Chemical profiling with cytokine stimulating investigations of *Sutherlandia frutescens L. R.* (*Br.*) (Fabaceae)

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Abstract

Sutherlandia frutescens, one of the medicinal plants of southern Africa, has been widely used to boost the immune system by various ethnic groups. This study aims to provide scientific evidence for *in vitro* immunemodulating activities of extracts of *S. frutescens*on cytokines, includinginterleukins 4, 6, 8, 10, 12p70 and TNF produced by the HL60 cell lines,as well as to identify possible compounds present therein. The chemical profile of extracts, fractions and compounds were determined using high performance liquid chromatography coupled to mass spectrometry. Results from experiments conducted supported the immunemodulating as well as anti-inflammatory activities of extracts from *S. frutescens*.The study showed that an ethanolic extract gave the best performance in recruiting various inflammatory cytokines to the site of infection upon stimulation with phorbol 12-myristate 13-acetate, where essentially the

non-polar compounds present in the ethanol extract contributed to most of the activity observed for this extract.

Keywords: *Sutherlandia frutescens*, cancer bush, immune stimulating, South African medicinal plants, cancer, cytokines

Abbreviations:BD = Beckton Dickinson; Ech = *Echinacea*; IR = Immune response; PMA = phorbol 12-myristate 13-acetate; Sa = *Siphonochilusaethiopicus.*

1. Introduction

Immunodeficiency is a condition in which the immune system, normally a first line of defence against unwanted pathogens, is unable to contain infectious diseases. As a result, an immune-compromised individual will often succumb to more severe infections of longer duration than normal, causing the body to become chronically emaciated (Farlex, 2012).

Various plants have been used for a variety of medicinal purposes, and have contributed significantly to the development of major medical drugs that are still in use today (Simpson and Ogorzaly, 2001). Among these are *Echinacea spp., Astragalus membranaceus, Zingiber officinale, Panax ginseng* and *Sambucus nigra* (Dewick, 2002; Spelman et al., 2006; Vukovic, 2004). Many scientific investigations have been conducted on these plants to demonstrate their immune boosting effects (Burger et al., 1997; Raduneret al., 2006; Spelman et al., 2006). Botanicals have also been used to suppress an over reactive immune system such as *Siphonochilus aethiopicus* (publication in process) and the drug cyclosporine initially isolated from the fungus *Tolypocladium inflatum* (Borel, 2002).

Southern Africa is rich in plant diversity and the use of traditional medicines is widespread and promoted by the Ministry of Health. The South African Department of Health has recommended two herbal remedies (*Hypoxis hemerocallidea* and *Sutherlandia frutescens*) for the management of many ailments, including cancer and for patients with human immunodeficiency virus (HIV) infection (Mills, Cooper et al., 2005).

Sutherlandia frutescens, one of the most widely used indigenous plants of southern Africa, is commonly found in the South West and Northern Cape provinces (**Figure 1**). Various tribes have used this plant for medicinal purposes, particularly chronic diseases, including cancer (Van Wyk and Albrecht, 2008). This study was undertaken to provide a scientific basis to support the use of this plant amongst traditional healers for immune-compromised and cancer-infected individuals.

Literature reports have also indicated that extracts from *S. frutescens* can also be used in the treatment of HIV/AIDS (Harnett et al., 2005; Mills, Forster et al., 2005), cancer (Chinkwo, 2005; Stander et al., 2007; Tai et al., 2004) as well as to treat diabetes (Chadwick et al., 2007; Sia, 2004). The anti-cancer and anti-viral properties of extracts from *S. frutescens* have been attributed to the presence of L-canavanine (Green, 1998). In addition, extracts have also been shown to have anti-bacterial, anti-oxidant (Katerere and Eloff, 2005), anti-inflammatory (Kundu et al., 2005) and anti-mutagenic (Reid et al., 2006) activities. However, most of these investigations have not provided conclusive data as to which compounds might be responsible for these actions. In addition, most studies did not focus on the immune modulating properties of extracts from *S. frutescens*, especially on cytokine production by the HL60 cell line. As such, modulation of cytokine secretion may offer alternative approaches in the treatment of a variety of diseases, especially in immune-compromised patients (Spelman et al., 2006). Cytokines are key mediators in an immune response (IR), which are produced by cells of the immune system in response to a stimulus and help to recruit or orchestrate other immune cells to the site of infection (Lydyard et al., 2004; O'Gorman and Donnenberg, 2008; Parkin and Cohen, 2001).

This research aims to show preliminary immune modulation activities of extracts prepared from fresh and dried *S. frutescens* plant material, using an HL60 model system with stimulated cytokine quantification; as well as to identify possible active compounds in the extracts using HPLC-MS analysis.

2. Materials and Methods

2.1 Chemicals

CP grade solvents were purchased from Merck and distilled before use. Solid phase extraction C-18 cartridges (Supelco 140 ml PP tubes) were purchased from Sigma Aldrich. Distilled water was used for all procedures. HPLC-grade solvents were purchased from Microsep (Romil pure chemistry solvents). RPMI-1640, Fetal Bovine Serum (FBS) and L-Glutamine were purchased from Whitehead Scientific. Gentamicin, Phorbol 12-Myristate 13-Acetate (PMA) and Etoposide were purchased from Sigma Aldrich. The Cytometric Bead Array™ Human Inflammation Cytometric Bead array (CBA) kit (551811) was purchased from Beckton Dickinson (BD) Biosciences and analysed using BD Biosciences' equipment and software.

2.2 Plant collection

Sutherlandia frutescens (L.) R. B.r. plant material was collected from a cultivation site in the Free State, South Africa (GPS coordinates: 29° 6.774′ S; 25° 24.305′ E). Identification of the specimen deposited at the South African National Biodiversity Institute (SANBI), Tshwane was confirmed (SANBI voucher specimen number: 428679).

2.3 Extract preparations

Fresh leaves (488 g) were boiled in 12.5L ofH_2O for 1 hr with occasional stirring. The suspension was then filtered and freeze-dried. The aqueous extract from the fresh leaves was labelled Extract A (47.8 g; 9.8 % yield). The air-dried leaves (200 g) were extracted with ethanol (EtOH; 1.6L of 96 % v/v) at room temperature, stirred, left overnight and then filtered and evaporated to give Extract B (11.17 g; 55.9 % yield). Another 200 g of the air-dried leaves were boiled in 2.0 L of H_2O for 1 hour with occasional stirring. This solution was filtered and freeze-dried (Extract C, 33.72 g; 16.9 % yield).

2.4 Preparation of fractions

The ethanol extract B was fractionated using solid phase cartridges. 10 g of extract B (EtOH) was dissolved in 20 ml H_2O and loaded on the activated C-18 (Supelco 140 ml PP tubes, Sigma Aldrich) cartridge. Different solvents were used to generate the fractions: 200 ml 100 % H_2O, 160 ml 20 % MeOH, 110 ml 40 % MeOH, 160 ml 60 % MeOH, 160 ml 80 % MeOH, 200 ml 100 % MeOH and 200 ml 100 % Acetonitrile (ACN). The resulting fractions were evaporated and freeze dried and combined to give fractions I to III according to TLC profiles.

2.5 Isolation of compounds

Sutherlandiosides A, B and D (**Figure 2**) were isolated from the plant extract following the procedure described by Fu et al. (2008). For structure elucidation of the compounds, NMR spectroscopy was performed using a 600 MHz Varian NMR.

Accurate mass analysis was performed on a WATERS Synapt G1 UPLC-TOF-MS system. Two column types were used namely, WATERS Acquity CSH C18 (150 x 2.1

mm, particle size: 1.7 µ) for the a polar compounds and WATERS Acquity HSS T3 (150 x 2.1 mm, particle size: 1.8 µ) for the more polar compounds.

2.6 HPLC MS analysis

For each extract and fraction, approximately 25 mg was weighed out into a vial and 2 ml of HPLC-grade solvent (methanol, acetonitrile or water depending on the solubility of the sample) was added. This mixture was then put in an ultrasonic bath for 10 minutes and filtered through Acrodisc GHP syringe filters, before being placed into 2 ml HPLC vials. The various samples were analysed using a WATERS 2695 HPLC separation module. Two Atlantis T3 columns (10 x 250 mm, 5 µ particle size) connected in series, were used for the separation. UV-VIS detection was done on a WATERS PDA scanning from 200 – 600 nm. The mobile phase used was 0.1 % (v/v) formic acid in water (A), methanol (B) and acetonitrile (C). The ratio of mobile phase prepared is given in the **Table 1** below. Additionally, mass spectrometry detection was performed using a WATERS SQD scanning from 100 – 1200 *m/z* with polarity (+/-) switching with a scan time of 0.20 seconds. The operating conditions in the ESI source were as follows: source temperature, 150 °C; desolvation temperature, 450 °C; capillary voltage, 3.00 kV; cone voltage, 30.0 V. Gas flow (N_2): desolvation, 800 L/hr; cone gas, 10 L/hr.

2.7 Quantification of cytokines with CBA

The HL60 cell line was obtained from the European Collection of Cell Culture (ECACC) and maintained in suspension at 37 °C in 5% carbon dioxide (CO_2) and 100% relative humidity in RPMI-1640 supplemented with 5 % foetal bovine serum (FBS), 2 mML-glutamine and 50 µg/ml gentamicin. Cells were counted and inoculated in a 96-

well microtiter plate at plating densities of 7 000 to 10 000 cells per well and incubated for 24 hrs. Test samples (50 µl) were added to specific wells at a concentration of 25 µg/ml and incubated for 48 hrs. Phorbol 12-myristate 13-acetate (PMA) (12.5 ng/ml) was used as a cytokine stimulant and added to the wells at 42 hrs, where needed, and placed in the incubator for the remaining 6 hrs. Cells without drug addition served as the control, while the blank contained complete cell culture media without cells. Ethanolic preparations of *Echinacea spp.* and *Siphonochilus aethiopicus* were used as positive (immune boosting) and negative (immune suppressant) controls respectively, and were also tested with and without PMA. After 48 hrs of incubation, the plate was removed and centrifuged for 2 min. at 1 000 g, supernatants (50 µl) from each well were removed and placed in Eppendorf tubes separately.

The IL12p70, TNF, IL10, IL6, IL1β and IL8 cytokines were detected using the human inflammation Cytometric Bead Array (CBA) kit (551811; BD Biosciences). Tests were performed according to the manufacturer's instructions available online. The six bead populations are resolved in a red channel of a BD FACSCalibur flow cytometer. For each set of experiments, a standard curve was generated. The results were expressed as pg/ml and then analysed for their relative expression (control versus treated samples). The lower limit for detection for each cytokine was determined as 10 pg/ml. In order to simplify results from the assays a numbering system as outlined in **Table 2** was used.

2.8 Statistical analysis

All determinations were done in quadruplicate, and the results were reported as mean ± standard deviation (sd). Graphs were plotted using Origin version 6.0 (Microcal Software, Inc.).

3. Results and discussion

All extracts were analysed using HPLC MS to obtain their chemical profiles. The resulting chemical profiles ofextracts A, B and C in ESI^- (electrospray ionisation negative mode) are shown in **Figure 3**.

From the chromatograms in **Figure 3**, differences between the various *S. frutescens* preparations are apparent. In extract A and C (aqueous extract of the fresh and dried leaves) a high concentration of polar compounds was seen, while in extract B (ethanol extract of the dried leaves), a higher concentration of the intermediate polarity compounds was apparent. Sutherlandiosides A, B and D (Fu et al., 2008) - isolated from extracts of the plant - are shown in the chemical profile of extract B and were found to elute at 57.24, 43.51 and 59.25 minutes, respectively (labelled **1**, **2** and **3** in **Figure 3**). This region contains the cycloartane triterpenoid type compounds, mainly found in *Sutherlandia*. The aqueous extracts were found to contain a higher concentration of the more polar compounds, possibly flavonol glycosides, as suggested in the literature (Avula et al., 2010; Fu et al., 2010). The chemical profiles of the fractions obtained from the ethanol extract are shown in **Figure 4**. From these chromatograms it can be seen that the majority of the polar compounds are found in fraction I, which could be mainly made up of flavonol glycosidic compounds similar to the ones isolated by Fu and co-workers (2010). The cycloartane triterpene-type compounds were found in higher quantities in fraction II, while the non-polar compounds appeared in fraction III.

The extracts and fractions of *S. frutescens* were analysed for their activities on the release of cytokines with PMA as a co-stimulant on HL60 cells. For immune cells to work effectively they need to be recruited to the sites of inflammation and appropriately

activated. This is achieved by cellular receptors and associated cytokines that bind to these receptors (Lydyard et al., 2004; O'Gorman and Donnenberg, 2008; Parkin and Cohen, 2001). The cytokines IL1β, IL6, IL10, IL12p70, IL8 and TNF, used in this assay, were selected due to their role in various pro- & anti-inflammatory actions involved in the first step of the IR i.e. inflammation.

In this experiment three controls were used namely, the blank control (number 1 in **Figures 5** and **6**), a positive control (*Echinacea* extract, number 15 in **Figures 5** and **6**) and a negative control (*Siphonochilus aethiopicus* extract, number 17 in **Figures 5** and **6**). No release of the six cytokines (i.e., initiation of an immune response) was observed when all the extracts and fractions of *S. frutescens* were applied to the HL60 cells without the co-stimulation of PMA (represented by odd numbers in **Figure 5 a** and **b**). When PMA (number 2 in **Figure 5 a** and **b**) was added to the cell culture system alone, there was a higher production of IL8 (4551.95 ± 410.85 pg/ml) and an improvement in the amount of TNF (129.69 ±19.21 pg/ml) being released into the supernatant when compared to the blank control (which had a concentration of 50.83 ± 7.74 pg/ml).

Specifically fraction III from the ethanol extract; together with PMA (number 14 in **Figure 5 a** and **b**), contributed to a marked increased release of the TNF and IL8 cytokines (229.45 ± 13.89 for TNF and 5967.93 ± 226.86 pg/ml for IL8). The aqueous extracts and fractions I and II, together with PMA, displayed a marked decrease of IL8 being released (concentrations ranging from about 2909 to 3260 pg/ml). As is apparent in **Figure 6**, it was observed that the *S. frutescens* extracts did not affect subsequent release of IL1β, IL6, IL10 and IL12p70 in the culture system, relative to PMA, after a 48 hour incubation period (concentrations varied from 3 to 20 pg/ml). From the results of the above experiment, the non-polar compounds found in the ethanol extract (fraction

III) gave the best performance in recruiting the various inflammatory cytokines (TNF and IL8) to the site of infection upon stimulation with PMA.

PMA (acting as a mitogen) was used as an initiator of the increased release of cytokines into the culture supernatant, which was taken as a measure of immune response initiation potential (**Figure 6 a** and **b**, sample number 2). This arrangement resulted in a co-stimulated release of TNF and IL8 into the culture supernatant well above that of the baseline levels found with the blank control.

As PMA forms part of the lipopolysaccharide (LPS) of gram negative bacterial cells, it can be recognised by pattern recognition receptors (PRR) present on the target cells used in this study (Lydyard et al., 2004). As such, the expression of TNF is stimulated by LPS interaction with PRR on the HL60 cell line and subsequently IL8 is activated by the release of TNF. TNF activation is consequently coupled with programmed cell death (apoptosis) initiated by infection with a particular pathogen and also coincides with the release of inflammatory mediators which stimulate recruitment of other immune cells to the site of infection (Lydyard et al., 2004). The release of IL8 is believed to help the immune system to increase chemotaxis for neutrophils (as well as for T cells and basophils) at the site of inflammation (Lydyard et al., 2004; Parkin and Cohen, 2001).

In contrast to the PMA alone, the effect of the non-polar compounds present in the ethanol extract on the target cells, with the co-stimulation of PMA, exhibited a greater release of TNF and IL8 into the culture supernatant. In essence, this observed effect, together with the up-regulated release of specific cytokines, could be taken to mimic a possible *in vivo* situation of a bacterial infection in a given host, being combated by the release of appropriate cytokines. From the chemical profiles, it was shown that the non-

polar compounds present in the ethanol extract were more important for the immune modulating effects.

The decreased influence of *S. frutescens* extracts on the release of IL8, together with the co-stimulation by PMA in the *in vitro* cell culture system, was mainly found in the aqueous extracts. This observation could possibly be related to the presence of flavonol glycosides and their polar interactions with cells of the *in vitro* cell culture system. As stated above, IL8 plays a role in inflammation and therefore the reduction of IL8 found with the aqueous extracts could possibly be related to the anti-inflammatory properties of *S. frutescens*. A crucial observation in this regard, was that for immune boosting effects, an ethanolic preparation would be more beneficial; however for an anti-inflammatory response an aqueous preparation would be the better option. A study done by Ngcobo (2008) showed that high concentrations of aqueous and ethanol extracts of *S. frutescens* could reduce the production of IL1β and TNFα, which are regarded as being helpful in fighting muscle wasting in cancer and HIV/AIDS patients. Anti-inflammatory agents have been shown to exhibit chemo-preventative activity (Surh et al., 2001; Surh, 2002); therefore, the anti-inflammatory properties attributed to the aqueous extracts of *S. frutescens* could possibly be used in a chemo-preventative setting as well.

However, the differential increase in IL8, together with the co-stimulation by PMA, of the non-polar compounds found in the ethanol extract could contribute to possible immune-stimulating effects. For this reason, preparations of *Sutherlandia* seem to exhibit many attributes that could be related to the complex nature of compounds present in the raw plant material. The different compounds present in the various extracts of *S. frutescens,* most likely justify its various traditional uses. The aqueous

extracts displayed a similar effect as the positive control (*Echinacea*) (Burger et al., 1997; Raduner et al., 2006; Sharma et al., 2010; Spelman et al., 2006; Vukovic, 2004), while the negative control (*S. aethiopicus*) displayed a complete suppression of IL8 and TNF, even with the stimulation of PMA, showing its immunosuppressive activities. Sa has been known to have immunosuppressive characteristics by suppressing the production of certain cytokines needed for the immune response, such as IL8 (publication in process). The plant contains an active sesquiterpenoid compound that is known to inhibit the nuclear transcription factor NF-κB involved in the regulation of many pro-inflammatory factors.

4. Conclusion

These results above have provided evidence that the non-polar compounds present in the ethanol extract of *S. frutescens* could amplify the release of specific, immune-modulating cytokines by cells already stimulated by a pathogenic micro-organism, such as a gram negative bacterium. These results also suggested that the cultured cells needed to be intrinsically releasing certain cytokines, so that when an a-polar fraction of an ethanolic preparation was subsequently added to the HL60 cells, they were able to up regulate and release more of the particular cytokines involved in the IR. This could be of great interest for the treatment of diseases that specifically attack the immune system, such as HIV/AIDS and cancer, resulting in a more rapid expulsion of the invader micro-organism and limit the likelihood of the infectious agent from spreading to other cells. HPLC-MS analysis made it possible to determine that the non-polar compounds were effective at stimulating the *in vitro* immune system, while the aqueous extracts (more polar compounds) were found to precipitate an anti-inflammatory type response.

Overall, particular attention needs to be pain to the correct choice of solvent used for an extract preparation of *S. frutescens*, in order to select for a specific biological effect. Further investigations into isolation and identification of the non-polar compounds could be of great value in future studies of this kind, especially where various in depth *in vivo* immune stimulating models are concerned.

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Figure Legends

Figure 1. Geographical distribution of *Sutherlandia* species throughout South Africa on the left (sahealthinfo.org) and on the right *S. frutescens* growing in the wild at Goegap Nature Reserve, Springbok, Northern Cape (photo taken by N. Harding).

Figure 2. Structures of sutherlandiosides A, B and D are respectively referred to as compounds 1, 2 and 3.

Figure 3. The HPLC ESI^- chromatograms for extracts A, B and C. 1, 2 and 3 in the chemical profile of extract B indicates the presence of sutherlandiosides A, B and D, respectively.

Figure 4. HPLC ESI^+ chromatograms for fractions I, II and III.

Figure 5. Release of TNF (a) and IL8 (b) for the blank control, PMA (at 12.5 ng/ml), extracts A to B, fractions I to III, positive and negative controls at 25 µg/ml for a 48 hour incubation period. For statistical analysis n = 4.

Figure 6. Release of IL1β, IL6, IL10 and IL12p70 cytokines for the blank control, PMA (at 12.5 ng/ml), extracts A to B, fractions I to III, positive and negative controls at 25 µg/ml for a 48 hour incubation period. For statistical analysis n = 4.

Table Legends

Table 1. Gradient timetable for the HPLC-SQD method used.

Table 2. Numerical values corresponding to the various samples used in the cytokine quantification assay.

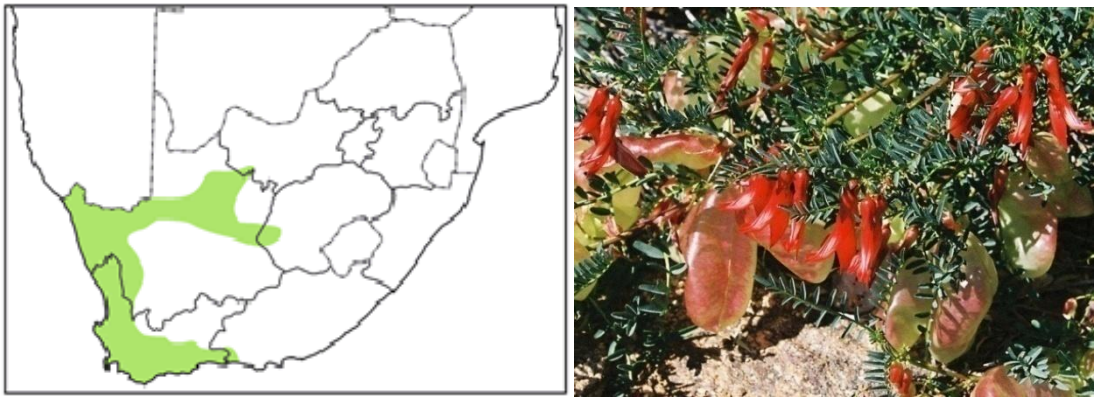
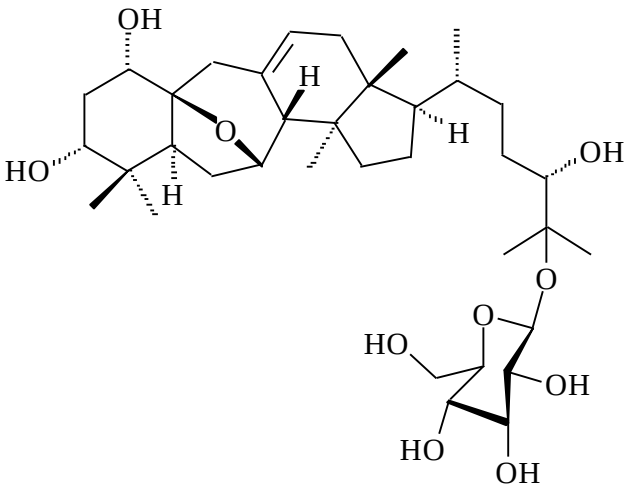
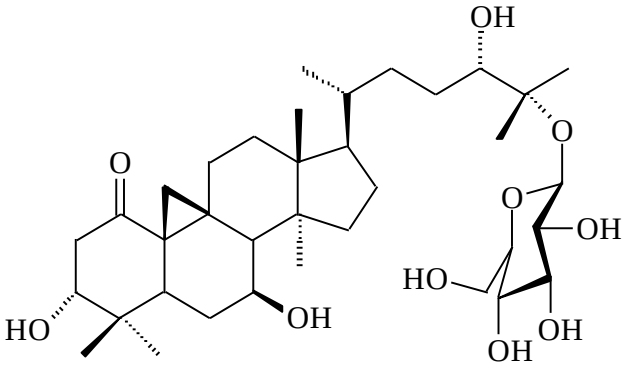


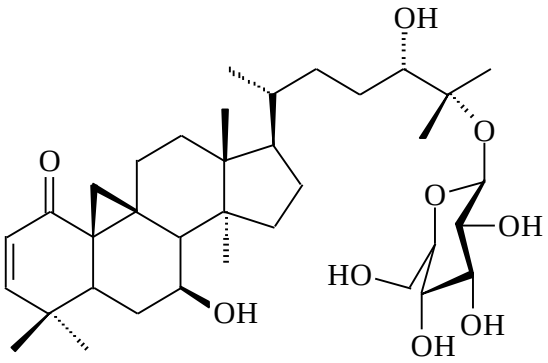
Figure 1.



Sutherlandioside A (compound number 1)



Sutherlandioside B (compound number 2)



Sutherlandioside D (compound number 3)

Figure 2.

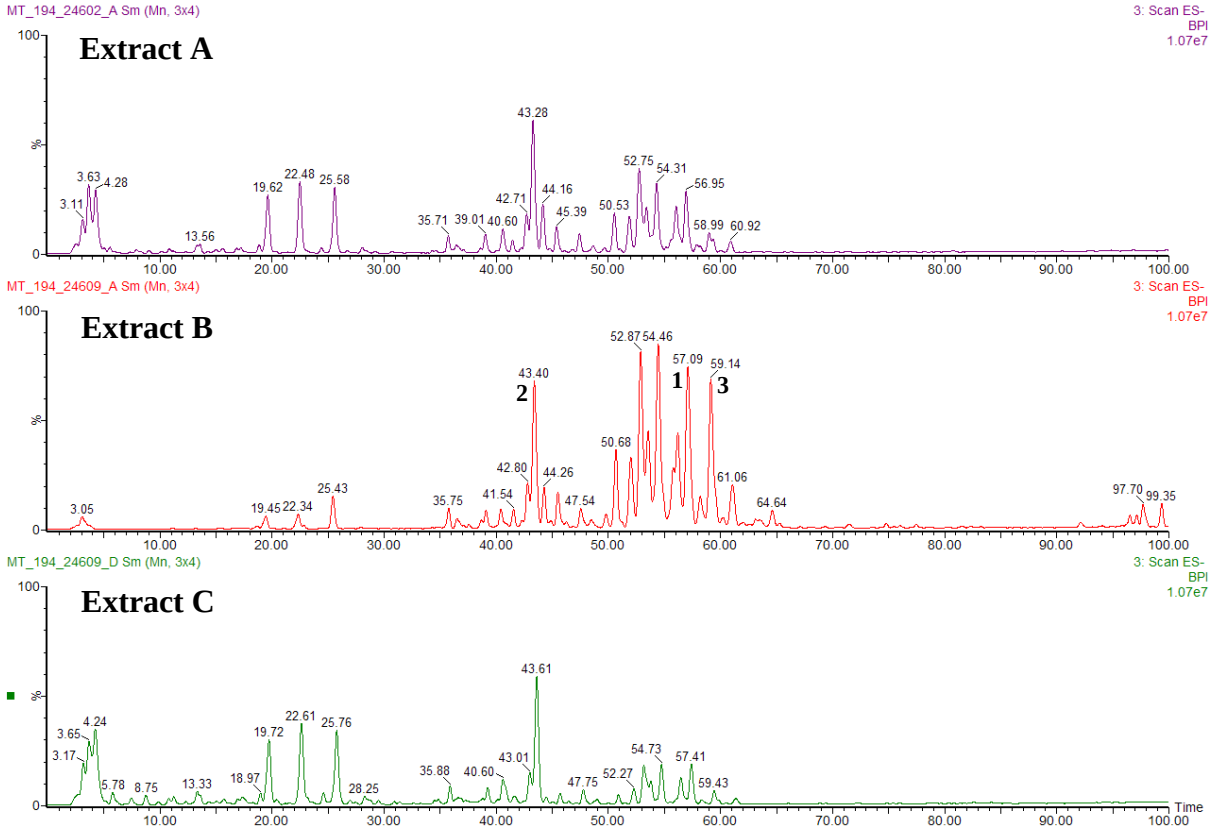

MT_194_24602_A Sm (Mn, 3x4)
3: Scan ES-
BPI
1.07e7
Extract A
MT_194_24609_A Sm (Mn, 3x4)
3: Scan ES-
BPI
1.07e7
Extract B
MT_194_24609_D Sm (Mn, 3x4)
3: Scan ES-
BPI
1.07e7
Extract C
Time

Figure 3.

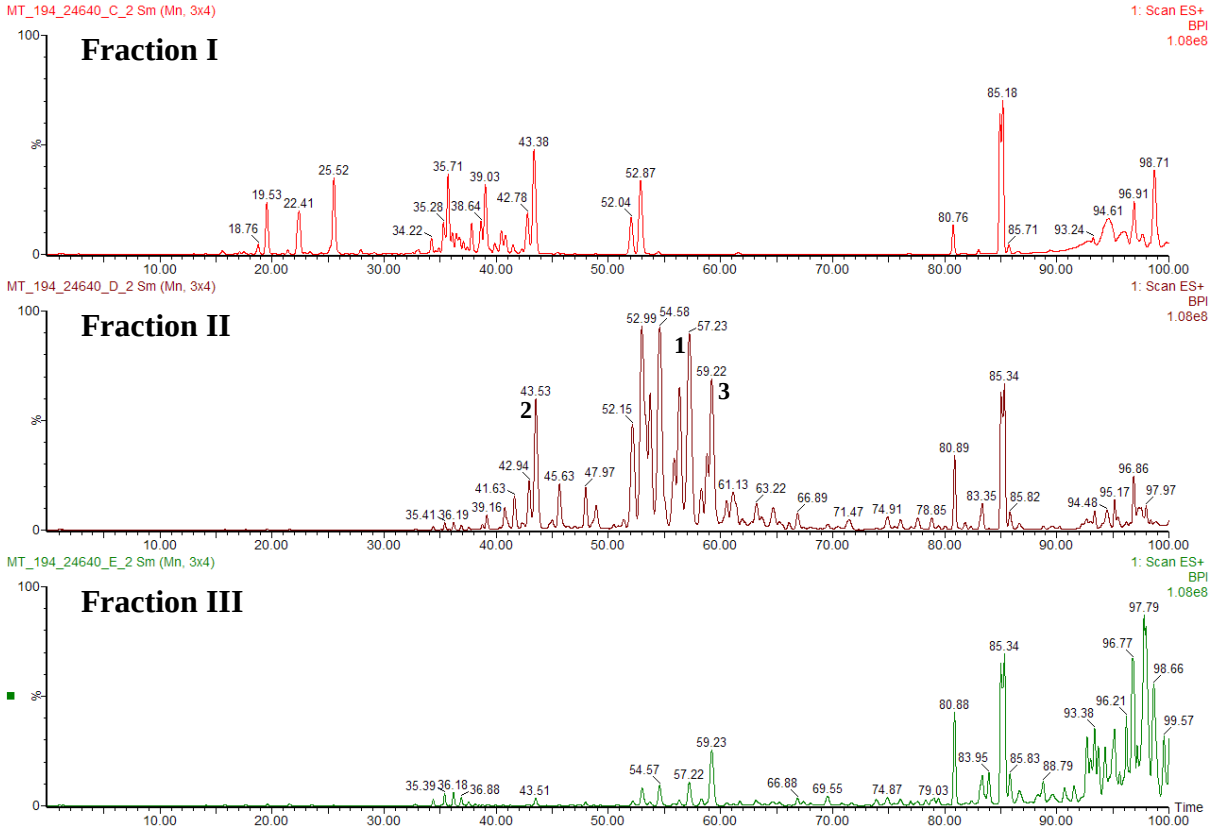
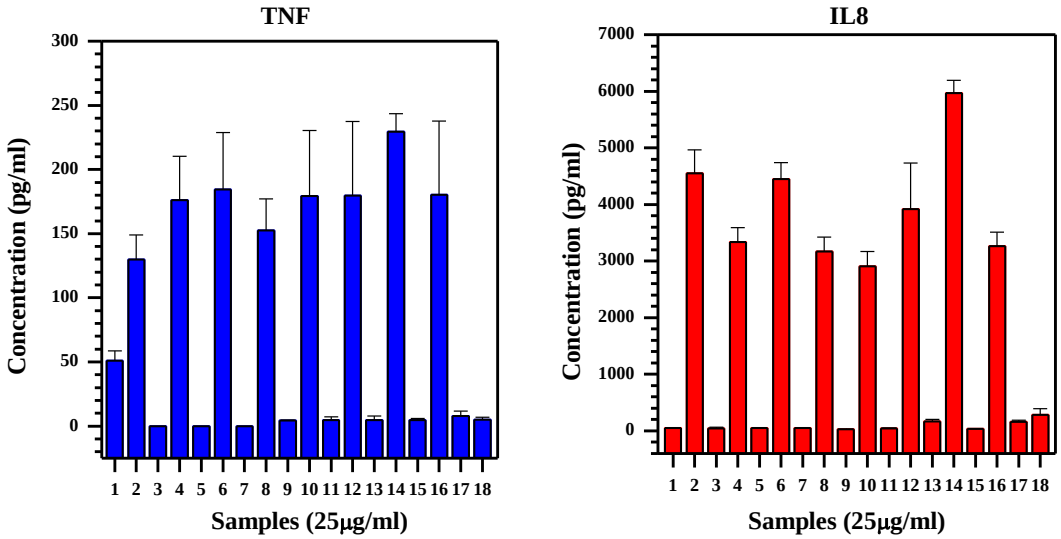

MT_194_24640_C_2 Sm (Mn, 3x4)
1: Scan ES+
BPI
1.08e8
Fraction I
MT_194_24640_D_2 Sm (Mn, 3x4)
1: Scan ES+
BPI
1.08e8
Fraction II
1
2
3
MT_194_24640_E_2 Sm (Mn, 3x4)
1: Scan ES+
BPI
1.08e8
Fraction III
Time

Figure 4.


TNF
Concentration (pg/ml)
300
250
200
150
100
50
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Samples (25µg/ml)
IL8
Concentration (pg/ml)
7000
6000
5000
4000
3000
2000
1000
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Samples (25µg/ml)

a **b**

Figure 5.

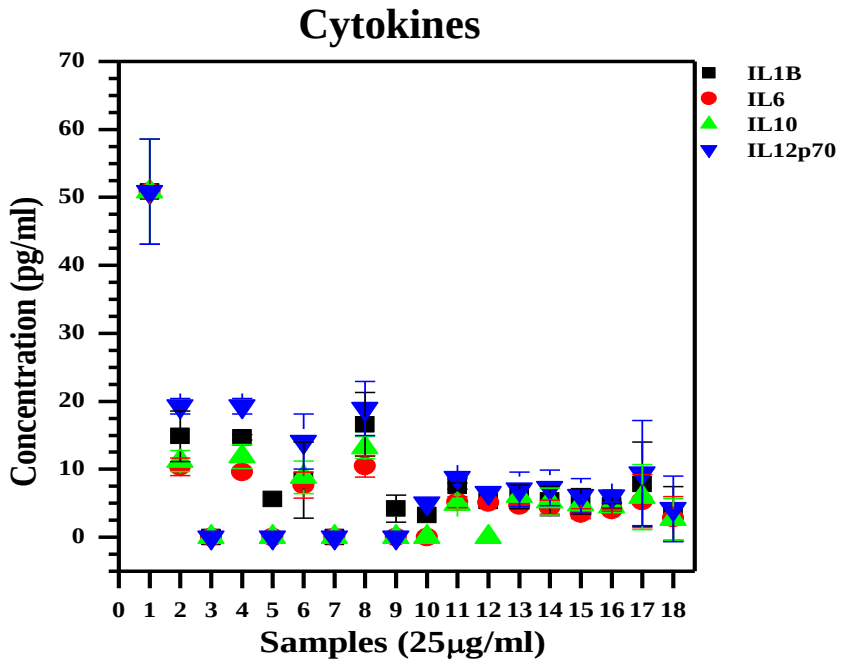

Cytokines
IL1B
IL6
IL10
IL12p70
Concentration (pg/ml)
Samples (25µg/ml)
0
10
20
30
40
50
60
70
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 6.

Time (min)	Flow rate (ml/min)	% A FA H_2O	% B MeOH	% C ACN
0	0.3	95	5	0
4	0.3	95	5	0
5	0.3	75	25	0
27	0.3	55	45	0
30	0.3	42	58	0
70	0.3	22	78	0
80	0.3	12	88	0
85	0.3	12	88	0
90	0.3	0	100	0
100	0.3	0	100	0
107	0.3	0	0	100
113	0.3	0	0	100
115	0.3	95	5	0

Table 1.

Number	Sample
1	Control (Ctrl.)
2	PMA (12.5 ng/ml)
3	Extract A (Fresh leaves)
4	A + PMA
5	Extract B (EtOH)
6	B + PMA
7	Extract C (Dry leaves)
8	C + PMA
9	Fraction I
10	I + PMA
11	Fraction II
12	II + PMA
13	Fraction III
14	III + PMA
15	*Echinacea* (Ech)
16	Ech + PMA
17	*Siphonochilus aethiopicus* (Sa)
18	Sa + PMA

Table 2.