



HHS Public Access

Author manuscript

Int Immunopharmacol. Author manuscript; available in PMC 2016 December 01.

Published in final edited form as:

Int Immunopharmacol. 2015 December ; 29(2): 254–262. doi:10.1016/j.intimp.2015.11.012.

Unveiling the anti-inflammatory activity of *Sutherlandia frutescens* using murine macrophages

Wei Lei¹, Jimmy D. Browning Jr.¹, Peggy A. Eichen¹, Korey J. Brownstein², William R. Folk², Grace Y. Sun², Dennis B. Lubahn², George E. Rottinghaus³, and Kevin L. Fritsche^{1,4}

¹Division of Animal Sciences, University of Missouri, Columbia, MO 65211, USA

²Department of Biochemistry, University of Missouri, Columbia, MO 65211, USA

³Veterinary Medical Diagnostic Laboratory, University of Missouri, Columbia, MO 65211, USA

⁴Department of Nutrition and Exercise Physiology, University of Missouri, Columbia, MO 65211, USA

Abstract

Sutherlandia frutescens is a botanical widely used in southern Africa for treatment of inflammatory and other conditions. Previously, an ethanolic extract of *S. frutescens* (SFE) has been shown to inhibit the production of reactive oxygen species (ROS) and nitric oxide (NO) by murine neurons and a microglia cell line (BV-2 cells). In this study we sought to confirm the anti-inflammatory activities of SFE on a widely used murine macrophage cell line (i.e., RAW 264.7 cells) and primary mouse macrophages. Furthermore, experiments were conducted to investigate the anti-inflammatory activity of the flavonol and cycloartanol glycosides found in high quantities in *S. frutescens*. While the SFE exhibited anti-inflammatory activities upon murine macrophages similar to that reported with the microglia cell line, this effect does not appear to be mediated by sutherlandiosides or sutherlandins. In contrast, chlorophyll in our extracts appeared to be partly responsible for some of the activity observed in our macrophage-dependent screening assay.

Keywords

Sutherlandia frutescens; inflammation; macrophage; chlorophyll

1. Introduction

Sutherlandia frutescens (L.) R. Br, commonly referred to as “cancer bush”, is a medicinal plant traditionally used in southern Africa for a variety of health conditions [1]. *S. frutescens* has been used to treat patients suffering from various chronic health conditions, such as rheumatoid arthritis, osteoarthritis, and gout where inflammation plays a significant role in

Correspondence to: Dr. Kevin L Fritsche, Professor, Division of Animal Sciences; Dept. Nutrition & Exercise Physiology, 110 Animal Sciences Research Center, University of Missouri, Columbia, MO 65211, Phone: 573-882-7240, FritscheK@missouri.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

the disease etiology or symptoms [2]. Fernandes and coworkers [3] suggested that the antioxidant properties of *S. frutescens* contribute to its anti-inflammatory actions. Using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging assay of acetone, aqueous, ethanol, methanol, and chloroform extracts of *S. frutescens* [4-6], antioxidant properties were demonstrated without direct evidence of anti-inflammatory activity. In fact, the evidence of anti-inflammatory activity in *S. frutescens* is fairly limited and mostly indirect. Kundu et al. [7] demonstrated that a methanolic extract of *S. frutescens* could decrease 12-O-tetra-decanoylphorbol-13-acetate-induced cyclooxygenase-2 (COX-2) expression in mouse skin. The proposed mechanism was a reduction in the activity of activator protein-1 (AP-1), a component of the ERK signaling pathway. Recently, we reported the ability for ethanol extract of *S. frutescens* to inhibit neuronal excitotoxicity and mitigate lipopolysaccharide (LPS)-induced ROS and NO production in BV-2 microglial cells [8].

Several biologically active compounds have been identified in extracts of *S. frutescens*, including: L-canavanine, pinitol, and gamma-aminobutyric acid (GABA) [9, 10]. Fu and colleagues [11] characterized two groups of compounds unique to *S. frutescens*: Sutherlandiosides (a group of cycloartane glycosides with structural variants denoted by designations A, B, C, D) and sutherlandins (a group of flavonol glycosides derivatives of 3-hydroxy-3-methylglutarate with structural designations of A-D, also). Investigation into the biological activities associated with these cycloartane- and flavonol-glycosides from *S. frutescens* has been limited [11-13]. No reports exist regarding the biological functions/activities of either sutherlandiosides or sutherlandins on innate immune cells.

Macrophages are innate immune cells that play critical roles in the inflammatory responses [14]. These cells are activated by a complex of immune-mediators including pathogen associated molecules (such as LPS from gram-negative bacteria) and host factors (e.g., TNF- α , IFN γ) [15, 16]. Co-stimulation with LPS and IFN γ has been widely used as a means of stimulating/activating macrophages. Following stimulation, the subunits of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases are assembled resulting in the production of large quantities of ROS [17]. ROS is known to play critical roles in anti-microbial activity and serves as secondary signaling molecules to modulate the inflammatory responses [18]. Additionally, LPS and IFN γ co-stimulation of murine macrophages promotes the release of nitric oxide (NO) through induction of nitric oxide synthase (iNOS), which depends on several signaling pathways, including: NF- κ B, MAP kinase (e.g. ERK1/2), and JAK-STAT1 [19-21]. However, over-production of ROS or NO can result in oxidative and nitrosative stress that can lead to health-related problems (e.g., cancer, inflammatory disease) [22-24]. The objective of this study was to investigate the anti-inflammatory activity of *S. frutescens* in macrophages, and to better define the putative bioactive compounds responsible for this activity.

2. Materials and Methods

2.1. Reagents

Lipopolysaccharide (LPS, from *E. coli* 0111:B4) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Murine interferon- γ (IFN γ) was purchased from R&D Systems (Minneapolis, MN, USA). Fetal bovine serum (FBS) was from Thermo Scientific (Logan,

Utah, USA). Antibodies used for Western blotting include: goat anti-rabbit IgG-horseradish peroxidase, goat anti-mouse IgG-horseradish peroxidase, and iNOS polyclonal from Santa Cruz Biotechnology (Santa Cruz, CA USA); phospho- and total-ERK1/2 antibodies from Cell Signaling Technology (Danvers, MA, USA); phospho-STAT1 α antibody from Thermo Fisher Scientific (Rockford, IL, USA); and total-STAT antibody from Millipore (Billerica, MA, USA). The 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) for ROS detection was purchased from Invitrogen, Inc. (Carlsbad, CA, USA). For the cytokines and chemokines analysis, a Multi-Plex kit (Cat. MCYTOMAG-70K-PMX) was obtained from EMD Millipore (Billerica, MA, USA). Protease inhibitor cocktail (P8340) was purchased from Sigma (St. Louis, MO, USA).

2.2. Preparation of ethanolic extract of *S. frutescens* (SFE)

S. frutescens (L.) R. Br. desiccated powder was purchased from Big Tree Nutraceutical (Fish Hoek, South Africa) and verified as described previously [25]. For preparation of SFE, 10 g of *S. frutescens* was stirred into 250 ml 95% ethanol. The mixture were left to stand overnight at room temperature, after which it was centrifuged, filter sterilized (0.2 μ m, Fisher Scientific, Pittsburgh, PA, USA), and then stored at -80°C until used. Fifty milliliters of extract was dried at 55°C . The yield of dry matter from the SFE was 4.16 mg/mL. Prior to use in cell culture experiments, the ethanolic extract was dried at low temperature under a vacuum (Centrivap Concentrator, LABCONCO, Kansas City, MO, USA). The dried extract was then re-suspended in a volume of dimethyl sulfoxide (DMSO) that was one-tenth of the original volume of the ethanolic extract. The addition of SFE to cells was carried out such that the final concentration of DMSO was always kept constant at 0.1% (vol/vol), including those cells designated “negative” and “positive” controls. Preliminary experiments demonstrated that this concentration of DMSO was without effect on cell viability as well as the other biological readouts measured (data not shown).

2.3. Determination and isolation of sutherlandiosides, sutherlandins and chlorophyll from *S. frutescens*

Sutherlandioside B, a mixture of sutherlandiosides A, C, D, a mixture of sutherlandins A and B, and a mixture of sutherlandins C and D were isolated from *S. frutescens* as described by Brownstein et al. [26], a modified isolation procedure originally described by Fu et al. [11, 27]. The sutherlandiosides and sutherlandins in the crude extracts and the quality of semi-purified fractions were determined using liquid chromatography (LC)-evaporative light scattering detection or LC-UV as described by Avula et al. [28]. The purity of sutherlandioside B was $> 95\%$ based on the analysis result, while the purity of fractions with sutherlandiosides A, C, D, sutherlandins A and B, and sutherlandins C and D was better than 90%. Each bioactive purified from these extracts as well as the original crude extract were screened for the presence of bacterial endotoxin using a highly-sensitive NF- κ B reporter cell line described below and were found to be free of such contamination.

The concentration of chlorophyll in the crude ethanolic extract of *S. frutescens* was estimated by spectrophotometry (Beckman, Brea, CA, USA) using the method described by Lichtenthaler [29]. Isolation of the native chlorophylls present in *S. frutescens* were conducted by following the method described by Bidigare and colleagues [30]. The purity of

chlorophylls (a mixture of chlorophyll a and chlorophyll b) isolated from *S. frutescens* by using this procedure was > 95%. Furthermore, neither sutherlandiosides nor sutherlandins were detected in the isolated chlorophyll fraction.

2.4. Cell culture

The murine macrophage cell line, RAW 264.7, was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (FBS), and maintained at 37 °C in an atmosphere of 5% CO₂. A colleague, Dr. Val Mossine, provided us with RAW 264.7 cells stably-transfected with a nuclear factor-κB (NF-κB) reporter that were generated as described previously [31]. The NF-κB reporter construct contained five copies of the element from the promoter of NF-κB, a copy of green fluorescent protein (GFP), and puromycin resistance genes. These cells were maintained under the same condition as wild-type RAW 264.7 cells.

2.5. Isolation of primary murine macrophages

Primary murine macrophages were obtained from healthy five to ten-week old male Balb/c mice (Jackson Laboratory, Bar Harbor, ME, USA) as described in a previous study [32]. Cells were then diluted to a final concentration of 2×10^6 cell/mL, and were seeded in 96-well plates (Corning® Costar®, Corning, NY, USA) at a density of 2×10^5 cells/well. After incubation for 2 h, non-adherent cells (e.g., lymphocytes, erythrocytes) were removed by gently washing twice with DMEM leaving a monolayer of murine macrophages ready for subsequent experiments.

2.6. Cell viability

Cell viability was measured using the resazurin assay and green fluorescent protein (GFP) assay as described previously [25]. The RAW 264.7 cells were exposed to different concentrations of SFE or fractions from *S. frutescens* for 1 h prior to co-stimulation with murine IFN γ and LPS at the concentration used for ROS and NO stimulation (i.e., 1 ng/mL and 100 ng/mL or 0.1 ng/mL and 10 ng/mL) for 20 h (resazurin assay) and 3 h (GFP assay). Cells without any treatment (neither stimuli nor *S. frutescens*) were included as a negative control, while cells co-stimulated with LPS and IFN γ but not treated with *S. frutescens* were considered as a positive control.

2.7. Analysis of reactive oxygen species (ROS) production

RAW 264.7 cells were cultured overnight in 96-well plates (1×10^5 cells/well) in DMEM contained 5% FBS to reach greater than 95% confluence. The cells were then cultured in DMEM with 1% FBS for 2 h, then pretreated with various dilutions of SFE for 60 min. Cells were then co-stimulated with LPS (100 ng/mL) and IFN γ (1 ng/mL) for 18 h. Intracellular ROS was measured using the oxidant-sensitive fluorescent probe CM-H₂DCFDA as described previously [25].

2.8. Nitric oxide (NO) measurement

The concentration of NO in the cell culture medium was measured using Griess reagents [33]. Similar to ROS assay, RAW 264.7 cells were cultured overnight in 96-well plates (1×10^5 cells/well) in DMEM/5% FBS. The cells were pretreated with various dilutions of SFE for 1 h prior to co-stimulation with LPS (10 ng/mL) and IFN γ (0.1 ng/mL) for 20 h. The cell culture medium was harvested for NO production and inflammatory cytokines/chemokines analysis. For the NO assay, 50 μ L of cell culture supernatants from each well were mixed with 50 μ L of fresh DMEM medium in a 96-well ELISA plate. Fifty microliters of each Griess reagent (7.5 mM sulfanilamide solution, 0.75 M HCL, 7.5 mM naphthyl ethylenediamine solution) were added sequentially to the wells. The absorbance at 548 nm was determined with a filter-based multi-mode microplate reader (Biotek, Winooski, VT, USA), and nitrite concentrations were calculated from a sodium nitrite standard curve that had a linear response in the range of 5 to 100 μ M.

2.9. Cytokine analysis

RAW 264.7 cells were treated with SFE and co-stimulated with inflammatory mediators as described above. Concentrations of cytokines/chemokines in culture media were measured using a MILLIPLEX magnetic bead panel kit (murine cytokines/chemokines, Cat. MCYTOMAG-70K-PMX, EMD Millipore, Billerica, MA, USA) according to the instructions provided by manufacturer. The plate was read using the MAGPIX[®] plate reader with xPONENT software (Luminex, Austin, TX, USA). Data were analyzed using the MILLIPLEX[™] Analyst software.

2.10. Western blot analysis

RAW 264.7 cells were plated in 6-well plates, and cultured overnight to > 95% confluence. Cells were pretreated with various concentrations of ethanolic extract of *S. frutescens* (SFE) for 1 h, and then co-stimulated with LPS (10 ng/mL) and IFN γ (0.1 ng/mL) for 30 min or 60 min. Samples harvested at 30 min were used for analysis of ERK1/2, and those harvested at 60 min were used for STAT1 α . The cells were washed with ice-cold PBS, and lysed in radio-immunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate and a protease inhibitor cocktail). The protein concentrations of each cell lysate were then measured with the BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Next, 2.4 μ g of protein from each sample was loaded on a lane in the gel and separated by 8% SDS-PAGE. The proteins were transferred onto 0.45 μ m nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA) at 300 mA for 3 h. The membranes were blocked for 1 h with 5% non-fat dried milk in Tris-buffered saline. Subsequently, the blots were probed with the primary antibodies in TBS containing 5% non-fat dried milk at 4°C overnight. After washing, antibody-antigen complexes were then detected using goat anti-rabbit or goat anti-mouse IgG-horseradish peroxidase (1:5000) conjugated antibodies and an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Monoclonal anti- β -actin peroxidase was used as the loading control. The blots were scanned, and the intensity of protein bands was quantified using the QuantityOne program (BioRad, Hercules, CA, USA).

2.11. NF- κ B activation assay

The impact of *S. frutescens* extract on NF- κ B activation was investigated using RAW 264.7 cells transfected with the NF- κ B reporter [31] as described by Lei et al [25]. Transfected cells were cultured overnight to reach confluence. The cells were exposed to various concentrations of *S. frutescens* extract or enriched fractions for 1 h prior to co-stimulation with IFN γ (0.1 ng/mL) and LPS (10 ng/mL) for 3 h to activate NF- κ B signaling. After removing the medium, 70 μ L lysis buffer was added to each well to lyse cells. Ten microliters of cell lysate was transferred into a white 96-well plate. Twenty microliters of BSA and 30 μ L luciferase substrates were added and the plate was read immediately to detect light output using a microplate reader (Biotek, Winooski, VT, USA).

2.12. Statistical analysis

All data shown represent the means \pm standard error of means from at least three independent experiments with each treatment condition conducted in triplicate. The post-comparisons were performed using the Tukey's multiple comparison tests. A $p < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. ROS production

To evaluate intracellular ROS production, RAW 264.7 cells were treated with SFE for 1 h prior to co-stimulation with LPS and IFN γ for 18h. After this time, ROS production was measured every 10 min for 3 h. Cells co-stimulated with LPS and IFN γ , but without SFE, were included as a positive control. The SFE extract did not affect cell viability as assessed by the resazurin assay and green fluorescent proteins (GFP) assay (data not shown). SFE significantly inhibited ROS production by RAW 264.7 cells co-stimulated with LPS and IFN γ in a dose-dependent manner (Fig. 1).

3.2. NO production and expression of iNOS

RAW 264.7 cells produced 76.3 ± 6.8 μ M NO after co-stimulating with LPS (10 ng/mL) and IFN γ (0.1 ng/mL) for 20 h. When cells were pretreated with SFE, LPS-induced NO production was reduced in a dose related pattern (Fig. 2A).

Exposure to SFE (200 μ g/mL) reduced NO concentration to 36.4 ± 7.4 μ M, which is about 47% less than the positive control. In an effort to identify whether the reduction of NO was due to inhibition of the enzyme, iNOS expression was evaluated by Western blot. As shown in Fig. 2B, iNOS expression was reduced by 60% in RAW 264.7 cells that were pretreated with 200 μ g/mL SFE and co-stimulated with LPS and IFN γ . The effects of ethanolic extract of *S. frutescens* on iNOS expression were consistent with the results of NO production and thus indicated that *S. frutescens* extract reduced LPS-induced NO production by diminishing the expression of iNOS.

3.3. Cytokines and chemokines production

Treating RAW 264.7 cells with LPS/IFN γ resulted in the production of a number of cytokines and chemokines including IL-1 α , TNF- α , IL-6, IL-10, IL-12p70, G-CSF, MIP-2,

and RANTES (Fig. 3). Exposure to 200 µg/mL SFE significantly decreased the production of interleukin (IL)-6 and TNF-α by 65% and 28%, respectively. On the other hand, treatment with SFE at 200 µg/mL increased secretion of MIP-2 and RANTES. The SFE also modulated other inflammatory cytokines and chemokines, such as IL-1α, IL-10, and G-CSF but the changes were not dose-dependent. Treatment of stimulated primary murine macrophages with SFE had similar effects on the production of most cytokines as in LPS/IFNγ stimulated primary murine macrophages (Table 1).

3.4. NF-κB activation

NF-κB has been regarded a major signaling pathway for mediating inflammatory responses. To evaluate the impact of SFE on NF-κB activation, RAW 267.4 cells containing a luciferase reporter for NF-κB were treated with SFE for 1 h, followed by 3 h stimulation with LPS and IFNγ, and cell lysates were analyzed using the luciferase assay protocol. Co-stimulation with LPS and IFNγ increased NF-κB activation by ~50-fold and exposure to SFE diminished the activation (Fig. 4). Nevertheless, with this protocol, significant reduction of 26% was only observed with SFE at 200 µg/mL.

3.5. Phosphorylation of ERK1/2 and STAT1α

In addition to the NF-κB signaling pathway, we also investigated the effect of SFE on the MAP kinase (i.e. ERK1/2) and JAK-STAT1 signaling pathways. Exposure to SFE significantly reduced the phosphorylation of ERK1/2 activated by LPS and IFNγ up to 70% without changing the expression of total ERK1/2, and this reduction was observed in all doses used (25 to 200 µg/mL) (Fig. 5A). Similar to the effect on ERK1/2, SFE had no effect on expression of total STAT1α, however, SFE also significantly and dose-dependently inhibited the activation of STAT1α induced by LPS and IFNγ (Fig. 5B).

3.6. Effects of sutherlandiosides and sutherlandins on responses induced by co-stimulation with LPS/IFNγ

To identify the bioactive compound(s) in *S. frutescens*, various fractions prepared from *S. frutescens* were tested on their ability to inhibit LPS/IFNγ-induced production of NO and ROS as well as activation of NF-κB using the murine macrophage (i.e. RAW 264.7 cells). The LC-ELSD analysis determined that the ethanolic extract of *S. frutescens* contained about 1442 ± 95 µg/mL sutherlandioside B, which was the most abundant cycloartanol glycoside in this medicinal plant. The concentrations of sutherlandioside C and sutherlandioside D were about 400 µg/mL. There was no detectable sutherlandioside A in the ethanolic extract.

Since the crude SFE was most potent at reducing ROS generation we evaluated each of the fractions that contained one or more of the sutherlandiosides or sutherlandins for its impact on LPS/IFNγ-induced ROS production by RAW 264.7 cells. As shown in Fig. 6A and 6B, sutherlandiosides possessed modest amounts of ROS reducing activity (i.e., 25-30%). However, when either sutherlandioside-enriched fraction was used at higher concentrations, the bioactivity fell off and was no longer statistically different from the untreated cells. The concentrations of sutherlandiosides tested reflect those present in the same dilutions of the crude SFE that we evaluated in earlier experiments (see Fig. 1). Treatment of RAW 264.7

cells with sutherlandins A and B significantly reduced the ROS production by 30-40% (Fig. 6C). On the other hand, treatment with sutherlandins C and D had much less activity on ROS production compared to sutherlandins A and B (Fig. 6D).

While sutherlandioside B possessed some antioxidant activity (i.e., reduced ROS generation), these compounds failed to significantly affect LPS/IFN γ -induced NO production or NF- κ B signaling in RAW 264.7 cells (data not shown). Similar results (i.e., no activity) were found when RAW 264.7 cells were treated with fractions enriched with sutherlandiosides A + C + D, sutherlandins A + B, or sutherlandins C + D (data not shown).

3.7. Anti-inflammatory activity of chlorophyll extracted from *S. frutescens*

The ethanolic extract of *S. frutescens* displayed a dark and vibrant green color suggesting the presence of chlorophyll in this extract. In order to evaluate the role of chlorophyll on the bioactivity we observed with crude *S. frutescens* extracts, we isolated chlorophyll from *S. frutescens* by preparative HPLC. Our purified chlorophyll preparation was a mixture of chlorophyll a and chlorophyll b isoforms at a ratio of ~2:1. The concentrations of purified chlorophyll used in this experiment matched those present in the dilutions of the crude ethanolic extract of *S. frutescens* used elsewhere. Our results show that the chlorophyll in SFE potently reduced LPS/IFN γ -induced ROS production (Fig. 7A), however, this effect was not dose-dependent. Similar to SFE, the chlorophyll-enriched fraction isolated from *S. frutescens* had only a modest impact on NO biosynthesis (Fig. 7B) and NF- κ B activation (Fig. 7C).

4. Discussion

In this study, we report that an ethanolic extract of *S. frutescens* (SFE) dose-dependently diminished the production of two key inflammatory mediators (i.e., ROS and NO), yet only had a modest impact on inflammatory cytokine (e.g., TNF- α and IL-6) production by murine macrophages. For much of this research we used a common murine macrophage cell line (i.e. RAW 264.7), while conducting a more limited set of experiments with macrophages isolated from healthy mice. Furthermore, we relied on a well-characterized method for inducing inflammation in macrophages (i.e., treatment with LPS and IFN γ). Overall, these experiments confirm and extend our previous findings with a murine microglia cell line (i.e., BV-2 cells) that SFE possesses both anti-oxidant and anti-inflammatory activities [8].

Another major objective of this research was to investigate the bioactivity of some of the novel compounds found in *S. frutescens* to better understand the immune-modulatory potential of this medicinal plant. Sutherlandiosides and sutherlandins have previously been identified as unique compounds found in *S. frutescens*, yet knowledge of their bioactivity is rudimentary [11, 27, 28]. In this study, we found that a sutherlandioside B-enriched fraction had no discernible impact on the production of NO or activation of NF- κ B, yet it significantly reduced the production of ROS in RAW 264.7 cells co-stimulated with LPS and IFN γ . Similarly, a SFE fraction containing sutherlandiosides A, C, and D was without activity in our assay system. The sutherlandins had no impact on production of NO or activation of NF- κ B, however, they were able to modestly reduce the production of ROS.

Not surprisingly the ethanolic extract of *S. frutescens* contained plant pigments, most notably chlorophylls. The *in vivo* and *in vitro* biological activities associated with chlorophyll has been the subject of considerable research [34]. In fact, others have reported that chlorophyllin, a synthetic, water-soluble form of chlorophyll, reduces the production of NO and the activation of NF- κ B in LPS-stimulated RAW 264.7 cells [35]. Natural chlorophylls, however, exists as a group of structurally-related, fat-soluble compounds that possess a magnesium atom in the center of a tetrapyrrole structure with a phytol-side chain of variable length. During the production of chlorophyllin the magnesium atom in the porphyrin ring is replaced with a copper atom and the phytol tail is removed. While these changes make “chlorophyll” more stable and water-soluble, they also have a significant impact on its bioactivity. Since native chlorophylls are lipid-soluble, their absorption and distribution within cells is likely to be restricted to cell membranes. The uptake of natural chlorophylls into cells in culture is relatively inefficient relative to the water-soluble derivative, chlorophyllin [34]. Since much of what is known about the bioactivity of chlorophylls is based on studies that use chlorophyllin [36, 37], rather than native chlorophylls, we thought it was important for us to directly assess the contribution of these plant pigments in our extract. We found that native chlorophylls extracted directly from *S. frutescens* only modestly reduced the production of NO and NF- κ B signaling in RAW 264.7 cells co-stimulated with LPS and IFN γ . In contrast, native chlorophyll diminished ROS production by ~50% independent of the dosage used to treat the cells. Whether this reduction of cellular ROS production was by direct quenching or modulation of the activity of NADPH oxidase was not determined in these experiments. Regardless, our results with native chlorophylls suggest that only some, but not all, anti-inflammatory activity in an ethanolic extract of this medicinal plant was mediated by this common plant pigment.

In this study we observed that the ethanolic extract of *S. frutescens* and some of the bioactive compounds isolated from this plant showed greater impact on the production of ROS than on NO or cytokine production. We believe that our research into some of the various signaling pathways provided some insight into the possible mechanism(s) for the anti-inflammatory activity of SFE. Upon LPS and IFN γ co-stimulation of macrophages, several protein kinases, such as protein kinase C, ERK, and p38, are activated. These activated kinases phosphorylate subunits of NADPH oxidase, such as p47^{phox} [38]. The activated subunits are recruited to the membrane resulting in the activation of NADPH oxidase to produce ROS for host defense [18]. We demonstrated that the phosphorylation of ERK was strongly reduced by treatment with SFE, and that this reduction seemed to correlate with the impact of SFE on ROS production. Stimulation with LPS and/or IFN γ also activates the NF- κ B pathway, which induces the expression of iNOS to produce NO [23]. The modest effect of SFE (and chlorophyll) on the activation of NF- κ B was consistent with the limited impact they had on macrophage NO production. We believe that additional experiments with alternative bioactives isolated from *S. frutescens* would improve our understanding of how this medicinal plant impacts human health.

There are a few limitations in the present study that are worth noting. First, in this study we only investigated the bioactivity of a limited number of constituents found in *S. frutescens*. Our rationale for this was based on the fact that both the sutherlandiosides and sutherlandins

are thought to be unique to this medicinal plant and also that these compounds were found in relatively high quantities such that they are being considered useful biomarkers for *S. frutescens*. In fact, to our knowledge this is the first research report describing biological activity for these unique botanical compounds on innate immune cells. However, this investigation was limited to macrophage-mediated production of ROS, NO, and cytokines. It is entirely possible that these compounds exhibit important biological activities other than those examined here and in cells other than macrophages. For example, collaborators have recently reported dose- and time-dependent growth inhibition in human prostate cancer cells from a methanolic extract from this botanical [39]. While others have recently reported that a *S. frutescens* extract down-regulated PI-3 kinase and Akt phosphorylation in human colon cancer cells [40]. Finally, some might find our investigation into the role that chlorophyll played in the *in vitro* antioxidant and anti-inflammatory activities of this plant extract to be limited in scope. This was purposeful, because our sole interest in chlorophyll was to account for its contribution to the immune modulating activities we were investigating in our crude botanical extract. We believe we did just that and that we can conclude that there exists some biological activity present in the SFE independent of the native chlorophylls present.

5. Conclusions

In conclusion, an ethanolic extract of *S. frutescens* (SFE) had a significant and dose-dependent inhibitory effect on select inflammatory responses by a murine macrophage cell line and in primary murine macrophages. We demonstrated that this anti-inflammatory activity was associated with diminished activation of ERK, STAT1 α and NF- κ B dependent signaling pathways. Furthermore, we found that neither sutherlandiosides nor sutherlandins accounted for this anti-inflammatory activity. Additional research is needed to identify and characterize the bioactive compound(s), other than chlorophyll, that are responsible for anti-inflammatory activity observed in SFE.

Acknowledgements

This publication and project was made possible by Grant Number P50AT006273 from the National Center for Complementary and Integrative Health (NCCIH), the Office of Dietary Supplements (ODS), and the National Cancer Institute (NCI) and the support of the University of Missouri's College of Agriculture, Food, and Natural Resources, and the Food-for-the-21st Century Program. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NCCIH, ODS, or NCI.

References

- [1]. van Wyk BE, Albrecht C. A review of the taxonomy, ethnobotany, chemistry and pharmacology of *Sutherlandia frutescens* (Fabaceae). *J Ethnopharmacol.* 2008; 119:620–9. [PubMed: 18761068]
- [2]. Ojewole JA. Anticonvulsant property of *Sutherlandia frutescens* R. BR. (variety Incana E. MEY.) [Fabaceae] shoot aqueous extract. *Brain Res Bull.* 2008; 75:126–32. [PubMed: 18158106]
- [3]. Fernandes AC, Cromarty AD, Albrecht C, van Rensburg CE. The antioxidant potential of *Sutherlandia frutescens*. *J Ethnopharmacol.* 2004; 95:1–5. [PubMed: 15374599]
- [4]. Katerere DR, Eloff JN. Antibacterial and antioxidant activity of *Sutherlandia frutescens* (Fabaceae), a reputed anti-HIV/AIDS phytomedicine. *Phytother Res: PTR.* 2005; 19:779–81. [PubMed: 16220570]

- [5]. Chen, YC. Bioactivities of selected *Sutherlandia frutescens* (L.) R. BR. leaf extracts. University of Missouri-Columbia; 2007. Master's Degree Thesis
- [6]. Tobwala S, Fan W, Hines CJ, Folk WR, Ercal N. Antioxidant potential of *Sutherlandia frutescens* and its protective effects against oxidative stress in various cell cultures. *BMC Complement Altern Med*. 2014; 14:271. [PubMed: 25070435]
- [7]. Kundu JK, Mossanda KS, Na HK, Surh YJ. Inhibitory effects of the extracts of *Sutherlandia frutescens* (L.) R. Br. and *Harpagophytum procumbens* DC. on phorbol ester-induced COX-2 expression in mouse skin: AP-1 and CREB as potential upstream targets. *Cancer Lett*. 2005; 218:21–31. [PubMed: 15639337]
- [8]. Jiang J, Chuang DY, Zong Y, Patel J, Brownstein K, Lei W, et al. *Sutherlandia frutescens* ethanol extracts inhibit oxidative stress and inflammatory responses in neurons and microglial cells. *PLoS One*. 2014; 9:e89748. [PubMed: 24587007]
- [9]. Tai J, Cheung S, Chan E, Hasman D. In vitro culture studies of *Sutherlandia frutescens* on human tumor cell lines. *J Ethnopharmacol*. 2004; 93:9–19. [PubMed: 15182898]
- [10]. Faleschini MT, Myer MS, Harding N, Fouchè G. Chemical profiling with cytokine stimulating investigations of *Sutherlandia frutescens* L. R. (Br.) (Fabaceae). *S Afr J Bot*. 2013; 85:48–55.
- [11]. Fu X, Li XC, Smillie TJ, Carvalho P, Mabusela W, Syce J, Johnson Q, Folk W, Avery MA, Khan IA. Cycloartane glycosides from *Sutherlandia frutescens*. *J Nat Prod*. 2008; 71:1749–53. [PubMed: 18808182]
- [12]. Muller AC, Patnala S, Kis O, Bendayan R, Kanfer I. Interactions between phytochemical components of *Sutherlandia frutescens* and the antiretroviral, atazanavir *in vitro*: implications for absorption and metabolism. *J Pharm Pharm Sci*. 2012; 15:221–33. [PubMed: 22579002]
- [13]. Prevo D, Swart P, Swart AC. The influence of *Sutherlandia frutescens* on adrenal steroidogenic cytochrome P450 enzymes. *J Ethnopharm*. 2008; 118:118–26.
- [14]. Gordon S. Alternative activation of macrophages. *Nat Rev Immuno*. 2003; 3:23–35.
- [15]. Schroder K, Sweet MJ, Hume DA. Signal integration between IFN gamma and TLR signalling pathways in macrophages. *Immunobiology*. 2006; 211:511–24. [PubMed: 16920490]
- [16]. An HJ, Kim IT, Park HJ, Kim HM, Choi JH, Lee KT. Tormentic acid, a triterpenoid saponin, isolated from *Rosa rugosa*, inhibited LPS-induced iNOS, COX-2, and TNF-alpha expression through inactivation of the nuclear factor-kappa pathway in RAW 264.7 macrophages. *Int Immunopharmacol*. 2011; 11:504–10.
- [17]. Nauseef WM. Assembly of the phagocyte NADPH oxidase. *Histochem Cell Biol*. 2004; 122:277–91. [PubMed: 15293055]
- [18]. Lam GY, Huang J, Brumell JH. The many roles of NOX2 NADPH oxidase-derived ROS in immunity. *Semin Immunopathol*. 2010; 32:415–30. [PubMed: 20803017]
- [19]. Chan ED, Riches DW. IFN-gamma + LPS induction of iNOS is modulated by ERK, JNK/SAPK, and p38(mapk) in a mouse macrophage cell line. *Am J Physiol-Cell Ph*. 2001; 280:C441–50.
- [20]. Hallman M, Ramet M, Ezekowitz RA. Toll-like receptors as sensors of pathogens. *Pediatr Res*. 2001; 50:315–21. [PubMed: 11518816]
- [21]. Honda K, Taniguchi T. IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. *Nat Rev Immunol*. 2006; 6:644–58. [PubMed: 16932750]
- [22]. Xiang M, Namani A, Wu S, Wang X. Nrf2: bane or blessing in cancer? *Journal of Cancer Research and Clinical Oncology*. 2014; 140:1251–9. [PubMed: 24599821]
- [23]. Aktan F. iNOS-mediated nitric oxide production and its regulation. *Life sciences*. 2004; 75:639–53. [PubMed: 15172174]
- [24]. Coleman JW. Nitric oxide in immunity and inflammation. *Int Immunopharmacol*. 2001; 1:1397–406. [PubMed: 11515807]
- [25]. Lei W, Browning J Jr, Eichen PA, Lu CH, Mossine VV, Rottinghaus GE, et al. Immunostimulatory activity of a polysaccharide-enriched fraction of *Sutherlandia frutescens* occurs by the toll-like receptor-4 signaling pathway. *J Ethnopharmacol*. 2015; 172:247–53. [PubMed: 26096188]
- [26]. Brownstein KJ, Rottinghaus GE, Knight M, Ito Y, Folk W. Isolation of Sutherlandioside B from by Spiral Countercurrent Chromatography. *J Liq Chromatogr Related Technol*. 2015; 38:423–9.

- [27]. Fu X, Li XC, Wang YH, Avula B, Smillie TJ, Mabusela W, et al. Flavonol glycosides from the south African medicinal plant *Sutherlandia frutescens*. *Planta Med.* 2010; 76:178–81. [PubMed: 19670162]
- [28]. Avula B, Wang YH, Smillie TJ, Fu X, Li XC, Mabusela W, et al. Quantitative determination of flavonoids and cycloartanol glycosides from aerial parts of *Sutherlandia frutescens* (L.) R. BR. by using LC-UV/ELSD methods and confirmation by using LC-MS method. *J Pharmaceut Biomed.* 2010; 52:173–80.
- [29]. Lichtenthaler HK, Kuhn G, Prenzel U, Meier D. Chlorophyll-protein levels and degree of thylakoid stacking in radish chloroplasts from high-light, low-light and bentazon-treated plants. *Physiol Plant.* 1982; 56:183–8.
- [30]. Bidigare RR, Kennicutt MCI, Keeney-Kennicutt WL. Isolation and purification of chlorophylls a and b for the determination of stable carbon and nitrogen isotope compositions. *Anal Chem.* 1991; 63:130–3.
- [31]. Mossine VV, Waters JK, Hannink M, Mawhinney TP. piggyBac transposon plus insulators overcome epigenetic silencing to provide for stable signaling pathway reporter cell lines. *PLoS one.* 2013; 8:e85494. [PubMed: 24376882]
- [32]. Feng C, Keisler DH, Fritsche KL. Dietary omega-3 polyunsaturated fatty acids reduce IFN-gamma receptor expression in mice. *J Interferon Cytokine Res.* 1999; 19:41–8. [PubMed: 10048767]
- [33]. Wang J, Mazza G. Inhibitory effects of anthocyanins and other phenolic compounds on nitric oxide production in LPS/IFN- γ -activated RAW 264.7 macrophages. *J Agric Food Chem.* 2002; 50:850–7. [PubMed: 11829656]
- [34]. Ferruzzi MG, Blakeslee J. Digestion, absorption, and cancer preventative activity of dietary chlorophyll derivatives. *Nutr Res.* 2007; 27:1–12.
- [35]. Kamat JP, Bolor KK, Devasagayam TP. Chlorophyllin as an effective antioxidant against membrane damage *in vitro* and *ex vivo*. *Biochim Biophys Acta.* 2000; 1487:113–27. [PubMed: 11018464]
- [36]. Sharma D, Kumar SS, Sainis KB. Antiapoptotic and immunomodulatory effects of chlorophyllin. *Mol Immunol.* 2007; 44:347–59. [PubMed: 16616780]
- [37]. Cho KJ, Han SH, Kim BY, Hwang SG, Park KK, Yang KH, et al. Chlorophyllin suppression of lipopolysaccharide-induced nitric oxide production in RAW 264.7 cells. *Toxicol Appl Pharmacol.* 2000; 166:120–7. [PubMed: 10896853]
- [38]. Bae YS, Oh H, Rhee SG, Yoo YD. Regulation of reactive oxygen species generation in cell signaling. *Mol Cells.* 2011; 32:491–509. [PubMed: 22207195]
- [39]. Lin H, Jackson GA, Lu Y, Drenkhahn SK, Brownstein KJ, Starkey NJ, et al. Inhibition of Gli/hedgehog signaling in prostate cancer cells by "cancer bush" [*Sutherlandia frutescens* extract]. *Cell Biol Int.* 2015 doi: 10.1002/cbin.10544.
- [40]. Leisching G, Loos B, Nell T, Engelbrecht A-M. *Sutherlandia frutescens* treatment induces apoptosis and modulates the PI3-kinase pathway in colon cancer cells. *SAMJ.* 2015; 100:20–6.

Highlights

- Extracts of *S. frutescens* (SFE) reduce macrophage production of ROS and NO
- SFE alters NF- κ B, ERK1/2, and JAK-STAT1 signaling pathways in macrophages
- Sutherlandiosides and sutherlandins were not responsible for these effects
- Native chlorophyll were partly responsible for the bioactivity in this extract

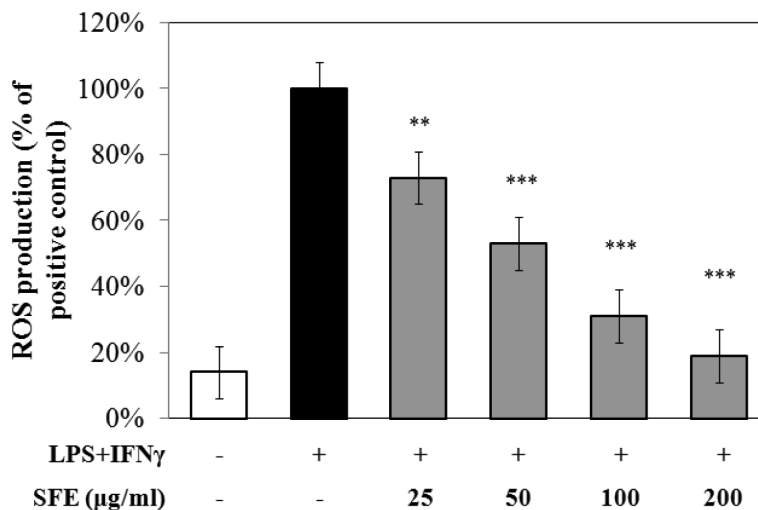


Fig. 1. Ethanolic extract of *S. frutescens* (SFE) reduced reactive oxygen species (ROS) production by murine macrophage cell line (i.e. RAW 264.7 cells) in a dose-dependent manner
 ROS production is expressed as percentage of that relative to cells co-stimulated with LPS (100 ng/mL) and IFN γ (1 ng/mL) after exposure to various concentrations of SFE for 18 h. The Y-axis is showing percentage of ROS production compared to the positive control, and X-axis shows different treatments. The data were from three independent experiments each conducted in triplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

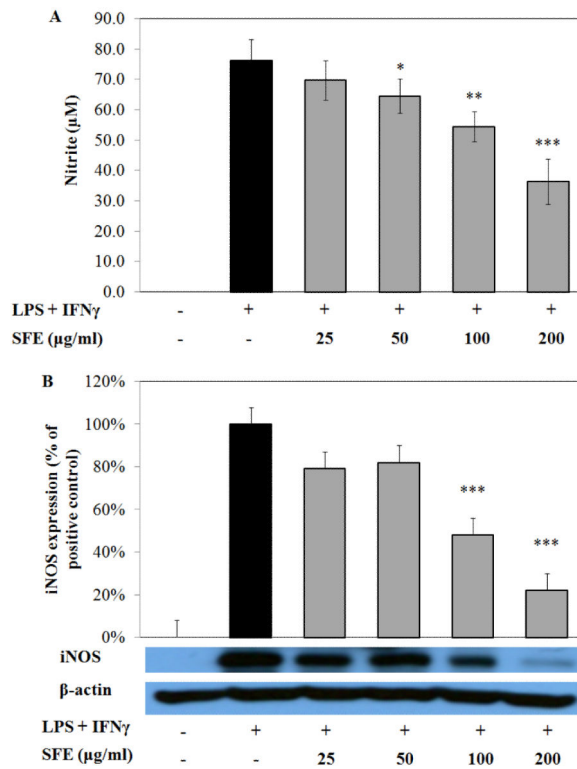


Fig. 2. Ethanolic extract of *S. frutescens* (SFE) dose-dependently reduced nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) expression murine macrophage cell line (i.e. RAW 264.7 cells) in a dose-dependent manner

Cells were pretreated with SFE, then costimulated with LPS (10 ng/mL) and IFN γ (0.1 ng/mL) for 20 h. NO production was significantly inhibited by exposure of SFE induced by inflammatory stimuli (A). Data were from three independent experiments each conducted in triplicate. The iNOS expression induced by the stimuli was significantly reduced by SFE(B). The Western blot figure is one representative experiment, and the bar graph is data from three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

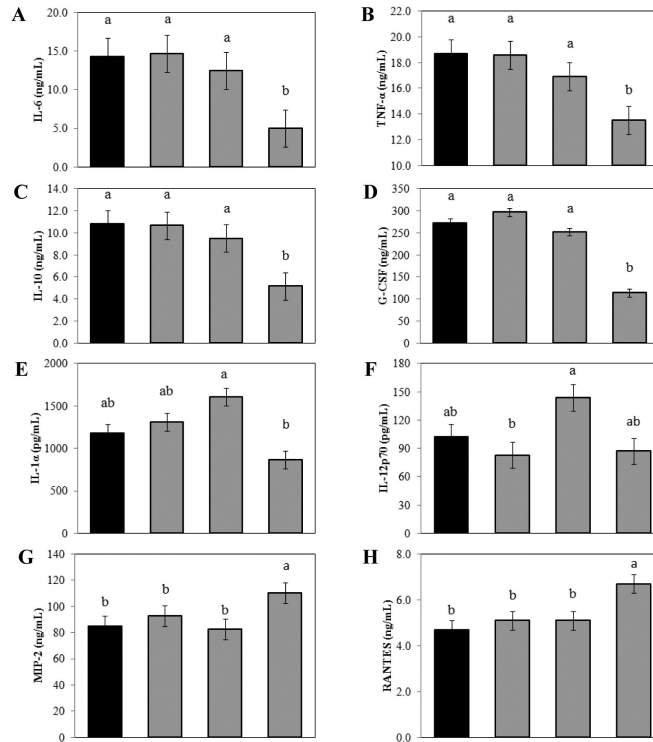


Fig. 3. Ethanolic extract of *S. frutescens* (SFE) modulated the production of inflammatory cytokines and chemokines by murine macrophage cell line (i.e. RAW 264.7 cells)
 Exposure to SFE (200µg/ml) significantly reduced the production of G-CSF, IL-6, TNF-α, and IL-10 (A to D). SFE increased the production of IL-1α, IL-12p70 at 25 or 50 µg/mL, however, the highest concentration of SFE reduced them to similar or less than that of the group without SFE (E and F). SFE significantly increased the production of MIP-2 and RANTES (G and H). Data are from three independent experiments, and are expressed as mean ± SME, and letters (a and b) are showing the difference among treatments based on the statistical analysis results.

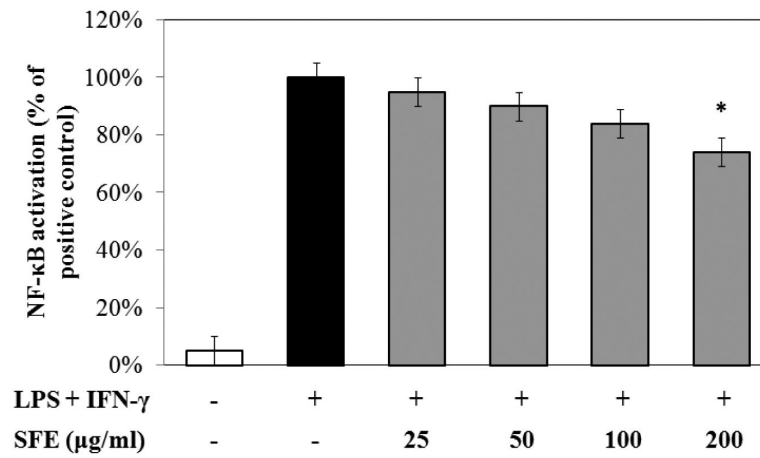


Fig. 4. Ethanolic extract of *S. frutescens* (SFE) inhibited NF- κB activation in RAW 264.7 cells
 The NF- κB activity was measured using the luciferase reporter assay. SFE had modest reduction effect on NF- κB activation. The bars indicated the percentage of activated-cells treated with different concentration of SFE compared to that of cells stimulated with LPS/IFN γ without any *Sutherlandia* treatment (100%) to calculate the percentage. * $p < 0.05$.

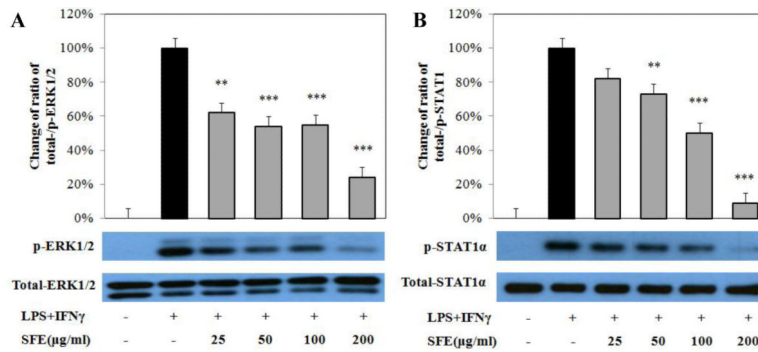


Fig. 5. Ethanolic extract of *S. frutescens* (SFE) inhibited the phosphorylation of ERK1/2 (A) and STAT1 α (B) activated by LPS and IFN γ

RAW 264.7 cells were pretreated with SFE for 1 h, followed by co-stimulation of LPS and IFN γ for 30 min (ERK1/2) or 60 min (STAT1 α). The phosphorylated- and total-ERK1/2 and STAT1 α was analyzed using Western blot. The Western blot band graphs showing a representative experiment of SFE pretreatment on the phosphorylation of ERK1/2 or STAT1 α induced by LPS/IFN γ in RAW 264.7 cells. The bar graphs are showing the ratios of p-ERK1/2 or p-STAT1 α to total ERK1/2 or STAT1 α from four independent experiments using samples from co-stimulation of LPS and IFN γ as control (100%). Data are expressed as mean \pm SD. ** $P < 0.01$, *** $P < 0.001$.

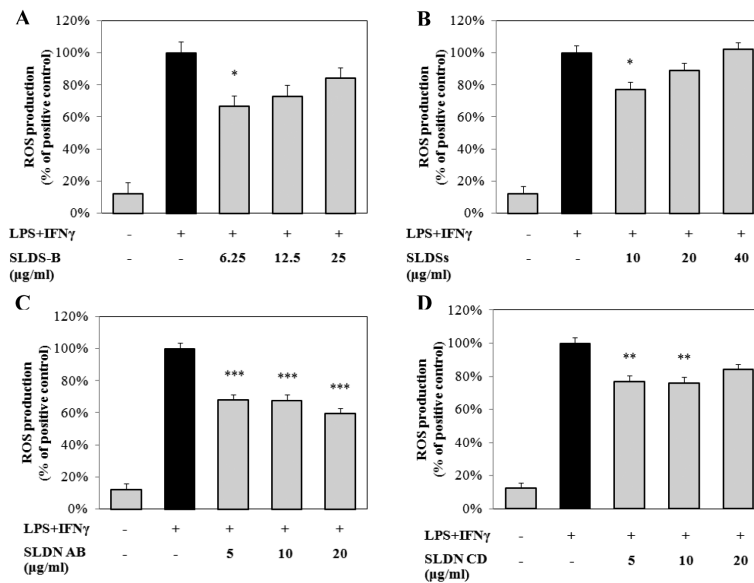


Fig. 6. Compounds isolated from *S. frutescens* reduced the production of reactive oxygen species (ROS) by murine macrophage cell line (i.e. RAW 264.7) Sutherlandioside B (SLDS-B)(A), combination of sutherlandiosides A + C + D (SLDSs) (B), combination of sutherlandins A + B (SLDN AB)(C), and sutherlandins C + D (SLDN CD) (D) significantly decreased the production of ROS induced by LPS/IFN γ . Data were expressed as mean \pm SME (n = 3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

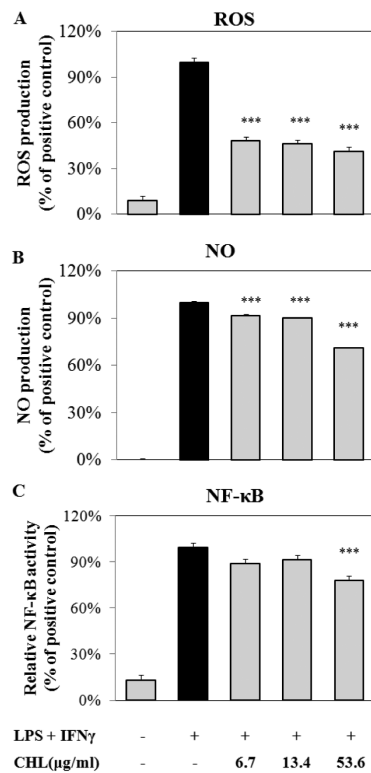


Fig. 7. Chlorophyll (CHL) isolated from *S. frutescens* exhibited anti-oxidant activity in murine macrophage cell line (i.e. RAW 264.7)

The highest concentration of chlorophyll was about 60 μM (based on the molecular weight of chlorophyll b). The LPS/IFN γ -induced ROS production (A) was significantly reduced by treatment of chlorophyll. Chlorophyll had modestly, but significantly, attenuated the production of NO (B) and activation of NF- κ B (C). Data were from three independent experiments and were expressed as mean \pm SME (n = 3). *** $p < 0.001$.

Table1

The impact of ethanolic extract of *S. frutescens* (SFE) on the production of cytokines and chemokines by LPS + IFN γ -stimulated primary murine macrophages (ng/ml)

	LPS+IFN γ	LPS+IFN γ +SFE25	LPS+IFN γ +SFE50	LPS+IFN γ +SFE200
IL-6	153 \pm 12	185 \pm 8	185 \pm 31	106 \pm 67
TNF-α	13.1 \pm 1.0	15.6 \pm 0.7	14.5 \pm 2.1	10.7 \pm 4.1
IL-10	0.27 \pm 0.09	0.26 \pm 0.11	0.27 \pm 0.08	0.29 \pm 0.03
G-CSF	4.78 \pm 0.86	8.09 \pm 0.28	8.61 \pm 1.95	5.73 \pm 3.88
IL-1α	2.46 \pm 0.28	1.85 \pm 0.74	2.27 \pm 0.34	1.46 \pm 0.49*
IL-12p40	0.98 \pm 0.16	0.58 \pm 0.12**	0.51 \pm 0.14**	0.27 \pm 0.10***
IL-12p70	0.91 \pm 0.16	0.81 \pm 0.30	0.69 \pm 0.29	0.37 \pm 0.11*
IL-9	2.62 \pm 0.73	1.79 \pm 0.52	2.23 \pm 0.65	2.40 \pm 1.29
IL-13	128 \pm 13	141 \pm 29	123 \pm 14	116 \pm 25
GM-CSF	1.74 \pm 0.49	1.86 \pm 0.59	1.58 \pm 0.88	1.21 \pm 0.28
KC	61.7 \pm 4.0	85.7 \pm 13.7	96.1 \pm 4.5	105.8 \pm 40.7
IP-10	1.58 \pm 0.38	1.37 \pm 0.31	1.75 \pm 0.56	1.66 \pm 1.44
MCP-1	33.8 \pm 11.0	34.5 \pm 10.4	39.4 \pm 11.3	30.1 \pm 22.5
MIP-1α	25.8 \pm 7.9	24.4 \pm 18.5	36.0 \pm 7.6	31.8 \pm 16.1
MIP-1b	25.2 \pm 4.4	25.8 \pm 4.3	28.5 \pm 4.7	28.1 \pm 14.4
MIP-2	144 \pm 17	199 \pm 29	223 \pm 21	210 \pm 106
RANTES	8.35 \pm 1.39	7.56 \pm 1.09	8.00 \pm 1.13	6.16 \pm 3.49

All the data were from three independent experiments.

GM-CSF, granulocyte-macrophage colony stimulating factor; IL, interleukin; IP, interferon gamma-induced protein; KC, chemokine (C-X-C motif) ligand 1, MCP, monocyte chemotactic protein, MIP, macrophage inflammatory protein, RANTES, regulated on activation, normal T cell expressed and secreted.

* $p < 0.05$,

** $p < 0.01$,

*** $p < 0.001$.