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The inner gel component of Aloe vera suppresses bacterial-induced pro-inflammatory cytokines from human immune cells

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Abstract

The present study was carried out to examine the anti-inflammatory activity of the inner leaf gel component of *Aloe barbadensis* Miller. A simple *in vitro* assay was designed to determine the effect of the inner gel on bacterial-induced pro-inflammatory cytokine production, namely TNF-α and IL-1β, from peripheral blood leukocytes stimulated with *Shigella flexneri* or LPS. This report describes the suppression of both cytokines with a freeze-dried inner gel powder and a commercial health drink from the same source. Comparison was made with a human monocytic cell-line (THP-1 cells) and a similar trend in responses was demonstrated.

Keywords: Aloe vera; Anti-inflammatory; Inflammation; Pro-inflammatory cytokines

1. Introduction

We have previously described the significant anti-microbial effects of the inner gel from *Aloe barbadensis* Miller (syn. *Aloe vera* (L.) *Burma. f.* or Aloe vera) [1]. Aloe vera is also reputed to have anti-inflammatory properties [2–4]. Compounds isolated from the inner gel, such as salicylates, magnesium lactate, bradykinin, thromboxane inhibitors, sterols and a beta linked acetyl mannan (acemannan) have been proposed as active anti-inflammatory components [5–7].

In this study, our aim was to investigate the effect of Aloe vera on immune cell responses induced by bacterial stimulation. The microbe used was *Shigella flexneri*, a non-motile rod belonging to the Enterobacteriaceae family. The species are an important cause of gastrointestinal illness, which is manifest by watery diarrhoea that progresses to mucoid bloody diarrhoea and shigellosis. A *Shigella* infection is characterised by induction of an acute host inflammation response, accompanied by increased infiltration of inflammatory cells into the colonic mucosa, particularly of cells producing pro-inflammatory cytokines [8–11]. Infection is usually restricted to the top layer of colonic mucosa, where it causes ulceration and severe tissue damage [12]. Pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), the interleukins (IL-1β, IL-6, IL-8, IL-12) and interferon gamma (IFN-γ) are systemically elevated during bacterial invasion [13] and in particular, TNF-α and IL-1β have been shown to play an important role in the inflammatory process in shigellosis and septic shock [8,14–16]. In addition, it is thought that these cytokines contribute to vascular lesions in blood vessels of the colon, kidneys and central nervous system [17], as a result of Shiga-toxin release. Treatment with TNF-α inhibitors has been shown to ameliorate such tissue damage [18]. We therefore assessed the therapeutic potential of Aloe vera using a simple *in vitro* assay (modified from [19]), consisting of

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human blood leukocytes stimulated by whole bacteria or lipopolysaccharide (LPS), in the presence and absence of Aloe vera inner gel. We also used another assay consisting of a human monocytic cell-line to compare anti-inflammatory responses in a single cell type, without anomalies from different donors [20].

2. Materials and methods

2.1. Materials

Packed blood cells were obtained from the Scottish Blood Transfusion Service, UK and consisted of partial donations. THP-1 cells were purchased from European Collection Animal Cell Culture (Porton Down, UK), subcultured and maintained in RPMI 1640, supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 250 IU/ml penicillin at 37 °C in a humidified atmosphere of air/CO_2. *S. flexneri* (NCTC 9950) was purchased from the Public Health Laboratory Services (Porton Down, UK), subcultured and maintained in tryptic soy broth (TSB; Difco Laboratories Ltd., Detroit, Mich., USA). A commercial health drink (Gel, Forever Living Products, USA) was passed through sterile Whatman no. 54 filter (it was not possible to accurately determine the loss of active material through this process). Unformulated, freeze-dried Aloe vera inner gel powder (AV, supplied by Aloe Vera of America Inc., USA or Rainbow Naturprodukte GmBH, Germany) was reconstituted in phosphate-buffered saline (pH 7.5) at 37 °C for 30 min and then sterile filtered through a 0.22 μm pore size filter unit (Schleicher and Schuell, Dassel, Germany). Lipopolysaccharide (LPS) and RPMI-1640 medium were purchased from Sigma–Aldrich Ltd., Poole, Dorset, UK. Cytokine ELISA kits (Diaclone Research) were kindly donated by Immunodiagnostic Systems Ltd., Newbury, Berkshire, UK. Plasticware was obtained from Greiner Bio-One Ltd., Stonehouse, Lanarkshire, UK; 96-well tissue culture plates from Techno Plastic Products AG (Trasadingen, Switzerland); 24-well culture plates from Costar, UK and Nunc-Immuno™ Modules from Nunc A/S (Roskilde, Denmark).

2.2. Methods

2.2.1. In vitro blood assay to assess anti-inflammatory responses

Packed blood cells (900 μl) were incubated in sterile 1.5 ml microfuge tubes with 100 μl lipopolysaccharide (LPS) or *S. flexneri*, for 24 h at 37 °C, 100% humidity and 5% CO_2. Dose and time curves were constructed. The samples were vortexed and centrifuged at 5000*g* for 4 min and the plasma decanted into fresh tubes and stored at −20 °C until assayed by ELISA, following the manufacturers instructions. For the TNF-α and IL-1β assays, at a final concentration of 0.2 μg/ml LPS or 1×10^9 and 1×10^3 cfu/ml *S. flexneri*, respectively, consistently gave detectable results and was used in subsequent experiments.

A preliminary ELISA was carried out with various plasma dilutions (using diluent buffer) for each new donation of blood. In general, for the TNF-α and IL-1β assays, a 1:100 and 1:200 dilution, respectively, was found to give values, which fell within the standard curve.

2.2.2. Assessment of Aloe vera on cytokine production and cell viability

The assay was set up as above with packed blood cells and LPS or bacteria. One hundred microliters AV per well (4.5 or 45 mg/ml) or Gel (5% v/v or 10% v/v) was incubated in the stimulation assay at 37 °C, 5% CO_2 and 100% humidity. At the end of the incubation period, the blood was vortexed gently and a drop of blood mixed with trypan blue for examination of cell viability to establish that the plant material was not toxic to the cells. The blood was then centrifuged at 5000*g* for 4 min and the supernatant assayed by ELISA for cytokine determination.

2.2.3. Assessment of Aloe vera on cytokine production in bacterial-stimulated THP-1 cells

THP-1 cells were seeded in 24-well plates at a concentration of 1×10^5 cells/well (1 ml), together with 10 μl *S. flexneri* (final concentration 1×10^3 cfu/ml) or LPS (ranging from 0.01 to 100 μg/ml), in the presence of 10 μl Aloe vera at a range of concentrations. Controls included medium only, cells only, stimulated cells without Aloe vera. The cells were incubated for 24 h at 37 °C, 5% CO_2 and 100% humidity. The contents of each well were centrifuged at 5000*g* for 4 min and the supernatant assayed by ELISA for cytokine determination.

2.2.4. Assessment of cytoxicity of Aloe vera on THP-1 cells

Toxicity of THP-1 cells was assessed using an Alamar Blue™ reduction assay. Cells (1×10^6 cells/well) were seeded in 96-well plates. Aloe vera was added to the first row (100 μl) and a serial dilution carried out across the plate. 10 μl Alamar Blue™ was added to each well. Cells in medium alone acted as a positive control, medium and Aloe vera was a background control, while cells in water was used as a negative control. After incubation with test agents for 24 h at 37 °C, 5% CO_2 and 100% humidity, the medium was replaced in all wells with fresh medium containing 10 μl Alamar Blue™. Measurements of the reduction of Alamar Blue™ were taken as absorbances at 570 and 600 nm; both at 24 and 48 h after initial addition of Aloe vera, using a microplate reader (UVSpectramax, Molecular Devices). These readings were used to calculate the percentage growth of the THP-1 cells in the presence of test agent. Results were verified using Trypan Blue dye exclusion.

2.2.5. Statistical analysis

A student *t*-test (unpaired, two-tailed) performed on Statview, v5.1 software.

3. Results

3.1. Effect of Aloe vera on bacterial induced TNF-α levels from stimulated blood cells

Fig. 1a and b show representative dose and time response curves for TNF-α produced from LPS stimulated leukocytes. The maximum TNF-α production of 450 ± 40 pg/ml was induced with 0.2 μg/ml LPS (Fig. 1). A maximum of 500 ± 20 pg/ml was measured after 12 h (Fig. 2) and remained at this level for the 24 h incubation period of the experiment.

In order to determine the effect of *Aloe vera* on cytokine production, the experiment was repeated with whole bacteria (*S. flexneri*, final concentration 1×10^9 cfu/ml) and LPS (0.2 μg/ml final concentration) for 24 h. These stimuli induced TNF-α concentrations of 991 ± 176 pg/ml (Fig. 3a) and 523 ± 45 pg/ml (Fig. 3b), respectively. Freeze-dried *Aloe vera* (examined at 4.5 and 45 mg/ml final concentrations), significantly ($p < 0.0001$) reduced bacterial induced TNF-α to base-line levels, whereas only 45 mg/ml significantly ($p < 0.001$) reduced LPS-induced TNF-α to 301 ± 62 pg/ml. Aloe vera Gel (10% v/v) significantly ($p < 0.0001$) reduced both LPS and bacterial-stimulated cytokine production to base-line levels. Although 5% (v/v) Aloe vera Gel reduced the cytokine production significantly ($p < 0.001$), it was not to base-line concentrations (250 ± 52 and 205 ± 7 pg/ml, in the presence of bacteria and LPS, respectively).

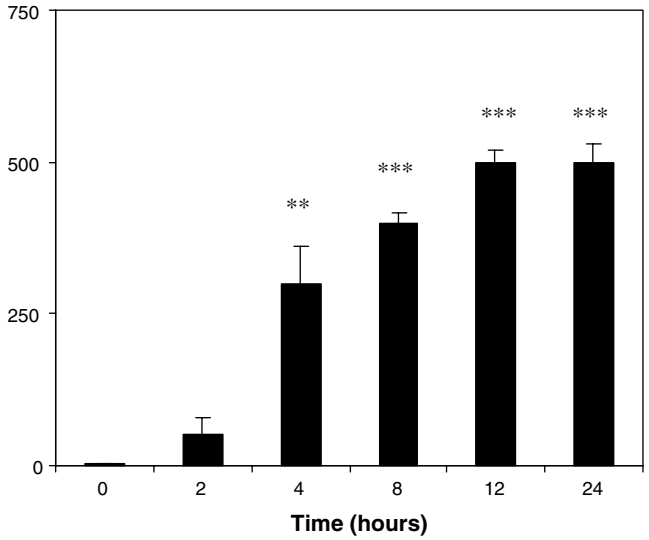

Fig. 2. Production of TNF-α from peripheral blood leukocytes, stimulated with a final concentration of 0.2 μg/ml LPS for varying times at 37 °C, 5% CO_2 and 100% humidity. The plasma was assessed by ELISA and the concentration of cytokine calculated from the A_{450}. The results are expressed as the mean of triplicate readings ± SD, $n = 3$, **significant increase in TNF-α compared with medium only control ($p < 0.001$), ***significant increase in TNF-α compared with medium only control ($p < 0.0001$).

3.2. Effect of Aloe vera on bacterial induced IL-1β concentration from stimulated blood cells

The same assay conditions as for TNF-α were used to determine IL-1β concentrations. 261 ± 14 and 195 ± 22 pg/ml (Fig. 4) were induced by whole bacteria (final concentration 1×10^3 cfu/ml) and LPS (0.2 μg/ml final concentration), respectively, compared with untreated controls (24 ± 3 pg/ml). AV at 45 mg/ml final concentration significantly ($p < 0.001$) reduced IL-1β levels to 103 ± 56 pg/ml in the presence of bacteria, whereas both concentrations significantly ($p < 0.0001$) and 10% (v/v) Aloe vera Gel reduced LPS-induced IL-1β (below 60 pg/ml), although not to base-line levels. However, only 10% (v/v) Aloe vera Gel reduced the cytokine to base-line levels in the presence of bacteria.

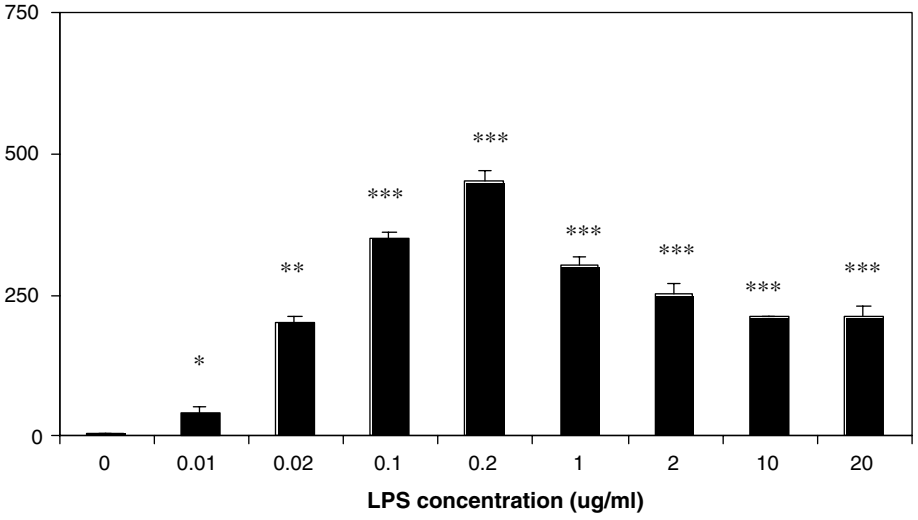

Fig. 1. Production of TNF-α from peripheral blood leukocytes, stimulated with varying concentrations of LPS for 24 h at 37 °C, 5% CO_2 and 100% humidity. The plasma was assessed by ELISA and the concentration of cytokine calculated from the A_{450}. The results are expressed as the mean of triplicate readings ± SD, $n = 3$, *significant increase in TNF-α compared with medium only control ($p < 0.01$), **significant increase in TNF-α compared with medium only control ($p < 0.001$), ***significant increase in TNF-α compared with medium only control ($p < 0.0001$).

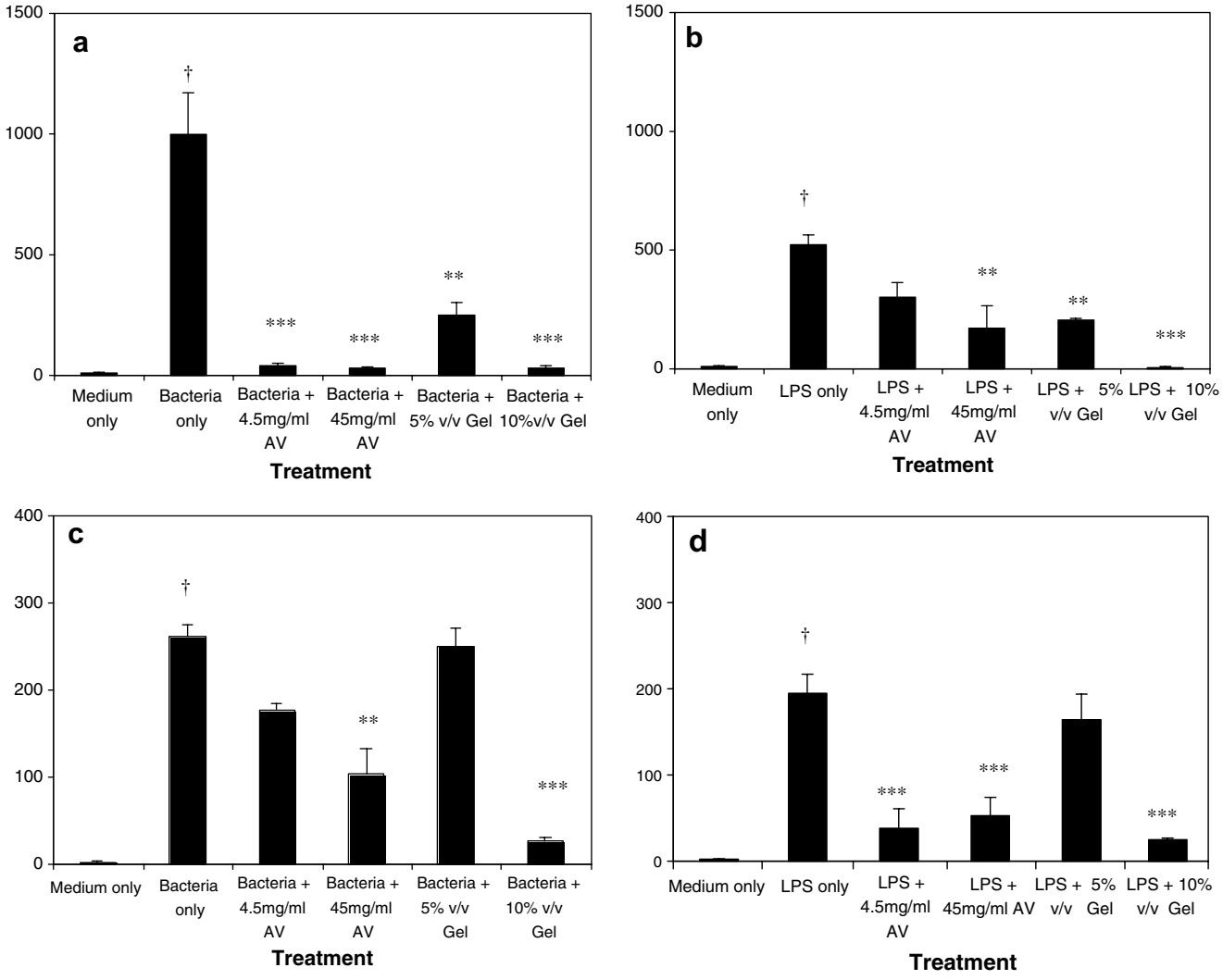

Fig. 3. (a) Production of TNF-α from peripheral blood leukocytes, stimulated with *S. flexneri* at a final concentration of 1×10^9 cfu/ml for 24 h at 37 °C, 5% CO_2 and 100% humidity. Test agents consisted of 100 μl freeze-dried inner gel from *Aloe vera* (AV, 4.5 and 45 mg/ml) and Aloe vera Gel (Gel, 5% v/v and 10% v/v). The plasma was assessed by ELISA and the concentration of cytokine calculated from the A_{450}. The results are expressed as the mean of triplicate readings ± SD, $n = 3$, †significant increase in TNF-α compared with medium only control ($p < 0.0001$), **significant decrease in TNF-α compared with the bacteria only control ($p < 0.001$), ***significant decrease in TNF-α compared with the bacteria only control ($p < 0.0001$). (b) Production of TNF-α from peripheral blood leukocytes, stimulated with LPS at a final concentration of (0.2 μg/ml) for 24 h at 37 °C, 5% CO_2 and 100% humidity. Test agents consisted of 100 μl freeze-dried inner gel from *Aloe vera* (AV, 4.5 and 45 mg/ml) and Aloe vera Gel (Gel, 5% v/v and 10% v/v). The plasma was assessed by ELISA and the concentration of cytokine calculated from the A_{450}. The results are expressed as the mean of triplicate readings ± SD, $n = 3$, † significant increase in TNF-α compared with medium only control ($p < 0.0001$), **significant decrease in TNF-α compared with the LPS only control ($p < 0.001$), ***significant decrease in TNF-α compared with the LPS only control ($p < 0.0001$). (c) Production of IL-1β from peripheral blood leukocytes, stimulated with *S. flexneri* at a final concentration of 1×10^3 cfu/ml for 24 h at 37 °C, 5% CO_2 and 100% humidity. Test agents consisted of 100 μl freeze-dried inner gel from *Aloe vera* (AV, 4.5 and 45 mg/ml) and Aloe vera Gel (Gel, 5% v/v and 10% v/v). The plasma was assessed by ELISA and the concentration of cytokine calculated from the A_{450}. The results are expressed as the mean of triplicate readings ± SD, $n = 3$, †significant increase in IL-1β compared with the medium only control ($p < 0.0001$), **significant increase in IL-1β compared with the bacteria only control ($p < 0.001$), ***significant decrease in IL-1β compared with the bacteria only control ($p < 0.0001$). (d) Production of IL-1β from peripheral blood leukocytes, stimulated with LPS at a final concentration of (0.2 μg/ml) for 24 h at 37 °C, 5% CO_2 and 100% humidity. Test agents consisted of 100 μl freeze-dried inner gel from *Aloe vera* (AV, 4.5 and 45 mg/ml) and Aloe vera Gel (Gel, 5% v/v and 10% v/v). The plasma was assessed by ELISA and the concentration of cytokine calculated from the A_{450}. The results are expressed as the mean of triplicate readings ± SD, $n = 3$, †significant increase in IL-1β compared with the medium only control ($p < 0.0001$), ***significant decrease in IL-1β compared with the LPS only control ($p < 0.0001$).

3.3. Effect of Aloe vera on THP-1 cells

Significant ($p < 0.0001$) TNF-α levels (290 ± 5 pg/ml) were released by THP-1 cells with 0.1 μg/ml LPS (Fig. 4), over a 24 h period (Fig. 5).

Higher levels of TNF-α, between 2- and 7-fold, were achieved in stimulation assays with *S. flexneri*. Nevertheless, the higher level of Aloe vera (45 mg/ml) was able to suppress cytokine levels to base-line, while the lower concentration of Aloe vera (4.5 mg/ml), caused

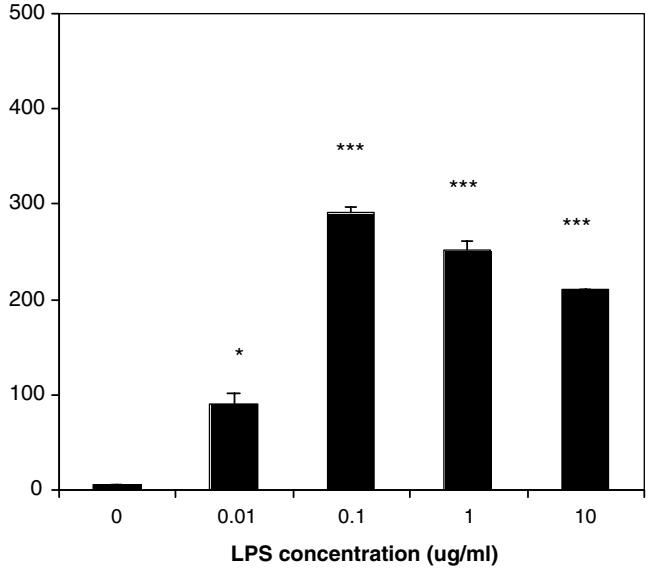

Fig. 4. Production of TNF-α fromTHP-1 cells, stimulated with varying concentrations of LPS for 24 h at 37 °C, 5% CO_2 and 100% humidity. The plasma was assessed by ELISA and the concentration of cytokine calculated from the A_{450}. The results are expressed as the mean of triplicate readings ± SD, $n = 3$, *significant increase in TNF-α compared with medium only control, ***significant increase in TNF-α compared with medium only control ($p < 0.0001$).

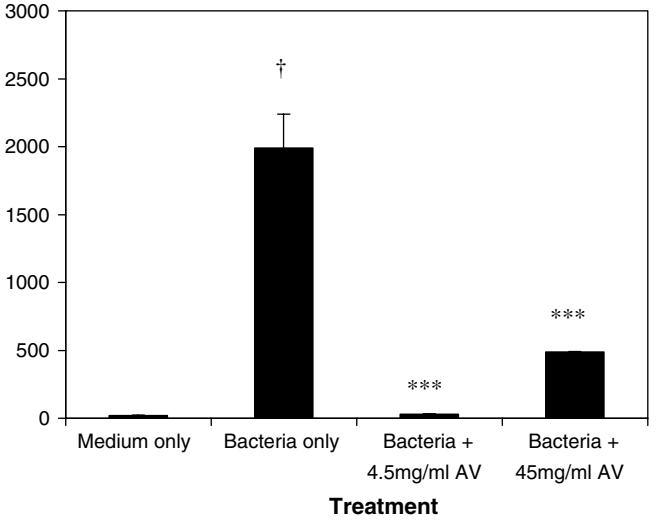

Fig. 6. Production of TNF-α fromTHP-1 cells, stimulated with *S. flexneri* at a final of 1×10^3 cfu/ml for 24 h at 37 °C, 5% CO_2 and 100% humidity. Test agents consisted of 100 μl freeze-dried inner gel from *Aloe vera* (AV, 4.5 and 45 mg/ml). The plasma was assessed by ELISA and the concentration of cytokine calculated from the A_{450}. The results are expressed as the mean of triplicate readings ± SD, $n = 3$, †significant increase in TNF-α compared with medium only control ($p < 0.0001$), ***significant decrease in TNF-α compared with the bacteria only control ($p < 0.0001$).

significant reduction by 75%. Fig. 6 shows a typical experiment.

Aloe vera was non-toxic to the cells at all concentrations tested, as determined by Alamar Blue™ and Trypan blue dye exclusion.

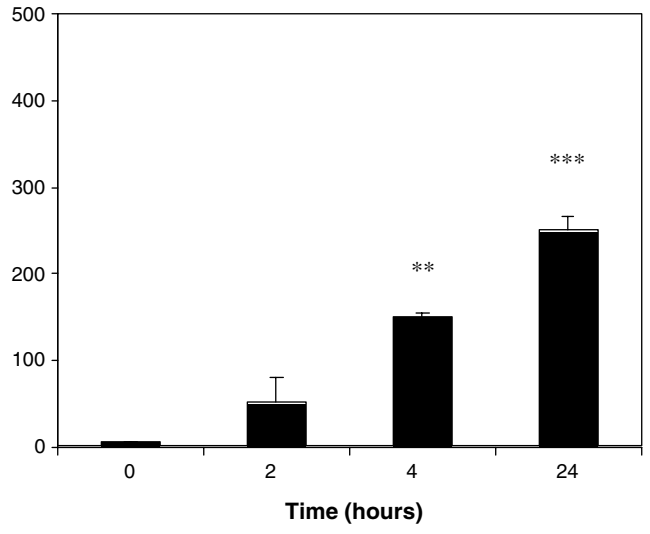

Fig. 5. Production of TNF-α fromTHP-1 cells, stimulated with LPS at a final concentration of 0.1 μg/ml for varying times at 37 °C, 5% CO_2 and 100% humidity. The plasma was assessed by ELISA and the concentration of cytokine calculated from the A_{450}. The results are expressed as the mean of triplicate readings ± SD, $n = 3$, **significant increase in TNF-α compared with medium only control ($p < 0.001$), ***significant increase in TNF-α compared with medium only control ($p < 0.0001$).

4. Concluding remarks

In view of the complexities of examining the pharmacology of natural products, simple assays, which can be easily replicated and have the potential for high throughput are important. This study therefore used a simple assay with blood leukocytes to show that both pro-inflammatory cytokines induced by whole bacterial stimulation can be suppressed with at least 45 mg/ml inner gel (final concentration in assay 4.5 mg/well), while a tenfold dilution was only effective against TNF-α. A similar trend was observed with Aloe vera Gel, whereby both concentrations (5% and 10%, v/v) effectively suppressed TNF-α induced by whole bacteria, but only the higher concentration showed significant activity against IL-1β. Overall, the pro-inflammatory response to whole bacterial stimulation was greater than to the bacterial cell wall component (LPS) and the trends observed were different. Both concentrations of AV effectively suppressed IL-1β stimulated by LPS, but only the high concentration suppressed TNF-α in the presence of LPS. Both concentrations of Aloe vera Gel were effective in suppressing TNF-α, but only 10% (v/v) suppressed IL-1β induced by LPS. It is conceivable that the multiple components within the bacteria, such as LPS, outer membrane proteins, flagelin and peptidoglycan, activate the cells via a number of different mechanisms, leading to variations in pro-inflammatory response. In addition, the greater response to the whole organism is particularly pertinent to LPS derived from *Shigella*, compared with other organisms [21]. The importance of LPS in *S.*

flexneri infections, is that it is a dominant inducer of polymorphonuclear (PMN) transmigration, which helps to further destabilise the epithelium [21]. LPS also activates the nuclear factor kappa B (NFκB) pathway, an important transcriptional regulator of genes involved in inflammation [22–24]. Although there has been anecdotal evidence for the anti-inflammatory activity of *Aloe vera*, there has been sparse examination of the mechanism of action, particularly with respect to the inner gel. The examination of various signal transduction pathways is one approach in this respect and since we have shown a positive anti-inflammatory response on both TNF-α and IL-1β, it would be pertinent for future experimentation to pursue this path. However, the use of a mixed cell population (as found in packed blood cells) and variation between experiments caused by the use of blood from different donors is not convenient for studying cell signalling pathways. Therefore, this has led us to develop another *in vitro* assay.

We have previously described the use of an assay, involving a human monocytic cell line (THP-1 cells) to determine the anti-inflammatory properties of another plant compound, cardamonin [20]. In the present paper, we demonstrate that THP-1 cells provide similar trends of responses to whole blood. With this system, we consistently have the same number of cells, can expect similar responses each time and therefore can compare different extracts and compounds. This assay therefore provides potential for high throughput pre-screening, prior to using the whole blood assay. Furthermore, the THP-1 assay will enable study of cell signalling pathways and mechanisms of action of Aloe vera in inflammation, once we have isolated individual compounds.

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