Original article:

An *in vitro* study to elucidate the effects of Septilin™ on immune pathways

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Abstract:

Objective: The use of herbal immunomodulatory preparations to prevent and treat immunological complications is increasing in popularity. Rigorous *in vitro, in vivo* and clinical trial studies are needed to ensure safety, quality and efficacy for the wellbeing of patients. Septilin™, a proprietary herbal medicinal product has been reported to have immunomodulatory effects. This study investigated the *in vitro* effects of Septilin™ on biomarkers of specific immune pathways by using whole blood culture assays (WBC). ***Materials and Methods:*** Stimulated and unstimulated WBC have been incubated with the product. Enzyme linked immunosorbent assays have been used to screen for IL-6, IL-10, and IFNγ as biomarkers for inflammation, humoral immunity, and cell mediated immunity, respectively. ***Results:*** Septilin™ had no effect on the release of IL-6 production by (lipopolysaccharide) LPS stimulated WBC across all concentrations tested. However, Septilin™ induced significant higher levels ($P<0.001$) of IL-6 release in unstimulated WBC across all concentrations between 0µg/ml-258µg/ml. Septilin™ had no effect on the release of IL-10 release in unstimulated WBC across all concentrations. However the presence of Septilin™ in phytohaemagglutinin (PHA) stimulated WBC induced significant higher release ($P<0.01$) of IL-10 release at concentrations between 64.5µg/ml-258µg/ml when compared to the control. The presence of Septilin™ in unstimulated WBC had no effect on the release of IFNγ production across all concentrations. The presence of Septilin™ in PHA stimulated WBC release of IFNγ is inconclusive. ***Conclusion:*** This study shows that Septilin™ has immunomodulatory effects on WBC *in vitro*.

Keywords: Septilin™; immune pathways biomarkers; immunomodulatory; herbal medicinal products; whole blood cultures

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Introduction

In the past few decades there has been a huge increase in the use of herbal medicine globally. Herbal medicines are defined as plants, parts of plants or extracts from plants that are used in health care as curative agents[1]. WHO recognizes the important therapeutic and economic role of herbal medicine for many developing countries. Herbal medicines are the main therapeutic interventions used in complementary and traditional medicine systems such as African traditional medicine, Unani-Tibb, traditional Chinese medicine, amongst others and are practiced and utilized by millions all over the world[2]. The use of plant immunostimulants has a well known history in these traditional medical systems[3]. Herbal immunomodulators have gained popularity amongst the public and researchers in the prevention and treatment of immunological complications[4,5]. Septilin™, a proprietary polyherbal formulation, has been reported to have immunomodulatory, antibacterial and wound healing properties. Septilin™ is a widely used health supplement claimed to strengthen immunity and to be effective against Gram-positive and Gram-negative infections[6,7]. Traditional herbal pharmacotherapy often combines immunomodulatory plants producing complex

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phytochemical mixtures which could potentially influence numerous molecular pathways. The sum effects of these polyherbal formulations may differ from the individual plants[8].

Confirmation on the quality, safety and efficacy of these polyherbal formulas are of utmost importance to reduce potential health risks to consumers[9]. In light of the above, this study was undertaken to investigate the *in vitro* effects of Septilin™ on biomarkers (IL-6, IL-10 and IFNγ) of specific immune pathways by using human WBC.

Materials and methods

This experimental study was conducted at the University of the Western Cape (South Africa), Medical Biosciences Department from June 2012 to December 2014.

Sample preparation

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 -80ºC.

Blood collection for preparation of WBC

The blood was collected at the University of the Western Cape's campus clinic. Blood from 4 healthy male volunteers, not on any medication, was used. Samples of blood were collected by venipuncture directly into heparinised vacuum tubes. The blood was stored at room temperature and used within 2 hours of collection. Whole blood cell cultures were prepared under sterile conditions in a laminar flow cabinet. The University of the Western Cape's ethics committee approved this study (Ethics number: ScRIRC2010/exco/09/02) and informed consent was obtained from all participants.

Preparation of unstimulated blood for WBC

Cultures were prepared under sterile conditions. The following media were prepared for the cultures: Medium for unstimulated cultures was RPMI1640 supplemented with 1% antibiotic/antimycotic (Sigma, Germany), 0.05% gentamycin (Sigma, Germany), and 1% glutamax (Sigma, Germany). Blood was diluted to a final concentration of 20 % in medium. Extracts of the herbal product (Septilin™) were diluted in normal medium to give a concentration range from 0-2000µg/ml. Three replicates of the diluted extracts were transferred to a 96 well plate at 100µl/well. This was followed by the addition of 100 µl/well of diluted blood in normal medium to each of the diluted extract replicates. After overnight incubation at 37ºC and 5% CO_2, culture supernatants were collected for IL-6, IFNγ and IL-10 assays.

Preparation of LPS stimulated blood WBC

Cultures were prepared under sterile conditions. The following media were prepared for the cultures: Medium for LPS stimulated cultures was RPMI1640 supplemented with 1% antibiotic/antimycotic (Sigma, Germany), 0.05% gentamycin (Sigma, Germany), and 1% glutamax (Sigma, Germany) and 1µg/ml LPS. Blood was diluted to a final concentration of 20 % in LPS enriched medium. Extracts of the herbal product (Septilin™) were diluted in normal medium to give a concentration range from 0-2000µg/ml. Three replicates of the diluted extracts were transferred to a 96 well plate at 100µl/well. This was followed by the addition of 100µl/well of diluted blood in LPS stimulated medium to each of the diluted extract replicates. After overnight incubation at 37ºC and 5% CO_2, culture supernatants were collected for IL-6 assays.

Preparation of PHA stimulated blood WBC

Cultures were prepared under sterile conditions. The following media were prepared for the cultures: Medium for (PHA) stimulated cultures was RPMI1640 supplemented with 1% antibiotic/antimycotic (Sigma, Germany), 0.05% gentamycin (Sigma, Germany), and 1% glutamax (Sigma, Germany) containing 16µg/ml PHA (Sigma, Germany). Blood was diluted to a final concentration of 20% in PHA enriched medium. Extracts of the herbal product (Septilin™) (net weight: 452mg in 35ml of distilled water) were diluted in normal medium to give a concentration range from 0-2000µg/ml. Three replicates of the diluted extracts were transferred to a 96 well plate at 100µl/well. This was followed by the addition of 100µl/well of diluted blood in PHA stimulated medium to each of the diluted extract replicates. After overnight incubation at 37ºC and 5% CO_2, culture supernatants were collected for IFNγ and IL-10 assays.

Cytokine analysis of Septilin™ (IL-6, IL 10 and IFNγ ELISAs) for WBC

Double antibody sandwich enzyme linked immunosorbent assay (DAS ELISAs) (e-Bioscience, Germany) was used to measure cytokine release from the supernatants of the whole blood cell cultures. Nunc maxisorp (Nunc™, Denmark) plates were used for the assays. This kit contained all the reagents, buffers and diluents needed for performing quantitative ELISAs. The ELISAs were carried out

according to the manufacturer's instructions. In summary: 96 well plates were coated with primary antibody against the respective cytokine 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 human cytokine 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 the respective cytokine. The plate was incubated for 1 hour at ambient temperature on a shaker followed by washing as before. The wells then received avidin horseradish peroxidase (Avidin-HRP) conjugate. The plate was then incubated for 30 minutes at ambient temperature on a shaker. After washing as before the bound peroxidase was monitored by addition of tetramethylbenzidine solution (substrate solution) to each well. The plate was then incubated for approximately 15 minutes. The reaction was stopped by adding 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. The standard curve was used to determine the cytokine concentrations of the culture supernatants.

Statistical analysis of WBC

All experiments were performed in triplicate to confirm reproducibility. The unstimulated WBC versus the stimulated WBC for each biomarker (IL-6,IL-10 and IFNγ) were compared. 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

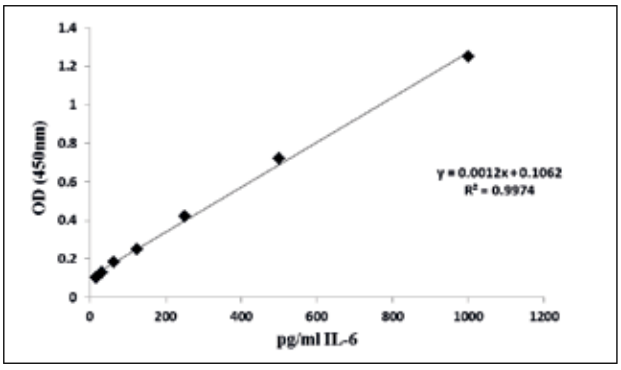

Figure 1a. Standard curve for IL-6 ELISA. This standard curve shows a good correlation (R2= 0.997) between absorbance readings and IL-6 concentration.

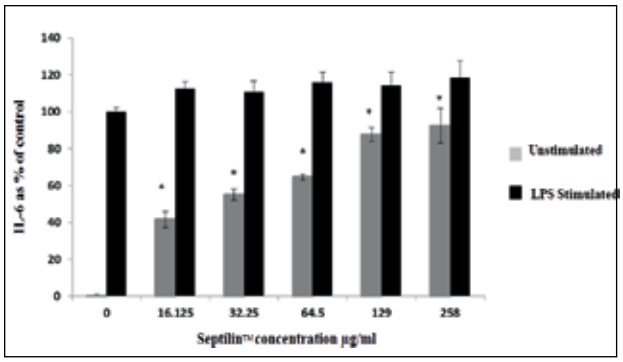

Figure 1b. Effects of Septilin™ on IL-6 production. IL-6 was used as a biomarker to determine the inflammatory response on stimulated (LPS) and unstimulated WBC in the presence of Septilin™ across various concentrations. The statistical significant (P<0.001) difference compared to the control is designated by an asterisk (*).

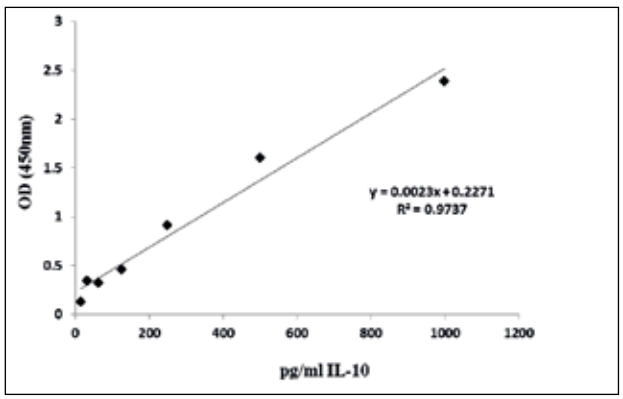

Figure 2a. Standard curve for IL-10 ELISA. This standard curve shows a good correlation (R2= 0.973) between absorbance readings and IL-10 concentration.

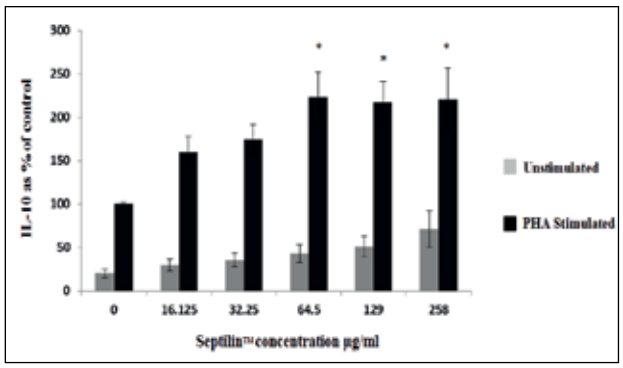

Figure 2b. Effects of Septilin™ on IL-10 production. IL-10 was used as a biomarker to determine the humoral immune response on stimulated (PHA) and unstimulated WBC in the presence of Septilin™ across various concentrations. The statistical significant (P<0.01) difference compared to the control is designated by an asterisk (*).

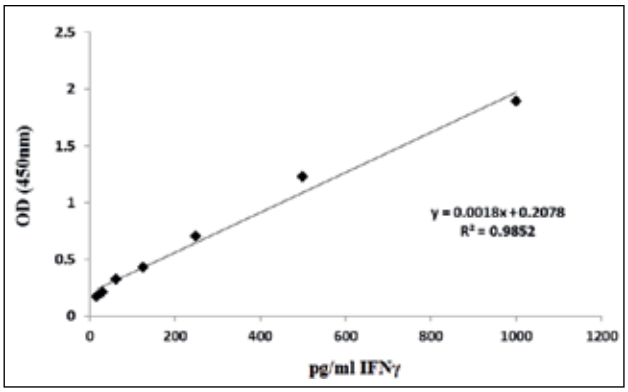

Figure 3a. Standard curve for IFNγ ELISA. This standard curve shows a good correlation (R2= 0.985) between absorbance readings and IFNγ concentration.

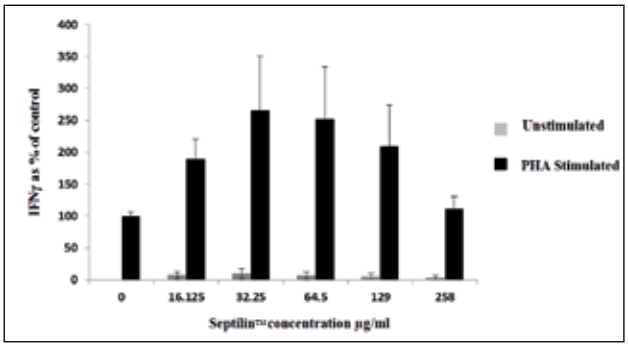

Figure 3b. Effects of Septilin™ on IFNγ production. IFN γ was used as a biomarker to determine the cellular immune response on stimulated (PHA) and unstimulated WBC in the presence of Septilin™ across various concentrations.

Discussion

Herbal medicines are extremely complex in nature. The use of highly sensitive and miniaturized assays is therefore essential alongside innovative technologies like immunochemical and enzymatic methods to elucidate the mode of action of these products. These techniques are used directly for a wide range of mechanism-based and cellular assays. A variety of *in vitro* bio-assays have been utilised to determine mechanisms of action, pharmacological actions, efficacy and safety, leading to more effective products in healthcare[1]. 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 have proved to be useful in evaluating the immunomodulatory effects of herbal constituents[10]. Pro-inflammatory cytokines like TNF-α, IL-1, IL-6, IL-8 and nitric oxide (NO) have been implicated in numerous immuno-pathological conditions[5]. The majority of *in vitro* experiments in herbal medicine make use of aqueous extractions as opposed to ethanolic extractions with varying concentrations of the wet or dried plants ranging between 0.3µg/ml to 1g/ml. Popular cell types used are splenoctyes, T cells, monocytes and macrophages and the most common stimulants utilized for immune pathway activations are Con A, PHA and LPS. *In vitro* cell culture incubation times varied from 6 hours to 4 weeks[8].

Stimulated monocytes produce IL-6, TNF-α, and IL-1, which promotes inflammation, T-cell growth and differentiation and the release of the anti-inflammatory cytokine IL-10. Monocyte cytokine production can be assessed *in vitro* by stimulating isolated monocytes but this method is time consuming, expensive and involves numerous steps which could compromise cell viability, stimulation and function[11]. The use of peripheral blood is a far simpler and an inexpensive procedure. WBC contain all the physiological requirements for cells to function naturally and therefore is ideal as a reference for immune activity. Other advantages of using WBC are that samples can be used immediately and only small volumes are needed. Cytokine production based on the WBC model is considered to be a reliable method as it has good reproducibility[11]. This system provides better outcomes over culture of single cell types because it creates a natural environment with physiological concentrations of factors influencing immune cell function which allows analysis of the effects of various experimental substances on inflammatory leucocytes. In other models the pattern of cytokine release may be altered by experimental procedures during cell isolation[12]. *In vitro* assays using WBC are ideal as they resemble the *in vivo* environment[13]. Biomarkers can be employed as an indicator to objectively measure and analyse biological, pathological or pharmacological responses to a therapeutic intervention. The pharmaceutical potency of an agent and the mechanism of its actions can be measured by the presence of biomarkers[14]. Some herbal immunomodulatory preparations have been observed to exert anti-inflammatory effects and modulate both humoral and cellular functions[5]. 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. Herbal formulas are often prescribed for inflammatory and immune-related illnesses[8].

According to the Therapeutic Index of the Himalaya herbal drug company, Septilin™ is a phytopharmaceutical formulation which is recommended for the treatment and management of various infections. Septilin™ has been reported

to exert anti-inflammatory action[15]. It is clear that the cytokine IL-6 is involved in the systemic changes associated with inflammation and infection. The availability of bioassays which are sensitive for IL-6 allows for the determination of this cytokine in clinical samples[16].

Effects of Septilin™ on IL-6 production in WBC

In this study IL-6 was used as a biomarker to determine the effects of Septilin™ on inflammatory activity in stimulated and unstimulated WBC. The standard curve for the IL-6 ELISA is shown in Figure 1a. 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. Blood of four donors were assayed in triplicate. Septilin™ has no effect on IL-6 production in LPS stimulated WBC (Figure 1b.). These findings are contrary to the majority of *in vitro* and *in vivo* studies which reported on the anti-inflammatory effects of Septilin™ via IL-6 inhibition/reduction[5,6,7,15,21].

In a review on the cytokine expression by herbal immunomodulators, Spelman *et al*, suggests that the inconsistencies noted in previous studies on the effects of several herbal products on cytokine expressions (immune activity) is due to a biphasic dose response. The biphasic effect explains that exogenous and endogenous compounds may have opposing, dose-dependent physiological effects. Paradoxical responses in cytokine activity by herbal products in both *in vitro* and *in vivo* studies is not an uncommon finding[17]. Divergent models, dosages, duration of exposure (incubation time), 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 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 significant 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 production in the absence of a stimulus. 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[18]. Septilin™ may have triggered an immune response against itself as cells may have recognised Septilin™ as a threat. 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[18]. This 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* WBC model.

Effects of Septilin™ on IL-10 production in WBC

This study also used IL-10 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 2a. 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. Blood of four donors were assayed in triplicate.

Septilin™ has no effect on the release of IL-10 release by unstimulated WBC (Figure 2b.). 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.

B cells are known to play an important role in the suppressive regulation of the immune system[19]. 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 induce IL-10 secretion[19]. 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[20]. During infection Septilin™ may regulate the inflammatory process by means of increased IL-10 production which may inhibit cytokine release of macrophages. This may be a beneficial for chronic inflammation as a regulatory mechanism but may hamper acute inflammation by down regulating the inflammatory process. IL-10 activates the proliferation and differentiation of B cells, and upregulates Ig production. These findings agrees with results of Sharma and Ray, who reported a significant increase ($p<0.001$) in both IgM and IgG concentrations in the Septilin™ treated group (*in vivo* rodent model)[21]. Very few studies are available on the effects of Septilin™ on IL-10 production by WBC in *vitro*.

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 3a. 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. Blood of four donors were assayed in triplicate.

Septilin™ has no effect on the release of IFNγ production by unstimulated WBC (Figure 3b.). The effect of Septilin™ on IFNγ synthesis by PHA stimulated WBC were 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 through NK cell activation. This ensures host defences against bacteria and viruses (cell mediated immunity) [21]. IL-10 may have suppressed IFNγ secretions as mentioned previously. 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 however further studies are needed to confirm this.

Conclusion

In summary, this *in vitro* study on WBC indicates the pro-inflammatory effect (basal) of Septilin™ in the absence of a stimulus. Septilin™ was shown to have anti-inflammatory effects by means of increased IL-10 production. The effect of Septilin™ on IFNγ production on stimulated WBC were inconclusive. This *in vitro* study indicates to the immunomodulatory effects of Septilin™ in WBC.

Conflict of interest: None declared

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