, MTT and CV bioassay systems. The aqueous and organic solvent extracts (acetone, hexane and dichloromethane) were used. 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. In addition, the effects of the different extracts were compared with the effects of HC.

HC was chosen as a pharmaceutical control for the plant extract because of its known anti-asthma effects. The pilot study showed that HC like other treatment agents caused statistically significant differences between most of the concentrations in the NR assay indicating that its effect on lysosomal membrane integrity was concentration dependent.

The differences observed between the concentrations in the MTT assays of most of the treatments in this study were not statistically significant, implying that cell viability was not dependent on concentration difference. The crystal violet (CV) assays however showed statistically significant differences between concentrations mostly in the water, HC and acetone treatments, indicating that cell number differences could be concentration dependent.

The concentration dependence of the effects of treatment agents in bioassay systems has been previously studied. Findings from this particular study are similar to many reported findings from previous studies. In a study by Castro-Garza et al., (2007) the effects of Triton X-100 (known to dissolve the cell membrane leading to cell death) on a macrophage (THP-1 cell line) monolayer were investigated. The study found that the effects depended on the concentration

used, starting with a 0.05% concentration and reaching a maximum at 0.1%, measured with the CV assay. Another study by Greil et al., (1999) reported that a polyvinyl chloride (PVC) extract concentration of 15% killed MRC-5 cells completely but did not cause a decrease in the neutral-red uptake of the MRC-5 cells in the NR test. In addition, PVC-extract concentrations of 4.5% or less showed no cytotoxicity on MRC-5 cells in the MTT test.

The biological effects of the individual organic solvents used to prepare the plant extracts (acetone, dichloromethane and hexane) appear to differ from the effects of the plant extracts prepared with them. Results from this study show that acetone treatment differed significantly ($p \leq 0.05$) from treatment with the acetone extract of *E.hirta*. Similar results were observed for the hexane solvent versus hexane extract comparisons but the differences between the dichloromethane solvent and the DCM extract were not statistically significant.

A previous study showed that out of all the active solvent extracts, the DCM extracts of *Baccharis grisebachii* and *Pluchea sagittalis* had the greatest inhibitory activity (Pérez-García et al., 2001). The cells were stimulated using hydrogen peroxide, phorbol-12-myristate-13-acetate (PMA) or formyl-methionyl-leucyl-phenylalanine (FMLP) for reactive oxygen species (ROS) generation and sodium nitroprusside (SNP) or PMA for reactive nitrogen species (RNS) generation

In the present study, the effects of the different organic solvent extracts were also found to differ significantly ($p \leq 0.05$) from those of the aqueous extract. Observations of differences in effects of the different extracts of the same plant as seen in this study are consistent with previous studies. One such study by Šipailienė et al., (2005) showed that the methanol extract had the most effective

antimicrobial activity followed by the acetone extracts whereas the aqueous extracts either showed the lowest effectiveness or did not possess inhibitory effect in all eight bacterial test cultures studied.

3.8. Conclusion

At the concentrations used in this study, treatment of MRC-5 cells with HC in the pilot study showed toxic effects with increasing concentration. On the other hand, acetone, dichloromethane and hexane solvents showed very limited toxic effects on the MRC-5 cells *in vitro*, and less toxic effects were observed when the cells were exposed to the acetone, dichloromethane and hexane extracts of *E. hirta*. However, the aqueous extract showed the least toxic effects compared to all three solvent extracts.

The above results prove the hypothesis set for this cell culture study. Further studies done with different assay probes at different concentrations of the plant extracts or using the individual active compounds, as well as mechanism-of-action studies will be required.

CHAPTER FOUR

Animal Experiments and Weight Studies

4.1. Introduction

The use of animals for modelling human diseases has been considered in the literature review chapter and the various procedures for inducing airway reaction to allergenic stimulation have been reported in literature. The wide variety of available sensitization and challenge protocols as well as the time point of assessment and readout systems has been reviewed by Kumar and Foster (2002). In addition, a comprehensive review of murine models of asthma by Kips et al., (2003) considered a number of critical factors that may affect experimental modelling of asthma in animals by introducing variation in results obtained.

The age of animals or differential response of the airways to various chemical agents used during the sensitization and challenge (nebulization) stages of the experiment could cause such variation. Also important as other sources of variation are the strain of mice used as well as the route and timing of exposure to allergen (Kips et al., 2003).

The procedures used in evaluating the experimental outcomes of modeled asthma also vary slightly depending on researcher discretion and aim of experiment, all targeted at establishing the usefulness of the asthma mouse model. Epithelial shedding, airway smooth muscle hypertrophy and hyperplasia, overproduction of mucus and airway inflammation have all been examined in different previous studies. In some of the review articles studied, there seemed to be suggestions that separate modelling of the various phenotypes or traits of asthma may be better than attempting to model the entire disease in a single experiment. Kips et al., 2003 concluded that the specific characteristics of the various asthma phenotypes could be clearly defined if modeled separately.

Besides the plethora of research on the inflammation aspect of asthma, studies that focus on other phenotypes of asthma such as studies on the ultrastructure of airway epithelium, shedding of epithelium (Shahana et al., 2003; Shebani et al., 2005) as well as airway remodelling (Bischof et al., 2003; Snibson et al., 2005) have been reported.

4.1.1. Aim of study

The aim of this present study was therefore to induce asthma in BALB/c mice using a previously described procedure (albeit with slight variations) and attempt to treat the condition with the aqueous extract of *E. hirta*. The focus in this chapter will be to determine the effects of hydrocortisone as well as the aqueous extract of *E. hirta* on progressive changes in the body weights of the BALB/c mice as this reflects the general wellbeing of the animals.

4.2. Materials and methods

4.2.1. Materials

4.2.1.1. BALB/c Mice

A total of sixty (60) six-week-old male BALB/c mice (mean weight 20g) were used in this study. These mice have previously been used to investigate a number of parameters involved in asthma (Bice et al., 2000).

4.2.1.2. Hydrocortisone (HC)

HC was used in this study as a pharmaceutical control for the extracts of *E. hirta* as used in the cell culture study. One hundred milligrams (100mg) of the sterile powder (Brand name Solu-Cortef®) which contains HC sodium succinate as the active ingredient was dissolved in 2 ml of bacteriostatic injection water, giving a

concentration of 50mg/ml. In this study, a high dose (125mg/kg) and a low dose (100mg/kg) of the 50mg/ml HC preparation were used. Fresh solution was prepared for each injection.

4.2.1.3. *Euphorbia hirta*

Only the aqueous plant extract of *E. hirta* was used for the animal studies. The plant material was collected in the Gezina region of Pretoria South Africa. A herbarium specimen was prepared and compared to an authentic specimen in the HGJW Sweikerdt herbarium at the University of Pretoria. The aboveground parts of the plant were allowed to dry at room temperature for one week in the Cell Biology laboratory of the Department of Anatomy, University of Pretoria, and the material was grounded into a fine powder. Shortly after, 50 grams of the sample was extracted in 500ml of double distilled water after which it was filtered, and dried on a rotary evaporator at 40 °C. A stock aqueous solution of 50mg/ml plant extract was prepared and stored in a fridge until used.

The doses of 62,5mg/kg and 25mg/kg were prepared from the stock solution and administered orally to the mice. This implied that each animal (average weight 20g) received 0.01 ml of plant material. This dose was decided upon after studying literature that mentioned physiological doses suggested by herbalists. Typically, a teaspoon of the herb is added to a teacup volume of water and allowed to simmer for 20 minutes (Lindsey et al., 2002). Alternatively, an extract of the plant could be prepared and the recommended adult dose range of the fluid extract is 0.2-0.3ml, taken three times daily and of the infusion, 120-300mg three times daily (Skidmore-Roth, 2001).

4.2.1.4. Reagents and equipment

The reagents and equipments used for this study include ovalbumin (OVA) purchased from Sigma-Aldrich Co., Phosphate Buffered Saline (PBS), alum, NaCl, KCl, $Na_2HPO_4.H_2O$, KH_2PO_4, DPSS, KLAVA ultrasonic nebulizer, oral-pharyngeal canula, injection syringes and needles.

4.2.2. Methods

This study was divided into short-term (ST) and long-term (LT) phases to assess both initial and advanced effects of the induced asthma on body weight changes, blood cell count, airway activity, airway morphology, among other characteristics.

4.2.2.1. Animal care and grouping

All the mice used in this study were obtained from the Biomedical Research Centre (Faculty of Veterinary Sciences of the University of Pretoria) and maintained in a pathogen-free environment at the Onderstepoort Animal Care facility. A temperature range of 20-24°C, a relative humidity of 40-60% and a 12-hour day light and 12-hour night were maintained. Polycarbonate Type III cages were obtained from Tecniplast and only one mouse was housed per cage containing autoclaved wood shavings as bedding and elite white facial tissue paper as enrichment (to reduce male mouse aggressive behaviour during handling). Animals were provided with OVA-free food (Balanced EpolT mice cubes and pellets obtained from EPOL - a division of Rainbow Farms PTY LTD, South Africa) and pre-boiled tap water was made available *ad libitum,* one bottle per cage. Mice were allowed to acclimatize for seven days before the experiments commenced. All experimental protocols complied with the requirements of the University of Pretoria's Animal Use and Care Committee (UPAUCC).

The mice were randomly assigned to two main groups, each for the short-term (ST) and (LT) long-term phases of the study. Each main group consisted of thirty animals and further assigned randomly into six sub-groups (each containing 5 mice) according to the treatment to be given – control (CT), asthma (AS), high HC-treated (HHC), low HC-treated (LHC), high *E. hirta* – treated (HEH) and low *E. hirta* - treated (LEH) groups respectively. Altogether, there were twelve groups as listed in *table 4.1*

Table 4.1: Groups for the short-term and long-term studies.

Groups for the short-term studies	
Control (CT)	5 mice; pure controls; neither sensitized nor challenged.
Asthma (AS)	5 mice; asthmatic controls; sensitized and challenged but not treated.
High HC (HHC)	5 mice sensitized and challenged, treated with high dose of HC.
Low HC (LHC)	5 mice sensitized and challenged, treated with low dose of HC.
High EH (HEH)	5 mice sensitized and challenged, treated with high dose of *E. hirta* plant extract.
Low EH (LEH)	5 mice sensitized and challenged, treated with low dose of *E. hirta* plant extract.
Groups for the long-term studies	
Control (CT)	5 mice; pure controls; neither sensitized nor challenged.
Asthma (AS)	5 mice; asthmatic controls; sensitized and challenged but not treated.
High HC (HHC)	5 mice; sensitized and challenged mice, treated with high dose of HC.
Low HC (LHC)	5 mice; sensitized and challenged mice, treated low dose of HC.
High EH (HEH)	5 sensitized and challenged mice, treated with high dose of *E. hirta* plant extract.
Low EH (LEH)	5 mice; sensitized and challenged, treated with low dose of *E. hirta* plant extract.

4.2.2.2. Experimental procedure

The process of inducing asthma involved sensitization (immunization) and nebulization (allergen challenge). The study was extended beyond day 18 (the long-term phase) to evaluate the effects of a much longer exposure of the mice to

the test agents (HC and *E. hirta* extract). All animals except those in the CT group were sensitized and nebulized before treatment with the two test agents. Asthma was induced in the AS group and the animals were left untreated whereas animals in all other test groups were treated with HC or *E. hirta* extract after exposure to the same asthma-inducing conditions as the animals in the AS group.

4.2.2.2.1. Sensitization

Sensitization (also described as immunization) is the procedure that prepared the immune system of the animals for the subsequent exposure to allergens in the nebulization chamber. Sensitization was done on days 0 and 5 respectively via intraperitoneal injection of a solution of 25mg OVA emulsified in 2mg aluminium hydroxide [Al $(OH)_3$] and dissolved in 0.5ml of 0.9% saline solution. All mice except those in the control and asthma groups (CT for LT and ST) were sensitized and all sensitized animals were allowed a duration of one week before exposure (challenge).

4.2.2.2.2. Nebulization

The nebulization procedure served to challenge the sensitized immune system of the animals to the presence of allergens. This procedure was carried out one week after immunization and involved placing the mice (except those in the pure control groups) in a Plexiglas chamber and exposing them to fumes generated via a KLAVA ultrasonic nebulizer from a 1% OVA in PBS solution (1mg OVA in 100 ml PBS).

In order to induce acute onset of asthma, eligible mice were nebulized for two consecutive 30-minute periods daily with one-hourly intervals on days 13, 14 and

15 for the short-term groups; and repeated on days 34, 35 and 36 for the long-term groups. The animals were carefully observed and monitored for basic asthmatic symptoms (wheezing and difficulty to breath) shortly after nebulization up to 10 minutes before treatment. Blood cell count was carried out as an additional source of proof of the presence of asthma.

4.2.2.3. Administration of the test agents

HC dosages of 75mg/kg (LHC) and 125mg/kg (HHC) were used in this study and administered to the mice was used in this study and administered to eligible short-term and long-term animal study groups via intraperitoneal injection. On the other hand, the HEH (62.5mg/kg) and LEH (25mg/kg) dosages of the plant extract were administered orally to the mice in designated groups. Each animal received the same dose of the treatment agents twice daily, with an hour' interval allowed between treatments.

For groups involved in the short-term studies, administration of treatment agents was done on days 15 (about 30 minutes after last nebulization), 16 and 17. Animals involved in the short-term studies, were terminated on day 18 to end the short-term studies. For groups involved in the long-term studies, administration of treatment agents was on days 15 (about 30 minutes after last nebulization), 16, 17, 18, 22, 25, 29 and 32. Treatment was stopped after day 32 to repeat nebulization (on days 34, 35, 36) and was continued daily for one week from days 39, 40, 41, 42, 43, 44 and 45.

A number of factors informed the choice of reference days on which to analyze and study the possible effects of the different experimental interventions. The selection took into account the days on which animals were sensitized (days 0 and

5), nebulized (days 13-15) as well as treated (days 15-17 for ST studies; days 22, 25, 29, 32, 39, 40, 41, 42, 43, 44, 45 for the LT studies respectively).

The reference days chosen for analysis included days 6, 13, 15, 18, 26, 32, 37, 42 and 46, as shown in *table 4.2*.

Table 4.2: Day of weighing and expected effects on weight changes

DAY	Expected effects of procedures on weight changes
1	Baseline weight
6	Early effects of immunization
13	Late effects of immunization (just before first nebulization)
15	Effects of first nebulization
18	Effects of first batch of treatment on nebulization (just before sacrifice)
26	Midterm effects of continuous treatment
32	Late effects of continuous treatment
37	Acute effects of repeated nebulization on weight changes
42	Early effects of post-nebulization treatment on weight changes
46	Terminal effects of post-nebulization treatment on weight changes

All treated animals were also observed for basic asthmatic symptoms (wheezing and difficulty to breath) shortly after nebulization, 10 minutes before treatment as well as one hour after each treatment exposure. As one of the criteria for the assessment of general health, animals were weighed at the beginning of the experiment and daily before any other routine procedures were carried out. Weighing continued until day 46 of the long-term study when the last batch of animals was terminated. The mean weights for all the groups on each day were expressed in grams ± standard deviation.

Mean weight values on day 1 were considered baseline weights and a 2-tailed paired-sample t-test was used to determine differences between the baseline

weights and other weight values recorded on all the reference days in each group. Since the starting weights varied between the groups, mean weight values per group per day were converted into percentages of the starting weights and the respective percentage weight differences (PWDs) were determined relative to the starting or baseline weights. This offered a more appropriate means of determining weight differences between groups as opposed to using the original weight values. Values for the percentage weight differences from baseline weights (PWDs) were obtained using the formula:

$$\mathbf{PWD} = \frac{[\text{Mean weight}_n - \text{Mean weight}_b]}{\text{Mean baseline weight}_b} \times 100;$$

Where:

Mean weight_n = mean weight of specified group on selected day and

Mean weight_b = mean baseline weight on the specified group.

At the end of the different stages of the experiment, animals were terminated by skilled UPBRC technical personnel on the morning of days 18 (ST) and 46 (LT) either by bleeding them to death or via cervical dislocation depending on whether a lot of blood was required for coagulation studies or not. During the course of the experiment, only one out of the five animals in the AS (LT) group died on day 1 and pathology report did not associate the cause of death with the experimental procedures. A second animal in the low HC (ST) group was perceived to be unhealthy by the technical personnel and was thus excluded from the experiment and terminated on experimental day 4.

4.3. Results and discussion

4.3.1. General effects of HC and *E. hirta* on asthmatic mice

Asthma was induced in BALB/c mice using OVA, the mice were then treated with high and low dosages of HC and *E. hirta.* Basic asthmatic symptoms including reduced physical activity, general discomfort, difficulty of breathing and wheezing, were made throughout the study by the principal investigator.

Although these symptoms were present in most of the animals just after the nebulization, these symptoms gradually eased out. Greater improvement was however observed after about 30 minutes following treatment with either HC or the plant extract.

4.3.2. Effects of HC and *E. hirta* on body weights of asthmatic mice

The co-existence or relationship between airway inflammation (including that seen in asthma) with either excessive body weight, anomalous body mass index, or obesity has been reported (Camargo et al., 1999; Hakala et al., 2000; Aaron et al., 2004; Weiss and Shore 2004; Beuther et al., 2006), but the underlying mechanisms remain obscure. Although it is known that obesity worsens asthmatic conditions, the cause-effect relationship (i.e. whether one of these two conditions can lead to the other) is not understood.

Shore (2006) in a review article, argued that the relationship between asthma and weight changes as reported in most previous studies, does not address the direction of causality, adding that one possible interpretation of these studies could be that asthma leads to obesity, perhaps because asthmatics adopt a sedentary lifestyle to avoid respiratory symptoms during exercise. A different opinion by

Hayman (2006) was that any factor that causes inflammation could lead to weight gain that could in turn lead to more inflammation.

In adult humans, a reduction in excessive body weight by medical treatment and surgical procedures has resulted in a reduction of asthma symptoms, medication usage, and severity, and an improvement of lung function, indicating a possible causal relationship (Macgregor and Greenburg, 1993; Dixon et al., 1999; Stenius-Aarniala et al., 2000).

Animal models of mice, guinea pigs, rats, dogs, cats, monkeys, sheep, and horses have been developed to study disease pathogenesis and for drug discovery (Epstein, 2004a, b). 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). BALB/c mice were used in this study primarily because so much is already known about their immune responses and genetics from literature. Results from previous studies have shown that despite a few shortcomings, the BALB/c mouse model still manages to paint a better pattern of the human airway disease 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).

Animal models of weight studies have also been reported in literature (Harris et al., 1998; Retana-Ma´rquez et al., 2003; Snibson et al., 2005) and the use of different plant extracts or herbal preparations in managing weight changes have also been reported. For instance, a study using plant extracts showed that the leaves of *Syzygium cordatum* did not cause any weight changes in diabetic rats (Musabayane et al., 2005) unlike the significant weight gains reported in

ovariectomised mice treated with extracts from the plant *Onobrychis ebenoides* (Dontas et al., 2006). Another study by Kyungah et al., (2004) showed that different medicinal plant extracts did not have significant effect on the body weight compared to the control group.

In this study, the possible effects of the plant extract *E. hirta* on asthma and weight change was investigated. *E. hirta* (Euphorbiacea) is found worldwide and in many parts of Africa. Extracts or a decoction of the flowering and fruiting plant have long been used (and are still being used) in East and West Africa and elsewhere for the treatment of many conditions (Oliver 1959; Hazleton and Hellerman 1954; Watt and Breyer Brandwijk 1962; Kokwaro, 1976; Le Strange 1977; Wong 1980; Lanhers 1990; 1991). The medicinal properties of *E. hirta* are possibly due to its content of many active ingredients including alkaloids, flavonoids, glycosides, sterols, tannins and triterpenoids (Gupta and Garg, 1966; Atallah and Nicholas, 1972; Sofowora, 1984; Galvez et al., 1993). Flavanoids are well known to have a high antioxidant activity (Kandaswami and Middleton, 1994).

The (bio) flavonoid in *E. hirta*, Quercitrin (3-rhamnosylquercetin) is usually converted to Quercetin (3-O-alpha-L-rhamnopyranoside - Quercetrin) in the alimentary canal and appears to be the compound that has given this plant its great therapeutic potential. Quercitrin is the glycosylated form of Quercetin and possesses antioxidant as well as anti-inflammatory properties (Comalada et al., 2005). Another flavonoid in *E. hirta,* Myricitrin also seems to be a powerful Nitric Oxide Synthase-inhibiting anti-oxidant. In addition, the sterols 24-methylene-cycloartenol and β-sitosterol have been reported to exert significant and dose-

dependent anti-inflammatory activity while the triterpene β-amyrin also showed anti-inflammatory effects (Martinez-Vazquez et al., 1999).

Free radicals are created in the body as a by-product of energy released by cells. Excessive amounts of free radicals can cause a wide range of diseases but antioxidants help the body fight and neutralize these reactive groups. 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). Since many weight dynamics in especially asthmatics are related to oxidative stress (Fenster et al., 2004; Johnson et al., 2007), the presence of antioxidants in *E. hirta* could have positive effects in eliminating free radicals associated with weight processes in animals treated with extracts of this plant.

The direct or indirect effects of drugs on weight changes have been reported. Sixty percentage of male wistar rats that received cortisone (5 mg/day) were reported to have diminished gain of body weight and total body protein Hausberger and Hausberger (1958). Similarly, a marked decrease in weight was observed in another study in mice receiving HC (Borovitskaya et al., 1971). In addition, following administration of a high-dose of systemic dexamethasone for 3 days, Kumar et al., (1997) reported a marked catabolic effect with weight loss in rats. Another study with Prednisolone showed that the drug caused reduced body weight in mice and guinea pigs (Nagao et al., 2004) while other studies with antipsychotic drugs showed varying results (Ganguli 1999; Goudie et al., 2002). In

line with the above, this current study investigates the possibility of HC and *E. hirta* having effects on weights of asthmatic BALB/c mice.

Day 6 was one day after the last immunization and therefore the acute effects of immunization could be determined on this day. Day 13 was analyzed because any possible effects of the immunization procedure on changes in animal weights could manifest on this day. Similarly, day 15 was considered because it is the last day of the first batch of nebulization and the day when administration of HC and the plant extract commenced. On this day, the acute effects of nebulization on weight changes could be determined. Finally, the weights recorded on day 18 (the last day of the ST studies) could provide information about the effects of all treatments given on days 15, 16 and 17.

Days of analysis for the LT studies included days 18, 26, 32, 37, 42 and 46 since administration of treatment agents continued on days 22, 25, 29, 32 and later days 39, 40, 41, 42, 43, 44, 45. Day 18 was studied to assess the early effects of treatments on animal weights. Day 26 was chosen to assess the midterm effects while day 32 was chosen to assess the late effects of treatments. Since treatment was suspended while the second batch of nebulization was done on days 34-36, treatment effects evaluated after day 36 will be related to the reciprocal effects of nebulization and treatment on the animal weights.

4.3.2.1. Analysis of intra-group weight changes on selected days

Tabulated summaries for mean weights of animals in the ST and LT study groups are given in (*Table 4.3a and b*). The complete daily data are given in the appendix. The mean weights in each experimental group that are significantly lower ($p \leq$

0.05) than the mean baseline or starting weights of same group on Day 1, are indicated by the asterisk symbol in *tables 4.3a* and *b*.

Table 4.3a: Intra-group mean weights on selected reference days (ST study)

DAY	Mean Weights (g) ± SD					
	CT	AS	HHC	LHC	HEH	LEH
1	22.47±0.71	18.29±1.85	21.43±1.53	18.47±2.82	18.89±0.76	17.95±2.56*
6	22.59±0.75	19.57±1.50*	22.23±1.26*	20.69±0.98*	19.59±0.66*	19.42±2.36*
13	23.01±0.64	20.62±1.42*	22.87±0.93*	21.19±0.72*	20.23±0.76*	20.26±2.25*
15	23.40±0.61*	20.98±1.33*	23.43±0.97*	21.72±0.64*	20.83±0.49*	20.84±2.24*
18	23.63±0.57*	21.10±1.81*	22.18±0.97*	19.85±0.82	20.22±0.27*	20.51±2.41*

Table 4.3b: Intra-group mean weights on selected reference days (LT study)

DAY	Mean Weights (g) ± SD					
	CT	AS	HHC	LHC	HEH	LEH
1	19.86±2.36	19.08±2.36	18.40±2.40	18.78±1.71	18.07±1.44	20.09±2.34
6	19.92±2.30	20.17±2.09*	19.47±2.00*	19.69±1.20*	19.19±1.54*	20.82±2.05*
13	20.53±2.46*	21.16±1.80*	20.39±1.77*	20.19±1.27*	19.88±1.55*	21.87±2.07*
15	20.74±2.19*	21.61±1.73*	21.07±1.93*	20.51±1.08*	20.27±1.55*	22.14±2.08*
18	21.11±2.12*	21.90±1.73*	19.79±1.44*	19.64±1.03	20.34±1.48*	22.15±2.13*
26	21.83±2.00*	22.63±1.42*	19.83±1.71*	19.94±1.09*	20.80±1.42*	21.96±1.78*
32	22.56±1.92*	23.52±1.43*	20.73±1.58*	20.72±1.15*	21.78±1.46*	23.50±2.16*
37	22.69±2.08*	23.53±1.43*	21.13±1.57*	21.00±0.87*	22.10±1.56*	23.79±2.15*
42	23.04±2.18*	23.87±1.43*	21.26±1.76*	21.54±1.28*	22.61±1.49*	24.21±2.40*
46	22.86±1.98*	24.46±1.64*	20.90±1.22*	21.31±1.32*	23.09±1.32*	24.37±2.31*

*difference significant compared with weight on day 1 (baseline weight) in same group

The absolute mean weight values were not used when comparing data by group since the starting weights vary between groups. Instead, respective percentage values (PWD) were determined as described in Section 4.2.2.3. Thus, all baseline weights became equivalent to 100% and all original mean weight values that were greater than the baseline weights became percentage values higher than 100% and vice versa for all lower original mean weight values. The respective change in percentage weights between groups on the same reference day were then compared by evaluating their closeness to (or their comparability with) the percentage weight values of the group under reference (*Table 4.4*).

Table 4.4: Percentage weights relative to baseline weights

ST Study						
Days	**CT**	**AS**	**HHC**	**LHC**	**HEH**	**LEH**
1	100.00	100.00	100.00	100.00	100.00	100.00
6	100.54	106.99	103.70	112.03	103.72	108.18
13	102.39	112.72	106.70	114.73	107.11	112.82
15	104.15	114.73	109.31	117.60	110.29	116.07
18	105.15	115.39	103.47	107.45	107.08	114.28
LT study						
Days	**Control**	**Asthmatic**	**HHC**	**LHC**	**HEH**	**LEH**
1	100.00	100.00	100.00	100.00	100.00	100.00
6	100.30	105.69	105.82	104.86	106.22	103.60
13	103.34	110.86	110.79	107.50	110.04	108.87
15	104.42	113.25	114.52	109.23	112.21	110.19
18	106.28	114.74	107.55	104.57	112.56	110.22
26	109.92	118.60	107.76	106.16	115.13	109.31
32	113.56	123.23	112.64	110.33	120.54	116.97
37	114.21	123.31	114.83	111.84	122.32	118.43
42	115.97	125.06	115.55	114.72	125.13	120.51
46	115.08	128.18	113.60	113.46	127.81	121.30

Line and column graphs were used to illustrate weight change trends within groups and to compare patterns between the different groups (*figure 4.1).* Intra-group comparisons of weight changes on selected days showed that most of the mean weights in each experimental group were significantly lower ($p \leq 0.05$) than the mean baseline (or starting weights) of the same group on Day 1 as indicated by the asterisk symbol (*table 4.3a and b*).

4.3.2.1.1. Early and late effects of immunization

Any possible effects of immunization on weight change could be evaluated on day 6. Since all animals received treatment only on a later day, any exaggerated experimental effects on day 6 could only be due to the immunization procedure. Analyses of animal weights in all the groups show that the mean weights on day 6 were significantly higher than baseline weights (*table 4.3a and b*). Unlike all other

weight values on same day, values for the controls in both the short-term and long-term study groups did not differ significantly from the respective baseline weights. Thus, the segment in the histograms for the controls corresponding to the period after immunization is relatively linear (*figure*s *4.3 and 4.4*). Since histograms for all other groups show remarkable percentage weight increase, it could be deduced that the early sensitization (immunization) procedure caused some weight gain in the mice.

Similarly, it is reasonable to examine the possible late effects of immunization on animal weights on day 13 because one week‘s interval was allowed between the first immunization procedure on day 5 and the next nebulization on day 13. Results show that on this day, there was remarkable weight gain in most of the groups but since a similar pattern was observed in the (unexposed) long-term controls, it could imply that the general weight gain observed may not be due to factors other than the immunization procedure. Previous studies involving different forms of animal sensitization showed mixed results, most of which seemed to be species and strain-specific (Curtis et al., 1990; Huneau et al., 1991; Saldanha et al., 2004).

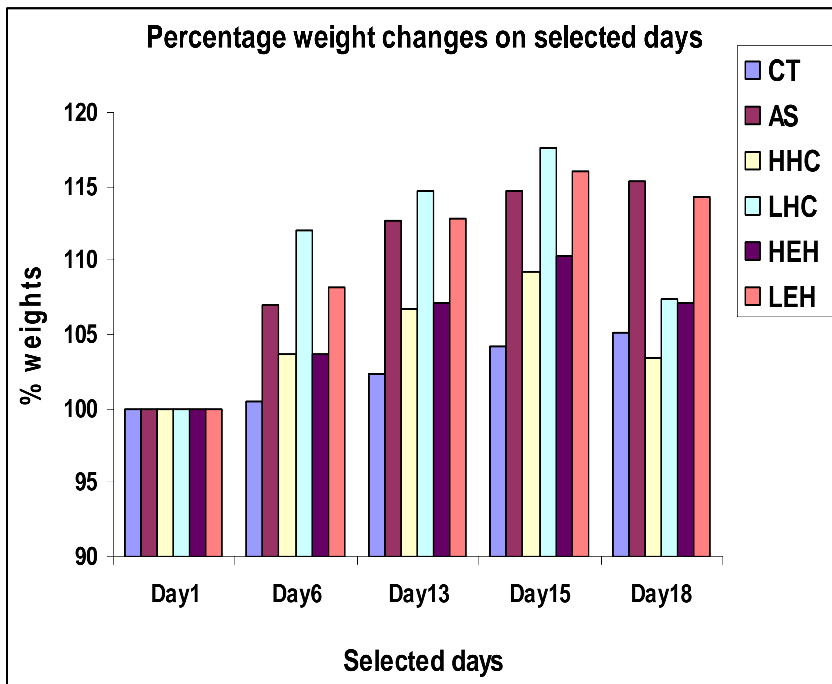


Figure 4.1: Histogram illustrating weight changes during the ST study for the control, asthma, high and low hydrocortisone, high and low E. hirta treatment groups.

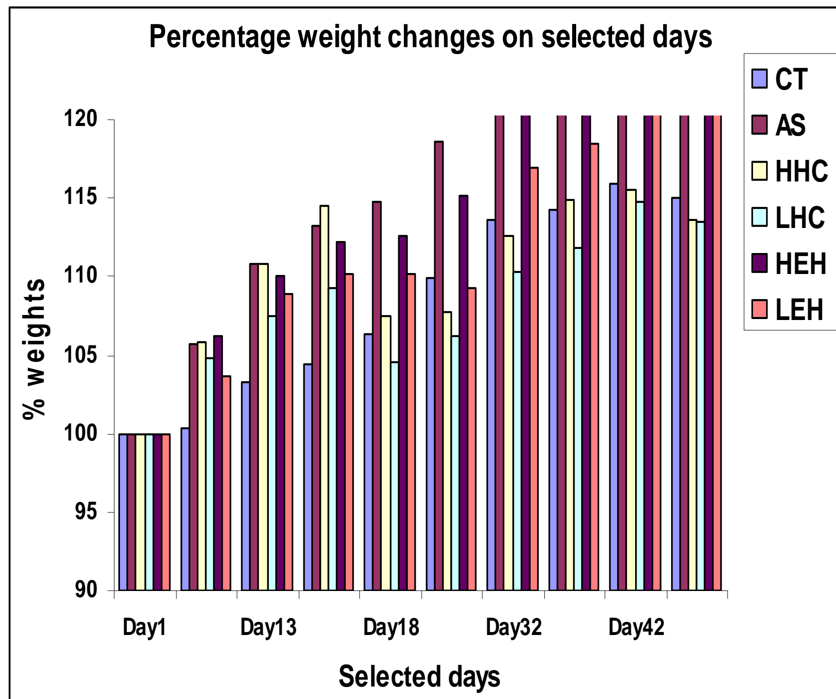


Figure 4.2: Histogram illustrating weight changes during the LT study for the control, asthma, high and low hydrocortisone, high and low E. hirta treatment groups.

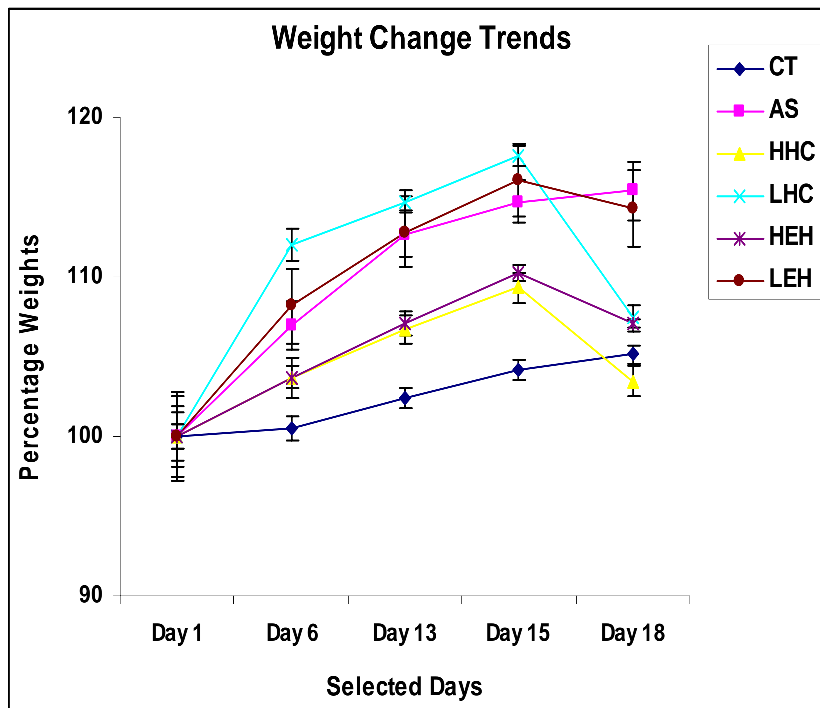


Figure 4.3: Line graph illustrating the trend of changes in weight during the ST study for the control, asthma, high and low hydrocortisone, high and low *E. hirta* treatment groups.

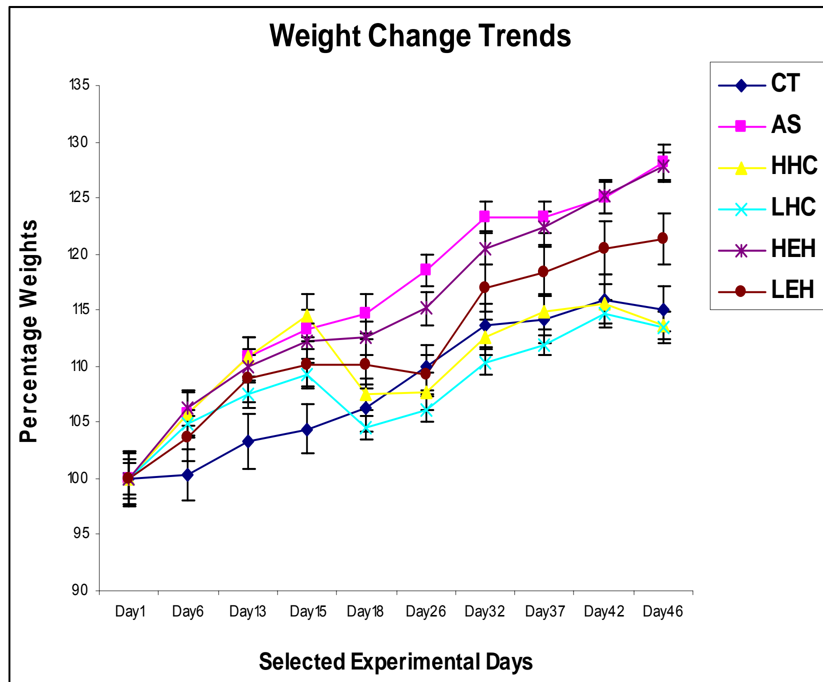


Figure 4.4: Line graph illustrating the trend of changes in weight during the LT study for the control, asthma, high and low hydrocortisone, high and low *E. hirta* treatment groups.

Nebulization was a very important experimental procedure in this study intended to induce the onset of asthma. The first batch of nebulization was on days 13, 14 and 15 before treatment on day 15. The acute effects of nebulization on animal weights could therefore be determined from the weight data taken on day 15 before treatment commenced. Results from *tables 4.3a and b* show that weights in all the groups on this day were significantly ($p \leq 0.05$) higher than baseline weights.

Figures 4.3 and 4.4 show that the histograms for all the groups in both the short and long-term studies were almost linear between day 13 and 15 in most of the groups although slight weight increases were observed. It could therefore be suggested that nebulization had minimal weight gain effects compared to immunization.

4.3.2.1.2. Effects of first time treatment on nebulization

The early effects of HC and *E. hirta* treatment on the nebulization procedure could be evaluated on day 18. Treatment on day 18 was expected to alter the 'asthmatic' effects of nebulization.

Results from *table 4.3a and b* and the histograms (*figures 4.3* and *4.4*) indicate that animals treated with HC and *E. hirta* in both the short and long-term studies lost varying amounts of weight on day 18, most of which was statistically significant. However, the short and long-term control and asthma groups continued to gain relatively small amounts of weight on this day, indicating that treatment with HC remarkably reduced the cumulative weights gained following prior experimental procedures (immunization and nebulization), and in some cases to values approximate to those of Day 6. On the other hand, weights of the two *E.*

hirta groups remained relatively similar to values on Day 15 in the LT study group. During the LT study, weights in the low *E. hirta* group reduced on Day 26 and later increased steadily along with weights in other groups. All these results tend to suggest that the weight gain induced mostly by prior experimental procedures like immunization and nebulization could be reduced remarkably by treatment with HC.

4.3.2.1.3. Midterm and late effects of continuous treatment

Treatment continued during Days 22, 25, 29 and 32 of the long-term study and was suspended during days 34, 35 and 36 in order to repeat the nebulization procedure. The midterm effects of continuous treatment could be assessed from data obtained on Day 26. Except for the LEH group, animals in most of the groups re-gained weight (albeit slightly) on this day after the plunge on day 18. The rate of weight gain was however more marked between days 26 and 32, indicating that a longer period of continuous treatment had remarkable cumulative effects on 'weight recovery'.

Although treatment with HC was observed to cause a sharp weight loss on day 18, prolonged treatment appeared to initiate and sustain an increase in the rate of weight gain as observed between days 26 - 32 (*figures 4.3* and *4.4*). HC is known to enhance improvement in the asthmatic condition (Landstra et al., 2003) while prednisolone was found to reduce body weight in mice and guinea pigs (Nagao et al., 2004).

4.3.2.1.4. Effects of repeated nebulization on weight changes

Effects of initial nebulization observed on Day 15 had minimal weight gain effects compared to immunization. Repeated immunization ended on day 36 of the LT term study and effects of repeating this process were determined on Day 37.

Results from table 2 and the graphs show that weight increase was only very slightly in almost all the test groups, confirming the minimal effects of nebulization on weight gain.

4.3.2.1.5. Early and terminal post-nebulization treatment effects after nebulization on weight changes

After nebulization, treatment resumed during days 39, 40, 41, 42, 43, 44, 45. Acute post-nebulization effects could be determined on day 42. Results from the histograms show that weights increased sharply between day 37 and 42 instead of causing a sharp reduction following treatment after repeated nebulization. This effect was however not sustained after day 42 as animals in especially the two HC groups lost weight slightly while *E. hirta* animals gained weight slightly. The reason for the weight disparity between the treatment groups on Day 46 is not clear but the weight loss effect observed in the HC-treated animals is consistent with one previous finding (Bernick and Zipkin, 1967).

4.3.2.2. Comparison of progressive inter-group weight changes

4.3.2.2.1. Control versus asthma group

Throughout the study, percentage mean weights in the control groups were generally lower than in the asthma group as shown in *Figures 4.3 and 4.4*. The weight gain in the controls was at a relatively slower rate compared to other groups since animals in this group were not exposed to any procedure. The rate of weight gain in the asthma group was faster than in other groups at different periods during the study indicating that the effects of the different experimental procedures on weight change were more pronounced in the asthma group.

4.3.2.2.2. Control versus treatment groups

Percentage mean weights in all the treatment groups were higher than the control weights during most of the ST study period. During the LT study period however, weights in the two HC groups were lower than control weights from Day 26 following prolonged treatment. Weights remained relatively low until the end of the study albeit at about the same rate of weight change. The weight gain in the two *EH* groups were consistently higher than the controls throughout the study. These results suggest that long-term administration of HC causes a low but sustained weight gain pattern while prolonged administration of *E. hirta* extracts causes only a slight but consistent weight gain.

4.3.2.2.3. Asthma versus other groups

The percentage weight values for the asthma group were higher than all other groups by Day 18. During the short-term study, the weights were lower than some of the groups and almost of same value as others. *Figures 3* and *4* shows that there is inconsistent weight change patterns during the short-term period of the experiment but the weights in the asthma group were highest during the long-term study period.

The higher weight values in the asthma group and the consistent rate of weight gain in this group throughout the study indicate that immunization causes a high steady and sustained rate of weight gain in mice, which can only be reduced by treatment. On the other hand, challenge causes only a mild effect on weight change.

During the ST study, administration of the low doses of both HC and *E. hirta* appeared to be less effective in reducing weights to values below the weights in

the asthma group but the rate of weight gain in the two *E. hirta* groups appears to approximate that in the asthma group during the long-term period. This implies that treatment with *E. hirta* causes less reduction in the weight gained due to induced asthmatic conditions, than treatment with HC. This finding is in line with a previous study using extracts of six medicinal plants *Cordyceps militaris* (CM), *Paecilomyces japonia* (PJ), *Phellinus linteus* (PL), *Ganoderma lucidum* (GL), *Grifola frondosa* (GF), and *Panax ginseng* (PG).

In this particular study, body weight, weight gain and food efficiency ratio (FER) were found to have no significant effect on the in treated mice compared to the control group (Jung et al., 2004). Also, extracts of the Brazilian plant *Cissus sicyoides* were found to further reduce the weight loss caused by alloxan in diabetic animals (Viana et al., 2004) implying that *Cissus sicyoides* extracts are less effective in weight reduction.

In *figures 4.1* and *4.2,* the lower the bars in the graph, the more effective is the treatment represented by the bar likely to cause reduction in weight. It was found that the lower doses of the treatment agents used in this study were more effective than the higher doses. The reason for this is unclear but the “low dose of 100mg/kg” used in this study is only described in the context of a relative weight, when considering the higher dose of 125 mg/kg. In a previous study by Hausberger and Hausberger (1958) variable results were reported produced following a 5 mg/day cortisone dose.

4.3.2.2.4. Low dose versus high dose groups

During the short-term study period, there appeared to be no clear weight change pattern as seen in the long-term period. Weight gain was much higher in the "high dose groups" implying that the weights tended towards the values observed in the asthmatic group. The low dose weight data for both the low HC and low *E. hirta* groups were however closer in value to the control group, indicating a low rate of weight gain compared to the corresponding high dose groups. As mentioned previously, these results indicate that 100mg/kg body weight dose of HC and 25mg/kg dose of *E. hirta* had a more effective impact on weight loss than the corresponding higher doses (125 mg/kg HC and 62.5 mg/kg *E. hirta*) respectively.

4.3.2.2.5. Cortisone versus *E. hirta* groups

Weight patterns were generally irregular during the short-term period. However, the plant extract groups appeared to have higher weight values that closely approximated the asthma group for most of the long-term study duration. These results again showed that cortisone administration produces a lower rate of weight gain than does administration of *E. hirta* extracts, indicating that after the animals became asthmatic, the HC-treatment was more effective in restoring conditions to normal (near control) states than *E. hirta*-treatment.

A number of factors including motivation, eating behaviour, amount of activity (especially exercise), overall health, metabolism and stress can cause weight loss or gain. Excessive body weight increases the risk of asthma (Camargo et al., 1999) and obese individuals with asthma may improve their lung-function symptoms and overall health status by engaging in a weight loss program. A controlled study found that weight loss resulted in significant decreases in

episodes of shortness of breath, increases in overall breathing capacity, and decreases in the need for medication to control symptoms (Stenius-Aarniala et al., 2000).

The effects of various forms of activity (experimental procedures, food intake, exercise, physical and chemical stress) on weight changes have been studied in humans and animals alike. Findings from these studies tend to suggest that weight changes that occur especially in relation to stress are usually in response to internal changes in animal physiology as induced by the stress exposure (Retana-Ma´rquez et al., 2003). In one study using restraint stress, rats lost weight and remained hypophagic until a few days after the stressor had ended (Harris et al., 1998).

In yet another study, stress effects on body weight were observed only with repeated exposure to the stressors, and less body weight gain (but not body weight loss) was observed compared to the control group in animals subjected to stress by immobilization or by immobilization plus tail shocks during three days. The loss in body weight observed in other studies by Ottenweller et al., (1992) and Marti et al., (1994) was due to a decrease in food intake.

During most of this study, animals experienced consistent weight increase but there were periods of weight reduction after weight gain. Some of the observed weight gain in all the groups in this study could be attributed to a number of factors including the various experimental interventions. Specifically, any weight changes in the control groups at any time during the study were assumed to be due to changes in normal body metabolic responses, growth as well as changes in food

and fluid consumption patterns. The control animals had no form of experimental intervention and were provided food and water *ad libitum*.

In yet another study, stress effects on body weight were observed only with repeated exposure to the stressors, and less body weight gain (but not body weight loss) was observed compared to the control group in animals subjected to stress by immobilization or by immobilization plus tail shocks during three days. The loss in body weight observed in other studies by Ottenweller et al., 1992 and Marti et al., 1999 was due to a decrease in food intake. One interesting observation was that weights in the control and asthma groups were generally among the lowest throughout the study, suggesting that the progressive higher animal weights observed in all other groups were caused by the different experimental procedures.

4.4. Conclusion

A guided stage-to-stage analysis of all data was undertaken to avoid excluding any possible contributions to weight change by the different experimental interventions, especially because the causes of weight changes are multifactorial. Data obtained show that animals in the control groups initially experienced weight losses that steadily became marginal weight increments (see appendix tables). The reason for the early weight losses is not clear but beyond day 6, mean weights in the control groups became regular and progressively higher throughout the remaining duration of the study. Data also show that animals in both the ST and LT control groups experienced slow but steady progressive weight increments throughout the duration of the study. In addition, mean weights in all other groups also increased progressively in value. After day 6, none of the animals lost weight

below their starting (baseline) weights on the selected days and only at certain stages during the study did animals lose weight relative to the weight values recorded on the previous study days.

It could be concluded that both immunization and nebulization had positive weight gain effects on the animals but the effects were more pronounced following immunization but were 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.

The above conclusions are based on the assumption that the control group values represented animals with normal, uninterrupted, physiological states and the asthma group values represented the animals with “the asthmatic symptoms”. Further studies with specific defined weight-related experimental goals would be required to clearly determine the possible effects of varying doses of especially the *E. hirta* plant extracts on animal weights, as well as to confirm the effects of HC observed in this study.

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.

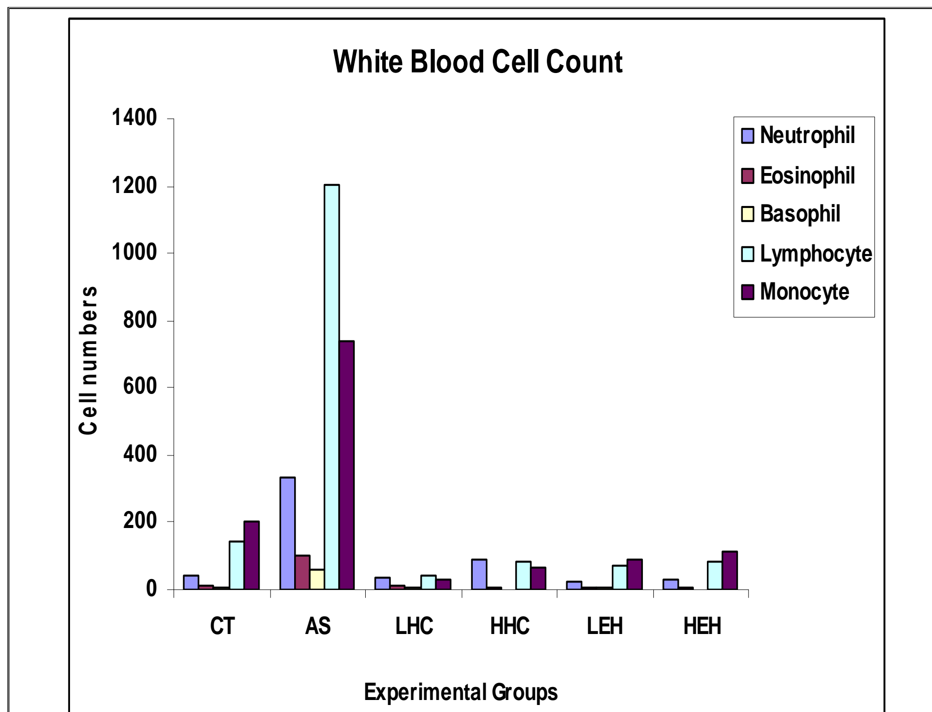


Figure 5.1a: Histogram showing the mean values obtained from white blood cell counts per group

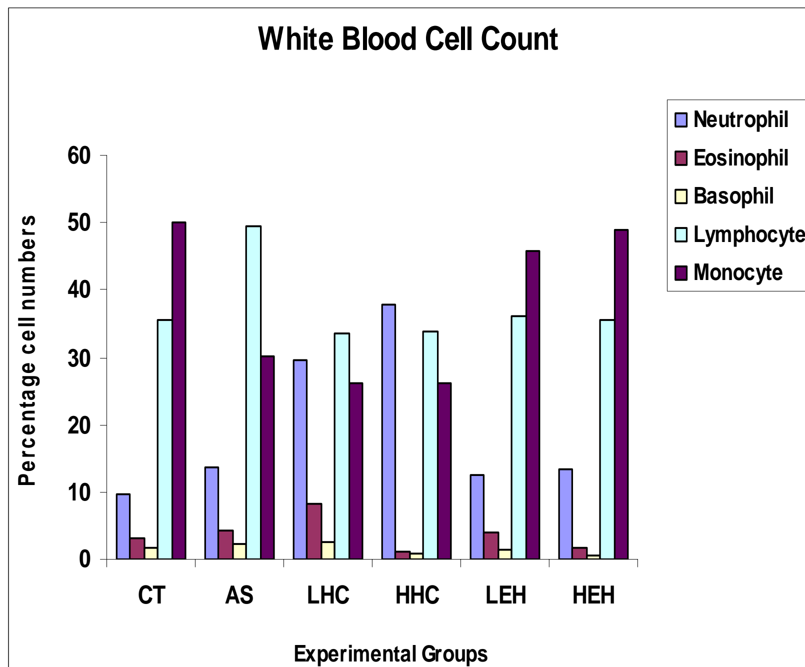


Figure 5.1b: Histogram showing the percentage white blood cell counts per group

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.

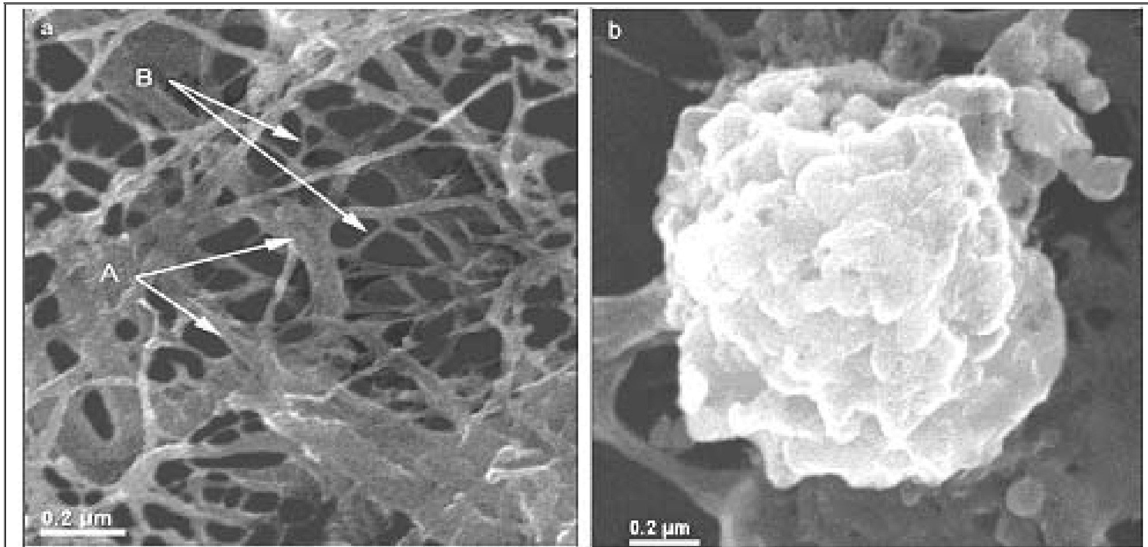


Figure 6.1. (a) Control fibrin network with thick, major fibres as well as thin, minor fibres. Label A ¼ thick, major fibres; Label B: ¼ thin, minor fibres. (b) Control platelet aggregate forming dense, round aggregate with pseudopodia.

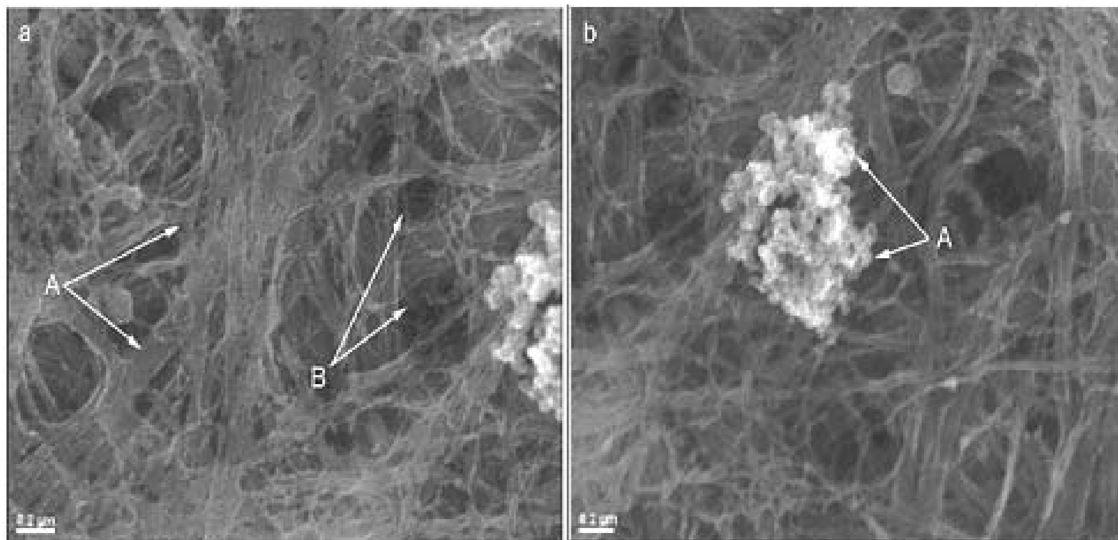


Figure 6.2. (a) Fibrin network from asthmatic BALB/c mice showing thick, major fibres as well as thin, minor fibres. Label A ¼ thick, matted, major fibres; Label B ¼ thin, minor fibre forming a dense network. (b) Platelet aggregate from asthmatic BALB/c mice forming course, granular aggregate

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.

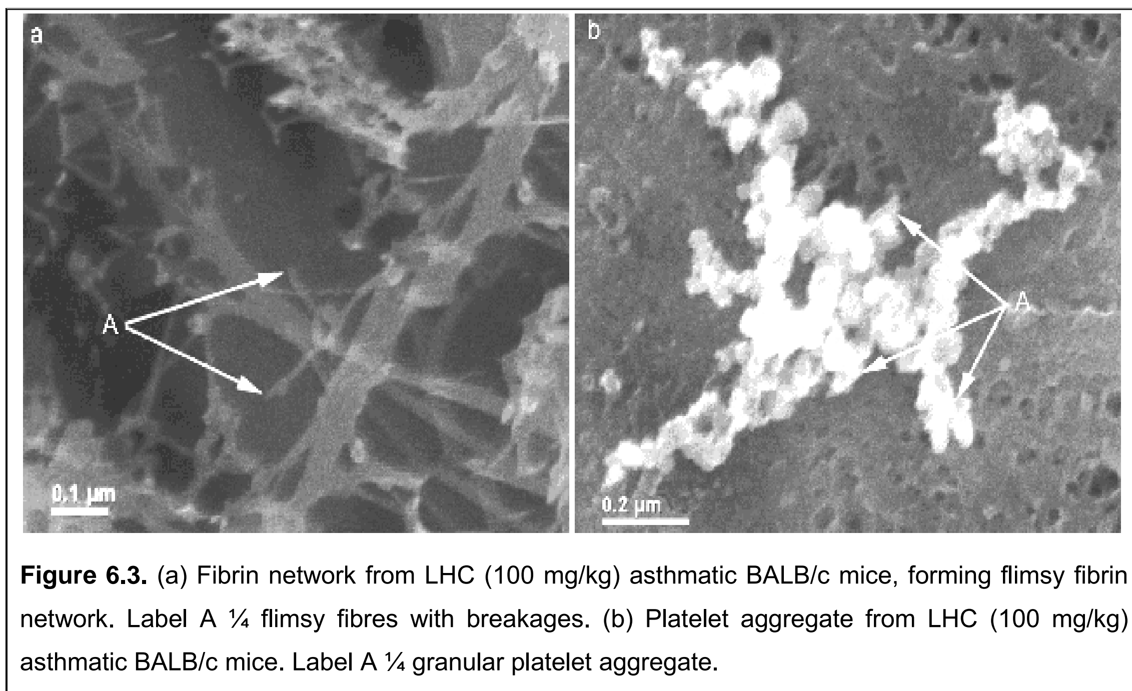


Figure 6.3. (a) Fibrin network from LHC (100 mg/kg) asthmatic BALB/c mice, forming flimsy fibrin network. Label A ¼ flimsy fibres with breakages. (b) Platelet aggregate from LHC (100 mg/kg) asthmatic BALB/c mice. Label A ¼ granular platelet aggregate.

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.

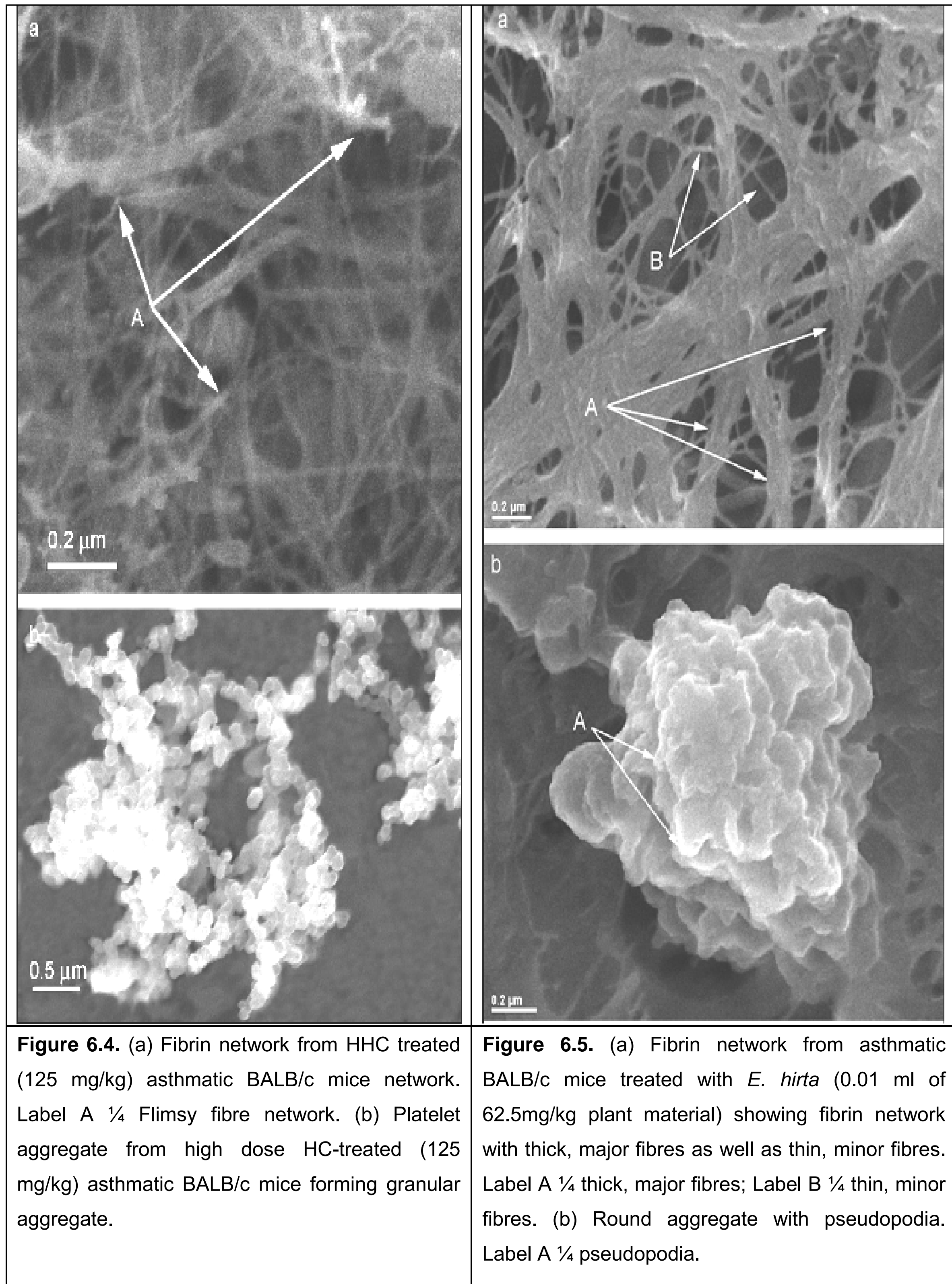


Figure 6.4. (a) Fibrin network from HHC treated (125 mg/kg) asthmatic BALB/c mice network. Label A ¼ Flimsy fibre network. (b) Platelet aggregate from high dose HC-treated (125 mg/kg) asthmatic BALB/c mice forming granular aggregate.

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.

CHAPTER SEVEN

Inflammatory Cell Infiltration and Structural Changes in the Airways

7.1. Introduction

Asthmatic inflammation usually affects the entire respiratory tract, from the nose down to the most distal (and therefore much smaller) respiratory passages as well as the alveolar tissue (Martin, 2002; Togias, 2003), causing a wide range of structural changes. Some of the common changes include thickening of the reticular basement membrane (Roche et al., 1989; Wilson and Li, 1997), thickening of the smooth muscle layer as well as infiltration of different cell types. Thickening of the basement membrane could be due to deposition of collagen types I, III and V (Roche et al., 1989).

Cell infiltration into the airway has also been considered a remarkable feature of asthma that causes structural changes. Some studies have indicated that the predominant cells in bronchial asthma were eosinophils and lymphocytes (Hoshi et al., 1995) and yet other studies have shown that CD4 T-lymphocytes, eosinophils and mast cells were the predominant cells in asthma (Saetta and Turato, 2001). Other studies reported the presence of eosinophils, neutrophils and macrophages in lung tissues (McKay et al., 2004). Infiltration of T-cells, macrophages and eosinophils into the proximal and distal lung tissues has also been reported in rare cases of sudden asthma death (dying within one hour of onset of symptoms) (Faul et al., 1997) and in soybean dust induced asthma (Synek et al., 1996).

The distribution pattern of eosinophilic infiltration was found to differ between the central and peripheral airways, suggesting important functional consequences (Saetta and Turato, 2001). A comprehensive review by Epstein (2006) stated that peril-bronchial and peril-vascular infiltrates were evident in all asthma models but the cellular composition, severity of overall inflammation and location of infiltrates

was protocol-dependent. The study also showed that although the protocol-dependent differences in asthma histopathology are poorly understood, and could provide new insights into the heterogeneity of disease.

Many previous studies have shown that the entire length of the airways is involved in asthma (Huber and Koessler 1922; Dunnill 1960; Dunnill et al., 1969; Kuwano et al., 1993). Using surgically resected lung tissue (Taha et al., 1999), trans-bronchial biopsies (Kraft et al., 1999) and autopsy lung specimens (Christodoulopoulos et al., 2000), more severe inflammatory and structural changes were observed in small airways (Faul et al., 1997) and in peripheral airways and lung parenchyma (Kraft et al., 1996) of 'asthmatic' persons when compared to the larger airways. Regional variations were also observed in inflammatory cell distribution within 'asthmatic' airways (Haley et al., 1998).

The types of inflammatory cells present in the airway walls and spaces could indicate the pattern of asthmatic progression. The present study examines the effects of HC and extracts of the medicinal herb *E. hirta* on the structural changes and cellular disposition and characteristics in especially the distal respiratory passages using the Balb/c mouse asthma model.

7.2. Mixed inflammatory infiltrate in the lung parenchyma

A mixture of inflammatory cells, notably eosinophils, lymphocytes, mast cells, basophils, macrophages, fibroblasts and platelets usually infiltrates the subepithelial layers of respiratory passages. Results from previous studies are given in the following sections.

7.2.1. Eosinophils

Tissue eosinophilia is a characteristic of asthma but it is not necessarily specific to asthma (Lacoste et al., 1993). The classic descriptions of asthma pathology include the presence of eosinophils (and neutrophils) in both the lamina propria and the airway lumen of patients with asthma (Kaliner et al., 1976). Biopsy specimens from 'mild asthma' patients have confirmed the presence of eosinophils in the mucosa, often beneath the basement membrane as well as in the epithelium (Djukanovic et al., 1990).

Eosinophils also appear to be important cells of airway remodelling as they release growth factors (Ohno et al 1992, Walz et al 1993), elastase (Lungarella et al., 1992) and metalloproteases (Ohno et al., 1997) that are all involved in the process of tissue remodelling and pulmonary fibrosis (Schlick et al., 1993), mostly through fibroblast stimulation (Pincus et al., 1987). Recruited eosinophils become activated during segmental allergen challenge and then release products that cause contraction of the human bronchial smooth muscle and increased vascular permeability (Collins et al., 1993; Rabe et al., 1994; Shaver et al 1997).

7.2.2. Lymphocytes

T-cells possibly play a role in controlling the chronic inflammation of allergic and non-allergic asthma via the release of Th2-cytokines (Ying et al., 1995, Robinson et al., 1992; Del Prete et al., 1993). After allergen challenge, the population of activated T-cells appears to increase in 'asthmatic' subjects (Robinson et al., 1993; Bentley et al., 1993) with only a few B-cells present in the bronchi.

7.2.3. Mast cells

Mast cells are found in the bronchi of normal subjects and "asthmatics" alike (Pesci et al., 1993; Koshino et al., 1995) that are often degranulated in the airways of "asthmatics" in both their stable phase and after allergen challenge (Laitinen 1985; Beasley et al., 1989). Mast cells appear to be critical "trigger" cells during episodes of acute asthma (Broide et al., 1991). These cells may be involved in airway remodelling because they appear to play an important role in pulmonary fibrosis (Jordana et al., 1993; Chanez et al., 1993) especially because they are potential sources of products that stimulate migration and proliferation of fibroblasts (Ruoss et al., 1991; Nagata et al., 1992). Research has also shown that mast cell lines can release components of basement membranes such as laminin and collagen IV (Thompson et al., 1991) as well as angiogenic growth factors (Meininger and Zetter, 1992). Mast cell products have also been reported to have anti-inflammatory properties (Green et al., 1993; Tyrell et al., 1995).

7.2.4. Basophils

The role of basophils in the pathogenesis of asthma appears poorly defined (Denburg, 1998). There has been some controversy over the extent to which basophils participate in the inflammatory processes of asthma (Walls et al., 2001) even though these cells are known to be responsible for the mediator release observed during the asthmatic response (Lichtenstein and Bochner, 1991). After anti-IgE challenge, most human blood basophils release histamine and other inflammatory mediators without degranulating (Youssef et al., 2007). Though often regarded as circulating counterparts of the mast cell, many basophils infiltrate the tissues and fluids that bathe mucosal surfaces (Walls, 2001).

7.2.5. Macrophages

A study by Calhoun et al (1992) showed that activation of alveolar macrophages occurred immediately after antigen challenge, indicating a major role for macrophages in asthma. Alveolar macrophages have been reported to exhibit enhanced capacity to release pro-inflammatory cytokines (John et al., 1998). In addition, macrophage numbers increased 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).

Phagocytic cells such as polymorphonuclear leukocytes and macrophages, respond to a variety of membrane stimulants via the production and extracellular release of a number of reactive oxygen reduction products (Santos et al., 2004). Alveolar macrophages may also contribute to inflammation via the release of reactive oxygen species such as superoxide (SO) anion, hydroxyl radicals, hydrogen peroxide, singlet oxygen and hypohalous acids, which may cause cellular injury and alterations in airway functions (Freeman and Crapo, 1982; Barnes 1990). These reactive groups may be involved in the regulation of airway remodelling through secretion of growth-promoting factors for fibroblasts, cytokines as well as growth factors possibly involved in fibrosis (Kovacs and DiPietro, 1994).

7.2.6. Polymorphonuclear neutrophils

The role of neutrophils in stable asthma remains unclear but neutrophil numbers were noted to have increased after an allergen challenge in the airways in the different forms of asthma during the late-phase reaction (Koh et al., 1993; Montefort et al., 1994). In one study, both bronchoalveolar lavage (BAL) and lung

tissue digestion were used to determine rat lung inflammatory cell contents following a 4-hour exposure to 2-ppm ozone. Immediately following the exposure, the neutrophil content of the lung tissue significantly increased and by three hours post-exposure, reached a value fourfold higher than air-exposed controls. Although lavage-recovered neutrophils were elevated 24 hour afterwards, tissue neutrophil numbers had returned to control values. This transient elevation of tissue neutrophils directly correlated with an elevation and subsequent decline of airway hyperresponsiveness (DeLorme et al., 2002).

7.2.7. Fibroblasts

Fibroblasts are often found in connective tissue and are responsible for the production of collagen, reticular and elastic fibres as well as for the synthesis of proteoglycans and glycoproteins of the amorphous intercellular substance (Sheppard and Harrison, 1992). Although these cells are regarded as fixed cells of connective tissue origin, they retain the capacity for growth and proliferation and are pluripotent cells (precursors for various other cell types), including smooth muscle cells (Gizycki et al., 1997).

7.2.8. Myofibroblasts

Myofibroblasts may potentially contribute to the regulation of bronchial inflammation via the release of cytokines (Zhang et al., 1996) and to tissue remodelling via the release of extracellular matrix (ECM) components such as elastin, fibronectin and laminin (Leslie et al., 1992). During the late-phase reaction after allergen challenge, myofibroblasts increase from the normal of 2% of cells to approximately 15% within a day (Gizycki et al., 1997). In asthma, myofibroblasts are increased in numbers beneath the reticular basement membrane and there is

an association between their numbers and the thickness of reticular basement membrane (Brewster et al., 1993; Gabbrielli et al., 1993).

7.2.9. Dendritic cells

Dendritic cells are receiving increasing scientific and clinical interest due to their key role in anti-cancer host responses and potential use as biological adjuvants in tumour vaccines, as well as their involvement in the immunobiology of tolerance and autoimmunity (Satthaporn and Eremin, 2001). Dendritic cells are a major source of many cytokines, namely, interferon-alpha (IFN-a), IL-1, IL-6, IL-7, IL-12 and IL-15 and also produce macrophage inflammatory protein (MIP1g), all of which are important in the elicitation of a primary immune response (Macatonia et al., 1995; Cella et al., 1996; Mohamadzadeh et al., 1996).

In humans, there is a network of dendritic cells within the epithelium of the conducting airways (Jahnsen et al., 2001). These cells may be critically important to the induction of immune responses within the airways as they are specialized in antigen processing and presentation. In animals, the dendritic cell population in the airway epithelium is renewed every 48 to 72 h (Hance, 1993) and it appears that the number of dendritic cells can be altered after exposure to both topical and systemic corticosteroids (Moser et al., 1995).

Dendritic cell numbers are greater in the airways of asthmatics compared with those of control subjects (Tunon-De-Lara et al., 1993; Moller et al., 1996). Their role in asthma remains under investigation. Mouse models and culture systems of human cells have been used to investigate the implications of the dendritic cell-epithelial interaction for the pathogenesis of asthma (Lambrecht and Hammad, 2003).

7.2.10. Aim of study

The aim of this study was to determine the presence of inflammatory cells in the airway walls and spaces in especially the distal respiratory structures using the BALB/c mouse asthma model, and to compare the findings in the control (CT), asthmatic (AS), HC-treated and *E. hirta*-treated specimens. Differences in cellular characteristics as well as structural changes in the airways were also analyzed and compared. Although the amount of inflammatory cells in the airway walls was not determined, their possible relationship to the findings in peripheral blood cell counts in chapter five was analyzed.

7.3. Materials and methods

7.3.1. Materials

7.3.1.1. BALB/c Mice

A total of sixty (60) six-week-old male BALB/c mice (mean weight 20g) were used in this study.

7.3.1.2. Hydrocortisone (HC)

HC was used in this study as a pharmaceutical control for the extracts of *E. hirta* as used iin the cell culture study. One hundred milligrams (100 mg) of the sterile powder (Brand name Solu-Cortef®) which contains HC sodium succinate as the active ingredient was dissolved in 2 ml of bacteriostatic injection water, giving a concentration of 50mg/ml. In this study, a high dose (125mg/kg) and a low dose (100mg/kg) of the 50mg/ml HC preparation were used. Fresh solution was prepared for each injection.

7.3.1.3. *Euphorbia hirta*

Only the aqueous plant extract of *E. hirta* was used for the animal studies. The plant material was collected in the Gezina region of Pretoria South Africa. A herbarium specimen was prepared and compared to an authentic specimen in the HGJW Sweikerdt herbarium at the University of Pretoria. The aboveground parts of the plant were allowed to dry at room temperature for one week in the Cell Biology laboratory of the Department of Anatomy, University of Pretoria, and the material was grounded into a fine powder. Shortly after, 50 grams of the sample was extracted in 500ml of double distilled water after which it was filtered, and dried on a rotary evaporator at 40 °C. A stock aqueous solution of 50mg/ml plant extract was prepared and stored in a fridge until used.

The doses of 62,5mg/kg and 25mg/kg were prepared from the stock solution and administered orally to the mice. This implied that each animal (average weight 20g) received 0.01 ml of plant material. This dose was decided upon after studying literature that mentioned physiological doses suggested by herbalists. Typically, a teaspoon of the herb is added to a teacup volume of water and allowed to simmer for 20 minutes (Lindsey et al., 2002). Alternatively, an extract of the plant could be prepared and the recommended adult dose range of the fluid extract is 0.2-0.3ml, taken three times daily and of the infusion, 120-300mg three times daily (Skidmore-Roth, 2001).

7.3.1.4. Reagents and equipment

The reagents and equipments used for this study include ovalbumin (OVA) purchased from Sigma-Aldrich Co., Phosphate Buffered Saline (PBS), alum, formaldehyde, assorted clearing agents, glutaraldehyde, epoxy resin, 1% OsO_4,

cacodylate buffer, Agar 100 Resin, DPSS, KLAVA ultrasonic nebulizer, oral-pharyngeal canula, injection syringes and needles, light microscope (Olympus Japan binocular BH2 (BHTU) model) and transmission electron of microscope (Multi-purpose TEM (Philips 301), 100kV, eucentric goniometer stage, heating holder).

7.3.2. Light microscopy

For light microscopy, samples from the lungs were processed using basic histological techniques. Tissues were fixed in 10% formaldehyde for 24 hours and later dehydrated in increasing grades of ethanol, cleared in xylene and infiltrated with molten paraffin wax. Thereafter, the specimens were embedded in paraffin wax to form tissue blocks and five-micron thick sections were cut from the blocks and stained with Haematoxylin and Eosin (H and E) dyes. The stained sections were later observed with the light microscope (Olympus Japan binocular BH2 (BHTU) model).

7.3.3. Transmission electron microscopy (TEM)

All specimens were taken to the central Laboratory for Microscopy and Microanalysis at the University of Pretoria. Intrapulmonary tissues were cut into very small sizes and fixed in glutaraldehyde for one hour. The samples were then put in buffer (0.075M sodium potassium phosphate at pH = 7.4) for 15 minutes and later washed twice in same buffer. Thereafter, samples were placed in a secondary fixative (1% osmium tetra-oxide solution: OsO_4) for one hour. Following OsO_4 fixation, the tissues were rinsed again thrice in the buffer as described earlier and later dehydrated through 30%, 50%, 70%, 90%, and then three changes of 100% ethanol. The dehydrated samples were later embedded in resin

and ultra-thin sections prepared and stained with uranyl acetate for 15 minutes followed by 10 minutes staining with lead citrate. Later the stained specimens were allowed to dry for a few minutes before examination under the multi-purpose transmission electron microscope (Philips 301 100kV, eucentric goniometer stage, heating holder). Ultra-photomicrographs of the internal morphology of the intrapulmonary respiratory tract were also taken.

7.4. Results

7.4.1. Light microscopy

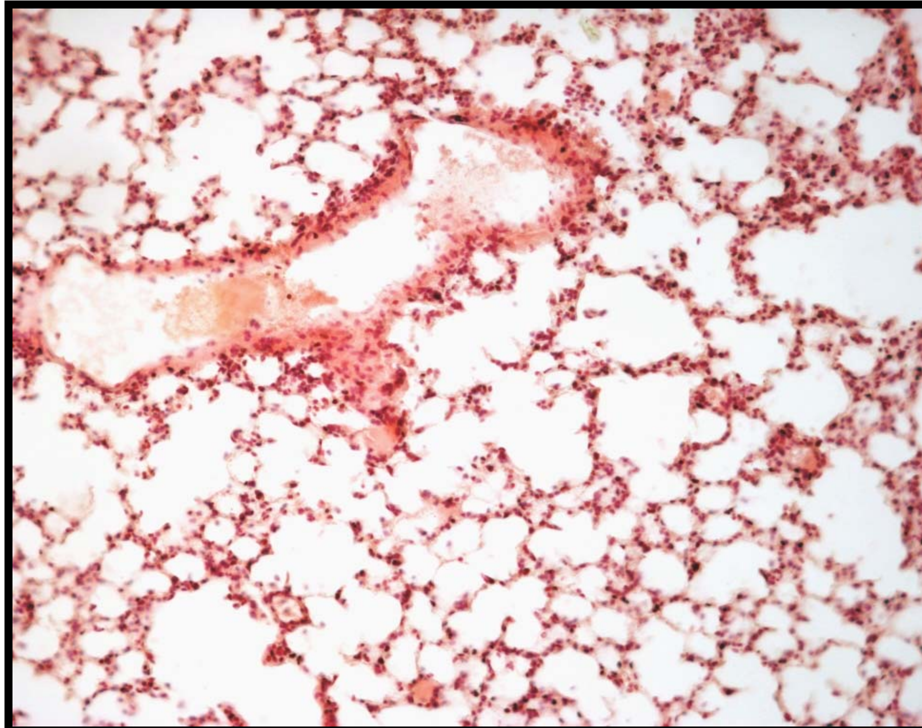
At an objective magnification of X10, the following photomicrographs were taken for the different study groups to show the possible effects of the two treatment agents on alveolar sizes, alveolar wall thickness, as well as the presence of other features as the presence of smooth muscle masses in the terminal bronchioles.

Results from the light microscopy studies show that the CT specimens had relatively thin and continuous (interconnected) alveolar walls with the alveoli being of regular shapes. In contrast, alveoli appear irregularly shaped in the asthma group, their walls very thick and discontinuous, with smooth muscle masses seen in the walls of distal airways. These findings appear to confirm the presence of asthma induced in mice of the AS group.

In the case of specimens of mice treated with the HHC, the alveoli were relatively compact, relatively small and of irregular shapes. Alveolar walls were also discontinuous and moderately thick. Smooth muscle thickenings of terminal airways were also seen. The LHC treatment specimens showed alveoli that were relatively thick and irregular in shape with walls that were discontinuous at some regions. Smooth muscle masses were also seen in the walls of distal airways.

These findings tend to indicate that at the level of light microscopy, both doses of HC did not ameliorate the effects of asthma on the lung microstructure in specimens of this group in terms of the characteristics earlier mentioned.

CONTROL A:



CONTROL B:

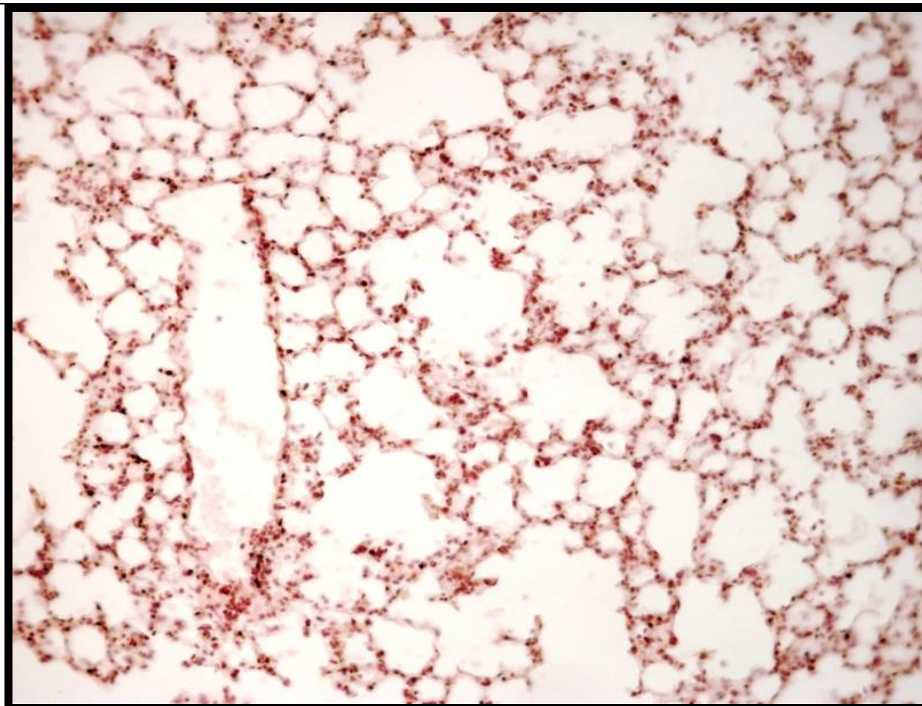
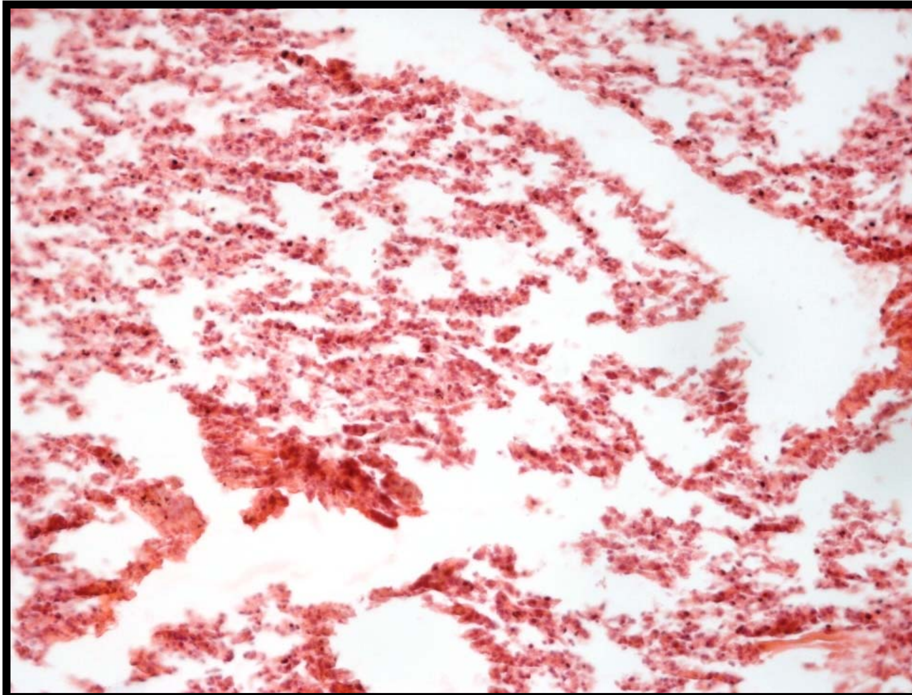


Figure 7.1: Histological cross sections of control mice lungs showing **A)** and **B)** thin and continuous alveolar walls; regular shaped alveoli. H and E staining. Magnification X 100

ASTHMA A:



ASTHMA B:

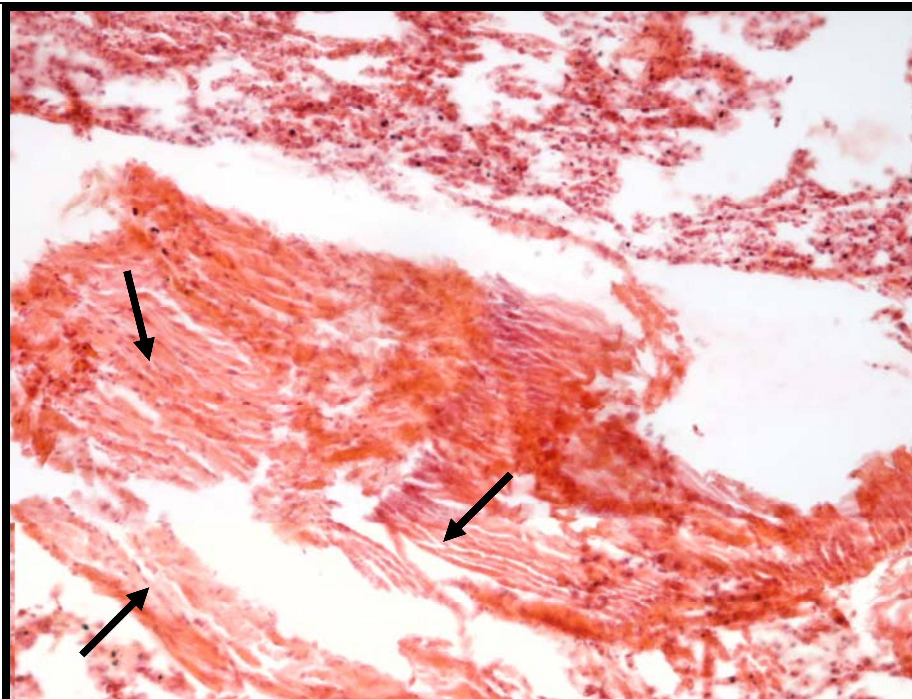
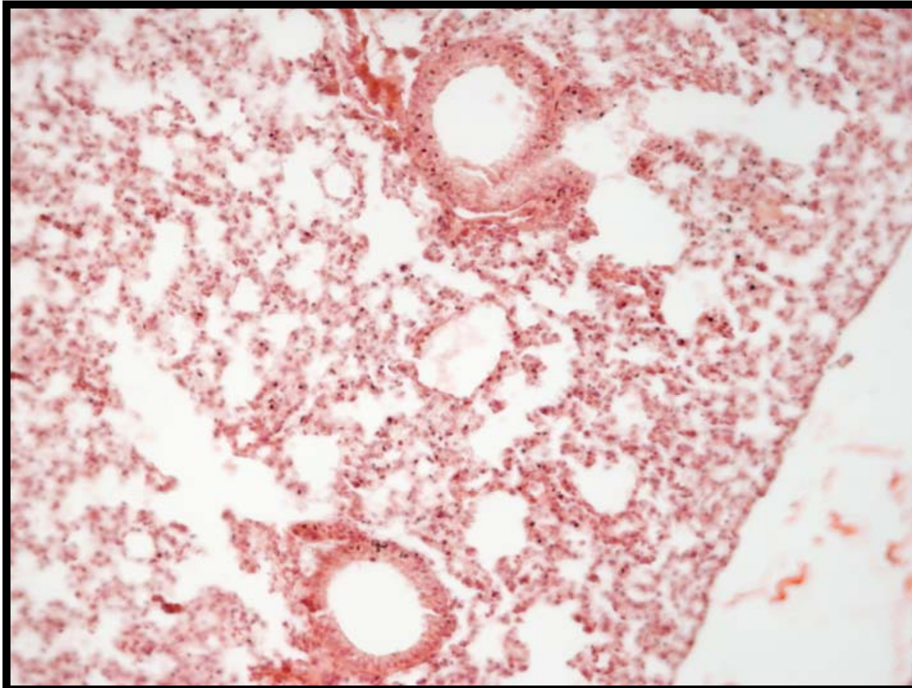


Figure 7.2: Histological cross sections of asthma mice lungs showing **A)** thick and discontinuous alveolar walls and **B)** thick smooth muscle mass in walls of distal airways indicated with arrows. H and E staining. Magnification X 100

HHC A:



HHC B:

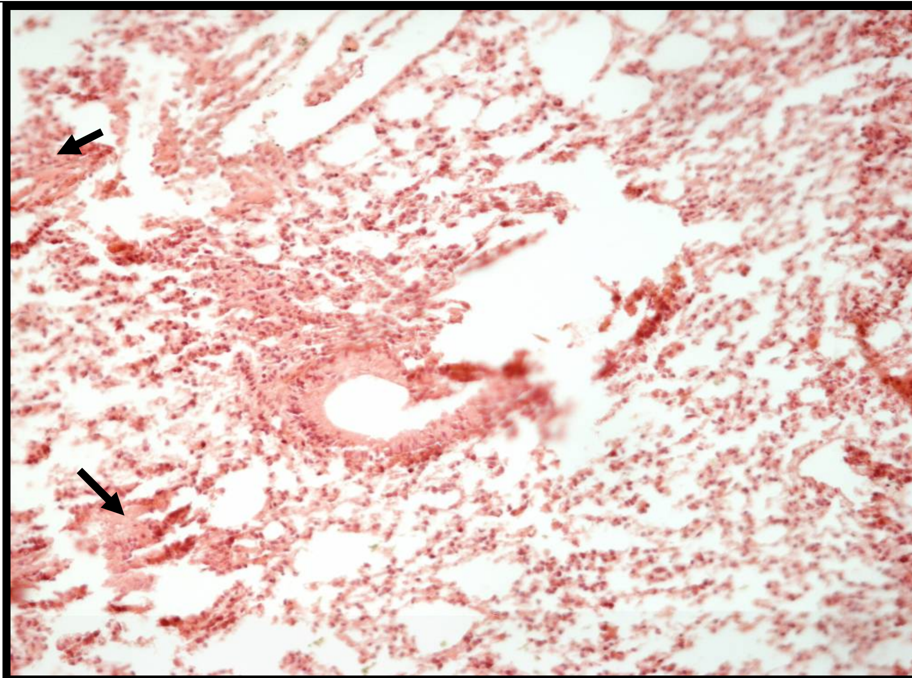
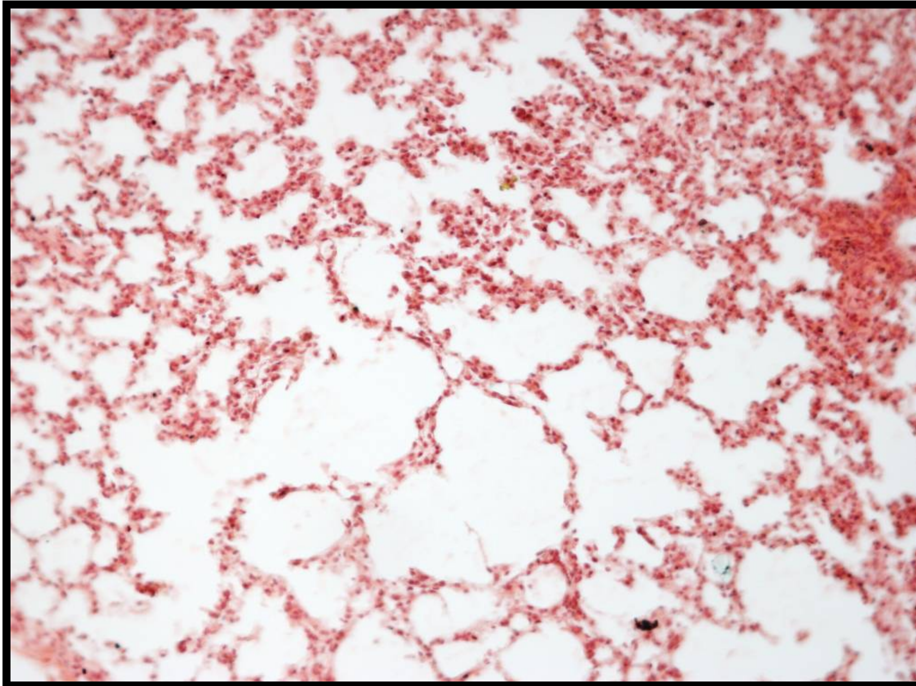


Figure 7.3: Histological cross sections of the lungs of mice treated with HHC showing **A)** and **B)** alveoli that are relatively small but numerous; alveolar walls moderately thick, alveoli compact and irregular in shape; alveolar walls discontinuous; smooth muscle thickenings of terminal airways seen, shown with arrows. H and E staining. Magnification X 100.

LHC A:



LHC B:

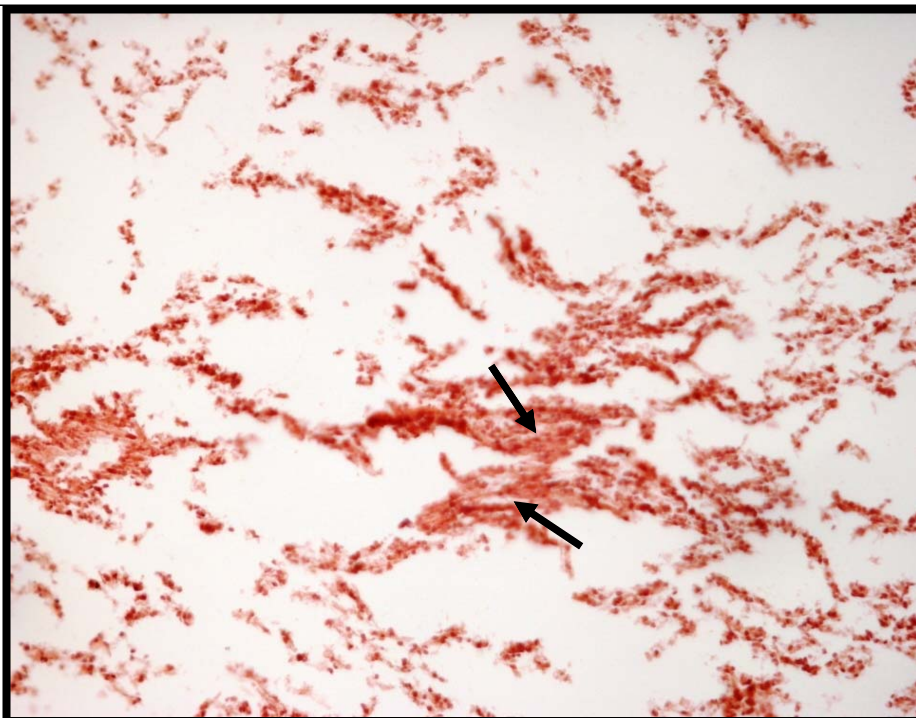
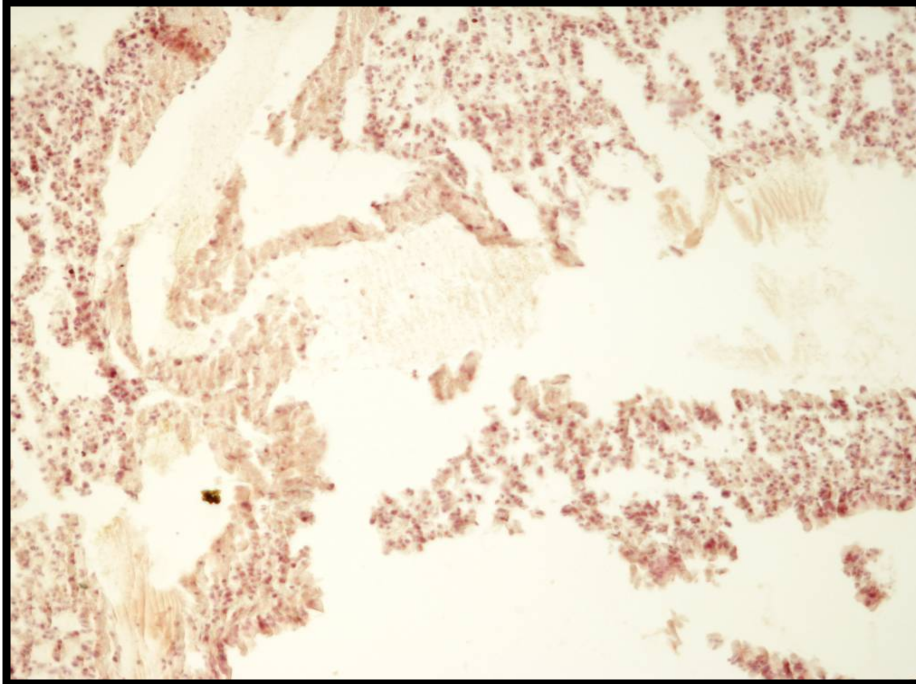


Figure 7.4: Histological cross sections of the lungs of mice treated with low HC showing **A)** and **B)** alveolar walls discontinuous and irregular; smooth muscle, masses seen in walls of distal airways) H and E staining. Magnification X 100

HEH A:



HEH B:

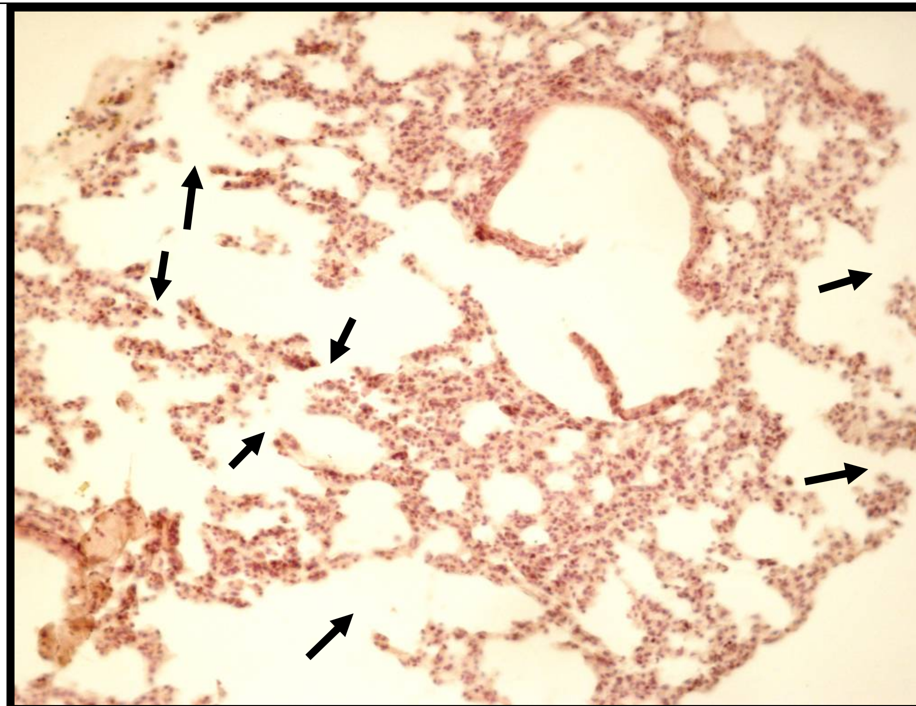
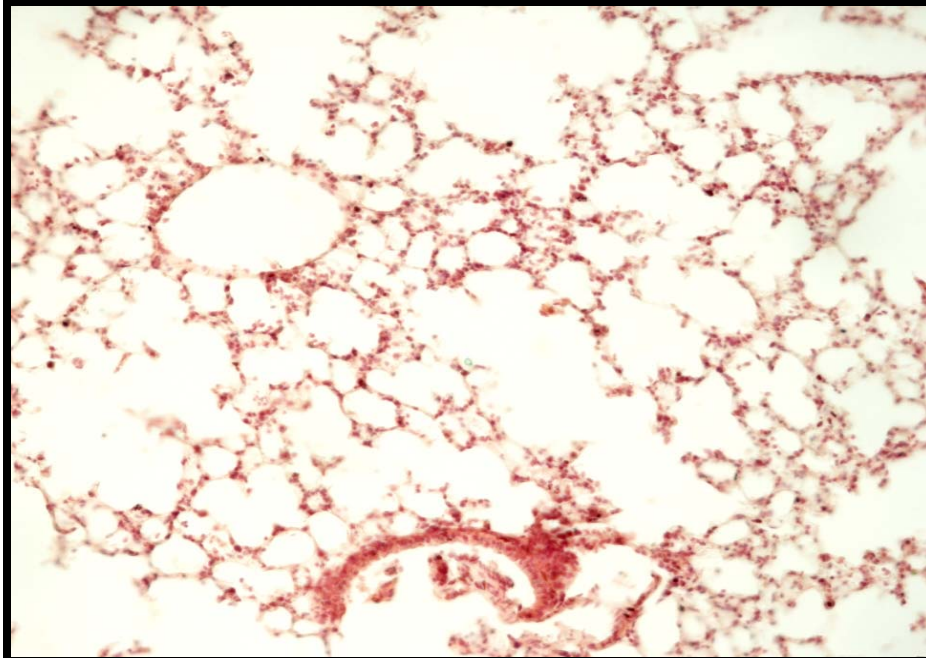


Figure 7.5: Histological cross sections of the lungs of mice treated with high EH showing **A)** and **B)** thick alveolar walls; alveoli very compact and irregular in shape; alveolar walls discontinuous (shown with arrows in B). H and E staining. Magnification X 100

LEH A:



LEH B:

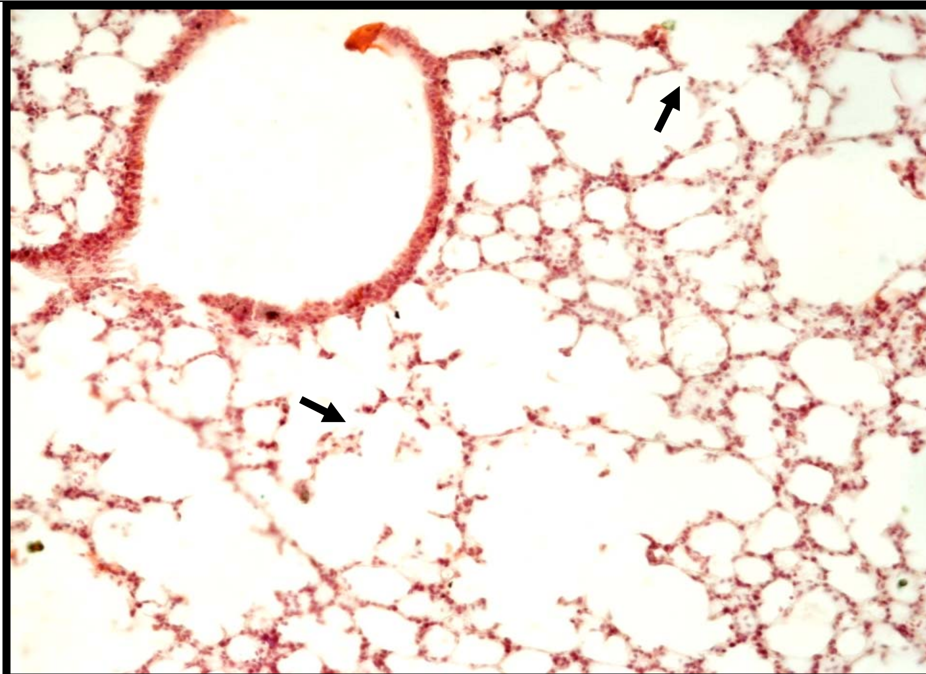


Figure 7.6: Histological cross sections of the lungs of mice treated with low EH showing **A)** thin alveolar walls; less compact and more patent alveoli; alveolar walls are only partially discontinuous (shown with arrows) and **B)** thinner alveolar walls; alveoli less compact and irregular in shape; alveolar walls only partially discontinuous (arrowed). H and E staining. Magnification X 100

The HEH treatment specimens showed compact, irregularly shaped alveoli having thick and discontinuous walls. In contrast, the low *E. hirta* specimens had thinner alveolar walls, less compact and more patent alveoli, with the alveolar walls only partially discontinuous. The features seen in the LEH specimens resemble those of the CT group specimens and therefore suggest that treatment with the LEH appears effective in ameliorating any histological alterations observed in the lung parenchyma in the presence of asthmatic conditions.

7.4.2. Transmission electron microscopy (TEM) analysis

Transmission electron micrographs revealed a variety of intra cellular and extracellular structures shown in the labelled figures below.

7.4.2.1. Cell types

Results from the photomicrographs below show that different cell types are present in distal respiratory passages and in lung parenchyma as follows:

Fibroblasts: were scanty in the CT specimens as well as in the low dose treatment specimens. However, these cells were as many in the “asthmatic” (AS) specimens as were in the high dose treatment groups.

Lymphocytes: were few in the specimens of the CT and LHC groups but were relatively scanty in specimens of the AS, HHC and HEH groups.

Monocytes: were absent in specimens from animals treated with the LHC.

Neutrophils: were generally not prominent but only very few were seen in CT group specimens.

Macrophages: were numerous in specimens of the asthmatic and LHC groups but only a few were present in specimens of the CT, HHC as well as in the HEH and LEH specimens.

Plasma cells: were scanty or absent in specimens of all groups except those from the low dose *E.hirta* treatment group. These cells were characterized by the elaborate presence of rough endoplasmic reticulum in their cytoplasm.

7.4.2.2. Other structures

Collagen fibres: were scanty in specimens from the CT and LHC groups. The remaining groups had varying amounts of collagen fibres.

Lamellar bodies: are usually associated with type 2 pneumocytes (great alveolar cells) and in this study, lamellar bodies were present in specimens of the low HC and low *E. hirta* doses.

Thick alveolar walls: were more prominent in specimens of the LHC group and less thick in the CT specimens.

Mitochondria: were prominent in specimens of the asthma and HHC groups.

Striations: were observed as special structures in some of the asthma group specimens, much similar to those observed in a typical striated muscle.

Mucous-secreting structures: were prominent in specimens of the LHC and LEH groups.

Smooth muscles: were relatively well developed in bronchiolar specimens of the asthma group compared to other treatment groups.

Filopodia: were seen in fibroblasts as cytoplasmic extensions only in specimens of the LHC and the HEH groups.

CONTROL

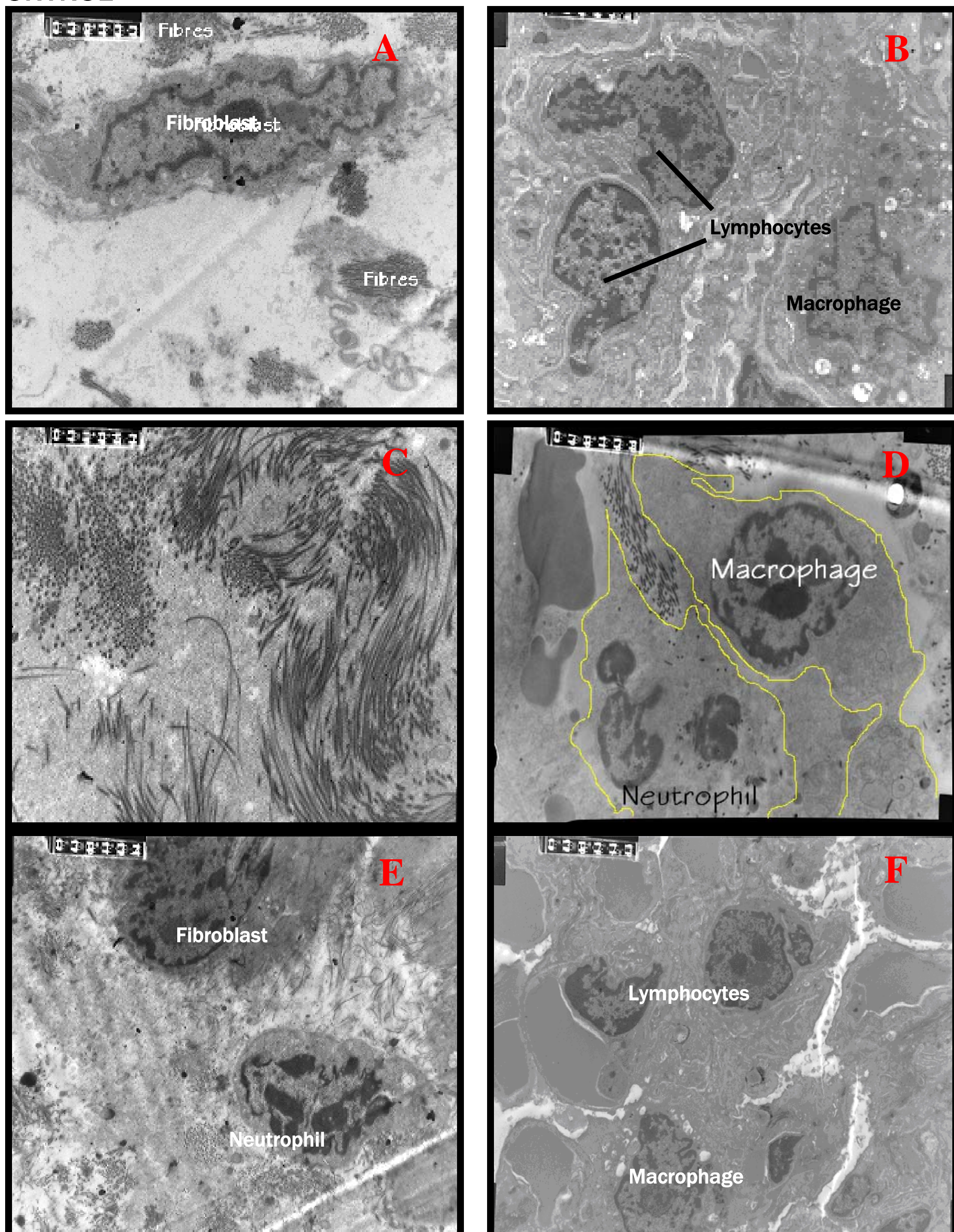


Figure 7.7: TEM micrograph of lung tissue from **control** mice showing **A** = Fibroblast (F), scanty collagen fibres [x7500]; **B** = Macrophage and lymphocytes in interstitium [x9800]; **C** = Collagen fibres [x13000]; **D** = Neutrophil, macrophage [x7500]; **E** = Fibroblast, neutrophils scanty fibres x5900; **F** = Lymphocytes and macrophage in lung interstitium [x4300].

ASTHMA

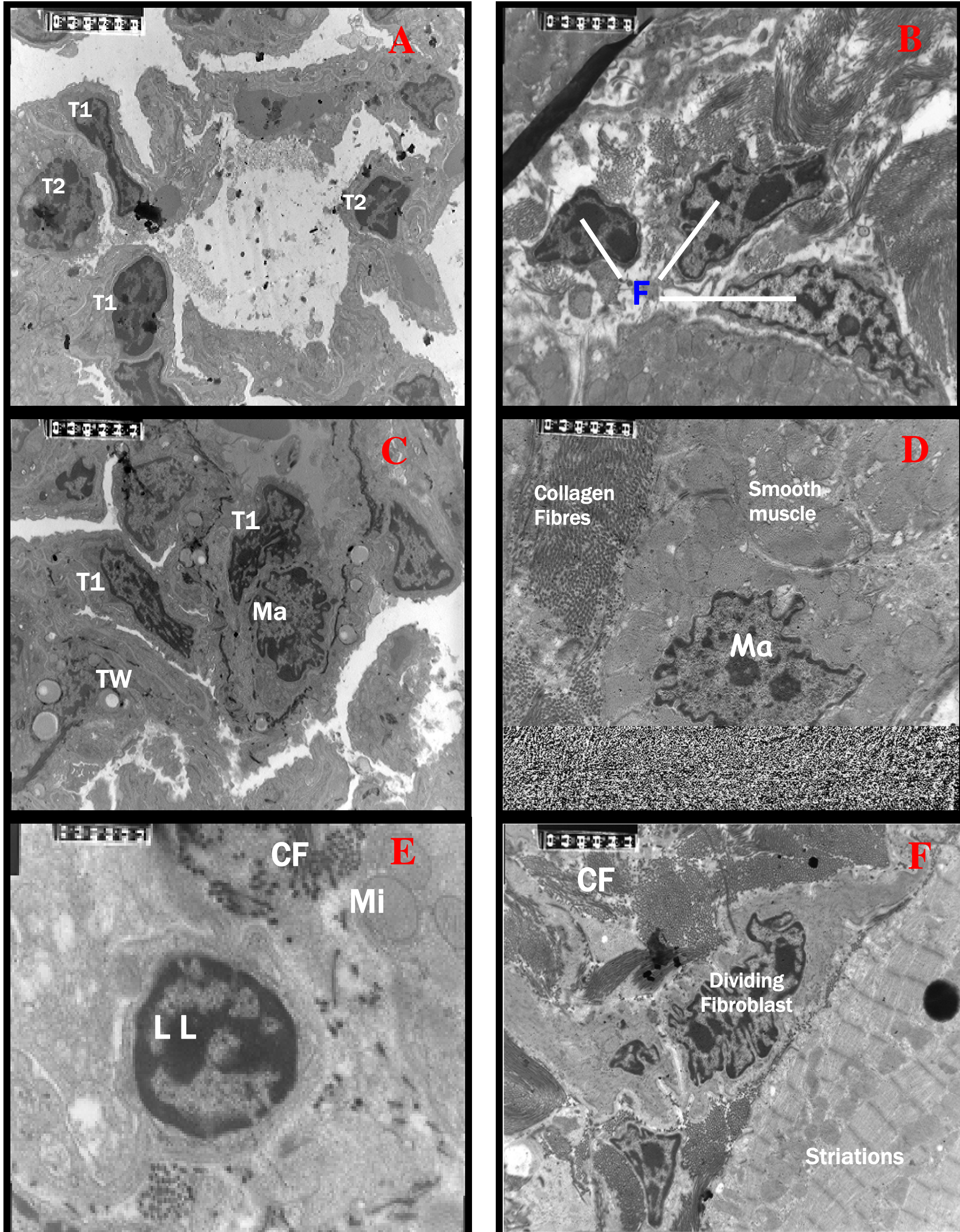


Figure 7.8: TEM micrograph of lung tissue from asthma mice showing **A** = Types 1 and 2 (T1; T2); thick walls [x3600]; **B** = Fibroblasts (F), many collagen fibres, smooth muscle [x5900]; **C** = Thick walls (TW), Type 1 cells (T1); macrophage (Ma) [x4300]; **D** = Macrophage (Ma), collagen fibres, smooth muscle [x9800]; **E** = Large Lymphocytes (LL); fibres (F), mitochondria (Mi) [x18000]; **F** = Many collagen fibres (CF), fibroblast, striations [x5900].

LHC

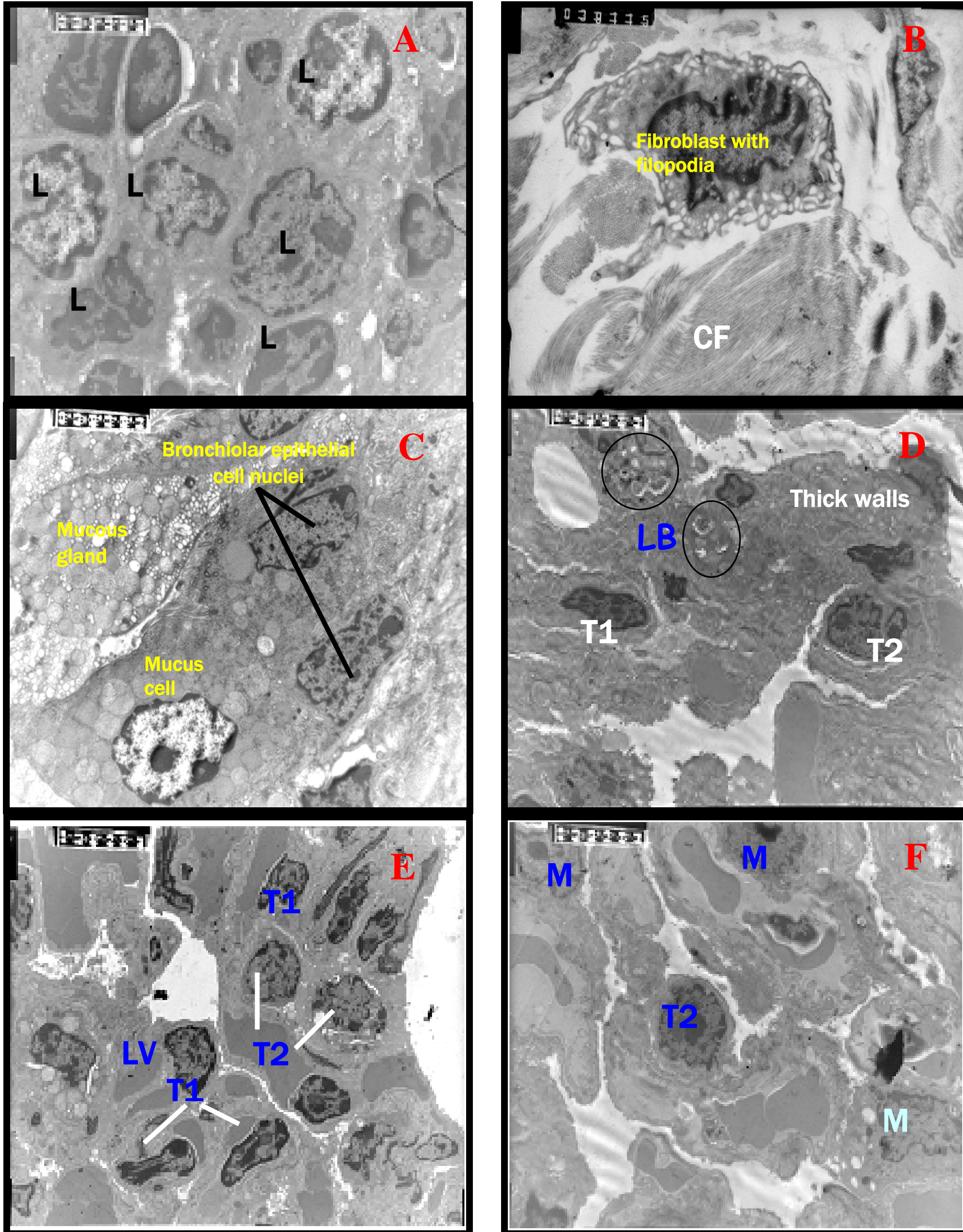


Figure 7.9: TEM micrograph of lung tissue from asthma mice treated with LHC showing **A =** Monocyte (Mo), lymphocytes (L) [X5900]; **B =** Fibroblast, collagen fibres (CF) [X7500]; **C =** Mucous secreting structures [X4300]; **D =** Thick walls, lamellar bodies LB, Types 1, 2 cells [X3600]; **E =** Lymphocyte with vesicles (LV), Types 1, 2 cells [X2800]; **F =** Thick walls, macrophages (M), T2 cells [x3600].

HHC

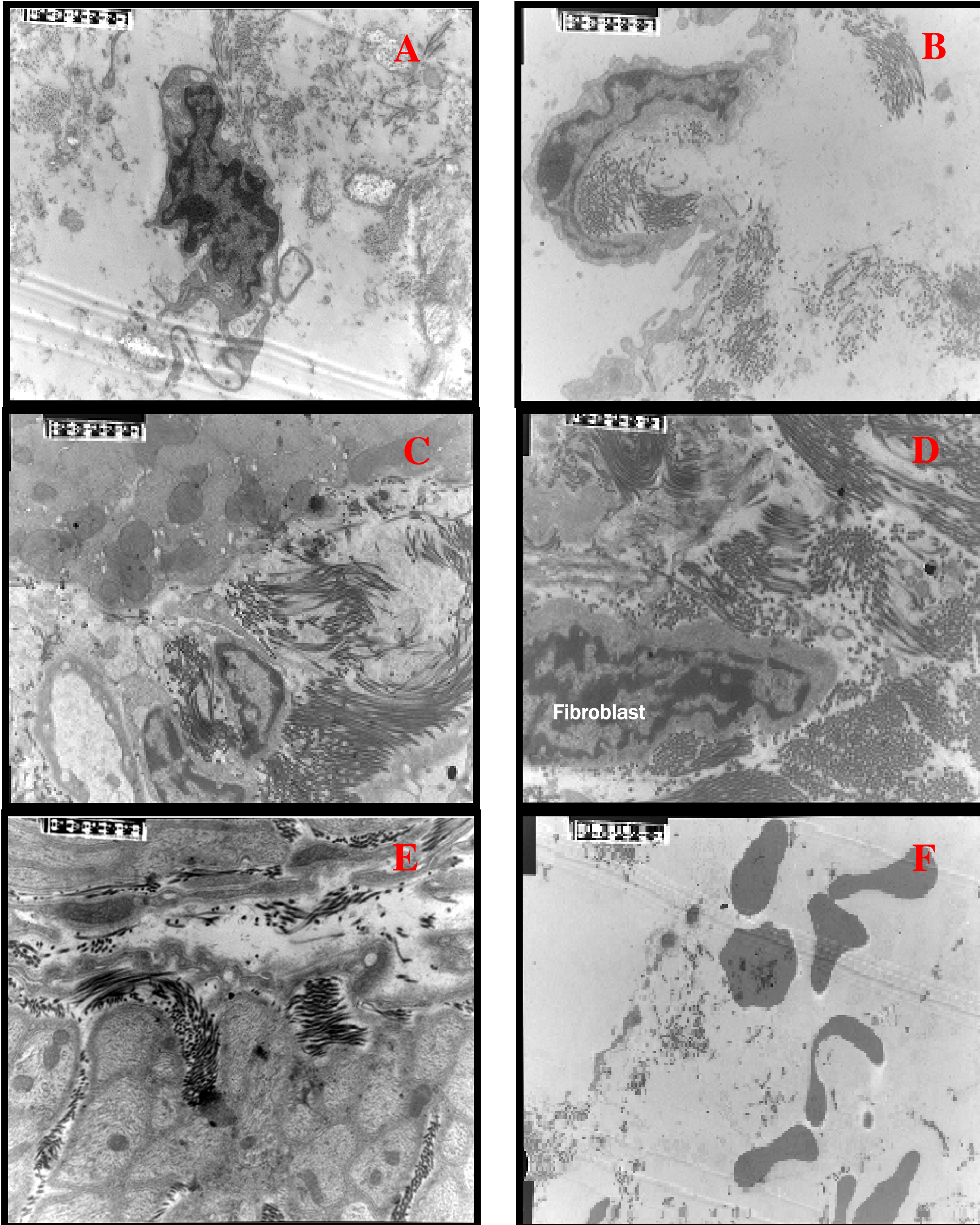


Figure 7.10: TEM micrograph of lung tissue from asthma mice treated with HHC showing **A =** Fibroblast and scanty collagen fibres [x9800], **B =** Fibroblast and scanty collagen fibres [x9800], **C =** Fibroblasts, few fibres, many mitochondria [x9800], **D =** Fibroblast (F), relatively more fibres (CF) [x9800], **E =** Unmyelinated nerves and collagen fibres [x13000], **F =** Very scanty collagen fibres, red blood cells [x4300]

HEH

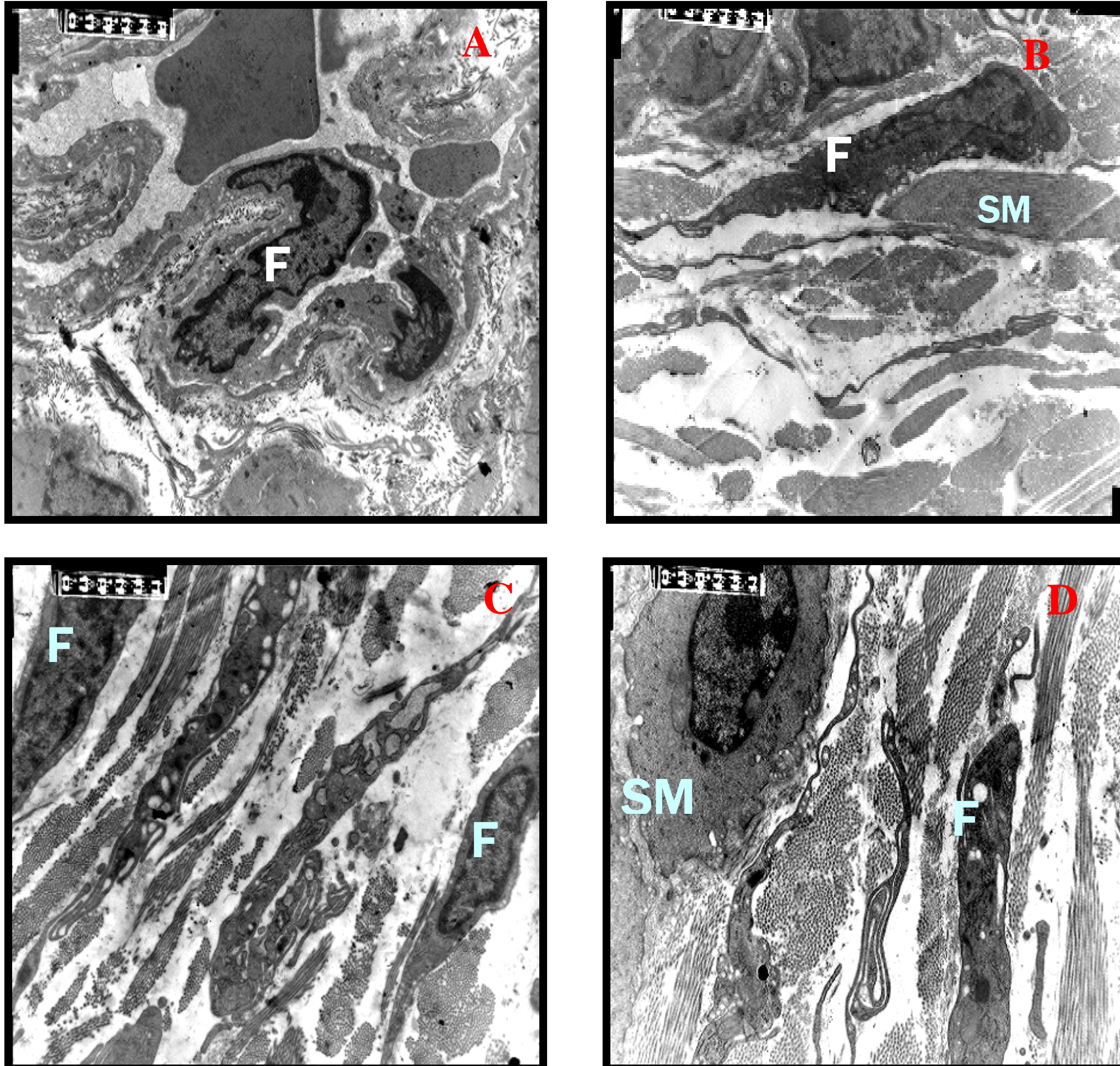


Figure 7.11: TEM micrograph of lung tissue from asthma mice treated with HEH showing **A** = Highly fibrous lung parenchyma, fibroblast [x5900]; **B** = Highly fibrous lung parenchyma, fibroblast [X5900]; **C** = Fibroblasts (F) and fibres [x7500]; **D** = Smooth muscle (SM) cell, many fibres [x9800]

LEH

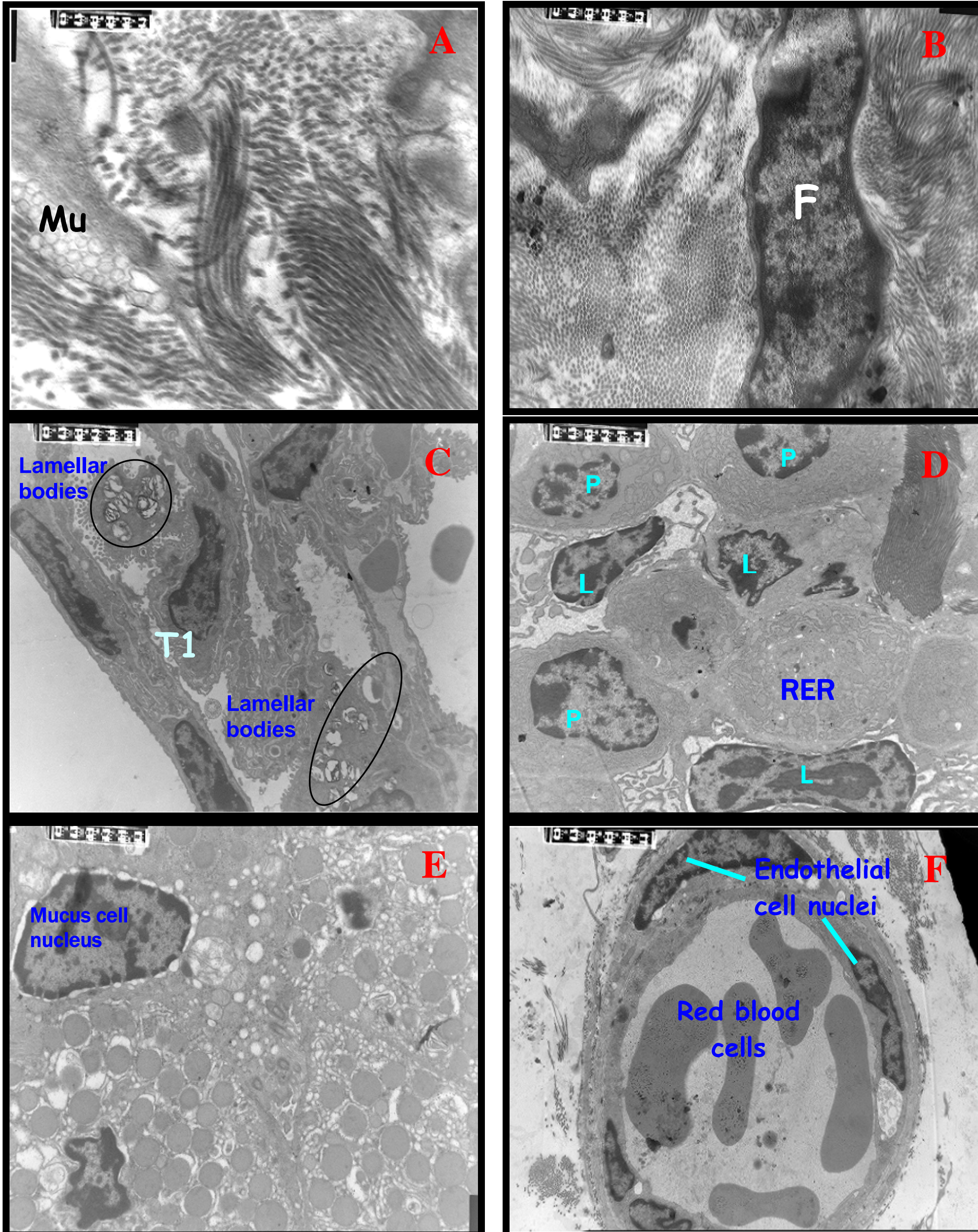


Figure 7.12: TEM micrograph of lung tissue from asthma mice treated with LEH showing **A =** Mucus gland (Mu) and many fibres [x3600]; **B =** Fibroblast (F), many fibres [x13000]; **C =** Alveolar walls, Type 1 (T1) cells, lamellar bodies [x4300]; **D =** Bronchioles: Plasma cell (P), lymphocyte (L), Rough ER [x4300]; **E =** Bronchial seromucous gland and cell [x7500]; **F =** Bronchial artery containing red blood cells [x5900]

7.5. Discussion

The airway wall consists of epithelial, interstitial, nervous and immunological components (Plopper 1996, Pinkerton 1997, Evans et al., 1999) all of which actively interact with one another (Kips et al., 2003). The common structural changes that characterize the asthmatic condition include an increase in epithelial goblet cells, mucous gland hyperplasia, subepithelial fibrosis (with deposition of collagen and fibronectin), smooth muscle hypertrophy and/or hyperplasia, formation of new vessels (Ordonez et al., 2001; Kips et al., 2003), among others.

Cell migration into the airway lumen and parenchyma is a common and very important feature of asthma and a wide range of cells are known to be involved. In prior experiments discussed in chapter five of this study, results from blood parameters have confirmed the presence of asthmatic conditions in the experimental mice used in this study.

7.5.1. Cellular structures

Fibroblasts

Results obtained from this study show that fibroblasts were scanty in both the LHC and LEH specimens respectively, almost as much as was seen in the CT specimens. This finding appears to indicate that treatment with the LHC and LEH could lower fibroblast proliferation. On the other hand, the HHC and HEH specimens had more fibroblasts but only as much as in the ‘asthmatic’ specimens. This finding may suggest that the high dose treatment with both HC and *E. hirta* has minimal or no effect on fibroblast proliferation since about same amount of fibroblasts was seen in the AS specimens.

It is not clear why the low dose treatments were more effective in reducing fibroblast proliferation in this study but previous studies have long shown that glucocorticoids inhibit fibroblast proliferation albeit through obscure molecular mechanisms (Kruse et al., 1978; Ponec et al., 1979; Ramalingam et al., 1997). The effects of many botanical extracts on fibroblast proliferation showed similar results. Van Wyk et al (1994) demonstrated that areca nut extract was toxic to cultured fibroblasts and inhibited their proliferation in a concentration-dependent manner. Valentiner et al (2003) also showed that six out of seventeen 'potentiated' plant extracts, had no effect on fibroblast proliferation while eleven of the plant extracts had a dose-dependent inhibitory effect.

Lymphocytes

These cells are among the most prominent cell types during asthma. The 'compartmental' distribution of these cells was studied in the Brown Norway rat asthma model and lymphocyte numbers were observed to have increased in the BALF and in the lung parenchyma (Schuster et al., 2000). The CD8 subtype of T-lymphocytes is also known to form a significant component of alveolar wall inflammatory infiltrate (Finkelstein et al., 1995; Saetta et al., 1999).

Lymphocyte migration to the airways and into airway tissue appears to occur via an indirect mechanism and the effects of the treatment agents used in this study on lymphocyte migration have been studied. It was found in one study, that in spite of their widespread effects during asthma, glucocorticoids were capable of reducing the effects of cytokine-induced recruitment and survival of inflammatory cells in general, of which the lymphocytes are a part (Schwiebert, et al., 1996).

In this study, a few lymphocytes were seen in the lung parenchyma of the LHC dose specimens but in all other groups, including the AS group, there were much fewer lymphocytes. The implication of this finding for the high HC group may possibly be that 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 unlike as observed in the low HC specimens in which the effect on inhibition seemed minimal.

The exact mechanisms by which the *E. hirta* extract acts on lymphocytes are not clear but both the high and low doses of this extract appear to exert similar pattern of effects on lymphocyte migration to the airway interstitium probably via alteration of adhesion molecules. Previous studies have shown that Bromelain, a natural proteinase preparation derived from pineapple stem, selectively removed certain cell surface molecules that possibly affected lymphocyte migration and activation following *in vitro* treatment (Hale and Haynes, 1992).

Neutrophils

Neutrophils were not seen in specimens of all other groups (including the asthma group) but very few were found in the CT group specimens. Neutrophils normally have a high turnover and rapid clearance via apoptosis, followed by macrophage phagocytosis (Cox et al., 1995; DeLeo 2004). The few neutrophils present in the CT specimens could be naïve residual cells normally attracted to the airways prior to an infection or inflammation. In the AS and all other treatment groups, an initial neutrophil accumulation could have occurred, followed by either migration away or apoptotic elimination from airways. A previous study by (DeLorme et al., 2002) showed that airway tissue neutrophil numbers were capable of reverting to control

values after 24 hours airway exposure to ozone. This could explain the observation for the AS group but not for the different treatment interventions. It is unclear whether the transient elevation of tissue neutrophils in the airways and the accompanying changes apply only to ozone exposure or is of general application.

The mechanism through which the different treatment applications contributed to the absence of neutrophils in the airways is not clear, but glucocorticoid treatment is generally known to inhibit neutrophil apoptosis in the airways (Cox 1995) implying that neutrophil numbers were supposed to be much higher in the airway tissue of the HC treated specimens. It thus appears that in this study even 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

In this study, monocytes were only seen in the LHC specimens but absent in all other specimens. This could be because in asthma, circulating monocytes are mobilized via differentiation into the macrophage pool that addresses any prevailing inflammatory conditions in the airways and even down to the alveoli (Landsman and Jung, 2007). Thus, there would be only few or no monocytes in especially the distal respiratory structures in all the other specimen groups. It is possible that unlike *E. hirta*, HC alters the monocyte-macrophage balance in a reverse dose-dependent manner.

Macrophages

There is much information in literature about the dynamics of macrophage involvement in non-asthmatic forms of inflammation than in asthmatic

inflammation. Macrophage infiltration into the airway and lung parenchyma has only been identified as a common feature in COPD and bronchitis (Saetta et al., 1993; Barnes, 2002) but little has been reported about their involvement in asthma. Results from this study showed that there were relatively more macrophages in specimens of the AS and LHC groups than in specimens of the CT, HHC as well as the HEH and LEH.

The presence of only a few macrophages in the CT specimens and relatively more of the cells in the AS group is understandable but in all the other treatment groups, it seems that only a few of the mobilized macrophages were able to penetrate the airway walls while the rest possibly remained within the airway spaces, including the alveolar spaces. The HHC and both doses of *E. hirta* plant extract appear to inhibit migration of the macrophages into the airway parenchyma while the LHC appeared to have little or no inhibitory effect on the ability of macrophages to penetrate the airway parenchyma.

Plasma cells

These cells are essential immunological cells in asthma and are known to be derived from B-lymphocytes. Under the influence of cytokines, plasma cells produce antigen specific IgE. Allergens induce T cells to activate B cells, which then develop into plasma cells that produce and release more of the IgE antibodies (Maddox and Schwartz, 2002). After their role in early immune reactions, plasma cells appear to migrate finally to the bone marrow where they survive without undergoing any further proliferation (Manz et al., 1997; Slifka et al., 1998).

In this study, plasma cells were easily identified by the elaborate presence of rough endoplasmic reticula in their cytoplasm. Plasma cell numbers were either very scanty or absent in all treatment specimens except those of the LEH group. The AS group also contained but only a few plasma cells in the airway and lung parenchyma, an indication that these cells are only prominent and active at the early stage of the inflammation process and not resident in the airways.

The presence of a few cells observed in the LEH group could indicate that this dose was inadequate to promote migration of plasma cells away from the airways but it is not clear whether this dose rather promoted the migration of the plasma cells into the airway interstitium. On the other hand, though plasma cells ultimately migrate away from the airways, treatment with the HEH as well as with both HC doses appears to be effective in promoting this migration, hence the fewness or absence of these cells in specimens of these treatment groups.

Pneumocytes

These alveolar cells are resident structural cells and therefore not expected to vary remarkably in number between the specimens of the respective treatment groups. In most species, there are generally more type-II cells than are type-I cells in the alveoli even though type-I cells cover 95% of the alveolar surface (Berthiaume et al., 2006). In the rat lungs for instance, type-II cells cover approximately 3.6% while type-I pneumocytes cover 96.4% of the entire alveolar surface (Travis and Tucker, 1986). In this study, there were more of the flat type-I cells than the cuboidal type-II cells in the alveolar parenchyma of all specimens indicating that the numerical composition of these resident cells was not distorted by the different experimental interventions.

7.5.2. Other structures

Collagen deposition

In the subepithelial "basement membrane" (SBM), collagen deposition is a known feature in the pathophysiology of asthma. Submucosal collagen deposition has also been studied especially because this airway region is larger and anatomically closer to smooth muscles than it is to the subepithelial "basement membrane" (Chu et al., 1998). The authors suggested from their findings that large airway collagen deposition might not be a key contributor to the symptoms and pathophysiology of asthma.

The present study explored the distal airways and the lung parenchyma. Collagen fibre deposition was minimal in the CT specimens as well as in specimens of the LHC groups. Specimens of the AS group had a high amount of fibre deposition compared to others. The remaining groups had varying amounts of collagen fibre deposition, albeit higher than observed in the CT specimens indicating that treatment with *E.hirta* extract as well as with the HHC did not cause significant reduction in collagen fibre deposition even after a possible increase at the onset of asthma prior to treatment. The LHC specimens had minimal collagen deposition possibly because the low dose was ineffective in reducing collagen deposition.

The exact mechanism involved in the reduction of collagen fibre deposition following HC or *E. hirta* treatment is not understood. Results from previous studies have been indeterminate regarding the effects of steroids on airway remodelling. Laitinen et al (1992) and Jeffery et al (1992) demonstrated that inhaled corticosteroids reduced airway inflammation, but could not reduce the thickness of the SBM in mild/moderate asthmatics. In contrast, other studies (Trigg et al., 1994;

Olivieri et al., 1997; Sont et al., 1997) showed reduction of the thickness of bronchial subepithelial basement membrane after a few months of inhaled corticosteroid treatment in mild/moderate asthmatics. Not much information is available on the effects of plant extracts on collagen deposition in airway parenchyma and no previous studies are available on the effects of *E. hirta*. However, extracts of *Ecklonia Cava* (a polyphenol/phlorotannin derived from Brown Algae) have been reported to cause reduction in airway epithelial hyperplasia by 75% as well as reduced collagen-causing fibrosis in lung interstitium by 20% (Levine, 2007).

Lamellar bodies

Alveolar type II cells synthesize, store and secrete pulmonary surfactant (King, 1974) and the final secretory product is segregated as concentric lamellar bodies, which are destined for secretion into the alveolar space to become part of the surfactant-lining layer (Chevalier and Collet, 1972). The presence of many lamellar bodies could therefore indicate increased surfactant secretion.

In this study, lamellar bodies in type II cells were more abundant only in the LHC and LEH specimens. A previous study showed cortisol-accelerated synthesis of pulmonary surfactant in the human fetal lung as early as the second trimester (Ekelund et al., 1975). Cortisol also caused a marked stimulation of synthesis and accumulation of lamellar body phosphatidylcholine in lung explants from both 21-day and 28-day fetal rabbits (Mendelson and Synder, 1985). Similarly, Asabe et al (2007) obtained results which suggested that maternal dexamethasone (a glucocorticoid) treatment accelerates the maturation of the surfactant system, especially the expression of lamellar bodies in type II pneumocytes, even in

hypoplastic lungs induced by oligohydramnios. It is not clear if the stimulatory effect of HC observed in this study was dose dependent.

There is no information available in literature on the effects of *E.hirta* extract administration on either the formation of lamellar bodies or surfactant production. However, the effects of other herbal products on the surfactant system have been reported. Amygdalin, which occurs naturally in a number of plant materials but usually commercially obtained from the kernel of *Prunus armeniaca L.* (apricot pits) was found to partially protect type II alveolar epithelial cells from the effects of hyperoxia-induced lung injury (Huaping et al., 2004). In another study, “Bakumondo-to” (a traditional Chinese medicinal prescription consisting of six herbs (*Ophiopogonis tuber, Pinelliae tuber, Zizyphi fructus, Glycyrrhizae radix, Ginseng radix, and Oryzae fructus*), was found to have characteristic secretory-enhancing effect on pulmonary surfactant and normalizes hypersecretion by the inhibition of superoxide (Miyata et al., 1999).

The absence of lamellar bodies in the asthmatic group specimens as well as specimens of both higher doses of the test agents used in this study is not understood. In the asthmatic group, it seems that induction of asthma in the mice did not influence a high type II cellular activity by way of lamellar body formation and surfactant secretion. This seeming absence of surfactant-producing cells possibly relates to the reasons the airways remain constricted and the alveoli collapsed during an asthmatic attack unlike what is seen in normal lungs. It is not clear why in this study there was a dose-related disparity in the effects of treatment on the presence of lamellar bodies. However, the absence of lamellar bodies in the high dose specimens could mean that treatment with these high

doses does not stimulate lamellar body formation and surfactant production in spite of other positive anti-asthmatic effects associated with these dose levels.

Alveolar wall thickness

Another feature observed in this study was the relative thinness of the alveolar walls in specimens of the CT group as well as the moderate thickness in all other groups with the thickest walls seen in specimens of the LHC group. This finding tends to suggest that all other treatment agents and doses had varying degrees of effect in reducing alveolar wall thickness except the low HC treatment, which appears to have a mild effect on collagen fibre deposition in the alveolar wall.

Mitochondria

An important role for mitochondria in the pathogenesis of inflammation and in the development of bronchial asthma has been reported (Heinzmann et al., 2003). In this study, increase in mitochondria number in the different cells studied showed that there were relatively higher numbers of mitochondria in the cells of the asthma and HHC specimens. Damage to mitochondria in asthma could affect clearance of any free radicals released in asthmatic inflammation and increased mitochondria numbers could point to the high-energy requirement of these cells. The number and activity of mitochondria in smooth muscle cells from patients with asthma was found to increase due to an altered calcium homeostasis that increases mitochondrial biogenesis (Trian et al., 2007). The reason why there were higher mitochondria numbers in the asthmatic group is not clear but much energy is apparently required in asthma by cells to respond to the inflammatory reactions associated with the sudden onset of experimental asthma and this will involve mitochondrial activity.

Results of experiments by (Lowe et al., 1954) have long indicated that the number of mitochondria per cell is significantly reduced in rat liver cells following parenteral administration of HC. It is therefore not understood why the HHC specimens had relatively higher numbers of mitochondria compared to specimens from other treatment groups. Perhaps organism specificity and activity levels are possible implicating factors since in this case HC was administered as a treatment for the asthma induced in the mice.

Striations

The presence of striated muscle cells in non-neoplastic lung parenchyma is an uncommon observation (Aterman and Patel, 1970; Chellam 1988) even though these cells have been previously detected in pulmonary anomalies. Studies with human specimens have shown many cases of proliferation of these cells in non-neoplastic lung tissue (Fraggetta et al., 2000) and the authors believed these findings are more common than usually reported.

Striations seen in this study were only present in specimens of the AS group and were much similar to those observed in a typical striated muscle. It is not clear why in this study, such rare finding occurred only in the AS group and therefore the association of this structure with asthma is uncertain. Although the exact origin of these cells is speculative, a wide morphogenetic error has been suggested (Drut et al., 1988; Fraggetta 2000). No previous studies on experimental asthma have shown similar results.

Mucus-secreting structures

Mucus plugs occur in airways of all sizes, from the second-generation airway to bronchioles (Roche et al., 1989). Although only relatively few mucous-secreting

structures were identified in the asthma group specimens, these structures were very prominent in specimens of the LHC and LEH groups respectively but absent in specimens of the respective high doses and in the controls. This finding tends to suggest that the low doses of the two treatment agents are ineffective in ameliorating or eliminating mucous cell proliferation and possibly mucus secretion. The presence of relatively few mucous-secreting structures in the AS group under the TEM may not imply that mucous secretion was not a feature of this model of asthma. It is possible that mucus secretion is not dependent solely on the number of cells but on the metabolic potency of the few mucous cells and glands present.

Smooth muscle cells

Smooth muscle cells surround the airways from the trachea down to the alveolar ducts and their constriction could be induced by a variety of stimuli. It is not understood why more smooth muscle is present in the airways of patients with asthma, although the commonly held paradigm suggests that mediators released during chronic inflammation induce airway smooth muscle (ASM) proliferation (Panettieri 1998; Hirst et al., 2000). The precise role of this muscle in the pathogenesis of asthma is not certain but it is known that ASM contraction certainly causes acute narrowing of the airway and airflow obstruction in asthma, and airways (Panettieri, 2004; Solway, 2007).

In this study, smooth muscle mass increased prominently in specimens of the AS group while specimens of other groups exhibited varying degrees of muscle hypertrophy that possibly relate to the respective treatment doses. This finding conforms to information from previous studies. Treatment with HC and the extract of *E. hirta* appear to be effective in preventing airway smooth muscle cell

proliferation. Glucocorticoids have been shown to inhibit the proliferation and migration of airway myocytes and suppress their expression of a number of proinflammatory cytokines (Bonacci et al., 2006; Solway, 2007). In one previous study with herbal products, the ethanolic extract of a Malagasy species *Euphorbia stenoclada* (ES) (Euphorbiaceae), traditionally used as a herbal remedy against asthma and acute bronchitis, was tested to evaluate possible anti-proliferative activity on human airway smooth muscle cells (HASMC) (Chaabi et al., 2007). This extract was found to completely abolish the interleukin-1β (IL-1β)-induced proliferation of HASMC. It is therefore possible that the absence of airway smooth muscle proliferation in the *E. hirta* group reflects similar effects as seen with the *Euphorbia stenoclada* treatment.

Filopodia

Filopodia are thin, dynamic cell extensions comprising tight bundles of long actin filaments covered with cell membrane. Filopodia allow cells to explore their environment (extracellular matrix and surfaces of other cells), to identify appropriate targets for adhesion, and then generate guidance cues and traction forces to move the cell body (Jacinto and Wolpert, 2001). The presence of these structures in cells may indicate a high cellular activity. In this study, only specimens of the LHC group had fibroblasts with many filopodia. It is not understood whether this low dose treatment promotes filopodia formation but its inability to prohibit filopodia formation appears certain. The absence of filopodia in the fibroblasts of the HHC dose specimens could indicate that this dose inhibits filopodia formation by fibroblast.

A previous study showed that Piperlactam S inhibited filopodia formation in macrophages among other effects (Chiou et al., 2003). Piperlactam S is an alkaloid isolated from *Piper kadsura*, an anti-inflammatory Chinese herbal medicine used for the treatment of asthma and rheumatic arthritis (Han et al., 1990). Since *E. hirta* extract also contains alkaloids, the absence of filopodia in the specimens of both the high and low doses of *E. hirta* extract in this study possibly denotes inhibition of filopodia formation by the extract via a mechanism similar to that described for Piperlactam S by Chiou et al (2003).

A summary of all the findings is given in *table 7.1.*

Table 7.1: Summary of the comparative effects of HC and *E hirta* extract treatments on inflammatory cell infiltration and structural changes in the airways as determined by TEM analysis.

Cellular structures

	CT	AS	LHC	HHC	LEH	HEH
Fibroblasts	Very few	Many	Scanty	Few	Scanty	Few
Lymphocytes	Very few	Very few	Few	Very few	Very few	Very few
Neutrophils	Very few	Absent	Absent	Absent	Absent	Absent
Monocytes	Absent	Absent	Very few	Absent	Absent	Absent
Macrophages	Very few	Many	Few	Very few	Very few	Very few
Plasma cells	Scanty	Few	Scanty	Scanty	Very few	Scanty
Pneumocytes	More type-I cells than type-II cells in all specimens; treatment did not alter the cell ratio.					

Other structures

	CT	AS	LHC	HHC	LEH	HEH
Collagen fibre deposition	Minimal	More than in CT	Few	More than in CT	More than in CT	More than in CT
Lamellar bodies	Absent	Absent	Many	Absent	Many	Absent
Alveolar wall thickness	Very thin	Very Thick	Thickest	Thick	Thick	Thick
Mitochondria	Few	Many	Few	Many	Few	Few
Striations	Absent	Present	Absent	Absent	Absent	Absent
Mucus-secreting structures	Absent	Few	Many	Absent	Absent	Many
Smooth muscle cells	Few	Many	Few	Few	Few	Few
Filopodia in fibroblasts	Absent	Absent	Present	Absent	Absent	Absent

7.6. Conclusion

The general effects of both treatment agents show that 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 whereas lymphocyte migration into the lung parenchyma was found to be inhibited by both HC and *E. hirta* in a dose-dependent manner. In addition, HC treatment appeared to be more effective than *E. hirta* in promoting monocyte differentiation into macrophages, albeit in a dose dependent manner.

This study also showed that the numerical composition of alveolar pneumocytes was not distorted by the different experimental interventions and abnormal muscle striations were observed only in the AS specimens but all treatment agents and doses were found to have varying effects on alveolar wall thickness. Treatment with both HC and *E. hirta* also appeared to abolish smooth muscle hypertrophy.

There were relatively more mitochondria in specimens of the ‘asthmatic’ mice than others and treatment with the LHC appeared more effective in promoting lamellar body formation and plasma cell migration into the airway parenchyma but caused reduced fibroblast proliferation. Treatment with the HHC dose appeared to promote migration of plasma cells into the airway parenchyma but inhibited filopodia formation by fibroblasts as well as migration of macrophages into the airway parenchyma. The HHC treatment also appeared to inhibit mucous cell proliferation but did not reduce collagen fibre deposition. In addition, there were relatively more mitochondria in the HHC specimens than in others.

The LEH extract not only caused a reduction in fibroblast proliferation but also inhibited the migration of macrophages into the airway parenchyma as well as filopodia formation by fibroblasts. However, treatment with this dose of *E. hirta* promoted lamellar body formation whereas treatment with the HEH dose appeared promote plasma cell migration into the airway parenchyma but inhibits migration of macrophages into the airway parenchyma. This HEH dose also inhibits filopodia formation by fibroblasts as well as mucous cell proliferation but did not reduce collagen fibre deposition.

When compared to the white cell counts in *tables 5.1* and *5.2*, no correlations were seen in terms of abundance. Neutrophils, lymphocytes and monocytes were all more abundant in the asthma group than in other cells, extreme variations from this were observed in the peripheral blood cell counts.

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.

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