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Does Sutherlandia frutescens influence the immune system via regulation of macrophage polarization and function?

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Sutherlandia frutescens (L) R. BR. belonging to the Family *Fabaceae,* subsp. *Microphylla* is a medicinal plant, indigenous to South Africa, that is used to treat various conditions, including type 2 diabetes and immune disorders[1,2,3]. It has also been classified as *Lessertia frutescens,* although there is still debate as to whether these are the same plant[4]. A randomized placebo based trial indicated that *S. frutescens/L. frutescens* is non-toxic and tolerated by humans[5].

In view of increasing evidence that low-grade chronic inflammation plays a role in the pathogenesis of type 2 diabetes and other metabolic disorders[6,7], we propose a case for a medicinal role of *S. frutescens* extracts in regulating the inflammatory process. The macrophage lineage is an important common component of inflammatory metabolic conditions and extracts of *S. frutescens* are thought to have the capacity to regulate macrophage differentiation, thus influencing the progression of metabolic immune diseases[8,9,10,11].

S. frutescens contains a complex mixture of free amino acids, the non-protein amino acids γ-aminobutyric acid (GABA) and L-canavanine, flavonoids, triterpenes, saponins and plant steroids, with no identified active component[1,12,13,14,15,16]. In addition, polysaccharide components have been purified from the leaves and their bioactivity analysed, including pectins, galactose-rich regions of the AGII and AGI type and fragments rich in xylose[4]. Zhang et al[4] concluded that these could contribute to immunomodulating activity, potentially via the complement system. A number of different extracts and purified components have been analysed, with hot aqueous and ethanolic extracts most commonly used traditionally and in animal studies and cell culture experiments. Extracts vary depending on the method of preparation and plant source, leading to discrepancies in reported action. For our work samples of plants identified by Professor E Campbell, Botany Department, Nelson Mandela University, were collected from a single source in the Karoo region of the Western Cape, to avoid any variation brought about by environmental conditions. A voucher specimen was deposited in the University Herbarium as PEU 14 800. We have analysed ethanolic, methanolic, hot and cold aqueous *S. frutescens* leaf extracts with proven anti-diabetic and macrophage regulating activity using untargeted triple-Time-of-Flight Liquid Chromatography-Mass Spectroscopy (Triple TOF LC-MS) separation in positive ionic mode[17]. All extracts contained measurable amounts of flavonoids, flavonols, tannins and phenols, with the organic extracts containing the highest amounts of flavonoids and flavonols[17]. Hydrophilic substances are primarily found in the aqueous extracts, which may account for discrepancies in relative potency, as reported by us[11,17] and in the literature[9,10,18]. Although we found no single compound with identified anti-diabetic activity that was common to all the extracts tested, combination profiles of known compounds were identified for each extract[17]. Therefore, we conclude that most likely a synergistic combination of compounds provides optimal medicinal activity and that differing profiles in different extracts can account for their divergent activity. We demonstrated *in vivo* biological activity of our hot aqueous extract

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as an anti-diabetic and immune modulatory agent, using male Wistar rats fed a high fat diet to induce obesity and a pre-diabetic insulin resistant state[8]. All our extracts have anti-diabetic activity shown in cell culture studies using liver cell lines[17,19] and immune regulatory action in macrophage cell lines (11, Fortuin-Seedat, unpublished data), although we found that the ethanolic extract containing hydrophobic molecules had the most potent anti-inflammatory activity[11].

Macrophages are a major cell type in the immune response, displaying phenotypic heterogeneity, which enables them to undertake different roles depending on the biological situation[20]. They develop from circulating blood monocytes and differentiate in the tissues during inflammatory responses[21]. A spectrum of many different macrophage populations has been characterized using combinations of membrane markers and gene expression profiles[22]. This has led to the identification of two main functionally distinct macrophage phenotypes: M1 pro-inflammatory and M2 anti-inflammatory macrophages following stimulation by microbial products, cytokines or other immunomodulatory molecules[23,24]. Their overall function is to maintain homeostasis, immune defence and tissue repair[22].

The effects of *S. frutescens* on macrophage development from monocytes, and the activation of differentiated macrophages has been investigated. Using the human THP-1 monocyte cell line (which differentiates into macrophages following induction with Phorbol Myristate Acid), we demonstrated that both aqueous and ethanolic extracts of *S. frutescens* significantly increased adherence and expression of the macrophage marker CD14 after 48 hours, compared to cells treated with PMA alone. Shorter treatment times did not alter either CD14 expression or cell adherence, suggesting that *S. frutescens* does not affect early macrophage differentiation, but is directed at later macrophage activation stages (Oosthuysen, unpublished data). In support of this hypothesis, Africa and Smith[25] demonstrated that *S. frutescens* increased the migration of an HIV Tat protein-stimulated primary human monocyte culture model, but not unstimulated cells.

Investigation into the regulation of macrophage function by *S. frutescens* has produced some conflicting results. Lei *et al*[9] reported that a polysaccharide-enriched fraction of an aqueous extract of *S. frutescens* was associated with dose-dependent elevations of the pro-inflammatory product nitric oxide (NO), the cytokine Tumor Necrosis Factor-α (TNF-α), several chemokines, and intracellular reactive oxygen species (ROS) in RAW 264.7 murine macrophages. However, later prevention studies by Lei *et al*.[10] showed that pre-treatment of the RAW 264.7 cell line with an ethanolic extract of *S. frutescens* limited an immune response stimulated by bacterial lipopolysaccharide (LPS) and interferon gamma (IFNγ) through the reduction of ROS and NO generation. They found that semi-purified sutherlandioside B and sutherlandins reduced ROS, but had no effect on NO and nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB), whilst sutherlandiosides A, C and D had no effect at al[10]. Only the complete ethanolic extract down regulated ROS and NO, acting on NFκB, ERK and STAT1α signalling molecules[10]. We have also investigated[11] the influence of our hot aqueous extract and the ethanolic extract upon the pro-inflammatory M1 and the anti-inflammatory M2 phenotypes of LPS stimulated RAW 264.7 cells. Using Flow cytometric analysis of the CD markers CD86 (M1) and CD206 (M2), we demonstrated that both *S. frutescens* extracts significantly down-regulated CD86 expression, and the ethanolic extract up-regulated CD206, indicating stimulation of the M2 phenotype over the M1 phenotype[11]. These extracts also significantly inhibited NO production, production of ROS and COX-2 expression in a dose dependent manner, with the ethanolic extract showing greatest activity[11], in agreement with Lei et al[10]. Patterns of cytokine production determined by ELISA assays indicated that the M1 pro-inflammatory cytokines TNF-α, IL-6, IL-1α, G-CSF and GM-CSF were all down regulated, whilst the M2 cytokine profile remained unchanged[11]. These studies again indicate that *S. frutescens* may down regulate an ongoing inflammatory response involving M1 macrophages, and also influence the polarisation of M1 and M2 macrophage sub-populations.

The molecular interactions and signaling pathway

involved in macrophage regulation by *S. frutescens* are not yet clear. We have investigated the effects on the NFκB and MAPK signaling pathways in the RAW 264.7 and the THP-1 cell lines. In RAW 264.7 reduced production of inflammatory mediators was associated with significantly decreased activity of the mitogen activated protein kinases (MAPKs) and NFκB signalling pathways[11]. The NFκB signalling pathway regulates the expression of an array of inflammatory genes linked to M1 macrophage activation[26]. In resting cells, the NFκB transcription factor is complexed with the inhibitory protein I_KB, masking the NFκB Nuclear Location Signal, preventing its translocation into the nucleus[27]. Upon pro-inflammatory stimulation, I_KB undergoes phosphorylation and is rapidly degraded, enabling NFκB phosphorylation and activation, followed by translocation from the cytosol to the nucleus, inducing up-regulation of M1 macrophage pro-inflammatory genes. LPS-stimulation increases phosphorylation and nuclear translocation of p65 (Ser 536) NFκB, but we demonstrated that treatment with both hot aqueous and ethanolic *S. frutescens* extracts inhibited translocation of NFκB in a dose dependant manner, associated with a dose-dependent decrease in p65 NFκB phosphorylation[11]. This finding correlates with a report from Lee *et al.*[28], who established that an ethanolic extract of *Solanum tuberosum L.* cv Jayoung inhibited NFκB activation in RAW 264.7 cells, indicating that plant phytochemicals can regulate NFκB signalling in macrophages.

Activation of NFκB and macrophage inflammation is also regulated by MAPK cellular kinases. The MAPK signaling pathways are themselves activated through phosphorylation of their Thr-Tyr residues[29] to mediate cell growth, differentiation, cell death and immune responses[30,31]. ERK1/2 and p38 MAPK are rapidly phosphorylated in response to LPS stimulation, to induce the expression of iNOS and COX-2, amongst other inflammatory mediators[32,33]. Activation of p38 MAPK is also directly linked to NFκB activation[18]. Camille and Dealtry[11] showed that *S. frutescens* attenuated LPS induced MAPKs ERK1/2 and p38 MAPK phosphorylation in RAW 264.7 cells in a dose-dependent manner, with the ethanolic extract being significantly more potent than the hot aqueous extract. Thus the MAPK signaling pathway is also influenced by *S. frutescens*, in agreement with studies of other cell types in which *S. frutescens* has been reported to inhibit activation of ERK1/2 and p38 MAPK[18]. Importantly, Camille and Dealtry[11] have shown that both aqueous and ethanolic *S. frutescens* extracts influence the reactive state of mature Raw 264.7 macrophages via NFκB and MAPK signalling. This leads to the hypothesis that several actions of *S. frutescens* extracts may be mediated by a reduction in M1 macrophage activity and redirection towards M2 anti-inflammatory functions, promoting tissue remodelling and immune regulation.

In order to investigate the induction of activated macrophages from inactive monocytes, our current work focuses upon the human THP-1 monocyte cell line. The monocytes are stimulated to differentiate into macrophages by PMA, as indicated by increased CD14; and activated along the M1 pathway by LPS, indicated by an increase in CD86. In these cells, the ethanolic extract of *S. frutescens* significantly decreased CD86 expression in a dose dependant manner, but did not alter expression of the M2 marker CD206 (Fortuin-Seedat, unpublished data).

Regulation by *S. frutescens* of the signalling pathways influencing the transcription factor NFκB is being investigated in THP-1 cells by Western blot analysis of phosphorylation of key signalling molecules. In agreement with Camille and Dealtry[11] and Lei *et al*[10], phosphorylation of p38 MAPK was decreased in a dose dependent manner by the ethanolic extract. However, high doses of the hot aqueous extract (greater than 100µg/ml) increased phosphorylation of p38 MAPK (Fortuin-Seedat, unpublished data). Phosphorylation of ERK1/2 (another component of the MAPK pathway) was increased by both aqueous and ethanolic *S. frutescens* extracts (Fortuin-Seedat, unpublished data). The parallel signalling pathway via phosphorylation of JNK was also stimulated by the ethanolic extract, as shown by an increase in phosphorylated JNK, although the hot aqueous extract had no significant effect (Fortuin-Seedat, unpublished data). Africa and Smith[25] also found that phosphorylation of JNK was increased in primary cultures of human monocytes by a hot aqueous extract of *S. frutescens*.

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Akt is also a regulator of NFκB and showed a reduction in phosphorylation following treatment with the hot aqueous extract, but an increase in phosphorylation after treatment with high doses of the ethanolic extract (Fortuin-Seedat, unpublished data). Phosphorylation of the transcription factor NFκB itself (p65 NFκB) was decreased by both the ethanolic and hot aqueous extracts (Fortuin-Seedat, unpublished data), in agreement with Camille and Dealtry[11] who showed that both the ethanolic and the hot aqueous extracts inhibited nuclear translocation of NFκB, a phosphorylation-dependent process.

We speculate that the observed differences in activity of *S. frutescens* extracts may reflect actions at different stages of macrophage differentiation and activation. In addition, although similar concentrations of the major phytocompounds (tannins, flavonoids and phenols) were found in both the hot aqueous and the ethanolic extracts, as expected more hydrophobic flavonols were found in the ethanolic extract[17]. These differences may underlie the increased potency of the ethanolic extract to down regulate inflammatory responses, but may also explain the stimulatory action of high doses of aqueous extract and will be investigated further. Furthermore, the differing activities may also reflect differences in specific compounds within the phytochemical groups in each of the extracts.

In conclusion, we and others have shown that *S. frutescens* influences the immune system via regulation of macrophage polarization and function, acting via down regulation of the NFκB and MAPK signalling pathways. The greatest activity is found with ethanolic extracts containing predominantly hydrophobic compounds, but aqueous extracts also have shown anti-inflammatory activity. Further investigations will help to define the roles of the different phytocompounds and to indicate which extracts are best used in medicinal therapies.

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