

**Investigating the effect of *Withania somnifera* on
coagulation, blood count and bronchial lavage of Balb/c
mice**

By

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Investigating the effect of *Withania somnifera* on coagulation, blood count and bronchial lavage of Balb/c mice

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Abstract

Withania somnifera is a phytomedicine that is an important component of the Ayurvedic Pharmacopoeia of India and has been used for over 400 years to treat all kinds of diseases and ailments. The aim of this study was to investigate the effect of *W. somnifera* on coagulation, blood count and bronchial lavage by using the asthmatic Balb/c mouse model. Because it's known that asthmatic patients have decreased selenium levels and since selenium is known to be a potent antioxidant, the effects of selenium alone and in combination with *Withania somnifera* were investigated in this study. Hydrocortisone is most commonly used in the treatment of asthma and therefore hydrocortisone was used as positive control in this study.

To establish the asthmatic Balb/c mouse model, the mice were sensitized, nebulized and treated over a period of 43 days and blood smears were made of each individual animal and bronchial lavage was performed by injecting 0.3ml of saline into the trachea of the mice on the day of termination. Light microscopy analysis of the bronchial lavage revealed a significant decrease in the number of eosinophils counted in the asthmatic and the different treatment groups. In the asthmatic group, numerous platelet clumps were found distributed between white blood cells. Platelets were also found in the other

treatment groups but are not as prevalent as in the asthmatic group. Results from the blood smears showed the same trends, where cell counts in control and hydrocortisone blood smears were decreased compared to that of the asthma group.

Platelets and fibrin play an important physiological role in allergic reactions and immunological mechanisms. In conditions like allergic asthma, platelets participate by acting as inflammatory cells, by releasing mediators, spasmogens and/or by interacting with other inflammatory cell types. Results indicate control mice possess major, thick fibers and minor thin fibers as well as tight round platelet aggregates with typical pseudopodia formation. Minor fibers of asthmatic mice have a netlike appearance covering the major fibers, while the platelets seem to form loosely connected, granular aggregates. Hydrocortisone made the fibrin more fragile and the platelet morphology changes from a tight platelet aggregate to a more granular aggregate not closely fused to each other. The plant extract separately and in combination with selenium did not impact on the fragility of the fibrin and reversed the formation of the dense minor netlike layer over the major fibers. A tight platelet aggregate was also seen in these mice. Asthmatic mice only treated with selenium showed a dense minor fibrin layer (as was seen in the asthmatic mice); however the platelets formed a dense aggregate.

It is concluded that *W. somnifera* and *W. somnifera* combined with selenium significantly decreased the white blood cells in both bronchial lavage as well as blood smears, suggesting that *W. somnifera* indeed has an anti-inflammatory potential and it, in combination with an anti-oxidant like selenium might successfully be used in the treatment of asthma. Also, the anti-inflammatory products impacts on the fibrin network but not on the platelet aggregate stability (seen with hydrocortisone). Anti-oxidants like selenium do not impact on the stability of the fibrin networks in asthma, but impacts on platelet stability. Herbal products like *W. somnifera*, which are potent antioxidants as well as anti-inflammatory product impacts on the stability of both the fibrin networks and the platelet aggregates. These results therefore suggests that asthmatic patients should indeed use an antioxidant supplement e.g. selenium, as it stabilizes platelet aggregates.

Declaration

I, Hester Magdalena Oberholzer, hereby declare that this research dissertation is my own work and has not been presented by me for any degree at this or any other University;

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When I look back, I am grateful, when I look forward, I am hopeful and when I look up, I am peaceful. Going on my knees, I thank God for guiding me every step of the way and for granting me the gift of people who have made enormous contributions to my life.

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List of abbreviations, Symbols and Chemical Formulae

%	Percentage
°C	Degrees centigrade
hr	Hour
g	Gram
mg	Milligram
ml	Milliliter
mg/kg	Milligram per kilogram
µg	Microgram
µg/ml	Microgram per milliliter
µl	Micro liter
µl/kg	Micro liter per kilogram
µm	Micrometer
nm	Nanometer
mM	Millimolar
M	Molar
w/v	Weight/ Volume
α	Alpha
β	Beta
δ	Delta
ADP	Adenosine diphosphate
Al(OH₃)	Aluminium Hydroxide
ATP	Adenosine Triphosphate
CAT	Catalase
CD4	Cluster of differentiation 4

CTX	Cyclophosphamide
CO₂	Carbon dioxide
CV	Crystal Violet
ddH₂O	Double distilled water
DMBA	7,12-dimethylbenz(a) anthracene
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate Buffered Saline
DTH	Delayed type hypersensitivity
G	Gauge
GABA	Gamma amino butyric acid
GP	Glucoproteins
GPx	Glutathione Peroxidase
GSH	Glutathione
GST	Glutathione S-transferase
H₂O₂	Hydrogen peroxide
HeLA	Human cervical Adenocarcinoma cells
IgE	Immunoglobulin E
IL	Interleukin
KCL	Potassium chloride
KH₂PO₄	Potassium dihydrogen phosphate
LDL	Low density lipoproteins
LPO	Lipid peroxidation
MeOH	Methanol
MRC-5	Human fetal lung fibroblast cells

MTT	3 -(4,5-Dimethylthiazol-2-yl)- 2,5- diphenyltetrazolium bromide
NaCl	Sodium chloride
NADH	Reduced form of Nicotinamide adenine dinucleotide
Na₂HPO₄	Disodium hydrogen phosphate
NADPH	Reduced form of Nicotinamide adenine dinucleotide (NADP ⁺)
NO	Nitric oxide
NOS	Nitric oxide synthase
NR	Neutral red
OCS	Open canalicular system
OsO₄	Osmium tetroxide
OVA	Ovalbumin
PA	Plasminogen activator
PAF	Platelet activation factor
PAI_{act}	Platelet activation inhibitor
PBS	Phosphate buffered saline
PRP	Platelet rich plasma
RANTES	Regulated upon Activation, Normal T cell Expressed and Secreted
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Refs per minute
SEM	Scanning electron microscope
SK- N- SH	Human neuroblastoma cells
SOD	Superoxide dismutase

TEM	Transmission electron microscope
TGF	Transforming growth factor
TH1	T- Helper cell 1
TH2	T- Helper cell 2
TNF- α	Tumor necrosis factor alpha
UPBRC	University of Pretoria Biomedical Research Centre
WHO	World Health Organization
WS	<i>Withania somnifera</i>
WSRE	<i>Withania somnifera</i> root extract
X	Times

Chapter 1: Introduction

Withania somnifera (*Physalis flexuosa* L) is a member of the family Solanaceae and is also known as Ashwagandha. It is most commonly known as Indian ginseng and winter cherry and has been an important component of the Ayurvedic Pharmacopoeia of India, the Indian Traditional Health care System which is the oldest medical system in the world and has been used for over 400 years in India. It is categorized as a rasayana in Ayurveda. This plant is a small, woody and erect perennial shrub that grows up to one meter high with pale green leaves that are covered with short, dense hair. The flowers are white-yellowish and are present in small clusters together with small, round orange-red berries that are enclosed in brown membranous structures. *Withania somnifera* (WS) has a wide distribution in many countries specifically in Africa, Europe and Asia and in South Africa it is indigenous and has become a weed in distributed places (Henderson and Anderson, 1966).

The chemical constitution of the plant is very complex since more than 80 compounds are known from it (Van Wyk *et al.*, 2000) such as the steroidal alkaloids and steroidal lactones which are the major biological constituents of the roots of WS. They belong to a class of constituents called the withanolides (Elsakka *et al.*, 1990; Mishra *et al.*, 2000). Investigation into the active chemical substance led to the isolation of Withaferin A from the roots of the plant, which represented the first member of a new class of phytosteroids (Lavie *et al.*, 1965).

WS has been in use for over 2500 years to treat all kinds of diseases and human ailments (Bhattacharya *et al.*, 2001). Popularly known as Indian ginseng, it is most commonly used in Ayurvedic medicine to improve cerebral function in the geriatric population, to augment the faculty of learning and memory retention and to provide non-specific host defense (Pandey, 1985; Ghosal *et al.*, 1989). Several studies from the past have indicated that *Withania* possesses antioxidant, antitumor, antistress, anti-inflammatory, immunomodulatory, hemapoetic and rejuvenating properties and was also reported to influence various neurotransmitter receptors in the central nervous system (Pattipati *et al.*, 2003). Numerous studies have also revealed the anti-ageing, anxiolytic and antidepressive effects of this medicinal plant.

Ashwagandha has also been shown to have a tranquilizing effect on the central nervous system in animals (Mishra *et al.*, 2000).

Numerous studies have been done over the past few years to investigate the antioxidant effect of WS. Various studies came to the conclusion that WS exhibits a potent antioxidant effect (Jayaprakasam *et al.*, 2003).

Ashwagandha is effective in a variety of rheumatologic conditions and this may be, in part, due to its anti-inflammatory activities that have been studied by several researchers over recent years. Some studies revealed that the anti-inflammatory and immunomodulatory properties of WS root extracts are likely to contribute to the chemo preventive action of WS (Prakash *et al.*, 2002).

Numerous studies revealed that WS does exhibit anti-inflammatory properties similar or even greater than that of anti-inflammatory drugs such as hydrocortisone (Begum and Sadique, 1988; Iuvone *et al.*, 2003; Davis and Kuttan, 2002). Asthma is an example of such an inflammatory disease.

Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. It is characterized by episodes of wheezing, chest tightness, breathlessness and coughing and the chronic inflammation is associated with airway hyper-responsiveness (GINA, 2006). Asthma is considered one of the most common respiratory complaints in the world today. The World Health organization (WHO) reported that approximately 300 million people worldwide 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. Deaths due to asthma are projected to increase by almost 20% in the next 10 years except if urgent action is taken (WHO, 2006).

Corticosteroids are currently the most effective asthma treatment as they reduce the occurrence of asthma symptoms. However, there are some serious potential adverse effects when using corticosteroids in the long-term treatment of asthma.

Side effects of cortisone injections include atrophy and depigmentation of the skin at the site of injection. The skin is very sensitive to the side effects of cortisone. The skin and the superficial blood vessels contain connective tissue that is easily affected by cortisone. The skin becomes fragile and local bleeding occurs due to broken blood vessels.

It is known that fibrin and coagulation products are intimately associated with asthma. 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 and may play a role in the pathogenesis of airway remodeling (Gabazza *et al.*, 1999). Human platelets can produce platelet activation factor (PAF) upon thrombin stimulation in the lungs (Touqui *et al.*, 1985). Fibrinogen can be produced by lung epithelia as a result of inflammatory stimulus (Lawrence and Simpson-Haidaris, 2004). Fibrin degradation products have also been found to increase pulmonary vascular smooth muscle contraction. Airway fibrin deposition occurs in inflammatory disorders of the lung, and it is known that fibrin inhibits surfactant function (Kern *et al.*, 1986). Fibrin is typically formed at sites of vascular damage and extra-vascular thrombin, fibrinogen, and fibrin has been found in the sputum of patients with asthma. Airway hyper-responsiveness 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. There is a decrease in plasminogen activator (PA) activity in asthma. 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).

Selenium is a powerful antioxidant. Selenium on its own is not used as a treatment supplement for asthma; however, it is known that asthma sufferers have decreased levels of selenium. WS is also proven to be a powerful antioxidant and as previously mentioned exhibit anti-inflammatory properties as well.

From this the question arises whether the phytomedicine, WS and the antioxidant selenium could play a role in the treatment and alleviation of asthma symptoms. The overall aim of this study was to investigate the effect of WS on coagulation, blood count and bronchial lavage by using the asthmatic Balb/c mouse model, and to use this

knowledge to emphasize the importance of the use of phytomedicine in the treatment and prevention of diseases like asthma.

Therefore, the following research objectives will be investigated:

- To determine the cytotoxicity of WS
- Establish the Balb/c asthmatic mouse model
- Investigate the effect of WS, selenium, WS and selenium in combination and hydrocortisone on the ultra-structure of platelets and fibrin fiber networks by using SEM
- Utilize Balb/c asthmatic mouse model to investigate the ultra-structure of platelets by using TEM

The mentioned research objectives were used to find the optimum concentration of the plant that is not cytotoxic in a permanent cell culture. The optimum concentration found was 330mg/ml. This cytotoxicity testing preceded the animal study.

The animal model was used to determine if there are morphological differences when platelets and fibrin networks of the asthmatic group are compared to the group treated with WS, selenium, WS and selenium in combination and hydrocortisone on ultra structural level by using SEM and TEM.

Chapter 2: Literature Review

2.1 INTRODUCTION

In the current thesis the effects of *W. somnifera* (WS) and the potent anti-oxidant selenium separately and in combination will be investigated using the Balb-c murine asthma animal model. In this literature review the chemicals identified from WS will be discussed as well as the diseases treated with the plant. It is also known that in patients with asthma, selenium levels are lowered. Finally, the role of animal models in the study of asthma will be discussed and reasons will be given for choosing the Balb/c murine asthma animal model.

2.1.1 *Withania somnifera*

Withania somnifera is a member of the family *Solanaceae* and is also known as Ashwagandha with synonym known as *Physalis flexuosa* L. It is most commonly known as Indian Ginseng and winter cherry. It has been an important component of the ayurvedic pharmacopoeia of India, the Indian traditional health care system that is the oldest medical system in the world and has been used for over 400 years in India and is an important herb in this ayurvedic and indigenous medical systems for over 3000 years (Henderson and Anderson, 1966; Ahmad *et al.*, 2005; Glaser, 1988). It is categorized as a rasayana in Ayurveda. The origin of the word “*Withania*” is not very clear whereas “*somnifera*” refers to the narcotic properties of the leaves of the plant (Scartezzini and Speroni, 2000). WS has been in use for over 2500 years to treat all kind of diseases and human ailments (Bhattacharya *et al.*, 2001). It is well known in folk medicine in India as a sedative, general tonic and antihypertensive agent (Thatte and Dahanukar, 1989). Popularly known as Indian Ginseng, it is most commonly used in Ayurvedic medicine to improve cerebral function in the geriatric population, to augment the faculty of learning and memory retention and to provide non specific host defense (Pandey, 1985; Ghosal *et al.*, 1989).

WS is a small, woody and erect perennial shrub that grows up to one meter high with distinctively hairy stems. The leaves are pale green and covered with short, dense hair. White-yellowish flowers are produced in small clusters and small, round, orange-red berries are also present. They are enclosed in brown membranous structures

(Henderson and Anderson, 1966). The fruit is harvested in the late fall and the bright yellow seeds are dried for planting in the following spring. One or more roots are present and a transversal section shows light brown thin cork. The cortex contains a thick central parenchymatous region, which has a storage function. Thick walled fibers form part of the outside of the wood and contain small patches of thin walled parenchyma as well as small groups of vessels and tracheids (Scartezzini and Speroni 2000). WS requires well-drained, moist soil. It cannot grow in the shade.

It is distributed worldwide and has a wide distribution in Africa, Southern Europe and Asia. It is considered to be indigenous to South Africa and is known as a weed (Henderson and Anderson, 1966). In South Africa the species occur mainly in KwaZulu-Natal, Free State and Eastern Cape provinces (Van Wyk *et al.*, 2000). It also grows in the drier tropical regions and is also found from Liberia to Nigeria and the Southeast Islands (Iwu, 1993).

The plant is chemically very complex and more than 80 compounds are known from it (Van Wyk *et al.*, 2000). The major biological constituents of WS roots are the steroidal alkaloids and steroidal lactones. They belong to a class of constituents called the withanolides (Elsakka *et al.*, 1990; Mishra *et al.*, 2000). Investigation on the active chemical constituents of the plant led to the isolation of Withaferin A by Lavie *et al.* in 1965. It has been reported that the withanolides exert immunosuppressive activities amongst others (Cassady *et al.*, 1981; Blasko and Cordell, 1988).

Withaferin A represented a class of phytosteroids (Lavie *et al.*, 1965). A number of related Withanolides have also been isolated since 1965, and they include some naturally occurring chlorinated derivatives (Chu *et al.*, 1988). The Sitoindoside IX and Sitoindoside X are glycosolated derivatives that occur in the roots of several varieties of WS have also been isolated (Ghosal *et al.*, 1988). Alkaloids, withanolides, flavonoids and reducing sugars are all found in the roots of WS. The active compounds reported in WS include the following: Withaferin A, sitoindosides VII-X, 5-dehydroxywithanolide-R, withasomniferin-A, 1-oxo-5beta,6beta-epoxy-witha-2-ene-27-ethoxy-olide, 4-(1-hydroxy-2,2-dimethylcyclopropanone)-2,3-dihydrowithaferin A, 2,3-dihydrowithaferin A, 24,25-dihydro-27-desoxywithaferin A, physagulin D (1-6)-beta-D-glucopyranosyl-(1-4)-beta-D-

glucopyranoside, 27-O-beta-D-glucopyranosylphysagulin D, physagulin D, withanoside I-VII, 27-O-beta-D-glucopyranosylviscosalactone B, 4,16-dihydroxy- β ,6 β -epoxyphysagulin D, viscosalactone B and diacetylwithaferin A (Uma Devi, 1996; Ganzera *et al.*, 2003; Jayaprakasam *et al.*, 2003., Kaur *et al.*, 2003; Matsuda *et al.*, 2001).

Because of the reported active components, particularly the withanolides, it was suggested that WS is a rich source of bioactive compounds. The withanolides are classified according to their structural skeleton. The wide array of pharmacological activities of the withanolides is due to their structural variation (Ray and Gupta, 1994). They serve as important hormone precursors and they can convert into physiological hormones in human as needed. It is also regarded as an amphoteric substance because it can help to regulate important physiological processes. The plant-based hormone precursor occupies cell membrane receptor sites when there is an excess of a certain hormone. By doing so, the actual hormone cannot attach to the receptor site and exert its effect. When the hormone level is low, the plant-based hormone will exert only a small effect (Schwartz, 1963).

Extracts from the roots of WS have particularly been used for the treatment of uterus, menstrual cycle and arthritis disorders (Mishra *et al.*, 2000; Upton, 2000). It also has shown to be effective in the treatment of osteoarthritis (Kulkarni *et al.*, 1991). Several studies from the past have indicated that *Withania* possesses antioxidant, antitumor, antistress, anti-inflammatory, immunomodulatory, hemopoetic and rejuvenating properties and is was also reported to influence various neurotransmitter receptors in the central nervous system (Pattipati *et al.*, 2003). Numerous studies have also revealed the anti-ageing, anxiolytic and antidepressive effects of this medicinal plant. Choudhary *et al.* in 1995 and Abou-Douh in 2002 also stated that Ashwagandha is a potential antimicrobial agent with antifungal activity and moderate antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Ali *et al.*, 2001).

A dosage of 3-6 grams of dried decocted root or 6-12 ml of liquid extract of Ashwagandha is recommended per day (Mills and Bone, 2000). Ashwagandha is a relatively safe herb when taken in the prescribed range of dosage. Large doses, however, have been shown to cause gastrointestinal upset, diarrhea and vomiting.

No toxicity has been seen after administering ashwagandha for 90 days to rats as in a study done by Aphale in 1998. The acute LD50 for withaferin A in Swiss mice is around 80mg/kg (Sharad, 1996). The leaves and root bark of the plant are abortifacient, adaptogen, antibiotic, aphrodisiac, deobstruent, diuretic, narcotic, strongly sedative and tonic. It is also used internally to tone the uterus after a miscarriage and also to treat post-partum difficulties (Siddique *et al.*, 2004). In 2004, Mohan and co-workers also revealed through their studies that the active compound of WS, Withaferin A, exhibits potent anti-angiogenic activities (Mohan *et al.*, 2004). In 2000, Dhuley studied the pharmacological and metabolic effects of Ashwagandha. Ashwagandha treatment resulted in significant increase in coagulation time, which attains normalcy 7 days after cessation of treatment. On the basis of these observations they concluded that WS possesses anticoagulant properties as well (Dhuley, 2000).

2.1.2 The effect of *Withania somnifera* on the nervous system

Ashwagandha has also been shown to have a tranquilizing effect on the central nervous system in animals (Mishra *et al.*, 2000). Ashwagandha has remarkable stress-relieving properties comparable to those obtained by powerful anxiolytics and antidepressants. It is also used in the treatment of a variety of degenerative diseases such as Alzheimer's disease and Parkinson's disease.

In an animal study to assess the anxiolytic and antidepressive actions of WS, they administered an extract of the root of WS orally to rats once daily for five days. The authors compared the results to commonly prescribed pharmaceuticals. For the anxiolytic activity, they compared the results with the benzodiazepine lorazepam and for the antidepressant activity they compared it to the tricyclic antidepressant imipramine. The ashwagandha group showed reduced brain levels of a marker of clinical anxiety comparable to that obtained by the lorazepam group. Ashwagandha also exhibited an antidepressant effect comparable to that caused by the prescribed pharmaceutical imipramine (Bhattacharya *et al.*, 2000).

Similar studies done by other researchers supported the use of ashwagandha as an antistress adaptogen (Bhattacharya *et al.*, 2001; Singh *et al.*, 2001; Archana and Namasivayam, 1999; Dhuley, 2000).

A number of physiological conditions can be the result of chronic stress. These include cognitive deficit, immunosuppression, sexual dysfunction, gastric ulceration, and irregularities in glucose homeostasis and changes in plasma corticosterone levels.

WS was tested for its ability to attenuate some effects of chronic stress in a rat model of chronic stress and the plant decreased the number and severity of chronic stress-induced ulcers, reversed chronic stress-induced inhibition of male sexual behavior and inhibits the adverse effects of chronic stress on retention of learned tasks. WS also reversed chronic stress-induced immunosuppression and increased peritoneal macrophage activity in rats (Bhattacharya and Muruganandam, 2003).

In 2001, Jain *et al.* investigated the neuroprotective effects of WS on stressed, adult female Swiss albino rats. The experimental rats were exposed to immobilization stress for 14 hr and they were treated with a root powder extract of WS. Serial coronal sections of the brain passing through the hippocampal regions were stained with Thionin and revealed 85% degenerative cells. Treatment with WS showed a significant reduction (80%) in the number of degenerating cells. This study supports the antistress neuroprotective effects of WS (Jain *et al.*, 2001).

Tohda and co-workers in 2000 did a study to explore drugs that activate neurite outgrowth and regenerate the neuronal network. They found that the methanol extract of Ashwagandha significantly increased the percentage of cells with neurites in human neuroblastoma SK-N-SH cells. With these results the authors suggested that the methanol extract of Ashwagandha promotes the formation of dendrites and this can compensate for and repair damaged neuronal circuits in the dementia brain (Tohda *et al.*, 2000). In 1991 it was revealed by Mehta and co-workers that WS extracts contains an ingredient that has a GABA-mimetic activity when they investigated the pharmacological effects of the plant root extract on GABA receptor complex (Mehta *et al.*, 1991).

Ashwagandha was also shown to be effective in the treatment of Parkinson and Alzheimer's diseases, both chronic neurodegenerative diseases. In a study in 2005, using one of the most widely used rat models for Parkinson's disease, the authors found that Ashwagandha reversed all the parameters of this type of neurodegeneration

significantly in a dose-dependant manner (Ahmad *et al.*, 2005). In another study in 2004, the researchers revealed that WS extracts inhibit acetylcholinesterase, which is the enzyme responsible for the breaking down of the brain's chemical messengers.

WS works on exactly the same way as the drugs currently used in the treatment of Alzheimer's disease, by slowing the progression of this disease (Choudhary *et al.*, 2004).

2.1.3 Anti-cancer and chemo preventative action of *Withania somnifera*

In addition to the neuroprotective effects of WS reported, the plant has also revealed through recent studies, the potential to stop cancer cell growth. In recent studies done on human breast, lung and colon cancer cell lines, Ashwagandha was shown to inhibit the growth of these cell lines. The researchers revealed that a specific extract from the plant, Withaferin A, was more effective in the inhibition than the common cancer chemotherapy drug, doxorubicin, they used to compare it with (Anon. Monograph, 2004; Jayaprakasam *et al.*, 2003).

In January 2006 Mathur *et al.* reported on the anti-angiogenic activities of WS. They revealed that with this anti-angiogenic activity, it has the ability to prevent cancer from forming new blood vessels to support its growth. This study supports the potential role of Ashwagandha in fighting cancer (Mathur *et al.*, 2006).

In another study done on experimental laboratory animals, with induced stomach cancer, the researchers administered an extract of the plant orally to these animals. This administration reduced tumor incidence by 60% and reduced the number by 92%. The same happened in a rodent model of skin cancer where Ashwagandha also reduced the incidence by 45% and the tumor multiplicity by 71% (Padmavathi *et al.*, 2005). The protective effect of WS against skin cancer has been reported in other studies as well (Mathur *et al.*, 2004).

Davis and Kuttan (2000) investigated the effect of WS on 20-methylchoanthrene induced fibrosarcoma in mice. They found that the administration of an extract from the plant root inhibit the 20-methylcholanthrene induced sarcoma development and also increased the life span of these animals. In the animals treated with WS, the survival rate was 100% after 15 weeks of carcinogen treatment, and with these results the authors concluded

that WS could inhibit the induced sarcoma development in mice (Davis and Kuttan, 2000a).

Some studies over the past years have revealed that WS may also have application as an adjunct to cancer chemotherapy treatment. An animal study demonstrated that Ashwagandha protect against the decline in neutrophils (neutropenia) which is one of the consequences of chemotherapy (Gupta *et al.*, 2001). The alcoholic extract from the root of the plant as well as the active component Withaferin A showed significant antitumor and radio sensitizing effects in experimental tumors *in vivo*. Research indicates that WS could prove to be a good natural source of a chemotherapeutic agent (Devi, 1996).

2.1.4 Antioxidant effects of *Withania somnifera*

Numerous studies have been done over the past few years to investigate the antioxidant effect of WS. Various studies came to the conclusion that WS exhibits a potent antioxidant effect.

The brain and nervous system are more susceptible to free radical damage than any other tissue because they are rich in lipids and iron that both are important in generating reactive oxygen species (ROS) (Halliwell and Gutteridge, 1989). Free radical damage of nervous tissue may cause neurodegenerative diseases such as Alzheimer's, Parkinson's, epilepsy and other diseases and it may also contribute to neuronal loss in cerebral ischemia (Jesberger and Richardson, 1991; Russo *et al.*, 1997). The active principles of WS have been tested for antioxidant activities using superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), which are the major free radical scavenging enzymes, in rat brain frontal cortex and striatum. They administered active glycowithanolides of WS one daily for 21 days and found a dose related increase in all enzymes. With this the authors then concluded that WS does have an antioxidant effect in the brain (Bhattacharya *et al.*, 1997b).

In 2006, Nishant and co-workers (Nishant *et al.*, 2006) investigated the antioxidant effects of WS in male albino rats. They administered 0.75 g/rat/day and 1.5 g/rat/day of WS root powder respectively to normal animals (NWS I) and NWS II (the higher

concentration) and to hypercholesteremic animals, HWS I and HWS II. There was a significant reduction in hepatic lipid peroxidation and an increase in the catalase and SOD activities as well as the ascorbic acid levels. The NWSI and NWSII groups also revealed a decline in hepatic lipid peroxidation and an increase in CAT activity and ascorbic acid levels. The authors attributed this antioxidant activity of WS to the sitoindosides VII-X and Withaferin A with reference to CAT and SOD activities (Bhattacharya *et al.*, 1997a).

Other potential sources of antioxidant compounds such as flavonoids, polyphenols and vitamin C present in the plant, can also contribute to the antioxidant efficacy of WS. These antioxidant compounds scavenge free radicals including hydroxyl and superoxide anions and reduce levels of lipid peroxidation and oxidation of LDL (Rice-Evans *et al.*, 1995; Jovanovic and Simic, 2000; Tiwari, 1999). They revealed that the presence of polyphenols and flavonoids can be the reason for the elevated levels of hepatic antioxidant activities in the HWS groups.

In another study done by Parihar *et al.* in 2004 (Parihar *et al.*, 2004) they examined whether the extracts of WS reduces the oxidative lesions in the brain of diabetic mice and if subsequently results in a reduction of oxidative damage of lipids and proteins. Their study revealed that the extract of WS given to diabetic mice were very effective since it reduced the oxidative burden in the hippocampus and cerebral cortex.

Several studies conducted over recent years also revealed that supplementation of phenolic antioxidants attenuate neuronal cell death caused by oxidative stress (Youdim and Joseph, 2001; Parihar and Hemnani, 2003; Shroeter *et al.*, 2000; Ishige *et al.*, 2001). Administration of an extract of WS to Swiss albino mice by Davis and Kuttan in 2001, showed a significant enhancement in antioxidant enzymes such as GSH, GST, Glutathione peroxides and catalases. The WS administration also inhibited an elevated level of lipid peroxide (Davis and Kuttan, 2001).

In 2001, Bhattacharya *et al.* assessed the antioxidant activity of WS glycowithanolides in chronic foot-shock stress induced changes in rat brain frontal cortex and striatum. Their study induced an increase in superoxide dismutase and lipid peroxidation activity with a

decrease in catalase and glutathione peroxidase activities in both brain regions (Bhattacharya *et al.*, 2001).

Russo and coworkers in 2001 investigated the free-radical scavenging capacity of the methanol extract of WS and the effect on DNA cleavage induced by H₂O₂ UV-photolysis. They also investigated whether WS is capable of reducing the hydrogen peroxide induced cytotoxicity and damage in human non-immortalized fibroblasts. The authors revealed that the methanol extract showed a dose dependant free radical scavenging capacity and a protective effect on DNA cleavage. Their results were confirmed by a significant protective effect on H₂O₂-induced cytotoxicity and DNA damage in human non-immortalized fibroblasts. They concluded that these antioxidant effects of the active principles of WS may explain in part the reported anti-stress, anti-inflammatory, immunomodulatory, cognition-fascilitating and anti-ageing effects produced by it in experimental animals and clinical situations (Russo *et al.*, 2001).

In 1999, Panda and Kar investigated the effects of daily administration of WS root extract for 20 days on thyroid function in female mice. They found a significant decrease in lipid peroxidation (LPO) and an increase in CAT activity (Panda and Kar, 1999).

2.1.5 Anti-inflammatory and immunomodulatory effects of *Withania somnifera*

Ashwagandha is effective in a variety of rheumatologic conditions and this may be, in part, due to its anti-inflammatory activities that have been studied by several researchers over recent years. As early as 1981, Anbalagan and Sadique investigated the anti-inflammatory properties of WS by administering powdered root of WS orally one hour before the induction of inflammation in rats. They continued this daily administration for three days. WS was found to cause a significant reduction in inflammation. The blood was monitored by crossed immunoelectrophoresis and it was found that there were changes in many serum proteins (Alpha-2 glycoprotein, major acute-phase alpha-1 protein and pre-albumin). The alpha-2 glycoprotein that is found only in inflamed rat serum was decreased to undetectable levels with administration of WS. The authors also revealed that WS influenced several modulator proteins in normal rats, which suggests that several plant chemicals in WS possibly interact with the liver protein synthesis process (Anbalagan and Sadique, 1981). Another study done by the same

authors in 1984 (Anbalagan and Sadique, 1984), revealed that WS cause a dose dependant suppression of alpha-2-macroglobulin, which is an indicator for anti-inflammatory drugs, in the serum of inflamed rats.

A clinical trial done by Kulkarni and co-workers in 1991 supports the use of WS in the treatment of arthritis. They did a double blind, placebo-controlled cross-over study on 42 patients with osteoarthritis. The patients received a formula containing ashwagandha and were evaluated for a month. They found that the herbal formula significantly reduced the severity of pain and disability (Kulkarni *et al.*, 1991).

The effect of WS on cyclophosphamide (CTX) induced urotoxicity was investigated in 2000 by Davis and Kuttan (Davis and Kuttan, 2000c). CTX administration causes nausea, vomiting, mucosal ulceration, interstitial pulmonary fibrosis, hepatic toxicity, lymphocytopenia and alopecia and higher doses of CTX can even produce severe urotoxicity. They administered an extract from the plant for 5 days along with CTX. WS was found to reduce the CTX- induced urotoxicity. Analysis of the bladders of the CTX-treated group on morphological level showed severe inflammation and dark coloration. The WS-treated group on the other hand showed normal bladder morphology. They also revealed that the extract reduced the protein level in the serum and also reduced the blood urea N₂ level which both was drastically higher in the CTX treated animals. The authors concluded that the morphological, histopathological, biochemical and enzyme analysis showed that WS could alleviate the severe urotoxicity induced by CTX.

The immunomodulatory activities of two extract of WS (WST and WS2) were studied for immune inflammation in mice (Agarwal *et al.*, 1999). Active paw anaphylaxis and delayed type hypersensitivity (DTH) were investigated. The authors assessed the immunomodulatory effect through the induction of paw edema by ovalbumin and then comparing the results with those observed by the standard drug disodium chromoglycate. The WS reduced the paw edema significantly. The DTH was assessed as potentiation or suppression of the reaction. Potentiation was observed in the groups treated with cyclophosphamide and the two extracts of WS whereas the groups treated with WST and WS2 showed a suppressed DTH reaction. They also found a protective

effect of WS on cyclophosphamide-induced myelosuppression in animals treated with the extracts of WS (Agarwal *et al.*, 1999).

In 2006 Senthilnathan and co workers investigated the immunomodulatory effects of extracts of WS root powder against benzo(a)pyrene-induced lung cancer in male Swiss albino mice. After the administration of WS for 30 days, the authors found that WS significantly alters the levels of immunocompetent cells, immune complexes and immunoglobulin. With their results they concluded that WS indeed has immunomodulatory activities (Senthilnathan *et al.*, 2006).

Davis and Kuttan tested for the immunomodulatory activities of WS in 2000 by administering a dose of an extract from the powdered root of the plant to Balb/c mice. WS was found to stimulate immunological activity. Treatment with 5 doses of the plant revealed an enhancement of the total white blood cell count, as well as an increase in the bone marrow cellularity and alpha-esterase positive cell number. It also inhibited DTH reaction in mice. An enhancement in phagocytic activity of peritoneal macrophages was also observed. With these results the authors confirmed the immunomodulatory activity of the WS extract (Davis and Kuttan, 2000b).

The induction of nitric oxide (NO) synthase expression by the methanolic extract of WS in macrophages was investigated by Iuvone and co workers in 2003. They found that WS produced a significant and concentration-dependant increase in NO production. Western blot analysis showed that WS increased, in a concentration dependant manner, inducible NOS protein expression. They came to the conclusion that the increased NO production by macrophages could account in part for the immunostimulant properties of this plant (Iuvone *et al.*, 2003).

In 2002, Prakash *et al.* investigated the chemopreventive effect of WS hydroalcoholic root extract (WSRE) on 7,12-dimethylbenz(a) anthracene (DMBA) induced skin cancer in Swiss albino mice. The skin lesions were induced by the application of DMBA twice weekly for 8 weeks and WSRE was administered at the maximum tolerated dose of 400mg/kg three times a week for 24 weeks. The results revealed a significant decrease in incidence and average number of skin lesions in mice when compared to those obtained by DMBA alone. When they assessed the biochemical parameters in the

lesions of WSRE treated and untreated mice, they noticed impairment in the levels of reduced glutathione, malondialdehyde, superoxide dismutase, catalase, glutathione peroxidase and glutathione S transferase in skin lesions of DMBA treated control mice. The authors revealed that the anti-inflammatory and immunomodulatory properties of WSRE are likely to contribute to the chemo preventive action of WS (Prakash *et al.*, 2002).

Begum and Sadique in 1988 examined the effect of WS root powder on paw swelling and bony degenerative changes in Freund's adjuvant-induced arthritis in rats. WS caused a significant reduction in both the swelling and degenerative changes in the bone. They found that these reductions were better than those produced by hydrocortisone, the reference drug. Thus, WS does exhibit anti-inflammatory properties similar or even greater than that of anti-inflammatory drugs such as hydrocortisone.

Asthma is an example of an inflammatory disease and corticosteroids are currently the most effective asthma treatment as they reduce the occurrence of asthma symptoms. However there are some serious potential adverse effects when using corticosteroids in the long-term treatment of asthma. Side effects of cortisone injections include atrophy and depigmentation of the skin at the site of injection. The skin is very sensitive to the side effects of cortisone. The skin and the superficial blood vessels contain connective tissue that is easily affected by cortisone. The skin becomes fragile and local bleeding occurs due to the broken blood vessels.

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 and may play a role in the pathogenesis of airway remodeling (Gabazza *et al.*, 1999). Human platelets can produce PAF upon thrombin stimulation in the lungs (Touqui *et al.*, 1985). Fibrinogen can be produced by lung epithelia as a result 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) and extra-vascular thrombin, fibrinogen, and

fibrin has been found in the sputum of patients with asthma (Pizzichini *et al.*, 1996; Wagers *et al.*, 2004).

Airway hyper-responsiveness 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. There is a decrease in plasminogen activator (PA) activity in asthma. 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).

One of the anti-oxidants that might have an important role to play in asthma includes selenium. Selenium and its uses in asthma will now be reviewed.

2.1.6 Selenium

In food, selenium is derived from both vegetable and animal products, particularly seafood, liver and cereals. In 1973, Rotruck and colleagues provided rationale for identifying the mineral selenium as an antioxidant. Later it was determined that selenium is indeed an essential component in the antioxidant defense systems and immune function. Also, it forms part of several major metabolic pathways, including thyroid hormone metabolism (Brown and Arthur, 2001).

Selenium is a powerful destroyer of free radicals created from air pollutants and is also involved in antioxidant defenses as a coenzyme in glutathione peroxidase (McKeever and Britton, 2004). The selenium-dependent enzyme glutathione peroxidase (GPX) recycles glutathione, reducing lipid peroxidation by catalyzing the reduction of peroxides, including hydrogen peroxide; this represents a crucial component of antioxidative potential in humans (Gazdik *et al.*, 2004). It is also known that selenium deficiency attenuates the host immune response, thereby increasing the risk of bacterial and viral infections (Jahnova *et al.*, 2002); furthermore, studies have shown that asthma sufferers have low blood levels of selenium (Powell *et al.*, 1994; Kadrobova *et al.*, 1996; Allam and Lucane, 2004). Furthermore, Kocyigit and co-workers in 2004 suggested that increased iron and decreased selenium concentrations in patients with childhood asthma may be responsible for the oxidant/antioxidant imbalance.

It is known that cigarette smoking, inhalation of airborne pollutants, such as oxidant gases (ozone, nitrogen dioxide, sulphur dioxide) or particulate in air is lung damage and elevated inflammatory responses in the lungs (Rahman and MacNee, 1996). There is also a link between these inhalation pollutions and selenium deficiency and Rubin and co-workers (2004) suggested that the selenium-asthma association was stronger in youth who were smoke exposed (Rubin *et al.*, 2004).

Shaheen and co-workers in 2001 also mentioned that selenium protected against asthma and that the intake of this mineral has been declining in Britain. Because of its antioxidative potential, selenium supplementation has become a new attractive approach to antiasthmatic complementary therapy (Gazdik *et al.*, 2004).

2.1.7 Animal models in asthma

Research done by using animal models have provided important information over the years on many aspects of the pathophysiology of human diseases. Potential benefits of proposed therapeutic interventions can be determined by well-performed animal studies. The results from experimental animal models have also served as the basis for many clinical trials (O'Neil *et al.*, 1999). There is no animal model that reproduces the exact pathology of human asthma but they play an important role in the development of novel therapies and the understanding of the pathogenesis of the disease (Isenberg-Feig *et al.*, 2003).

There is a wide range of different animal models available and choosing the appropriate model for a specific study could be difficult. According to Zosky and Sly (2007) the most popular models that are used are inbred mice and rats and also guinea pigs, which have the benefit of being easy to handle. Antigen-induced mouse allergic asthma is a useful model for testing novel therapeutics (Epstein, 2006). Experimental mouse models offer excellent tools for studying the effects of allergens *in vivo* in their natural target organs (Epstein, 2004a, b). *In vivo* animal models have been used successfully during the past few years to study diseases like asthma (Epstein, 2004a, b). The study of disease pathogenesis and development of new therapeutics have been made possible by these experimental mouse models and has been used for testing many novel agents aimed at

reducing lung inflammation, mucus hyper secretion, airway hyper responsiveness and IgE profiles.

Asthmatic animal models have been developed to study the immune and inflammatory mechanisms of the disease as well as to investigate the efficiency of new drugs in the treatment of asthma. There is a correlation between the allergic response in sensitive mice and that of human asthmatics. In fact, both bronchoconstriction and non-specific airway hyper-reactivity not only correlate with IgE levels, but are also induced. (Eum *et al.*, 1995). Airway remodeling also occurs. This includes mucus cell metaplasia, shedding of the epithelium, thickening of the basement membrane and edema (Blyth *et al.*, 1996; Hogan *et al.*, 1996). Mosmann and coworkers in 1986 revealed that the TH1/TH2 cytokine profile of mice resemble those of humans. The Balb/c murine asthma model can be used successfully to study the effects of Phytomedicine (Pretorius *et al.*, 2007). Common protocols for the sensitization process of mice involve the immunization of mice with allergen together with aluminium hydroxide as well as allergen challenge in the airways. The allergic response is usually characterized by allergen-specific IgE antibodies, eosinophilic inflammation in the lungs and a Th2-type of T-cell response to the sensitizing allergen (Epstein, 2004). This model is a reliable, clinically relevant facsimile of human disease (Epstein, 2006).

According to Kumar and Foster, studies in murine experimental models have contributed greatly to understanding the mechanism of allergic inflammation underlying asthma (Kumar and Foster, 2002).

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 Roberto and Kheradmand *et al.* in 2005 mouse models are not exact replicas of human asthma but they have helped to understand some of the basic mechanisms involved in the production of this disease (Roberto *et al.*, 2005).

Because it seems as if WS as well as selenium has great potential in the treatment of asthma, the current study therefore aims to investigate the anti-asthmatic effect of WS

and selenium separately and in combination on the white blood cell counts and fibrin networks using the Balb/c asthmatic mouse model, with positive control, hydrocortisone.

The following research questions will therefore be investigated in the current study:

1. What volume of *W. somnifera* is not cytotoxic to a permanent cell culture?
2. Can the Balb/c mouse be used to establish an asthmatic mouse model?
3. What is the effect of WS, selenium, WS and selenium in combination and hydrocortisone on the ultrastructure of the platelets and fibrin networks of the Balb/c mouse model?
4. What are the effect of WS, selenium, a combination of WS and selenium, and hydrocortisone on the white blood cells as well as the bronchial lavage of the Balb/c mouse model?

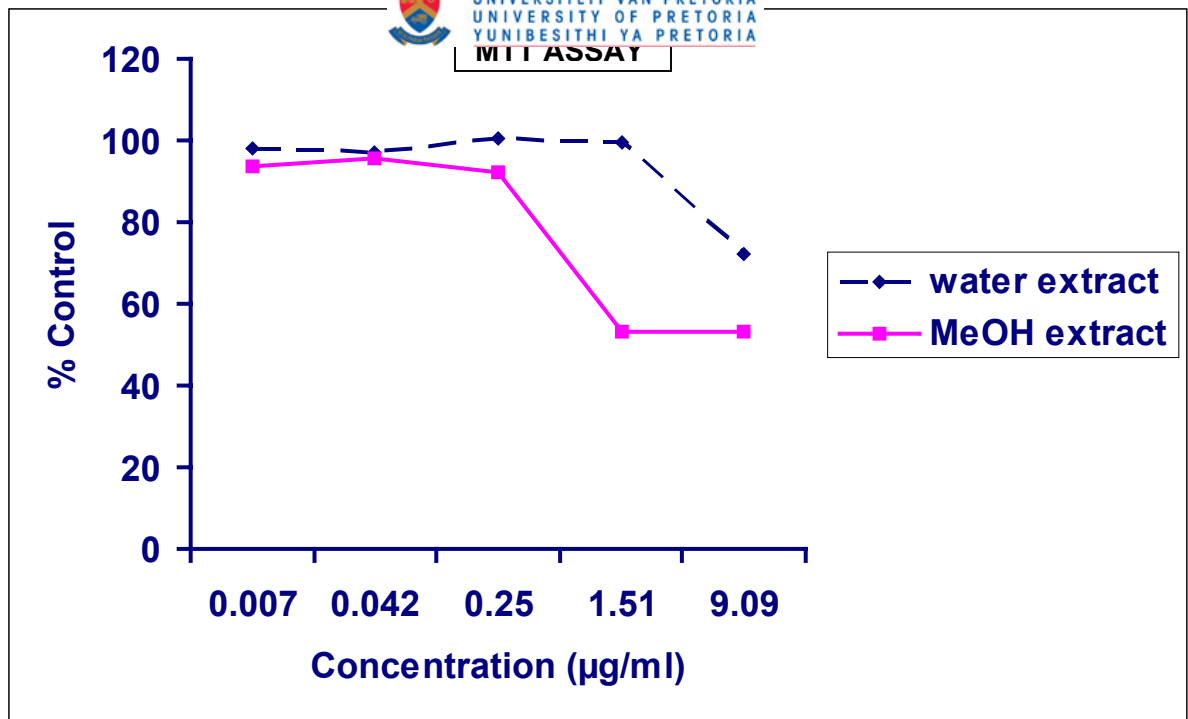
Chapter 3: The cytotoxic effects of *Withania somnifera* on MRC-5 cells in culture

3.1 INTRODUCTION

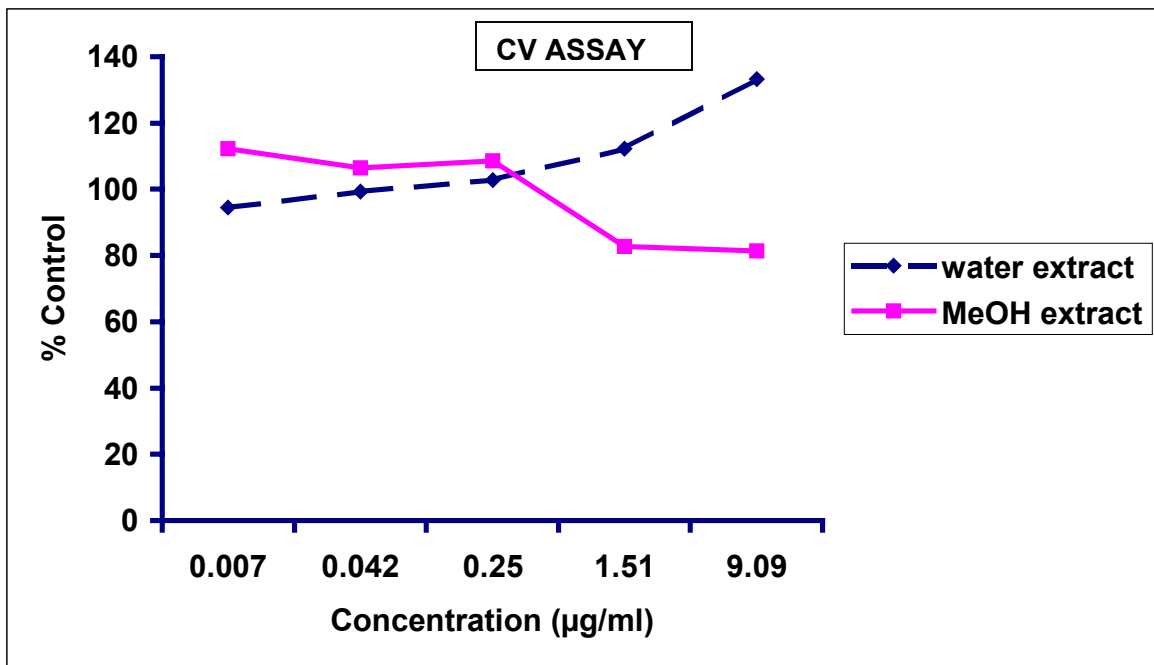
Cell culture assays are used to study the cytotoxicity of a substance or material referred to as the *in vitro* assessment. *In vitro* evaluations by using cell culture are the precursors for animal studies. Two types of cell culture can be distinguished: Primary cultures and permanent cultures. Primary cultures are obtained directly from an animal and can keep the differentiated state only for a short period of time. Primary culture is that stage after isolation of the cells but before the first subculture. Primary cell cultures that are functional and differentiated have a limited life span. Permanent cultures such as HeLa (Human Cervical Adenocarcinoma Cells) and MRC-5 cells amongst others have unlimited proliferation capacity. Permanent cultures originate from embryos, tumors or transformed cells (Freshney, 2005). MRC-5 cells are human fetal lung fibroblast cells and are commonly used for virology research, vaccine development and *in vitro* cytotoxicity testing. The MRC-5 cell line is derived from human lung tissue of a 14-week-old male fetus. MRC-5 cells grow adherently in culture and exhibit fibroblast morphology. It may double in population size 42-46 times before senescence. (<http://microscope.fsu.edu>).

Fibroblasts are the most ubiquitous cells found in complex organisms. They are the main cells of stromal tissues, and are thought to be derivatives of the mesoderm (Chang *et al.*, 2002). Fibroblasts are elongated, spindle-shape cells that produce collagen and fibronectin. They do not express markers for other cell lineages. Chang *et al.*, 2002 and Han and Amar (2002) revealed that different populations of fibroblasts differ in gene expression profile depending on their tissue of origin (Chang *et al.*, 2002; Han and Amar, 2002). In culture studies, fibroblasts are used as a support layer for proliferation of embryonic stem cells (Stojkovic *et al.*, 2005).

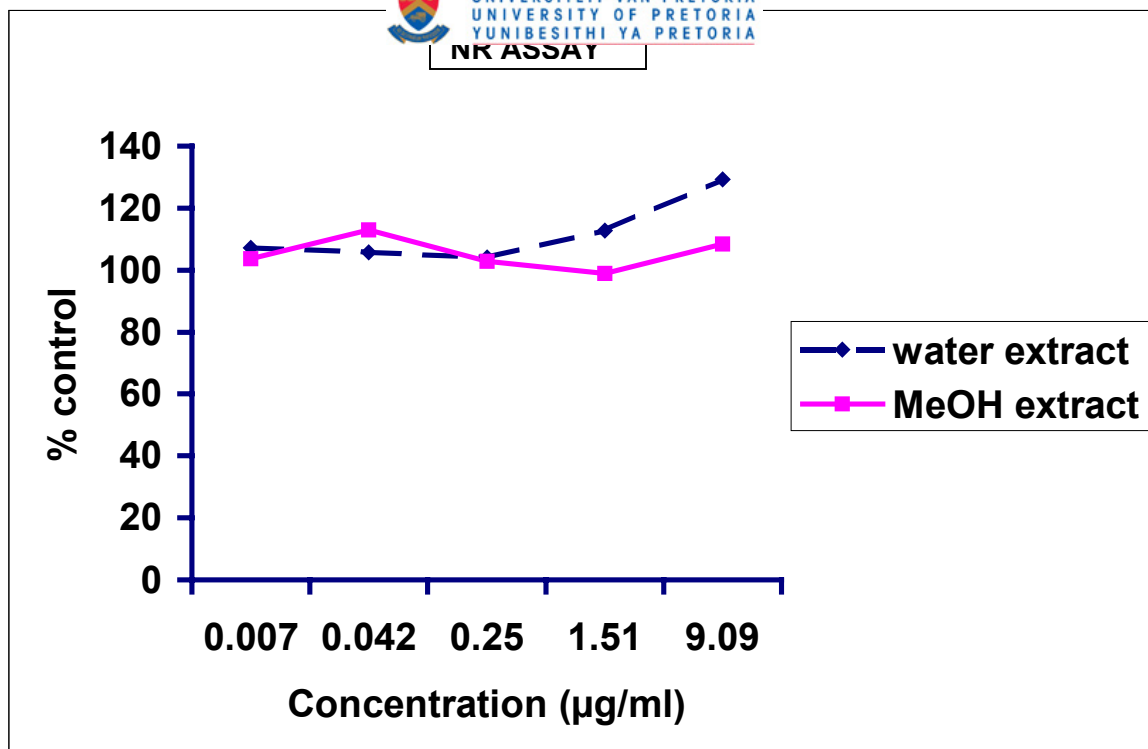
Numerous studies have been done over many years to investigate the different properties and uses of WS. In 2005, Marx have shown cytotoxicity of the water and methanol extracts of WS in MRC-5 cell line in a concentration range of 0-9.09 µg/ml.



Graph 3.1: Effects of 5 concentrations of *W. somnifera*, extracted in ddH₂O and MeOH and prepared in cell culture medium on MRC-5 cell viability after 48 hours exposure (Marx, 2005)



Graph 3.2: Effects of 5 concentrations of *W. somnifera*, extracted in ddH₂O and MeOH and prepared in cell culture medium on MRC-5 cell number after 48 hours exposure (Marx, 2005)



Graph 3.3: Effects of 5 concentrations of *W. somnifera*, extracted in ddH₂O and MeOH and prepared in cell culture medium on MRC-5 lysosomal integrity after 48 hours exposure (Marx, 2005)

In the CV assay the two extracts react similarly over the first 3 concentrations but behave very differently over the last 2 concentrations. The water extract causes an increase in cell number while the methanol extract caused a decrease in cell number at the higher concentrations.

In the NR assay the first 3 concentrations react similarly for both extracts and differ slightly over the last two higher concentrations.

In the MTT assay the first 3 concentrations did not differ significantly but differ from the last 2 concentrations that also differ from each other. Cell viability decreases when using the highest concentrations of both the water and methanol extracts.

The question now arises whether the methanol extract of WS will present with the same or greater toxicity when evaluated across a wider concentration range.

3.2 MATERIALS AND METHODS

3.2.1. Plant material

Root plant material were collected in the Pretoria area (South Africa) and identified by botanists for the Department of Botany, University of Pretoria, South Africa.

3.2.2. Media, supplements and reagents

MTT (1-(4,5- Dimethylthiazol- 2- yl) – 3,5 diphenylformazan), Crystal Violet (CV) powder and Neutral Red (NR) powder were form Sigma-Aldrich, Atlasville, South Africa. Water was double distilled and deionized (ddH₂O) with a Continental Water System and sterilized through a Millex 0.2µm filter. Glassware was sterilized at -140°C in a HL-340 Series vertical Type Steam Sterilizer Autoclave. 24-well and 96-well plates, 25cm² and 75cm² cell culture flasks, 5ml and 10ml pipettes, 15ml and 50ml centrifuge tubes, micro centrifuge tubes and eppendorf tubes were from NUNCTM supplied by AEC- Amersham, Johannesburg, South Africa.

3.2.3. Preparation of extracts

Leafs, stems and roots were fragmented and dried at 45°C for 48 hours and MeOH as well as water extracts made. The aboveground parts of the plant were allowed to dry at room temperature for 1 week. The material was grounded into a fine powder and subdivided into four 50 g samples. The samples were subsequently extracted with 500ml of water, after which it was filtered and dried on a rotary evaporator at 40 °C. The water extract was freeze-dried. This procedure was repeated three times for each sample. A stock solution of 330 mg/ml was prepared. A dilution series were made and the concentrations were 0µg/ml, 0.08µg/ml, 0.4µg/ml, 2.0µg/ml, 10.0µg/ml, 50.0µg/ml.

3.2.4. MRC-5 Permanent cell line

MRC-5 cells were obtained from Highveld Biological Company, Johannesburg, South Africa. The cells were plated at a cell concentration of 2x10⁴ cells per ml in 24 flat well plates and were kept for 24 hours at 37°C and 5% CO₂ before conducting the experiments.

Solution of 100mg/ml of each of the plant extracts was prepared either in ddH₂O or MeOH and MRC-5 cells were exposed to this solution for 48 hours. The cytotoxicity of MeOH alone was also determined.

3.2.5. The Crystal Violet assay

To determine the number of cells adherent to the surface of the tissue culture surface following the exposure of WS, a 300µl volume of 0.1% (weight/volume (w/v)) Crystal Violet (CV) dye solution prepared in 200mM of formic acid pH 3.5 was added to each well. The cells were stained for 60 minutes after which the bound dye was dissolved by shaking for 10 minutes in 200µl of 10% acetic acid solution. The dye solution was transferred into a 96-well plate and the absorbency was measured at 570nm, using a spectrophotometer plate reader.

3.2.6 The MTT assay

The MTT assay measures cell viability. A 100µl volume of a 0.1mg/ml 1-(4,5-Dimethylthiazol- 2- yl) – 3,5 diphenylformazan (MTT) solution prepared in DPBS was added to each well. The cell culture plates were maintained for 60 minutes at 37°C and 5% CO₂. The medium was then removed and the MTT formazan crystals were dissolved by adding 200ul DMSO to the wells. After shaking the plates for 20 minutes, the solution was transferred into a 96-well plate and the absorbency was measured at 570nm with a spectrophotometer.

3.2.7 The Neutral Red assay

The Neutral Red (NR) assay was used to determine lysosomal membrane integrity. A 100µl volume 0.15% NR prepared in DPBS. A 10x DPBS stock solution was prepared by dissolving 2g/l KCL, 2g KH₂PO₄, 80g/l NaCL and Na₂HPO₄ in ddH₂O that was diluted 1:9 with ddH₂O prior to use was added and incubated for 60 minutes at 37°C and 5% CO₂. The medium was then discarded and the cells were fixed for 10 minutes with 200µl of 1% acetic acid and 1% formaldehyde solution. The solution was then removed and the dye was solubilized with a 1% acetic acid and 50% ethanol solution. The dye solution was then transferred into a 96-well plate and the absorbency of the solubilized dye was measured at 570nm by using a spectrophotometer.

3.2.8 Statistical analysis

At each concentration each assay was done in quadruple and four experiments were done on the MRC-5 cell line.

3.2 RESULTS AND DISCUSSION

This study was undertaken to investigate the toxicity of WS on MRC-5 cell number, cell viability as well as lysosomal membrane integrity. The effect of the plant was measured using the CV, MTT and NR assays. Crystal Violet stains cellular proteins and the nuclei of fixed cells with a direct correlation between protein staining and cell number is being reported (Kueng *et al.*, 1989). Toxic effect of the plant material to the cell can cause cell detachment or cell damage with the resulting leakage of protein and therefore a corresponding decrease in protein staining (Timbrell, 2000; Kanduc *et al.*, 2002).

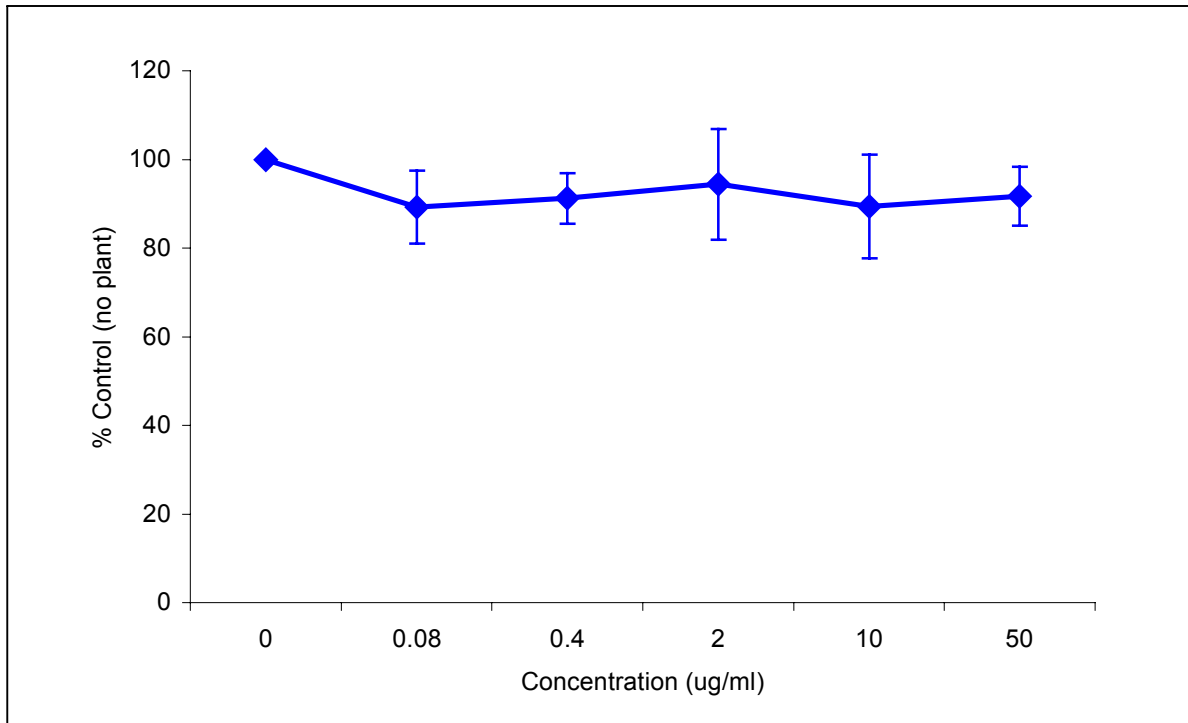
The MTT assay is a bioassay that is used to determine cell viability or the metabolic state of the cell by demonstrating the cells oxidative enzymes. The MTT is a soluble yellow dye that is converted into water insoluble blue formazan derivative by mitochondrial reductase enzymes. This conversion only takes place when the mitochondrial reductase enzymes, such as succinate dehydrogenase, are active. Most cellular MTT reduction does not occur as a direct effect of the specific dehydrogenase, but occurs outside the mitochondrial inner membrane and involves NADH and NADPH-dependant mechanisms (Mossman, 1983; Parish *et al.*, 1983; Cook *et al.*, 1989; Sgouras and Duncan, 1990; Munetaka *et al.*, 1996; Pariete *et al.*, 2002).

The NR assay is a measurement of lysosomal membrane integrity. It is a cell survival chemo sensitivity assay. The NR assay is based on the ability of viable cells to accumulate NR in their lysosomes. NR is a weak cationic dye that easily penetrates cell membrane by non-ionic diffusion. The NR accumulates intracellularly in the lysosomes by binding to anionic sites in the lysosomal matrix. This process requires intact cell membrane and active cell metabolism (Ishiyama *et al.*, 1996).

The Crystal violet assay is used to determine the amount of cells adherent to the tissue culture surface. It is reported as a percentage of the control where there is no plant

extract added. Crystal violet stains the proteins in the cell and is therefore an indication of the number of viable cells in the tissue culture.

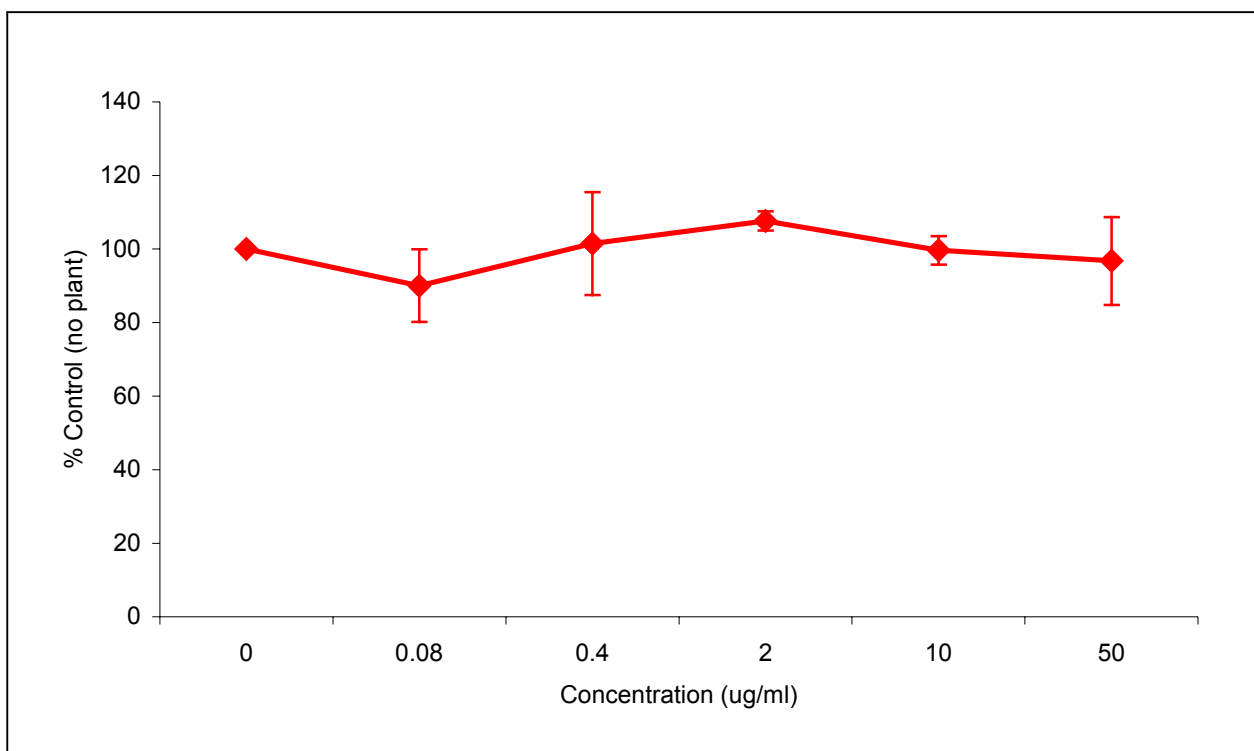
The Crystal Violet assay



Graph 3.4: Effects of 5 concentrations of *W. somnifera*, extracted in MeOH and prepared in cell culture medium on MRC-5 cell number after 48 hours exposure.

The Crystal Violet assay measures the amount of cells and it is reported as a percentage of the control. From this graph it is clear that with an increase in concentration there is no significant decrease in the cell number.

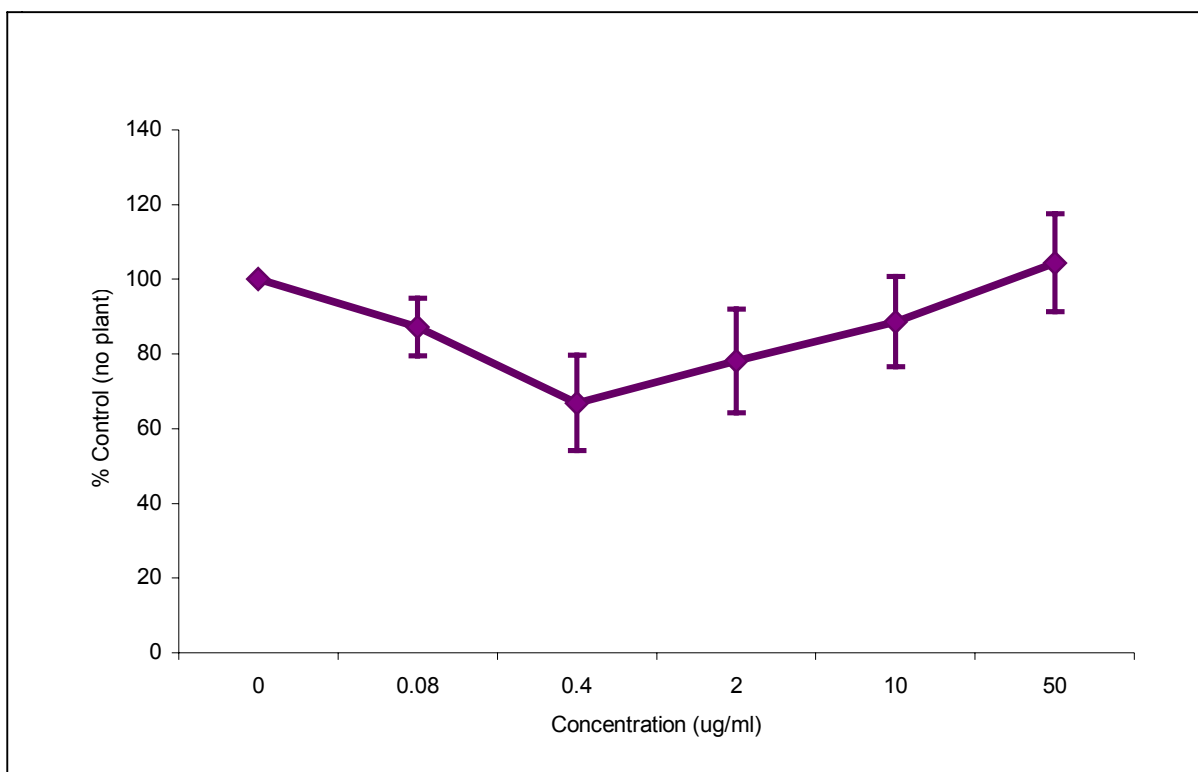
The Neutral Red assay



Graph 3.5: Effects of 5 concentrations of *W. somnifera*, extracted in MeOH and prepared in cell culture medium on MRC-5 lysosomal integrity after 48 hours exposure

The Neutral Red assay measures the effect on the lysosome membrane integrity. In other words, does the plant extract have an effect on the integrity of the lysosomal membrane? If the plant material is toxic to the cells there will be a decrease in the number of cells. From this graph it is clear that there is no significant decrease in the number of cells as the concentration of the plant increases.

The MTT assay



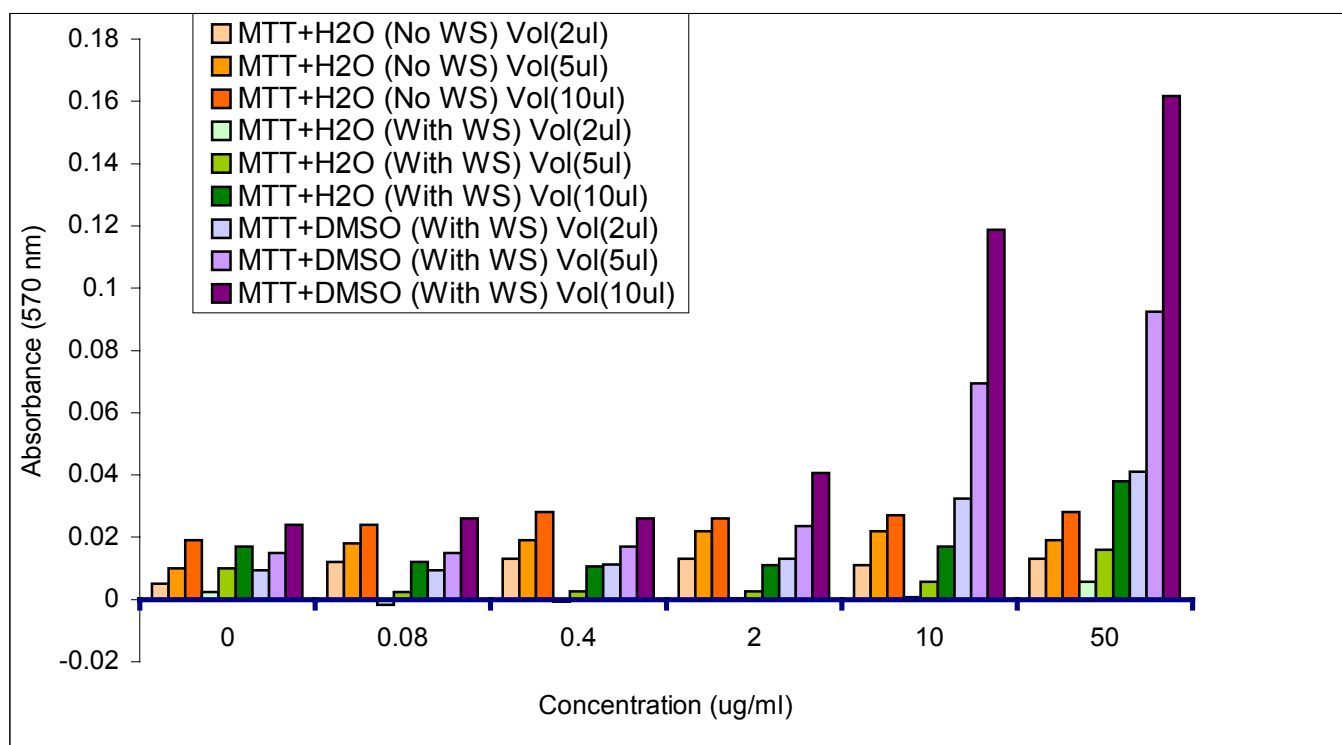
Graph 3.6: Effects of 5 concentrations of *W. somnifera*, extracted in MeOH and prepared in cell culture medium on MRC-5 cell viability after 48 hours exposure

The MTT assay measures cell viability. This graph reveals that there is toxicity over the first three concentrations. The lowest point on the graph is significantly lower than the control, which indicates that there is a significant decrease in cell viability. At the last two concentrations (10 µg/ml and 50µg/ml) there is a significant increase in cell viability when compared to the lowest point on the graph. Significance was determined at $p < 0.05$ using ANOVA. This increase in cell viability can be due to one of three factors. The first is contamination by residual plant material. This happens when some of the plant material remains attached to the surface of the wells when the medium is removed and this can lead to an increase in absorbance. The second factor that can lead to the increase in absorbance is interaction of the plant material with the MTT assay. Thirdly it can simply be a cellular effect, which means that there was an increase in the mitochondrial activity.

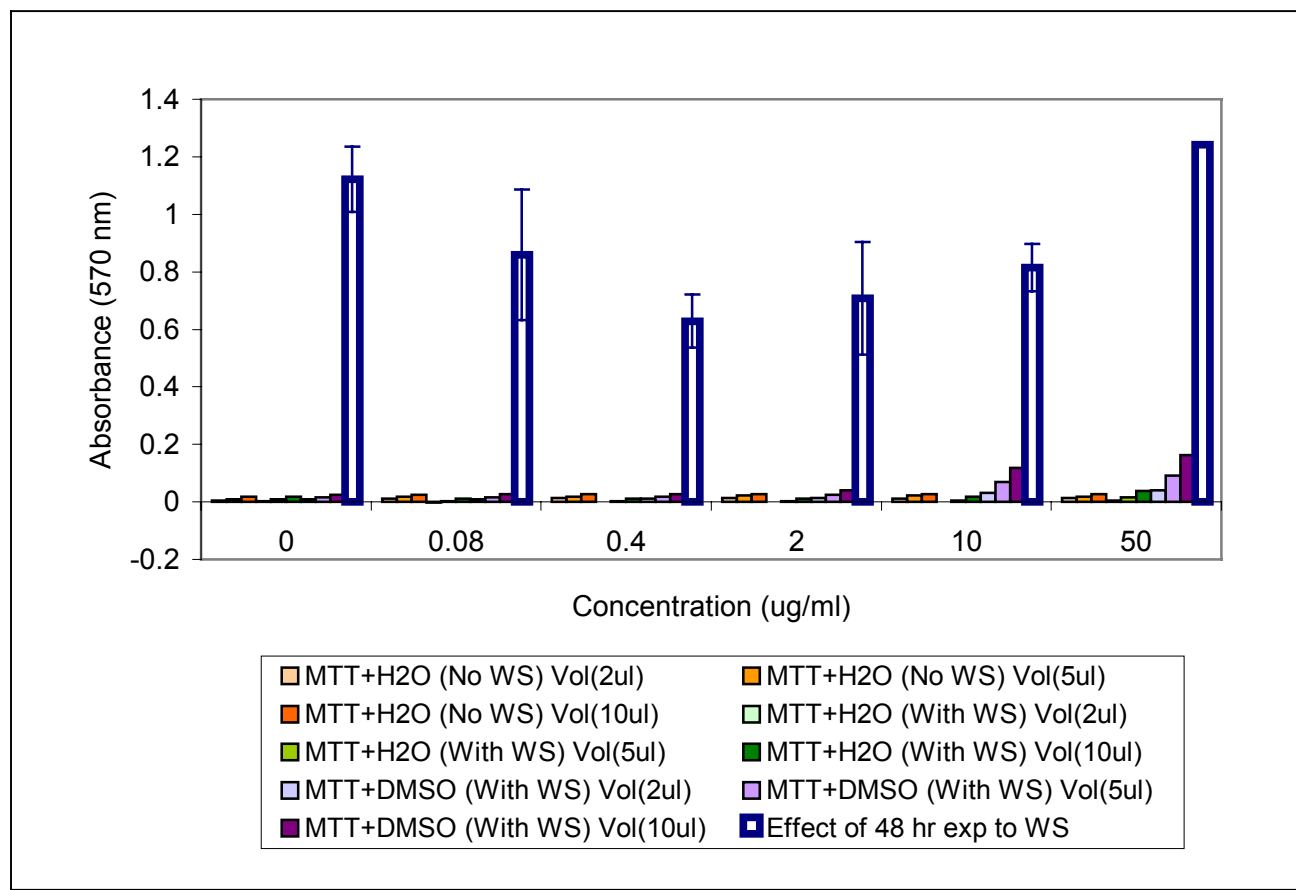
To investigate which one of the three factors caused the increase in absorbance, the following experiment was done in three different concentrations. The effect of MTT alone, MTT and plant in combination and MTT, plant and DMSO in combination were measured and the results were plotted on a graph.

MTT alone	Water + MTT dilution series
MTT and Plant in combination	MTT+ Plant dilution series
MTT, Plant and DMSO in combination	MTT+Plant+DMSO dilution series

Table 3.1: The effect of MTT alone, MTT and plant in combination and MTT, plant and DMSO in combination and the dilutions



Graph 3.7: The effect of three different concentrations of MTT alone, MTT and Plant in combination and MTT, Plant and DMSO in combination.



Graph 3.8: The effect of three different concentrations of MTT alone, MTT and Plant in combination and MTT, Plant and DMSO in combination revealing that the contribution of the plant material to the increased absorbance is only minimal.

In Graph 3.7 the first three bars at each concentration represent the effect of MTT alone. The second three bars at each concentration represent the effect of MTT and plant in combination and the last three at each concentration are an indication of the effect of MTT, plant and DMSO in combination.

Although there is an increase in absorbance with the MTT, DMSO and plant in combination, as revealed in graph 3.7, indicating that the plant did interact with the DMSO and caused an increase in absorbance, graph 3.8 shows that the contribution of the plant material to the increased absorbance is only minimal. This would imply that the plant on its own cannot be responsible for the observed increase in absorbance.

Therefore, as mentioned earlier, some other factors caused the increased absorbance observed in Graph 3.6. Most possibly this could be due to a cellular effect which will only be confirmed by further and more intense research on this subject.

3.4 CONCLUSION

WS has been an important herb in the Ayurvedic and indigenous medical systems. This small woody shrub with a very complex chemical constitution has been used for a variety of different conditions over many years such as stress, fatigue, pain, asthma, anxiety and depression and is also a potent antioxidant. Numerous studies have shown the effects of the plant in animal models as well as in cell culture. MRC-5 cells were used in a combination assay where lysosomal integrity, cell viability and cell number were measured with the NR, MTT and CV assays respectively and the absorbance was measured. The CV and NR assays revealed no significant decrease in cell number as the concentration increased whereas the MTT assay showed a significant decrease in cell viability over the first three concentrations and a significant increase in cell viability over the last two concentrations. With the additional experiment done to investigate what factor caused the increase in absorbance, it was found that the contribution of the plant material to the increase in absorbance was only minimal and therefore it can be concluded that the increase in absorbance could possibly be caused by a cellular effect that should be further investigated.

Chapter 4: Establishing the Balb/c asthmatic mouse model and investigating the effect of Withania somnifera, Selenium and Hydrocortisone on blood count and bronchial lavage

4.1 INTRODUCTION

Asthma is an inflammatory disease of the airways and involves a wide range of cell types and cellular mediators. Inflammation in asthma is a consistent feature and the pattern of inflammation in the airways appears to be similar in all clinical forms of asthma. However according to Cohn *et al.* (2004) and Bousquet *et al.* (2004) the relationship between the intensity of the inflammation in asthma and the severity of asthma is not clearly understood (Cohn *et al.*, 2004; Bousquet *et al.*, 2004). Tulic *et al.*, (2001) then also suggested that the inflammatory process on pathological and physiological level extends beyond the central airways to the peripheral airways and lung parenchyma.

Chemokines play an important role in the recruitment of inflammatory cells into the airways, which are mainly expressed in airway epithelial cells (Miller, 2001). Cysteinyl leukotrienes are derived from mast cells and eosinophils. They are potent bronchoconstrictors and pro-inflammatory mediators and are the only mediators whose inhibition has been associated with an improvement in lung function and asthma symptoms (Leff, 2001). Cytokines are the main mediators of the inflammatory process and determine the severity of asthma (Barnes, 1998). 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. Histamine is released from mast cells and plays a role in bronchoconstriction and the inflammatory process. 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). The action of inducible nitric oxide synthase in airway epithelial cells produces Nitric oxide (NO) that is a potent vasodilator (Ricciardolo *et al.*, 2004). The effectiveness of asthma treatment is

monitored by using exhaled NO because of its reported association with the presence of inflammation in asthma (Smith and Taylor, 2005). Prostaglandin D₂ is a bronchoconstrictor derived predominantly from mast cells and is involved in Th2 cell recruitment to the airways.

The cells present in the blood smears and bronchial lavage of asthmatic mice includes the following: Mast cells, Eosinophils, T lymphocytes, dendritic cells, Macrophages and Neutrophils. Mast cells release bronchoconstrictor mediators such as histamine, cysteinyl leukotrienes and prostaglandin D₂ (Galli *et al.*, 2005). According to Robinson (2004), airway hyperresponsiveness cause an increase in mast cell numbers in airway smooth muscle.

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 remodeling (Kay *et al.*, 2004). T lymphocytes are usually present in increased numbers in the airways and they 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). The dendritic 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). There is an increased number of macrophages 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). The 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).

Numerous studies have been done over the past few years to investigate the antioxidant effect of *W. somnifera* (WS) and came to the conclusion that WS exhibits a potent antioxidant and anti-inflammatory effect (Anbalagan and Sadique, 1984; Davis and Kuttan, 2000; Gazdik *et al.*, 2004). However, no research has been done previously using an *in vivo* murine model to determine the effect of the plant on asthma. Little information is also available of the *in vivo* effect of selenium on asthma. In the current

chapter the effect of WS, antioxidant selenium, WS and selenium in combination and hydrocortisone as positive control on the number of white blood cells in blood smears and bronchial lavage smears as well as the platelet distribution of asthmatic Balb/c mice are discussed.

4.2 MATERIALS AND METHODS

4.2.1 Implementing the Balb/c asthma model

36 Six-week-old (female) Balb/c mice each of average weight 20g maintained in the University of Pretoria Biomedical Research Center (UPBRC) and provided OVA-free food and water *ad libitum*, were used. All experimental protocols complied with the requirements of the University of Pretoria's Animal Use and Care Committee.

Mice were divided in the following groups (6 animals per group):

- Control mice
- Asthmatic mice
- Mice exposed to physiologically comparable levels of *W. somnifera* (45µl/kg of body mass)
- Mice exposed to anti-oxidant (Selenium) (0.02µg/100µl)
- Mice exposed to combination *W. somnifera* and anti-oxidant (45 µl + 100µl)
- Mice exposed to low dose hydrocortisone (100mg/kg)

Sensitization (on day 0 and day 5) of mice was via intraperitoneal injection of 25mg ovalbumin (OVA) (grade V; Sigma-Aldrich) and 2mg Al(OH)₃ that was dissolved in 0.5ml of 0.9% saline solution. All mice except the control mice (which were left untreated) were sensitized.

Nebulisation with 1% OVA in PBS (1mg OVA in 100 ml PBS) was performed twice daily, for 1 hour on days 13, 14 and 15. WS, selenium, the combination of WS and selenium as well as hydrocortisone was administered on day 15 to day 18, again on days 21, 22, 25 and 28. Animals were again nebulised on days 34, 35 and 36 and treated daily from day 39 to day 42 and the animals were terminated on day 43.

4.2.2 Techniques of bronchial lavage

After termination, a small skin incision was made in the skin of each mouse anterior to the trachea. The trachea was exposed by blunt dissection and a small transversal incision was made below the larynx. Through the sheath of a 21G venous catheter 0.3 ml of saline was injected into the trachea and aspirated with a syringe. The bronchial lavage fluids collected for the individual groups were pooled, centrifuged for 2 minutes at 1000rpm and smears were made. The smears were stained with Giemsa Wright stain. White blood cells were counted under a 100x magnification and average 18 fields per slide were counted.

4.2.3 Techniques of blood smears

Blood samples of mice from each group were collected by cardiac puncture on the day of termination, pooled and Citrate added (11µl citrate per 100µl of blood) and histological blood smears were prepared and stained with Giemsa Wright stain and an average of 18 fields were counted per slide.

4.3 RESULTS

Figure 4.1 shows the different white blood cells in bronchial lavage found in mice. Figure 4.2 shows platelet distributions amongst the different groups. Table 4.1 shows the different counts of white blood cells in bronchial lavage in control, asthmatic mice and those treated with WS alone and in combination with selenium and also shows that of mice treated with hydrocortisone. Table 4.2a shows the different number of white blood cells counted in the blood smears; up to hundred cells were counted. Table 4.2b shows the percentages of white blood cells counted per zone in the bronchial lavage. The number of zones counted in each treatment group is indicated in the table.

Graph 4.1 shows the total number of white blood cells counted in the bronchial lavage. T- tests performed revealed a significant difference between the asthma group and the control group as well as between the asthma group and all the other treatment groups with the **P-value < 0.05**. Graph 4.2 shows the distribution of eosinophils between the different groups. T- tests performed revealed a significant increase in the number of eosinophils between the asthma and the other groups. Two-tailed significance was

observed in each case with the **P-value** < **0.05**. Graph 4.3 shows the percentages of the white blood cells counted in the blood smears.

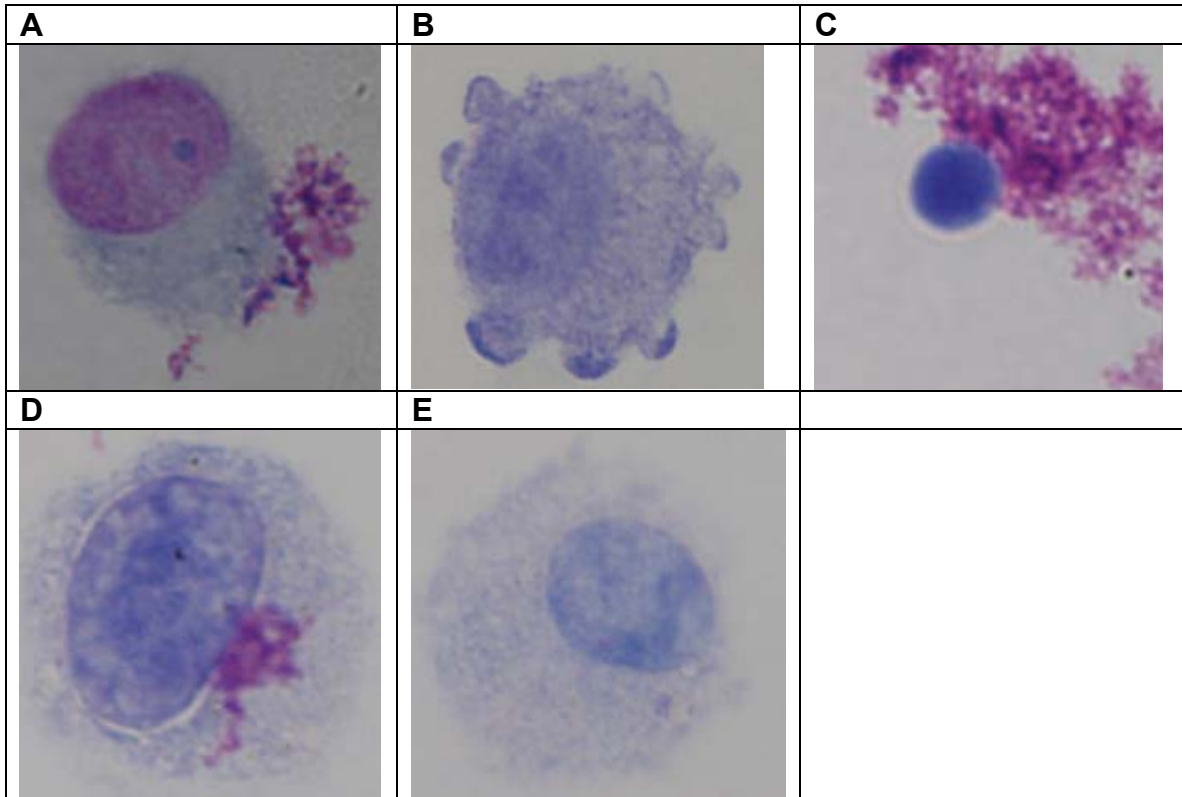


Figure 4.1: White blood cells in the bronchial lavage of Balb/c mice. Eosinophil (**A**), Monocyte (**B**), Lymphocyte (**C**), Basophil (**D**), Neutrophil (**E**)

	Control	Asthma	<i>Withania</i>	Selenium	<i>Withania</i> + Selenium	Cortisone
Lymphocyte	5	87	109	27	144	18
Monocyte	37	80	129	93	131	48
Neutrophil	0	18	19	7	24	6
Basophil	0	10	7	1	3	2
Eosinophil	0	86	32	40	32	5

Table 4.1: Number of white blood cells counted in the bronchial lavage of Balb/c mice

	Control	Asthma	<i>Withania</i>	Selenium	<i>Withania</i> + Selenium	Cortisone
Lymphocyte	32	53	42	32	28	70
Monocyte	57	19	38	30	44	18
Neutrophil	7	15	8	21	17	10
Basophil	3	0	2	3	0	1
Eosinophil	1	13	10	14	11	1

Table 4.2a: White blood cell count in the blood smears of Balb/ c mice (100 cells counted)

	Control	Asthma	<i>Withania</i>	Selenium	WS + Sel	Cortisone
Lymphocyte	3.55	17.67	7	6.4	4.66	8.75
Monocyte	6.3	6.3	6.3	6	7.33	2.25
Neutrophil	0.77	5	1.3	4.2	2.83	1.25
Basophil	0.33	0	0.3	0.6	0	0.125
Eosinophil	0.11	4.33	1.7	2.8	1.83	0.125
Nr of zones counted	9	3	6	5	6	8

Table 4.2b: Percentage of white blood cells counted per zone in the blood smears of Balb/ c mice

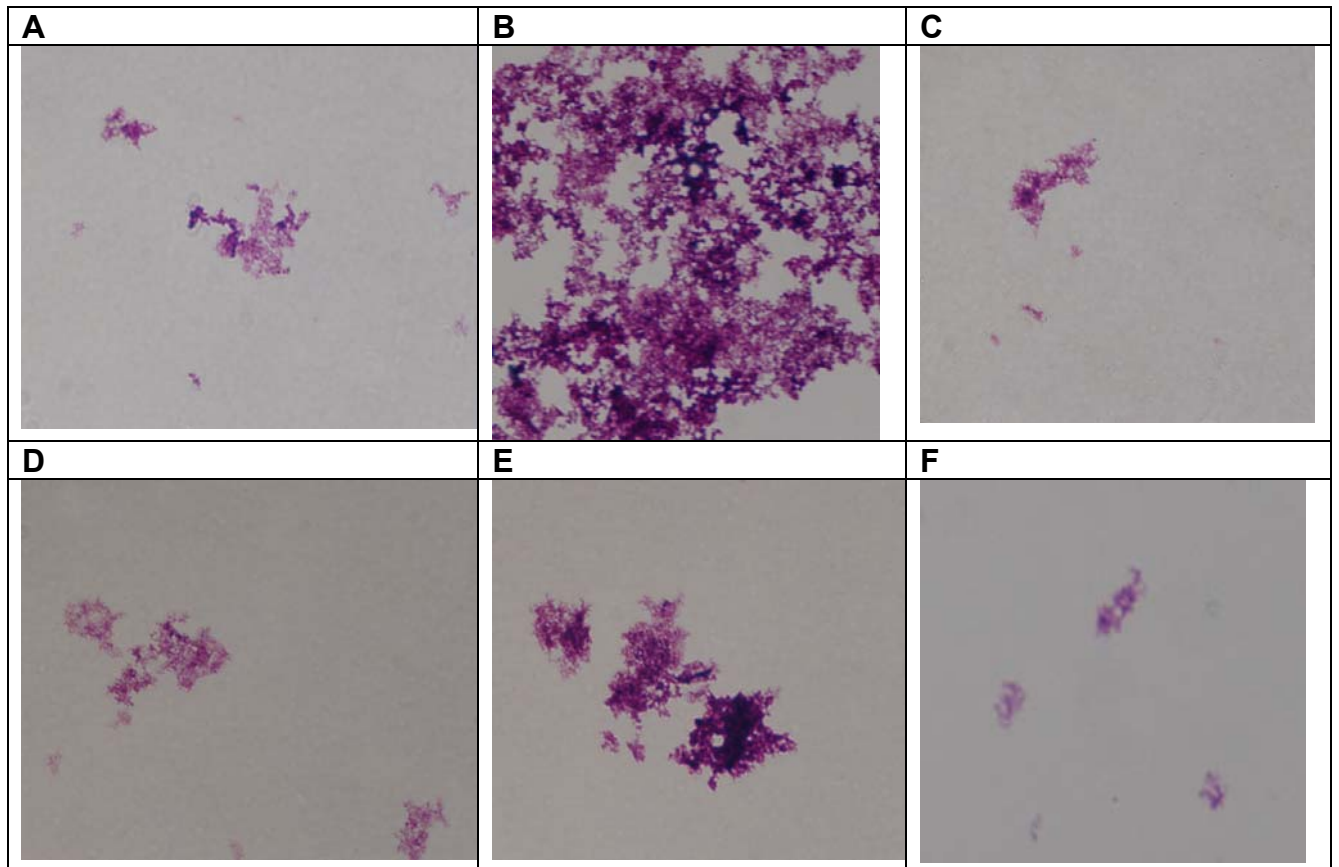
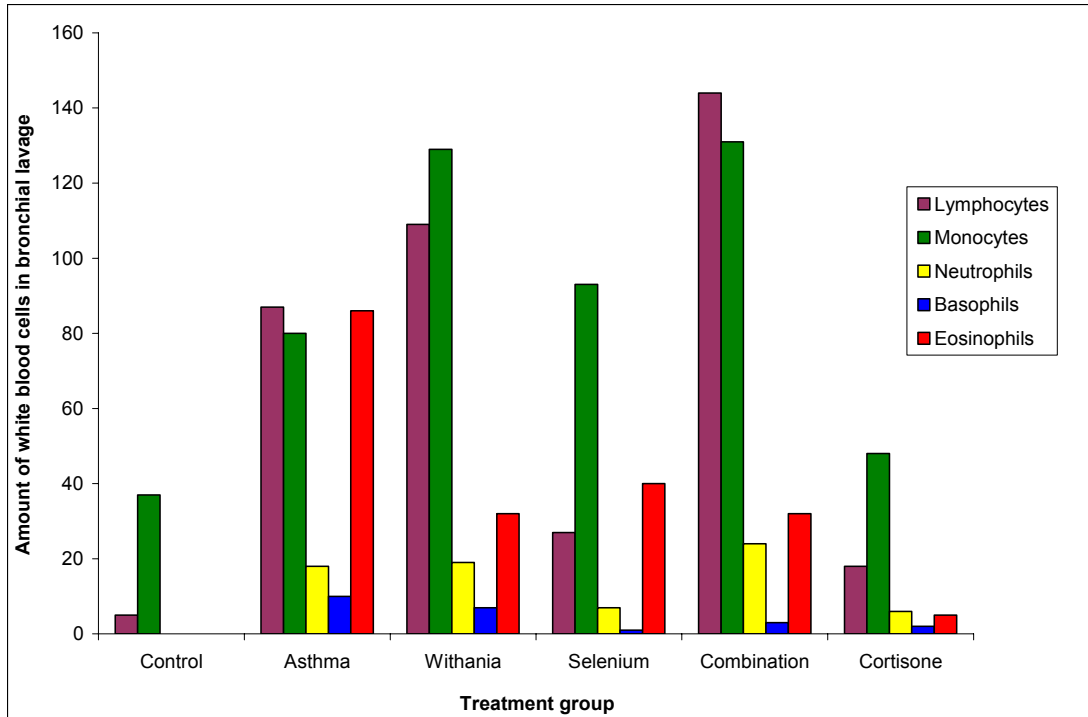
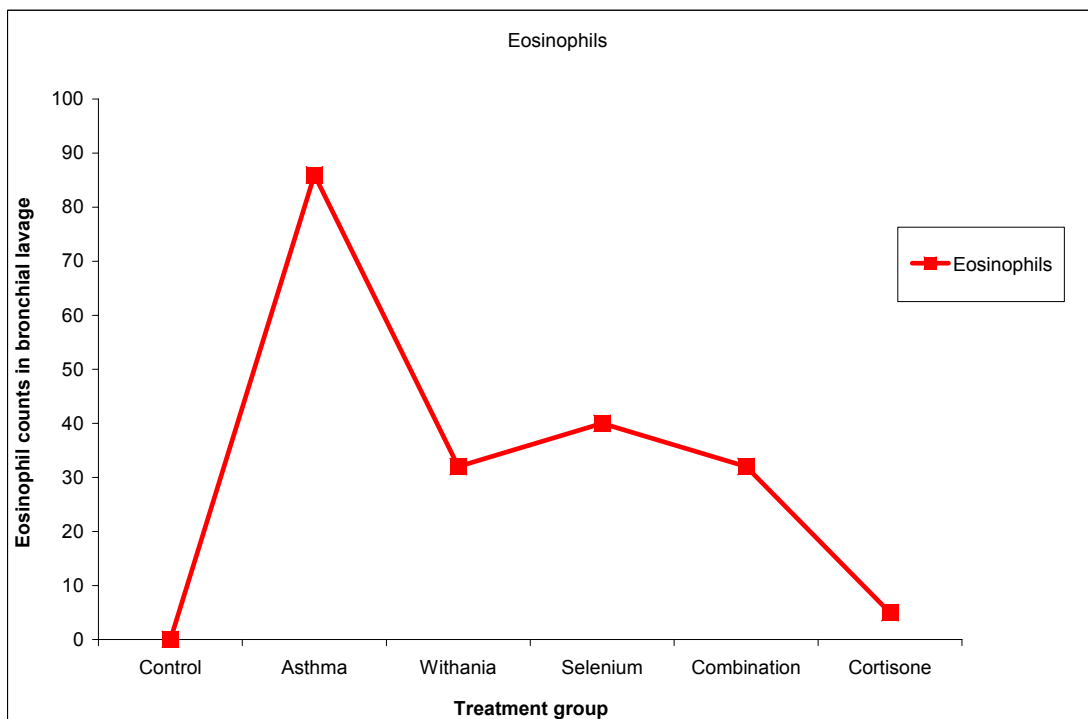


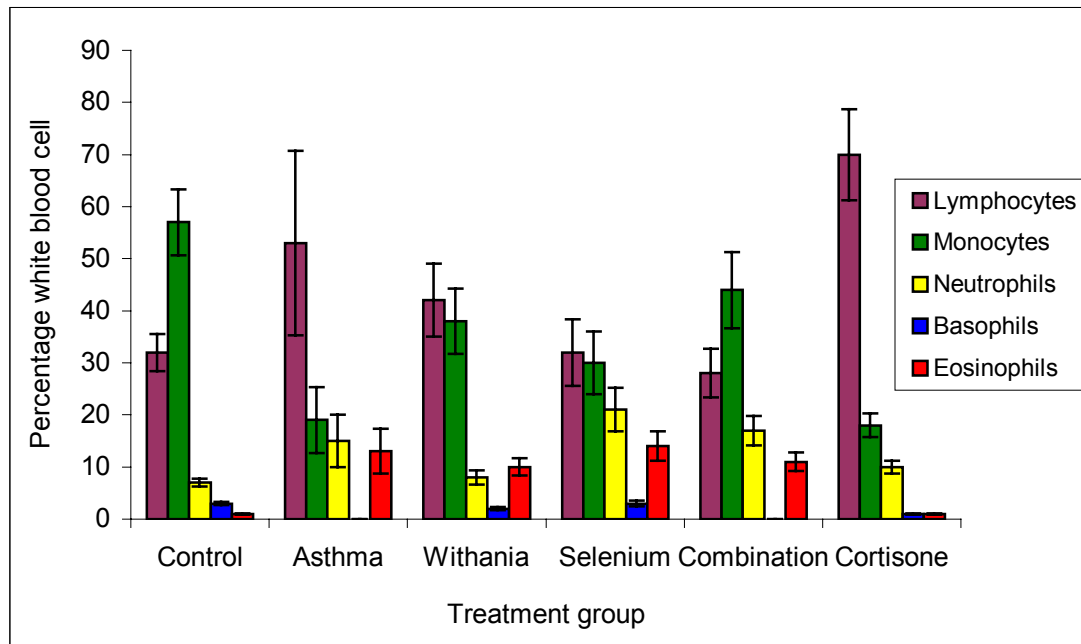
Figure 4.2: Distribution of platelets between different treatment groups in the bronchial lavage of Balb/c mice. Control **(A)**, Asthma **(B)**, Cortisone **(C)**, *Withania* **(D)**, Selenium **(E)**, Combination **(F)**



Graph 4.1: Amount of white blood cells counted in the bronchial lavage of asthmatic Balb/c mice in the different treatment groups



Graph 4.2: Eosinophil counts in the bronchial lavage in the different treatment groups



Graph 4.3: Percentage of white blood cells counted in the blood smears

4.4 DISCUSSION

Asthma is a chronic inflammatory disorder of the airways, characterized by episodes of wheezing, coughing, and shortness of breath. There is an increase in mucus production and airway hyper-responsiveness which result in a decrease in air flow. Asthma is a multifactorial disease that is associated with genetic, allergic, environmental, infectious, emotional, and nutritional components. The underlying pathophysiology of asthma is airway inflammation and T – cells play an important role in this inflammatory process (Miller, 2001)

In asthma, white blood cells play an important role. The process of inflammation in asthma is described by an inflammatory cascade which is dividable into seven phases: sensitization, stimulation, cell signaling, 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 process 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 and chemokines such as eotaxin and RANTES was observed in the airways of allergic asthmatics (Kon and Kay, 1999; Renauld, 2001).

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 (interleukin-4 (IL-4), IL-5, IL-9 and IL-13) (Barnes *et al.*, 1998). The released cytokines influence conversion of B-lymphocytes to plasma cells, which 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. Antigen-specific IgE is partly responsible for initiation of an allergic response in asthma. The antigens cross-link with IgE on the mast cells. The mast cells then release their contents, histamine and leukotrienes and continue with the inflammatory response by damaging local tissue and attracting other lymphocytes to the area. Interactions between antigen-presenting cells and B and T lymphocytes regulate IgE production. Macrophages and dendritic cells, which are antigen-presenting cells, present an antigen

to CD 4⁺ Th2 cells, which secrete cytokines that magnify the immune response. Th2 cells produce IL-4 which stimulates IgE production in B-cells, and IL-5 stimulates eosinophil differentiation and mobilization to inflammatory sites. IL-10 enhances the growth and differentiation of mast cells (Erb *et al.*, 1996; Kon and Kay, 1999; Selgrade *et al.*, 1997; Kline and Hunninghake, 1994; Umetsu and De Kruyff, 1997; Ray and Cohn 1999; Bodey *et al.*, 1999; Le Gros *et al.*, 1998; Bell *et al.*, 1996; Coyle *et al.*, 1996).

According to Page *et al.* (2001), mast cells also play an important role in the pathophysiology of asthma. Mast cells respond to non-IgE stimuli in the airway such as changes in the osmotic cellular environment associated with exercise, hypertonicity, and hypotonicity. The mediators released from activated mast cells include prostaglandins, leukotrienes, cytokines (such as IL1, IL 2, IL4, IL10, and IL13), growth factors (such as platelet-derived growth factor and transforming growth factor b) and potent proteases (such as tryptase).

Kassel *et al.* in 1999 suggested that the secretion of stem cell factor could perhaps cause the increased presence of mast cells in the airway smooth muscle of patients with asthma by the muscle. Stem-cell factor is chemo attractant for mast cells that is responsible for regulating the growth, function, and survival of the mast cells.

Increase in eosinophil numbers and T lymphocytes in the bronchial mucosa and bronchoalveolar lavage 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, tumor 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 (Dombrowicz and Capron, 2001; Gleich, 2000).

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 chemo-attractant substances (Busse and Lemanske, 2001; Prescott, 2003; Lampinen *et al.*, 2004).

In humans, eosinophil 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 remodeling (Kay *et al.*, 2004). Also, T lymphocytes are usually present in increased numbers in the airways and they 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).

Macrophages are also 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). Grootendorst *et al.*, (1997) compared the cellular compositions of hypertonic saline-induced sputum, bronchoalveolar lavage fluid and bronchial biopsies in 18 clinically stable patients with mild to moderate atopic asthma. Patients were treated with inhaled short-acting bronchodilators or regular inhaled steroids (Grootendorst *et al.*, 1997). The authors found that sputum cell differentials were not different between the patients with and without inhaled steroids, and showed a median value of 19.4% squamous cells, with 1.0% eosinophils, 3.3% lymphocytes, 28.7% neutrophils, 49.4% macrophages and 6.9% cylindrical epithelial cells (in percentage non-squamous cells).

Typically in bronchial lavage of control mice, up to 90% of cells are macrophages. In the Balb/c mouse model, Larsen and co-workers (2007) reported that with inflammation the total number of white blood cells increased, but especially the distribution of cells is changed, with an increase in eosinophils, neutrophils and sometimes lymphocytes - cells that is very rarely seen in bronchial lavage from healthy mice (Larsen *et al.*, 2007). Pinto *et al.*, (2004) also mentioned that in control mice, no significant neutrophils were found in bronchial lavage (Pinto *et al.*, 2004).

This was also found in the current study where, in control mice, most of the cells were late stage monocytes (precursor macrophages) (Figure 4.1B) and the cell counts showed that as well (Table 4.1 and Graph 4.1). Since these monocytes had such a clear macrophage differentiated appearance, henceforth they will be referred to as

macrophages, although the micrographs and graphs of cell counts will refer to these cells as monocytes due to leukocyte terminology.

4.4.1 Bronchial lavage and platelet distribution

Platelet distribution changed significantly in the asthmatic group, when compared to the control bronchial lavage (Figure 4.2A and 4.2B). In the asthmatic group, numerous platelet clumps were found distributed between white blood cells. While in the control group, platelets were present, but not as prevalent and did not form the clumps present in the asthmatic group.

It is known that hydrocortisone decreases white blood cells in bronchial lavage in humans. The hydrocortisone treated mice also showed a significant decrease in white blood cells in the bronchial lavage, and platelet clumps were also not present as was found in the asthmatic group (Figure 4.2C).

Selenium on its own is not used as a treatment supplement for asthma, however, as previously mentioned, selenium is a powerful anti-oxidant and it is known that asthma sufferers have decreased levels of selenium. It was also found that the platelet distribution did not differ as much between the selenium and the asthmatic group as between the asthmatic group and the other treatment groups.

WS also decreased the number of eosinophils significantly in the bronchial lavage and the effect of selenium and WS revealed more or less the same distribution of white blood cells and a significant decrease in the number of eosinophils compared to the asthmatic group. Platelet clumping was also not as prevalent in the distributions WS, selenium and the combination (Figure 4.2D, 4.2E, 4.2F).

4.4.2. Blood smears

In the asthmatic group, the distribution changed where there is an increase in the number of all white blood cells and especially a significant increase in the number of eosinophils. (Table 4.1). A t- test performed revealed a significant increase in the number of eosinophils between the asthmatic group and the control group as well as between the asthmatic group and each of the other treatment groups (Graph 4.2).

In the current study, it was found that selenium decreased the number of lymphocytes, neutrophils, and basophils and significantly decreased the number of eosinophils.

Results from the blood smears of the WS, selenium, WS and selenium in combination and hydrocortisone showed the same trends as in the bronchial lavage where cell counts in control and hydrocortisone blood smears were decreased compared to that of the asthma group (Table 4.2a and 4.2b). WS and the combination WS and selenium also showed decreased cell numbers, whereas selenium alone did not significantly decrease cell numbers in blood.

4.5 CONCLUSION

It is concluded that WS and WS combined with selenium significantly decreased the white blood cells in both bronchial lavage as well as blood smears, suggesting that WS indeed has an anti-inflammatory potential and it, in combination with an anti-oxidant like selenium might successfully be used in the treatment of asthma. However, further studies should be embarked upon to investigate the effect of this Phytomedicine on immunological parameters.

Chapter 5: Scanning Electron Microscopic analysis of the formation of fibrin networks in the asthmatic Balb/c mouse model

5.1 INTRODUCTION

Platelets and fibrin play an important physiological role in allergic processes and immunological mechanisms. In conditions like allergic asthma, platelets participate by acting as inflammatory cells, by releasing mediators, spasmogens and/or by interacting with other inflammatory cell types. Platelets are activated by a number of stimuli resulting in the expression and/or activation of surface receptors, secretion of vaso-active substances, adhesion, aggregation, and finally thrombus formation (Lazarus *et al.*, 2003). The activation may be due to, amongst others, inflammatory processes (Camera, 1999; Butenas and Mann, 2002). Fibrin and platelets are two factors that play an important role in asthma. Fibrinogen itself is also widely recognized as a marker for systemic inflammation as it is considered an acute phase protein. Pitchford *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.

The association between thrombin, fibrin and asthma can be summarised as follows:

- 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 and may play a role in the pathogenesis of airway remodelling (Gabazza *et al.*, 1999)
- Human platelets can produce PAF upon thrombin stimulation in the lungs (Touqui *et al.*, 1985)
- Fibrinogen can be produced by lung epithelia as a result 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) and extra-vascular thrombin, fibrinogen, and fibrin has been found in the sputum of patients with asthma (Banach-Wawrzenczyk *et al.*, 2000; Pizzichini *et al.*, 1996; Wagers *et al.*, 2004).
- Airway hyper-responsiveness 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. There is a decrease in plasminogen activator (PA) activity in asthma. 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).

Mice are used widely for *in vivo* models of allergy and asthma (Kips *et al.*, 2003). 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 hyper-secretion, airway hyper-responsiveness and IgE profiles. Experimental mouse models of allergic asthma have been established almost ten years ago. The study of disease pathogenesis and development of new therapeutics have been made possible by these experimental mouse models (Epstein, 2004). In previous research Pretorius *et al.* (2007) showed that the murine asthma Balb/c model can be used successfully to study platelets and fibrin. The effects of the plant *Euphorbia hirta* (a phytomedicine traditionally used to treat asthma) on platelet and fibrin formation and morphology were studied to determine how these products affect the coagulation system. Hydrocortisone was used as positive control because it is a known anti-inflammatory product that is used in the treatment of asthma.

In the current chapter the Balb/c murine asthma model was used to study the effect of the phytomedicine WS as well as the well-known anti-oxidant, selenium, separately and in combination, on the ultrastructure of platelets and fibrin networks. Hydrocortisone is used as positive control as hydrocortisone is used most commonly in the treatment of asthma.

5.2 MATERIALS AND METHODS

5.2.1 Preparing water extracts of *W. somnifera*

Plant material was collected in the Pretoria region of 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 1 week. The material was grounded into a fine powder and subdivided into four 50 g samples. The samples were subsequently extracted with 500ml of water, after which it was filtered and dried on a rotary evaporator at 40°C. The water extract was freeze-dried. This procedure was repeated three times for each sample. A stock solution of 330 mg/ml was prepared that was administered orally to the mice. This implied that each animal (average weight 20g) received 45µl of plant material. This dose was decided upon after studying literature that mentioned physiological doses suggested by herbalists. Rasool and Varalakshmi (2006) did an animal study where they used WS root powder at a dosage of 500 and 1000mg/kg of body weight. In the current study this dosage was used and converted back to the average weight of a Balb/c mouse.

5.2.2 Implementing the Balb/c asthma model

36 Six-week-old (female) Balb/c mice, each of average weight 20g, maintained in the University of Pretoria Biomedical Research Centre (UPBRC) and provided OVA-free food and water *ad libitum*, were used. All experimental protocols complied with the requirements of the University of Pretoria's Animal Use and Care Committee.

Mice were divided in the following groups (6 animals per group):

- Control mice
- Asthmatic mice
- Mice exposed to physiologically comparable levels of *W. somnifera* (45µl/kg of body mass)
- Mice exposed to anti-oxidant (Selenium) (0.02µg/100µl)
- Mice exposed to combination *W. somnifera* and selenium (45 µl + 100µl)
- Mice exposed to low dose hydrocortisone (100mg/kg)

Sensitization (on day 0 and day 5) of mice was via intraperitoneal injection of 25mg OVA (grade V; Sigma-Aldrich) and 2mg $\text{Al}(\text{OH})_3$ that was dissolved in 0.5ml of 0.9% saline solution. All mice except the control mice (which were left untreated) were sensitized.

Nebulisation with 1% OVA in PBS (1mg OVA in 100 ml PBS) was performed twice daily, for 1 hour on days 13, 14 and 15. WS, selenium, the combination of WS and selenium as well as hydrocortisone was administered on day 15 to day 18, again on days 21, 22, 25 and 28. Animals were again nebulized on days 34, 35 and 36 and treated daily from day 39 to day 42 and the animals were terminated on day 43.

5.2.3 Preparation of fibrin clots

On day 43 during termination, 100 to 500 μl of blood was drawn from each of the Balb/c mice in each of the groups and 11 μl citrate for every 100 μl of blood, was added. Blood was then centrifuged at 1000 rpm for 2 minutes to obtain platelet rich plasma (PRP).

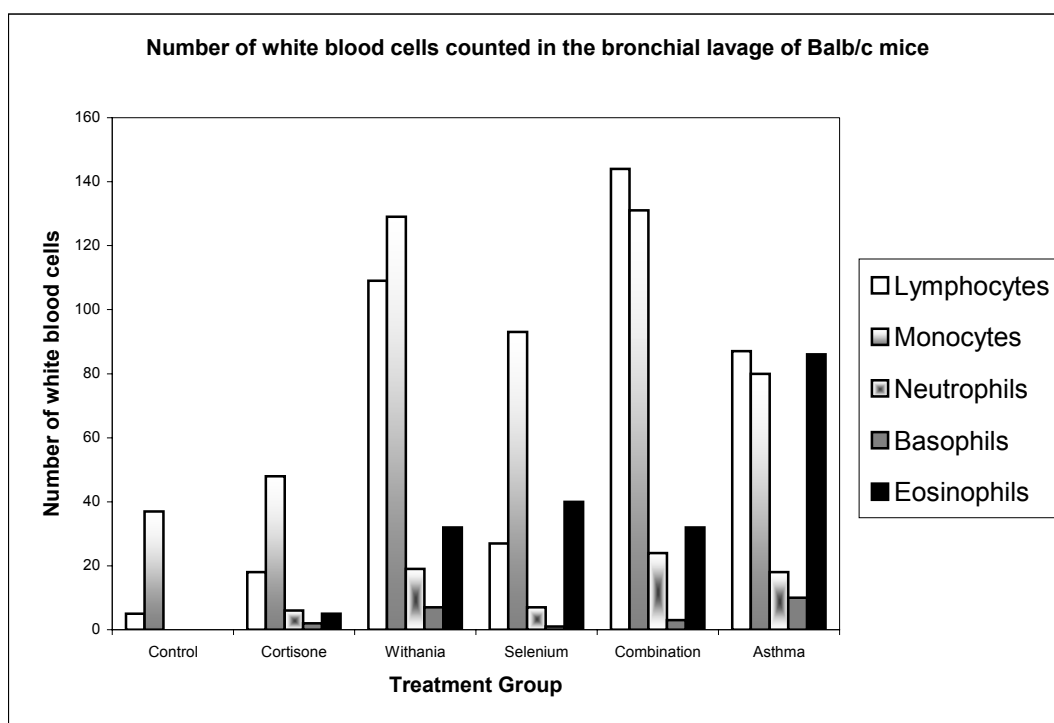
Human thrombin (provided by The South African National Blood Services) was used to prepare fibrin clots. The thrombin is 20 U/ml and is made up in biological buffer containing 0,2% human serum albumin. When thrombin is added to PRP, fibrinogen is converted to fibrin and intracellular platelet components e.g. transforming growth factor, platelet derived growth factor and fibroblastic growth factor are released into the coagulum.

10 μl of mouse PRP was mixed with 10 μl of human thrombin. The PRP and thrombin mix was immediately transferred 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 and placed at 37°C for 10 minutes. A washing process where the millipore membranes with the coagula were placed in PBS and magnetically stirred for 120 minutes followed this. This was done to remove any blood proteins trapped within the fibrin network.

5.3.4 Preparation of washed fibrin clot for SEM

Washed fibrin clots were fixed in 2.5% glutaraldehyde in Dulbecco's Phosphate buffered saline (DPBS) buffer with 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 tetroxide (OsO_4). The samples were rinsed thrice with distilled water for 5 minutes and were dehydrated serially in 30%, 50%, 70%, 90% and three times with 100% ethanol. The SEM procedures were completed by critical point drying of the material, mounting and examining the tissue with a ZEISS ULTRA 55 FEG.SEM

5.3: RESULTS AND DISCUSSION



Graph 5.1: Amount of white blood cells present in the bronchial lavage of Balb/c mice in the different treatment groups

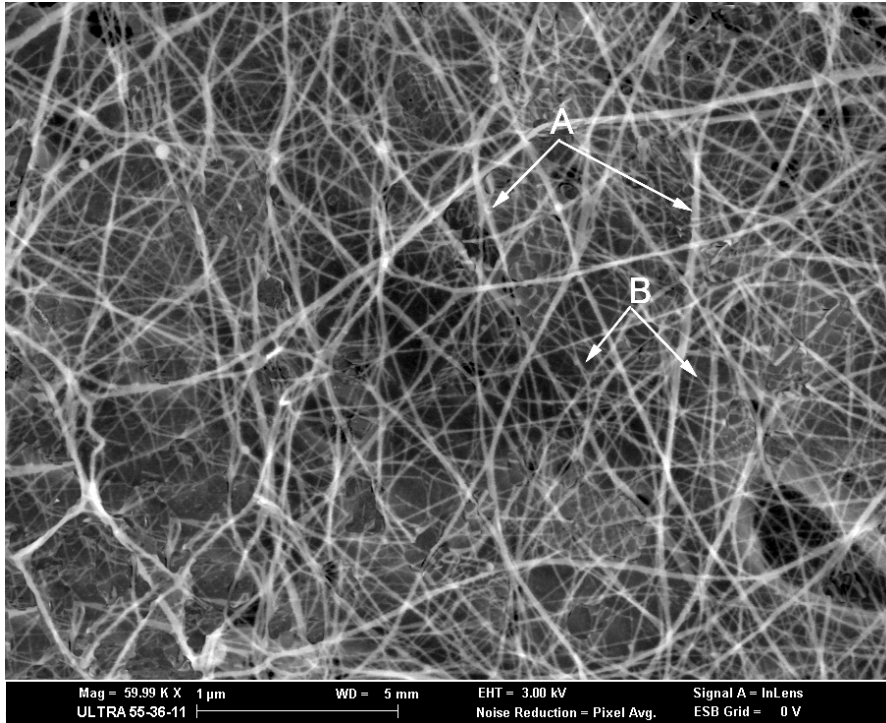


Figure 5.1a: Control fibrin network with thick, major fibers as well as thin, minor fibers. Label A = thick, major fibers; Label B = thin, minor fibers.

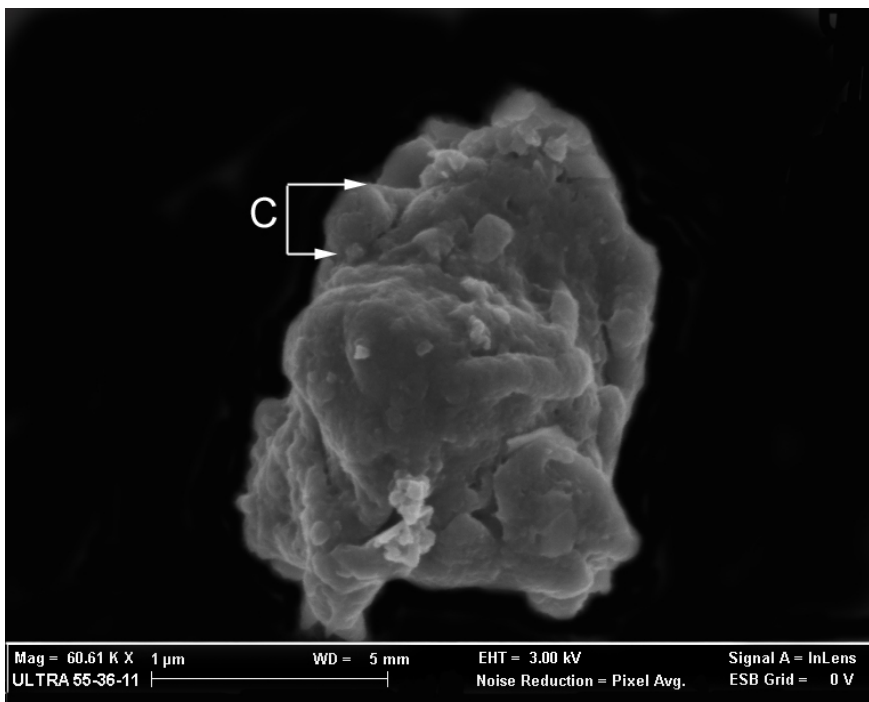


Figure 5.1b: Control platelet aggregate forming dense, round aggregate. Label C = pseudopodia.

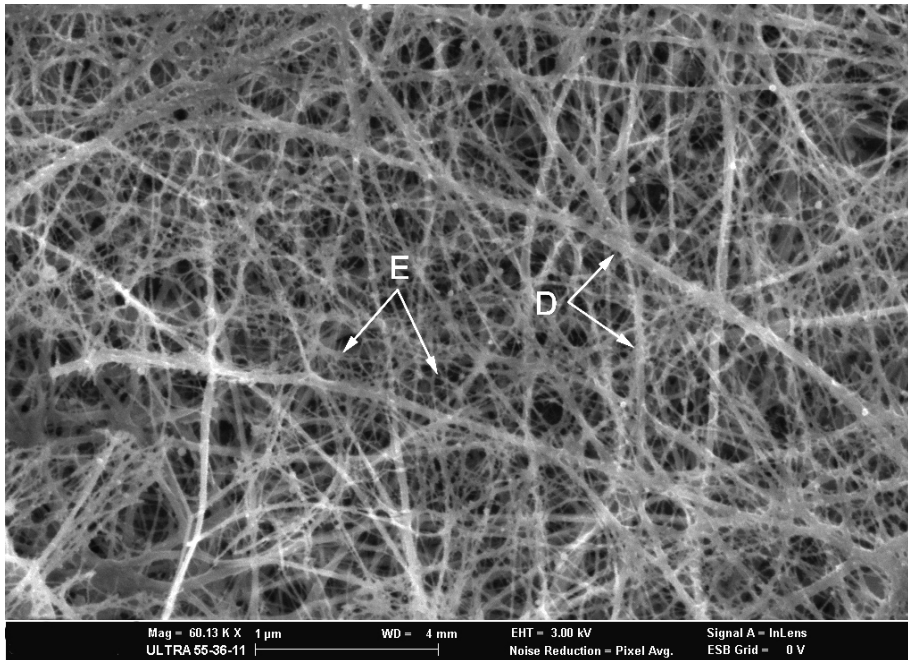


Figure 5.2a: Fibrin network from asthmatic Balb/c mice showing thick, major fibers as well as thin, minor fibers. Label D = thick, major fibers; Label E = thin, minor fiber forming a dense network.

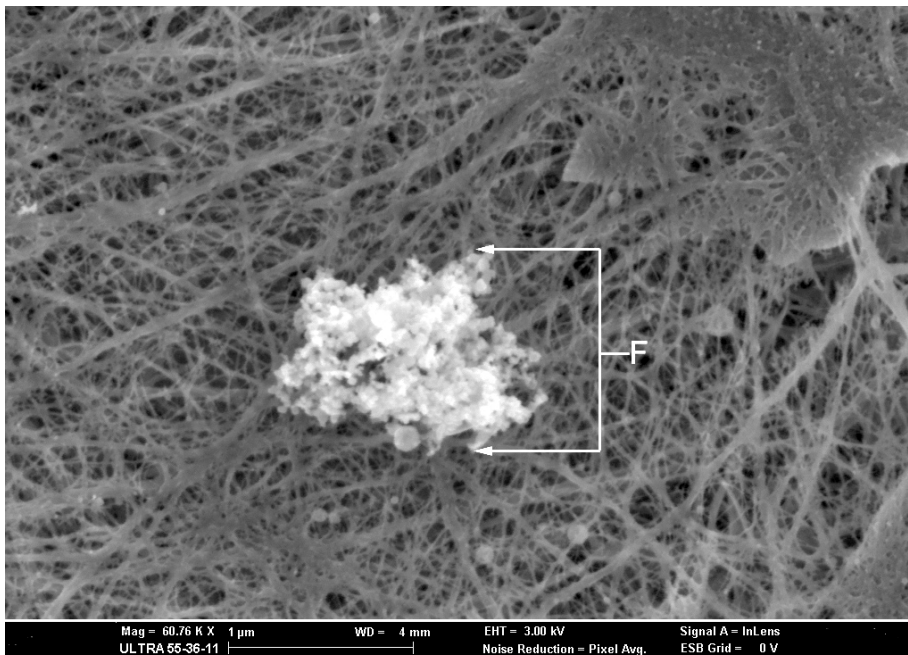


Figure 5.2b: Platelet aggregate from asthmatic Balb/c mice. Label F = granular, loosely arranged aggregate.

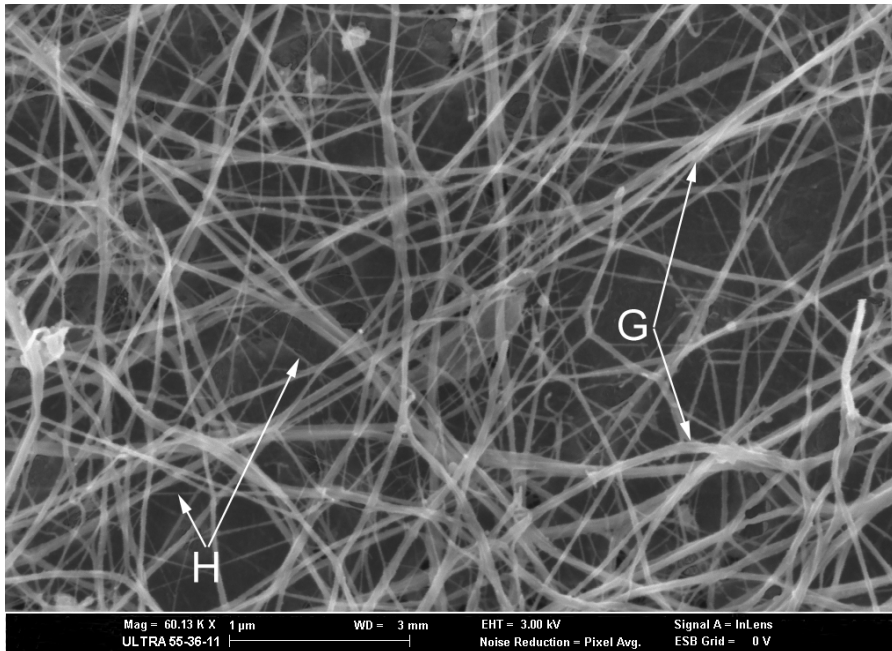


Figure 5.3a: Fibrin network from hydrocortisone-treated asthmatic Balb/c mice, forming flimsy fibrin network (100mg/kg). Label G = thick, major fibers; Label H = matted, thin minor fibers.

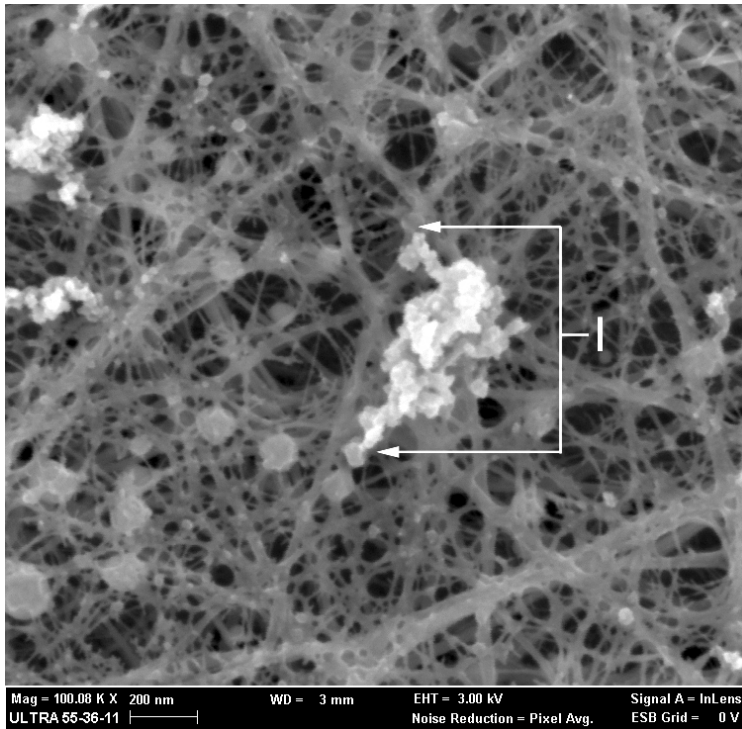


Figure 5.3b: Platelet aggregate from hydrocortisone-treated asthmatic Balb/c mice (100mg/kg). Label I = granular platelet aggregate.

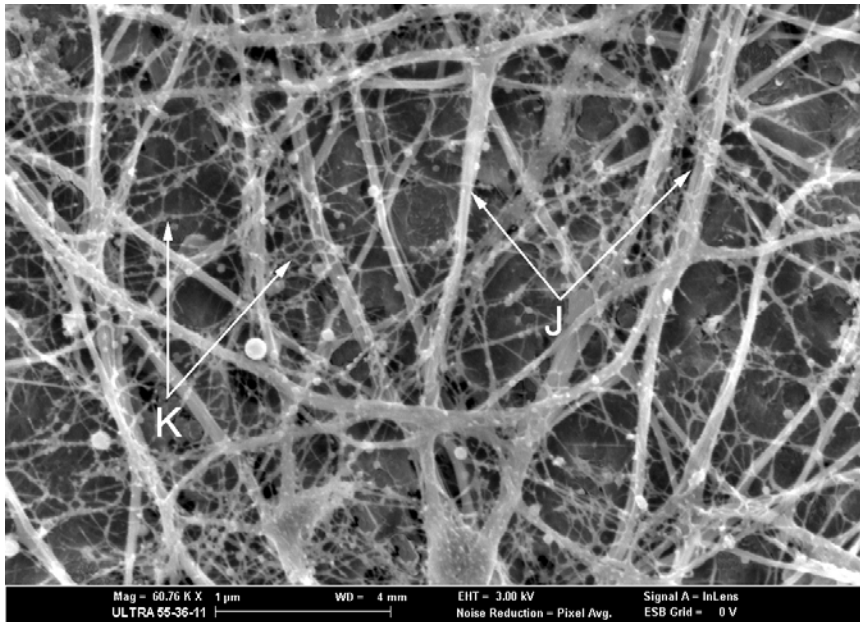


Figure 5.4a: Fibrin network from asthmatic Balb/c mice treated with *Withania somnifera* showing fibrin network with thick, major fibers as well as thin, minor fibers. Label J = thick, major fibers; Label K = thin, minor fibers

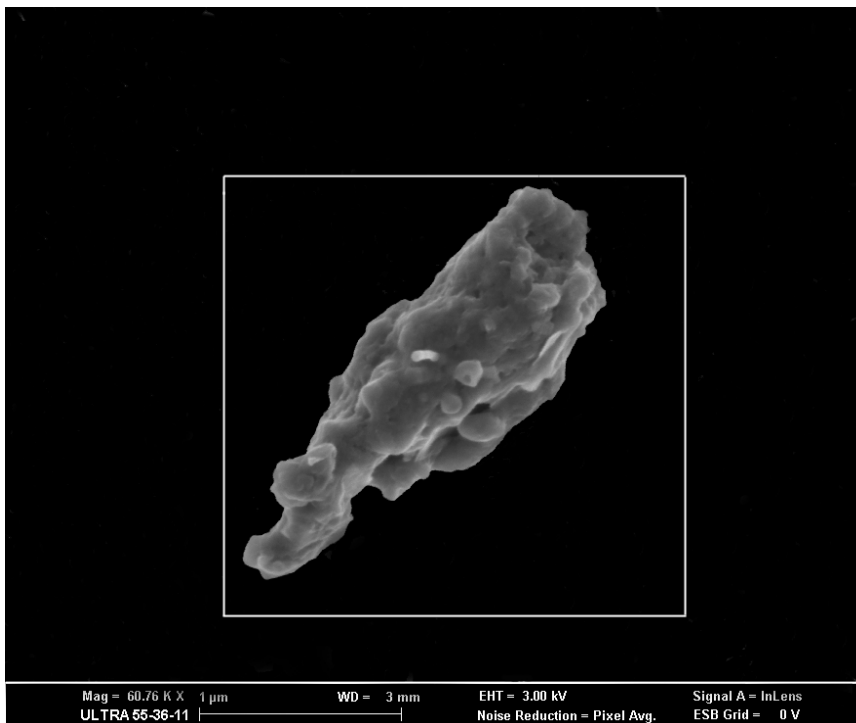


Figure 5.4b: Platelet aggregate from asthmatic Balb/c mice treated with *Withania somnifera*, forming tight aggregates with pseudopodia. Aggregate in block.

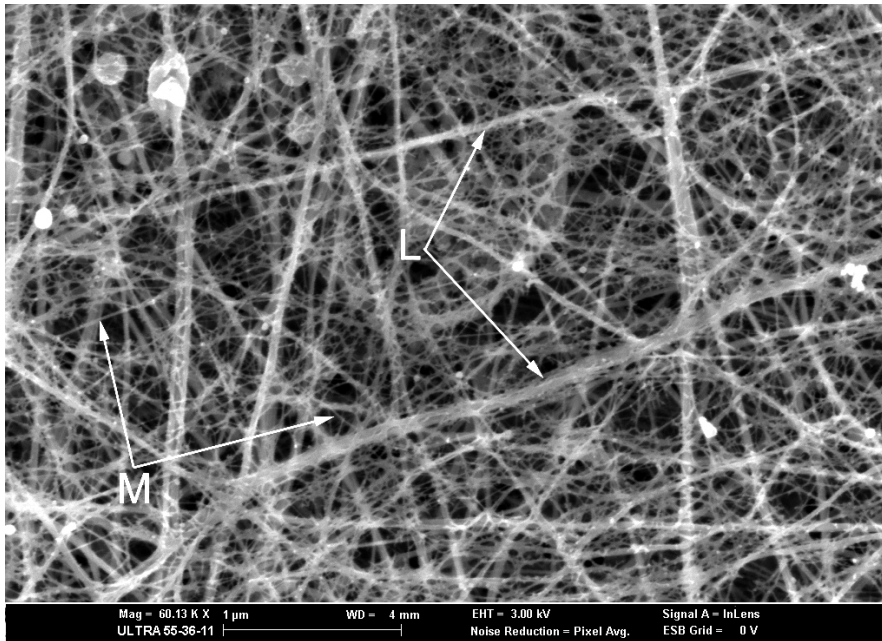


Figure 5.5a: Fibrin network from asthmatic Balb/c mice treated with selenium showing fibrin network with thick, major fibers as well as thin, minor fibers. Label L = thick, major fibers; Label M = thin, minor fibers

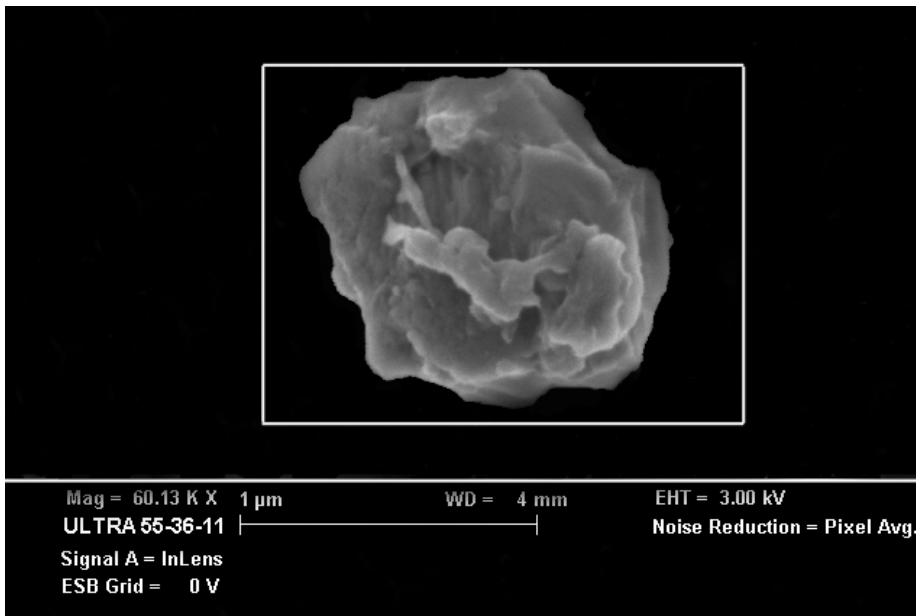


Figure 5.5b: Platelet aggregate from asthmatic Balb/c mice treated with selenium, forming tight aggregates with pseudopodia. Aggregate in block.

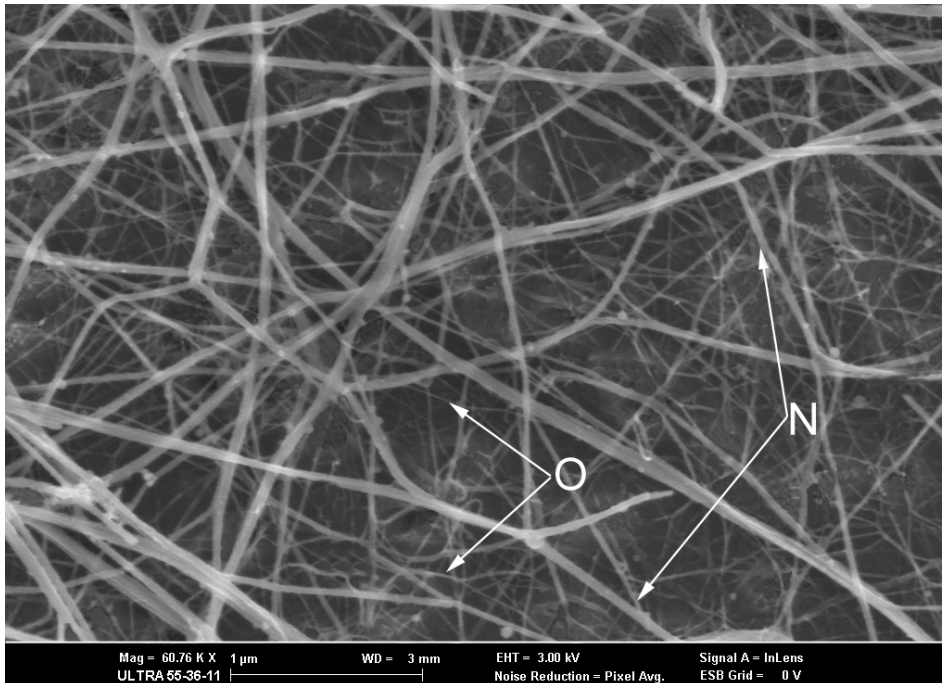


Figure 5.6a: Fibrin network from asthmatic Balb/c mice treated with *Withania somnifera* and selenium, showing fibrin network with thick, major fibers as well as thin, minor fibers. Label N = thick, major fibers; Label O = thin, minor fibers

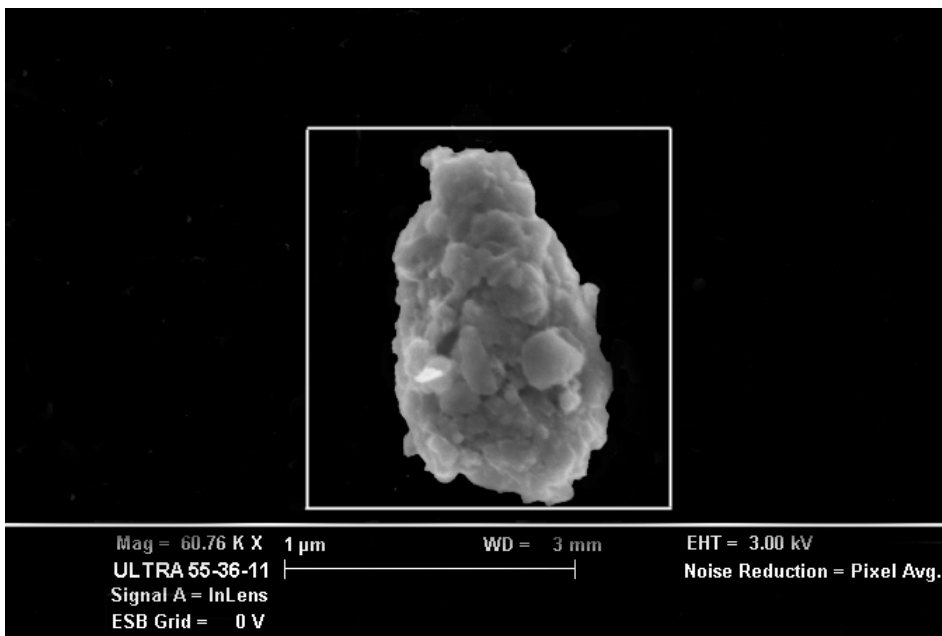


Figure 5.6b: Platelet aggregate from asthmatic Balb/c mice treated with *Withania somnifera* and selenium, forming tight aggregates with pseudopodia. Aggregate in block.

The results obtained in chapter 4 gave rise to the research objective investigated in the current chapter. Seeing that the mice were indeed asthmatic, the question arose whether there will be an effect on platelet morphology and the fibrin networks in the different treatment groups.

Focusing on eosinophil counts, Graph 5.1 shows that bronchial lavage in the asthmatic animals had a significant increase in the number of eosinophils [Oberholzer *et al.* (submitted for publication)]. Also, bronchial lavage smears of the asthmatic group showed numerous platelet clumps that were distributed between white blood cells. Platelets were also found in the other treatment groups but are not as prevalent as in the asthmatic group. Results from the blood smears showed the same trends, where cell counts in control and hydrocortisone blood smears were decreased compared to that of the asthma group. It was concluded that WS and WS combined with selenium significantly decreased the white blood cells in both bronchial lavage as well as blood smears, suggesting that WS indeed has an anti-inflammatory potential and it, in combination with an anti-oxidant like selenium might successfully be used in the treatment of asthma.

Previous research by Pretorius *et al.* (2007) showed control mice possesses major, thick fibers and minor thin fibers as well as tight round platelet aggregates with typical pseudopodia formation. Minor fibers of asthmatic mice have a netlike appearance covering the major fibers, while the platelets seem to form loosely connected, granular aggregates. Hydrocortisone, however, made the fibrin more fragile and platelet morphology changed from a tight platelet aggregate to a more granular aggregate not closely fused to each other. It was concluded that hydrocortisone impacted on the fragility of the fibrin and that it prevents the minor fibers to form the dense netlike layer over the major fibers, as is seen in untreated asthmatic mice.

However, the question that is addressed in the current chapter is whether WS and selenium separately and in combination is able to stabilize the fibrin networks and platelet aggregates in asthmatic mice. Figure 5.1a and b show control fibrin networks and platelets, Figure 5.2a and b show an asthmatic fibrin networks and platelets, Figure 5.3a and b show the fibrin network and platelet aggregate of asthmatic mice treated with

hydrocortisone, Figure 5.4a and b, 5.5a and b and 5.6a and b show asthmatic mice treated with WS, selenium and a combination.

Control fibrin networks have major, thick and minor thin fibers (Figure 5.1a; label A and B), while platelet aggregates form tight masses with pseudopodia protruding from the platelet aggregate (Figure 5.1b; label C) (Pretorius *et al.*, 2007).

Fibrin networks from asthmatic animals show thick, major fibers (Figure 5.2a; label D) and a thick, matted layer of thin, minor fibers (Figure 5.2a; label E). Platelets do not aggregate in a tight mass (Figure 5.2b; label F) (Pretorius *et al.*, 2007).

Fibrin networks from hydrocortisone treated animals showed a flimsy fiber network easily prone to breakages and both major and minor fibers are present (Pretorius *et al.*, 2007) (Figure 5.3a; labels G and H). The minor fibers are thinner than the normal minor fibers and even breakages could be observed in some places. Platelet aggregates also did not have a tight round appearance and was visible as granular, loosely arranged clumps and not attached closely to each other as seen in the controls (Figure 5.3b; label I).

Asthmatic mice treated with WS had a similar appearance as that of the control mice, with major, thick fibers and few thin, minor fibers. Therefore, WS stabilized the fibrin networks in the asthmatic mice (Figure 5.4a; label J and K). Platelets also formed a tighter aggregate, similar to that of the controls. Quite a lot of plasma proteins can be observed on the minor fibers in Figure 5.4a with small coagulants dispersed here and there.

The fibrin network of asthmatic mice treated with selenium appear similar to that of the asthmatic group, whereas the platelets look different from that of the asthmatic group and similar to the platelets found in the control group where they formed tight aggregates. (Figure 5.5a and 5.5b).

Fibrin networks and platelets of asthmatic animals treated with WS in combination with selenium showed the same ultrastructure as that of the WS treated group (Figure 5.6a and b), where the thin fibers of the fibrin networks appeared the same as that of the controls and the platelet aggregates were arranged in a tight aggregate.

Although selenium seems to play an important role in stabilizing asthma and it is known that asthma patients have decreased selenium levels, it is known that taking selenium supplementation on its own will not decrease the symptoms of asthma. Results from this study showed that the fibrin networks appeared similar to that of the asthmatic mice. However, platelet morphology of animals treated with selenium did indeed differ in ultrastructure from that of asthmatic animals (Figure 5.5a and 5.5b). The platelets once again resumed the tight aggregate as found in the controls. Therefore, selenium promoted platelet stickiness. WS treated plants also showed this tight aggregate, and it is known that the plant has an anti-oxidant as well as anti-inflammatory potential. This therefore suggests that the anti-oxidant function might be the reason why platelets of asthmatic mice treated with selenium and WS and also the plant *Euphorbia hirta* (also a potent anti-oxidant as well as anti-inflammatory phytomedicine) regain their tightly bound platelet structure. (Pretorius *et al.*, 2007). Where in hydrocortisone-exposed asthmatic animals, the platelets still had the loosely arranged aggregate, as was found in the asthmatic animals. Furthermore, it is well known that hydrocortisone does not have an anti-oxidant potential.

From previous bronchial lavage and blood cell counts [Oberholzer *et al* (Submitted for publication)] (Graph 5.1), it was seen that WS on its own and in combination with selenium does have an anti-inflammatory potential. The current research shows that the phytomedicine, alone and in combination with selenium, does have a stabilizing effect on fibrin networks and platelet aggregate structure. Selenium alone does not impact on the fibrin networks but platelet aggregates were stabilized.

5.4 CONCLUSION

It is therefore concluded that the anti-inflammatory potential of WS and its possible uses in the treatment of asthma, should be investigated further. Furthermore, it is suggested that the effect of WS on the fibrin networks is because of the anti-inflammatory potential, and the stabilizing effect on the platelets because of the anti-oxidant potential. Therefore it was seen that selenium has a stabilizing effect on the platelets but not on the fibrin networks. It is known that selenium is a scavenger of free radicals, and

previous research has shown that asthmatic patients have decreased selenium levels (Gazdik *et al.*, 2004). Its specific role in the physiology of asthma, however, should be investigated further.

Chapter 6: Ultrastructural analysis of platelets by Transmission Electron Microscopy using the Balb/c asthmatic mouse model

6.1 INTRODUCTION

The coagulation process as well as the ultrastructure of cellular components involved in haemostasis like platelets and fibrin networks might be studied successfully using the murine experimental animal model. By comparing the effects of hydrocortisone and Phytomedicine on the ultrastructure of platelets and the formation of fibrin networks, using this model, might shed light on what effects these products have on the coagulation system.

Platelets are the smallest blood cells and they are able to activate and cooperate with leukocytes and endothelial cells by biologically active substances released from their granules (Badzenta-kukaszuk *et al.*, 2002). Platelets are formed from the remains of larger cells in the bone marrow, called megakaryocytes (Larson and Watson, 2006). They contain RNA, mitochondria, a canalicular system, and several different types of granules; lysosomes (containing acid hydrolases), dense bodies (containing ADP, ATP serotonin and calcium) and alpha granules (containing fibrinogen, factor V, vitronectin, thrombospondin and von Willebrand factor), the contents of which are released upon activation of the platelet, and upon entering the blood stream, they act similar to the leukocytes in their ability to produce their own movement. There are approximately 250 000 to 450 000 platelets present in one cubic millimeter of blood and they survive in the blood stream for 5 to 9 days. (www.medicallook.com).

According to Italiano *et al.* in 1999, terminally differentiated megakaryocytes acquire a significantly polyploid DNA content and enlarge to 50 to 100 μm in diameter before releasing their platelet load. When in the release phase, the cytoplasm of the megakaryocyte converts into long branched protrusions, and disc-shaped platelets are assembled de novo within these proplatelet extensions (Italiano *et al.*, 1999). The core of each proplatelet consists of microtubule bundles and their distal tips organize into the

repetitively coiled structure of the platelet marginal band (Italiano *et al.*, 1999; Kenney and Linck, 1985; Schwer *et al.*, 2001).

Platelets play an important role in inflammation in particular vascular inflammation by the release and expression of regulatory molecules. As early as 1989, Page described platelets as the repository of a host of physiologically active substances classified as spasmogens (5-hydroxytryptamine, platelet activating factor, (PAF) thromboxane A₂, cationic proteins), chemotactic factors (platelet factor 4, growth factors, lipoxigenase products) and proteolytic enzymes.

Other regulatory molecules have also been revealed; platelet derived growth factor, the selectin family and platelet histamine (Page, 1989).

Many aspects of the clinical and pathological features seen in asthmatic airways can be induced by PAF such as airway oedema, the accumulation of eosinophils in the airway wall, and bronchial hyper-responsiveness. PAF is a potent chemotactic agent and activator of eosinophils, which are prominent cells in asthmatic airways, through the activation of specific surface receptors. The interaction between PAF and eosinophils may play an important role in the pathogenesis of bronchial hyper-responsiveness in asthma (Chung and Barnes, 1991).

Other normal features of platelet anatomy and physiology are the close contacts that exist between platelet membranes and membrane systems (White, 1987). The association between platelets and damaged vascular surfaces play an essential role in haemostatic plug formation (Sixma and Wester, 1977; Sixma *et al.*, 1995; White, 1987). The activation of platelets result in the communication of alpha granules with membrane lining channels of the open canalicular system (OCS) and this is followed by the secretion of contents into the external environment (Escobar *et al.*, 1989; White and Krumwiede, 1987). The membranes of the OCS and the dense tubular system (DTS) are often in association in membrane complexes and they resemble the sarcoplasmic reticulum of embryonic muscle cells (White, 1972; Ezerman and Ishikawa, 1967). As early as 1968, Behnke did an electron microscope study on the megakaryocyte and revealed that the OCS appears to originate during a membrane sequestration process

where the cytoplasm of the parent megakaryocyte gets divided into small units, which become circulating platelets (Behnke, 1968).

In 1978, Fedarko revealed that each channel in the OCS represent a single tubular invagination of the platelet surface membrane that follows deep into the cytoplasm (Fedarko, 1978). Studies over many years have shown that the channels of the OCS serve as conduits for the uptake and transfer of particles to platelet storage organelles and act as egress routes for products secreted during the platelet release reaction (White, 1970; White, 1968; White and Estensen, 1972; White, 1972; White, 1973).

Investigations by Milton and Frojmovic (1979) have suggested that channels of the OCS represent a reservoir of sequestered membrane that can be evaginated following platelet activation and shape change.

In the current chapter the effects of WS, selenium, WS and selenium in combination and hydrocortisone on the ultrastructure of platelets will be discussed. The platelets and granules of the different treatment groups will be compared to that of the control group as well as with the diagrammatic representation of a typical platelet.

6.2 MATERIALS AND METHODS

6.2.1 Preparing the water extract of *Withania somnifera*

Plant material was collected in the Pretoria region of 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 1 week. The material was grounded into a fine powder and subdivided into four 50 g samples. The samples were subsequently extracted with 500ml of water, after which it was filtered and dried on a rotary evaporator at 40°C. The water extract was freeze-dried. This procedure was repeated three times for each sample. A stock solution of 330 mg/ml was prepared that was administered orally to the mice. This implied that each animal (average weight 20g) received 45µl of plant material. This dose was decided upon after

studying literature that mentioned physiological doses suggested by herbalists. Rasool and Varalakshmi (2006) did an animal study where they used WS root powder at a dosage of 500 and 1000mg/kg of body weight. In the current study this dosage was used and converted back to the average weight of a Balb/c mouse (Rasool and Varalakshmi, 2006)

6.2.2 Implementing the Balb/c asthma model

36 Six-week-old (female) Balb/c mice each of average weight 20g maintained in the University of Pretoria Biomedical Research Centre (UPBRC) and provided OVA-free food and water *ad libitum*, were used. All experimental protocols complied with the requirements of the University of Pretoria's Animal Use and Care Committee.

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- Mice exposed to combination *W. somnifera* and selenium (45 µl + 100µl)
- Mice exposed to low dose hydrocortisone (100mg/kg)

Sensitization (on day 0 and day 5) of mice was via intraperitoneal injection of 25mg OVA (grade V; Sigma-Aldrich) and 2mg Al(OH)₃ that was dissolved in 0.5ml of 0.9% saline solution. All mice except the control mice (which were left untreated) were sensitized.

Nebulisation with 1% OVA in PBS (1mg OVA in 100 ml PBS) was performed twice daily, for 1 hour on days 13, 14 and 15. WS, selenium, the combination of WS and selenium as well as hydrocortisone was administered on day 15 to day 18, again on days 21, 22, 25 and 28. Animals were again nebulised on days 34, 35 and 36 and treated daily from day 39 to day 42 and the animals were terminated on day 43.

6.2.3 Preparation of fibrin clots

On day 45 during termination, 100 to 500 μ l of blood was drawn from each of the Balb/c mice in each of the groups and 11 μ l citrate for every 100 μ l of blood, was added. Blood was centrifuged at 1000 rpm for 2 minutes to obtain platelet rich plasma (PRP). Mouse thrombin was used to prepare the clots. Mouse alpha thrombin was obtained from Heamatologic Technologies Incorporated (USA). The original mouse thrombin concentration was 3.9 g/l.

2,5 μ l of mouse PRP was mixed with 7 μ l of 100nm/l mouse thrombin (final concentration of thrombin after mixing with PRP = 75 nm/l) and this produced platelet aggregates. Also, 5 μ l of mouse PRP was mixed with 10 μ l of mouse thrombin (5 nm/l) mouse thrombin produced platelet aggregates (final mouse thrombin concentration = 3nm/l).

The human thrombin is 20 U/ml and was 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, platelet derived growth factor and fibroblastic growth factor are released into the coagulum.

The PRP and thrombin mix was immediately transferred with a pipette tip to a 0.2 μ m 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 and placed at 37°C for 10 minutes. A washing process where the Millipore membranes with the coagula are placed in PBS and magnetically stirred for 120 minutes follow this. This was done to remove any blood proteins trapped within the fibrin network (Pretorius *et al.*, 2006).

6.2.4 Preparation of the washed fibrin clot for TEM

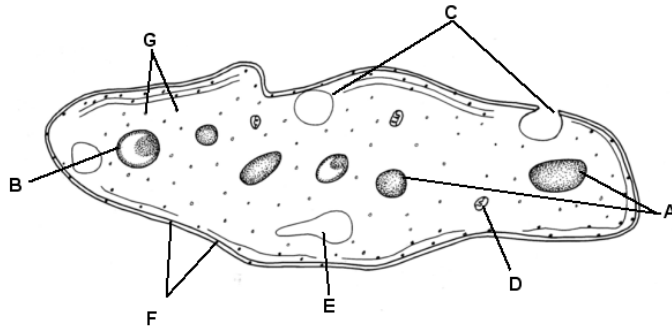
The Millipore membranes with the PRP and thrombin mix were fixed in 2.5% glutaraldehyde for 1 hour, rinsed three times in 0.075M sodium potassium phosphate buffer (pH=7.4) for 15 minutes before being placed in secondary fixative, 1% osmium

tetraoxide solution for 1 hour. Following fixation, the tissues were rinsed again as described above. The tissues were then dehydrated in 30%, 50%, 70%, 90% and three changes of 100% ethanol.

The samples were then embedded in resin and ultra-thin sections (80-100nm), cut with a diamond knife using an ultramicrotome, were contrasted with uranyl acetate for 15 minutes followed by 10 minutes of contrasting with lead citrate, after which samples were allowed to dry for a few minutes before examination with the Philips EM 301 transmission electron microscope. Photomicrographs revealing the morphology of the platelet cell membrane and cell organelles were taken.

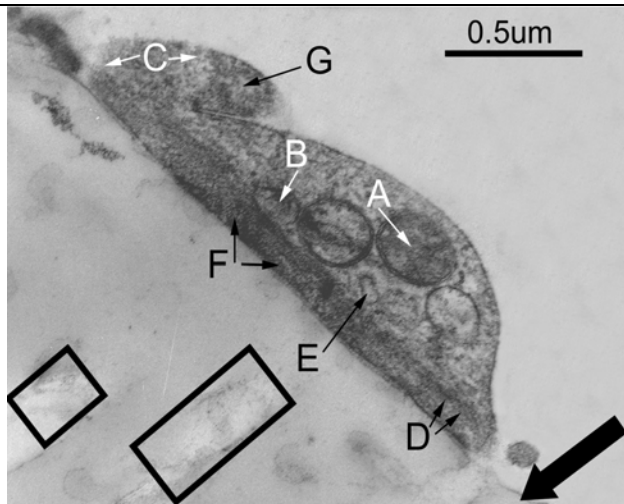
6.3: RESULTS

A: Diagrammatic representation of a platelet and its granules



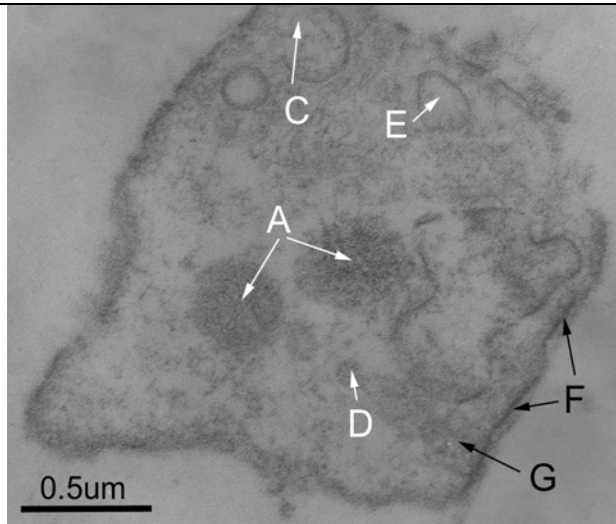
- A-** Alpha granules
- B-** Dense bodies
- C-** OCS
- D-** Mitochondria
- E-** Dilated channels
- F-** Microtubules
- G-** Glycogen

B: Control platelet



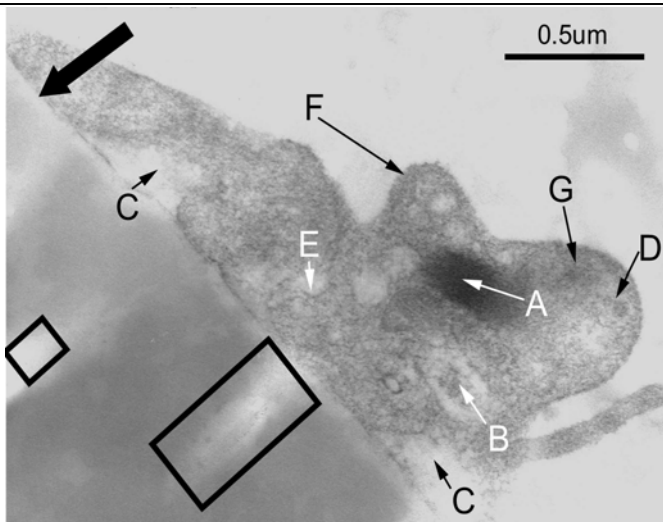
- A-** Alpha granules
- B-** Dense bodies
- C-** OCS
- D-** Mitochondria
- E-** Dilated channels
- F-** Microtubules
- G-** Glycogen

C: Platelet of asthmatic mice



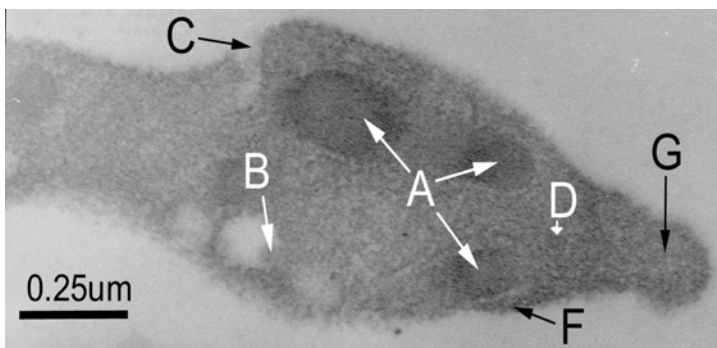
- A-** Alpha granules
- B-** Dense bodies
- C-** OCS
- D-** Mitochondria
- E-** Dilated channels
- F-** Microtubules
- G-** Glycogen

D: Platelet of mice treated with WS



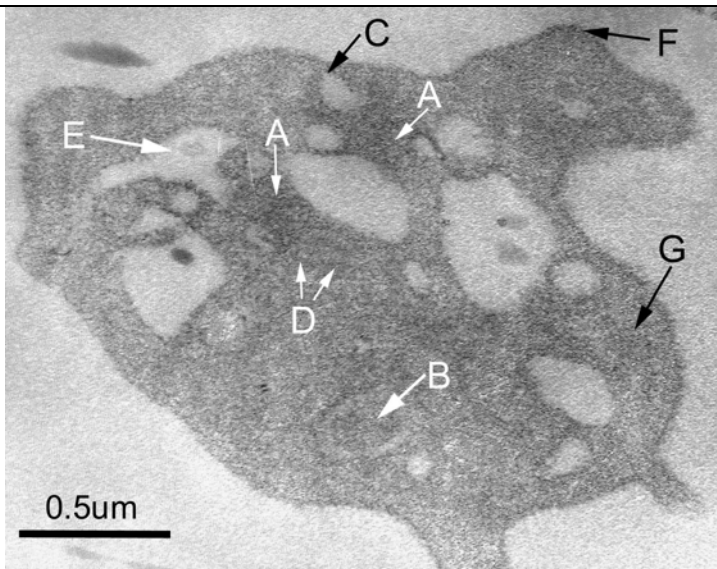
- A-** Alpha granules
- B-** Dense bodies
- C-** OCS
- D-** Mitochondria
- E-** Dilated channels
- F-** Microtubules
- G-** Glycogen

E: Platelet of mice treated with Selenium



- A-** Alpha granules
- B-** Dense bodies
- C-** OCS
- D-** Mitochondria
- E-** Dilated channels
- F-** Microtubules
- G-** Glycogen

F: Platelet of mice treated with WS and Selenium



- A-** Alpha granules
- B-** Dense bodies
- C-** OCS
- D-** Mitochondria
- E-** Dilated channels
- F-** Microtubules
- G-** Glycogen

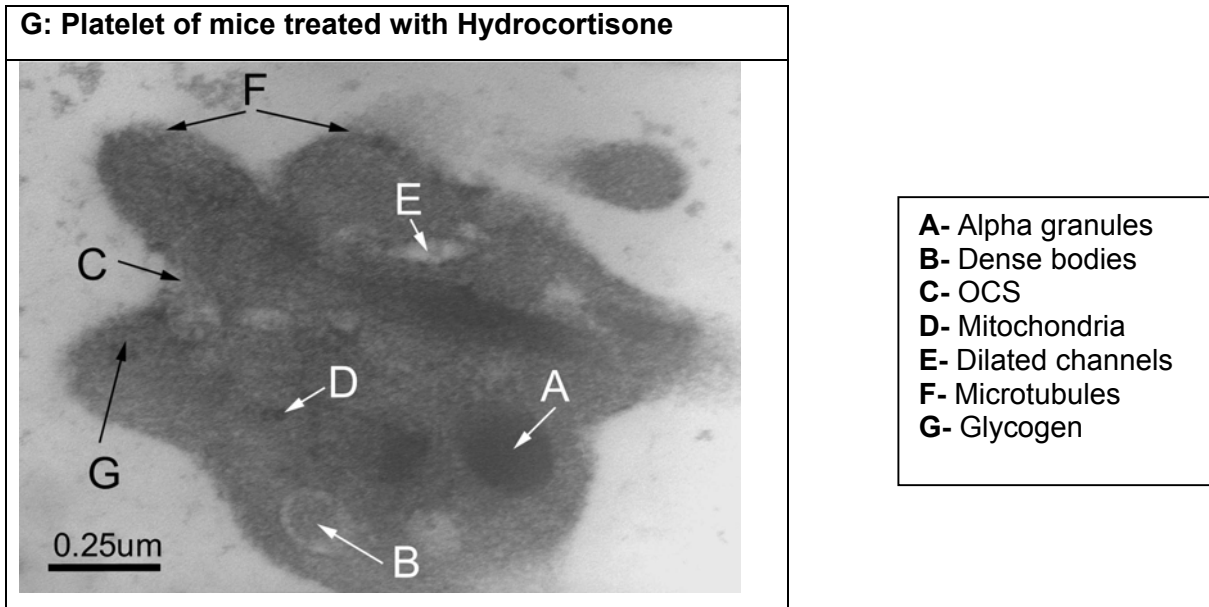


Figure 6.1: Transmission electron micrographs of platelets.

A= Diagrammatic representation, **B=** Control, **C=** Asthma, **D=** WS, **E=** Selenium, **F=** Combination, **G=** Hydrocortisone

6.4 DISCUSSION

Figure 6.1 A is a diagrammatic representation of a typical platelet including its contents. The **α granules** (A) are 300-500nm in size. They are located in the granulomere (the central darker region of the platelet). α Granules contain fibrinogen, platelet-derived growth factor, platelet thromboplastin, thrombospondin and coagulation factors which facilitate vessel repair, platelet aggregation and coagulation of blood (Gartner and Hiatt, 2007). Alpha granules possess the metabolic or cytoplasmic pool for reactions, including fibrinogen, thrombospondin, von Willebrand factor, beta-thromboglobuline and factor 4 and 5. On activation, platelets will release the contents of these alpha granules.

The **dense bodies** (δ granules) (B) are 250-300nm in size and are located in the granulomere. Dense bodies contain calcium, ADP, ATP, serotonin, histamine and pyrophosphatase. These factors facilitate platelet aggregation and adhesion as well as vasoconstriction.

Also hosted in the granulomere is a system of enzymes that enables the platelet to catabolise **glycogen** (G), consumes oxygen and generates ATP and is therefore the energy source of platelets.

The **open canalicular system** (OCS) (C) is formed via the invagination of the platelet plasma membrane and is a system of cytoplasmic channels. (Kierszenbaum, 2007)

This surface opening system communicates with the outside and is a continuation of the outer surface of the platelet, increasing the platelet surface area. (Gartner and Hiatt, 2007). The channels of the OCS are sometimes transformed into **dilated channels** (E).

The **mitochondria** (D) are located in the granulomere together with the rough endoplasmic reticulum, Golgi apparatus and granules where they perform their normal cellular function.

The electron micrographs of platelets display **microtubules** (F) that are arranged parallel to each other forming a ring within the hyalomere (the peripheral clear region of the platelet.) The microtubules are responsible for the discoid shape of the platelet. Actin and myosin monomers are also associated with these bundles of microtubules and can rapidly assemble to form a contractile apparatus (Gartner and Hiatt, 2007).

Platelets also contain the following membrane receptors: (Ramasamy, 2004)

- GPIIb/IIIa receptor (structure: β_1 integrins, $\alpha_2\beta_1$) - collagen receptor
- GPIIb/IIIa receptor (structure: $\alpha_{11b}\beta_3$) – receptor for fibrinogen, von Willebrand factor, fibronectin, vitronectin and thrombospondin
- GPIb-IX-V receptor for insoluble von Willebrand factor and mediates initial deposition of platelets in subendothelium
- GPVI nonintegrin receptor – receptor for collagen
- $\alpha_5\beta_1$ - fibronectin receptor
- $\alpha_6\beta_1$ - laminin receptor

When thrombin, ADP and collagen related peptides stimulate platelets, GPIIb/IIIa is activated to form a high affinity soluble collagen (Ramasamy, 2004). GPVI are also involved as a collagen receptor. Also, activation of the platelets causes changes in the

GPIIb/IIIa membrane receptor allowing the binding of integrin type proteins. This receptor is expressed on non-stimulated platelets and is a major adhesion receptor, and is one of the most abundant surface receptors approximately 80 000 per platelet (Ramasamy, 2004). It is also present in a low activation resting state. It also supports platelet to platelet interaction and aggregation by binding fibrinogen (Nachman and Leung, 1982) and aggregation is also facilitated by von Willebrand interaction with this receptor.

Also, at high shear rates, GPIb α -subunit-von Willebrand factor interaction may promote inter-platelet interaction regardless on activation. This subunit also has a binding site for thrombin and the domain overlaps with that of the von Willebrand binding domain. GPIIb/IIIa interacts with at least three different adhesive ligands, namely fibrinogen, fibronectin, and von Willebrand factor (Andrieux *et al.*, 1989). These interactions are critical for platelet adhesion, spreading, and aggregation. Collagen also plays an important role in aggregation as it enhances the ability of platelets to bind the fibronectin. Fibronectin is a large glycoprotein in the extracellular matrix and provide scaffolding for platelet adhesion and aggregation and other repair reactions.

Figure 6.1 B is an electron micrograph of a platelet from the control group. α Granules and dense bodies are present in the granulomere. The OCS and dilated channels seems to be smaller compared to that of the asthmatic group, which seems to be releasing more particles. When platelets are activated a communication occur between the α granules and the OCS and more particles are released into the environment because the channels of the OCS serve as exit routes for products to be secreted during the platelet release (Figure 6.1 C label C and E). Also, the α granules of the platelet from the asthmatic group is not as well defined comparing to the control group. The α granules facilitate platelet aggregation. This may possibly contribute to the increased formation of platelet clumps that are formed in asthmatic animals (Figure 6.1 B and C label A). The α granules, dense bodies and OCS of the hydrocortisone group seem to be similar to that of the control group which may imply that hydrocortisone had no effect on these specific platelet structures (Figure 6.1 B and G labels A, B and C).

Glycogen is present in the granulomere of the control platelet whereas less glycogen is seen in that of the asthmatic group (Figure 6.1 B and C label G). Various mitochondria

can be seen in the granulomere of both the control and asthmatic groups (Figure 6.1 B and C label D). Microtubules are seen in the control platelet, contributing to the discoid shape of the platelet (Figure 6.1 B label F). Microtubules are also present in the platelet from the asthmatic group (Figure 6.1 C label F) but the plasma membrane of the platelet seems to be disrupted at some places. The big black arrow in Figure 6.1 B indicates the Millipore membrane that was used to prepare the fibrin clot on and the rectangles indicate the pores of the Millipore membrane.

The platelets of the mice treated with the Phytomedicine WS, the antioxidant selenium and WS and selenium in combination appear to be similar to that of the control group which implies that neither the plant, nor selenium had an effect on the platelet contents (Figure 6.1 D, E and F). The platelet plasma membranes are intact in all of these three groups, which is the result of the parallel arrangement of the microtubules in the hyalomere of the platelet (Figure 6.1 D, E, F label F).

Selenium plays an important role in asthma and asthma patients have decreased selenium levels. Selenium is a potent antioxidant and *W. somnifera* also have antioxidant activities, which indicate that it is the antioxidant properties of these two treatments that protect the platelet plasma membrane. In Figure 6.1 D the big black arrow shows the Millipore membrane and the black rectangles indicate the pores of the Millipore membrane. α Granules, dense bodies, glycogen, mitochondria, OCS and dilated channels are present and look similar to those of the control group.

	α Granules	Dense Bodies	OCS	Mitochondria	Dilated Channel	Micro- tubules	Glycogen
CONTROL	✓	✓	✓	✓	✓	✓	✓
ASTHMA	✓		✓	✓	✓	✓	✓
WS	✓	✓	✓	✓	✓	✓	✓
SELENIUM	✓	✓	✓	✓		✓	✓
COMBINATION	✓	✓	✓	✓	✓	✓	✓
CORTISONE	✓	✓	✓	✓	✓	✓	✓

Table 6.1: Indication of structures and organelles present in the platelet electron micrographs of the different treatment groups

In Table 6.1 the presence and absence of some of the structures and organelles within the platelets can be seen. Everything is present in the control group as well as in the WS, combination and cortisone groups. In the asthma group no dense bodies could be identified and in the selenium group no dilated channels were seen. An explanation for the absence of dense bodies in the asthmatic group and dilated channels in the selenium-treated group could simply be that the sample investigated was sectioned in such a way that these structures could not be identified.

Alpha granules were present in the asthmatic group although not as well defined as in the control group. The alpha granule seen in the hydrocortisone group looks similar to that of the asthma group.

The alpha granules release coagulation factors that facilitate platelet aggregation. Previous studies done on the ultrastructure of platelets from asthmatic mice showed that asthma affects the initial stages of platelet aggregation, keeping the platelets discoid and impacts on the “stickiness” of the platelets. Integrin proteins like fibrinogen and Thrombospondin-1 are up regulated in the plasma of asthmatics [Pretorius *et al.*, (submitted for publication)].

In previous research Mantzoros *et al.*, (2004) revealed that corticosteroids inhibit collagen synthesis both *in vivo* and *in vitro* by interfering with normal inflammatory phase, stabilising membranes of the intracellular lysosomes of the white blood cells and reducing inflammatory cell infiltration and blood vessel and fibroblast ingrowth. All these actions are mediated by a cytokine transforming growth factor beta (TGF- β 1). TGF- β 1 is a polypeptide member of the transforming growth factor beta super family of cytokines. TGF- β 1 is stored in large amounts as a latent peptide in the secretory α -granules of circulating platelets and is one of the molecules released during platelet activation. It has been hypothesized that steroids block the action of this cytokine at a molecular level by intercepting the expression of the TGF- β 1 gene (Mantzoros *et al.*, 2004).

When asthmatic mice are treated with hydrocortisone, hydrocortisone causes a down-regulation of integrin proteins. This results in sufficient treatment of the negative effects of abundance of these circulating proteins, but without influencing the inability of the platelets to form proper aggregates and pseudopodia as can be seen in the previous chapter as well [Pretorius *et al.*, (submitted for publication)]. In a previous study it was hypothesized that asthma influences platelet membrane receptors, therefore binding proteins released in plasma do not have a binding site. Therefore analysis of plasma levels will show increases in these proteins. Hydrocortisone treatment reduces the levels of these proteins, but without changing influencing the receptors damaged due to asthma [Pretorius *et al.*, (submitted for publication)].

Open canicular systems can be seen in all of the groups. In the asthmatic and hydrocortisone groups the OCS seems to be enlarged, compared to that of the control and other treatment groups as mentioned earlier. Although it cannot be confirmed, the enlarged OCS may possibly be due to an apoptotic process because apoptosis is a process where cells swell whereas necrosis is cell shrinkage. Performing tests to

determine whether the cell had undergone apoptosis can only prove this, but from a morphological point of view, it may possibly be apoptosis.

6.5 CONCLUSION

Therefore it can be concluded that asthma do have an influence on platelets and its granules compared to control platelets, platelets treated with WS, selenium, WS and selenium in combination as well as hydrocortisone when evaluated on ultrastructural level.

Chapter 7: Concluding discussion

Withania somnifera (WS) is reported as a potent antioxidant by different researchers over many years (Jesberger and Richardson, 1991; Scarfiotti *et al.*, 1997; Halliwell and Gutteridge, 1989). It has also been reported to exhibit anti-inflammatory properties similar or even greater than that of anti-inflammatory drugs such as hydrocortisone (Prakash *et al.*, 2002; Begum and Sadique, 1988).

Although many studies have been done over many decades to investigate the anti-inflammatory and antioxidant properties of this phytomedicine, no research has been done previously using an *in vivo* murine model to determine the effect of the plant on asthma. Little information is also available of the *in vivo* effect of the antioxidant selenium on asthma. To investigate this anti-inflammatory effect of WS on asthma, it was necessary to establish an asthmatic Balb/c mouse model. This was done by sensitization of the animals with OVA and Al(OH)₃ followed by a series of nebulization with OVA and treatment of the animals with WS, selenium, a combination of WS and selenium and hydrocortisone over a period of 43 days. The asthmatic status of the animals was confirmed by eosinophil counts in the bronchial lavage of the animals. The effect of WS, the antioxidant selenium, WS and selenium in combination on the number of white blood cells in blood smears and in the bronchial lavage as well as platelet distribution were studied with this model and hydrocortisone was used as positive control.

Research showed that typically in bronchial lavage of control mice, up to 90% of cells are macrophages, (late stage monocytes). Larsen and co-workers (2007) used the Balb/c model and reported that with inflammation the total number of white blood cells increased, but especially the distribution of cells is changed, with an increase in eosinophils, neutrophils and sometimes lymphocytes - cells that is very rarely seen in bronchial lavage from healthy mice (Larsen *et al.*, 2007). Light microscopy analysis of the bronchial lavage revealed a significant decrease in the number of eosinophils counted in the asthmatic and the different treatment groups. Lymphocytes are the second most abundant cells in the human body (Kierszenbaum, 2007) and this explains the increase in lymphocyte counts as seen in Figure 5.1. In this study it was found that in

control mice, most of the cells were macrophages. The distribution of cells in the asthmatic group changed where there were an increase in the number of all white blood cells and especially a significant increase in the number of eosinophils. Statistical analysis confirmed a significant increase in the number of eosinophils between the asthmatic group and the control group as well as between the asthmatic group and each of the other treatment group.

Platelet distribution in the asthmatic group differed greatly from the other treatment groups. Numerous platelet clumps were found distributed between white blood cells in the asthmatic group while in the control group, platelets were present, but not as prevalent and did not form the clumps present in the asthmatic group.

It is known that hydrocortisone decreases white blood cells in bronchial lavage in humans. In the hydrocortisone treated group there were also a significant decrease in white blood cells in the bronchial lavage, and platelet clumps were also not present as was found in the asthmatic group.

Selenium decreased the number of lymphocytes, neutrophils, and basophils and significantly decreased the number of eosinophils. It was also found that the platelet distribution did not differ as much between the selenium and the asthmatic group as between the asthmatic group and the other treatment groups.

WS also decreased the number of eosinophils significantly in the bronchial lavage and the effect of selenium and WS revealed more or less the same distribution of white blood cells and a significant decrease in the number of eosinophils compared to the asthmatic group. Platelet clumping was also not as prevalent in the distributions WS, selenium and the combination.

Results obtained from the blood smears showed the same trends, where cell counts in control and hydrocortisone blood smears were decreased compared to that of the asthma group. WS and the combination WS and selenium also showed decreased cell numbers, whereas selenium alone did not significantly decrease cell numbers in blood.

From these results it was concluded that WS indeed has an anti-inflammatory potential and it, in combination with an anti-oxidant like selenium might successfully be used in the treatment of asthma.

Platelets and fibrin also play an important physiological role in allergic processes and immunological mechanisms like allergic asthma where platelets participate by acting as inflammatory cells, by releasing mediators, spasmogens and/or by interacting with other inflammatory cell types. The next question that arose was whether *Withania somnifera* and selenium separately and in combination is able to stabilize the fibrin network and platelet aggregates in asthmatic mice since it was reported by Pretorius *et al.*, (2007) that control mice possess major, thick fibers and minor thin fibers as well as tight round platelet aggregates with typical pseudopodia formation. They revealed that the minor fibers of asthmatic mice have a netlike appearance covering the major fibers, while the platelets seem to form loosely connected, granular aggregates. Hydrocortisone, however, made the fibrin more fragile and platelet morphology changed from a tight platelet aggregate to a more granular aggregate not closely fused to each other. They concluded that hydrocortisone impacted on the fragility of the fibrin and that it prevents the minor fibers to form the dense netlike layer over the major fibers, as is seen in untreated asthmatic mice.

The results obtained from this study showed the same trend. Fibrin networks from the control group had major, thick and minor thin fibers and the platelet aggregates form tight masses with pseudopodia protruding from the platelet aggregate. Fibrin networks from asthmatic animals show thick, major fibers (Figure 5.2a; label D) and a thick, matted layer of thin, minor fibers and the platelets do not aggregate in a tight mass.

In the hydrocortisone treated group the fibrin networks appeared flimsy and easily prone to breakages and both major and minor fibers are present. Platelet aggregates also did not have a tight round appearance and was visible as granular, loosely arranged clumps and not attached closely to each other as seen in the controls. WS was found to stabilize the fibrin networks in the asthmatic mice and the platelets formed a tighter aggregate. Platelets also formed a tighter aggregate, similar to that of the controls.

The fibrin networks of the selenium-treated group appeared similar to that of the asthmatic mice but the platelet morphology was different from that of asthmatic animals. The platelets once again resumed the tight aggregate as found in the controls. From this it can be concluded that selenium promoted platelet stickiness and it can therefore be suggested that the anti-oxidant function of WS might be the reason why platelets of asthmatic mice treated with selenium and WS and also the plant *Euphorbia hirta* (also a potent anti-oxidant as well as anti-inflammatory phytomedicine) regain their tightly bound platelet structure (Pretorius *et al.*, 2007). Where in hydrocortisone-exposed asthmatic animals, the platelets still had the loosely arranged aggregate, as was found in the asthmatic animals. Furthermore, it is well known that hydrocortisone does not have an anti-oxidant potential.

Fibrin networks and platelets of asthmatic animals treated with the combination showed the same ultrastructure as that of the WS treated group where the thin fibers of the fibrin networks appeared the same as that of the controls and the platelet aggregates were arranged in a tight aggregate.

In this part of the study it was concluded that the phytomedicine, alone and in combination with selenium, does have a stabilizing effect on fibrin networks and platelet aggregate structure. Selenium alone stabilized the platelet aggregates but does not impact on the fibrin networks. Furthermore, it can be concluded that the effect of WS on the fibrin networks is because of the anti-inflammatory potential, and the stabilizing effect on the platelets because of the anti-oxidant potential.

Transmission electron microscopy was used to further investigate the effect of this phytomedicine on the ultrastructure of platelets and its granules.

In the platelet from the asthmatic group, all the granules are present except for dilated channels that could not be identified. The alpha granules of the asthmatic group platelet appeared to be not as well defined as those present in the control group. The OCS of the asthmatic group seemed bigger compared to the control group. The α granules, dense bodies and OCS of the hydrocortisone group seem to be similar to that of the control group. Glycogen is present in the granulomere of the control platelet as well as in the WS, selenium, combination and hydrocortisone groups whereas less glycogen is seen in that of the asthmatic group. Microtubules are also present in the platelet from the

asthmatic group but the plasma membrane of the platelet seems to be disrupted at some places.

The platelets of the mice treated with WS, the antioxidant selenium and WS and selenium in combination appear to be similar to that of the control group. From this results it is suggested that asthma do have an influence on platelets and its granules compared to control platelets, platelets treated with WS, selenium, WS and selenium in combination as well as hydrocortisone when evaluated on ultrastructural level.

After evaluating all the results it is concluded that WS and WS combined with selenium significantly decreased the white blood cells in both bronchial lavage as well as blood smears, suggesting that WS indeed has an anti-inflammatory potential and it, in combination with an anti-oxidant like selenium might successfully be used in the treatment of asthma.

Also, anti-inflammatory products such as WS impacts on the fibrin network but not on the platelet aggregate stability (seen with hydrocortisone). Anti-oxidants like selenium do not impact on the stability of the fibrin networks in asthma, but impacts on platelet stability.

Herbal products like the Phytomedicine investigated in this study, which are potent antioxidants as well as anti-inflammatory products impact on the stability of both the fibrin networks and the platelet aggregates. These results therefore suggests that asthmatic patients should indeed use an antioxidant supplement e.g. selenium, as it stabilizes platelet aggregates.

Chapter 8: References

Abou-Dough A.M. New withanolides and other constituents from the fruit of *Withania somnifera*. *Arch Pharm*. 2002; **335**: 267-276

Agarwal R., Diwanay S., Patki P., *et al*. Studies on immunomodulatory activity of *Withania somnifera* (Ashwagandha) extracts in experimental immune inflammation. *J Ethnopharmacol*. 1999; 67(1): 27-35

Ahmad M., Saleem S., Ahmad A.S., *et al*. Neuroprotective effects of *Withania somnifera* on 6-hydroxydopamine induced Parkinsonism in rats. *Hum Exp Toxicol*. 2005; 24(3): 137-147

Akbari O. J. , Faul L., Hoyte E. G., Berry G. J., Wahlstrom J., Kronenberg, M., *et al*. CD4+ invariant T-cell-receptor+ natural killer T cells in bronchial asthma. *N Engl J Med*, 2006; **11**: 1117-29

Ali N.A., Julicch W.D., Kusnick C., *et al*. Screening of Yemeni medicinal plants for antibacterial and cytotoxic activities. *J Ethnopharmacol*. 2001; **74**: 173-179

Allam M.F., Lucane R. A. Selenium supplementation for asthma. *Cochrane Database Syst Rev*. 2004; **2**: CD003538

Anbalagan K., Sadique J. Influence of an Indian medicine (Ashwagandha) on acute-phase reactants in inflammation. *Indian J Exp Biol*. 1981; **19**: 245-249

Anbalagan K., Sadique J. Role of prostaglandins in acute phase proteins in inflammation. *Biochem Med*. 1984; **31**: 236-245

Andrieux A., Hudry-Clergeon G., Ryckewaert J., Chapel A., Ginsberg M. H., Plow E. F., Marguerie G. Amino Acid Sequences in Fibrinogen Mediating Its Interaction with Its

Platelet Receptor, GPIIb/IIIa" *The Journal of Biological Chemistry*. The American Society for Biochemistry and Molecular Biology, Inc Val. 1989; 264 (**16**): 9258-9265.

Andriopoulou P., Navarro P., Zanetti A., Lampugnani M.G., Dejana E. Histamine induces tyrosine phosphorylation of endothelial cell-to-cell adherens junctions. *Arterioscler Thromb Vasc Biol*. 1999; 19(**10**): 2286-2297.

Anon. Monograph, *Withania somnifera*. *Altern Med Rev*. 2004; 9(**2**): 211-214

Aphale A.A., Chhibba A.D., Kumbhakarma N.R., *et al*. Sub acute toxicity study of the combination of ginseng (*Phanax ginseng*) and ashwagandha (*Withania somnifera*) in rats: a safety assessment. *Indian J Physiol Pharmacol*. 1998; 42(**2**): 299-302

Archana R., Namasivayam A. Antistressor effect of *Withania somnifera*. *J Ethnopharmacol*. 1999; **64**: 91-93

Badzenta-Kukaszuk A., Kowal K., Pampuch A. The role of platelets in allergic inflammation. *Przeql Lek*. 2002; 59(**11**): 903-6

Banach-Wawrzenczyk E., Dziedziczko A., Rosc D. Fibrinolytic system in bronchial asthma after prednisone treatment. *Pol. Merkurisuz Lek*. 2000; **7**: 9-11.

Barnes P.J., Chung K. F., Page C. P. Inflammatory mediators of asthma: an update, *Pharmacol Rev*. 1998; **4**: 515-596.

Begum V.H., Sadique J. Long term effect of herbal drug *Withania somnifera* on adjuvant induced arthritis in rats. *Indian J Exp Biol*. 1988; **26**: 877-882

Behnke O. An electron microscope study of the megacaryocyte of the rat bone marrow: The development of the demarcation membrane system and the platelet surface coat. *J Ultrastruc Res*. 1968; **24**: 412-433

Bell S.J., Metzger W.J., Welch C.A., Gilmour M.I. A role for Th2 T-memory cells in early airway obstruction. *Cell Immunol*. 1996; **170**:185-194.

Bhattacharya A., Ghosal S., Bhattacharya S.K. Antioxidant effect of *Withania somnifera* glycowithanolides in chronic foot shock induced perturbations of oxidative free radical scavenging enzymed and lipid peroxidation in rat frontal cortex and striatum. *J Ethnopharmacol.* 2001; **74**: 1-6

Bhattacharya S.K., Bhattacharya A., Sairam K., et al. Anxiolytic-antidepressant activity of *Withania somnifera* glycowithanolides: an experimental study. *Phytomedicine.* 2000; **7**: 463-469

Bhattacharya S.K., Muruganandam A.V. Adaptogenic activity of *Withania somnifera*: an experimental study using a rat model of chronic stress. *Pharmacol Biochem Behav.* 2003; **75**: 547-555

Bhattacharya S.K., Satyan K.S., Chakrabarti A. Effect of Trasina, an Ayurvedic herbal formulation, on pancreatic islet superoxide dismutase activity in hyperglycaemic rats. *Indian J Exp Biol.* 1997a; **35**: 297-299

Bhattacharya S.K., Satyan K.S., Ghosal S. Antioxidant activity of glycowithanolides from *Withania somnifera*. *Indian J Exp Biol.* 1997b; **35**: 236-239

Bice D.E., Seagrave J., Green F.H.Y. Animal models of Asthma: Potential usefulness for studying Health effects of Inhaled Particles. *Inhal Toxicol.* 2000; **12(9)**: 829-862

Blasko G., Cordell C.A. Chemistry of plant derived anti-cancer agents. In Economic and Medicinal Plant Research, H Wagner, H Hikino and NR Farnsworth, eds, Vol 2. *Academic Press. London.* 1988; 119

Blyth D. I., Pedrick M. S., Savage T. J., et al. Lung inflammation and epithelial changes in a murine model of atopic asthma. *Am J Respir Cell Mol Biol.* 1996; **14**: 425-438

Bodey K.J., Semper A.E., Redington A.E., et al. Cytokine profiles of BAL T cells and T-cell clones obtained from human asthmatic airways after local allergen challenge. *Allergy.* 1999; **54**: 1083-1093.

Bousquet I.J., Ansotegui R., van Ree P.G., Burney T., et al. European Union meets the challenge of the growing importance of allergy and asthma in Europe. *Allergy*. 2004; **59**: 1-4

Brown K.M., Arthur J.R. Selenium, selenoproteins and human health: a review, *Public Health Nutr*. 2001; 4(**2B**), 593-599.

Busse W.W., Lemanske R. F. Asthma. *N Engl J Med*. 2001; **5**: 350-62.

Butenas S., Mann K.G. Blood coagulation. *Biochem*. 2002; **671**: 3-12.

Camera M., Giesen P.L., Fallon J., Aufiero B.M., Taubman M., Tremoli E., Nemerson Y. Cooperation between VEGF and TNF- α is necessary for exposure of active tissue factor on the surface of human endothelial cells. *Arterioscler Thromb Vasc Biol*. 1999; **19** 531–537.

Caramori G., Pandit A., Papi A. Is there a difference between chronic airway inflammation in chronic severe asthma and chronic obstructive pulmonary disease? *Curr Opin Allergy Clin Immunol*. 2005; **5**: 77-83

Cassady J.M., Chang C.J., McLaughlin J.L. Recent advances in the isolation and structure elucidation of antineoplastic agents from higher plants. In Natural Products as Medicinal Agents, JL Beal and E Reinhard eds. *Hippokrates verlag, Stuttgart*. 1981; 119

Chang H.Y., Chi J.T., Dudoit S., Bondre C., van de Rijn M., Botstein D., Brown P.O. Diversity, topographic differentiation, and positional memory in human fibroblasts. *Proc Natl Acad Sci USA*. 2002; **99**: 12877–12882.

Choudhary M., Dur-e-Shahwar I., Parveen Z., et al., Antifungal steroidal lactones from *Withania coagulans*. *Phytochemistry*. 1995; **40**: 1243-1246

Choudhary M.I., Yousuf S., Nawaz S.A., et al. Cholinesterase inhibiting withanolides from *Withania somnifera*. *Chem Pharm Bull. (Tokyo)*. 2004; 52(**11**): 1358-1361

- Chu D.T., Wang W., Mavlight G.M. *J clin lab immunol.* 1988; **25**: 119
- Chung K .F., Barnes P. J. Role for platelet activating factor in asthma. *Lipids.* 1991; 26 (**12**): 1277-1279
- Cohn L., Elis J. A., Chupp G. L. Asthma: mechanisms of disease persistence and progression. *Annu Rev Immunol.* 2004; **22**: 789–815
- Cook J.A., Mitchell J.B. Viability measurements in mammalian sell systems. *Anal Biochem.* 1989; **179**: 1-7
- Coyle A. J., Bertrand C., Tsuyuki S., *et al.* IL-4 differentiates naive CD8+ T cells to a "Th2-like" phenotype: a link between viral infections and bronchial asthma. *Ann N Y Acad Sci.* 1996; **796**: 97-103.
- Davis L., Kuttan G. Effect of *Withania somnifera* on DMBA induced carcinogenesis. *J Ethnopharmacol.* 2001; **75**: 165-168
- Davis L., Kuttan G, Effect of *Withania somnifera* on 20-methylcholanthrene induced fibrosarcoma. *J Exp Clin Cancer Res.* 2000a; 19(**2**): 165-167
- Davis L., Kuttan G. Immunomodulatory activity of *Withania somnifera*. *J Ethnopharmacol.* 2000b; 71(**1-2**): 192-200
- Davis L., Kuttan G. Effect of *Withania somnifera* on cyclophosphamide-induced urotoxicity. *Cancer Lett.* 2000c; **148**: 9-17
- Davis L., Kuttan G. Effect of *Withania somnifera* on cell mediated immune responses in mice. *J. Exp Clin Cancer Res.* 2002; 21(**4**): 585-90

Devi P.U. *Withania somnifera* Dunal (Ashwagandha): potential plant source of a promising drug for cancer chemotherapy and radiosensitization. *Indian J Exp Biol.* 1996. 34(10): 927-932

Dhuley J.N. Adaptogenic and cardioprotective action of ashwagandha in rats and frogs. *J Ethnopharmacol.* 2000; **70**: 57-63

Dombrowicz D., Capron M. Eosinophils, allergy and parasites, *Curr Opin Immunol.* 2001 ; **13** : 16-20.

Elsakka M., Grigorescu E., Stanescu U., et al. New data referring to chemistry of *Withania somnifera* species. *Rev Med Chir Soc Med Nat Iasi.* 1990; **94**: 385-387

Epstein M.M. Do mouse models of allergic asthma mimic clinical disease? *Int Arch Allergy Immunol.* 2004a; **133**: 84-100.

Epstein M.M. Modeling allergic asthma: in vitro assays to virtual patients. *Drug Discovery Today, Disease Models.* 2004b; **1**: 387-394

Epstein M.M. Are mouse models of allergic asthma useful for testing novel therapeutics? *Exp Toxicol Pathol.* 2006; 57(2): 41-4

Erb K.J., Le Gros G. The role of Th2 type CD4+ T cells and Th2 type CD8+ T cells in asthma. *Immunol Cell Biol.* 1996; **74**: 206-208

Escolar G, Leistikow E, White JG: The fate of the open canalicular system in surface and suspension activated platelets. *Blood.* 1989; 74 (6): 1983-1988

Eum S. Y., Lefort J., Huerre M., Vargaftig B. Eosinophil recruitment into the respiratory epithelium following antigenic challenge in hyper IgE-mice in accompanied by interleukin 5-dependant bronchial hyperresponsiveness. *Proc Natl Acad Sci USA.* 1995; **92**: 12290-12294,

Ezerman E. B., Ishikawa H. Differentiation of the sarcoplasmic reticulum and T system in developing chick skeletal muscle *in vitro*. *J Cell Biol.* 1967; **35**: 405–20.

Fedarko M.E. Morphologic and functional observations on bone marrow megakaryocytes, *The Year in Hematology-1978*. Edited by R Silber, J LoBue, AS Gordon. New York, *Plenum Medical Book Company*. 1978; 171-209

Fireman P. Understanding asthma pathophysiology, *Allergy Asthma Proc* . 2003; **2**: 79-83

Freshney I. R. *Culture of Animal Cells: A manual of basic technique*, Fifth edition 2005.

Gabazza E.C., Taguchi O., Tamaki S., Takeya H., Kobayashi H., Kobayashi T., Hataji O., Urano H., Zhou H., Suzuki K., Adachi Y. Thrombin in the airways of asthmatic patients. *Lung*. 1999; **177**: 253–262.

Galli S.J., Kalesnikoff J., Grimbaldston M.A., Piliponsky A.M., Williams C.M., Tsai M. Mast cells as "tunable" effector and immunoregulatory cells: recent advances. *Annu Rev Immunol*. 2005;**23**: 749-86.

Ganzera M., Chaudhary M.I., Khan I.A.. Quantitative HPLC analysis of withanolides in *Withania somnifera*. *Fitiotherapy*. 2003; **74**: 68-76

Gartner L. P., Hiatt J. L. *Color textbook of histology*. Third edition, 2007. *Saunders Elsevier*, 232-233

Gazdik F., Pijak M.R., Gazdikova K. Need of complementary therapy with selenium in asthmatics, *Nutrition*. 2004; 20(**10**): 950-2.

Ghosal S., Kaur R., Srivastava R.S. Sitoindosides IX and X, new glycowithanolides from *Withania somnifera*. *Indian Journal Nat Prod*. 1988; **4**: 12-13

Ghosal S., Lal J., Srivastava R.S., Bhattacharya S.K., Upadhyay S.N., *et al.* Immunostimulatory and CNS effects of sitoindosides IX and X, two new glycowithanolides from *Withania somnifera*. *Phytother Res.* 1989; **3**: 201-206

Glaser J.L. Maharishi Ayurveda: an introduction to recent research. *Modern Science and Vedic Science.* 1988; 2 (**1**): 89-108

Gleich G.J. Mechanisms of eosinophil-associated inflammation, *J Allergy Clin Immunol.* 2000; **105**: 651-63

Grootendorst D.C., Sont J. K., Willems L. N., Kluin- Nelemans J. C., Van Krieken J. H., Veselic- Charvat M., Sterk P. J. Comparison of inflammatory cell counts in asthma: induced sputum vs bronchoalveolar lavage and bronchial biopsies. *Clin Exp Allerg.* 1997; **7**: 769-79

Gupta Y.K., Sharma S.S., Rai K., *et al.* Reversal of paclitaxel induced neutropenia by *Withania somnifera* in mice. *Indian J Physiol Pharmacol* . 2001; 45(**2**): 253-257

Halliwell B., Gutteridge J.M.C. Free Radicals in Biology and Medicine, 2nd Edition, Oxford, Clarendon Press. 1989.

Han X., Amar S. Identification of genes differentially expressed in cultured human periodontal ligament fibroblasts vs. human gingival fibroblasts by DNA microarray analysis. *J Dent Res.* 2002; **81**: 399–405.

Henderson M., Anderson J.G. Common Weeds in South Africa. *Memoirs Botanical Survey of South Africa* 37, 1966; 286

Hogan S. P., Koskinen A., Foster P. S. Interleukin 5 and eosinophils induce airway damage and bronchial hyperreactivity during allergic airway inflammation in Balb/c mice. *Immunol Cell Biol.* 1996; **75**: 284-288.

Holt P.G., Macaubas C., Stumbles P.A., Sly P.D. The role of allergy in the development of asthma. *Nature.* 1999; **402**: 12-17

<http://microscope.fsu.edu>

Isenberg-Feig H., Justice J. P., Keane-Myers A. Animal models of allergic asthma. *Curr Allergy Asthma Rep.* 2003; 3(1): 70-78

Ishige K., Schubert D., Sagara Y. Flavonoids protects neuronal cells from oxidative stress by three distinct mechanisms. *Free Radic Biol Med.* 2001; 30(4): 433-446

Ishiyama M., Tominaga H., Masanobu S., Sasamoto K., Ohkura Y., Ueno K. A combined assay of cell viability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. *Biol Pharm Bull.* 1996; **19**:1518-1520.

Italiano J.E., Lecine P., Shivdasani R.A., Hartwig J.H. Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes. *J Cell Biol.* 1999; **147**: 1299-1312

Iuvone T., Esposito G., Capasso F., *et al.* Induction of nitric oxide synthase expression by *Withania somnifera* in macrophages. *Life Sci.* 2003; 72(**14**): 1617-1625

Iwu M.M. Handbook of African Medicinal Plants. Florida, USA: CRC Press, 1993

Jahnova E., Horvathova M., Gazdik F., Weisssova S. Effects of selenium supplementation on expression of adhesion molecules in corticoid-dependent asthmatics, *Bratisl Lek Listy.* 2002; **1**: 12-6.

Jain S., Shukla S.D., Sharma K., *et al.* Neuroprotective effects of *Withania somnifera* Dunn. in hippocampal sub-regions of female albino rat. *Phytother Res.* 2001; 15(**6**): 544-548

Jayaprakasam B., Zhang Y., Seeram N.P., Nair M.G. Growth inhibition of human tumor cell lines by withanolides from *Withania somnifera* leaves. *Life Sci.* 2003; **74**: 125-32

Jesberger J.A., Richardson J.S. Oxygen free radicals and brain dysfunction. *Int J Neurosci*. 1991; **57**: 1-17

Jovanovic S.V., Simic M.G. Antioxidants in nutrition. *Ann Ny Acad Sci* . 2000; **899**: 326-334

Kadrobova J., *et al*. Selenium status is decreased in patients with intrinsic asthma. *Biol Trace Elem Res*. 1996; **52**: 241-248

Kanduc D., Mittelman A., Serpico F., *et al*. Cell death: Apoptosis versus necrosis (review). *Int J Oncol*. 2002; **21**: 165-170

Kassel O., Schmidlin F., Duvernelle C., Gasser B., Massard G., Frossard N. Human bronchial smooth muscle cells in culture produce stem cell factor. *Eur Respir J* .1999;**13**: 951-4.

Kaur P., Sharma M., Mathur S., *et al*. Effect of 1-oxo-5beta, 6beta-epoxy-with a-2-ene 27-ethoxy-olide isolated from the roots of *Withania somnifera* on stress indices in Wistar rats. *J Altern Complement Med*. 2003; **9**: 897-907

Kay A.B., Phipps S., Robinson D. S. A role for eosinophils in airway remodeling in asthma. *Trends Immunol* . 2004; **9**: 477-82

Kenney D.M., Linck R.W. The cytoskeleton of unstimulated blood platelets: structure and composition of the isolated marginal microtubular band. *J Cell Sci*. 1985; **78**: 1-22.

Kern D.F., Saldeen K., Saldeen T., Malik A.B. Pulmonary vascular effects of a fibrin (ogen)-derived vasoactive peptide. *Thromb Res*. 1986; **42**: 783–788.

Kierszenbaum A. L. Histology and cell biology: An introduction to pathology. Second edition, 2007. *Mosby Elsevier*, 173-175

Kips J. C., Anderson G. P., Fredberg J. J., *et al*. Murine models of asthma. *Eur J Respir Dis*. 2003; **22**: 374-382

Kline J.N., Hunninghake G.W. T-lymphocyte dysregulation in asthma. *Proc Soc Exp Biol Med.* 1994; **207**: 243-253.

Kocyigit A., Armutcu F., Gurel A., Ermis B. Alterations in plasma essential trace elements selenium, manganese, zinc, copper and iron concentrations and the possible role of these elements on oxidative status in patients with childhood asthma. *Biol Trace Elem Res.* 2004; 97(1): 31-41.

Kon O.M., Kay A. B. T cells and chronic asthma, *Int. Arch. Allergy Immunol.* 1999; **118**: 133-135

Kueng W., Silber E., Eppenberger U. Quantification of cells cultured on 96-well plates. *Anal Biochem.* 1989; **185**:16-19

Kuipers H., Lambrecht B.N. The interplay of dendritic cells, Th2 cells and regulatory T cells in asthma. *Curr Opin Immunol.* 2004; 16(6):702-8.

Kulkarni R.R., Patki P.S., Jog V.P., *et al.* Treatment of osteoarthritis with a herbomineral formulation: a double blind, placebo-controlled, cross over study. *J Ethnopharmacol.* 1991; **33**: 91-95

Kumar R.K., Foster P.S. Modelling allergic asthma in mice. *Am J Respir Cell Mol Biol.* 2002; **27**: 267-272

Lampinen M., Carlson M., Hakansson L. D., Venge P. Cytokine regulated accumulation of eosinophils in inflammatory diseases, *Allergy.* 2004; **59**: 793-805.

Larche M. D., Robinson S., Kay A. B. The role of T lymphocytes in the pathogenesis of asthma, *J Allergy Clin Immunol.* 2003; **3**: 450-63.

Larson M. K., Watson S. P. Regulation of proplatelet formation and platelet release by integrin $\alpha_{IIb}\beta_3$. *Blood.* 2006; 108 (5):1509-1514

Larsen S.T., Hansen J. S., Hansen E. W., Clausen P. A., Nielsen G. D. Airway inflammation and adjuvant effect after repeated airborne exposures to di-(2-ethylhexyl) phthalate and ovalbumin in BALB/c mice. *Toxicology*. 2007; **3**: 119-29

Lavie D., Glotter E., Shro Y. Constituents of *Withania somnifera* Dun. The structure of Withaferin A. *J Chem Soc*. 1965; **30**: 7517-7531

Lawrence SO., Simpson-Haidaris PJ. Regulated de novo biosynthesis of fibrinogen in extrahepatic epithelial cells in response to inflammation. *Thromb Haemost*. 2004; **92**(2): 234-43.

Lazarus A.H., Song S., Crow A.R. Understanding platelet function through signal transduction. *Transfus Med Rev*. 2003; **17**: 45-56.

Leff A. R. The biology of leukotrienes, eosinophils, cytokines and nitric oxide in airway inflammation. *Clin Exp Allergy Rev*. 2001; **1**(2): 133-136

Le Gros G., Erb K., Hams N., *et al*. Immunoregulatory networks in asthma. *Clin Exp Allergy*. 1998; **28**: S92-S96.

Maddox I., Schwartz D.A. The pathophysiology of asthma, *Annu Rev Med*. 2002; **53**: 477-98.

Mantzoros I., Kanellos I., Demetriades H., Christoforidis E., Kanellos D., Pramateftakis M. G., Zaraboukas T., Betsis D. Effects of steroid on the healing of colonic anastomoses in the rat, *Tech Coloproctol*. 2004; **1**: 180-183.

Marone G., Granata F., Spadaro G., Onorati A.M., Triggiani M. Anti-inflammatory effects of oxatomide. *J Investig Allergol Clin Immunol*. 1999; **9**(4): 207-214

Marx J. Unpublished data. 2005.

Masoli M., Fabian D., Holt S., Beasley R. The Global Burden of Asthma Report, 2004

Mathur S., Kaur P., Sharma M., *et al.* The treatment of skin carcinoma induced by UVB radiation, using 1-oxo-5beta, 6beta-epoxy-with a-2-enolide, isolated from the roots of *Withania somnifera* in a rat model. *Phytomedicine*. 2004; 11(5): 452-460

Mathur R., Gupta S. K., Singh N., Mathur S., Kochupillai V., Velpandian T. Evaluation of the effect of *Withania somnifera* root extracts on cell cycle and angiogenesis. *J Ethnopharmacol*. 2006; 105(3): 336-341

Matsuda H., Murakami T., Kishi A., Yoshikawa M. Structures of Withanolides I, II, III, IV, V, VI and VII, new withanolide glycosides, from roots of Indian *Withania somnifera* DUNAL and inhibitory activity for tachyphylaxis to clonidine in isolated guinea-pig ileum. *Bioorg Med Chem*. 2001; 9: 1499-507

McKeever TM., Britton J. Diet and asthma. *Am J Respir Crit Care Med*. 2004; 170: 725–729

Metha A.K., Binkley P., Ghandi S.S., *et al.* Pharmacological effects of *Withania somnifera* root extract on GABAA receptor complex. *Indian J Med Res*. 1991; 94: 312-315

Miller A.L. The etiologies, pathophysiology and alternative/complementary treatment of asthma, *Altern Med Rev*. 2001; 1: 20-47.

Mills S., Bone K. Poke root. *Principles and Practice of Phytotherapy*. Churchill Livingstone, 2000; 515–18.

Milton J.G., Frojmovic M.M. Invaginated plasma membrane of human platelets: Evagination and measurement in normal and "giant" platelets. *J Lab Clin Med*. 1979; 93:162-170

Mishra L.C., Singh B.B., Dagenais S. Scientific basis for the therapeutic use of *Withania somnifera* (Ashwagandha). *Altern Med Rev*. 2000; 5: 334-46

Mohan R., Hammers H.J., Bargagna- Mohan P., *et al.* Withaferin A is a potent inhibitor of angiogenesis. *Angiogenesis*. 2004; 7: 115-122

Morley J., Sanjar S., Page C.P. The platelet in asthma. *Lancet*. 1984; **1**: 1142-1144.

Mosmann T. R., *et al.* Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol*. 1986; **136**: 2348-2357.

Mossman T. Rapid calorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods*. 1983; **65**: 55-63

Munetaka I., Tominaga H., Shiga M., *et al.* A combined assay of cell viability and in vitro cytotoxicity with a highly water soluble tetrazolium salt, neutral red and crystal violet. *Biol Pharm Bull*. 1996; **10**: 1518-1520

Nachman R. L., Leung L. L. K. Complex formation of platelet membrane glycoprotein IIb and IIIa with fibrinogen. *J Clin Invest*. 1982; **69**: 263-269.

Nishant P., *et al.* Hypocholesteremic and antioxidant effects of *Withania somnifera* (Dunal) in hypercholesteremic rats. *Journal Phymed*. 2006; 3- 5

Oberholzer H.M., Pretorius E., Smit E., Ekpo O.E., Humphries P., Auer R.E. Investigating the effect of *Withania somnifera*, selenium and hydrocortisone on blood count and bronchial lavage of Balb/c mice. *Scand J Lab Anim Sci*. [Submitted for publication]

O'Neil B. J., Kline J. A., Burkhart K., Younger J. The use of Laboratory Animal Models in Research. *Acad Emerg Med*. 1999; **6**(1): 75-82

Padmavathi B., Rath P.C., Rao A.R., *et al.* Roots of *Withania somnifera* inhibit fore stomach and skin carcinogenesis in mice. *Evid Based Complement Alternat Med*. 2005; **2**(1): 99-105

Page C. P. Platelets as inflammatory cells. *Immunopharmacology*. 1989; **17**: 51-9

Page S., Ammit A.J., Black J.L., Armour C.L. Human mast cell and airway smooth muscle cell interactions: implications for asthma. *Am J Physiol Lung Cell Mol Physiol*. 2001; **281**:1313- 1323.

Panda S., Kar A. *Withania somnifera* and *Bauhinia purpurea* in the regulation of circulating thyroid hormone concentrations in female mice. *J Ethnopharmacol*. 1999; **67**: 233-239

Pandey V.N. Phytochemical Investigation of Certain Medicinal plants used in Ayurveda. Central Counsel for Research in Ayurveda and Sidda (Govt of India), New Dehli, 1985; 31-33

Panettieri R.A., Hall I.P., Maki C.S., Murray R.K. alpha-Thrombin increases cytosolic calcium and induces human airway smooth muscle cell proliferation. *Am J Respir Cell Mol Biol*. 1995; **13**: 205–216.

Pariete J.L., Kim B.S., Atala A. In vitro biocompatibility evaluation of naturally derived and synthetic biomaterials using normal human bladder smooth muscle cells. *J Urol*. 2002; **164**: 1867-1871

Parihar M.S., Chaudhary M., Shetty R., *et al*. Susceptibility of hippocampus and cerebral cortex to oxidative damage in streptozotacin treated mice: prevention by extracts of *Withania somnifera* and *Aloe vera*. *J Clin Neurosci*. 2004; **11**(4): 397-402

Parihar M.S., Hemnani T. Phenolic antioxidant attenuates hippocampal neuronal cell damage against kainic acid induced excitotoxicity. *J Biosci*. 2003; **28**: 121-128

Parish C.R., Mullbacher M. Automated calorimetric assay for T cell cytotoxicity. *J Immunol Methods*. 1983; **58**: 225-237

Pattipati S., Amanpreet S., Shrinivas K. Effect of *Withania somnifera* root extract on Haloperidol Induced Orofacial Dyskinesia: Possible mechanism of action. *J Med Food*. 2003; **6** (2): 107-114

Peters-Golden. M The alveolar macrophage: the forgotten cell in asthma, *Am J Respir Cell Mol Biol.* 2004; **1**: 3-7

Pitchford S. C., Riffo-Vasquez Y., Sousa A., Momi S., Gresele P., Spina D *et al.* Platelets are necessary for airway wall remodeling in a murine model of chronic allergic inflammation, *Blood.* 2004; 103 (2): 639–647.

Pinto L.A., *et al.* Effect of clarithromycin on the cell profile of bronchoalveolar lavage fluid in mice with neutrophilpredominant lung disease, *Rev Hosp Clín Fac Med S Paulo.* 2004; **3**: 99-103

Pizzichini E., Pizzichini M.M.M., Efthimiadis A., *et al.* Measurement of inflammatory indices in induced sputum: effects of selection of sputum to minimize contamination. *Eur Respir J.* 1996; **9**:1174-1180

Powell C.V., Nash A. A., Powers H. J., Primrak R. A. Antioxidant status in asthma, *Pediatr Pulmonol.* 1994; **18**: 34-38.

Prakash J., Gupta S.K., Dinda A.K. *Withania somnifera* root extract prevents DMBA-induced squamous cell carcinoma of skin in Swiss albino mice. *Nutr Cancer.* 2002; **42**(1): 91-7

Prescott S.L. New concepts of cytokines in asthma. Is the Th2/Th1 paradigm out of the window? *J Paediatr Child Health.* 2003; **39**: 575-9.

Pretorius E., Ekpo O. E., Smit E. Comparative ultrastructural analyses of platelets and fibrin networks using the murine model of asthma, *Exp Toxicol Pathol.* 2007; **70**(9): 823-27

Pretorius E., Wallner B., Oberholzer H. M. Ultrastructural changes in platelets seen in asthma and Hydrocortisone treatment: a hypothesis. *Medical Hypothesis* (submitted for publication)

Pretorius E., Briedenhann S., Marx J., Franz R.C. Structural changes in the fibrin network of a Pretoria family with dysfibrinogenaemia: a scanning electron microscopical study. *Ultrastruct Pathol.* 2006; **30**(3): 167-76

Rahman I., MacNee W. Role of oxidants/antioxidants in smoking-induced lung diseases. *Free Radic Biol Med.* 1996; **21**: 669–681.

Ramasamy I. Inherited bleeding disorders: disorders of platelet adhesion and aggregation. *Critical Rev Oncol.* 2004; **49**: 1-35.

Rasool M., Varalakshmi P. Suppressive effect of *Withania somnifera* root powder on experimental gouty arthritis: An *in vivo* and *in vitro* study. *Chem Biol Interact.* 2006; 164(3): 174-80

Ray A., Cohn L. Th2 cells and GATA-3 in asthma: new insights into the regulation of airway inflammation. *J Clin Invest.* 1999; **104**:985-993.

Ray A. B., Gupta M., “Progress in the Chemistry of Organic Natural Products,” Vol. 63, ed. by Herz W., Kirby G. W., Moore R. E., Steglich W., Tam Ch., *Springer Verlag, New York*, 1994; 1-106.

Renauld J.C. New insights into the role of cytokines in asthma, *J Clin Pathol.* 2001; **54**: 577-589

Ricciardolo F.L., Sterk P.J., Gaston B., Folkerts G. Nitric oxide in health and disease of the respiratory system. *Physiol Rev.* 2004; 84(3):731-65.

Rice-Evans C.A., Miller N.J., Bolwell P.G., *et al.* The relative antioxidant activity of plant derived polyphenolic flavonoids. *Free Rad Res.* 1995; **22**: 375-383

Roberto J., Barrios M.D., Farrah Kheradmand M.D., LaKeisha Batts B.S., *et al.* Asthma: Pathology and Pathophysiology. *Arch Pathol Lab Med.* 2005; 130 (4): 447–451.

Robinson D.S. The role of the mast cell in asthma: induction of airway hyperresponsiveness by interaction with smooth muscle? *J Allergy Clin Immunol.* 2004; 114(1): 58-65.

Rotruck J.T., Pope A.L., Ganther H.E. *et al.* Selenium: biochemical role as a component of glutathione peroxidase, *Science.* 1973; **179**: 588-590

Rubin R.N., Navon L., Cassano P.A. Relationship of serum antioxidants to asthma prevalence in youth. *Am J Respir Crit Care Med.* 2004; 169(3): 393-8.

Russo A., Lzzo A.A., Scarfiotti C., Fabris F., Cestaro B., *et al.* Free radicals atherosclerosis, ageing, and related dysmetabolic pathologies: pathological and clinical aspects. *Eur J Cancer Prev.* 1997; **6**: 31-36

Russo A., Lzzo A. A., Cardile V., Borrelli G., Vanella A. Indian medicinal plants as antiradicals and DNA cleavage protectors. *Phytomedicine.* 2001; **8**: 125–132.

Scarfiotti C., Fabris F., Cestaro B., Giuliani A. Free radicals, atherosclerosis, ageing, and related dysmetabolic pathologies: pathological and clinical aspects. *Eur J Cancer Prev.* 1997; **6**:31-36.

Scartezinni P., Speroni E. Review on some plants of Indian traditional medicine with antioxidant activity. *J Ethnopharmacol.* 2000; **71**: 23-43

Schmidt D., Ruehlmann E., Branscheid D., Magnussen H., Rabe K.F. Passive sensitization of human airways increases responsiveness to leukotriene C4. *Eur J Respir Dis.* 1999; 14(2): 315-319.

Schwarting A.E. The Alkaloids of *Withania somnifera*, *Lloydia.* 1963; 26(4):258-73

Schwer H.D., Lecine P., Tiwari S., Italiano J.E., Hartwig J.H., Shivdasani R.A. A lineage-restricted and divergent beta-tubulin isoform is essential for the biogenesis, structure and function of blood platelets. *Curr Biol.* 2001;**11**: 579-586

Selgrade M.K., Lawrence D.A., Ullrich S.E., *et al.* Modulation of T-helper cell populations: potential mechanisms of respiratory hypersensitivity and immune suppression. *Toxicol Appl Pharmacol.* 1997; **145**:218-229.

Senthilnathan P., Padmarathi R., Banu S.M., *et al.* Enhancement of antitumor effect of paclitaxel in combination with immunomodulatory *Withania somnifera* on benzo(a)pyrene induced experimental lung cancer. *Chem Biol Interact.* 2006; 159(**3**): 180-185

Sgouras D., Duncan R. Methods for evaluation of biocompatibility of soluble synthetic polymers which have potential for biomedical use: 1- Use of tetrazolium-based colorimetric assay (MTT) as a preliminary screen for evaluation of *in vitro* cytotoxicity. *J Material Sci Material Med.* 1990; **1**: 61-68

Shaheen S.O., Sterne J.A., Thompson R.L., Songhurst C.E., Margetts B.M., Burney P.G. Dietary antioxidants and asthma in adults: population-based case-control study, *Am J Respir Crit Care Med.* 2001; 164(**10**): 1823-8.

Sharad A.C., Solomon F.E., Devi P.U., *et al.* Antitumor and radiosensitizing effects of Withaferin A on mouse Ehrlich ascites carcinoma in vivo. *Ata Oncol.* 1996; 35(**1**): 95-100

Shroeter H., Williams R.J., Matin R., *et al.* Phenolic antioxidants attenuate neuronal cell death following the uptake of oxidized low-density lipoprotein. *Free Radic Biol Med.* 2000; 29(**12**): 1222-1233

Siddique N.A., Bari M.A., Shahnewaz S., *et al.* Plant regeneration of *Withania somnifera* (L) Dunal (Ashwagandha) from Nodal Segments Derived Callus on Engangered Medicinal Plant in Bangladesh. *J Biol Sci.* 2004; 4(**2**): 219-223

Singh B., Saxena A.K., Chandon B.K., *et al.* Adaptogenic activity of a novel, withanolide-free aqueous fraction from the root of *Withania somnifera*. *Phytother Res.* 2001; **15**: 311-318

Sixma J. J., van Zanten G. H., Banga J. D., Nieuwenhuis H. K., DeGroot P. G. Platelet adhesion. *Sem Hematol.* 1995; **32**: 1–6.

Sixma J. J., Wester J. The haemostatic plug. *Sem Hematol.* 1977; **14**: 265–9.

Smith A.D., Taylor D.R. Is exhaled nitric oxide measurement a useful clinical test in asthma? *Curr Opin Allergy Clin Immunol.* 2005;5(1):49-56.

Stojkovic P., Lako M., Stewart R., Przyborski S., Armstrong L., Evans J., Murdoch A., Strachan T., Stojkovic M. An autogenetic feeder cell system that efficiently supports growth of undifferentiated human embryonic stem cells. *Stem Cells.* 2005; **23**: 306–314.

Thatte U.M., Dahanukar S.A. Immunotherapeutic modification of experimental infections by Indian medicinal plants. *Phytother Res.* 1989; **3**:43-49.

Tillie-Leblond I., Gosset P., Tonnel A. B. Inflammatory events in severe acute asthma. *Allergy.* 2005; **1**: 23–29

Timbrell J. Principles of biochemical toxicology. Third Edition, by UK: Taylor and Francis 2000

Tiwari A. Natural product antioxidants and their therapeutic potential in mitigating peroxidative modification of lipoproteins and atherosclerosis: recent development. *J Med Aroma Plant Sci.* 1999; **21**: 730-741

Tohda C., Kuboyama T., Kamatsu K. Dendrite extension by methanol extract of Ashwagandha (roots of *Withania somnifera*) in SK-N-SH cells. *Neuroport.* 2000; 11(9): 1981-1985

Touqui L., Hatmi M., Vargaftig B.B. Human platelets stimulated by thrombin produce platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) when the degrading enzyme acetyl hydrolase is blocked. *Biochem J.* 1985; **229**: 811–816.

Tulic M. K., Christodoulopoulos P., Hamid Q. Small airway inflammation in asthma. *Respir Res.* 2001; **2**: 333–339.

Uma Devi P. *Withania somnifera* dunal (Ashwagandha): potential plant source of promising drug for cancer chemotherapy and radiosensitization. *Indian J Exp Biol.* 1996; **34**: 927-32

Umetsu D.T., De Kruffyff R.H. Th1 and Th2 CD4+ cells in the pathogenesis of allergic diseases. *Proc Soc Exp Biol Med.* 1997; **215**:11-20.

Upton R. Ashwagandha Root. Santa Cruz, California: American Herbal Pharmacopoeia, 2000.

Van Wyk B.E., Van Oudtshoorn B., Gericke N. Medicinal plants of South Africa. Briza publications, 2000; 274

Wagers S., Norton R., Bates J., Irvin C.G. Role of Fibrin in Determining Airway Closure. *Chest.* 2003; 123: 362-363

Wagers S.S., Norton R.J., Rinaldi L.M., Bates J.H.T., Sobel B.E., Irvin C.G. Extravascular fibrin, plasminogen activator, plasminogen activator inhibitors, and airway hyperresponsiveness. *J Clin Invest.* 2004; 114(1): 104–111.

Walker C., Kaegi M. K., Braun P., Blaser K. Activated T cells and eosinophilia in bronchoalveolar lavages from subjects with asthma correlated with disease severity, *J Allergy Clin Immunol.* 1991; **88**: 935–942

Wenzel S. Mechanisms of severe asthma. *Clin Exp Allergy.* 2003; **12**: 1622-8

White J. G., Krumwiede M. Further studies of the secretory pathway in thrombin-stimulated human platelets. *Blood*. 1987; **69**:1196–1203.

White J. G. An overview of platelet structural physiology. *Scanning Microsc*. 1987; **1**: 1677–1700.

White J. G. Interaction of membrane systems in blood platelets. *Am J Pathol*. 1972; **66**: 295–312.

White J. G. Platelet structural physiology: The ultrastructure of adhesion, secretion and aggregation in arterial thrombosis. Thrombosis and Platelets in Myocardial Ischemia. *Cardiovasc Clin*. 1987; **18**:13–33.

White J.G., Estensen R.D., Degranulation of discoid platelets. *Am J Pathol*. 1972; **68**:289-302

White J.G., A search for the platelet secretory pathway using electron dense tracers. *Am J Pathol*. 1970; **58**: 31-49

White J.G. Identification of platelet secretion in the electron microscope. *Ser Hematol*. 1973; **6**: 429-459

White J.G. The transfer of thorium particles from plasma to platelets and platelet granules. *Am J Pathol*. 1968; **53**: 567-575

White J.G. Uptake of latex particles by blood platelets: Phagocytosis or sequestration? *Am J Pathol*. 1972; **69**: 439-458

WHO Media centre, 2006: Asthma. Fact sheet No.307 August 2006. Available online: www.who.int/mediacentre/factsheets/fs307/en/index.html.

www.ginasthma.com.

www.medicallook.com

Youdim K.A., Joseph J.A. A possible emerging role of phytochemicals in improving age related neurological dysfunction: a multiplicity of effects. *Free Rad Biol Med.* 2001; **30**: 583- 594

Zosky G. R., Sly P. D. Animal models of asthma. *Clin Exp Allergy.* 2007; 37(7): 973-988.