Leaf gel from several Aloe species shows anti-inflammatory properties through the inhibition of lipopolysaccharide (LPS) mediated activation of Toll-like receptor 4 (TLR4) signaling

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Leaf gel from several Aloe species shows anti-inflammatory properties through the inhibition of lipopolysaccharide (LPS) mediated activation of Toll-like receptor 4 (TLR4) signaling

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**ARTICLE INFO**

**Abstract**

Background: Gel extracted from the succulent inner leaf mesophyll of species from the plant genus Aloe have been used in traditional medicine for centuries as a topical treatment for a wide range of skin conditions. Aim of the study: The aim of this study was to investigate the potential anti-inflammatory properties of leaf gel from 23 different Aloe species. In this study we have focused on the pathways activated by the toll-like receptors TLR4 (activated by LPS), and TLR2/TLR6 (activated by FSL-1) by measuring transcriptional activity of NF-κB.

Materials and methods: Gel extractions from the inner leaf mesophyll material of 23 different Aloe species, pure acemannan obtained from Aloe vera, and pH. Eur. reference material from Aloe vera and Aloe ferox, were tested on the cell line J774-Dual. NF-κB activity was measured after 24 h.

Results: We found that 8 out of the 23 tested Aloe gel extracts significantly decreased LPS-induced NF-κB-activity, but no significant effect was seen with FSL-1.

Conclusion: Based on these findings we can confirm that extracted gel from some of the tested Aloe species but not all, have anti-inflammatory properties.

**Abbreviations**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMF</td>
<td>N-demethylformamide</td>
</tr>
<tr>
<td>FSL-1</td>
<td>Fibroblast-stimulating lipopeptide-1</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
</tr>
<tr>
<td>MTT</td>
<td>Methyl-Thiazolyl-Tetrazolium</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa beta</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Ph. Eur.</td>
<td>European Pharmacopoeia</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
</tr>
</tbody>
</table>

**Introduction**

The plants of the genus Aloe, and the species Aloe vera (L.) Burm.f. (Asphodelaceae) in particular, have been used in traditional medicine for centuries (Grace et al., 2009). The origin of aloes is in Southern Africa, from where the genus spread northwards primarily along the East Coast, with A. vera most likely originating from the Arabian Peninsula (Grace et al., 2015). Today, the genus comprises over 600 species, with up to 20% of them used in local traditional medicine in their native areas (Grace et al., 2009). The earliest known European descriptions of the genus and its topical use and pharmaceutical applications can be found
in the herbal “De Materia Medica” written by Dioscorides in the first century CE (Upton et al., 2012).

Today, two medicinal products are obtained from A. vera – the gel and the exudate. The often yellow exudate is produced in aloetic cells found adjacent to the vascular bundles in the leaves (Beaumont et al., 1985), and it is used as a laxative and cathartic (Boudreau and Beland, 2006). The gel is used for its wound healing effects, and it has been recorded that it has immunomodulating effects on skin inflammations when given topically (Sánchez et al., 2020). The many different effects and uses have given rise to the popularity of A. vera for cosmetics.

Biologically, all aloe are succulent species characterized by a thick layer of water storing cells (inner leaf mesophyll) referred to as the hydrenchyma. The hydrenchyma tissue is found in the center of their leaves and varies in thickness and polysaccharide composition between species. It has been found that this tissue can dramatically alter the polysaccharide composition and micromolecular structure of their cell walls when exposed to extended periods of drought (Ahl et al., 2019). Complex carbohydrates, polysaccharides, are the main building blocks of plant cell walls, and depending on the tissue and its function, the composition of the cell wall and the polysaccharides used in its construction differ. In Aloe hydrenchyma the most prominent hemi-celluloses, and the ones that have been most studied for their medicinal properties, are the glucosaminans. One of these mannans polymers (acemannan) have a backbone of β-(1,4)-mannose that can be acetylated in various amounts depending on their function and placement in the cell. The acetylated mannans has been associated with a range of clinical effects such as wound healing, anti-inflammatory and immunomodulatory activity (Egger et al., 1996a, 1996b; Fry, 2004; Grace et al., 2013; Xing et al., 2014). Even though Aloe leaf gel has been used for decades, the pharmacological effects of its components are not well known. According to a recent review by Sánchez et al. (2020), on A. vera and skin protection activities its constituents have not been very thoroughly studied to make firm conclusions on their effects (Li et al., 2017; Thunyaktipsal et al., 2017). More testing of isolated compounds extracted from A. vera is encouraged using both in vivo and in vitro assays as well as clinical trials related to skin protection.

The human body has a constant need for repair and protection from both internal and external stressors. The immune system contains the repair and defense mechanisms, that keeps a tight control with inflammations. The process and control required for the repair of damaged tissue and wound healing is highly dependent on alternative polarization of macrophages as a checkpoint for inflammatory resolution. Inflammation is a reaction by the body caused by infection or injury, and the immunological reaction is characterized by swelling, pain, redness, heat, and loss of function. This natural response by the body can delay healing, but it may also be detrimental to suppress inflammation before its purpose is accomplished (Agger et al., 2015; Ferrer et al., 2017). In the innate immune system, toll-like receptors (TLRs) are found in the cell membrane of macrophages. TLRs are a class of proteins that play a key role in the response by the innate immune system (O’Neill and Bowie, 2007). TLRs are homo- and heterodimeric transmembrane proteins that stimulate transcriptional activation of nuclear factor kappa beta (NF-kB) and other pathways. TLRs recognize Pattern-Associated Molecular Patterns (PAMPs), which leads to a release of pro-inflammatory cytokines. The study of NF-kB activity in response to inflammatory processes can be done by measuring the secretion of Secreted Embryonic Alkaline Phosphatase (SEAP) in a reporter cell assay. Here, NF-kB secretion can be initiated by adding lipopolysaccharides (LPS) that will then bind to TLR4 or by adding fibroblast-stimulating lipopeptide-1 (FSL-1) binding to TLR2/TLR6 in the macrophage membrane (Abdeladis and Trad, 2011; Kim et al., 2005). Stimulation of TLR4 by LPS during microbial infection plays an important role in immune responses, such as phagocytosis, antigen provision, and secretion of cytokines, chemokines, and other factors (Prymas et al., 2020).

A. vera is often described and studied for its anti-inflammatory properties. Over 20% of the genus is used in traditional medicinal practices, yet all the other medicinally used Aloe species are rarely included in these studies. In this study, the aim was to investigate the potential anti-inflammatory properties of leaf gel from 23 different Aloe species, pure acemannan and pH. Eur. reference material. Focus has been on the pathways activated by the toll-like receptors TLR4 and TLR2/TLR6 by measuring transcriptional activity of NF-kB in the cell line J774-Dual.

Materials and methods

Plant material and extraction

In this study 25 different gel extracts were included representing 23 different Aloe species in triplicate dilution series and controls accounting for n=144 (25 sample types used on two cell lines, all samples run in triplicates. For the FSL-1 experiments Aloe hildebrandtii and Aloe fucunda did not work. Calculations as follows: ((25×2)×3) – 6 failed samples = 144). For all analyses, three control samples (PBS and DMEM) were included. The control samples were all without the addition of Aloe gel. One control had LPS added, another FSL-1 and one contained PBS and DMEM. Aloe inner leaf mesophyll (containing both gel and insoluble structural material) was sampled from the living collections in the Botanical Garden of the Natural History Museum of Denmark, University of Copenhagen and vouchers were deposited in Herbarium C (Table 1). The water-soluble part of Aloe inner leaf mesophyll used for medicinal purposes will hereafter be referred to as gel, whereas the material containing both water soluble and insoluble material will be referred to as inner leaf mesophyll. Sampling was conducted as part of a study on polysaccharide diversity across the genus Aloe and a study on seasonal variation in Aloe inner leaf mesophyll (Ahl et al., 2019) following the protocol described in Ahl et al. (2018). In addition to the freshly harvested and freeze-dried material, Ph. Eur. reference material for A. vera and A. ferox (European Pharmacopeia, 2016) was obtained from Alfred Galke (Bad Grund, Germany), and a pure extract of acemannan was purchased from Elicityl OligoTech (Crolles, France). The complete sample list can be found in Table 1S in the Supplementary material. The freeze-dried inner leaf mesophyll was weighed and cut into smaller pieces and dissolved in phosphate buffered saline (PBS). The final stock solution of inner leaf mesophyll and PBS had an inner leaf mesophyll concentration of 2% for each sample. Samples and PBS were mixed on a ThermoMixer® C (Eppendorf, Hamburg, Germany) for 20min at 36°C and maximum speed (1000 x g). The mixture was then filtered through Falcon™ CellStrainer (BD, Franklin Lakes, NJ, USA) 70mm nylon and into a 50ml Falcon tube to separate undissolved material from the liquid. The extracted Aloe leaf gel will hereafter be called Aloe gel extracts. Samples were stored at -22°C prior to use in the assays.

Cell line experiments

The cell line J774-Dual™ was used for experiments. Cells were obtained from InvivoGen (San Diego, CA, USA). All cell work was done in a sterile laminar air flow bench to prevent contamination. Cells were plated out in 24-well plates with 350,000 cells/well and incubated overnight at 5% CO$_2$ and 37°C. 50 μl of the 2% Aloe stock solution was then added to either 450 μl DMEM, or 445 μl DMEM and 5 μl LPS or FSL-1 making the final concentration of Aloe in the plates 0.2%. The effect of Aloe gel extracts on cell viability were tested by the Methyl-Thiazolyl-Tetrazolium (MTT) assay as described previously (Mosmann, 1983). In brief, cells were pre-incubated for 24h with increased concentrations of Aloe gel extracts prior to addition of MTT buffer (5mg/ml MTT in PBS (phosphate buffered saline), Sigma Aldrich, M5655–1G). After 2–4h of incubation, MTT lysis buffer (195g SDS, 300ml DMF (N-dimethylformamide), 5ml 2.5N HCl, 23ml 100% acetic acid and 240ml H$_2$O) was added and the plate was incubated overnight at 37°C to dissolve formazan crystals. Absorbance at 570nm was measured by a spectrophotometer (Thermo Scientific Multiskan FC, Waltham, MA, USA).
Transcriptional activity of NF-kB was measured by reporter assay QUANTI-Blue™ as described by the manufacture (InvivoGen). Absorbance was measured at 620nm. Lipopolysaccharides (LPS) and fibroblast-stimulating lipopeptide-1 (FLS-1) were used as ligand stimuli. Positive controls were included where only ligands were added to the buffers.

Screening tests with two ligand stimuli

For Complex 1 experiments the method was the same as used for testing the concentration gradient and concentration screen 0.2% of the different Aloe species. In the Complex 2 method, Aloe gel extract and medium (DMEM) were added to cell and then incubated at 37°C and 5% CO₂ for 90min and then either LPS or FSL-1 was added for the two different receptors and incubated again for 24h before collecting the supernatant for measurement. For the Complex 3 method, Aloe, DMEM and LPS or FSL-1, were premixed in an Eppendorf tube and stored at room temperature for 90min before added to the cells and incubated for 24h before collecting the supernatant for measurement.

Pure acemannan from A. vera was dissolved in PBS to a stock concentration of 2mg/ml. The acemannan was tested with concentrations ranging from 5 to 500μg/ml and analysed by QUANTI-Blue solution. Controls as described in the section on plant material and extraction were included for all the Complex (1–4) experiments. See Fig. 1 for a schematic drawing of the experimental setup.

Statistical analyses

Statistical analysis of the raw data (absorbance) was generated with GraphPad Prism version 8.3.1. Absorbance data were converted to fold change to show the differences between the controls and a measurement. Fold change is the ratio between the two quantities. Data either as raw LMNI data or as fold change were tested for normality using a Shapiro-Wilk normality test (significance p < 0.05), before statistical significance was determined using Student’s t-tests (and nonparametric Kruskal-Wallis tests where necessary) between individual species, using both raw and fold change datasets independently. Bar-charts were created using the ggplot2 package (Wickham, 2016) within the R computing environment (v1.2.1335).

Results

In this study we tested 25 different extracts representing 23 different species of Aloe. In Fig. 2 all the raw data (control and with Aloe gel extract) from the LPS experiment is shown with significant changes in absorbance between the control and test sample marked with a star. In Fig. 3 the LPS data converted to fold change has been depicted in the same way with the significant changes marked with a star. In Fig. 4 results from the MTT assay are shown for A. vaombe and A. vera showing decreased cell viability compared with the control. FSL-1 data are not shown as graphs but included in Table 1 as only one significant result was found for Aloe mzimbana in the raw data. In the fold change dataset, no significant results were found for FSL-1.

In Table 1, all the results from the statistical analyses have been compiled showing both LPS and FSL-1 raw data and fold change, and significant changes are marked in bold.

Table 1

<table>
<thead>
<tr>
<th>Species</th>
<th>Voucher (Herbarium C)</th>
<th>LPS</th>
<th>FSL-1</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Absorbance</td>
<td>Fold change</td>
<td>Absorbance</td>
</tr>
<tr>
<td>Aloe arborescens Mill.</td>
<td>Ahl 51</td>
<td>2.513</td>
<td>0.0362</td>
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<tr>
<td>Aloe decaryi Gaillardin</td>
<td>Ahl 61</td>
<td>3.432</td>
<td>0.0089</td>
</tr>
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<td>Aloe deserti A.Berger (Shrubby)</td>
<td>Ahl 62</td>
<td>3.72</td>
<td>0.0181</td>
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<td>Aloe dhuarensis Lavranos</td>
<td>Ahl 14</td>
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<td>Aloe elegans Tod.</td>
<td>Ahl 15</td>
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<td>0.0255</td>
</tr>
<tr>
<td>Aloe ferox Mill.</td>
<td>Ahl 67</td>
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<td>0.0658</td>
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<tr>
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<td>NA</td>
<td>2.703</td>
<td>0.0192</td>
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<tr>
<td>Aloe fœtissimum Lavranos &amp; L.E.Newton</td>
<td>Ahl 17</td>
<td>1.802</td>
<td>0.1018</td>
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<td>Aloe hildebrandti Baker</td>
<td>Ahl 21</td>
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<td>0.0099</td>
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<td>Culti. P2015-5030</td>
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<td>0.0301</td>
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<td>Ahl 24</td>
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<td>0.0061</td>
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<td>Aloe macrocarpa Tod.</td>
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<td>Aloe massarwana Reynolds</td>
<td>Ahl 26</td>
<td>1.236</td>
<td>0.2628</td>
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<td>Aloe mzimbana Verd. &amp; Christian</td>
<td>Ahl 29</td>
<td>6.712</td>
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<tr>
<td>Aloe penduliflora Baker</td>
<td>Ahl 34</td>
<td>7.164</td>
<td>0.0004</td>
</tr>
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<td>Aloe secundiflora Engl.</td>
<td>Ahl 38</td>
<td>3.38</td>
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<td>Aloe striata Haw.</td>
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<td>Aloe speciosa Baker</td>
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<td>Aloe vaombe Decorce &amp; Poiss.</td>
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<td>Aloe vera L. (Ph.Eur)</td>
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<td>2.956</td>
<td>0.012</td>
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Fig. 1. Schematic drawing of the method used for complexity analysis when screening with two ligand stimuli - Fibroblast-stimulating lipopeptide-1 (FSL-1) and lipopolysaccharides (LPS). The Complex analyses were carried out to investigate the affinity by extract cells straight away. The used method being the same as with the concentration gradient and concentration screen of analyses were performed and are depicted adjacent to the numbers respectively. 1. Complex 1: mixture of Aloe gel extracts, ligand stimuli and DMEM are added to the cells straight way. The used method being the same as with the concentration gradient and concentration screen of analyses were performed and are depicted adjacent to the numbers respectively. 2. Complex 2: Aloe gel extract + DMEM are added to cells and incubated for 90 min before ligand LPS or FSL-1 is added and incubate for 24 h. 3. Complex 3: mix Aloe, LPS or FSL-1 and DMEM and let it sit in an Eppendorf tube for each sample for 90 min. Add the mixture to the cells and incubate for 24 h. 4. Complex 4: LPS or FSL-1 and DMEM are added to cells and incubated for 90 min before Aloe gel extracts are added. Then left to incubate for 24 h.

The results show that for the LPS experiments, 13 individual Aloe species had significantly lower absorbances (Fig. 2, Table 1), and similarly, 9 individual Aloes varied when converted to fold changes (Fig. 3, Table 1).

The most significant changes (Table S2) were measured in A. mzimbana (absorbance was 0.159 with a standard deviation of ±0.152), A. penduliflora (absorbance was 0.209 with a standard deviation of ±0.245) and A. secundiflora (absorbance was 0.288 with a standard deviation of ±0.325) with absorbance value of the samples differing particularly from controls.

Looking at the differences between species, 13 were found to produce significant changes in the absorbance data where only 9 species varied significantly in the fold change data (Figs. 2 and 3). Six out of the 22 Aloe gel extracts showed significant changes in both absorbance (Fig. 2) and fold change (A. arborescens, A. dhufarensis, A. ferox Ph. Eur., A. hildebrandii, A. penduliflora, and A. secundiflora) (Fig. 3). The remaining 7 species extracts went from being significant in the absorbance dataset to not being significant in the fold change dataset (Fig. 3). At the same time, 3 gel extracts did not change significantly in the absorbance dataset, yet once the data was converted into fold change they did. For the fold change dataset, the most significant changes were seen (Table S2) in A. secundiflora, A. dhufarensis, and A. vaombe.

From the FSL-1 data, only A. mzimbana varied significantly in the absorbance data, but not in the fold change data. For the remaining (vast majority) of species no significant variation was seen (Table 1).

Discussion

Eight out of the 23 tested Aloe species (A. ferox as a species is only counted once as a response was seen for both collected material and Ph. Eur. Material) showed a significant decrease in NF-κB-activity when extractions were added together with LPS (Fig. 3). None of the species tested with FSL-1 showed a significant decrease (Tables 1, and S1). Overall, the two tested ligands showed a similar result with a decrease in NF-κB-activity. A. dhufarensis showed a significant decrease in LPS induced NF-κB activity, but an increase in NF-κB-activity induced with FSL-1. Other species like A. decaryi and A. deserti also showed an increase in NF-κB-activity with FSL-1 but showed a decrease in NF-κB-activity with LPS (Table 1).

In a study of the polysaccharide composition of the genus Aloe from which the samples for this study were obtained, a correlation was found between the thickness of the succulent tissue and the polysaccharide composition. The correlation between leaf thickness and polysaccharide composition could likely be part of the explanation for the different anti-inflammatory effects observed between the different species. Acetylated mannan has been suggested as a possible component in aloe gel with therapeutic properties, focusing on its ability to accelerate wound healing and immune stimulation (Zhang et al., 1996). Absorbance and fold change data from the tested pure acemannan in different concentrations showed inhibition of LPS with the lowest concentration 5μg ml⁻¹ indicating anti-inflammatory effect (Figs. 2 and 3). When added in higher concentrations there was little to no inhibition. In addition, the higher concentrations (50μg ml⁻¹ and 500μg ml⁻¹), of acemannan induced NF-κB activity indicating acemannan can trigger the release of pro-inflammatory cytokines and therefore can give an immune stimulation.

Ph. Eur. material caused an increased cell viability in the MTT assay (Fig. 5) whereas samples from the botanical garden decreased cell viability. This could indicate a difference in the chemical composition of the Ph. Eur. material compared to the inner leaf mesophyll of the garden-collected samples. Aloe leaves are rich in phenolic compounds, and it is therefore important to differentiate between whole leaf and gel extracts. The Ph. Eur. reference standards are most likely whole leaves, whereas the monographs specify either exudate or inner leaf mesophyll. This raises concerns about the availability of relevant reference material as it was also discussed by Ahl et al. (2019).

In this study, inhibition of NF-κB activity was highest in Aloe gel extracts prior to the addition of LPS or FSL-1 (Fig. 1, S3 and S4). This
indicates that either Aloe gel extract attached to, or blocked, the TLR4 or that TLR2/TLR6 ligands, LPS or FSL-1 respectively, were unable to bind (Fig. 5). Components in the Aloe gel extracts could also have a higher affinity to the receptor than to the ligand. The effect was not as pronounced when Aloe gel material, DMEM and LPS or FSL-1 were added to cells at the same time. The Aloe gel extract did not make a complex with neither LPS, nor FSL-1 as seen by the similar results in the test where the mixture was added to the cells straight away (Fig. 1). The set up for the complexity analysis have been included in Fig. 4. Data from this experiment (Complex 4) could support that Aloe gel extracts and ligands are competing for the binding site of the receptor. When LPS was added and had the time to bind to the receptor TLR4, prior to the addition of Aloe gel extracts, the results were similar to the pattern seen for the Complex 1 analysis (Fig. 4). These findings could suggest that Aloe gel extracts might have a higher affinity binding to the TLR4 receptor or blocking the receptor as depicted in Fig. 5.

It is important to bear in mind that in vivo conditions are much more complicated to work with than in vitro. Additionally, inflammation is a very complex biological response of the immune system that can trigger multiple inflammatory signaling pathways, and these were not covered in this study. Here, a limited response of Aloe gel was investigated, meaning it is possible that species not showing anti-inflammatory properties in this study could be stimulating other receptors and pathways. Finally, inflammatory cytokines such as IL-1β, IL-6, and TNF-α also play important roles in the progression of inflammation and could potentially serve as biomarkers for disease diagnosis and thus be used to elucidate the anti-inflammatory properties of Aloe gel.

Conclusions

The main findings of this study were that leaf gel of several Aloe species showed anti-inflammatory properties and results indicated that it could be specific to TLR4 mediated responses. It was found that 8 of the 23 Aloe species (35%) at gel concentration 0.2% decreased the NF-κB activity significantly with LPS, but no significant effect was seen towards FSL-1. A tendency was also seen that aloe gel extracts were more...
effective against LPS-mediated activation of the TLR4 pathway. When tested for complexity with TLR4 or TLR2/TLR6 pathways it was found that aloe gel extracts either compete or interfere with the receptors of both TLR pathways when added first, but still seemed more efficient against LPS than FSL-1. Based on these findings, we can confirm anti-inflammatory properties of some aloes, possibly representing a

Fig. 3. Lipopolysaccharide (LPS) absorbance data converted to fold change data for all analysed Aloe species. Absorbance was measured at 570 nm on a spectrophotometer with the measured absorbance indicating the effect of Aloe gel extracts on cells from the J774-Dual™ cell line when lipopolysaccharide (LPS) had been added to simulate inflammation. On the left-hand side of the bar graphs “Control” refers to samples containing buffers and LPS and “Aloe” refers to solutions containing buffers, LPS and Aloe gel extracts. Species marked with a star have significant p-values with LPS in the fold change data set.

Fig. 4. Methyl-Thiazolyl-Tetrazolium (MTT) assay showed a decrease in cell viability in comparison with the control (CTRL) (blue arrow). Graph a. shows measures with species A. vaombe and controls 30 µl PBS. Graph b. shows A. vera and control 30 µl PBS (blue arrow) and control 15 µl PBS. For A. vera a lower absorbance was measured in control 30 µl PBS compared to control 15 µl PBS. Both the controls and samples with Aloe gel extract were stimulated with LPS. Data are shown with absorbance values and was performed in technical triplicates.
 mechanism for the reported therapeutic action of gel from A. vera and other Aloe species used medicinally. Pure acemannan from A. vera gel was also tested for its recorded effect on acceleration of wound healing and immune stimulation. The results obtained here were conflicting depending on the level of mannan concentrations added to the cells.

**Author agreement statement**

We the undersigned declare that this manuscript is original and has not been published before and is not currently. Being considered for publication elsewhere. We can confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfy the criteria for authorship. The order of the authors listed in the manuscript has also been approved by all.

We understand that the corresponding author is the sole contact for the Editorial process and that she is responsible for communicating in formation regarding the paper to co-authors.

**CRediT authorship contribution statement**

L.I.A., M.P.S., A.W. and N.R. jointly conceived and designed the project. L.I.A selected the plant material. M.P.S. conducted the experiments under supervision by L.H. and A.W. C.J.B. designed and conducted the statistical analysis. L.I.A and M.P.S. drafted the manuscript with C.J.B. and N.R. All authors commented on the manuscript and approved the final version.

**Declaration of Competing Interest**

The authors declare no financial or personal conflict of interest in this work.

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