Original article:

An *In Vitro* Study to elucidate the Effects of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ on Immune Pathways

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Abstract

Objective: Herbal immunomodulatory preparations are increasing in popularity. *In vitro, in vivo* and clinical trial studies are needed to ensure safety, quality and efficacy of these herbal medicines. Septilin™, a proprietary herbal medicinal product has been reported to have immunomodulatory effects. *Aspalathus linearis* (rooibos) is a commercialised South African (SA) tea recognised for its phytopharmaceutical potential. *Artemisia afra* is a well known SA herbal medicine used for various inflammatory conditions. This study assessed the effects of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ on inflammatory biomarkers using RAW 264.7 cells, a murine macrophage cell line. ***Materials and Methods:*** RAW 264.7 cells and lipopolysaccharide (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). ***Results:*** *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ were shown to be non-cytotoxic on unstimulated RAW 264.7 cells across all concentrations tested (31-1000µg/ml). Addition of *Aspalathus linearis* (rooibos) to unstimulated RAW 264.7 cells significantly up regulated ($P<0.001$) NO and IL-6 production at concentrations of 500µg/ml and 1000µg/ml when compared to the control, whilst Septilin™ and *Artemisia afra* had no effect. *Artemisia afra* and *Aspalathus linearis* (rooibos) were shown to be non-cytotoxic on stimulated RAW 264.7 cells across all concentrations tested (31-1000µg/ml). However, Septilin™ significantly ($P<0.001$) decreased metabolic activity at the highest concentration tested (1000µg/ml). Addition of *Artemisia afra* to stimulated RAW 264.7 cells significantly down regulated ($P<0.001$) NO and IL-6 production when compared to the control. *Aspalathus linearis* (rooibos) and Septilin™ samples had no effect on the synthesis of NO and IL-6 in stimulated RAW 264.7 cells when compared to the controls. ***Conclusion:*** *Artemisia afra* has anti-inflammatory effects while *Aspalathus linearis* (rooibos) up regulated the immune system. This study also shows that Septilin™ had no effects on RAW 264.7 cells.

Keywords: *Artemisia afra*, *Aspalathus linearis* (rooibos), Septilin™, nitric oxide, interleukin 6 (IL-6), immunomodulatory, RAW 264.7 cells

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Introduction

Herbal immunomodulatory preparations have been observed to exert anti-inflammatory effects[1]. These formulas may modify the actions of the immune system by influencing the regulation of messenger molecules like cytokines, nitric oxide, hormones, neurotransmitters, and other peptides. These herbal medicinal products (HMPs) are often prescribed for inflammatory and immune-related illnesses[2]. *Aspalathus linearis* (rooibos) tea is a commercialized popular health drink from SA well known for its numerous health benefits including anti-inflammatory and antioxidant effects[3,4,5]. *Artemisia afra* remains one of the most popular SA herbal medicines used for a variety of immune related illness conditions[6]. Septilin™

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is a phytopharmaceutical formulation which is recommended for the treatment and management of several immune related illnesses[6]. Despite the popularity of the above mentioned HMPs there are limited *in vitro* and *in vivo* studies available. In light of the above, this study was undertaken to investigate the *in vitro* effects of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ on inflammatory biomarkers (IL-6 and NO) using RAW 264.7 cells, a murine macrophage cell line. In this study *Artemisia afra* (ethanolic extract), *Aspalathus linearis* (rooibos) (aqueous extract) and Septilin™ (aqueous extract) were tested according to the traditional (common) methods of preparations. *In vitro* dosages of the above mentioned herbal medicines were calculated in relation to common use.

Materials and methods:

This experimental study was conducted at the University of the Western Cape (South Africa), Medical Biosciences Department from June 2013 to December 2014.

Sample preparation:

A 20% (w/v) plant extract of *Artemisia afra* was prepared using 94.4% ethanol (Parceval (Pty) Ltd pharmaceuticals, South Africa). The aerial parts of the *Artemisia afra* plant were milled (sieve size 2-3mm) and mixed with 94.4% ethanol (20g *Artemisia afra*: 100ml ethanol). The milled leaves were separated from the remaining tincture. The tincture was sterilised by filtration using a 0.50nm sterile filter and stored at 4°C. The final extract was air dried and re-suspended in DMSO. Final samples contained a 50% (wet leaf w/v) extract. *Aspalathus linearis* (rooibos) in a tea bag form (net weight: 25g; manufacture date 02/06/2013; expiry date 01/06/2014) was seeped in 500ml of boiling water. The sample was allowed to cool to room temperature. The sample was sterilised by filtration using a 0.50nm sterile filter. Aliquots of the extract 1ml/vial were stored at -80 °C. Septilin™ (net weight: 452mg; batch nr: E281004; manufacture date: 10/08/2011; expiry date: 04/2014) in tablet form was crushed by means of a sonicator then diluted in 35ml of distilled water. The sample was incubated on a shaker for 1 hour at ambient temperature. The sample was then centrifuged at 40 000 rpm for 10mins. After that it was sterile filtered using 0.50nm sterilized filters and stored in 1ml aliquots at -80°C.

Preparation of RAW 264.7:

Cultures were prepared under sterile conditions. Mouse macrophage RAW 264.7 cell line (ATCC-TB-71) was cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat inactivated foetal bovine serum (FBS), 1% antibiotic/antimycotic (Sigma, Germany), 0.05% gentamycin (Sigma, Germany), and 1% Glutamax™, at 37ºC and 5% CO_2. The cells were cultured in 96 well plates at a density of $5x10^5$ cells/ml till they were almost confluent. At this stage the following solutions were prepared: control medium for unstimulated cultures was just the normal culture medium, while stimulation medium for cultures was supplemented with 1µg/ml LPS from *Escherichia coli* 0111:B4 (Sigma, Germany). Extracts of the various products (*Aspalathus linearis*, *Artemisia afra* and Septilin™) were diluted in normal medium to give a concentration range from 0-2000µg/ml. At confluence half the plate received normal medium (unstimulated cultures), while the other half plate received LPS containing medium (stimulated cultures) at 100µl/well. This was followed by the addition of a further 100µl/well of the medium containing various extract concentrations. Final concentration ranges of the extracts were between 0-1000µg/ml. After overnight incubation at 37ºC and 5% CO_2, culture supernatants were collected for NO and IL-6 assays. The cells on a plate were used for cell viability assays.

Metabolic activity and cytotoxicity (WST-1):

The cell metabolic activity and cytotoxicity of RAW 264.7 cells were evaluated using the WST-1 Cell Proliferation Reagent (Rosche, Almere, the Netherlands). The WST-1 conversion assay is based on the mitochondrial integrity of whole cells which allows them to metabolise the stable tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) to a soluble violet formazan product. The metabolic conversion of WST-1 by the cells exposed to various concentrations of the above extracts was assessed in a 96-well microtitre plate. The assay was conducted according to the manufacturer's specifications. Briefly, cells were exposed to various concentrations (0-1000µg/ml) of the above extracts for 24 hours and subsequently incubated with WST-1 reagents for 1 hour. Absorbance was measured using a SpectraMax® spectrophotometer at a wavelength of 450nm. The absorbance of the

extracts in the culture medium, measured in the absence of cells, was subtracted from the total absorbance of the extract treated cells.

Measurement of nitrite formation: Nitrite production was determined in the supernatant of the media by Griess reaction. The reagents for the Griess assay were purchased from Sigma (USA). After the 24 hour incubation of the test cells, cell culture supernatant (100µl/well) was added to a solution of Griess reagent (1% sulfanilamide, 0.1% naphythyl ethylene diaminedihydrochrolide in 5% H^3PO^4), incubated at ambient temperature for 15 minutes to form a purple azodye. The absorption reading at 540nm was determined using a SpectraMax® spectrophotometer. Excel was used to generate a standard curve.

Cytokine analysis (IL-6 ELISA):

The release of the inflammatory biomarker IL-6 was measured in the supernatant of the RAW 264.7 cells after exposure to various concentrations of the plant extracts and Septilin™ for 24 hours at 37°C in a humidified atmosphere of 5% CO_2. The effects of the extracts on IL-6 released from the RAW264.7 cells were assessed using the Mouse Cytokine IL-6 ELISA kit (E-Bioscience kit, Biocom biotech). Cytokine analysis was performed according to the manufacturer's instructions. Briefly, 96 well plates were coated with primary antibody against the IL-6 and incubated overnight at -4˚C. After incubation, the plates were washed with phosphate buffered saline containing 0.05 % Tween-20. Non-specific binding sites were then blocked with assay diluent for 1 hour at ambient temperature after which the wells received either recombinant mouse IL-6 standards or sample. The plate was sealed and incubated for 2 hours at ambient temperature on a shaker. After incubation the wells were washed. The wells then received Biotin-conjugated antibody against IL-6. The plate was incubated for 1 hour at ambient temperature on a shaker followed by washing as before. The wells then received Avidin-HRP conjugate. The plate was incubated for 30 minutes at ambient temperature on a shaker followed by washing as before. After the last wash, the bound peroxidase was monitored by addition of Tetramethyl benzidine substrate (Sigma) solution to each well, after which the plate was incubated for approximately 15 minutes. The reaction was stopped by adding 50µl of 2M H^2SO^4 to each well. The absorbance was read at 450nM on an ELISA plate reader. Excel was used to generate a standard curve for each ELISA plate. This was then used to determine the cytokine concentrations of the culture supernatants.

Statistical analysis:

RAW 264.7 cells experiments was performed in triplicate to confirm reproducibility. All data was captured on excel spreadsheets and were expressed as mean ± standard deviation (SD). The statistical significance of data was analysed via one-way analysis of variance and regression analysis (ANOVA).

Results:

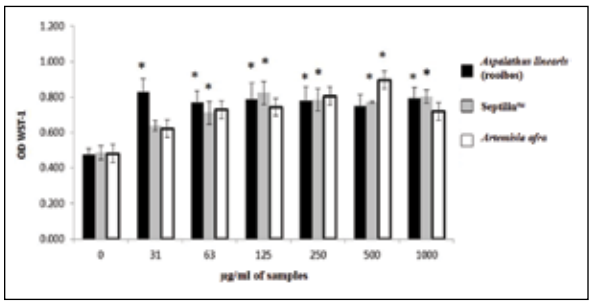

Figure 1a. Cell metabolic activity of unstimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra, Aspalathus linearis* (rooibos) and SeptilinTM. The statistical significance (P<0.001) compared to the control is designated by an asterisk (*).

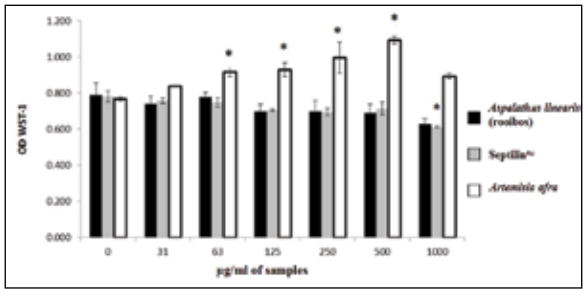

Figure 1b. Cell metabolic activity of LPS stimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra, Aspalathus linearis* (rooibos) and SeptilinTM. The statistical significance (P<0.001) compared to the control is designated by an asterisk (*).

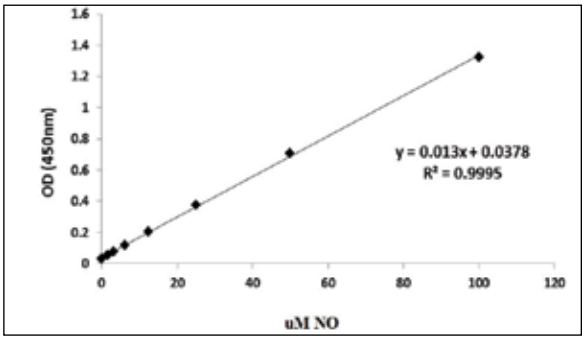

Figure 2a. Standard curve for NO assay. This standard curve shows a good correlation (R2= 0.9995) between absorbance readings and NO concentration.

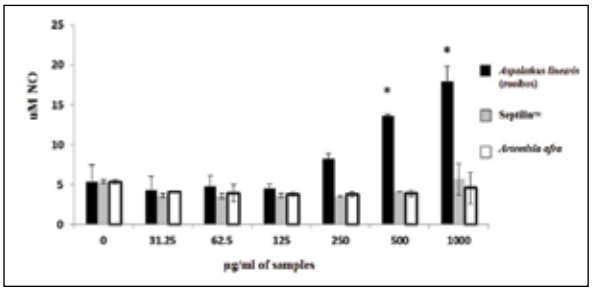

Figure 2b. NO production in unstimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra, Aspalathus linearis* (rooibos) and SeptilinTM. The statistical significant (P<0.001) differences designated by an asterisk (*).

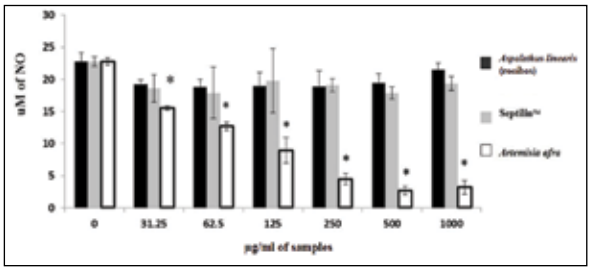

Figure 2c. NO production in LPS stimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra, Aspalathus linearis* (rooibos) and SeptilinTM. The statistical significant (P<0.001) difference compared to the control is designated by an asterisk (*).

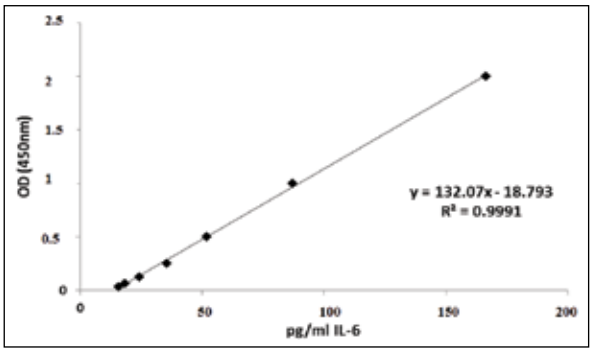

Figure 3a. Standard curve for IL-6 ELISA. This standard curve shows a good correlation (R2= 0.9991) between absorbance readings and IL-6 concentration.

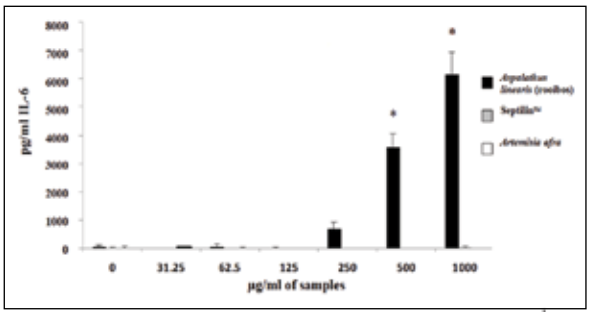

Figure 3b. IL-6 production in unstimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra, Aspalathus linearis* (rooibos) and SeptilinTM. The statistical significant (P<0.001) difference designated by an asterisk (*).

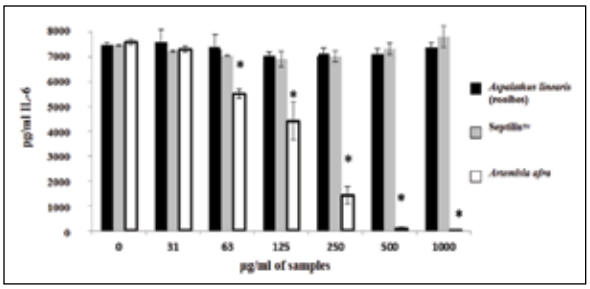

Figure 3c. IL-6 production in LPS stimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra, Aspalathus linearis* (rooibos) and Septilin™. The statistical significant (P<0.001) difference compared to the control is designated by an asterisk (*)

Discussion:

LPS, a bacterial antigen, is a potent activator of a wide range of signalling pathways particularly pathways of inflammation. Inflammatory mediators includes pro-inflammatory cytokines like TNF-α, IL-1, IL-6, IL-8 and also NO and prostaglandins amongst others. NO is a metabolite produced by enzymes, which include inducible nitric oxide synthase (iNOS). The enzymatic activity of iNOS in diverse cell types contributes to the overproduction of NO which is responsible for inflammation in several pathophysiological conditions like cancer, rheumatoid arthritis, diabetes and liver cirrhosis amongst others[8]. An unstable molecule that has lost an electron is referred to as a free radical. Free radicals can oxidise DNA, nucleic acid, proteins or lipids which contributes to degenerative illnesses like cardiovascular diseases and cancers. The production of reactive oxygen species is mechanistically linked to inflammation[9]. The excessive

production of NO and its oxidation product, peroxynitrite has been implicated in several inflammatory conditions. Inhibition of NO has become the main focus area in the field of anti-inflammatory research[10]. Macrophages and monocytes play a crucial role in innate and adaptive immunity. Macrophages affect various immune responses when encountering invading pathogens. The versatile role of macrophages includes antigen recognition, capture, clearance and transport of foreign products. Macrophages stimulated by LPS and microbes elicit the release of various proteins like iNOS which leads to the production of NO[11].

The use of RAW 264.7 mouse macrophage cell lines is a well-established model to determine NO production[9]. The LPS stimulated cell system has become popular in the area of new anti-inflammatory drug discovery[1].

The effect of *Artemisia afra, Aspalathus linearis* (rooibos) and Septilin™ on the metabolic activity of unstimulated and LPS stimulated RAW 264.7 cells:

Cellular proliferation refers to an increase in the number of cells due to cell growth and cell division which result in the increase in subcellular organelles like mitochondria. In all living organisms, tissue growth is dependent on a balance between cell proliferation and cell death. Many drugs affect particular stages of the cell cycle. Cell injury and cell death is a consequence of specific interferences with cell metabolism. Abnormal cell proliferation is the underlying factor to many pathological conditions. Alterations to the cell cycle and cell proliferation plays and important role in immunity[12].

The results on metabolic activity of unstimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ were evaluated using the WST-1 Cell Proliferation Reagent (Figure 1a.). *Artemisia afra* induced a significant increase in metabolic activity ($P<0.001$) at a concentration of 500µg/ml whilst *Aspalathus linearis* (rooibos) induced significant increases in metabolic activity ($P<0.001$) across the concentrations of 31-250 and 1000µg/ml in unstimulated RAW 264.7 cells. Septilin™ induced significant increases in metabolic activity ($P<0.001$) across the concentrations of 63-1000µg/ml in unstimulated RAW 264.7 cells.

These findings suggest that *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ are non-cytotoxic at the above mentioned concentrations in unstimulated RAW 264.7 cell activity. These findings agree with a study on the toxicity of *Artemisia afra* by Mukinda and Syce, who reported that the extract of *Artemisia afra* is non-toxic in acute doses[13]. In a similar study on scoparone, a major constituent of *Artemisia capillaris*, no cytotoxic effects were reported in unstimulated macrophage cells[14]. Another study on the inhibition potential of the extract of *Artemisia capillaris* on cytokine-induced nitric oxide formation and cytotoxicity on RINm5F cells reported no significant difference in cell viability in the absence of a stimulus even at the highest concentrations[15]. Possible differences that exist amongst studies could be due to differences in activities of these two species of *Artemisia.*

These current findings with regards to *Aspalathus linearis* (rooibos) also agrees with previously mentioned studies which confirmed the safety of *Aspalathus linearis* (rooibos). *Aspalathus linearis* (rooibos) has shown no cytotoxic effects at all concentration tested *in vitro* using whole blood cell cultures[16]. *Aspalathus linearis* (rooibos) has gained popularity globally as an accepted nutraceutical. The health-promoting benefits of *Aspalathus linearis* (rooibos) have been confirmed in several *in vitro* and *in vivo* studies[4]. These findings also agree with previously mentioned studies which refer to the safety of Septilin™ as it is widely used as a health supplement[1,7]. A non-randomized non-placebo controlled pilot study using Septilin™ in chronic periodontitis reported no adverse effects in patients indicating to its relative safety[35].

The results of the cell metabolic activity of LPS stimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ were evaluated using the WST-1 Cell Proliferation Reagent (Figure 1b.). Figure 1b (stimulated RAW 264.7) compared to Figure 1a. (unstimulated RAW 264.7) shows that the cell metabolic activity of RAW 264.7 cells exposed to LPS (control) increased cell proliferation. LPS is a well known mitogen (a substance which induces cell mitosis)[33]. *Artemisia afra* induced significant increases in metabolic activity ($P<0.001$) across concentrations of 63-500µg/ml in LPS stimulated RAW 264.7 cells. These findings agree with the previously mentioned study on *Artemisia capillaris* which reported no cytotoxic effects on stimulated macrophage cells[33]. In the previously mentioned study on the extract of *Artemisia capillaris* on RINm5F cells it was reported that the extract restored the cell proliferation potential proportional to its concentration[15]. These findings are more significant than that of the effect of *Artemisia afra* in unstimulated RAW 264.7 cells (Figure 1a.).

Septilin™ significantly decreased metabolic activity ($P<0.001$) at the highest concentration tested (1000µg/ml) in LPS stimulated RAW 264.7 cells. These findings are contrary to the results in unstimulated RAW 264.7 (Figure 1a.). Septilin™ may be cytotoxic at high doses however further investigation would be needed. This could indicate to the importance of dosage

optimisation when prescribing this medication for infectious conditions. Septilin™ is indicated for acute infectious conditions as mentioned in previous studies. Wiesner and Knoss reports that globally, most patients believe that HMPs are safe which improves compliance. The misconception regarding the safety of HMPs may cause patients to misuse these medicines[37]. This is the first study to note decreased metabolic activity at the above mentioned dose (1000 µg/ml) in LPS stimulated RAW 264.7 cells. No toxicological studies have been done on this herbal preparation and the current literature lacks sufficient evidence regarding 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[36]. 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[36]. 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.

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[10]. 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 herbal products. Research on herbal medicines in whole, standardized or extract forms are frequently investigated with regards to nitric oxide activity[38].

The standard curve for the NO assay is shown in Figure 2a. 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 2b). 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 who reports on the antioxidant effects of *Aspalathus linearis* (rooibos) *in vitro* and *in vivo*[3,4,5,8,9]. However, these findings agrees 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 0-730µg/ml[39]. 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[40]. 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[41]. *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[1].

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[1]. 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 2c.). *Artemisia afra* significantly decreased NO production ($P<0.001$) at all concentrations tested (31,25-1000ug/ml) in LPS stimulated RAW 264.7 cells. These findings agree with the previously mentioned study on *Artemisia capillaris* which reported significant ($P<0.01$) inhibition of NO production in LPS stimulated macrophage cells[14]. The previously mentioned study on the extract of *Artemisia capillaris* on RINm5F cells reported potent dose dependant inhibition of NO secretion[15]. Three *in vitro* studies on *Artemisia* species has reported on the inhibition/reduction of NO secretion in macrophages[15,42,43]. These results suggest the anti-inflammatory potential of *Artemisia* species. The results supports the anecdotal uses of *Artemisia* species for inflammatory conditions as previously mentioned.

In depth 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[44]. *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* who reported significant inhibition ($P<0.001$) of NO in LPS stimulated macrophages by Septilin™[1]. The findings of Varma *et al* were tested at concentrations of 2.5% and 5% of Septilin™ which are 25 to 50 fold higher than the concentrations of Septilin™ (31-1000ug/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[45]. 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 which is a common problem found in *in vitro* and *in vivo* studies on herbal medicines[31].

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 up 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. Dose-finding studies before formal animal studies are crucial in the preliminary phase to establish efficacy of HMPs[46].

In a comparative study on the nitric oxide (NO) scavenging activities of traditional polyherbal drugs, Septilin™ was tested at the same concentrations (31-1000ug/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[46]. The results of Jagetia *et al* showed far less efficacy of NO inhibition by Septilin™ to that of Varma *et al*. This could be due to the differences in the concentrations tested.

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 pharmacodynamics and/or pharmacokinetic factors[46]. Several *in vitro* and *in vivo* studies on the individual ingredients of Septilin™ were conducted on various models with varying effects on NO activity. *Commiphora mukul*, *Rubia cordifolia*, *Emblica officinalis* and *Moringa pterygosperma* has shown decreased NO secretion in previous studies[49,50,51,52]. Most studies of *Tinospora cordifolia* reported increased NO production[53,54]. *Glycyrrhiza glabra* studies reported either increased NO production or decreased NO production[55,56]. 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)[2]. 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[2]. The current results are contrary to several *in vitro* and *in vivo* studies reporting on the antioxidants and/or ant 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*., also reported on the pro-oxidant activity of the aqueous extracts of *Aspalathus linearis* (rooibos)[57].

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, IL-6 is involved in the systemic changes associated with inflammation and infection[28]. IL-6 concentrations were determined using a DAS-ELISA. The standard curve for the IL-6 ELISA is shown in Figure 3a. 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 3b.). The significant difference (P<0.001) is designated by an asterisk (*). *Artemisia afra* had no effect on IL-6 production in unstimulated RAW 264.7. These findings correspond to the previously mentioned results for Figure 2b.

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*[3,4,5,8,9]. 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[58]. Studies on the pro-inflammatory effects of *Aspalathus linearis* (rooibos) are few[4]. 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 2b., 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* (rooibos) tea[16]. 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[1]. IL-6 is involved in the systemic changes associated with tissue damage, inflammation and infection[59]. In an *in vitro* whole blood culture study on unstimulated WBC, *Aspalathus linearis* (rooibos) also induced higher IL-6 secretion at concentrations between 7.8125µg/ml - 250µg/ml[16]. 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 2b.).

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 3c.). *Artemisia afra* significantly decreased (P<0.001) production of IL-6 by LPS stimulated RAW 264.7 cells in a concentration dependant manner (63-1000ug/ml). These results suggest the anti-inflammatory potential of *Artemisia afra* which also corresponds to the results in Figure 2c. 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 for infectious conditions as seen in previous studies and anecdotal uses mentioned previously[15,42,43]. 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 amongst others[6,60]. These constituents amongst several others were present in the *Artemisia afra* ethanolic extract tested in this study (GCMS analysis of *Artemisia afra* extract data not included) 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 2c. (*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 [4,59,61].

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™. The anti-inflammatory effect of Septilin™ has been observed in previous studies which indicated that Septilin™ suppressed various inflammatory mediators like TNF-α, IL-6 and IL-8 in LPS stimulated *in vitro* cell culture models[1,7,21,22]. 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[1]. 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[62]. An anti-cancer *in vitro* study compared the effects of fifteen crude aqueous herbal extracts 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[63]. Further studies should include both ethanol and aqueous extracts of Septilin™, *Aspalathus linearis* (rooibos) and *Artemisia afra* within the same model.

Conclusion:

The overall findings of this study suggest 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.

Conflict of interest:

None declared

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