

BEST PRACTICE PROGRAMME IN THE STANDARDISATION OF TRADITIONAL MEDICINES:
EVALUATION OF AN IMMUNE BOOSTER FORMULATED BY TRADITIONAL HEALERS OF THE
VAAL TRIANGLE, SOUTH AFRICA

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Abstract

Background: Regulation and standardization of African traditional medicines (ATM) prescribed by traditional healers in South Africa is still far from being implemented. This is despite the fact that more people are using ATM products than ever. In an effort to demonstrate that collaboration with traditional health practitioners (THPs) can yield standardized TM products, this study aimed to evaluate the immunomodulatory effects of an herbal immune booster formulated by traditional healers from the Vaal Region, South Africa.

Materials and Methods: Using normal and lipopolysaccharide (LPS) stimulated human peripheral blood mononuclear cells (PBMCs) models, doses of the immune booster ranging from 1000 to 10 µg/mL were evaluated for their cytotoxicity, inflammatory cytokines and chemokines secretion, nitric oxide (NO) secretion, malondialdehyde (MDA) assay, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay.

Results: The immune booster induced a dose dependent cytotoxic effect on both normal and LPS stimulated PBMCs with higher doses showing cytotoxicity while lower dose from 100µg/mL did not show any cytotoxicity. When re-dissolved in aqueous PBS immune booster doses up to 100 µg/mL showed better DPPH radical inhibition (41%) than ascorbic acid at 40µg/mL (33%). The immune booster also decreased lipid peroxides significantly ($p < 0.05$) and this was comparable to ascorbic acid. There was also a significant ($p < 0.05$) increase in nitrite (NO) after treatment of LPS stimulated PBMCs with immune booster doses when compared to untreated samples. The immune booster stimulated inflammatory cytokines secretion in normal PBMCs (IL 1α, IL 1β, IL 6, IL 10 and TNFα while showing a decrease in IFNγ at the higher dose) while in LPS stimulated PBMCs some cytokines were decreased (IL 1α, IL 17α and at lower doses IL 10 and TNFα) and others were increased (IFNγ, TNFα and GM-CSF) depending on the dose used. In both normal and LPS stimulated PBMCs the immune booster significantly ($p < 0.05$) increased (MIP 1α) while causing significant ($p < 0.05$) decreases in IP 10 (high dose), I-TAC and MIG secretion.

Conclusions: This immune booster showed potential immunostimulatory effects by increasing nitric oxide, inflammatory cytokines and chemokines secretion in both normal and LPS stimulated PBMCs. This TM also showed promising antioxidant potential in the MDA and DPPH assays. Further *in vitro* and animal studies are warranted.

Keywords: Traditional healers, traditional medicines, regulation, standardization, immune stimulation

Introduction

Owing to countries' efforts to institutionalize traditional medicine practice in health systems and calls made by the WHO Regional Office of Africa over the past twenty years, more than half of the countries in the Africa Region have developed national policies on traditional medicine and regulation is one of the components of such policies (Kasilo and Trapsida, 2010). South Africa is one of the countries that have formulated national policies to regulate the practice of traditional healing and traditional medicines (TM) products. The Traditional Health Practitioners Act Number 22 of 2007 (THP Act No. 22, 2007) provide for a regulatory framework to register, regulate and control the practice of traditional healing. The THP Act mainly aimed to establish an Interim THP Council which was to implement the regulatory framework (Department of Health, 2007). Although the government has gone some way in implementing the THP Act (2007) by establishing the Interim THP Council in 2013, the regulation of TM products is far from being implemented.

South Africa has developed a draft policy on African traditional medicine which was published in the Government Gazette in 2008 (Department of Health, 2008). This draft policy makes a number of recommendations with regards to regulation of ATM, development of a national pharmacopoeia and cultivation and conservation of medicinal plants. Unfortunately, this draft policy has never been implemented which has resulted in the proliferation of unregulated commercial TM products and those prescribed by traditional healers. A number of publications have suggested possible models that can be followed in the regulation of TM and its practice (Ngcobo et al., 2012; Pinkoane et al., 2012). In a continuation of the study by Pinkoane et al. (2007) which documented a collaboration with traditional healers to develop a model for their integration into the National Health Care Delivery System of South Africa, the current study sought to demonstrate that traditional healers from the same area can come together to formulate a standardized TM product to be prescribed to patients with related ailments. This TM product with immunomodulatory activities was

formulated by traditional healers from the Vaal Triangle, South Africa and has been prescribed to patients for over two years. The health status of these patients has been tracked through an ongoing observational study in which the traditional healers play an integral part. In a form of adopting the Indian model of studying TM products which is termed 'reverse pharmacology' (Vaidya, 2007), this study aimed to evaluate the immunomodulatory effects of this immune booster using in vitro normal human peripheral blood mononuclear cells.

Materials and Methods

Materials

Human whole blood reagents were kindly donated by the South African National Blood Service (SANBS) (Human Research Ethics Committee Certificate Number: 2012/07). RPMI-1640 with L-glutamine, foetal calf serum (FCS), penstrepfungizone (PSF), L-glutamine, Opti-MEM, Hepes buffer were purchased from Lonza (SA). The lipopolysaccharide (LPS) from *Salmonella typhosa*, Cyclosporine, 1,1-Diphenyl-2-picryl-hydrazyl (DPPH), Ascorbic acid, 4-nitroquinoline-1-oxide (4-NQO), and Polymyxin B sulphate were purchased from Sigma Aldrich (USA). The Promega CellTiter-Glo™ Luminescent Cell Viability assay kit and the Griess Reagent System were products of Promega (USA). Multi-analyte Cytokines/Chemokines ELISArray™ kits were purchased from Qiagen (USA). The OxiSelect™ TBARS Assay Kit (MDA Quantitation) kit was purchased from Cell Biolabs Inc (USA). Automated cell counter was from BioRad (USA). Modulus™ Microplate Luminometer was from Turner BioSystems (USA). Colometric plate reader was from Zenyth (UK). All other reagents and equipment were purchased from standard commercial sources and were of the highest available purity.

Methods

Preparation of the Immune Booster Formulation

The immune booster is formulated and prepared by the traditional healers in single manufacturing place and then distributed to all those participating in the project. The immune booster is formulated by combining materials from 35 indigenous medicinal plants found in different areas of South Africa. The plants were purchased at a local market for medicinal plants by traditional healers who have an in-depth knowledge. The standard preparation method involves boiling the medicinal plant material in water for the whole day followed by cooling using natural air. The cooled extract is then filtered once with steel sift followed by removal of finer particles using a sifting net and then packaged into 1 or 5 liters containers.

To prepare the extract for *in vitro* studies, the liquid extract was further sterile filtered and then freeze dried to powder. The powdered plant material was then reconstituted at 10 mg/mL in phosphate buffered saline (PBS) and this was further sterile filtered with 0.22 µm filters. Working concentrations of 1000, 500, 100, 50 and 10 µg/mL was constituted using complete culture media. Polymyxin B sulphate (10 µg/mL) was added to reduce the immunostimulatory effects due to endotoxin contamination.

Cell Culture

Normal human whole blood was carefully layered onto equal amounts of Histopaque 1077 then centrifuged at 600 g for 30 minutes at 25 °C. After centrifugation, the buffy coat layer containing PBMCs was isolated and washed twice in phosphate buffered-saline (PBS, 5 mL) (300 g for 20 minutes at 25 °C). The final pellets were re-suspended in complete culture media at 1×10^6 cells/mL and then different models of PBMCs were prepared according to Leung et al., (2007) with a few variations. Briefly, the PBMCs were stimulated with LPS from *Salmonella typhosa* for 2 hours or left unstimulated. Without removing the stimulation, the cells were aliquoted to 6 well plates and treated with doses of the immune booster ranging from 1000 µg/mL to 10µg/mL at a ratio of 1:1. The treated PBMCs were then incubated for 24 hours at 37 °C, 5% CO₂ and 95% humidity. At the end of the incubation period, the cells and their supernatants were used for further experiments.

Cell Viability Assay

The luminescent cell viability ATP assay kit from Promega uses recombinant luciferase to catalyse the following reaction: $\text{ATP} + \text{d-Luciferan} + \text{O}_2 \rightarrow \text{Oxyluciferan} + \text{AMP} + \text{PPi} + \text{CO}_2 + \text{Light (560 nm)}$.

When ATP is the limiting component in the reaction, the intensity of the emitted light is proportional to the concentration of ATP. Based on these principles, the levels of ATP in PBMCs stimulated with LPS from *S. typhosa* for 2 hours or left unstimulated followed by treatment treated with doses of the immune booster were analysed according to manufacturer's instruction. Cyclosporine (20µg/mL) was used as a positive control for cytotoxicity against immune cells. Briefly, a sample (100 µL) of 24 hour treated/control cell suspension was pipetted into three different wells of a white opaque 96-well plate. The working CellTiter-Glo™ Reagent (cat number: G7570) was prepared immediately before use and was added to the wells with treated cells at 100 µL per well. The plate was agitated on a plate shaker for 2 minutes at 150 g and incubated in darkness for 10 minutes at room temperature. At the end of the incubation period, the plate was loaded into the luminometer and the relative light units (RLU) of the samples were measured. Background signals of cell culture media and the immune booster doses (negative control) were subtracted from each average read. A dose response curve was also generated for the ATP levels using RLU versus different concentrations of samples. The cell viability assay was done in triplicates and repeated three times before the follow up assays were undertaken.

Cytokines and Chemokines Secretion Assay

The Multi-Analyte Profiler ELISArray assay kit (Qiagen, USA) is designed to be used with supernatants from treated cells or with serum from whole blood. For the purpose of this research, inflammatory cytokines were analyzed from supernatants of treated models of PBMCs and control samples. Each kit included 96-well plate coated with antibodies for the various chemokines in the microarray. Each row of the plate from 1 to 12 represented a single cytokines in the following order: IL 1α, IL 1β, IL 2, IL 4, IL

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6, IL 8, IL 10, IL 12, IL 17 α , interferon gamma (IFN- γ), tumor necrosis factor alpha (TNF- α), and granulocyte-macrophage colony-stimulating factor (GM-CSF). Similarly, each row of the chemokines kit plate from 1 to 12 represented a single chemokine in the following order: interleukin 8 (IL 8), monocyte chemoattractant proteins 1 (MCP-1), regulated upon activation normal T cell-expressed and secreted (RANTES), microphage inflammatory protein 1 α and β (MIP-1 α and MIP-1 β), interferon (IFN) inducible protein 10 (IP-10), IFN-inducible T-cell α chemoattractant (I-TAC), monokine induced by gamma IFN (MIG), Eotaxin, thymus and activation-related chemokine (TARC), macrophage-derived chemokine (MDC), and GRO α . The kits had negative and positive controls. Each sample was assayed in duplicate.

The ELISA analyses were performed according to the manufacturer's instructions. Briefly, incubation of the samples in the 96-well plates allowed the capture antibodies to bind their specific protein of interest. Samples from PBMCs stimulated with LPS from *S. typhosa* for 2 hours or left unstimulated and then treated with the immune booster (100, 50 and 10 $\mu\text{g}/\text{mL}$) and untreated control samples were analyzed. After washing away unbound protein with wash buffer, biotinylated detection antibodies (50 μL) were added to the wells to also bind the captured analyte. Following another wash, an avidin-horseradish peroxidase conjugate (100 μL) was added. The wells were again washed and the colorimetric substrate solution was added, developing to a blue color in direct proportion to the amount of protein analyte present in the initial sample. The color development was stopped by adding the stop solution, and the absorbance was read at 450 nm with reference at 570 nm in a microplate reader as per manufacturer's instructions. Secretion of cytokines was measured in duplicates and two independent experiments were done.

Nitric Oxide Secretion

The Griess reagent system from Promega (USA) was used to measure nitrite (NO_2^-), which is one of two primary, stable and nonvolatile breakdown products of NO. To perform assay, 50 μL of supernatants from PBMCs stimulated with LPS from *S. typhosa* for 2 hours or left unstimulated and control samples were plated in triplicates 96 well plates. The samples were left to equilibrate to room temperature after which 50 μL of Sulfanilamide solution was dispensed to all experimental samples and incubated for 10 minutes at room temperature away from light. The NED solution (50 μL) was then dispensed to all sample wells and the plate incubated for another 10 minutes at room temperature protected from light again. Absorbance was then measure within 30 minutes in a Zenyth200 plate reader at 540 nm. Nitrite standards at doses ranging from 100 to 1.56 μM were included as part of the samples and were used to draw a reference curve. All samples and standards were done in triplicates and the experiments were repeated twice.

Malondialdehyde Quantification

To measure lipid peroxides levels in treated PBMCs and supernatants, the thiobarbituric acid reactive substances (TBARS) assay was used. First, the treated and control PBMCs in PBS were homogenized on ice and supernatants were centrifuged at 10,000 g for 5 minutes to remove insoluble particles. Cyclosporine at 20 $\mu\text{g}/\text{mL}$ and ascorbic acid at 40 $\mu\text{g}/\text{mL}$ were used as a positive control for lipid peroxidation. MDA standards (100 μL of each sample) at doses ranging from 125 to 7.8 μM were added into separate micro-centrifuge tubes followed by 100 μL of sodium dodecyl sulphate (SDS) lysis solution. The tubes were mixed thoroughly and incubated for 5 minutes at room temperature. The TBA reagent (250 μL) was added to each sample and standard and the tubes were incubated at 95 $^\circ\text{C}$ for 1 hour. After this the tubes were cooled in an ice bath and then centrifuged at 700 g for 15 minutes. The samples and standards supernatants (200 μL per well of a 96 well plate) were analyzed using a Zenyth200 spectrophotometer at 532nm. All samples and standards were read in triplicates and each experiment repeated twice. A blank control was included to substrate background noise.

DPPH Free Radical Scavenging Activity

The DPPH assay was performed according to Sharma and Bhat (2009) with a few deviations. Doses of the immune booster ranging from 10 to 100 $\mu\text{g}/\text{mL}$ from unstimulated models of PBMCs were used for this assay. Briefly, samples (3000 μL of different doses of the immune booster, PBS or positive control) and methanolic DPPH solution (1000 μL , 200 μM) were combined and kept in the dark at 37 $^\circ\text{C}$ for 30 minutes. The absorbance of samples was measured at 517 nm on a Zenyth200 plate reader. All tests were performed in triplicates. Ascorbic acid was used as a positive control and was reconstituted in PBS at a concentration of 1 mM.

Statistical Analyses

Data analyses were done on *Microsoft Excel* to obtain descriptive statistics. The IC_{50} for cytotoxicity, different levels of significances within the separate treated groups were analyzed using one-way analysis of variance (ANOVA) and the differences between the treated cells, the untreated cells and the negative control samples were analyzed using *GraphPad Prism* (version 5) software with the *Tukey-Kramer* multiple comparison test. Differences with $p \leq 0.05$ were considered statistically significant.

Results

Cell Viability

To assess the cytotoxicity of the immune booster in normal and LPS stimulated PBMCs, the metabolic generation of ATP was assayed under doses ranging from 1000 $\mu\text{g}/\text{mL}$ to 10 $\mu\text{g}/\text{mL}$. In both models, the immune booster induced a dose dependent cytotoxic effect with higher doses causing a significant decrease in cell viability ($p < 0.05$) when compared to untreated control cells. These higher doses were less cytotoxic than the negative control, cyclosporine, but the difference was not significant ($p > 0.05$) in the normal PBMCs model. Lower doses of the immune booster were less cytotoxic when compared to untreated control cells and these

doses (100 to 10 μ g/mL) were used for further experiments. Cyclosporine significantly ($p < 0.05$) reduced cell viability while PHA stimulated cell replication as expected respectively (Figure 1).

Cytokines and Chemokines Secretion Assay

Cytokines Secretion

i. Normal PBMCs

The immune booster doses significantly increased ($p < 0.05$) the secretion of IL 1 α , IL 1 β , IL 6, IL 10 and TNF α while showing a decrease in IFN γ at the higher dose when compared to untreated normal PBMCs. Cyclosporine, a known immune suppressor, significantly ($p < 0.05$) decreased the secretion of all cytokines while PHA either stimulated or did not change the concentration of cytokines when compared to untreated normal PBMCs (Figure 2).

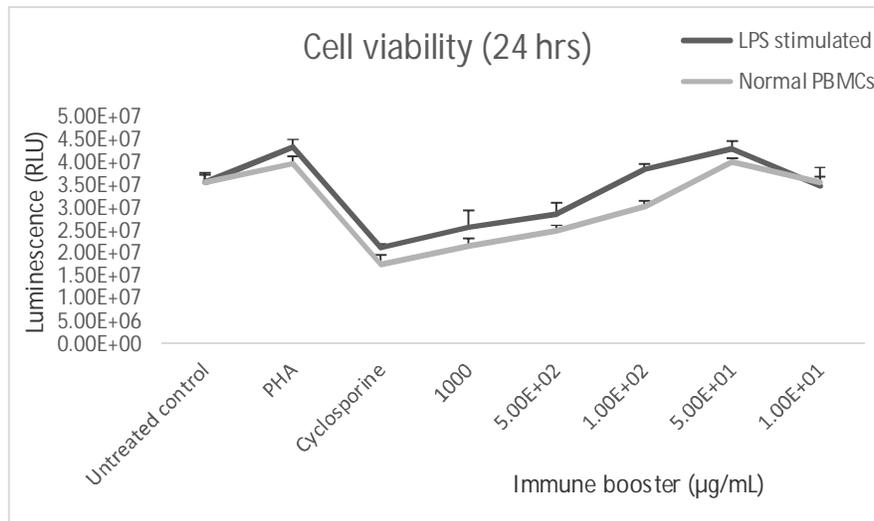


Figure 1: The cytotoxic effects of the immune booster on normal and LPS stimulated PBMCs over 24 hours. The immune booster induced a dose dependent cytotoxic effect on both models with higher doses from 500 to 1000 μ g/mL showing significant ($p < 0.05$) cytotoxicity when compared to untreated controls. Cyclosporine was significantly cytotoxic as expected while PHA increased cell viability but this was not significant.

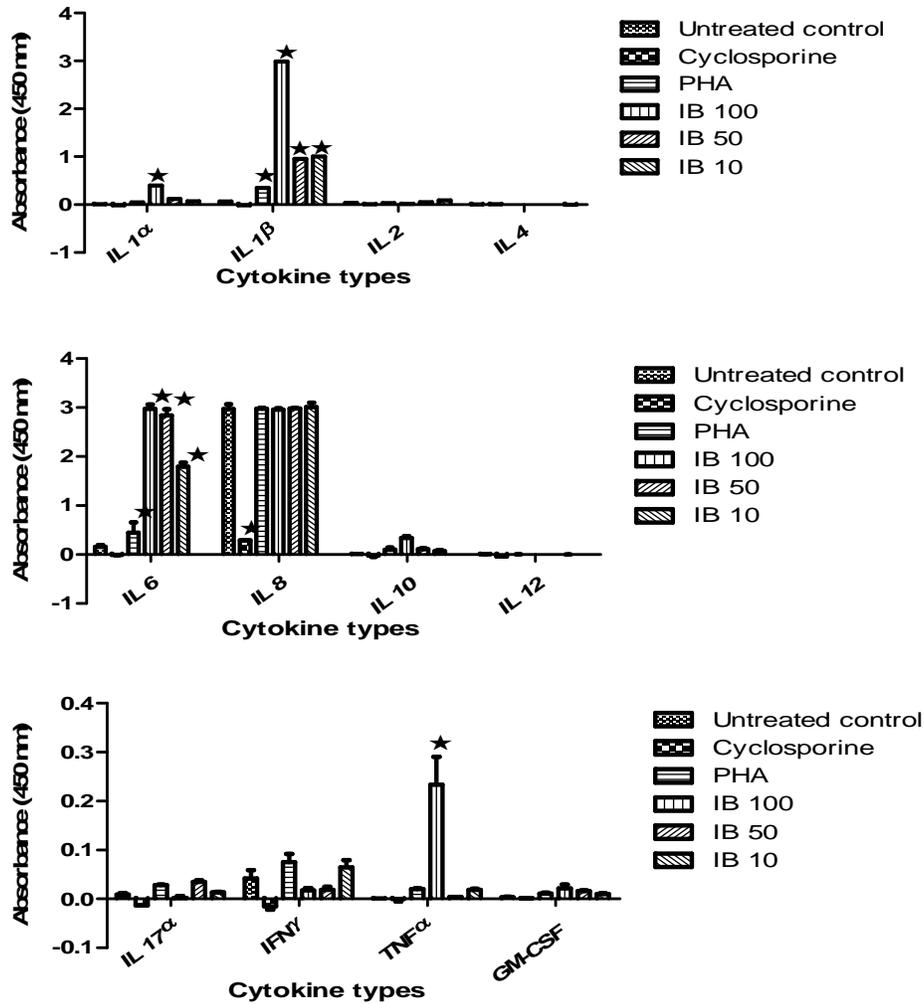


Figure 2: Effects of the immune booster doses (100, 50 and 10 µg/mL) on 12 inflammatory cytokines secretion over 24 hours in normal PBMCs. The immune booster doses significantly increased ($p < 0.05$) the secretion of IL 1α, IL 1β, IL 6, IL 10 and TNFα while showing a decrease in IFNγ at the higher dose when compared to untreated normal PBMCs. Cyclosporine significantly ($p < 0.05$) decreased the secretion of all cytokines while PHA either stimulated or did not change the concentration of cytokines when compared to untreated normal PBMCs.

ii. LPS Stimulated PBMCs

When compared to normal PBMCs (Figure 2), secretion of inflammatory cytokines was significantly increased after LPS stimulation (Figure 3). Treatment with the immune booster doses caused a decrease ($p < 0.05$) in IL 1α, IL 17α and at lower doses IL 10 and TNFα. High doses (100µg/mL) increased ($p < 0.05$) secretion of IFNγ, TNFα and GM-CSF when compared, PBMCs only stimulated with LPS. Cyclosporine significantly suppressed secretion of all cytokines (Figure 3).

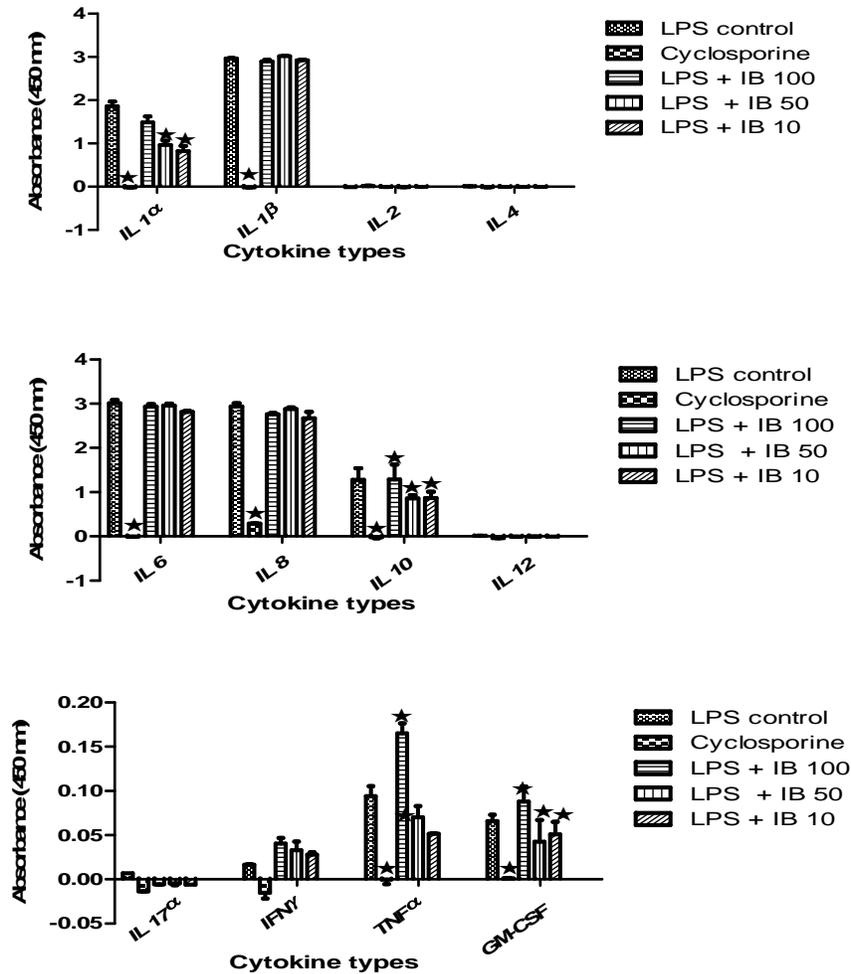


Figure 3: Effects of the immune booster doses (100, 50 and 10 µg/mL) on LPS stimulated PBMCs after 24 hours incubation. Treatment with the immune booster doses caused a decrease in IL 1α, IL 17α and at lower doses IL 10 and TNFα. High doses (100 µg/mL) increased secretion of IFNγ, TNFα and GM-CSF when compared LPS stimulated PBMCs. Cyclosporine significantly suppressed secretion of all cytokines.

Chemokines Secretion

i. Normal PBMCs

In normal PBMCs, the immune booster doses increased secretion of MIP-1α while causing significant ($p < 0.05$) decreases in IP 10 (at 100 µg/mL), I-TAC and MIG. Cyclosporine decreased ($p < 0.05$) all chemokines as expected (Figure 4).

ii. LPS Stimulated PBMCs

LPS stimulation of PBMCs followed by addition of the immune booster doses caused a decrease in the secretion of some chemokines when compared to normal PBMCs (Figure 5). Treatment with doses of the immune booster caused a significant ($p < 0.05$) decrease in the secretion of IP 10 and I-TAC while there were no significant changes in the levels of the other 10 chemokines measured when compared to samples stimulated with LPS only. Cyclosporine significantly ($p < 0.05$) decreased chemokines secretion as expected (Figure 6).

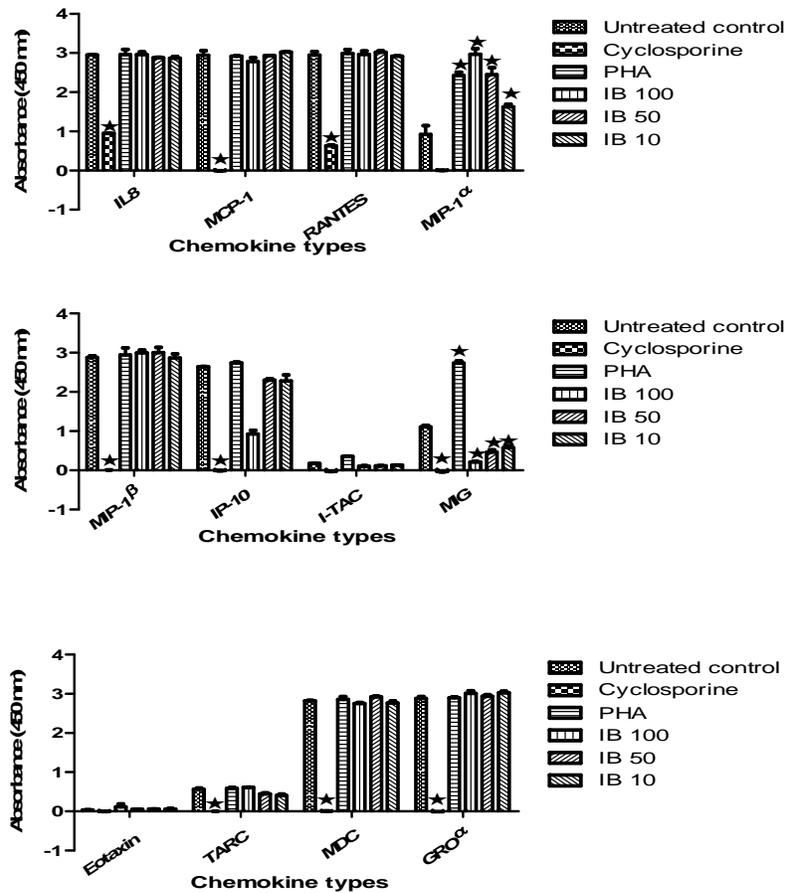


Figure 4: Effects of the immune booster doses on chemokines secretion in normal PBMCs over 24 hours. Immune booster doses increased secretion of MIP 1 α while causing significant ($p < 0.05$) decreases in IP 10 (high dose), I-TAC and MIG. Cyclosporine decreased ($p < 0.05$) all chemokines as expected.

Nitric Oxide Secretion

Treatment with doses of the immune booster showed a dose dependent effect on the levels of nitrite radicals in treated supernatants with the higher doses tested (100 and 50 $\mu\text{g/mL}$) significantly ($p < 0.05$) increasing nitrite radicals when compared samples stimulated with the lowest dose decreased nitrite levels but this was not significant ($p > 0.05$). Cyclosporine significantly ($p < 0.05$) decreased nitrite levels when compared to control and immune booster treated samples (Table 1). The increase in nitrite levels, as a result of treatment with the immune booster, is important in that nitrite is a radical and this should be considered together with the inhibition of DPPH and lipid peroxides results.

Table 1: Evaluation of nitrite as a measure of nitric oxide secretion from LPS stimulated PBMCs. Treatment with noncytotoxic doses of the immune booster showed a dose dependent effect on the levels of nitrite radicals in treated supernatants with the higher doses tested (100 and 50 $\mu\text{g/mL}$) significantly ($p < 0.05^{**}$) increasing nitrite radicals when compared to LPS stimulated samples. Cyclosporine significantly ($p < 0.05^*$) decreased nitrite levels.

	LPS	Cyclosporine	Immune booster ($\mu\text{g/mL}$)		
			100	50	10
Average readings (570 nm)	0.0037 \pm 0.010	-0.0003 \pm 0.0032	0.0157 \pm 0.025	0.0083 \pm 0.0065	0.002 \pm 0.002
Nitrite (μM)	0.3901	-0.0355*	1.6667**	0.8865**	0.2128

** Significant increase vs untreated cells

* Significant decrease vs untreated cells

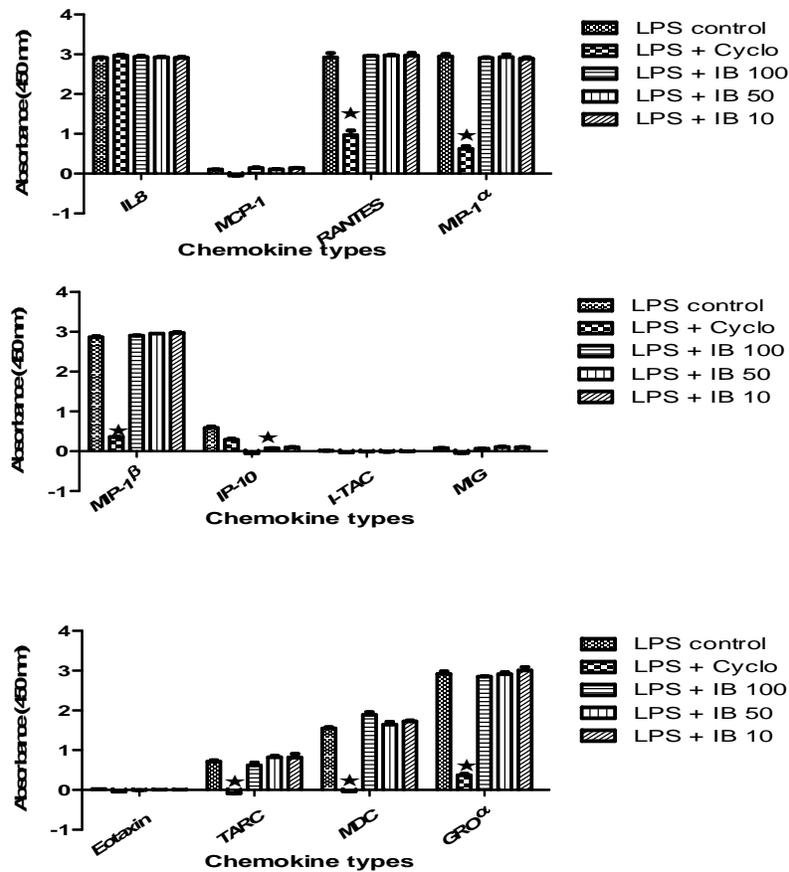


Figure 5: Effects of the immune booster doses on chemokines secretion in LPS stimulated PBMCs over 24 hours. Treatment with doses of the immune booster caused a significant ($p < 0.05$) decrease in the secretion of IP 10 and I-TAC while there were no significant changes in the levels of the other chemokines when compared to samples stimulated with LPS only. Cyclosporine significantly ($p < 0.05$) decreased chemokines secretion.

Malondialdehyde quantification

Lipid peroxidation was measured in normal PBMCs treated with noncytotoxic doses of the immune booster and compared to effects of cyclosporine and ascorbic acid. In PBMCs treated with the immune booster lipid peroxides were decreased in a dose dependent manner with the higher dose (100 $\mu\text{g/mL}$) showing significant ($p < 0.05$) lipid peroxide removal when compared to untreated PBMCs and those treated with cyclosporine. Ascorbic acid showed comparable significant lipid peroxide removal. In supernatants there were no significant difference ($p > 0.05$) between immune booster treated samples and those of untreated, cyclosporine and ascorbic acid samples (Figure 6).

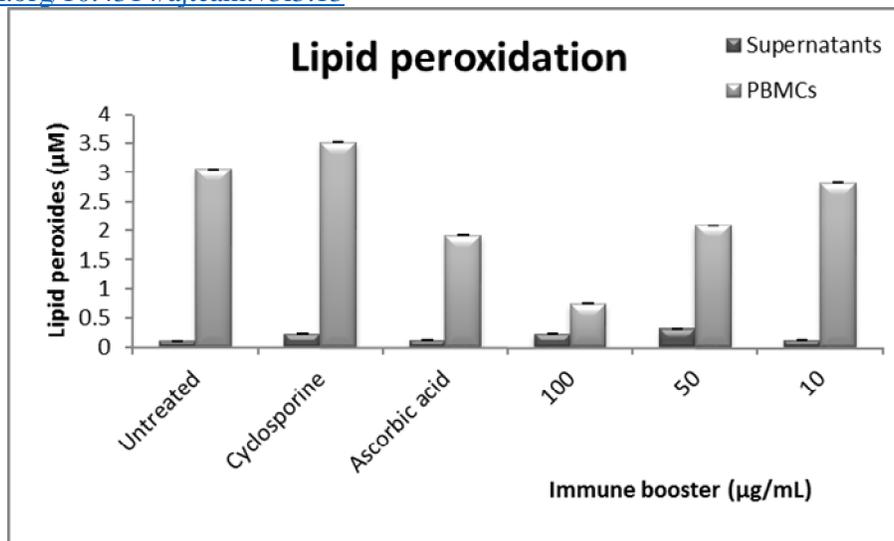
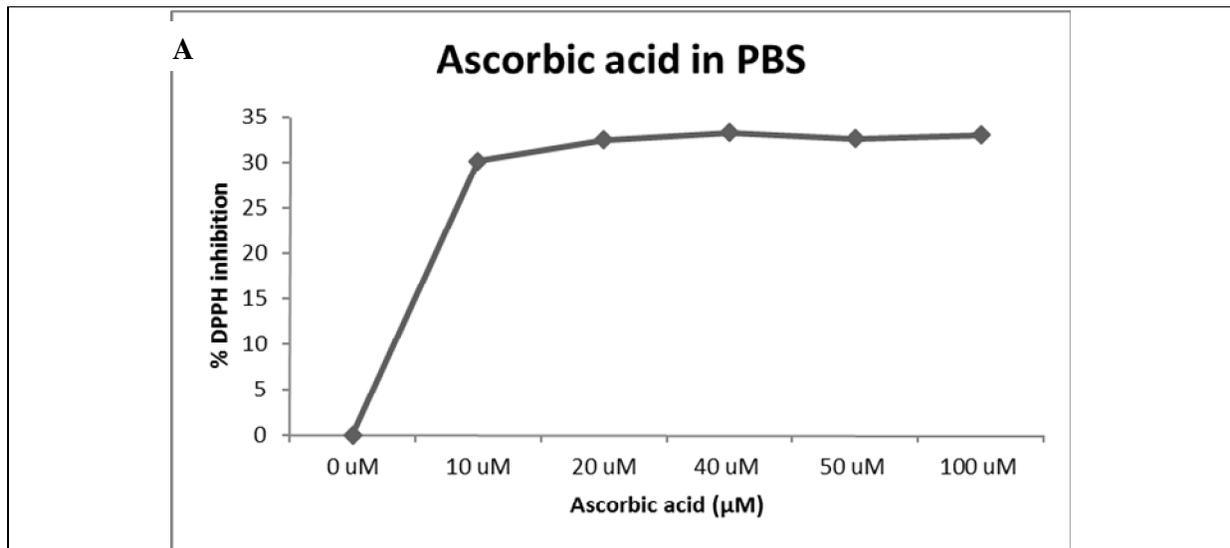


Figure 6: Effects of noncytotoxic doses of the immune booster on levels of lipid peroxides levels in normal PBMCs and their supernatants. In PBMCs treated with the immune booster lipid peroxides were decreased in a dose dependent manner with the higher dose (100 µg/mL) showing significant ($p < 0.05$) lipid peroxide removal when compared to untreated PBMCs and those treated with cyclosporine. Ascorbic acid showed comparable significant lipid peroxide removal. In supernatants there were no significant difference ($p > 0.05$) between immune booster treated samples and those of untreated, cyclosporine and ascorbic acid samples.

DPPH Free Radical Scavenging Activity

DPPH salt at 200 µM in methanol was mixed with noncytotoxic doses of the immune booster in PBS ranging from 100 to 10 µg/mL and the changes in absorbance were measured as an indicator of free radical scavenging activity. Ascorbic acid was reconstituted in the same medium as the immune booster (PBS) to ensure that the results of the free radical scavenging activities were comparable. Ascorbic acid had a reduced antioxidant effect when dissolved in PBS with a plateau being reached at 40 µM at 33% reduction of DPPH. Therefore, the IC_{50} for free radical scavenging activity for ascorbic acid was not reached when PBS was used as reconstitution medium (Figure 2 A). There was a dose dependent increase in DPPH reduction by the immune booster with highest reduction of DPPH seen at 100 µg/mL (41%) with a decreased reducing power at higher doses of the immune booster (Figure 2 B). Similar to ascorbic acid, the immune booster also did not reach an IC_{50} dose, with the aqueous PBS medium limiting the DPPH reducing powers of both test agents.



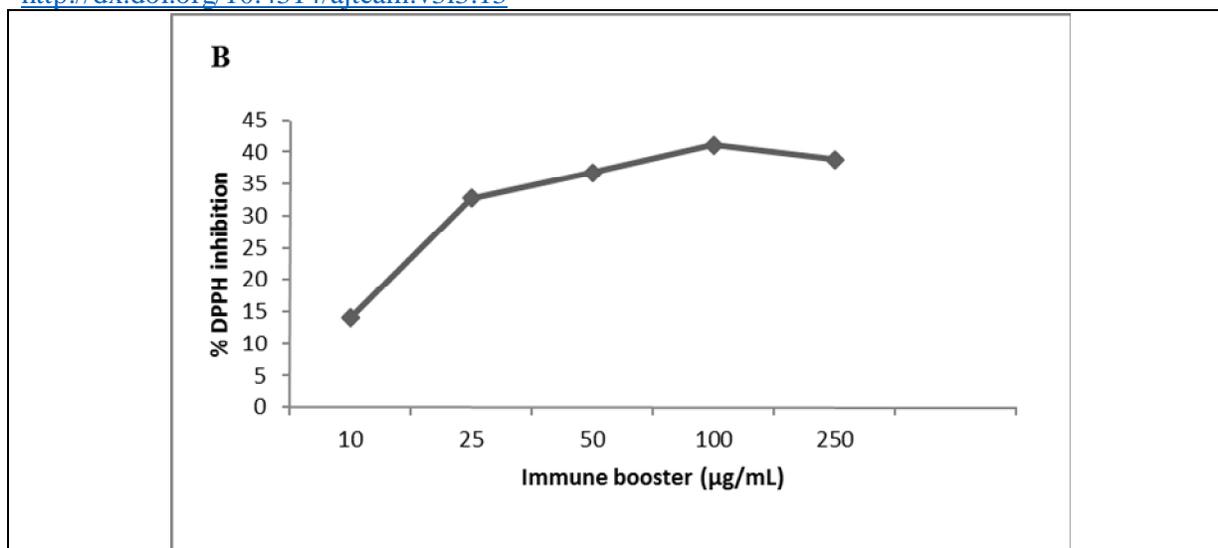


Figure 7: Reduction of the DPPH salt (200 µM) as a measure of the antioxidant potential of ascorbic acid doses (A) and the immune booster doses (B). Ascorbic acid had a reduced antioxidant effect when dissolved in PBS with a plateau being reached at 40 µM at 33% reduction of DPPH. There was a dose dependent increase in DPPH reduction due to the immune booster with highest DPPH reduction seen at 100 µg/mL (41%) showing better antioxidant potential than ascorbic acid when both are dissolved in PBS.

Discussion

The regulation and registration of TM continues to present challenges to many countries regardless of the fact that an increased number of the population utilises TM for their health care needs (Ngcobo et al., 2012). In 2013, an amendment to the Medicines and Related Substances Act of South Africa regulations put a requirement that herbal medicines on the shelf that promise a cure from disease, or improved sexual performance should provide to the Medicines Control Council (MCC) that they are safe for human consumption (Child, 2013). This is an indication that similar requirements will soon apply to TM products prescribed by traditional healers. Therefore, this unique collaborative study where traditional healers have come together to formulate a standardized immune booster demonstrates that it is possible to develop a common treatment prescribed by all traditional healers. The immune booster evaluated in this *in vitro* study showed that it was not significantly cytotoxic to PBMCs even at high doses (Figure 1), stimulates secretion of cytokines and chemokines in normal immune cells (Figures 2 and 4) while not causing a significant decrease in cytokines and chemokines in LPS stimulated immune cells (Figures 3 and 5). It also increased the secretion of nitric oxide (Table 1) as an indication of immune stimulation. This immune booster also showed promising antioxidant scavenging activities in the lipid peroxidation and DPPH assays (Figures 6 and 7).

Immune boosting is a relatively new term in traditional healing and its popularity is largely related to high prevalence of HIV infections in South Africa. An immune booster is described as an additional immunizing agent given to increase and sustain the immune response of the body (Lekhoaa et al., 2012). Such products are made using so-called tonic plants which are believed to support and tone either specific organs or the whole body by strengthening and stimulating the immune system, the nerve and/or hormonal system (Mowrey, 1998). Using normal human PBMCs, this study has shown that an immune boosted formulated by traditional healers can stimulate the immune response increasing the secretion of inflammatory cytokines and chemokines (Figures 2 and 4) while able to decrease lipid peroxidation (Figure 6). These results assert the thinking that immunostimulants can be used both by healthy individuals and persons with impaired immune systems. In healthy individuals, they are expected to act as prophylactic agents and in individuals with impairment of the immune response as immunotherapeutic agents (Agarwal and Singh, 1999).

Many advances have been made recently in the understanding of the functioning of the immune system. Knowledge of the specific components of cytokine networks and signaling pathways and their role in regulation of immune responses is important in designing strategies to augment these responses (Tzianabos, 2000). Stimulation of PBMCs with LPS increased the secretion of inflammatory mediators including cytokines and chemokines. Lipopolysaccharide activates cells through Toll-like receptor 4 (TLR4) as the central recognition and signal proteins. Engagement of cellular receptors leads to synthesis of new proteins through alteration of the pattern of gene expression ((Freudenberger et al., 2008). Addition of the immune doses to the LPS stimulated PBMCs did not significantly decrease the majority of inflammatory cytokines and chemokines (Figures 3 and 5). This maintained inflammatory state was confirmed by the significant increase in nitric oxide secretion in these LPS stimulated PBMCs (Table 1). Cyclosporine, a known inhibitor of inducible nitric oxide synthase (iNOS) (Amin et al., 1999), suppressed inflammatory cytokines, chemokines and nitric oxide. Cyclosporine inhibits inflammatory responses through the inhibition of transcription factors such nuclear factor-kappa β (NF-κβ) and activator protein-1 (AP-1) (Matsuda and Koyasu, 2000). It is therefore possible that the immune booster induces its immunostimulatory effects through the activation of NF-κβ and AP-1.

When generated in high concentrations, NO is rapidly oxidized to reactive nitrogen oxide species (RNOS) that mediate most of the immunological effects of NO. RNOS can reduce thiols to modify key signaling molecules such as kinases and transcription factors (Coleman, 2001). The increase in nitrite levels, which indicate an increase in secretion of NO, after treatment with doses of the immune booster (Table 1) is therefore an indication of an increase in levels of oxidants. While this increase in

nitrite radicals was as a result of LPS combined with the stimulatory effects of the immune booster, the same immune booster doses significantly reduced lipid peroxides in normal human PBMCs (Figure 6). The antioxidant potential of the immune booster doses was confirmed in DPPH assay where this TM showed better radical scavenging potential than ascorbic acid when dissolved in PBS (Figure 7). Immune boosters are used for specific outcomes such as reducing fatigue, improving general health (during or after illness), reducing stress and cleansing the blood. Some aphrodisiacs for men are also made from tonic plants (Olivier, 2012). The antioxidant activity of plant secondary metabolites has been widely established in *in vitro* systems. Numerous alkaloids, saponins, cardenolides, anthraquinones, polyphenols and terpenes with antioxidant effects have been reported from *in vitro* cultures (Matkowski, 2008).

Complex mixtures of traditional medicines are not a new phenomenon but their existence has only been recognized now due to increased interest in the activities of traditional healers (Gqaleni et al., 2012). Multi-herbal TM is based on centuries of knowledge and it is assumed that the ingredients contribute and undefined range and balance of pharmacologically active compounds to their overall therapeutic use. Herbalists have known for centuries the value of using a combination of herbal remedies, single extracts and combined extracts to switch on the body's defense mechanisms and self-healing and protective processes (Busia, 2005). The potential uses of immunomodulators such as the immune booster reported here in clinical medicine include treatment of immunodeficiency caused by AIDS and suppression of immune response in autoimmune disease (Alamgir and Uddin, 2010). Although the medicinal plants constituting the immune booster reported in this study are not identified, all of them are known and have been botanically identified. This TM immune booster is currently undergoing research and development to produce an authentic, standardized and effective traditional medicine product.

Conclusion

This research study is part of a number of studies aimed at proving the safety and efficacy of an immune booster formulated by traditional healers of the Vaal Region, South Africa. The immune booster evaluated in this *in vitro* study showed that it was not significantly cytotoxic to PBMCs even at high doses, stimulates secretion of cytokines and chemokines in normal immune cells while not causing a significant decrease in cytokines and chemokines in LPS stimulated immune cells. It also increased the secretion of nitric oxide as an indication of immune stimulation. This immune booster also showed promising antioxidant scavenging activities in the lipid peroxidation and DPPH assays. Further *in vitro* and animal studies are planned to further understand the effects and mechanism of action of this immune booster.

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References

1. Agarwal, S.S. and Singh, V.K. (1999). Immunomodulators: a review of studies on Indian medicinal plants and synthetic plants. *PINSA B65* **3 & 4**: 179-204.
2. Alamgir, M. and Uddin, S.J. (2010). Recent advances on the ethnomedicinal plants as immunomodulatory agents. In: *Ethnomedicine: A Source of Complementary Therapeutics* (Eds. Debprasad Chattopadhyay), 227-244.
3. Amin, A.R., Attur, M. and Abramson, S.B. (1999). Nitric oxide synthase and cyclooxygenases: distribution, regulation, and intervention in arthritis. *Curr. Opin. Rheumatol.* **11**:202-209.
4. Busia, K. (2005). Medical provision in Africa- past and present. *Phytother. Res.* **19**: 919-923.
5. Child, K. (2013). Natural cures to face safety scrutiny. *The Times*, November 20. Available from: www.timeslive.co.za.
6. Coleman, J.W. (2001). Nitric oxide in immunity and inflammation. *Int. Immunopharmacol.* **1**: 1397-1406.
7. Department of Health, (2007). Traditional Health Practitioners Act (35 of 2007). Pretoria: Republic of South Africa: <http://www.info.gov.za/view/DownloadFileAction?id=77788>.
8. Department of Health, (2008). Draft policy on African traditional medicine for South Africa. *S. Afr. Gov. Gazette Notice* **906**: 1-47.
9. Freudenberg, M.A., Tchaptchet, S., Keck, S., Fejer, G., Huber, M., Schutze, N., Beutler, B. and Galanos, C. (2008). Lipopolysaccharide sensing an important factor in the innate immune response to Gram-negative bacterial infections: Benefits and hazards of LPS hypersensitivity. *Immunobiol.* **213**: 193-203.
10. Gqaleni, N., Ngcobo, M., Parboosing, R. and Naidoo, A. (2012). *In vitro* testing of African traditional medicines for cytotoxic, immune modulatory and anti-HIV activities. *Afr. J. Tradit. Complement. Altern. Med.* **9**: 2-11.
11. Kasilo, O.M.J. and Trapsida, J.M. (2010). Regulation of traditional medicine in the WHO African region. *The African Health Monitor* **13**, 25-31.
12. Lekhooa, M., Walubo, A., Du Plessis, J.J.B. and Matsabisa, M.G. (2012). Evaluation of traditional medicines III: the mechanism of immune modulation by *PHELA*. *Afr. J. Tradit. Complement. Altern. Med.* **9**: 47-63.
13. Leung, T.F., Wong, K.Y., Wong, C.K., Fung, K.P., Lam, C.W.K., Fok, T.F., Leung, P.C. and Hon, K.L.E. (2007). *In vitro* and clinical immunomodulatory effects of a novel Pentaherbs concoction for atopic dermatitis. *Br. J. Dermatol.* **158**: 1216-1223.
14. Matkowski, A. (2008). Plant *in vitro* culture for the production of antioxidants- a review. *Biotechnol. Adv.* **26**: 548-560.
15. Matsuda, S. and Koyasu, S. (2000). Mechanisms of action of cyclosporine (Review). *Immunopharmacol.* **47**: 119-125.

16. Mowrey, D.B. (1998). Herbal tonic therapies. McGraw-Hill, New York.
17. Ngcobo, M., Nkala, B., Moodley, I. and Gqaleni, N. (2012). Recommendations for the development of regulatory guidelines for registration of traditional medicines in South Africa. *Afr. J. Tradit. Complement. Altern. Med.* **9**:59-66.
18. Olivier, D.K. (2012). The ethnobotany and chemistry of South African tonic plants: PhD Thesis. University of Johannesburg, 1-481.
19. Pinkoane, M.G., Greeff, M. and Koen, M.P. (2012). A model for the incorporation of the traditional healers into the national health care delivery system of South Africa. *Afr. J. Tradit. Complement. Altern. Med.* **9**:12-18.
20. Sharma, O.P. and Bhat, T.K. (2009). DPPH antioxidant assay revisited. *Food Chem.* **113**: 1202-1205.
21. Tzianabos, A.O. (2000). Polysaccharide immunomodulators as therapeutic agents: Structural aspects and biologic function. *Clin. Microbiol. Rev.* **13**: 523-533.
22. Vaidya, A.D.B. (2007). Reverse pharmacology and registration of Ayurvedic drugs. Presentation: International Arogya Conference, New Dehli.