Investigating the Effect of *Withania somnifera*, Selenium and Hydrocortisone on Blood Count and Bronchial Lavage of Experimental Asthmatic BALB/c Mice

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Summary

The murine asthmatic Balb/c mouse model was used to investigate the effect of *W. somnifera* L., the antioxidant selenium, *W. somnifera* 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. The mice were sensitised, 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, both 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. 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.

Introduction

W. somnifera is a member of the family Solanaceae and is also known as Ashwagandha, Indian ginseng and winter cherry. It is distributed worldwide with a wide distribution in Africa, Southern Europe and Asia, and is considered to be indigenous to South Africa (*Henderson & Anderson, 1996*).

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 *Withania* 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*), with the main active chemical constituent Withaferin A, a phytosteroid (*Lavi et al, 1965*). *W. somnifera* has been in use for over 2500 years to treat all kind of diseases and human ailments (*Bhattacharya et al, 2001*). Several studies indicated that *Withania* possesses antioxidant, antitumor, antistress, anti-inflammatory, immunomodulatory, hemapoetic and rejuvenating properties and also influences various neurotransmitter receptors in the central nervous

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system (*Pattipati et al, 2003*). Numerous studies have also revealed the anti-ageing, anxiolytic and antidepressive effects of this medicinal plant and the potential to stop cancer cell growth. In recent studies done on human breast, lung and colon cancer cell lines, plant extracts inhibited 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 (*Jayaprakasam et al, 2003*). Studies revealed that the anti-inflammatory and immunomodulatory properties of *W. somnifera* root extracts are likely to contribute to the chemo preventive action of *W. somnifera* (*Prakash et al, 2002*).

Selenium is also known to be a potent anti-oxidant and authors like 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 (*Shaheen et al, 2001*). Selenium is a powerful destroyer of free radicals created from air pollutants and is also involved in antioxidant defences as a coenzyme in glutathione peroxidase (*McKeever & Britton, 2004*). The selenium-dependent enzyme glutathione peroxidase (GPX) recycles glutathione, reducing lipid peroxidation by catalyzing the reduction of peroxides, including hydrogen peroxide; this represent a crucial component of antioxidative potential in humans (*Shaheen et al, 2001*). 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; Kadrabova et al, 1996; Allam & Lucane, 2004*). Also, Kocyigit and co-workers in 2004 suggest that increased iron and decreased selenium concentrations in patients with childhood asthma may be responsible for the oxidant/antioxidant imbalance (*Kocyigit et al, 2004*).

Numerous studies have also been done over the past few years to investigate the antioxidant effect of *W. somnifera*. Various studies came to the conclusion that *W. somnifera* also exhibits a potent antioxidant and anti-inflammatory effect. 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. The current study therefore uses the Balb/c murine asthma model to investigate the effects of *W. somnifera* alone and in combination with selenium on white blood cell counts in blood and in bronchial lavage and also platelet clumping. Hydrocortisone is used as positive control of asthma was this current research. 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. The Balb/c murine asthma model can be used successfully to study the effects of phytomedicine (*Pretorius et al, in press*).

Although the anti-inflammatory potential of the *W. somnifera* and the anti-oxidant effect of selenium is well-known, little is known of the effect of the plant on white blood cell counts in blood and bronchial lavage. In the current study, the effects of *W. somnifera* and selenium, separately and in combination, on platelet clumping, white blood cell counts in blood and in bronchial lavage was studied using the murine asthma Balb/c model.

Materials and Methods

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 plant material was air-dried, ground into a fine powder and 50g of this material was extracted in 500ml boiling double-distilled water (100mg/ml)

for 20 minutes with continues stirring. The water extract was allowed to cool, filtered and then dried using a rotary evaporator at 40°C. The final dry mass was 3480mg (6.96mg/ml or 6.92 of plant material extracted). A stock solution of 330mg/ml (47 times concentrated) was prepared and stored at -70°C. Mice with an average mass of 20g were divided into two groups and received either 750mg/ml (15mg/mouse (45µl per mouse)) equivalent to a volume of 2115µl of the original plan extract derived from 211.5mg plant material.

Implementing the Balb/c asthma model

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(ovalbumin)-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.

The animals were kept in polycarbonate type III cages that were obtained from Tecniplast (Buguggiate, Italy). A temperature range of 20-24°C, a relative humidity of 40-60% and a 12-hour day light and 12-hour night were maintained. Six mice were housed per cage, and autoclaved Pine Wood shavings, produced and supplied by JA Booysen Inc. (Pretoria, South Africa) were used as bedding while elite white facial tissue paper was added per cage for enrichment.

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 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 (phosphate-buffered-saline) was performed twice daily, for 1 hour, on days 13, 14 and 15 *W. somnifera,* selenium, the combination of *W. somnifera* 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.

Techniques of bronchial lavage

After termination, a small skin incision was made in the skin of each mouse in ventral of 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 an average18 fields per slide were counted.

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 (11 µl of citrate per 100 µl blood) and histological blood smears were prepared and stained with Giemsa Wright stain. A total of hundred leucocytes were counted in each blood smear.

Results and Discussion

Figure 1 shows the different white blood cells in bronchial lavage found in mice. Figure 2 shows platelet distributions amongst the different groups. Table 1 shows the different counts of white blood

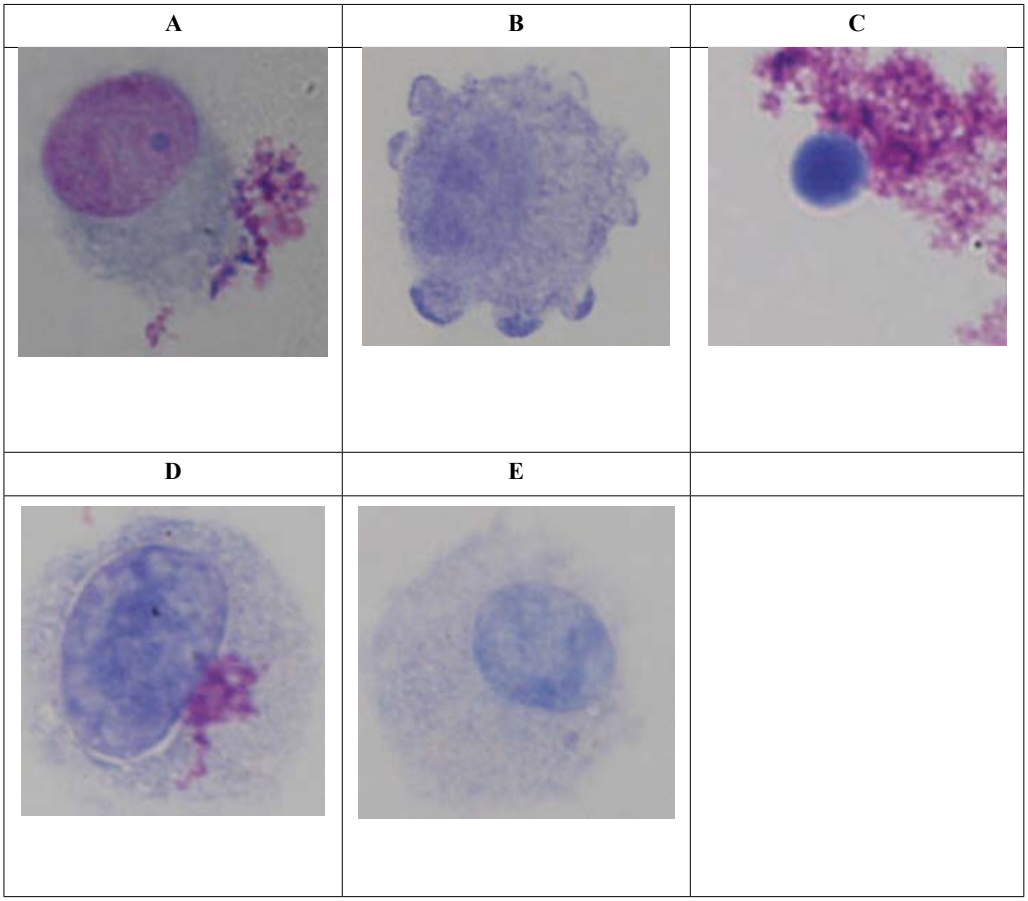

Figure 1. White blood cells in the bronchial lavage of Balb/c mice. Eosinophil (**A**), Monocyte (**B**), Lymphocyte (**C**), Basophil (**D**), Neutrophil (**E**)

Table 1. Number of white blood cells counted in the bronchial lavage of Balb/c mice

	Control	**Asthma**	***Withania***	**Selenium**	***Withania*** **+ 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

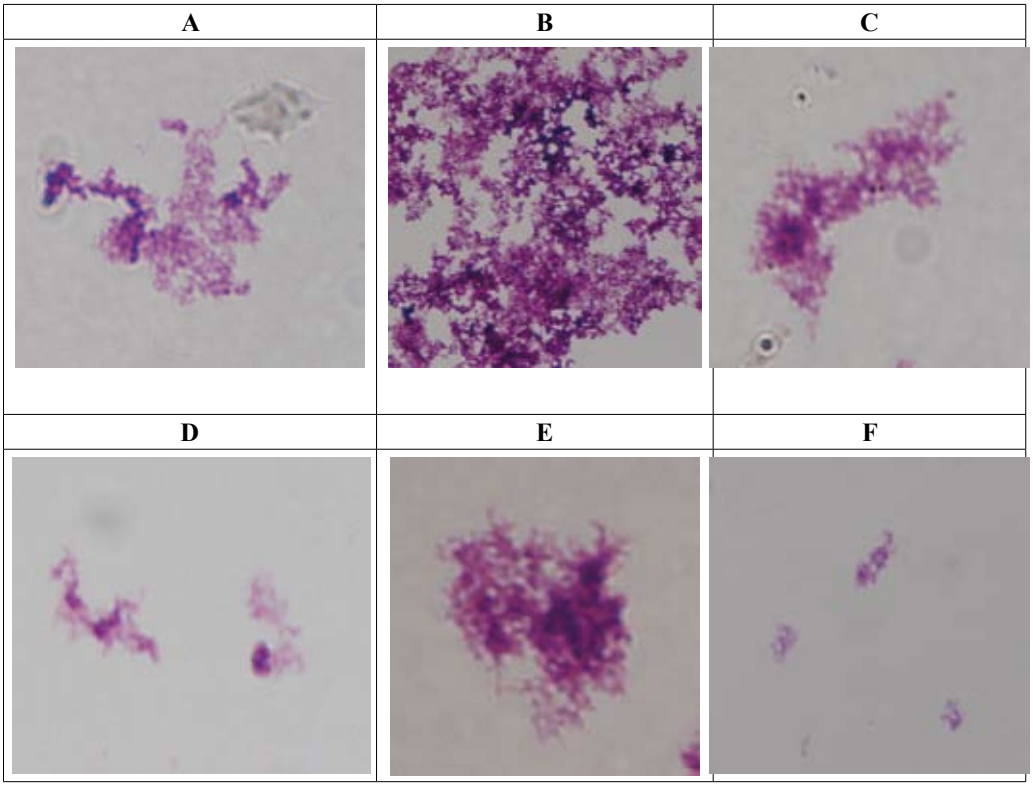

Figure 2. Distribution of platelets between different treatment groups in the bronchial lavage of Balb/c mice. Control **(A),** Asthma **(B),** Cortisone **(C),** *Withania* **(D),** Selenium **(E),** Combination **(F)**

Table 2a. White blood cell count in the blood smears of Balb/ c mice (100 cells counted)

	Control	**Asthma**	**Withania**	**Selenium**	***Withania*** **+ 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

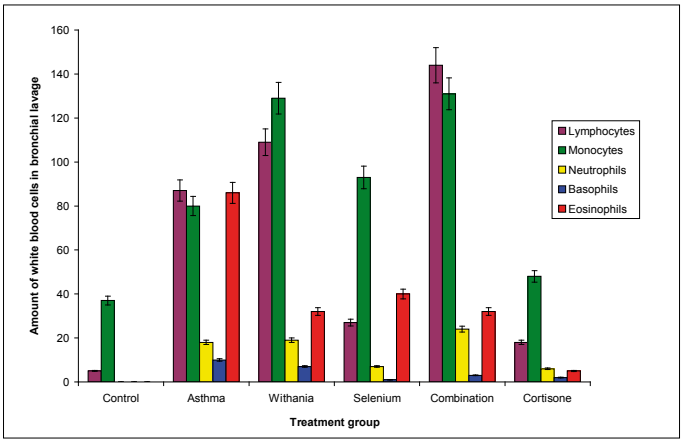

Figure 1. Amount of white blood cells counted in the bronchial lavage of asthmatic Balb/c mice in the different treatment groups

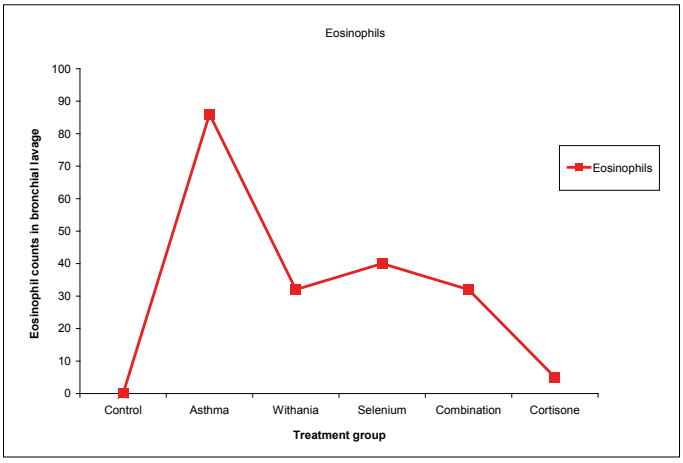

Figure 2. Eosinophil counts in the bronchial lavage in the different treatment groups

cells in bronchial lavage in control, asthmatic mice and those treated with *W. somnifera* alone and in combination with selenium and also shows that of mice treated with hydrocortisone. Table 2 shows the different number of white blood cells counted in the blood smears.

Figure 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. Figure 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.

In asthma, white blood cells play an important role. An inflammatory cascade, which is divisible into seven phases, describes the process of inflamma-

tion in asthma: 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 (Regulated upon Activation, Normal T-cell Expressed and Secreted) was observed in the airways of allergic asthmatics (*Kon & 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 & Schwartz, 2002*). The IgE then attach mostly to mast cells where it can bind allergens, thereby completing the first step in the inflammatory cascade.

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 interleukin-1, interleukin-5, tumour necrosis factor-alpha (TNF-α), and chemokines such as eotaxin and interleukin -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 & 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 & Lemanske, 2001; Prescott, 2003; Lampinen et al, 2004*).

In humans, eosinophils numbers are always increased in the airways and these cells are known to release basic proteins and growth factors that may damage airway epithelial cells and cause airway remodelling (*Kay et al, 2004*). 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*). Also, 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*). 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% cylindric epithelial cells (in percentage non-squamous cells).

It is also known that platelets play an important role in asthma, 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, 2001*). The activation may be due to, amongst others, inflammatory processes (*Camera et al, 1999; Butenas & Mann, 2002*).

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 are very rarely seen in bronchial lavage from healthy mice (*Larsen et al, 2007*). Pinto et al., 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 macrophages (Figure 1B) and the cell counts showed that as well (Table 1 and Figure 1). 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 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 (Figure 2). Platelet distribution also changed significantly in the asthmatic group, when compared to the control bronchial lavage (Figure 2A and B). 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 2C). It is known that 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. In the current study, it was found that 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.

W. somnifera also decreased the number of eosinophils significantly in the bronchial lavage and the effect of selenium and *W. somnifera* 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 *W. somnifera*, selenium and combination (Figure 2D, E, F) treatments.

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 (Table 2). *W. somnifera* and the combination *W. somnifera* and selenium also showed decreased cell numbers, whereas selenium alone did not significantly decrease cell numbers in blood.

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. However, further studies should be embarked upon to investigate the effect of this phytomedicine on immunological parameters.

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