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IN VITRO EFFECTS OF A COMMERCIAL HERBAL MEDICINE USED AS AFRICAN TRADITIONAL MEDICINE ON HUMAN NEUTROPHILS

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Abstract

Background: Commercial herbal medicines (CHMs) being marketed as immune boosters or tonics, have gained widespread popularity. The many herbal mixtures sold have not been tested for efficacy and safety, despite their modern packaging and presentations. It is imperative that these herbal mixtures be investigated for their effects on human neutrophils.
Methods: The selected herbal mixture (HM), *Stametta*™ Body healing liquid, is common in retail outlets in Pretoria, South Africa (SA) and is used as an immune booster or intended to strengthen the body. Isolated neutrophils as well as those in whole blood phagocytes were obtained from blood samples collected from consenting healthy adult volunteers. The neutrophils were incubated with the HM at different strengths, and taken through a luminol-enhanced luminescence assay, using activators- phorbol myristate acetate and N-formyl-methionyl-leucyl-phenylalanine.
Results: The HM had variable stimulatory and inhibitory effects on the luminescence activity of healthy isolated and non-isolated human neutrophils. The effects, ranging from weak to potent were either directly or inversely related to the concentration of the HM and were mediated through a direct protein kinase C activating mechanism and an indirect formyl peptide receptor-linked mechanism.
Conclusion: The findings have shown the immunomodulatory potential of *Stametta* ™. The *in vitro* inhibitory and stimulatory effects on neutrophils which are furthermore time-based, suggest variable effects on the immune system, which may be beneficial as well as risky. The effects at different concentrations highlight the importance of appropriate dosing. It would therefore be prudent to caution users of this commercial herbal medicine accordingly.

Keywords: herbal medicines, luminescence, neutrophils, inhibition, stimulation

Introduction

African traditional medicines (ATMs) are used for the treatment of a wide variety of conditions, illnesses and disease states and for general health and wellbeing (Hughes et al., 2013). Commercial herbal medicines (CHMs) being marketed as immune boosters or tonics, have gained widespread popularity. The use of these CHMs with modern packaging and marketing practices was reported to be a booming trade in SA, generating revenue for various sectors, including the pharmaceutical sector (Gqaleni et al., 2007). While the Department of Health in South Africa (SA) has made strides in the regulation of complementary and alternative medicines (CAMs), there is still no regulation for HMs used as ATMs (Mbedzi, F.B., Department of Health, SA, personal communication). As explained by Cano and Volpato (2004), herbal mixtures are simply concoctions of two or three species with the same popular medicinal use, and these may be prepared jointly to enhance the known beneficial effects of each species.

The immune system is a complex physiological system that serves to defend the body against foreign and abnormal cells. It involves both non-specific innate response and an acquired or adaptive immune system. Neutrophils are key components of the innate immune system and the inflammatory response. They form the largest components of leucocytes and are part of the first line of defense against invading microorganisms, generally circulating freely and passively until they are recruited into inflamed tissue (Bei et al., 1998; Cowburn et al., 2008). The role of neutrophils as effector cells is achieved by phagocytosing pathogens and destroying them using reactive oxygen species (ROS) generated by the intracellular NADPH oxidase (NOX) system and hydrolytic granule proteins (Dahlgren & Karlsson, 1999; Bylund et al., 2010).

The many herbal mixtures sold in many outlets and over the counter (OTC) in pharmacies for use as immune boosters, have not been tested for efficacy and safety the way conventional medicines are tested before use, despite their modern packaging and presentations (Ndhlala et al., 2011; Ndhlala & Van Staden, 2012). Although they are readily available and used, their safety and toxicity profiles remain unknown. It therefore calls for research by the scientific community, which would support the rational basis for the use of the herbal medicines. The aim of this study was therefore to investigate the effects on human neutrophils, of a commercial herbal medicine used commonly as an ATM to boost the immune system.

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materials and Method

Reagents and instruments

A biochemiluminescence assay was performed on the Orion™ L Microplate Luminometer supplied by Berthold Detection Systems, making use of standard 96-well white microplates. All the reagents used including Hanks' balanced salt solution (HBSS), phorbol myristate acetate (PMA), Percoll solution, dimethyl sulfoxide (DMSO), N-formyl-methionyl-leucyl-phenylalanine (fMLP) and luminol were supplied by Sigma Aldrich.

Collection of blood samples

Permission and ethical clearance (MREC/M/09/2011:PG) for the study was obtained from Sefako Makgatho Health Sciences University (SMU) Research Committee (SMUREC). The blood samples were collected from eight consenting adult healthy volunteers who were non-smokers, not on any medication and had not taken any self-medication such as painkillers, vitamin supplements or any herbs or herbal mixtures in the two weeks preceding the day of collection of sample. Each blood sample was processed immediately upon collection and taken individually through the whole process of analysing within the same day of collection.

Isolation of neutrophils

Neutrophils were isolated from each whole blood sample by using the Percoll sedimentation method outlined below, which was developed and adapted in the Department of Pharmacology laboratory of SMU (Kahler, 2000). Approximately 10 ml of each heparinised blood sample was carefully layered onto the Percoll working solution, such that the blood floated on top of the solution and was not submerged. The sample was centrifuged at 2000 rpm for 30 minutes, and the supernatant was removed by aspiration. Lysis buffer was added to the dark red layer at the bottom to lyse the red blood cells. After two successive centrifugations at 1000 rpm and the supernatant being discarded, the cell pellet at the bottom was resuspended in HBSS and kept on wet ice until used. A neutrophil cell count was performed for each sample at the National Health Laboratory Services haematology section.

Preparation of whole blood samples for luminescence

A whole blood sample was collected in a heparinised tube, and then a full blood count test with differential count was performed on the sample. Then a 50 times dilution of the sample was made with HBSS, for use in the luminescence determination. The sample was kept on ice-water until used.

Preparation of the herbal mixture (HM5)

The selected commercial herbal mixture *Stametta*™ (HM5) was one of the common HMs in retail outlets in Pretoria, (Gauteng, SA) used as an immune booster or intended to strengthen the immune system (Mothibe, 2015). Four serial dilutions of the herbal mixture were prepared, from 10x, 100x, 1000x to 10 000x with the HBSS.

Procedure for luminescence measurement

In a 96-well microplate, the following were added: 25 µl HBSS in each control well; 25 µl test solution in each test well (neat HM, followed by the 10x, 100x, 1000x and 10000x diluted solutions) and 25 µl of the cell suspension or 25µl of the whole blood sample in all the wells. The control wells contained cells and other reagents except the HM5 or its diluted solutions. The plate was covered tightly with parafilm wrap and incubated in a water-bath at 37^0C for 30 minutes, but not submerged in the water. Then the luminometer injectors were used to add 25 µl working luminol, 25 µl of either fMLP or PMA and 100 µl of HBSS, to make the final volume up to 200 µl in each well. This was followed immediately by reading the luminescence of each well (time = 0 minutes), measured in relative luminescence units (RLU), with total integral values set with repeated scans at 30 s intervals and 1 second points measuring time. Further readings for luminescence were taken for a total of 60 minutes, at time intervals of 5, 10, 15, 20, 25, 30, 45 and 60 minutes. Each sample was tested in triplicate.

Data collection, handling and analysis

The raw data from the luminometer were entered into a Microsoft Excel™ program to obtain descriptive statistics. The cell count for the luminescence activity measured were corrected for 1 x 10^3 cells/µl. The average luminol-enhanced luminescence (LEL) activity of cells for each test solution and controls were calculated per time interval. The control cells served as a reference for the LEL activities of cells in the absence of the HM5. The percentage inhibition (% inh) for each HM standard were calculated compared to the controls. The % inhibitions were graded as potent (≥ 80%), moderate (50-79%) and low (< 50%). Then average (mean) LEL activities for the 60 minutes and the corresponding % inhibitions were also calculated and displayed in tables.

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Statistical analysis

The LEL activity of each test standard was compared with the controls using the Microsoft Excel™ data analysis tool, t-Test: Two sample Assuming Unequal Variance. Statistical significance was declared where the differences had $p \leq 0,05$.

Results

Luminol-enhanced luminescence activity

The LEL activities of whole blood phagocytes (WBPs) and isolated human neutrophils after being incubated with the neat and the diluted standards of HM5 are shown in figures 1 and 2 respectively. The graphs showed how the activities of the cells changed in 60 minutes when compared with the control (CTRL) cells and how the effects vary with the different strengths of the HM.

The LEL activities of the fMLP-induced WBPs (A) incubated with the higher dilution standards (100X, 1000x and 10000x) and PMA-induced WBPs (B) incubated with the 100x diluted standard were significantly higher than the CTRL cells. The activities decreased gradually through the 60 minutes of reading (Figure 1). The LEL activities of all the isolated neutrophils exposed to the HM were lower than those of the CTRL cells, whether they were activated with PMA or fMLP (Figure 2).

The percentage inhibition of cells

The percentage inhibitions of the neat and the diluted standards of HM5 on WBPs and isolated neutrophils are shown in Figures 3 and 4 respectively. The graphs showed the variable effects of HM5 on the cells in 60 minutes when compared to the CTRL cells. The higher dilution standards (low concentrations) (100x, 1000X and 10000X) of HM5 had weak, moderate and potent stimulatory effects via both the fMLP and PMA pathways on WBPs (Figure 3). All the HM5 standards had general inhibitory effects on isolated neutrophils, ranging from weak to moderate through both pathways (Figure 4).

The average effects in 60 minutes of the neat HM and diluted standards were calculated and expressed in Tables 1 and 2. The WBPs were stimulated by the lower concentration standards in a directly concentration dependent manner via the fMLP pathway, as well as the PMA pathway (Table 1). The inhibitory effects on isolated neutrophils were also directly concentration dependent through the fMLP pathway by all the HM5 standards, and by the three higher concentration standards (the neat, 10X and 100X dilutions) via the PMA pathway (Table 2).

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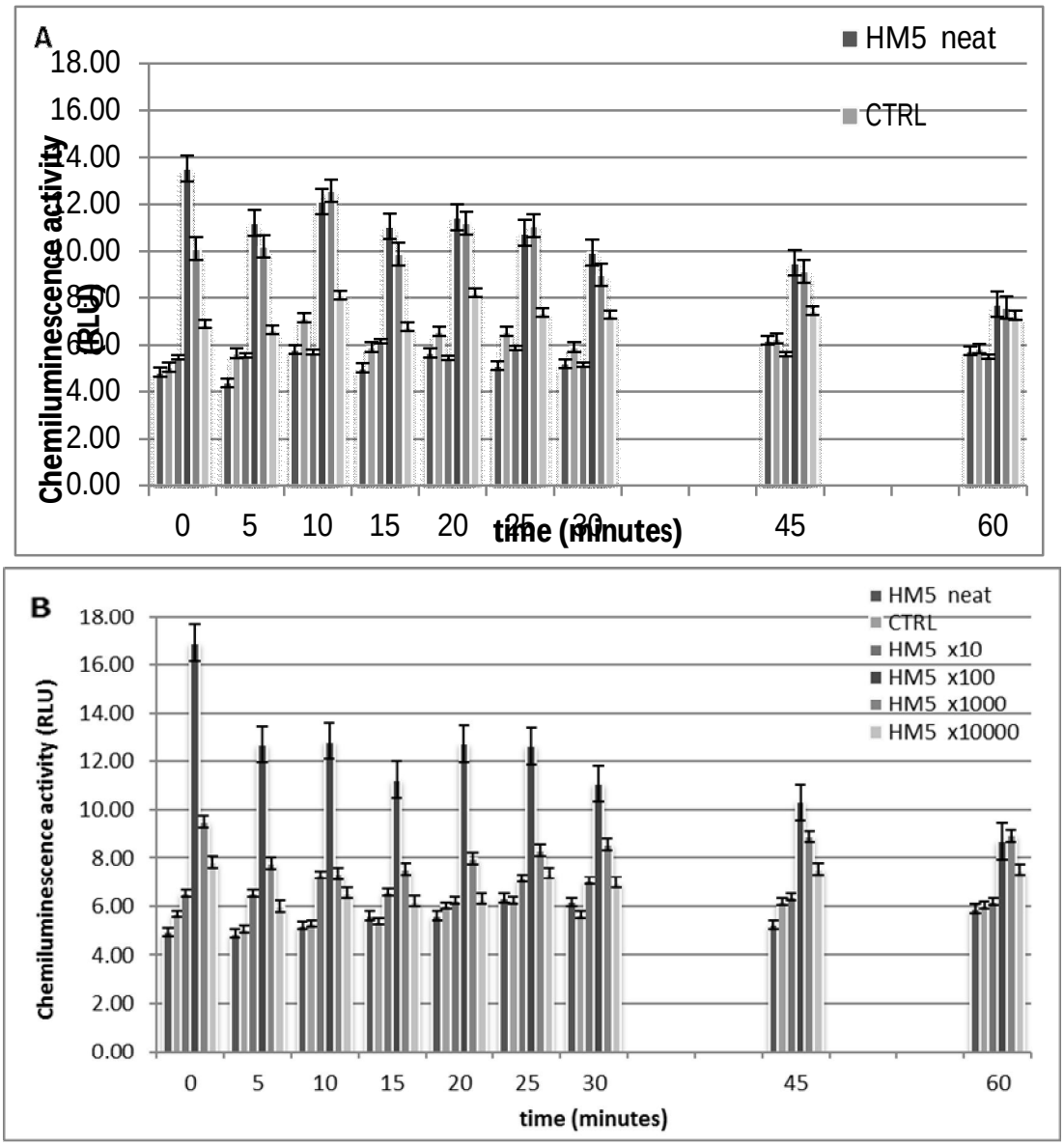

Figure 1: Luminol-enhanced luminescence (LEL) activity of whole blood phagocytes over time, after incubation with the neat HM5 and its diluted standards and stimulation with (A) fMLP and (B) PMA. (Each point displayed as mean ± SD, n = 8). The bars represent from left to right: neat HM5, CTRL, HM5 x10, HM5 x100, HM5 x 1000 and HM5 x 10000.

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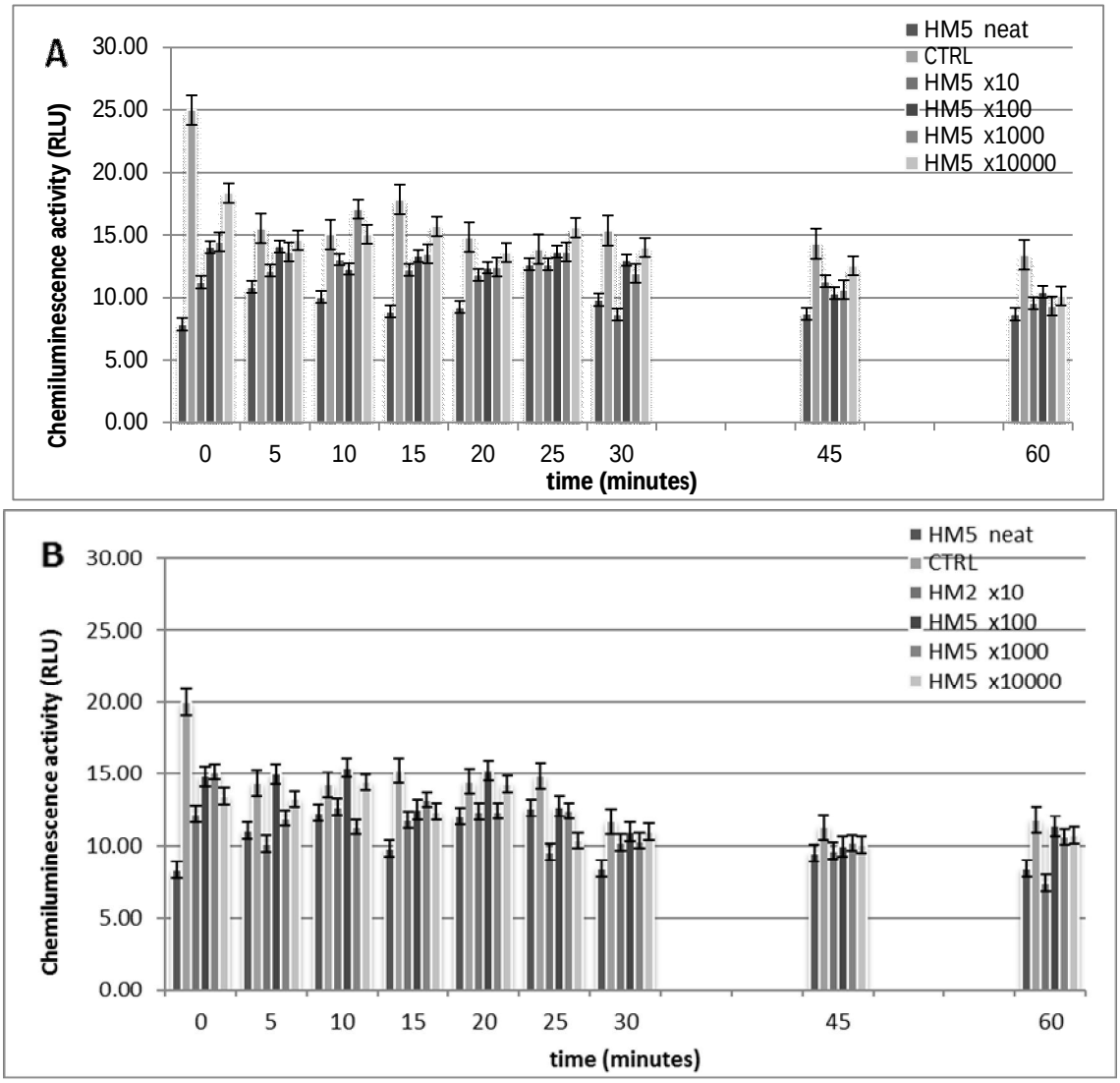

Figure 2: Luminol-enhanced luminescence activity of isolated human neutrophils over time, after incubation with the neat HM5 and its diluted standards and stimulation with (A) fMLP and (B) PMA. (Each point displayed as mean ± SD, n = 8). The bars represent from left to right: neat HM5, CTRL, HM5 x10, HM5 x100, HM5 x 1000 and HM5 x 10000.

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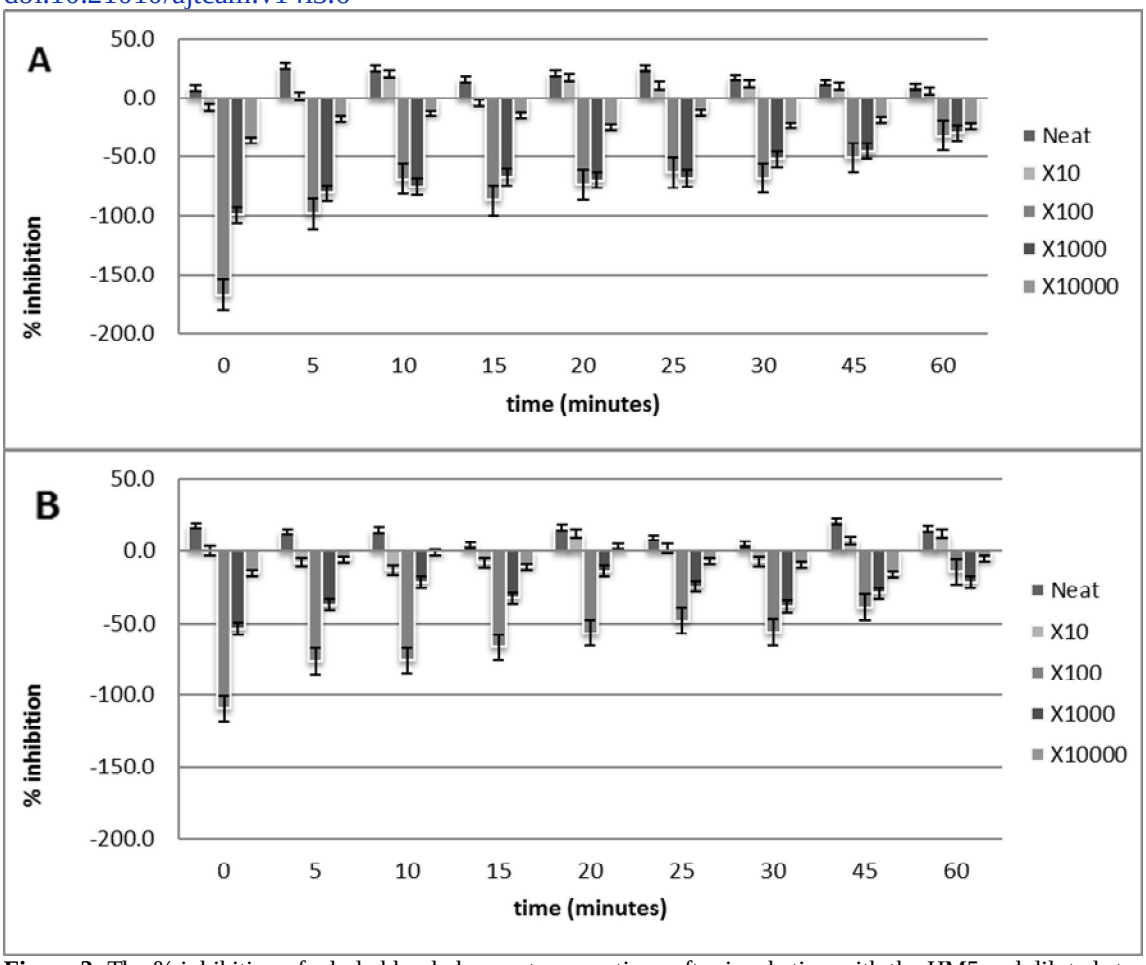

Figure 3: The % inhibition of whole blood phagocytes over time, after incubation with the HM5 and diluted standards, and stimulation with (A) fMLP and (B) PMA. (Each point displayed as mean ± SD, n = 8). Negative values indicate stimulation, positive values indicate inhibition. The bars represent from left to right: neat HM5, HM5 x10, HM5 x100, HM5 x 1000 and HM5 x 10000 diluted standards.

Table 1: The average LEL activity in 60 minutes and percentage inhibitions of whole blood phagocytes

Standard tested	fMLP-induced cells		PMA-induced cells	
	LEL activity (RLU)	% inhibition	LEL activity (RLU)	% inhibition
Control (CTRL)	6.07 ± 0.59		5.73 ± 0.43	
HM5 Neat	5.34 ± 0.56*	18.1 ± 6.9	5.53 ± 0.53	12.7 ± 5.8
HM5 10x dil	5.61 ± 0.28	7.4 ± 9.5	6.65 ± 0.41*	-0.6 ± 9.4
HM5 100x dil	10.81 ± 1.64*	-79.1 ± 38.2	12.13 ± 2.27*	-60.4 ± 26.5
HM5 1000x dil	10.09 ± 1.45*	-65.6 ± 20.5	8.31 ± 0.73*	-30.7 ± 11.8
HM5 10000x dil	7.34 ± 0.55*	-20.7 ± 7.5	6.91 ± 0.67*	-7.9 ± 6.3

dil- dilution; Results displayed as mean ± SD, * $p \leq 0.05$). Negative values indicate stimulation, positive values indicate inhibition.

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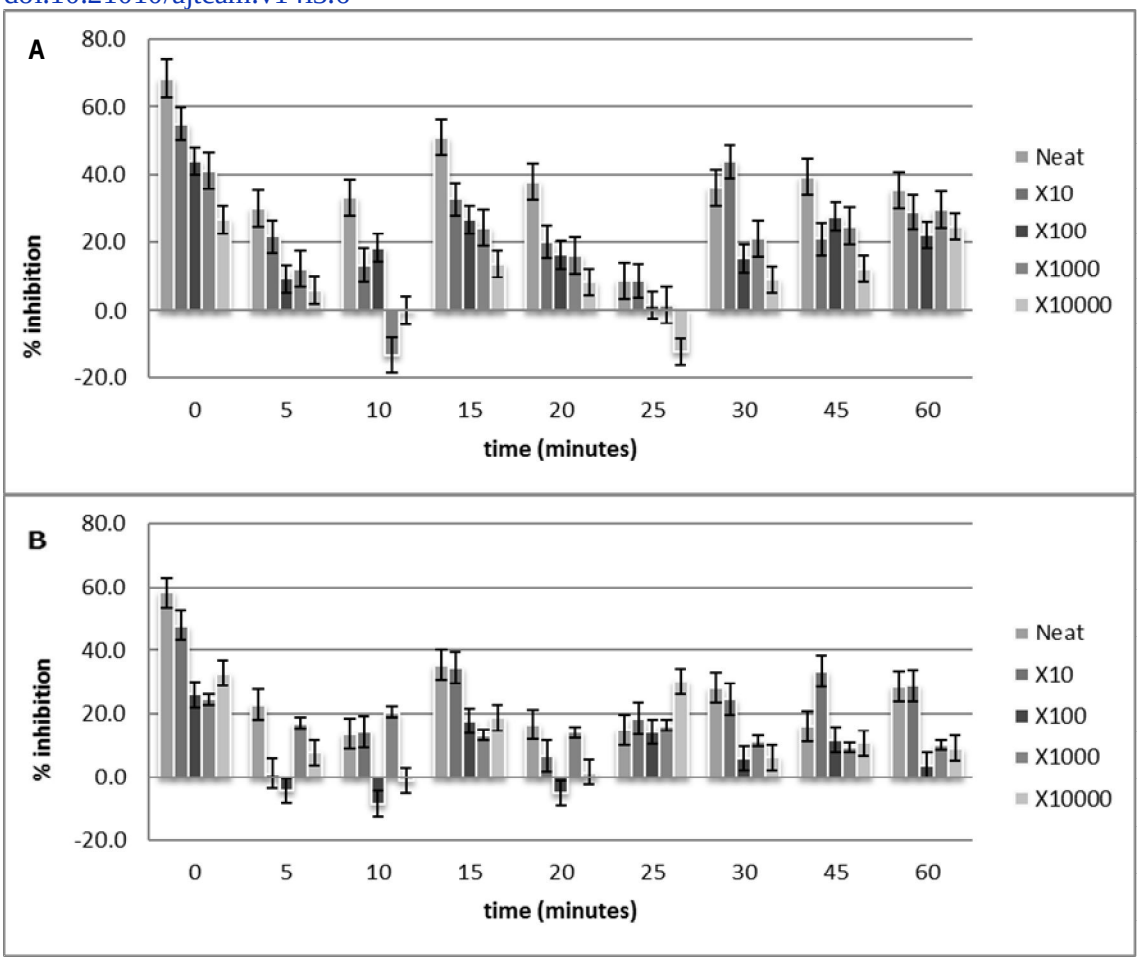

Figure 4: The % inhibition of isolated human neutrophils over time, after incubation with the HM5 and diluted standards, and stimulation with (A) fMLP and (B) PMA. (Each point displayed as mean ± SD, n = 8). Negative values indicate stimulation, positive values indicate inhibition. The bars represent from left to right: neat HM5, HM5 x10, HM5 x100, HM5 x 1000 and HM5 x 10000 diluted standards.

Table 2: The average LEL activity in 60 minutes and percentage inhibitions of isolated human neutrophils

Standard tested	fMLP-induced cells		PMA-induced cells	
	LEL activity (RLU)	% inhibition	LEL activity (RLU)	% inhibition
Control (CTRL)	16.17 ± 3.57		14.21 ± 2.64	
HM5 Neat	9.64 ± 1.44*	37.7 ± 16.0	10.29 ± 1.76*	26.0 ± 14.2
HM5 10x dil	11.41 ± 1.45*	27.2 ± 14.7	10.79 ± 2.33*	23.3 ± 14.7
HM5 100x dil	12.62 ± 1.41*	20.1 ± 12.1	13.12± 2.07	6.8 ± 11.6
HM5 1000x dil	12.96 ± 2.24*	17.5 ± 16.0	11.96 ± 1.57*	15.2 ± 5.0
HM5 10000x dil	14.39 ± 2.28	9.8 ± 11.9	12.21 ± 1.71	12.8 ± 12.0

dil- dilution; Results displayed as mean ± SD, * $p \leq 0.05$). Negative values indicate stimulation, positive values indicate inhibition.

Discussion

The determination of chemiluminescence activity is a direct measurement of the amount of light emitted by cells when stimulated. The light emitted is derived from the respiratory burst of cells resulting in the formation of reactive oxygen species (ROS). Therefore the light emitted is directly proportional to the amounts of ROS formed, and indicates whether the cells are stimulated or inhibited and to what extent. The two chemotactic agonists/activators – PMA and fMLP act via different mechanisms on the cells to stimulate production of ROS by the cells. PMA directly activates protein kinase C (PKC) resulting in activation of NADPH oxidase (NOX). The agonist, fMLP binds to specific G-protein-linked formyl peptide receptors on the cell membrane, initiating a cascade of reactions starting with the activation of phospholipase C (PLC) leading to activation of PKC, which in turn activates NOX. Although all the phagocytes are present in the whole blood sample, the luminescence signal obtained in whole blood is a function of the

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quantity and activity of neutrophils (Ristola & Repo, 1989), and is also indicative of their metabolic activity (Nordman et al., 1994).

The findings in this study showed that *Stametta*™ Body healing liquid (HM5) had variable effects on the luminescence activity of healthy isolated and non-isolated (whole blood phagocytes) human neutrophils; effects that ranged from weak to potent, and were mediated through a direct PKC activating mechanism as well as an indirect formyl peptide receptor-linked mechanism. The HM had general concentration dependent inhibitory and stimulatory effects on non-isolated neutrophils. The effects recorded in 60 minutes were highest initially and dissipated with time, suggesting a time-based mechanism. The herbal mixture had concentration dependent inhibitory effects on isolated neutrophils; effects which were directly related when mediated through the formyl receptor-linked pathway. The effects were inversely related to the concentration when lower concentrations of the HM were used, via the direct PMA pathway and fluctuated with time.

Inhibition of the neutrophils translates to reduced amount of ROS while stimulation means increased formation thereof. As postulated by Mahomoodally et al. (2012), modifying of both fMLP and PMA mechanisms suggests that the HM does not affect a specific pathway; instead it may directly act on a common biochemical target site such as NOX or scavenge the ROS. The stimulatory effects of the HM on non-isolated neutrophils suggested that the HM interacted with one or more other blood or plasma molecules, resulting in the increased formation of ROS by the neutrophils.

Stametta™ BHL is one of the popular CHMs used as ATM in Tshwane townships in Gauteng, SA (Mothibe, 2015). It was listed as one of the herbal remedies used by HIV patients in Kwazulu-Natal, SA, before and during antiretroviral therapy (ART) (Peltzer et al., 2011). It was found to have high levels of total phenolic compounds and lower levels of flavonoids, had relatively lowest toxicity on human liver cells and had indirect mutagenic activity when tested using the Ames test (Ndhlala et al., 2010). It is stated on the package that it contains aloe (1.667 g), ascorbic acid (1.667 g), aniseed oil (0.1817 g) and magnesium sulphate (71.667 g) per 500 ml. It is also said that regular use will improve general well-being and strengthen the immune system and is recommended to be used at a dose of 50 – 100 ml three or four times a week. Ascorbic acid is known for its antioxidant activities (Mainardi et al., 2009) and is an intracellular and extracellular scavenger of free radicals (Stamp et al., 2005). Aloe has antioxidant, anti-inflammatory and immunostimulatory activity, amongst its other pharmacological activities (Steenkamp & Stewart, 2007; Street & Prinsloo, 2013; Amoo et al., 2014). Compounds that have been isolated in aloe include classes such as the anthrones, flavonoids and alkaloids together with bioactive compounds such as aloin, aloesin and aloemodin, dithranol, magnesium lactate (Rodriguez-Fragoso et al., 2008; Amoo et al., 2014). As Spelman et al. (2006) stated, the pharmacological effects and therapeutic efficacies of medicinal plants are derived from several compounds acting in synergy, rather than from a single compound. Hence the activities in the study could be from the multiple components in aloe as well as those in the other ingredients. Flavonoids are the plant compounds proven to have an inhibitory effect on the respiratory burst (ROS production) of neutrophils. They are also known as powerful antioxidants as they are able to scavenge a diverse range of ROS (Ciz et al., 2012).

Therefore the inhibition of formation of ROS by the HM is warranted since two of its components have antioxidant activity. In healthy cells this effect plays a protective role against tissue damage caused by oxygen free radicals. Hence this may be indicative of the role of this HM as an antioxidant or scavenger of ROS, which may benefit the users as prophylaxis against the onset and progress of various conditions such as respiratory disease syndrome, asthma and rheumatoid arthritis (Caldefie-Chezet et al., 2002; Tintinger et al., 2008). However, the inhibition of neutrophils may also have other effects. As stated earlier the effectiveness of neutrophils as phagocytes is determined by a functional NOX system which generates ROS. ROS serve as potent antimicrobial agents that play an important role of defence against pathogens. Inhibited neutrophils may be unable to respond to stimuli as effector cells of the immune system. Therefore the intake of HM5 may compromise the immune system, leading to individuals being more susceptible to infections. Possible adverse drug interactions may also occur if there is need for use of antimicrobials as well as anti-inflammatory drugs. The success of bacteriostatic drugs as treatment depends on a viable immune system; hence there may be failure of bacteriostatic drug therapy; while the concomitant use of anti-inflammatory medicines may result in a severely depressed immune system.

Stimulation of neutrophils by the HM may be beneficial as part of immune-boosting effects. The rationale is that activated neutrophils would respond rapidly and favourably on stimulation, and be more efficient as elements of the immune system. Hence it may assist in the body resisting and managing infections and diseases. The presence of ROS may have both negative and positive attributes in a system. ROS have been said to be a threat to bystander host cells and tissues in the vicinity of an inflammatory reaction, and that they may cause damage by destroying surrounding tissue and inducing apoptosis in other immune reactive cells (Dahlgren & Karlsson, 1999; Tintinger et al., 2008). Inappropriate activation of neutrophils and extensive release of intracellular oxidising agents contributed to the development and progress of many acute and chronic inflammatory disorders of infective and non-infective origin (Caldefie-Chezet et al., 2002; Tintinger et al., 2008). Droge (2002) stated that an excessive and /or sustained increase in ROS production was associated with the pathogenesis of many diseases, and as such the functions of neutrophils must be regulated appropriately, to avoid tissue damage and to achieve proper host defense. It therefore needs to be noted that while it is suggested that the stimulation of neutrophils may be beneficial for healthy individuals, it may also lead to the development of acute and chronic inflammatory disorders and immune diseases mentioned. Healthy individuals are simply persons undiagnosed with any specific condition; however these people may be at risk of, or have a genetic

predisposition to undiagnosed conditions. The intake of this HM may therefore accelerate progression towards or trigger the onset of any of the mentioned conditions.

Conclusion

The findings in this study have shown the immunomodulatory potential of *Stametta* ™, which may explain its popularity as an immune boosting herbal medicine. The *in vitro* inhibitory and stimulatory effects on neutrophils which are furthermore time-based, suggest variable effects on the immune system, which may be beneficial as well as risky. The effects at different concentrations highlight the importance of appropriate dosing. It would therefore be prudent to caution users of this commercial herbal medicine accordingly.

Acknowledgements

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