its safety and toxicity therefore further studies are recommended. *Aspalathus linearis* (rooibos) had no effects on the metabolic activity of LPS stimulated RAW 264.7 cells at all concentration tested indicating that this herbal extract is non-toxic even at high concentrations. The popular use of *Aspalathus linearis* (rooibos) over time has contributed to the assumption of its relative safety (Joubert, *et al*., 2008). Many studies have looked at aspects of safety and toxicity of *Aspalathus linearis* (rooibos) however no toxicological studies have been done as yet. The minor component of *Aspalathus linearis* (rooibos), quercetin is suggested to be implicated in its mutagenic effects. However these effects were seen in concentration of 220-230 times more than that of the normal tea drinking quantities (Joubert, *et al*., 2008). The present study provides *in vitro* evidence suggesting that the product is non-toxic. A limitation to this study was that *Aspalathus linearis* (rooibos) was introduced to cells after stimulation which mimics the therapeutic approach to infection. *Aspalathus linearis* (rooibos) is most commonly consumed for prolonged periods as a daily beverage or health drink therefore to have tested *Aspalathus linearis* (rooibos) as a preventative would add more value for *in vivo* application.

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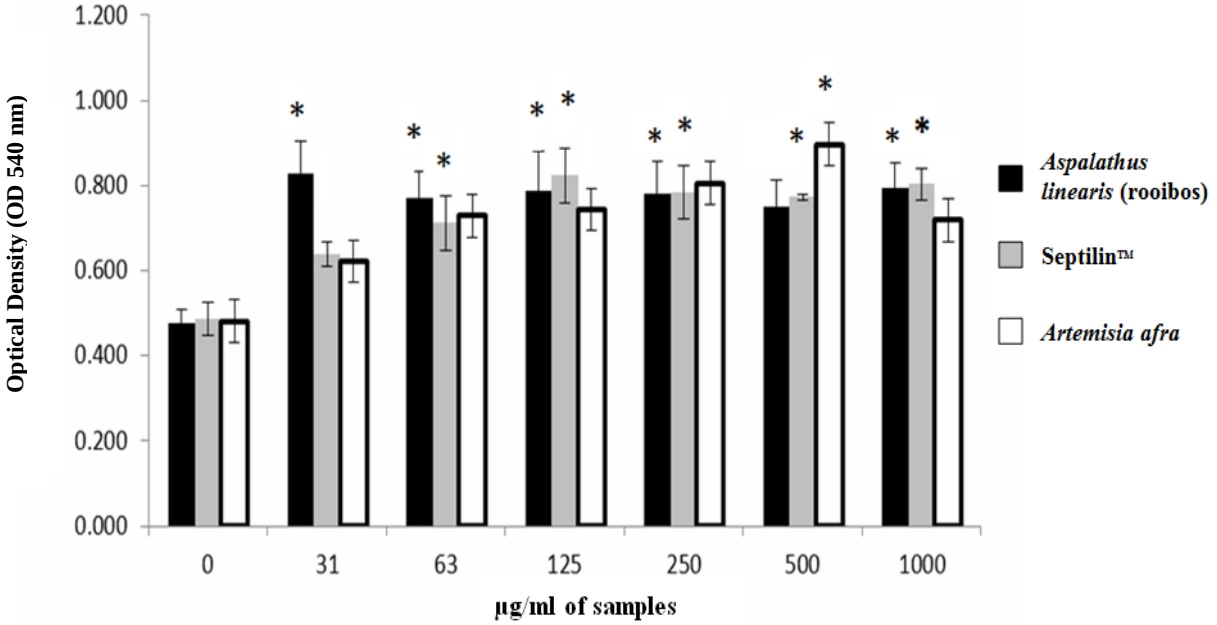

Figure 3.1. Cell metabolic activity of unstimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™. The statistical significant($P<0.001$) difference compared to the control is designated by an asterisk (*).

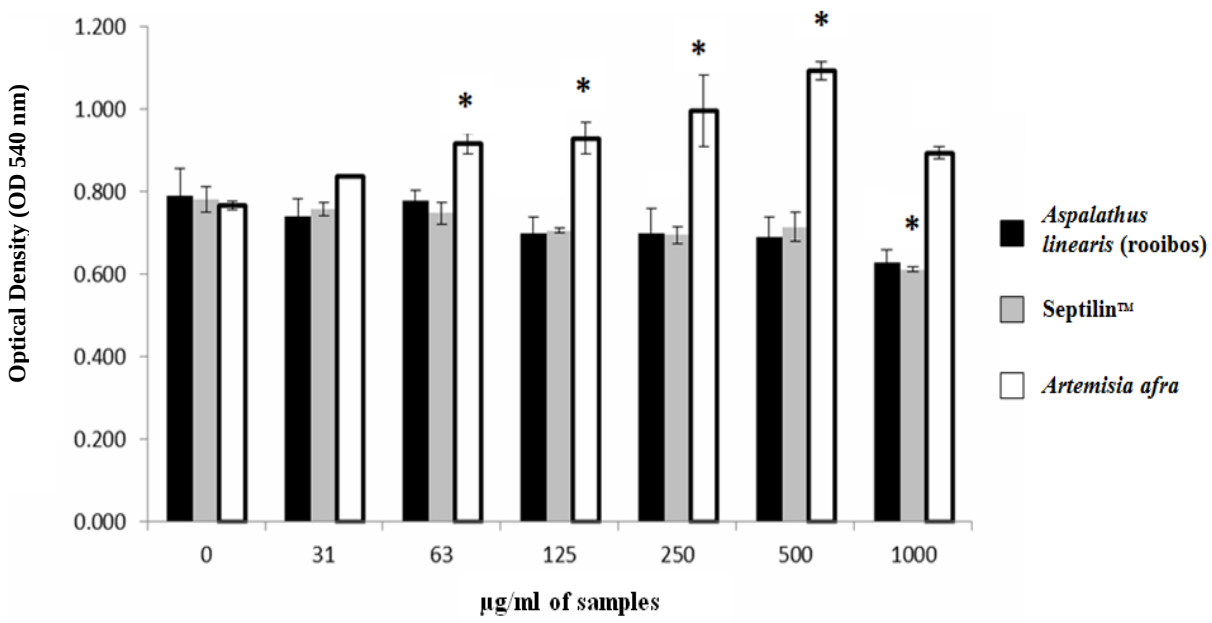

Figure 3.2. Cell metabolic activity of LPS stimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™. The statistical significant ($P<0.001$) differences compared to the control is designated by an asterisk (*)

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3.4.2 The effects of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ on NO production in unstimulated and LPS stimulated RAW 264.7 cells

The overproduction of NO is responsible for inflammation in several pathophysiological conditions like cancer, rheumatoid arthritis, diabetes, liver cirrhosis and septic shock. Inhibition of NO has become the main focus area in the field of anti-inflammatory research (Konkimalla *et al*., 2008). Herbal medicines may be valuable in the modulation of NO. iNOS is a popular investigated enzyme system utilised for *in vitro, ex vivo, in vivo,* animal, or human research on HMPs. Research on herbal medicines in whole, standardized or extract forms are frequently investigated with regards to nitric oxide activity (Bouchard *et al*., 2012).

The standard curve for the NO assay is shown in Figure 3.3. The standard curve was used to calculate the concentrations of NO in samples. The standard curve displays a good correlation (R^2= 0.9995) between the absorbance and NO concentration. Nitrite production, a marker of NO synthesis, was determined in the supernatant of unstimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ (Figure 3.4). There were no significant differences on NO secretion in unstimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra*.

Aspalathus linearis (rooibos) significantly increased (P<0.001) NO production at concentrations of 500µg/ml and 1000µg/ml in unstimulated RAW 264.7 cells. This suggests that *Aspalathus linearis* (rooibos) possess pro-oxidant potential at these concentrations in absence of a stimulus. These findings are contrary to several studies reporting on the antioxidant effects of *Aspalathus linearis* (rooibos) *in vitro* and *in vivo* (Nel *et al*., 2007; Snijman *et al*., 2007; Joubert *et al*., 2008; Baba *et al*., 2009; Marnewick *et al*., 2011; Chen *et al*., 2013; Mahomoodally, 2013; Ku *et al*., 2015; Waisundara and Hoon, 2015; Smith and Swart, 2016). However these findings agrees

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with Persson *et al.*, who reported increased NO production of *Aspalathus linearis* (rooibos) *in vitro* on cultured human umbilical veins endothelial cells at doses of 0-730 µg/ml (Persson *et al.*, 2006). In a follow up *in vivo* study, Persson *et al.*, reported no effect on NO activity in human subjects who consumed 400ml of *Aspalathus linearis* (rooibos) per week for 4 weeks in a randomized three-phase crossover design. Differences between the *in vitro* and *in vivo* studies may be due to differences in the content of the flavonoids or/and the metabolism of the components in the different teas as well as the use of different models (Persson *et al*., 2010). Waisundara and Hoon reported on the antioxidant effects of *Aspalathus linearis* (rooibos) but cautioned against the *in vivo* application of these findings due to the pro-oxidant reports of *Aspalathus linearis* (rooibos) in other studies (Waisundara and Hoon, 2015). *Aspalathus linearis* (rooibos) is mainly consumed as a health promoting beverage as mentioned in previous studies. Its pro-oxidant potential should be considered especially in chronic inflammatory conditions. NO stimulation is responsible for cellular and tissue damage which contributes to numerous inflammatory conditions affecting different organs (Varma *et al*., 2011).

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In this study, Septilin™ had no effect on NO secretion in unstimulated RAW 264.7 Septilin™ had no significant anti-inflammatory effects (NO inhibition) in unstimulated RAW 264.7 cells (Varma *et al*., 2011). This is the second known study which followed a similar model to that of Varma *et al.*, 2011 by assessing anti-inflammatory effects (NO inhibition) of Septilin™ and hence its importance since this herbal preparation is widely used as an anti-inflammatory agent.

The NO production was also determined in the supernatant of LPS stimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ (Figure 3.5). *Artemisia afra* significantly decreased NO production ($P<0.001$) at all concentrations tested (31,25-1000µg/ml) in LPS

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stimulated RAW 264.7 cells. These findings agree with the study of Jang *et al*., (2005) which reported on significant (P<0.01) inhibition of NO production of *Artemisia capillaris* in LPS stimulated macrophage cells. The previously mentioned study on the extract of *Artemisia capillaris* on RINm5F cells reported potent dose dependant inhibition of NO (Kim *et al*., 2007). Three *in vitro* studies on *Artemisia* species reported on the inhibition/reduction of NO secretion in macrophages which suggest the anti-inflammatory potential of *Artemisia* species (Kim *et al*., 2006; Yoon *et al*., 2010; Froushani *et al*., 2016). The results support the anecdotal uses of *Artemisia* species for inflammatory conditions.

An indepth study of the effect of herbal medicine on the immune system requires the use of both *in vitro* and *in vivo* experimentation. *In vitro* models are valuable in evaluating the immunomodulatory effects of herbal constituents (Silliman and Wang, 2006). *Aspalathus linearis* (rooibos) and Septilin™ showed no effect on NO activity on stimulated RAW 264.7 cells. These findings are contrary to that of Varma *et al*., (2011) who reported significant inhibition (P<0.001) of NO in LPS stimulated macrophages by Septilin™. The findings of Varma *et al*., (2011) were tested at concentrations of 2.5% and 5% of Septilin™ which are 25 to 50 fold higher than the concentrations of Septilin™ (31-1000µg/ml) used in this study. Such high concentrations of the herbal product could be unrealistic and problematic if these concentrations were to be extrapolated for *in vivo* application. Mansour *et al*., reported on the reduction of NO secretion in an *in vivo*, radiation induced rat model. In this study liquid preparation of Septilin™ was injected intraperitonially (100 mg/kg b.wt.) for five consecutive days (Mansour *et al*., 2014). Sharma and Ray, 1997 conducted a study using an oral dose of 500mg/kg of Septilin™ in rodents which is equivalent to an intake of 25-50g in humans. These dosages are clearly too high

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which is a common problem found in *in vitro* and *in vivo* studies on herbal medicines (Gertsch *et al*., 2010).

Pre-clinical evaluation of HMPs should begin with *in-vitro* models, by testing cytotoxicity, mutagenicity and acute and sub-chronic safety. These safety studies should be followed by *in-vivo* models at appropriate doses of the HMP's according to internationally accepted standards. Extrapolating doses of the HMPs for *in vivo* application proves to be challenging. Gericke (2011), states that dose-finding studies before formal animal studies are crucial in the preliminary phase to establish efficacy of HMPs

In a comparative study by Jagetia *et al*., (2004) on the nitric oxide (NO) scavenging activities of traditional polyherbal drugs, Septilin™ was tested at the same concentrations (31-1000µg/ml) as this current study. It was reported that Septilin™ inhibited the production of NO in a dose dependent manner up to 125 µg/ml (69.66%) which was followed by a gradual increase of NO production thereafter at the higher doses. The results of Jagetia *et al*., (2004) showed far less efficacy of NO inhibition by Septilin™ to that of Varma *et al*., (2011). This could be due to the differences in the concentrations tested.

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Another contributing factor to differences in findings of these two studies could be attributed to variations that exist in different batches of HMPs. The chemical composition of HMPs differ depending on various factors which includes the botanical species, the anatomical part of the plant used, storage methods, sun, humidity, type of soil, time of harvest, geographic location amongst others. Batch to batch variations can be found within the same manufacturing company which can result in significant variations in pharmacological activities influenced by pharmacodynamic and/or pharmacokinetic factors (Firenzuoli and Gori, 2007).

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Several *in vitro* and *in vivo* studies on the individual ingredients of Septilin™ were conducted on various models with varying effects on NO activity (refer to chapter 2). *Commiphora mukul* (Matsuda *et al*., 2004; Zhang *et al*., 2016), *Rubia cordifolia* (Ghosh *et al*., 2010), *Emblica officinalis* (Yokozawa *et al.,* 2006), *Moringa pterygosperma* (Yokozawa *et al.,* 2006) decreased NO secretion. Most studies of *Tinospora cordifolia* (Desai *et al*., 2007; Upadhyaya *et al*., 2011; Aranha *et al*., 2012; Sharma *et al.,* 2012) reported increased NO production. *Glycyrrhiza glabra* studies reported either increased NO production (Li and Zhou, 2012) or decreased NO production (Franceschelli *et al*., 2011; Thiyagarajan *et al*., 2011). Many studies on the molecular modes of activities of individual herbs have little relevance to its practical application as most herbal medicines are formulations (combinations of several herbs) (Burns *et al*., 2009). These formulas introduce extremely complex mixtures of compounds that may act synergistically to produce therapeutic effects. The overall effect of the formulation may be different to the sum of the individual effects of each herb which makes the study on herbal medicines extremely challenging due to its complex chemistry (Burns *et al*., 2009). The current results are contrary to several *in vitro* and *in vivo* reporting on the antioxidants and/or anti-inflammatory effects of *Aspalathus linearis* (rooibos) previously mentioned. Most of these *in vitro* studies used the ethanolic extract of *Aspalathus linearis* (rooibos) which may account for differences in findings. However in a previous study, Joubert *et al*., (2005) reported on the pro-oxidant activity of the aqueous extracts of *Aspalathus linearis* (rooibos).

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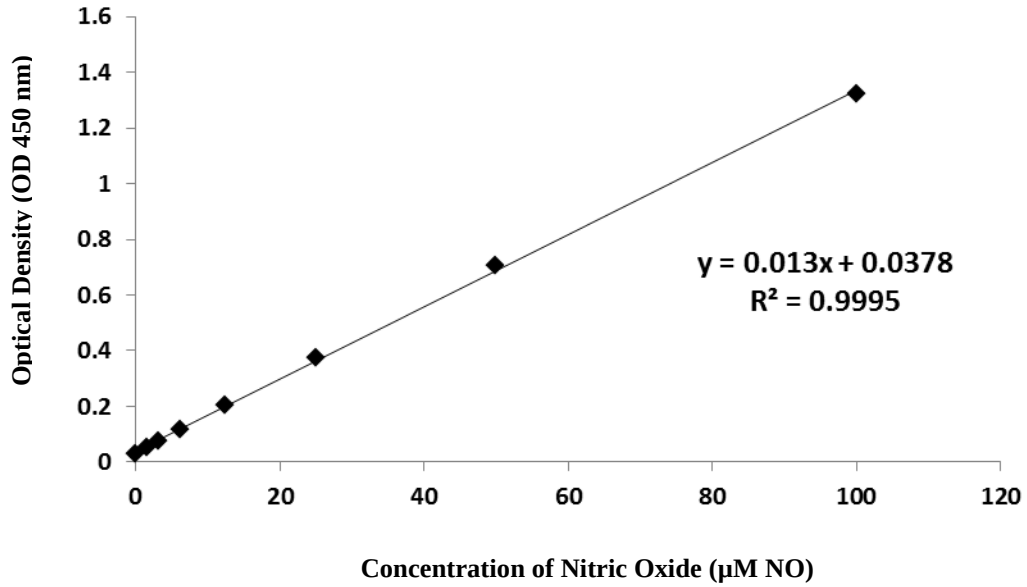

Figure 3.3. Standard curve for NO assay. This standard curve shows a good correlation (R^2= 0.9995) between absorbance readings and NO concentration.

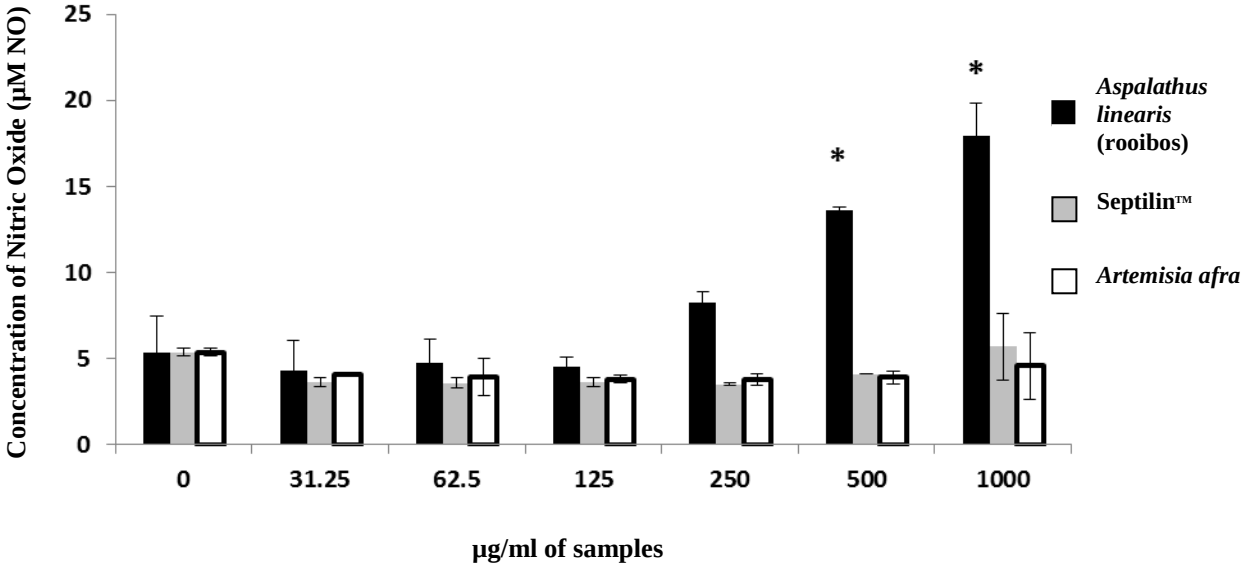

Figure 3.4. NO production in unstimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™. The statistical significant (P<0.001) differences designated by an asterisk (*).

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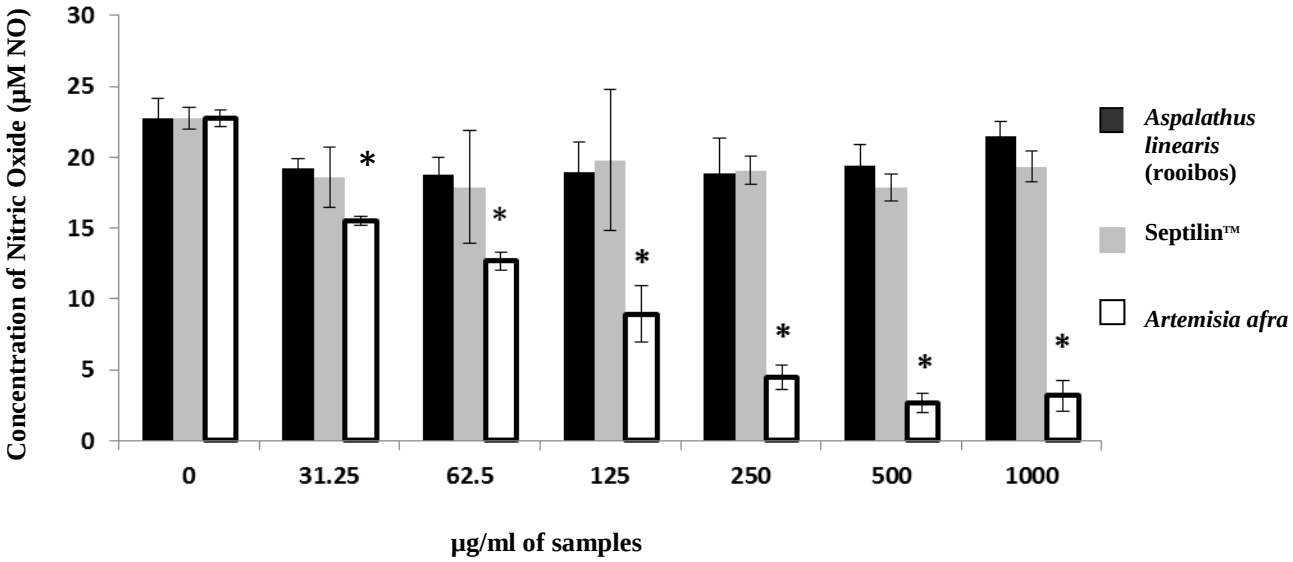

Figure 3.5. NO production in LPS stimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™. The statistical significant ($P<0.001$) difference compared to the control is designated by an asterisk (*).

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3.4.3 The effects of *Artemisia afra, Aspalathus linearis* (rooibos) and Septilin™ on IL-6 production in unstimulated and LPS stimulated RAW 264.7 cells

The cytokine, interleukin-6 (IL-6) is involved in the systemic changes associated with inflammation and infection (Kumar *et al*., 2007). IL-6 concentrations were determined using a DAS-ELISA. The standard curve for the IL-6 ELISA is shown in Figure 3.6. The standard curve was used to calculate the concentrations of IL-6 in samples. The standard curve displays a good correlation (R^2= 0.9991) between the absorbance and IL-6 concentration. IL-6 was used as a biomarker to determine the inflammatory response of LPS on unstimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ (Figure 3.7).

Artemisia afra had no effect on IL-6 production in unstimulated RAW 264.7 cells. These findings correspond to the previously mentioned results for Figure 3.4 which

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shows no effect of *Artemisia afra* on NO production in unstimulated RAW 264.7 cells.

Aspalathus linearis (rooibos) significantly increased (P<0.001) IL-6 production at concentrations of 500µg/ml and 1000µg/ml in unstimulated RAW 264.7 cells. These findings are contrary to most of the previous studies which reports on the anti-inflammatory properties of *Aspalathus linearis* (rooibos) *in vitro* and *in vivo* (Nel *et al*., 2007; Snijman *et al*., 2007; Joubert *et al*., 2008; Baba *et al*., 2009; Marnewick *et al*., 2011; Chen *et al*., 2013; Mahomoodally, 2013; Ku *et al.*, 2015; Waisundara and Hoon, 2015; Smith and Swart, 2016). Most of these *in vitro* studies were conducted using similar concentrations of *Aspalathus linearis* (rooibos) (0-1000µg/ml) as this study however within different models which may account for variations in findings. Mueller *et al.*, conducted a similar study on *Aspalathus linearis* (rooibos) on RAW 264.7 macrophages. Results showed decreased IL-6 at concentrations of 500µg/ml (Mueller *et al.*,2010). Studies on the pro-inflammatory effects of *Aspalathus linearis* (rooibos) are few (Smith and Swart, 2016). However these studies tested the aqueous extract of *Aspalathus linearis* (rooibos) whilst the majority of anti-inflammatory studies on *Aspalathus linearis* (rooibos) were conducted on the ethanolic extract. This maybe due to the presence of different bio-actives in aqueous extracts compared to ethanol extracts.

These current findings suggest the pro-inflammatory effects of *Aspalathus linearis* (rooibos) *in vitro* in absence of a stimulus which corresponds to the results in Figure 3.4, showing that *Aspalathus linearis* (rooibos) induced IL-6 production at concentrations of 500µg/ml and 1000µg/ml in unstimulated RAW 264.7 cells. Up regulation of IL-6 could potentially activate hepatocytes to produce acute phase proteins leading to complement activation allowing phagocytosis. Cellular responses to microbial pathogens could be improved by consuming *Aspalathus linearis*

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(rooibos) tea (Hendricks and Pool, 2010). This suggests that the consumption *Aspalathus linearis* (rooibos) tea could potentially be used for prophylactic purposes. However, important consideration should be given to its possible pro-inflammatory action in midst of inflammation which could lead to or worsen tissue damage. IL-6 is well known to mediate the involvement of inflammatory cells in acute and chronic inflammation (Varma *et al*., 2011). IL-6 is involved in the systemic changes associated with tissue damage, inflammation and infection (Hack *et al*., 2014). In an *in vitro* whole blood culture study on unstimulated WBC, *Aspalathus linearis* (rooibos) also induced higher IL-6 secretion at concentrations between 7.8125 µg/ml - 250 µg/ml (Hendricks and Pool, 2010).

Septilin™ had no effects on unstimulated RAW 264.7 cells. These findings correspond to the previously mentioned results which reported that Septilin™ did not effect NO secretion in unstimulated RAW 264.7 cells (Figure 3.4).

IL-6 was used as a biomarker to determine the inflammatory response on LPS stimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ (Figure 3.8). *Artemisia afra* significantly decreased ($P<0.001$) production of IL-6 by LPS stimulated RAW 264.7 cells in a concentration dependant manner (63-1000µg/ml). These results suggest the anti-inflammatory potential of *Artemisia afra* which also corresponds to the results in Figure 3.5 showing that *Artemisia afra* significantly decreased NO production of stimulated RAW 264.7 cells. These results supports the use of *Artemisia afra* as an anti-inflammatory agent for infectious conditions (Kim *et al*., 2006; Yoon *et al*., 2010; Froushani *et al*., 2016). Several previous studies reported on the anti-infective properties of the active constituents of *Artemisia afra* which includes; camphene, 1,8-cineole, Artemisia ketone, camphor, borneol, terpineol, chrysanthenyl acetate, amyrin amougst others (Abad *et al*., 2012; Liu *et al*., 2009). These constituents amongst

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several others were present in the *Artemisia afra* ethanolic extract tested in this study (see addendum 1) which may have contributed to the anti-inflammatory effects.

Aspalathus linearis (rooibos) did not induce significant changes in IL-6 secretion by stimulated RAW 264.7 cells. These findings are consistent with the results shown in Figure 3.5. (*Aspalathus linearis* did not induce significant changes in NO secretion) but inconsistent with the majority of previous studies which reported on the anti-inflammatory effects of *Aspalathus linearis* by inhibiting/reducing IL-6 secretion (Mueller *et al.*,2010; Swart *et al.*, 2013; Lee and Bae, 2015; Smith and Swart, 2016). In this study Septilin™ showed no effects in IL-6 secretion by stimulated RAW 264.7 cells. These findings are contrary to that of Varma *et al.*, 2011 and others who reported significant inhibition ($P<0.001$) in IL-6 secretion in LPS stimulated macrophages by Septilin™ (Varma *et al*., 2011). The anti-inflammatory effect of Septilin™ has been observed in previous studies (Kumar *et al*., 1997; Sharma and Ray, 1997; Khanna and Sharma, 2003; Daswani and Yegnanarayan, 2002; Jagetia and Balinga, 2004; Varma *et al*., 2011; Manal, 2014) which indicated that Septilin™ suppressed various inflammatory mediators like TNFα, IL-6 and IL-8 in LPS stimulated *in vitro* cell culture models. Studies also showed that Septilin™ inhibits iNOS gene expression, COX-2 enzyme activity and PDE4B gene expression. These are suggested to be the anti-inflammatory modes of action of this herbal product (Varma *et al*., 2011). The current findings are contrary to the previously mentioned studies with regards to the anti-inflammatory effects of Septilin™. A possible reason for this could be due to the use of an aqueous preparation of Septilin™ in this study. A study by Raveendran Nair and Chanda, on the efficacy of medicinal plants against pathogenic bacterial strains reported greater effects by the ethanol extract of the samples than the aqueous extract (Raveendran Nair and Chanda, 2006). An anticancer *in vitro* study compared the effects of fifteen crude aqueous herbal extracts

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to the ethanol herbal extracts against human cancer cell lines. This study reported that the aqueous herbal extracts decreased cell proliferation by more than 50% when compared to the ethanol herbal extracts. Another study also suggested that the ethanol extracts contained the herbal active constituents responsible for the significant results (Sun *et al*., 2007). Further studies should include both ethanol and aqueous extracts of Septilin™, *Aspalathus linearis* (rooibos) and *Artemisia afra* within the same model.

The overall findings of this study suggests the anti-inflammatory effects of *Artemisia afra* and pro-inflammatory effects of *Aspalathus linearis* (rooibos) in RAW 264.7 cells. Septilin™ showed no effects in RAW 264.7 cells.

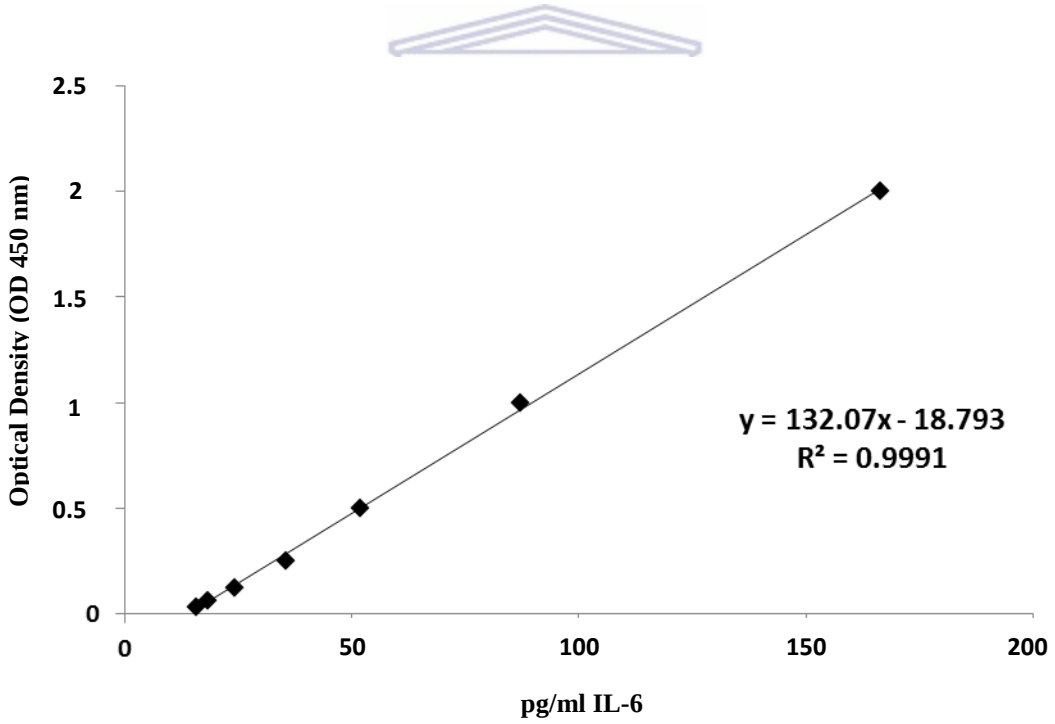

Figure 3.6. Standard curve for IL-6 ELISA. This standard curve shows a good correlation (R^2= 0.9991) between absorbance readings and IL-6 concentration.

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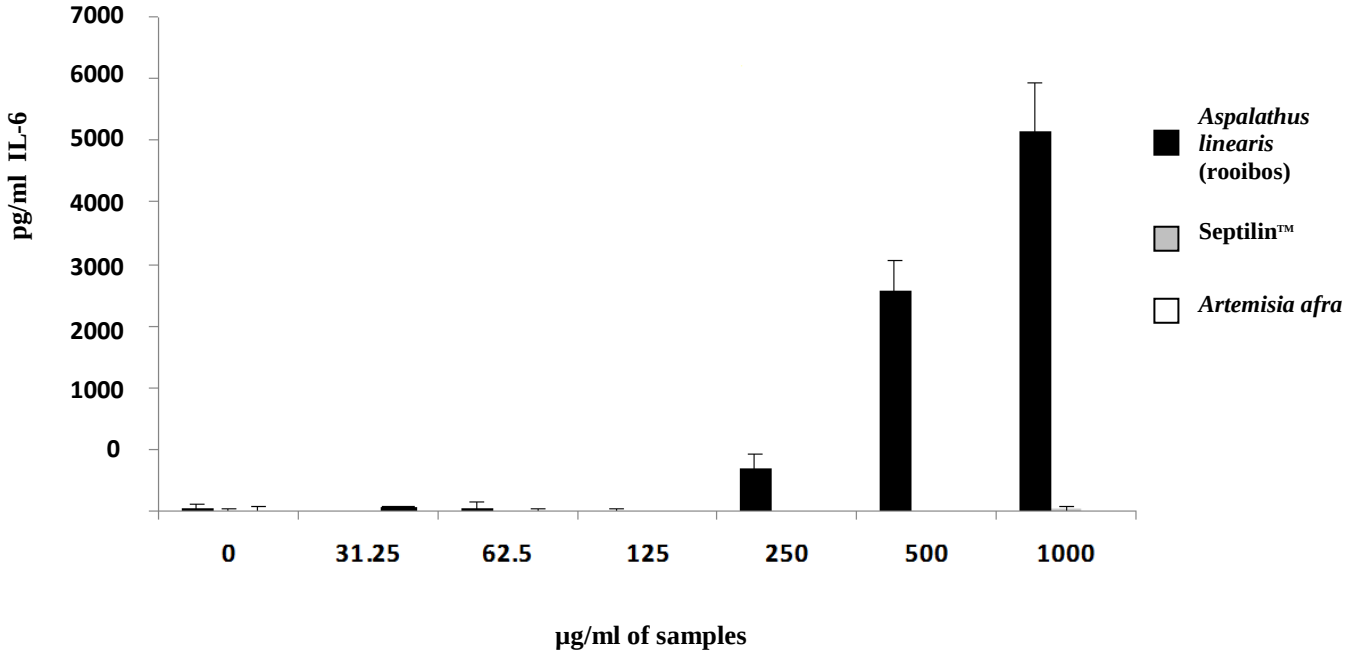

Figure 3.7. IL-6 production in unstimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™. The statistical significant (P<0.001) difference designated by an asterisk (*).

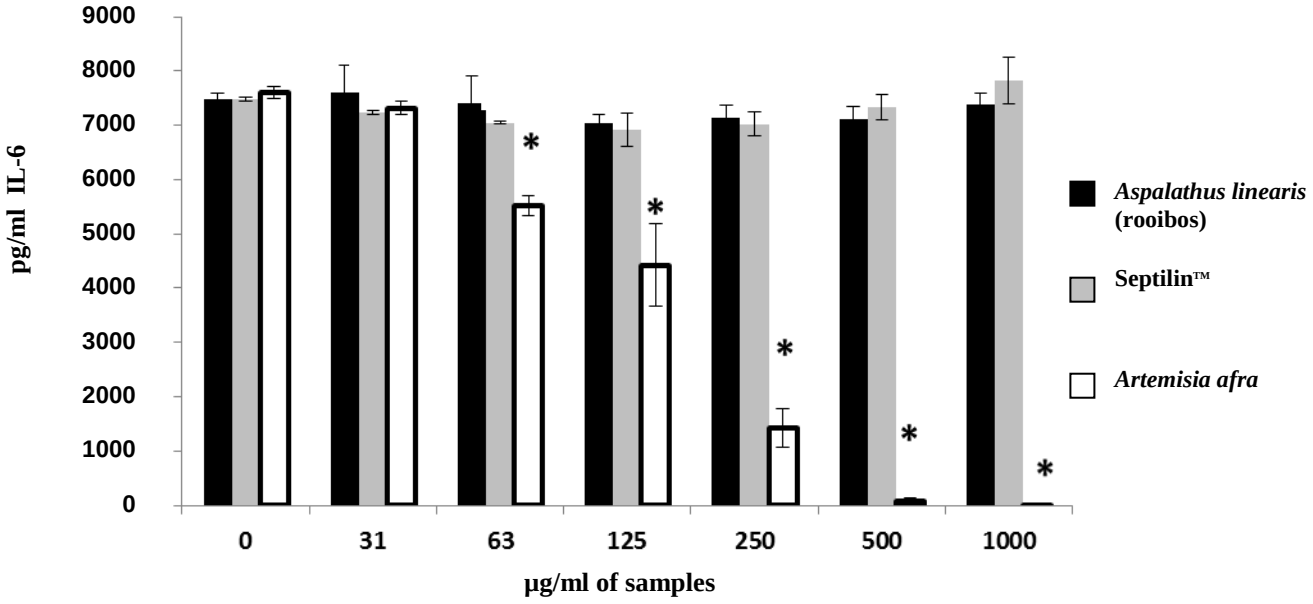

Figure 3.8. IL-6 production in LPS stimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™. The statistical significant (P<0.001) difference compared to the control is designated by an asterisk (*)

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3.4.4 Effects of Septilin™ on IL-6 production in WBC

IL-6 was used as a biomarker to determine the effects of Septilin™ on inflammatory activity in stimulated and unstimulated whole blood cultures (WBC). The standard curve for the IL-6 ELISA is shown in Figure 3.9. The standard curve was used to calculate the concentrations of IL-6 in samples. The standard curve displays a good correlation (R^2= 0.997) between the absorbance and IL-6 concentration. Septilin™ has no effect on IL-6 production in LPS stimulated WBC (Figure 3.10). These findings are contrary to the majority of *in vitro* and *in vivo* studies previously mentioned which reported on the anti-inflammatory effects of Septilin™ via IL-6 inhibition/reduction. The biphasic effect (refer to chapter 2) which refers to the paradoxical responses in cytokine activity by herbal products in both *in vitro* and *in vivo* studies is not an uncommon finding (Spelman *et al*, 2006). Divergent models, dosages, duration of exposure and method of administration of Septilin™ are factors which may explain differences in cytokine expression. In this experimental design blood was first diluted in LPS enriched medium before Septilin™ was added. This mounted an immune response indicated by IL-6 release before the addition of Septilin™. Septilin™ had no effect on IL-6 release in stimulated WBC which suggests that Septilin™ may not be potent enough to serve as a therapeutic intervention during or after infection. However this does not rule out the possibility that Septilin™ may be effective as a preventative treatment. Septilin™ as with many similar herbal products are prescribed as daily health supplements used for preventative treatment. This *in vitro* experimental design only assessed Septilin™ as a therapeutic intervention and not as a preventative treatment.

Addition of Septilin™ to unstimulated WBC resulted in a significantly higher release of IL-6 across all concentrations (16.125µg/ml-258µg/ml) of Septilin™ when compared to the control. This suggests that Septilin™ has a stimulatory effect on IL-6

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production in the absence of a stimulus. These findings differ from the previous data on RAW 264.7 cells which shows no effect of Septilin™ in both stimulated and unstimulated conditions. Activation of the immune system may be valuable in preventative treatment. On the other hand an overactive immune system is implicated in many pathologies including autoimmunity, chronic inflammatory diseases, systemic vasodilatation, carcinogenesis sepsis and the anaphylactic shock (Gertsch *et al.,* 2010). Residual bacterial endotoxins are known to be highly potent pro-inflammatory agents. A few molecules may induce cytokine expression. Plant extracts and herbal preparations have been reported to contain endotoxin contaminants (Gertsch *et al.,* 2010). The above is an important consideration especially in patients with chronic inflammatory conditions. IL-6 secretion in absence of a stimulus has been noted in previous studies on other herbal products. However, very few studies have reported this on Septilin™ using an *in vitro* whole blood culture model.

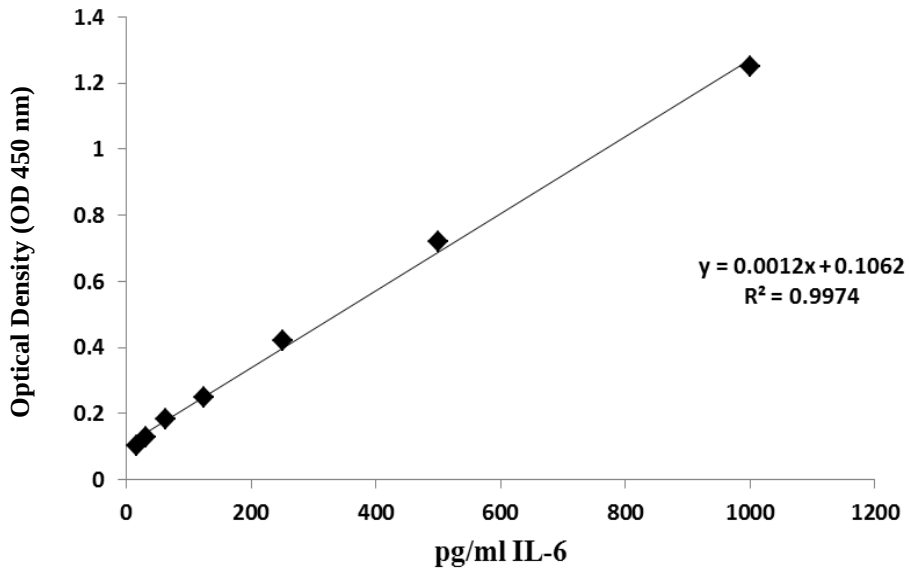

Figure 3.9. Standard curve for IL-6 ELISA. This standard curve shows a good correlation (R^2= 0.997) between absorbance readings and IL-6 concentration.

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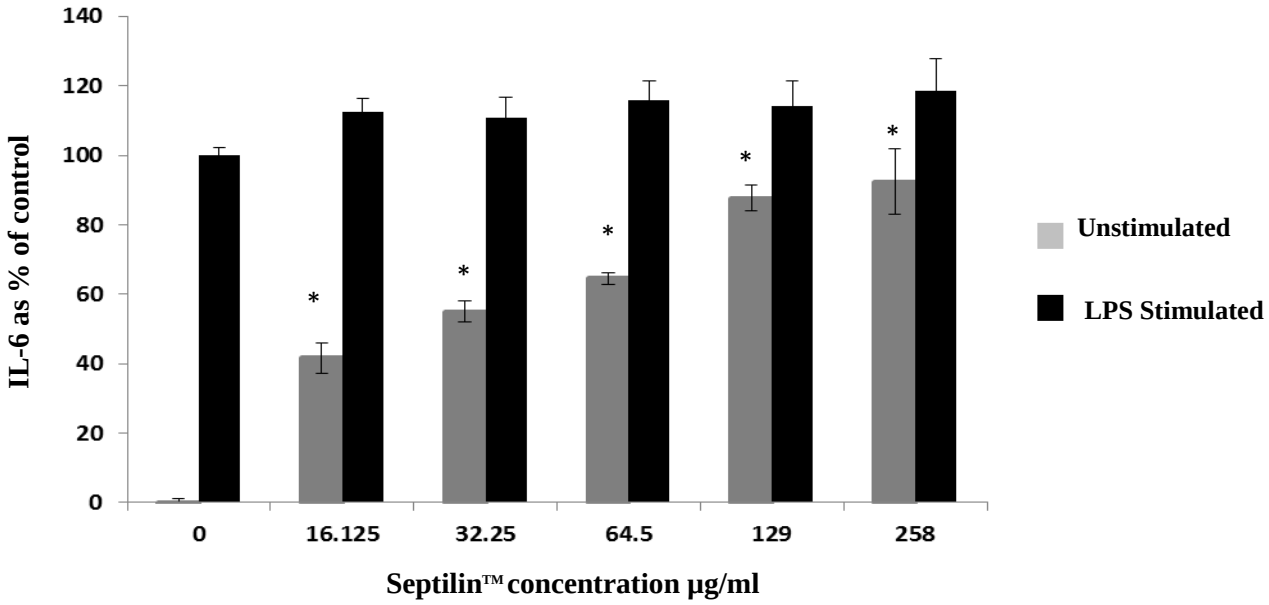

Figure 3.10. Effects of Septilin™ on IL-6 production. IL-6 was used as a biomarker to determine the inflammatory response on stimulated (LPS) and unstimulated WBC in the presence of Septilin™ across various concentrations. The statistical significant ($P<0.001$) difference compared to the control is designated by an asterisk (*)

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3.4.2 Effects of Septilin™ on IL-10 production in WBC

IL-10 was used as a biomarker to determine the effect on humoral immunity of stimulated and unstimulated WBC in the presence of Septilin™. The standard curve for the IL-10 ELISA is shown in Figure 3.11. The standard curve was used to calculate the concentrations of IL-10 in samples. The standard curve displays a good correlation (R^2=0.973) between the absorbance and IL-10 concentration.

Septilin™ has no effect on the release of IL-10 release by unstimulated WBC (Figure 3.12). Addition of Septilin™ to PHA stimulated WBC resulted in a significantly ($P<0.01$) higher release of IL-10 between 64.5µg/ml-258µg/ml of Septilin™ when compared to the control.

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B cells are known to play an important role in the immune system (Mion, *et al*., 2014). IL-10 (Th2-type response) is a well known immunosuppressive and anti-inflammatory cytokine which counteracts the effects of IL-6. IL-10 inhibits IFNγ production and Th1 cells. Both exogenous infectious signals and endogenous immune mediators induces IL-10 secretion (Mion, *et al*., 2014). The results obtained for this study suggests that Septilin™ may induce anti-inflammatory effects by means of IL-10 secretion. IL-10 is a known anti-inflammatory cytokine that acts on macrophages and may regulate the release of pro-inflammatory cytokines (Murphy, 2012). During infection Septilin™ may regulate the inflammatory process by means of increased IL-10 production. IL-10 activates the proliferation and differentiation of B cells, and upregulates Immunoglobulin (Ig) production. These findings agrees with results of Sharma and Ray (1997), who reported a significant increase ($p<0.001$) in both IgM and IgG concentrations in the Septilin™ treated group (*in vivo* rodent model). Very few studies are available on the effects of Septilin™ on IL-10 production by WBC in *vitro*.

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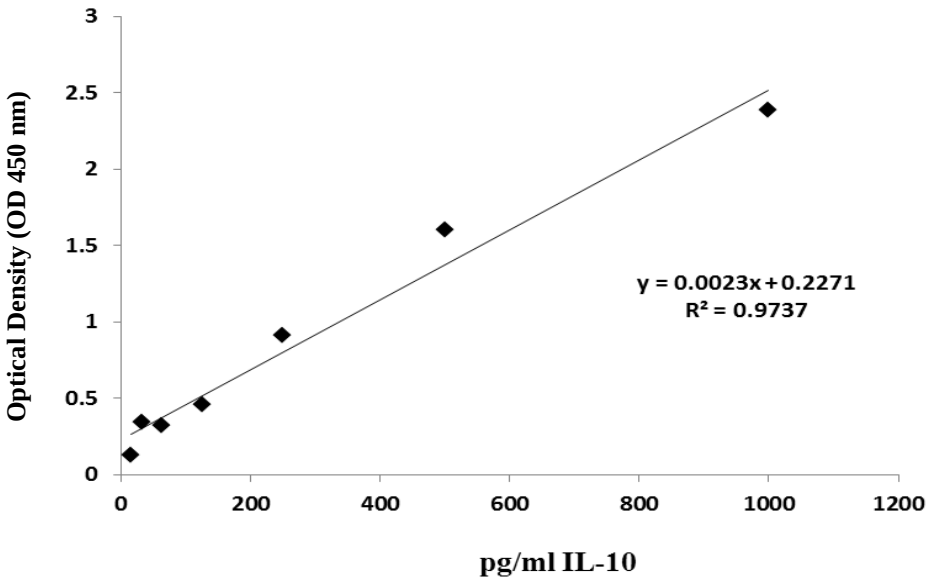

Figure 3.11. Standard curve for IL-10 ELISA. This standard curve shows a good correlation (R^2= 0.973) between absorbance readings and IL-10 concentration.

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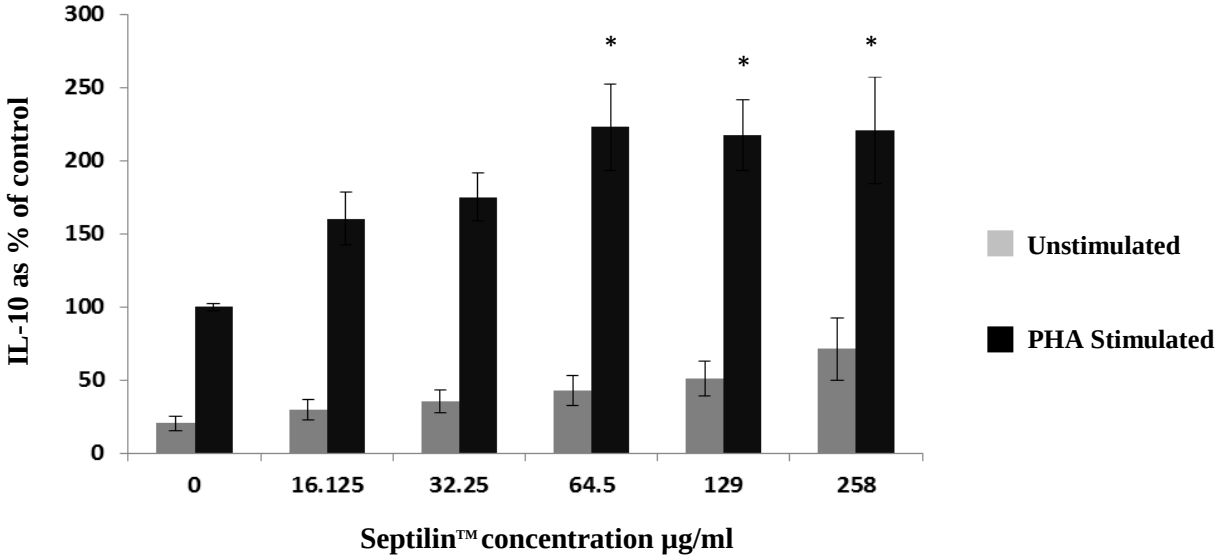

Figure 3.12. Effects of Septilin™ on IL-10 production. IL-10 was used as a biomarker to determine the humoral immune response on stimulated (PHA) and unstimulated WBC in the presence of Septilin™ across various concentrations. The statistical significant ($P<0.01$) difference compared to the control is designated by an asterisk (*).

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3.4.3 Effects of Septilin™ on IFNγ production in WBC

IFNγ was used as a biomarker to determine the effect of Septilin™ on cellular immunity of stimulated and unstimulated WBC. The standard curve for the IFNγ ELISA is shown in Figure 3.13. The standard curve was used to calculate the concentrations of IFNγ in samples. The standard curve displays a good correlation (R^2=0.985) between the absorbance and IFNγ concentration. Septilin™ has no effect on the release of IFNγ production by unstimulated WBC (Figure 3.14).

The effect of Septilin™ on IFNγ synthesis by PHA stimulated WBC was inconclusive. These findings are contrary to previous *in vitro* and *in vivo* (rodent models) findings which assessed the effects of Septilin™ on cell mediated immunity. These studies did not look at IFNγ as a marker of cellular immunity. IFNγ plays an important role in innate and adaptive immunity by increasing macrophage and anti-viral activity

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through Nuclear Kappa (NK) cell activation. This ensures host defences against bacteria and viruses (cell mediated immunity) (Murphy, 2012). Septilin™ is commonly prescribed for colds, influenza and respiratory conditions. This current *in vitro* study indicates that Septilin™ may not be effective in these conditions.

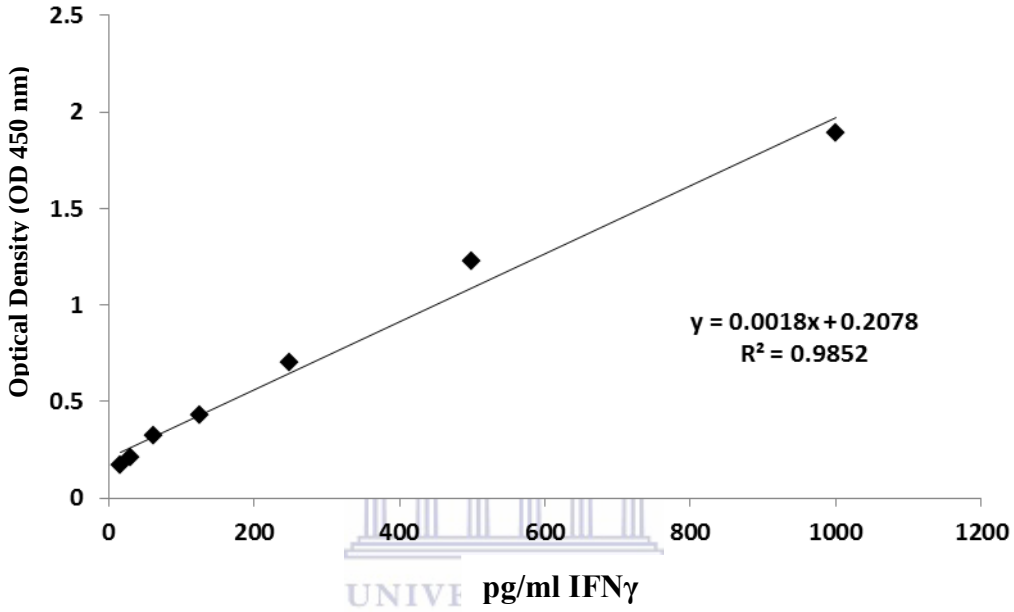

Figure 3.13. Standard curve for IFNγ ELISA. This standard curve shows a good correlation (R^2= 0.985) between absorbance readings and IFNγ concentration.

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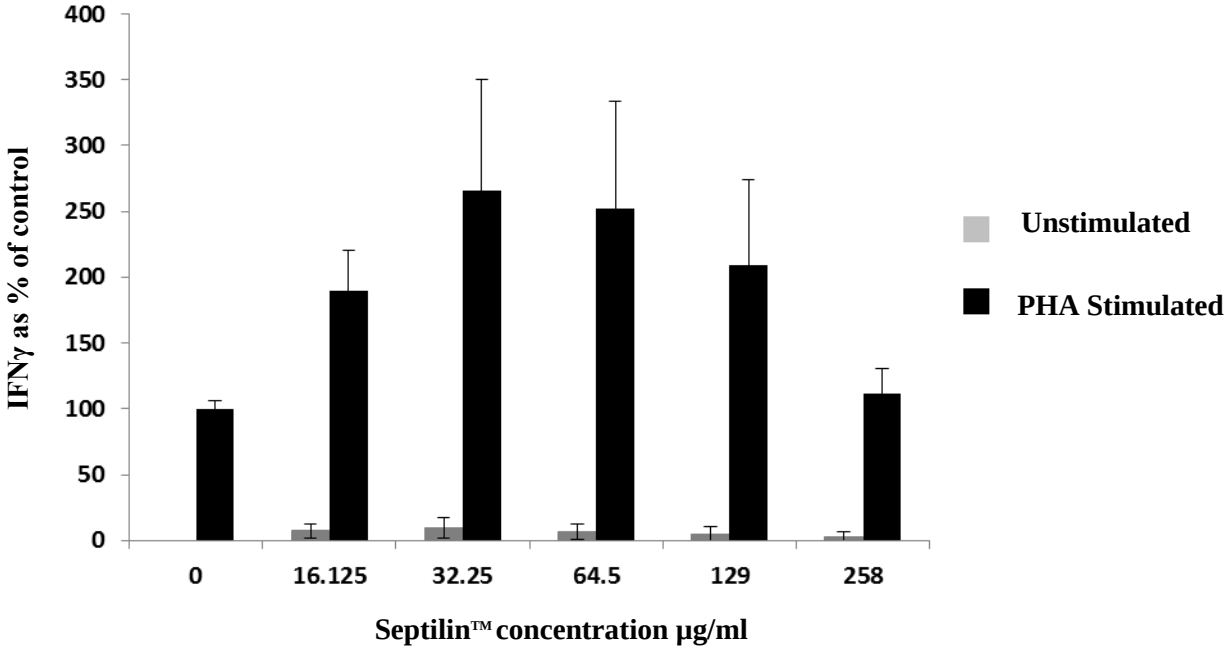

Figure 3.14. Effects of Septilin™ on IFNγ production. IFNγ was used as a biomarker to determine the cellular immune response on stimulated (PHA) and unstimulated WBC in the presence of Septilin™ across various concentrations.

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4. Concluding remarks

This study assessed the effects of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ on inflammatory biomarkers using RAW 264.7 cells. LPS activated RAW 264.7 cells were treated with various concentrations of the above mentioned samples after which the culture supernatants were assayed for specific inflammatory biomarkers namely, IL-6 and nitric oxide (NO). Septilin™ significantly decreased metabolic activity ($P<0.001$) of LPS stimulated RAW 264.7 cells at 1000µg/ml. This has not been reported before. These findings have to be confirmed in follow up studies to ensure patient safety when high doses are prescribed. Most patients consider HMPs to be safe and free of adverse effects which increase their use of these medicines.

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The anti-inflammatory findings of *Artemisia afra* in this study agree with previous studies as well as with anecdotal uses. *Artemisia afra* may be effective in the treatment of arteriosclerosis, rheumatoid arthritis, sciatica, neoplastic metastasis and non-insulin dependant diabetes mellitus, all of which are marked by elevated IL-6 levels. The pro-inflammamatory findings of *Aspalathus linearis* (rooibos) have been observed in a few previous studies however most studies reports otherwise (anti-inflammatory effects of rooibos). These differences in findings could be due to the extracts (aqueous vs ethanolic) used in these studies. Most reports on the anti-inflammatory effects of *Aspalathus linearis* (rooibos) were conducted using ethanolic extract of *Aspalathus linearis* (rooibos). On the other hand most of the reports on the pro-inflammamatory effects of *Aspalathus linearis* (rooibos) were conducted on aqueous extracts.

This study is contrary to previous reports suggesting the IL-6 inhibitory effects of Septilin™ (Varma *et al*., 2011; Shetty *et al*., 2015). This could be due to the vast differences in concentrations of Septilin™ used in this study when compared to other studies as well as the use of an aqueous preparation of Septilin™. This *in vitro* study shows that Septilin™ was not effective as a therapeutic intervention during infection. However its possible preventative capabilities has not been explored in this study. Therefore further studies are recommended.

This current *in vitro* study on WBC indicates the pro-inflammatory effect (basal) of Septilin™ in the absence of a stimulus. Septilin™ could potentially activate the immune system and contribute to healing and tissue repair. This could activate hepatocytes to produce acute phase proteins leading to activation of complement and allows for the phagocytosis of pathogens. These findings could support the anecdotal use of Septilin™ for prophylactic purposes. Septilin™ is commonly prescribed as a preventative for colds and flu before the winter season. However chronic

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inflammation is implicated in several pathological conditions. Basal pro-inflammatory effects are detrimental in patients with increased allostatic load. This basal pro-inflammatory effect may be due endotoxin contamination. HMPs have been reported to contain various contaminants and residues which could potentially be dangerous to the health of consumers. These could include naturally occurring radionuclides, toxic metals or bacteria (WHO guidelines for assessing quality of herbal medicines with reference to contaminants and residues, 2007). This study indicated an increase in IL-10 production by stimulated WBC exposed to Septilin™. This may contribute to stimulation of B-cells and synthesis of Ig which are consistent with previous *in vitro* and *in vivo* studies. Septilin™ was shown to have anti-inflammatory effects by means of increased IL-10 production which may be beneficial during infection. The effect of Septilin™ on IFNγ production on stimulated WBC were inconclusive. Septilin™ is a polyherbal formulation. Many of its herbal constituents have known immunomodulatory effects when tested as single ingredients. Possible herbal interactions should be investigated for future studies. This *in vitro* study indicates to the immunomodulatory effects of Septilin™ in WBC. This study also indicates that *Artemisia afra* has anti-inflammatory effects while *Aspalathus linearis* (rooibos) up regulated the immune system and Septilin™ showed no effects on RAW 264.7 cells.

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Chapter 4

Summary, concluding remarks and recommendations

4.1 Summary

The increased popularity of the use of herbal medicinal products (HMP) is a global phenomena. HMPs include raw herbs, herbal preparations and herbal teas which are used to treat various illness conditions. Herbal immunomodulators can alter immune function and may be used as alternatives or as adjunct treatment to allopathic medicines that possess harmful adverse effects. Many of these herbal immunomodulators are prepared from combinations of medicinal plants which may stimulate, down regulate, inhibit or have no effect on immune pathways. These effects can be easily monitored and understood on isolated constituents and single herbal extracts. However, polyherbal formulations present a complex mixture of chemistry into a human system. Septilin™ a polyherbal HMP, has been reported to have immunomodulatory properties. Septilin™ is prescribed by many complementary and alternative medicine (C&TM) practitioners for inflammatory and infectious conditions. Septilin™ is also available as an over the counter medication in health stores and pharmacies. Most of the studies on Septilin™ are conducted by its manufacturing company with very few citations on its immunomodulatory action using suitable *in vitro* models. According to the Medicine Control Council (MCC) regulation on C&TM in South Africa (SA), Septilin™ falls within the high risk level category due to the claims of efficacy by the manufacturer and therefore requires continuous scientific validation via relevant *in vitro, in vivo* and clinical trial studies. Scientific data on Septilin™ and other HMPs are crucial to the safety of patients and to the regulation of the practice of C&TM in SA, Africa and the rest of the world.

This study was undertaken to assess the effects of Septilin™ on immune pathways (*in vitro*) by means of using whole blood cultures (WBC) and RAW 264.7 cells. Enzyme

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linked immunosorbent assays were used to screen for IL-6, IL-10, and IFNγ as biomarkers for inflammation, humoral immunity, and cell mediated immunity, respectively. Results show that the presence of Septilin™ in LPS stimulated whole blood cultures (WBC) has no effect on the release of IL-6 and IFNγ production but stimulated IL-10 production. Septilin™ in unstimulated WBC has no effect on the release of IL-10 and IFNγ production but stimulatory effects on IL-6 production. These findings agree with previous *in vitro* studies on the effects of Septilin™ on IL-10 and IFNγ production. However, it disagrees with previous studies with regards to its anti-inflammatory action using IL-6 as a biomarker for inflammation.

This study also assessed the effects of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ on inflammatory biomarkers namely, IL-6 and nitric oxide (NO) using RAW 264.7 cells, a murine macrophage cell line. The results of this study indicate that *Artemisia afra* has anti-inflammatory effects while *Aspalathus linearis* (rooibos) up regulated the immune system. The study also shows that Septilin™ has no immunomodulatory effects on RAW 264.7 cells. These findings were inconsistent with previous *in vitro* studies on Septilin™.

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4.2 Concluding remarks

The LPS stimulated cell system has become an accepted tool in the detection of new anti-inflammatory drugs. *In vitro* whole blood cell cultures and RAW 264.7 cells are effective instruments used for assessing the effect of HMPs on the immune system. The *in vitro* data produced from this study support the humoral stimulating effects of Septilin™ as reported by its manufacture and previous studies. Stimulation of humoral immunity may increase antibody production and improve host defence against bacterial and parasitic infections. Septilin™ had no effect in the release of IFNγ in PHA stimulated WBC. Septilin™ up regulated IL-6 production in LPS unstimulated

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WBC and displayed no effect in LPS stimulated WBC. This is contrary to previous studies that reported anti-inflammatory effects. This study suggests that Septilin™ may have prophylactic benefits in conditions that requires up regulation of IL-6. This study also differed to previous studies in relation to the effects of Septilin™ on inflammatory biomarkers namely, IL-6 and nitric oxide (NO). One possible reason for this is the differences in dosages of the herbal product used in previous studies. Many of the previous *in vitro* studies used high to extremely high dosages of the herbal product. High doses used in previous studies may be cytotoxic when applied *in vivo*. Previous studies have reported on the vast differences between the efficacy of ethanol and aqueous herbal extract *in vitro*. Future studies conducted on these herbal medicines should include both ethanol and aqueous extracts.

The results of this study indicate that *Artemisia* has anti-inflammatory effects which agree with all previous studies as well as anecdotal uses. *Aspalathus linearis* (rooibos) up regulated the immune system which agrees with a few previous studies whilst most studies reports on its anti-inflammatory effects. These current findings suggests that the aqueous extract of *Aspalathus linearis* (rooibos) may account for the its pro-inflammatory effects *in vitro*.

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4.3 Recommendations

This is the first study to report on the immunomodualtory effect of Septilin™ using WBC and RAW 264.7 *in vitro* in one study. This is the first study that observed that high doses of Septilin™ decreased metabolic cellular activity of stimulated RAW 264.7 cells *in vitro*. Further studies on toxicity are recommended. This is an important factor to consider when prescribing this medication as incorrect/high dosages could have detrimental effects in patients. However further studies are needed to confirm these findings. This study also differed with previous studies with

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regard to the anti-inflammatory activities of Septilin™. *In vitro* studies on HMPs should be standardised and regulated in terms of dosages used. Concentrations used *in vitro* should be calculated based on anecdotal dosages with the objective of extrapolating *in vitro* data for *in vivo* application. This could contribute to an efficient system of researching HMPs in Africa which could be cost effective and less time consuming. Future studies on these HMPs should consider that different batches of the product may produce varying results due to the complex chemistry of HMPs. Also, there are vast pharmacological differences between aqueous and ethanol extracts of HMPs therefore studies should include both forms. Divergent *in vitro* cell models produce varying results regarding cytokine and NO activity by HMPs.

Future research into African HMPs should include collaborations between C&TM practitioners, microbiologists and phytochemists to ensure better research outcomes. This is essential to ensure the safety and efficacy of African HMPs.

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Addendum 1 **EPool_UWC_150130**

Central Analytical Facilities (Stellenbosch University)

Requested by: Edmund Pool
Company: UWC

Scope: GCMS Analysis of Artemisia Afra Extract Sample

ArtemisaAfra Extract			
RT	Library/ID	Qual	Area Pct
2.4254	tricyclene (Tricyclo[2.2.1.0.(2,6)]heptane, 1,7,7-trimethyl)	96	0.35
2.7712	Camphene	97	7.53
3.3356	1,3,5-Trimethylbenzene (Mesitylene)	97	0.25
3.4132	2,5,5-trimethyl-3,6-heptadien-2-ol (yomogi alcohol)	87	0.36
3.6178	alpha-Terpinene	98	0.47
3.7236	Benzene, 1-methyl-2-(1-methylethyl)- (p-Cymene)	97	3.97
3.8224	1,8-CINEOLE	99	18.15
3.8859	CIS-OCIMENE	97	0.17
4.2316	ARTEMISIA KETONE	87	36.05
4.4362	ARTEMISIA ALCOHOL	72	0.15
4.4926	alpha-terpinolene	98	0.20
4.6196	ISOAMYL-2-METHYL BUTYRATE	86	0.53
4.662	2-Methylbutyl 2-methylbutyrate	72	1.01
4.9089	Unkown	47	0.42
4.9583	CIS-SABINENEHYDRATE	35	0.17
5.1417	CAMPHOR	98	6.78
5.2476	Verbenyl ethyl ether	55	0.15
5.3605	endo-Borneol	94	0.83
5.4733	4-Terpineol	96	0.64
5.5298	Verbenyl ethyl ether	50	0.76
5.685	Unkown	94	0.62
5.7555	2-(1-Methylpropyl)-5-methylcyclohexanone	43	0.18
5.8261	6-methylene-6,7-dihydro-4H-thiazolo[2,3-c][1,2,4]triazin-4-one	52	0.15
5.8755	1,5-dimethyl-2-pyridithione	22	0.45
6.0448	Unkown	50	0.31
6.1154	Cuminic aldehyde	91	0.13
6.32	CHRYSANTHENYL ACETATE	64	0.58
6.5669	iso-bornyl acetate	99	0.28
6.6375	Nonanoic acid, ethyl ester	91	0.30
7.4065	.alpha.-Copaene	99	0.23
7.4276	Nonanal diethyl acetal	56	0.35
7.4912	BENZYL ISOVALERATE	83	0.24
7.5405	ETHYL CAPRATE	93	0.16
7.5617	N-TETRADECANE	93	0.19
7.8016	beta.-Caryophyllene	99	0.43
7.9568	beta-Phenylethyl butyrate	78	0.12

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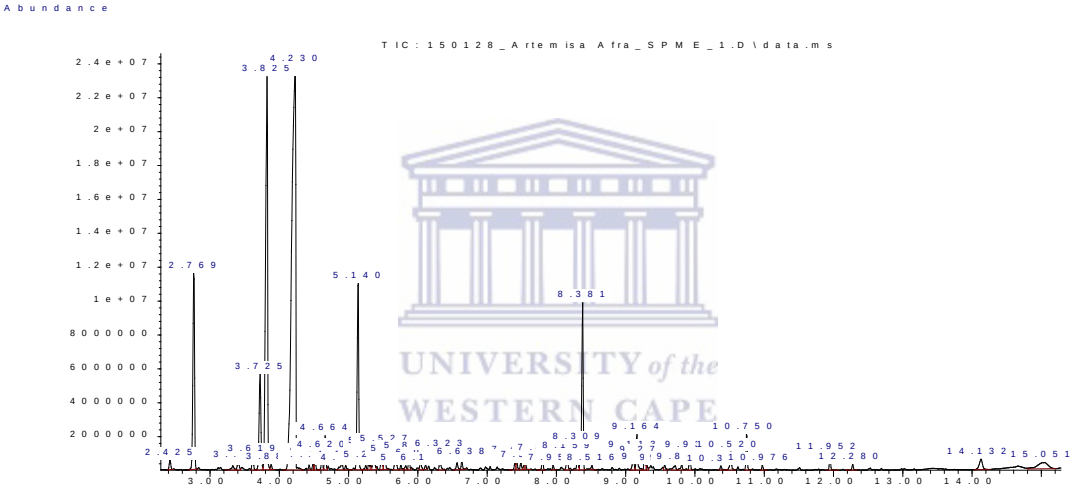
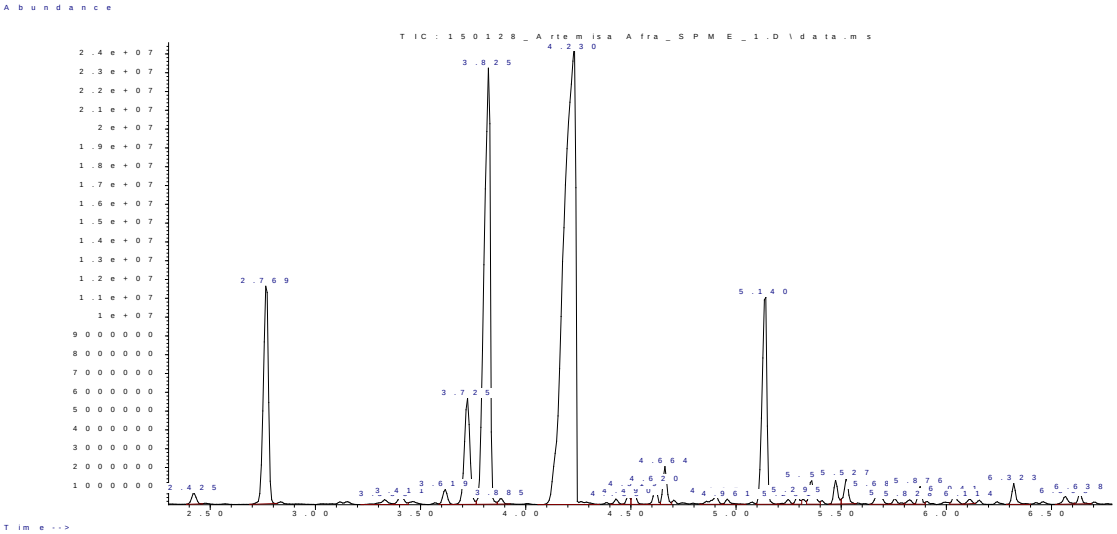
8.1614	ALLOAROMADENDRENE	**99**	**0.56**
8.3096	AR-CURCUMENE	**98**	**0.82**
8.3801	beta-Selinene	**99**	**5.46**
8.5142	beta-Bisabolene	**97**	**0.14**
9.1139	(+) spathulenol	**93**	**0.61**
9.1632	CARYOPHYLLENE OXIDE	**95**	**1.24**
9.2761	n-Butyl-.beta.-phenylpropionate	**49**	**0.44**
9.5654	Tetracyclo[6.3.2.0(2,5).0(1,8)]tridecan-9-ol, 4,4-dimethyl-	**64**	**0.20**
9.8123	alpha-Cedrene oxide	**38**	**0.25**
9.9323	NOOTKATONE	**96**	**0.54**
10.3768	Unkown	**50**	**0.18**
10.5179	Unkown	**90**	**0.65**
10.7507	Unkown	**90**	**1.08**
10.9765	6,10,14-trimethyl-2-Pentadecanone	**97**	**0.15**
11.9501	Ethyl palmitate	**99**	**0.46**
15.0544	beta-Amyrin (beta.-Amyrenol)	**90**	**1.54**





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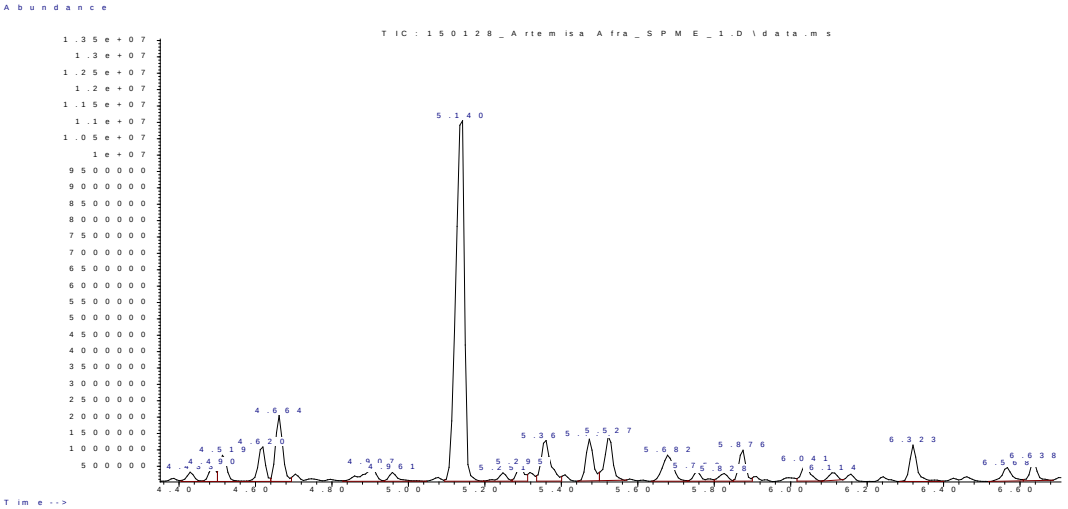

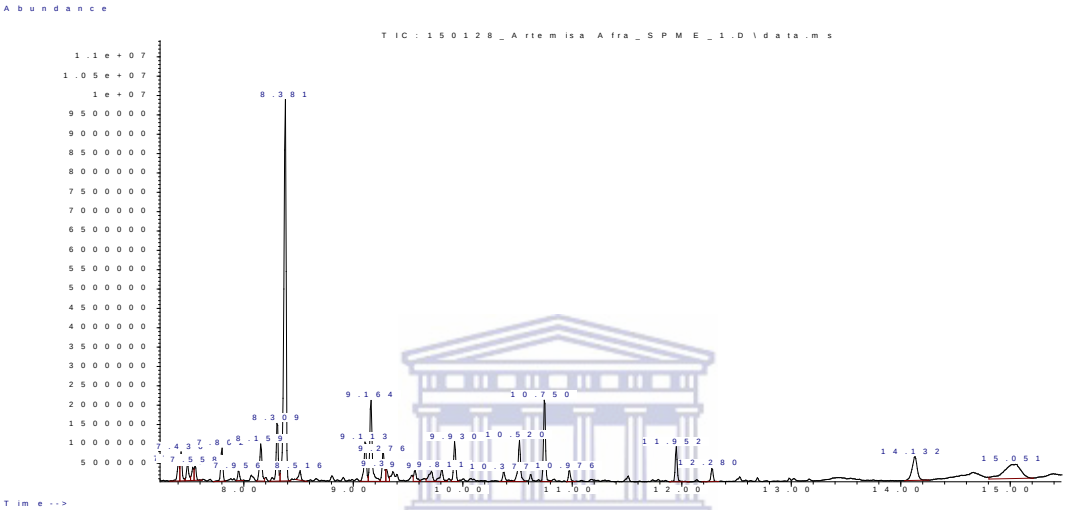

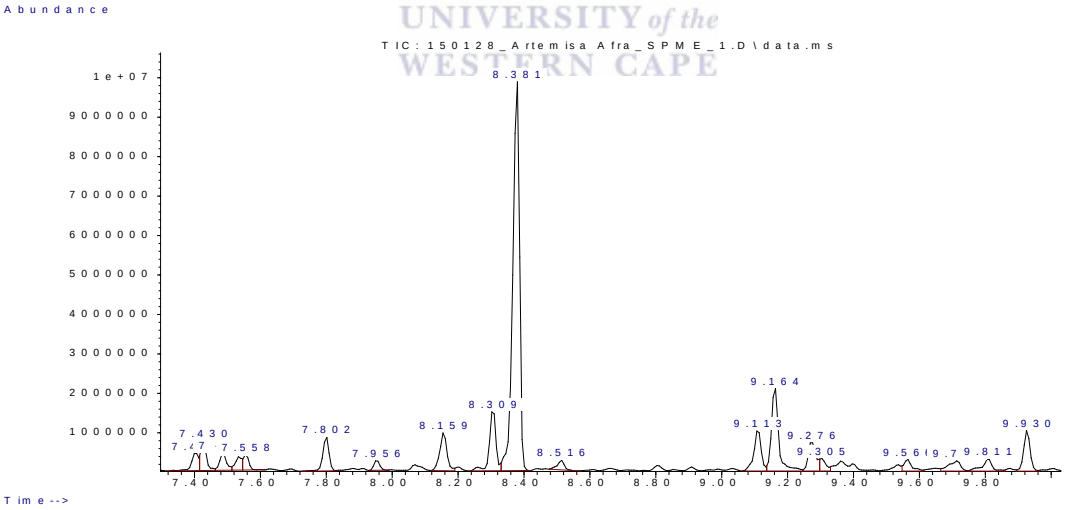

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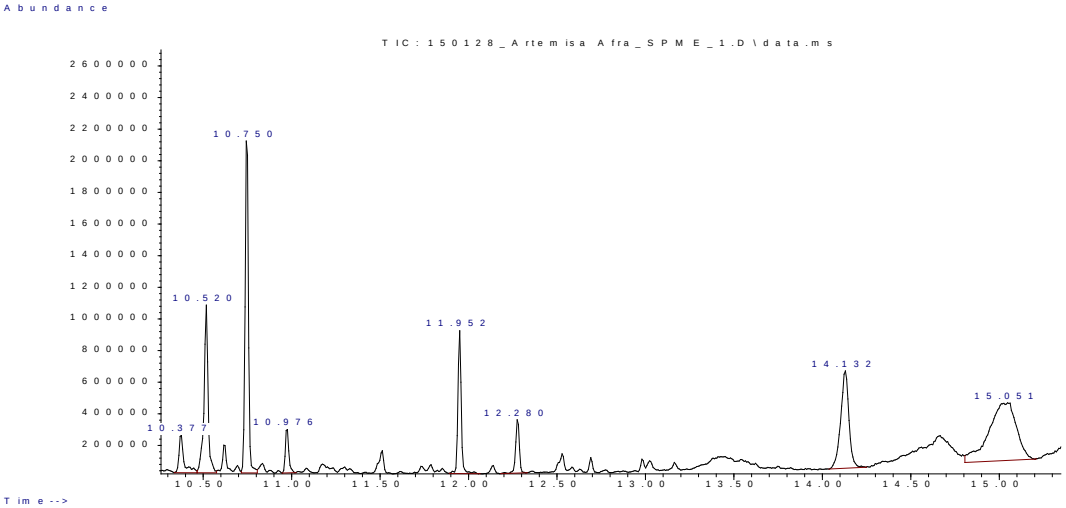

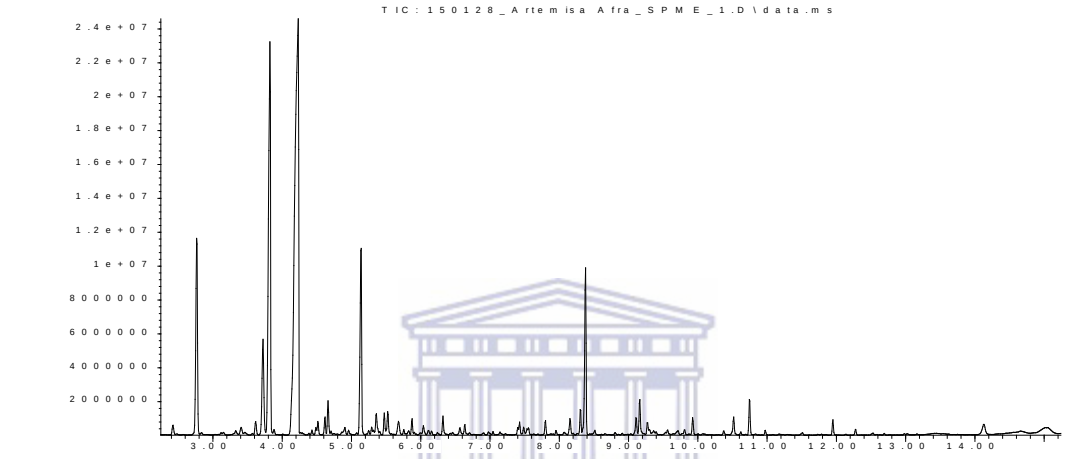

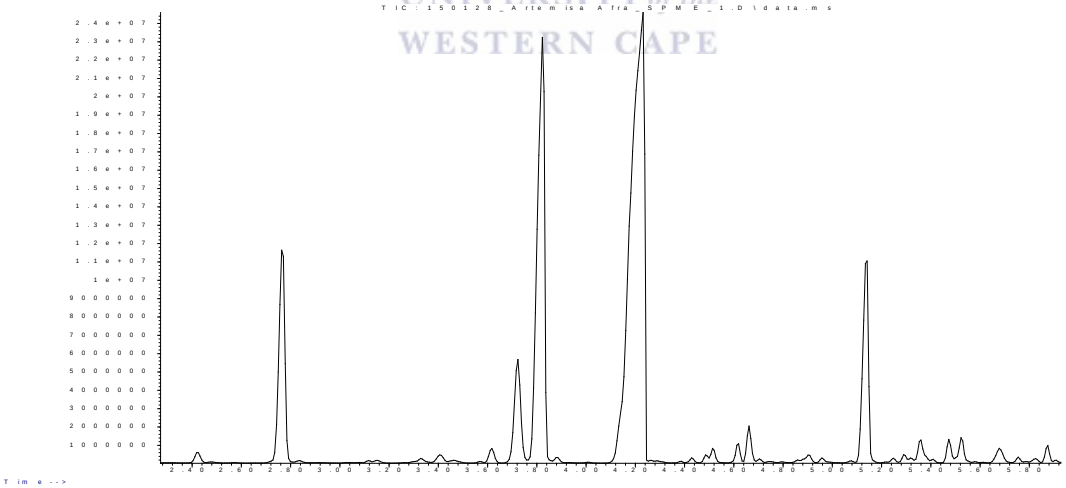

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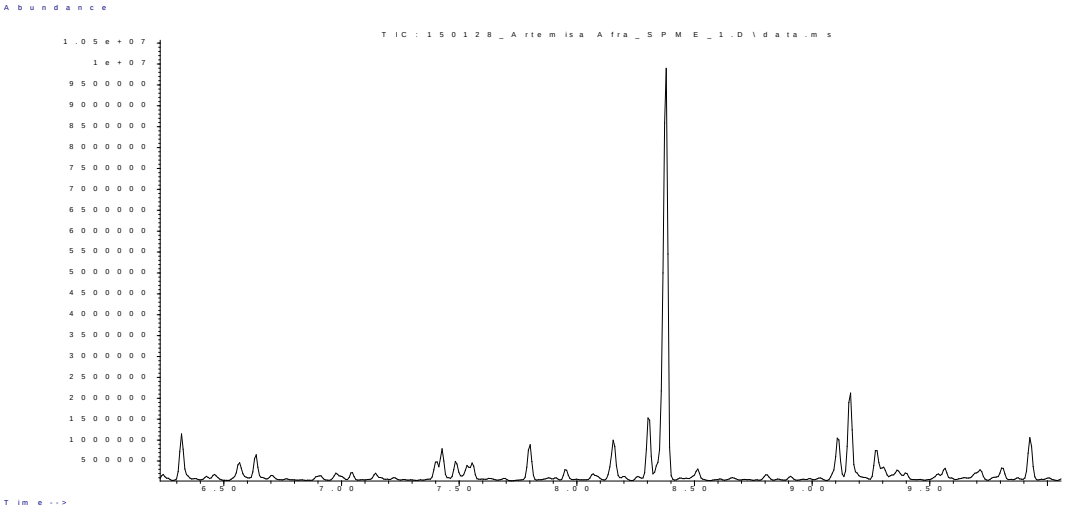

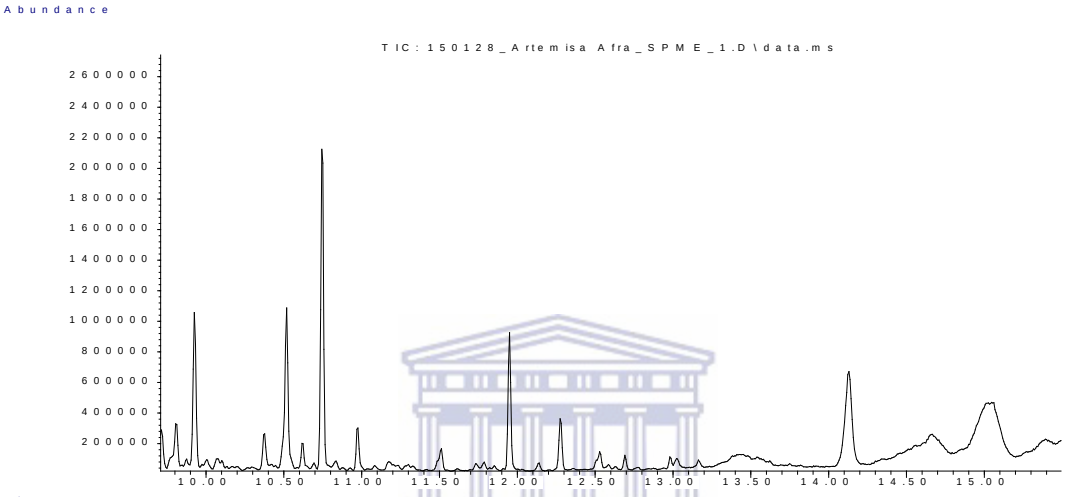

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2. All data and/or samples provided by the Client will be treated as confidential.
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4. Although the greatest care is taken by SU during analysis, SU accepts no responsibility for the loss of any work, samples or data provided by the Client.
5. Data files will not be kept for longer than one week after delivery of the results to the Client.
6. (Please advise the laboratory staff within one week after results have been received if any additional analysis or processing of data is required. It remains the responsibility of our Clients to make proper backups of data.)
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