An investigation of the anti-oxidant, antimicrobial and wound healing properties of whole leave juice and gel powders of *Aloe ferox* and *Aloe vera*

by

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Thank you so much, your positive contribution is highly appreciated.

Declaration

The work described in this dissertation was carried out at the Department of Pharmacology, University of Pretoria, Pretoria, South Africa, under the supervision of Dr AD Cromarty. I declare that this dissertation is my own work submitted for the degree of Masters of Science and that this dissertation does not incorporate, without written acknowledgement, any material that has previously been submitted for the award of any other degree or diploma in any university, college, or other educational institution and to the best of my knowledge, this dissertation does not contain any material previously published or written by another person except where due reference is made in the text, including the disclosure of contributions for any work based in joint research or publications.

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Signed **Date**

Abstract

Aloe vera is found in the Northern Africa and the Mediterranean areas while *Aloe ferox* is found in Southern Africa. *Aloe ferox* and *Aloe vera* prepared by different methods have been shown to possess the following properties: Stimulatory effects on different cell types (e.g human fibroblasts, rat adrenal cells, calf pulmonary artery endothelial cells etc.), wound healing, antimicrobial, antioxidant, antidiabetics etc.

In this study solvent extracted gel powders and whole leaf juice of *Aloe ferox* and *Aloe vera* prepared specifically without bitter components were tested. The aim was to assess if the samples could be used orally for therapeutic purposes with regards to wound healing, antimicrobial and antioxidant properties avoiding the laxative effects of the bitter components.

The following were used: Human lymphocytes cells to determine cytotoxicity effects, chicken fibroblasts cells for potential wound healing properties, *Candida albicans, Staphylococcus aureus* and *Pseudomona aeruginosa* microorganisms for antimicrobial properties and ORAC, DPPH, TEAC and chemiluminescence assays for antioxidant properties.

Most of the results obtained were contrary to the bulk of the literature available about these beneficial plants' extract. Bitter components have been reported to stimulate different cell types and to have antimicrobial and antioxidant properties. Thus the removal has been suggested as the main reason why the effects of the tested extracts did not correspond to much of the reported literature. From the results obtained from various aspects of this study it could be concluded that the removal of bitter components contributed to the apparently contradictory results.

From this study it might be concluded that the four Aloe extract samples tested could not be used therapeutically for wound healing, antimicrobial or antioxidant properties. However they could still be effective for cosmetics purposes as obtained from the literature.

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Chapter 1

1 Introduction

1.1 Background

Aloe vera is a succulent plant found in Northern Africa and the Mediterranean areas and is widely used as a herbal medicine. It grows best in dry chalky soil or in a sandy loam and requires semi–tropical conditions [1]. It is a stemless, drought-resisting succulent of the lily family having thick thorn covered fleshy leaves [2]. It has pendent flowers with a tubular yellow perianth [1].

Extracts of *Aloe vera* leaves consist of an orange–yellow sap called bitter juice and *Aloe vera* gel which is a clear mucilaginous substance that contains fibres, water and ingredients to retain water in the leaves [3]. Aloe leaf derived bitter juice has been proven to have many biological effects. Namely antimicrobial properties, antioxidant properties and laxative effects [4].

Aloe vera has been claimed to have more than 70 biologically active compounds [1].
These are divided into different classes as follows:
Anthraquinones/anthrones (this is found in bitter juice [5]): Aloe-emodin, aloetic-acid, anthranol, aloin A and B (or collectively known as barbaloin), isobarbaloin, emodin, esters of cinnamic acid.
Carbohydrates: cellulose, pure mannan, acetylated mannan, acetylated glucomannan, glucogalactomannan, galactan, galactogalacturan, galactoglucoarabinomannan, arabinogalactan, pectic substances and xylan.
Chromones: 8-C-glucosyl-(2'-O-cinnamoyl)-7-O-methylaloediol A, 8-C-glucosyl-(S)-aloesol, 8-C-glucosyl-7-O-methyl-(S)-aloesol, 8-C-glucosyl-7-O-methylaloediol, 8-C-glucosyl-noreugenin, isoaloeresin D, isorabaichromone, neoaloesin A.
Saccharides: Mannose, glucose, rhamnose, aldopentose.
Vitamins: B1, B2, B6, C, β-carotene,folic acid, α-tocopherol [6].
Other components include alkaloids, saponins, fatty acid materials, glycoproteins, resins, sterols, gelonins, minerals, amino acids, enzymes, glucosyl chromone [6;7], polymers, pectic

acid and acemannan [8], proteins, lipids and organic compounds (triglycerides, triterpenoid, gibberillins, lignins) [6] and potassium.

Aloe vera derived products have been shown to have anti-inflammatory [1;2;9;10] anti- anti-cancer, healing and anti-diabetic properties [2]. The culture technique and chemiluminescence assays were used.

Whole leaf juice of *Aloe vera* contains the following identified components with anti-inflammatory activity: salicylates, magnesium lactase, bradykinin, thromboxane inhibitors and beta linked acetyl mannan [11].

Aloe vera gel which was stored in an air tight container at 4°C has been shown to be effective in treating inflammatory bowel disease. It was claimed to have an inhibitory effect on the production of reactive oxygen metabolites and prostaglandin E_2. Furthermore it reduced the interleukin-8 level which is a pro-inflammatory cytokine implicated in aggravating inflammatory bowel disease [2].

The lipoxygenase pathway which leads to the synthesis of leukotrienes or lipoxins [12] has been shown to be blocked by certain *Aloe vera* extracts during the acute inflammation process, especially when applied topically to minor burns and skin ulcers [13].

Aloe vera gel is usually used as one of the ingredients in cosmetics, this is because it has been proven to have a valuable moisturising emollient effect. It is also included in shaving creams for the purpose of treating cuts [1]. The external use of Aloe (*Aloe vera* and *Aloe ferox*) as a cosmetic ingredient has been approved by FDA [4].

Aloe vera has been proven to have healing effects on gastric lesions [4]. An *in vivo* study using *Aloe vera* has shown gastro protective properties at lower concentration (100mg/kg) against mucosa of the stomach and dose dependently inhibits gastric acid secretion, thus is can be used in gastric ulcer treatment [14].

Carrageenan induces oedema and causes inflammation at the site of injection in rats, thus it causes neutrophil migration into the inflamed area. *Aloe vera* has been proven to reduce the induced oedema and decreases the neutrophil migration [15].

Aloe vera gel components (particularly Aloesin) have been proven to prevent UV skin reactions by preventing the immune suppression caused by radiation. It has also restored cellular immune responses in immunosuppressed mice [4]. Aloeride which is a component of *Aloe vera* has been proven to activate macrophages implying that *Aloe vera* demonstrates immunostimulating properties. Further research is required to assess aloe extracts as possible adjuvants for antiviral medication used in immunocompromised patients.

Aloe vera has been proven to have antidiabetic properties as follows:
Aloe vera sap has shown hypoglycaemic effect in clinical trials [16] *Aloe vera* leaf pulp extract has shown hypoglycaemic effect in both Type I and Type II diabetic rat models [17]. Ethanol extract of *Aloe vera* gel decreased blood glucose levels in streptozotocin-induced diabetic rats [18].

Aloe vera is used in food supplements and is regulated as a dietary supplement in the USA [4].

An aqueous extract of *Aloe vera* inhibited *in vitro* conversion of arachidonic acid to PGE2 [15], thus it has cyclooxygenase inhibitory properties and reduces the production of thromboxane A2 synthase [19] which is responsible for platelet aggregation.

Aloe vera has been shown to prolong life in rats with acute myocardial ischeamia [20], thus it might be effecive in patients with cardiovascular risk although further research is required.

Available literature about *Aloe vera* proves that the plant has many medicinal properties in different preparations and that isolated components from the extracts also show specific medicinal properties.

Aloe vera

Therapeutic area	**Type of extract**	**Positive/Negative results**
Inflammatory bowel disease	*Aloe vera* gel without being filtered [2]	Positive
Gastric lesions	Ethanol extract of *Aloe vera* [14]	Positive
Carrageenan induced oedema	*Aloe vera* gel was harvested from the green leaves, minced, homogenised and dried at 35°C. Aqueous and chloroform extracts were used [15].	Positive
Prevent UV skin reactions	Aloesin which is a component from *Aloe vera* gel [4]	Positive
Antidiabetic properties		
Hypoglycaemic effect in clinical trials	Dried *Aloe vera* sap (bitter juice) [16]	Positive
Hypoglycaemic effect in both type I and type II diabetic rat models	*Aloe vera* leaf pulp [17]	Positive

Adverse effects of *Aloe vera*

Long term ingestion of moderate levels of *Aloe vera* (1.5 and 5.5 months) has no apparent adverse effects on body weight, food intake, gastrointestinal transit time and gross pathology in rats, however at high dose, diarrhoea and a decrease in body weight occurs [21].

Long term use and high doses have been associated with dermatitis (even though the irritants products are apparently removed by manufacturing companies) [22] and hepatitis [23] in humans.

It has been reported that the interaction of *Aloe vera* and sevoflurane causes suppression of cyclooxygenase leading to a decrease in thromboxane A_2 levels and inhibition of the aggregation of platelets that can result in severe bleeding [24].

Aloe ferox

Aloe ferox is indigenous to South Africa [25]. It is commonly found in the Eastern and Western Cape provinces [26]. It is a robust, single stemmed plant with fleshy leaves that are green with brown spines along the edges [25] and has persistent dry leaves on the lower portion of the single stem [27]. The dried product of *Aloe ferox* leave exudate is commercially known as Cape aloe. Aloe gel originates from the water retaining inner part of the leaf.

Aloe ferox components are divided as follows:

Bitter components of *Aloe ferox* [28]

Aloe bitters: Anthrones and chromones (major classes) found in the leaf exudates
Anthrones: Aloin and barbaloin
Chromones: Aloesin and aloeresin A
Other components: anthraquinones , naphthalene, alkaloids

***Aloe ferox* gel components**

Major components: glucomannoglycan polysaccharides containing acetylated monosaccharides, acemannan (mannose), glucomannan (glucose) [29]
Other components: amino acids, salicylic acid, lignins, saponin, sterols, fatty acids, vitamins, enzymes, minerals [27, 29].
Aloe ferox components include antioxidant polyphenols, indoles and alkaloids [30].

Aloe ferox has been proven to contain phytosterols in a high concentration. Due to the fact that phytosterols are known to have cholesterol lowering effect, it is predicted that *Aloe ferox* may decrease the risk of cardiovascular diseases [30]. Apart from this, phytosterols have also been associated with other health benefits like lowering glucose levels in diabetic patients, immune boosting properties and anti-tumour properties [30].

Fatty acids are proved to be associated with decreasing the risk of inflammatory diseases [30]. According to Du Tiot *el al* [30], fatty acids contained in *Aloe ferox* are at a very low concentration thus perhaps they are contributing less to the health benefits of *Aloe ferox*.

Research has shown that *Aloe ferox* has been harvested for almost 250 years particularly for bitter sap [26] . South Africa is one of the developing countries, thus the government and private agencies are harvesting more of this plant as a source of income for the poor in the rural areas. South Africa earns foreign currency through the export of harvested *Aloe ferox* to Europe and North America for use in the health industry and for cosmetic ingredients [26].

Cape aloe extract has been reported to possesses these pharmacological effects: anti-inflammatory, anti-bacterial, anti-fungal and protective against liver injury [31].
Aloe ferox is used for arthritis, eczema, conjunctivitis, hypertension, stress and the bitter components as a laxative. Glycoproteins which are components of *Aloe ferox* extracts, are thought to be responsible for the reported healing properties due to its ability to increase collagen matrix content of the granular tissue [32, 33].

Two constituents of *Aloe ferox* i.e. aloeresin-1 and aloesin reduce *in vivo* oedematous response induced by croton oil in the mouse ear inflammation model [34].

In the current literature, adverse effects of *Aloe ferox* have not been reported. A study was done to evaluate the toxicological effects of aqueous leaf extract on haematological parameters, liver and kidney function indices in loperamide-induced constipated rats. The following concentrations of Aloe leaf extract were used 50, 100, 200 mg/kg body weight. None of these concentrations demonstrated toxicity. Based on this *Aloe ferox* may be safe for use as an oral remedy for constipation [35].

Aloe ferox

Therapeutic area	Type of extract	Positive/Negative results
Cardiovascular disease	Phytosterols derived from *Aloe ferox* [30]	Positive
Wound healing properties	Glycoproteins which are components of *Aloe ferox* [32,33]	Positive
Croton oil induced Oedema	Two constituents of *Aloe ferox* namely aloeresin 1 and aloesin [34]	Positive

Differences in chemical composition of *Aloe ferox* and *Aloe vera*

Literature has demonstrated these differences: [29],[36]

Aloe ferox produce approximately 20 times more bitter sap than *Aloe vera*

Aloe ferox leaves contain more amino acid content than *Aloe vera*

Aloe vera loses its viscosity much more rapidly after extraction than that of *Aloe ferox*

Thus *Aloe ferox* and *Aloe vera* have different reported chemical and physical properties.

Preparation of *Aloe ferox* and *Aloe vera* extracts differed from most of the studies reported using these two Aloe species. In this study the bitter juice was extracted from the leaf derived products so that the preparations could be used orally without the laxative side effects.

This study was the first reported study done on *Aloe ferox* and *Aloe vera* preparations with the bitter juice removed from the test products. Whole leaf juice extract and solvent extracted gel powder of *Aloe ferox* and *Aloe vera* were used.

Whole leaf juice: Juice obtained from the original leaves with bitter juice removed by washing with water and the leaf gel then homogenised in food processor after which a small amount of citric acid was added for stabilisation.

Solvent extracted gel powder: Was prepared by processing the liquid from the inner fleshy part of the leaf that is rich in polysaccharides and other plant secondary metabolites. Excess methanol was added to remove all the bitter components from *Aloe ferox* whereas

isopropanol was used for the *Aloe vera* product as precipitation of the polysaccharides using methanol did not result in an insoluble product.

1.2. Aim

To investigate cytotoxic, potential wound healing properties, antimicrobial and antioxidant properties of bitter component free whole leaf juice and solvent extracted gel powder of *Aloe ferox* and *Aloe vera*.

1.3. Objectives

The objectives of this study were:

1. To test bitter component free whole leaf juice and solvent extracted gel powders of *Aloe ferox* and *Aloe vera* on human lymphocyte cell cultures to determine either cytotoxicity or stimulation of proliferation of human lymphocytes.

2. To test bitter component free whole leaf juice and solvent extracted gel powder of *Aloe ferox a*nd *Aloe vera* on primary cultures of chicken fibroblasts to determine either cytotoxicity or stimulation of fibroblasts.

3. To test bitter component free whole leaf juice and solvent extracted gel powders of *Aloe ferox* and *Aloe vera* extract for antimicrobial properties with respect to *Staphyloccoccus aureus, Candida albicans* and *Pseudomonas aeruginosa* to determine if they might control infections in wounds.

4. To test bitter component free whole leaf juice and solvent extracted gel powders of *Aloe ferox a*nd *Aloe vera* for free radical scavenging properties.

Chapter 2

In vitro cytotoxicity assessment using human lymphocytes

2.1. Introduction

Lymphocytes are white blood cells involved in the innate and cellular immune responses that fight off infection in the body. They are activated when they encounter an antigen [37] . The process of lymphocyte formation is called lymphopoiesis [38]. Lymphocytes attack cells invaded by foreign substances or vectors (e.g T cytotoxic cells), or aid other components of the immune system (e.g T helper cells). Inhibition of lymphocyte proliferation and function would have a direct effect on the inflammatory response [38;39] .
Three distinct types of lymphocytes are: natural killer cells, B cells and T cells. Natural killer cells destroy tumour cells and virally infected cells. They are activated in response to inteferons (cytokines) [37]. T Helper cells are involved in transferring antigen information to B lymphocytes where antigen specific antibodies are produced. The activated B cells express antibody in response to a second infection by a specific antigen encoded by that B lymphocyte. In general T cells mature in the thymus and B cells mature in the bone marrow [38].

Aloe vera and *Aloe ferox* both contain glycoproteins as a component in their leaf gels [14;40] and glycoproteins have been reported to stimulate cell growth [41].

This study was done to confirm whether whole leaf juice and solvent extracted gel powder from *Aloe ferox* and *Aloe vera* stimulate or inhibit healthy human lymphocytes at different concentrations.

Currently, no studies have been reported where extracts from *Aloe ferox* and *Aloe vera* whole leaf juice or whole leaf gel were tested for cytotoxicity on lymphocytes.

In this study, bitter component free whole leaf juice and the solvent extracted gel powders of the two Aloe species were tested on both resting and PMA stimulated lymphocytes.
The resting lymphocytes represent normal lymphocytes in the body before activation by an infection. The stimulated lymphocytes represent lymphocytes in the body during an infection or mitogenic stimulus.

2.2. Aim:

To determine whether water extract of bitter component free whole leaf juice and solvent extracted gel powders from *Aloe ferox* and *Aloe vera* inhibit or stimulate the growth of isolated human lymphocytes.

2.3. Materials and Methods

Initial preparation of different Aloe extracts

The plant extracts was obtained from Alcare Company in Albertina which is located in the Western cape in South Africa.
The products from these preparation steps were used in the different experimental procedures during this study.

Test samples from both plants were prepared in a similar way except for the solvent used in the final removal of the bitter components.

1. **Whole leaf juice (*Aloe ferox* and *Aloe vera*)**

Leaves were cut and skinned (peeled) to remove the rind where most of the bitter compounds are found. The gel (parenchyma) was rinsed in deionised water to remove traces of bitter juice.
Leaf gel was homogenised in a food processor and citric acid added as a stabiliser.
Gel was course filtered through a 1 mm sizing sieve to remove all remaining solids, then filtrate was aliquoted into 25 ml cubes and frozen at -20°C overnight. Frozen filtrate was then freeze dried and the dried material stored at -20°C until used.
Prior to being used in an experiment known weights of the powder were added to deionised water and the suspension sonicated in a water bath to ensure solubilisation. These solutions were filter sterilised through 0.2 µm filters for use in cell culture work.
All solutions used for experiments were assayed gravimetrically for dry mass concentration before being used in experimental procedures.

2. **Solvent extracted gel powder of *Aloe ferox***

Leaves were weighed then scored and tapped to remove the bitter sap, tapped leaves were shredded and pressed to obtain the highest yields of leaf juice. Leaf juice was collected into large clean containers and the volume determined.
Three parts of methanol were added to the liquid *Aloe ferox* leaf juice and mixed well to ensure complete mixing, then allowed to settle. The supernatant was decanted and the precipitated pulp was further centrifuged at low g force to isolate the precipitate. The white precipitate was resuspended in methanol and recentrifuged three times or until the methanol was colourless to ensure complete removal of the bitter components.

Solvent extracted gel powder of *Aloe vera*

This was prepared the same way as for *Aloe ferox* described above except that isopropanol was used instead of methanol because precipitation with methanol resulted in a thick gel rather than a precipitate.

Both alcohol extracted gel powders were air dried completely to ensure complete evaporation of the alcohol from the powders to avoid that these solvents affected results when using these extracted gel powders.

The plant extracts were prepared in three different ways.

A. First Preparation

For each extract, 250 mg dried powder was added to 50 ml deionised water. The tube with the mixture was then, sonicated for 1 hour, incubated for 1 hour at 60°C, sonicated for 1 hour again, centrifuged at 800 *g* for 30 min and supernatant was collected into a clean tube.

The dry mass of each extract was determined gravimetrically. Empty pre-marked and oven dried petri dishes were weighed. For each extract, three replicates of 1, 0 ml were placed into dried and weighed pre-marked petri dishes. They were left to dry overnight in an oven at 90°C. Petri dishes were cooled in a desiccator with dry silica gel and weighed again to determine the weight difference (which gives the mass of the solids in each extract) between the empty petri dishes and petri dishes with 1, 0 ml of each extract.

Concentrations of extract used for experimental procedures

Whole leaf juice: 37.5 µg/ml, 75 µg/ml, 150 µg/ml and 300 µg/ml

Gel powder: 25 µg/ml, 50 µg/ml, 100 µg/ml and 200 µg/ml

B. Second preparation

Similar in all ways to the first preparation except that the mixture was not incubated at 60°C for 1 hour and only sonicated for 2 hours then centrifuged at 800 *g* for 30 min

Concentrations tested

Whole leaf juice: 2.0 µg/ml, 4 µg/ml, 8 µg/ml, 16 µg/ml, 31.3 µg/ml, 62.5 µg/ml, 125 µg/ml and 250 µg/ml

Solvent extracted powder: 0.4 µg/ml, 0.8 µg/ml, 1.6 µg/ml, 3.13 µg/ml, 6.25 µg/ml, 12.5 µg/ml, 25 µg/ml and 50 µg/ml.

Lymphocyte preparation

Heparinized peripheral blood from a healthy adult human volunteer was overlaid onto 15 ml Histopaque 1077, this was then centrifuged for 25 min at 625 *g* at room temperature. The top plasma layer was removed. Visible lymphocytes layer were transferred to sterile 50 ml tubes and filled the tubes with sterile RPMI- 1640 medium and then centrifuged for 15 min at 200 *g* (to remove contaminating platelets). Supernatant fluid was discarded, filled with sterile RPMI-1640 and centrifuged for 10 min at 200 *g*. Discarded supernatant fluid, filled with sterile, ice cold 0.83% ammonium chloride lysing solution and left for ±10 min on ice and centrifuged for 10 min at 200 *g*. Discarded supernatant, filled up with RPMI-1640 and centrifuged for 10 min at 200 *g* then supernant was discarded. Exactly 1 ml RPMI supplemented with 10% FCS (RPMI+) was resuspended.

Counting of cells

An aliquot of 50 µl of the cell suspension prepared as described above was added to 450 µl of white cell counting fluid and mixed well. A small volume of suspension was put onto a Haemocytometer and the cells were counted using a Reichert Jung Micro Star microscope.
Dilutions of the cell suspension were then made with the tissue culture medium supplemented with 10% bovine FCS to achieve the required cell concentration for the cell proliferation assay.

Cells were plated into 96 well culture plates and allowed to acclimatise for 1 hour. A set of untreated cells were accommodated for both the resting and the stimulated lymphocytes. The different concentrations of the test compounds were added from the stock of each to give final concentrations in the wells as follows: 2 µg/ml, 4 µg/ml, 8 µg/ml, 16 µg/ml, 31.3 µg/ml, 62.5 µg/ml, 125 µg/ml and 250 µg/ml for the whole leaf juice preparations and 0.4 µg/ml, 0.8 µg/ml, 1.6 µg/ml, 3.13 µg/ml, 6.25 µg/ml, 12.5 µg/ml, 25 µg/ml and 50 µg/ml for the gel powders.
The stimulated cells were then treated with 20 µl PHA giving a final concentration of 10 µg/ml while the resting cells had the equivalent volume of media only added.
The lymphocytes were then incubated at 37˚C in an atmosphere of 5% CO_2 with relative humidity of 95% for 72 hours.

After 3 days of incubation the cells were assayed using the MTT (3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide) assay to determine their cell number compared to untreated control cultures to assess cell sensitivity to the Aloe extracts.
The controls were not treated with any of the plant extract.

MTT staining principle

The MTT assay is a colorimetric assay that measures the activity of the enzyme that reduce MTT to formazan dyes. This assay is used to determine the viability, proliferation of cells and may also be used to determine cytotoxicity of different agents on particular cells. MTT is changed to a purple formazan in active living cells [42]

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) cell enumeration method.

Exactly 20 µl of a MTT solution (5 mg/ml in PBS) was added to each well. The plate was re-incubated for $3^1/_2$ - 4 hrs at 37°C in a 5% CO_2 incubator, then centrifuged at 400 *g* for 10 min and supernatant was removed without disturbing the pellet.
Washed pellets twice with 150 µl phosphate buffered saline (PBS) centrifuging the cells into a compact pellet each time. After the final wash the plates were allowed to dry for a few hours before the next step. Exactly100 µl DMSO was added and shaken gently for ± 1 hr on a rotary shaker and then measured spectrophotometrically at a wavelength of 570 nm using a reference wavelength of 630 nm. Each experiment was repeated at least three times.

2.4. Results

According to the results summarised in the figures below, whole leaf juice and solvent extracted gel powder inhibit lymphocytes proliferation as the concentration increases following a dose related response.
For all the graphs with regards testing the 4 samples on resting and stimulated lymphocytes, two way ANOVA statistical tests were used to determine statistical difference from results obtained. IC_{50} could not be obtained from all results.

First preparation

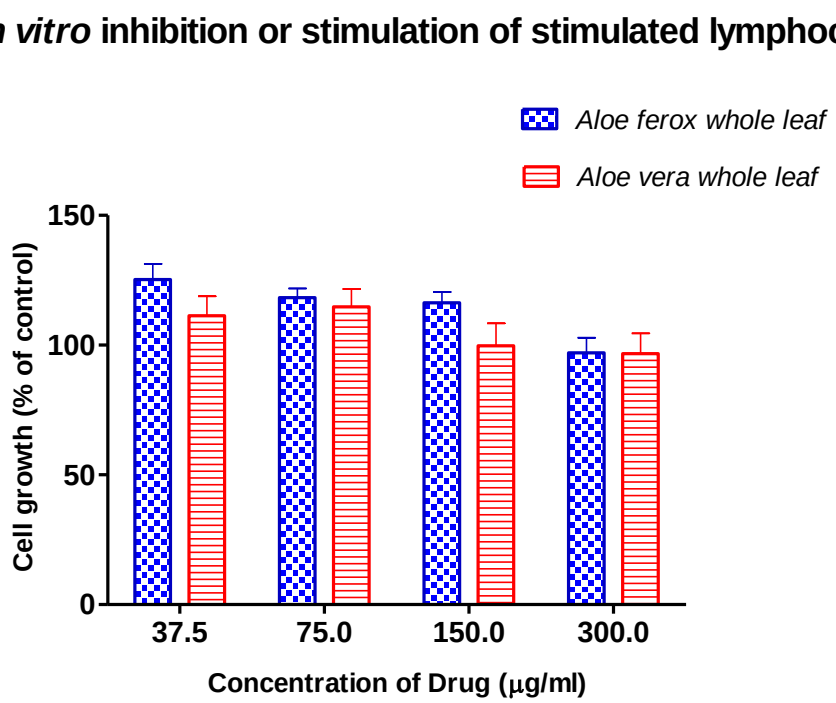


Figure 2.5.1: Whole leaf juice of *Aloe vera* and *Aloe ferox* on stimulated lymphocytes

Whole leaf juice of *Aloe vera* did not have any significant effect on inhibition or lymphocytes proliferation on stimulated lymphocytes, whereas whole leaf juice of *Aloe ferox* slightly inhibited stimulated lymphocytes as the concentration increased.

In vitro inhibition or stimulation of resting lymphocytes

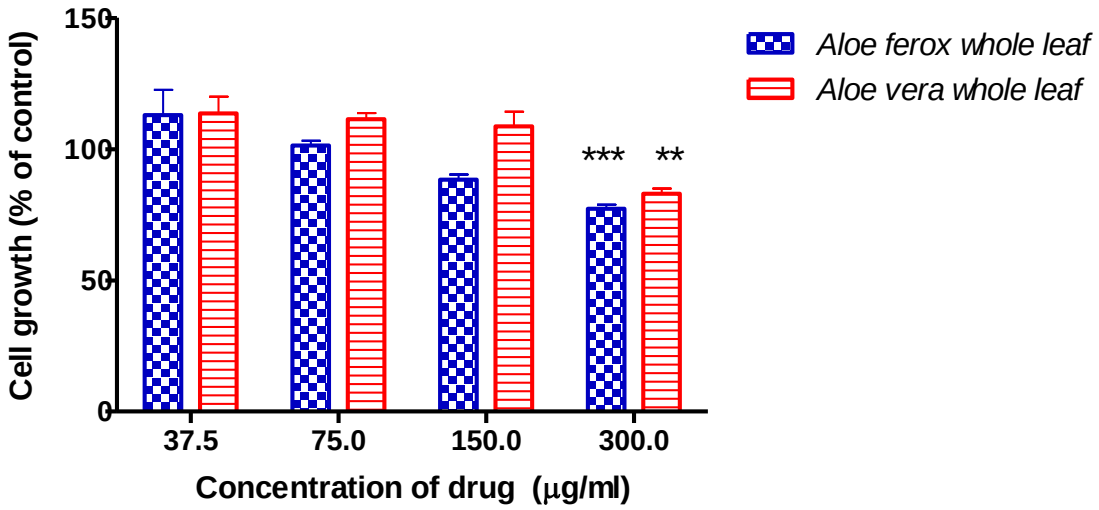


Figure 2.5.2: Whole leaf juice of *Aloe vera* and *Aloe ferox* on resting lymphocytes

P<0.001 = ***

P<0.01 = **

Whole leaf juice of both the Aloe species inhibited resting lymphocytes as the concentration increased following a dose related response.

In vitro inhibition or stimulation of stimulated lymphocytes

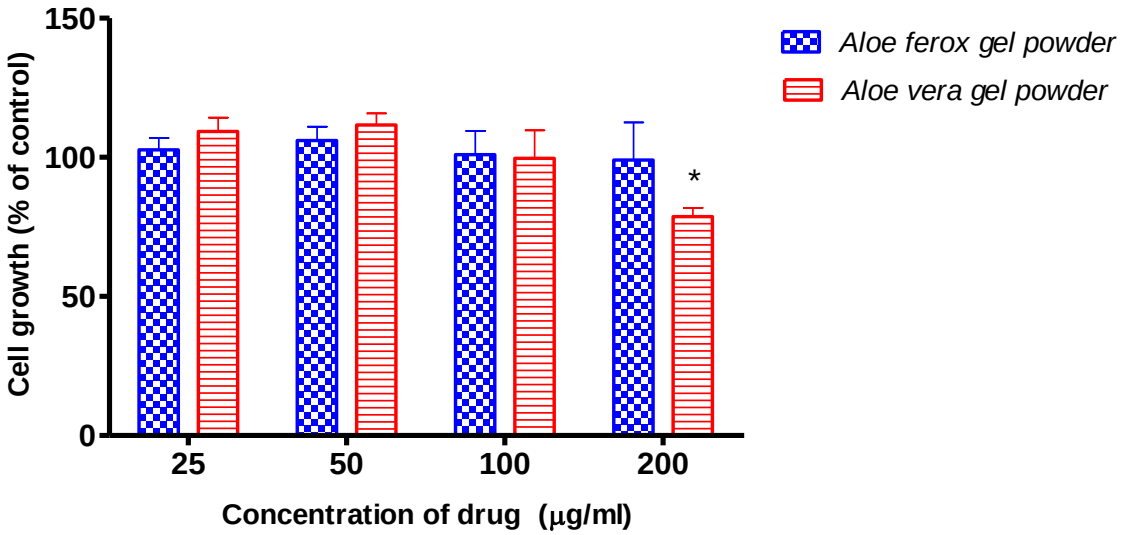


Figure 2.5.3: Solvent extracted gel powder of *Aloe vera* and *Aloe ferox* on stimulated lymphocytes

P<0.05 = *

Solvent extracted gel powder of *Aloe vera* inhibited stimulated lymphocytes as the concentration increased, whereas solvent extracted gel powder of *Aloe ferox* had no significant effect on inhibition or lymphocytes proliferation on stimulated lymphocytes. Only the highest concentration of *Aloe vera* showed a significant reduction in proliferation of the stimulated lymphocytes.

***In vitro* inhibition or stimulation of resting lymphocytes**

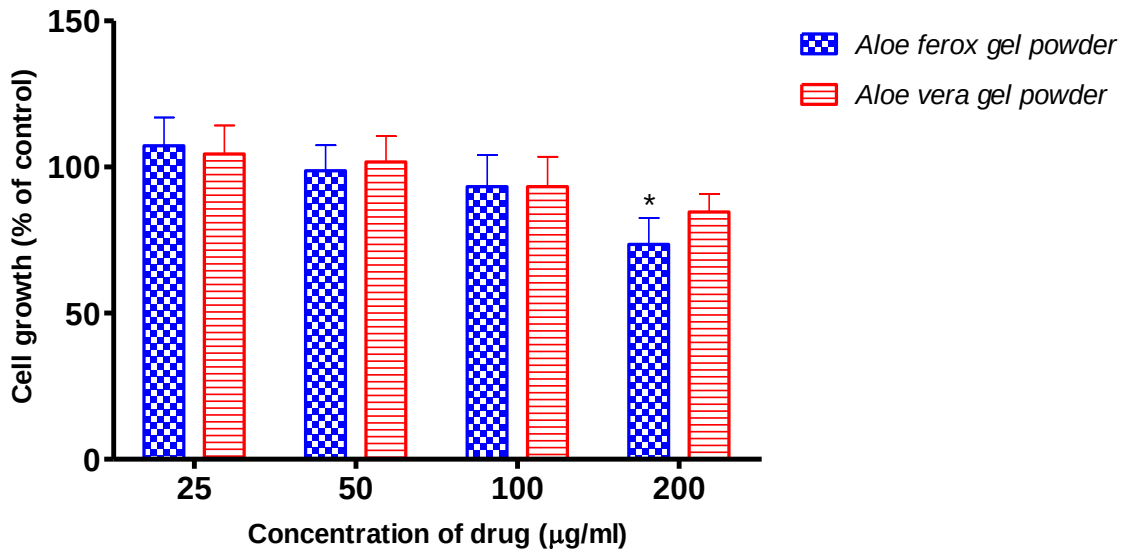


Figure 2.5.4: Solvent extracted gel powder of *Aloe vera* and *Aloe ferox* on resting lymphocytes

P < 0.05=*

Solvent extracted gel powder of *Aloe ferox* inhibited resting lymphocytes slightly as the concentration increased, whereas solvent extracted gel powder of *Aloe vera* had no significant effect on inhibition or lymphocytes proliferation on resting lymphocytes.

According to the assays summarised by graphs (Figure 2.5.1 to Figure 2.5.4) whole leaf juice and solvent extracted gel powder solution for the two Aloe species inhibited lymphocytes significantly only at the highest concentrations tested which are above the physiologically attainable levels. No significant inhibitory effect was observed on either stimulated or resting lymphocytes at lower concentrations.

Second preparation

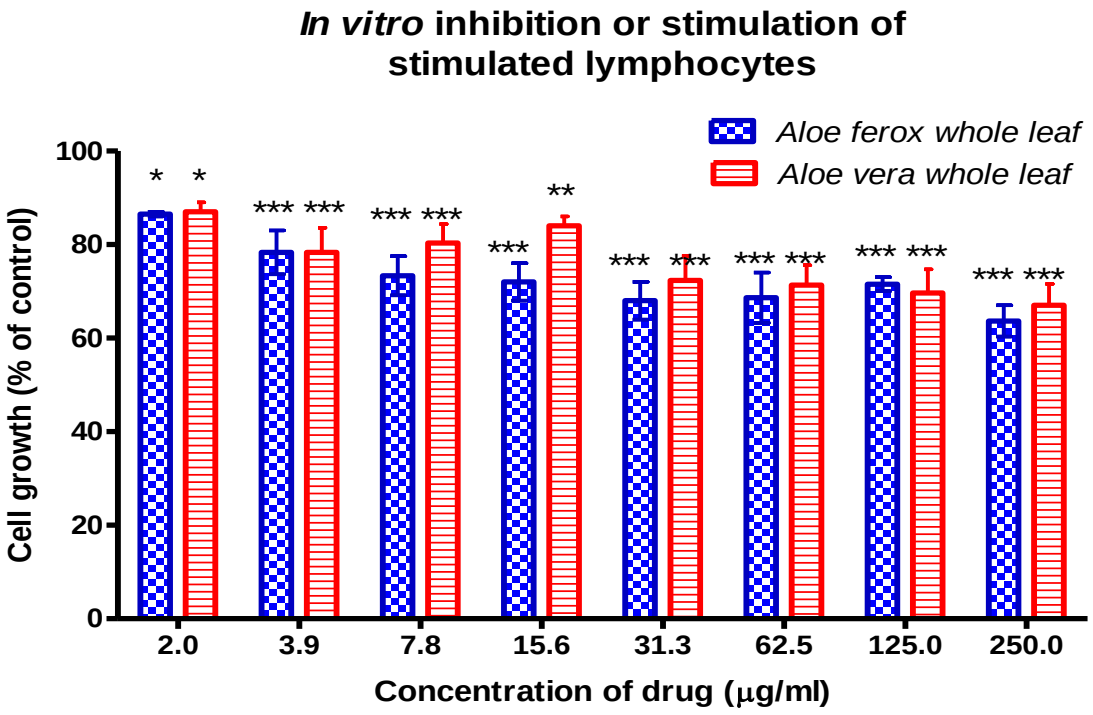


Figure 2.5.5: Whole leaf juice of *Aloe vera* and *Aloe ferox* on stimulated lymphocytes

P < 0.001= ***
P < 0.01 = **
P < 0.05 = *

Whole leaf juice of both *Aloe ferox* and *Aloe vera* significantly inhibited proliferation of stimulated lymphocytes as the concentration increased,

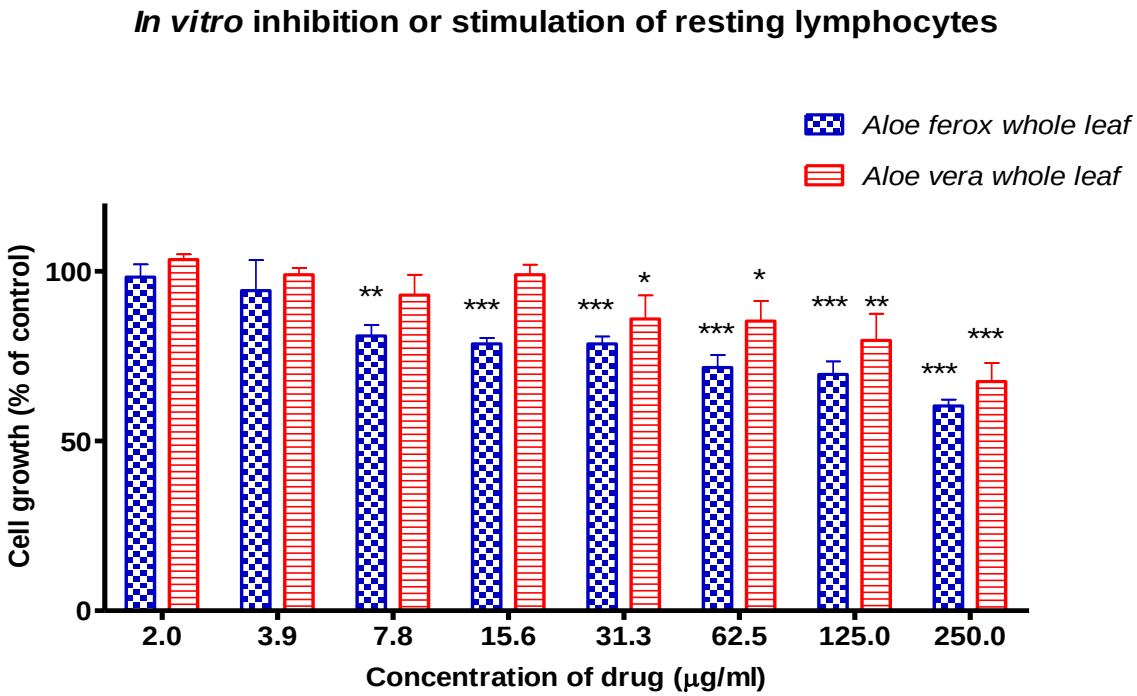


Figure 2.5.6: Whole leaf juice of *Aloe vera* and *Aloe ferox* on resting lymphocytes

P < 0.001= ***
P < 0.01 = **
P < 0.05 = *

Whole leaf juice of both *Aloe ferox* and *Aloe vera* statistically significantly inhibited resting lymphocytes at the highest concentrations with a distinct dose related response showing the typical trend of increasing inhibition as the concentration increased, however whole leaf juice of *Aloe ferox* seems to be more cytotoxic than whole leaf juice of *Aloe vera*.

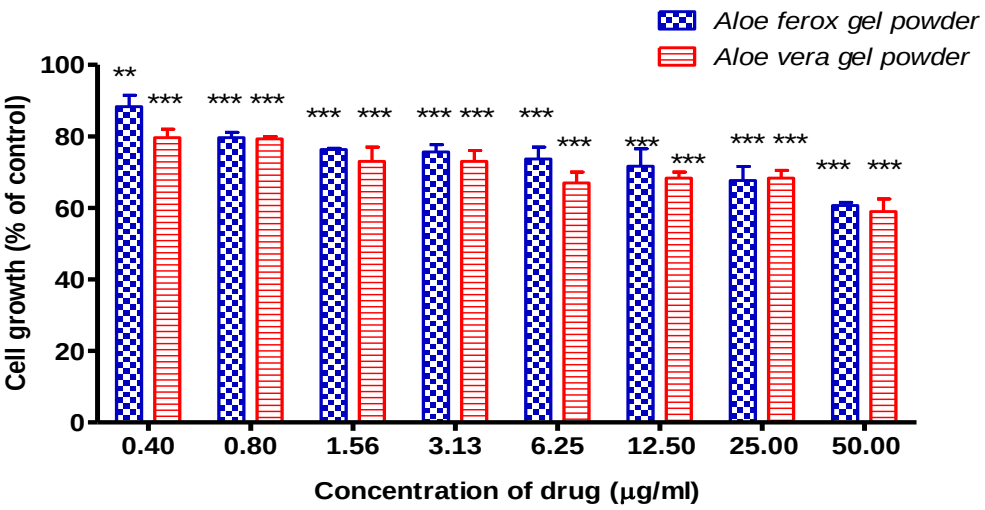


Figure 2.5.7: Solvent extracted gel powder of *Aloe vera* and *Aloe ferox* on stimulated lymphocytes

P < 0.001= ***
P < 0.01 = **
P < 0.05 = *

Solvent extracted gel powder of both *Aloe ferox* and *Aloe vera* showed a highly significant inhibition of the proliferation of stimulated lymphocytes at all concentrations tested.

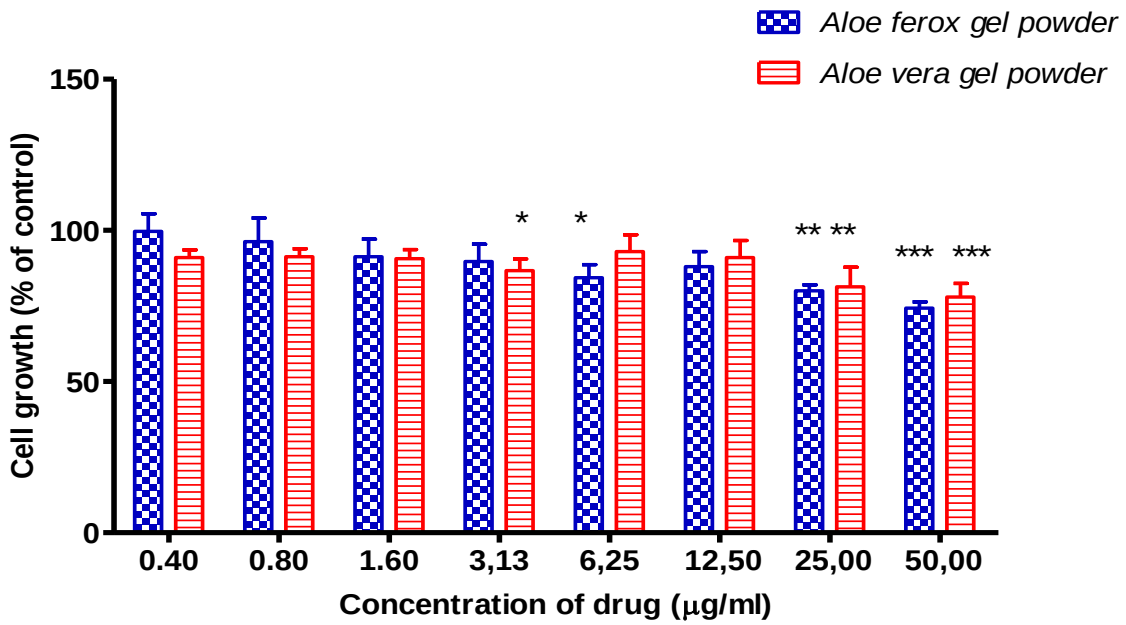


Figure 2.5.8: Solvent extracted gel powder of *Aloe vera* and *Aloe ferox* on resting lymphocytes

P < 0.001= ***
P < 0.01 = **
P < 0.05 = *

Solvent extracted gel powder of both *Aloe ferox* and *Aloe vera* inhibited proliferation of resting lymphocytes in culture at the highest concentrations tested to a statistically significant amount. Thus solvent extracted gel powder of both the two Aloe species does exhibit an inhibitory effect on resting lymphocyte proliferation under conditions of *in vitro* culture.

When comparing of effects of the four samples on resting and stimulated lymphocytes in culture, it is evident that whole leaf juice and gel powder solution of the two aloe species has a greater effect on stimulated lymphocytes by decreasing lymphocytes proliferation compared to equivalent cells grown under identical conditions without PHA stimulation. Despite the differences being statistically different the observed effects were reasonably small.

2.5. Discussion

First preparation

Whole leaf juice and solvent extracted gel powder from both Aloe species have inhibited proliferation slightly in either resting or stimulated human lymphocytes at which the highest concentration tested showed statistically significant inhibition.

Second preparation

Whole leaf juice and solvent extracted gel powder from both the Aloe species did show a significant inhibitory effects of both stimulated and resting lymphocytes in a distinct dose related response with increasing inhibition as the concentration of the tested extracts increased.

The resting lymphocytes represent normal lymphocytes in the body before activation by an infection. The stimulated lymphocytes represent activated lymphocytes in the body as would be found during an infection. This might imply that when there is no infection the extracts of the two Aloe species will affect lymphocytes and they would exhibit an inhibitory effect their proliferation. Potentially the effect during an infection or inflammatory response would be to inhibit the lymphocyte proliferation and would not stimulate them to divide which is a desired effect to assist in fighting off the infection.

Based on the above results, it can be concluded that whole leaf juice and solvent extracted gel powder of *Aloe ferox* and *Aloe vera* that have been prepared to specifically exclude the bitter juice compounds have a significant inhibitory effect on both resting and stimulated human lymphocytes in culture. Currently no studies have been reported with respect to the effects of Aloe extracts from *Aloe ferox* or *Aloe vera* on isolated human lymphocytes in culture.

According to the literature different components *Aloe vera* (aloesin, mannose-6-phosphate, glycoproteins, aloe-emodin, lectin-like substances) are proven to have growth promoting factors as they have demonstrated proliferation of several cells including normal foetal lung cells (*in vitro*), normal dermal and baby hamster kidney cells (*in vitro*), rat adrenal cells (*in vivo*), calf pulmonary artery endothelial cells (*in vitro*) and squamous carcinoma cells (*in vitro*) [43]. The same constituents are also contained in *Aloe ferox* and although fewer experiments were done using *Aloe ferox* components, it can be assumed that the activity should be the same but attenuated according to the difference in the concentrations of the active compounds. This is contrary to our study as inhibitory effects of both the Aloe species on the different preparations have been demonstrated. Thus components lost during the preparation of the extracts (as some of the active components commonly mentioned for Aloe are part of the bitter components like aloesin, aloe-emodin), could be the reason for the contradictory effects seen during this study when cultured lymphocytes were being assessed.

According to Winters *et al* [44] fresh *Aloe vera* juice has been found to contain a high level of lectin-like substances and this is believed to have contributed to the enhancement of growth of human normal foetal lung cells in tissue cultures. In this experiment lectin-like substances could have been removed with the bitter juice, or during the filtration process required to render the samples sterile for cell culture applications thus the remaining sample may have had too low lectin-like concentrations to have any proliferative effect on lymphocytes.

From the graphs, it is evident that first preparation whereby the extracts were incubated for an hour at 60°C did not show any effect or showed a lower effect (particularly at the highest concentration), whereas second preparation whereby the extracts were not incubated at elevated temperature showed a highly significant inhibitory effect at most of the concentrations used. In addition, concentrations tested from first preparation are higher than those of second preparation, however a greater inhibitory effect has been noted for the extracts made using the second preparation. Thus during the incubation process for the first preparation some components which contribute to the inhibitory properties could have been destroyed.

From the results, it is also evident that at low concentrations tested (for both first and second preparation) there was less inhibitory effect or no effect at all on tested human lymphocytes,

however as the concentration increased, inhibitory effects also increased. Thus it becomes more evident that during preparation process for all the samples used for this experiment, some components which are responsible lymphocyte proliferation could have been removed and perhaps the toxic components left are enhanced in their activity.

2.6. Conclusion

Water extracts of whole leaf juice and solvent extracted leaf gel powder from *Aloe ferox* and *Aloe vera* prepared without bitter components have significant inhibitory effect on resting lymphocytes and stimulated lymphocytes. However inhibitory effects have been more evident on stimulated lymphocytes than resting lymphocytes.

Chapter 3

In vitro Cytotoxicity – Chicken Fibroblasts

3.1. Introduction

Wound healing is the process in which the skin repairs itself after an injury which could be internal or external [45]. Wounds may be caused by many factors some of them being cuts, disease such as sugar diabetes and infections. Repair of the injured tissues is a complex process which includes inflammation, migration of different cell types and proliferation [46]. During the inflammation phase, invading bacteria are phagocytised prior to the cell proliferation phase when cell division occurs and which is characterized by angiogenesis, collagen deposition, granulation tissue formation, wound contraction and re-epithelialisation [47].

Fibroblasts are structural cells that express growth factors, which cause proliferation and differentiation of a wide variety of other cells and tissues. They are involved in wound healing through the production of collagen and extra cellular matrix [48] and are responsible for the wound contraction due to cell activity and the newly formed connective tissue to bring together the edges of the wound [49].

Aloe vera prepared by different methods has been proven to have wound healing properties [32;33;50;51]. It is believed that the wound healing properties of *Aloe vera* is due to its ability to increase the collagen content of the granular tissue which is the major protein of the extracellular matrix and is the component which contributes to wound strength. This was observed after both topical and oral administration of *Aloe vera* water extract [32;33]. Compared to the conventional treatments, *Aloe vera* has been proven to increase the rate of healing and the rate of re-epithelialisation in first and second degree burns. This was reported in a review where four studies with a total of 371 participants were included [51].

The glycoprotein isolated from *Aloe vera* has been shown to stimulate growth of human normal dermal and baby hamster kidney cells *in vitro* [41]. This could be explained by reports that indicate that glycoproteins have healing properties [52].

Acemannan containing hydrogel wound dressings derived from *Aloe vera* is approved by the Food and Drug Administration for the management of Stages I through IV pressure ulcers after the study evidenced complete healing of ulcers in 19 of 30 subjects during the 10 week observed period [53].

Aloe vera water extract has been proven to stimulate proliferation of diabetic fibroblasts, hence it has a positive impact on wound healing. *Aloe vera* may contain growth promoting factors, based on the fact that its extracts or components (e.g. aloesin, mannose-6-phosphate, glycoproteins, aloe-emodin, lectin-like substances) have been proven to stimulate *in vitro* proliferation of several cell types including rat adrenal cells, leukocytes, calf pulmonary artery endothelial cells etc [54].

Whole leaf juice of *Aloe ferox* and *Aloe arborescens* have been found to improve wound healing when applied topically to rat and rabbit wound models [55]. One of the components of *Aloe ferox* which is a glycoprotein is thought to be responsible for the healing properties due to its ability to increase the collagen matrix content of the granular tissue [32;33;40].

3.2. Aim

To determine whether water extract of bitter component free whole leaf gel and solvent extracted gel powders from *Aloe vera* and *Aloe ferox* stimulate or inhibit growth of chicken fibroblasts in culture.

3.3. Materials and methods

The plant products were prepared in two different ways

First preparation

For each extract, 250 mg was dissolved in 50 ml of water then sonicated for 2 hours, centrifuged at 800 *g* for 30 min and then filter sterilised.

Second preparation

For each extract, 250 mg was dissolved in 50 ml of water then sonicated for 2 hours, centrifuged at 800 *g* for 30 min and γ irradiated (for sterilization purposes)

Concentrations used for all three preparations:

Whole leaf juice: 2.0 µg/ml, 4 µg/ml, 8 µg/ml, 16 µg/ml, 31.3 µg/ml, 62.5 µg/ml, 125 µg/ml and 250 µg/ml

Gel powder juice: 0.4 µg/ml, 0.8 µg/ml, 1.6 µg/ml, 3.13 µg/ml, 6.25 µg/ml, 12.5 µg/ml, 25 µg/ml and 50 µg/ml

Growing chicken fibroblasts

Fertilized eggs were incubated at 37°C in a humid atmosphere for 6-10 days.

All procedures were carried out under sterile conditions, the eggs were cleaned with 70 % ethanol and placed with its blunt end facing up. With sterile forceps, the top of the egg was cracked, opened and the embryo removed, placed in a sterilized Petri-dish and decapitated.

The forceps were re-sterilized and used to remove fat and necrotic material. Embryos were cut with crossed scalpels into very fine pieces and transferred to a sterile 50 ml tube, the fine pieces were washed twice with RPMI medium (containing 10% FCS and antibiotics). Tissue was allowed to settle before the medium was discarded.

Trypsin solution was diluted to 1/10 using pre-warmed PBS (concentration of 9.2 mg/ml), 50 ml tube containing the pieces of the embryo was filled with 1/10 trypsin solution. The tube was left on the shaker for 7, 14 and 20 min and the suspension was left to settle for a minute and the typsin solution containing the detached cells were transferred into another 50 ml tube and filled with RPMI. The tube was centrifuged for 5 min at 200 *g* and supernatant was removed and the cells were resuspended in 1 ml of RPMI containing 10% FCS.

The 1 ml suspension was placed in a 25 cm^2 culture flask and 14 ml of medium added.

Cells were incubated for 8 hours before gently agitating the media to suspend non-attached cells and the media removed and replaced with new media. The cells were then cultured until 80% confluence was reached.

Detaching chicken fibroblasts from the flask

The medium was removed by decanting. The flask containing medium was briefly rinsed with 3 ml of trypsin/versene solution and then 5 ml of typsin/versene solution was added to the flask and incubated at 37°C until the cells detached (approximately 10 min).

Detached cells were transferred into a 15 ml tube and the tube was filled with fresh culture medium containing 10% FCS and then centrifuged for 5 min at 200 *g*, supernatant was removed and the pellet was re-suspended in 1 ml RPMI medium.
The concentrations of cells were counted by adding 50 µl of the cell suspension to 450 µl of counting fluid and determining the cell concentration by haemocytometer.
The cells were diluted to appropriate concentrations for the cytotoxic assays

Experimental procedure

Round-bottomed 96 well sterile tissue cultures plates were used and eighty microlitres of medium was added to all the wells. One hundred microlitres of cell suspension (ranging from 2 x 10^5/ ml → 0.5 x 10^5/ ml) was added to all wells and cells were incubated for 60 min at 37°C.
Twenty microlitres of a respective Aloe extract was added to the experimental wells and 20 µl medium to the control wells. The plates were incubated for 3 days at 37°C in a 5% CO2 atmosphere in an incubator at 95% relative humidity.
After 72 hours incubation, MTT solution was added to all wells and the plates further incubated for 3 – 4 hours. After washing the cells twice with PBS the plates were allowed to dry for at least two hours before adding 100 µl of DMSO and shaking the plate for about an hour to solubilise the coloured formazan. The plate was then measured at 570 nm and corrected using a reference wavelength of 630 nm.

3.4. Results

Based on all the concentrations tested, whole leaf juice and solvent extracted gel powder showed inhibitory properties of chicken fibroblasts in a dose related manner with inhibition increasing as the extract concentration increased. For all the graphs with regards samples tested on chicken fibroblasts, Wilkosein Friedman statistical tests were used on the obtained results to determine statistical significance.
IC_{50} could not be obtained from any results. For the control, chicken fibroblasts were not treated with any plant extract. The control is 100% growth, thus a decrease in growth after treating cells with extracts demonstrate cell inhibition.

First Preparation

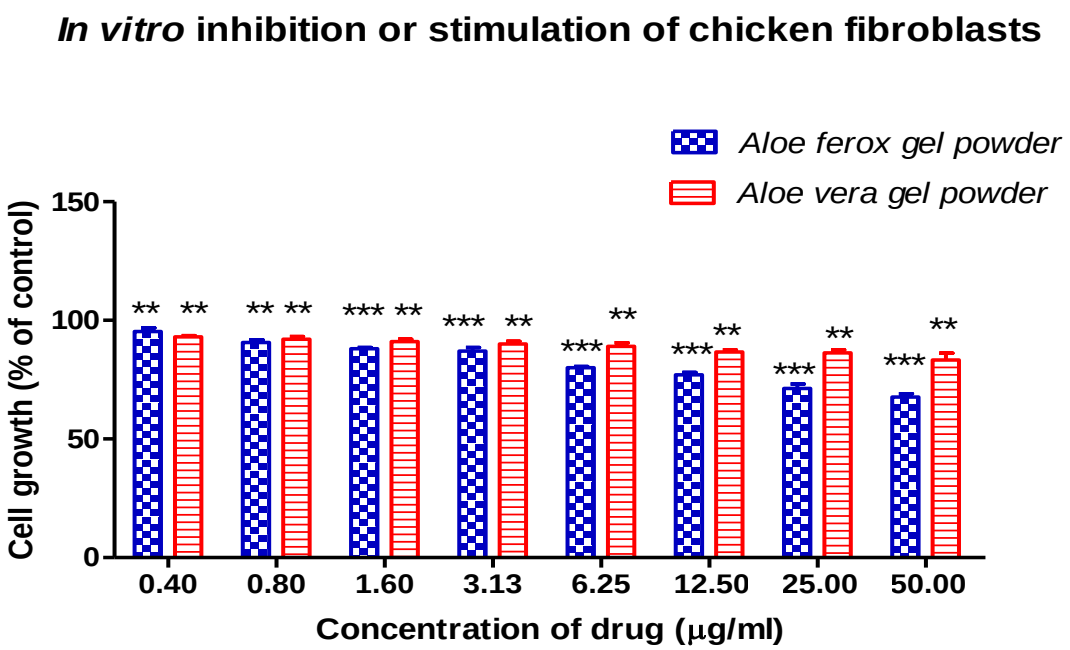


Figure 3.4.1: Solvent extracted gel powder of *Aloe vera* and *Aloe ferox* on chicken fibroblasts

P < 0.001= ***
P < 0.01 = **
P < 0.05 = *

Solvent extracted gel powder of *Aloe ferox* and *Aloe vera* showed a significant inhibitory effect on chicken fibroblasts at all the concentrations tested.

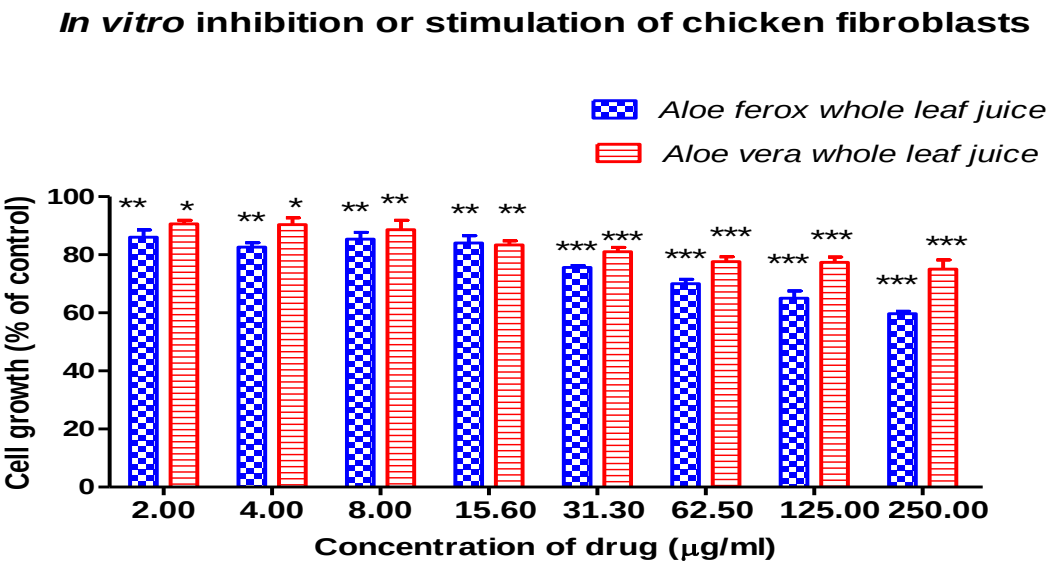


Figure 3.4.2: Whole leaf juice of *Aloe ferox* and *Aloe vera* on chicken fibroblasts

P < 0.001= ***
P < 0.01 = **
P < 0.05 = *

Whole leaf juice of *Aloe ferox* and *Aloe vera* showed a significant inhibitory effect on chicken fibroblasts on all the concentrations tested.

Second preparation

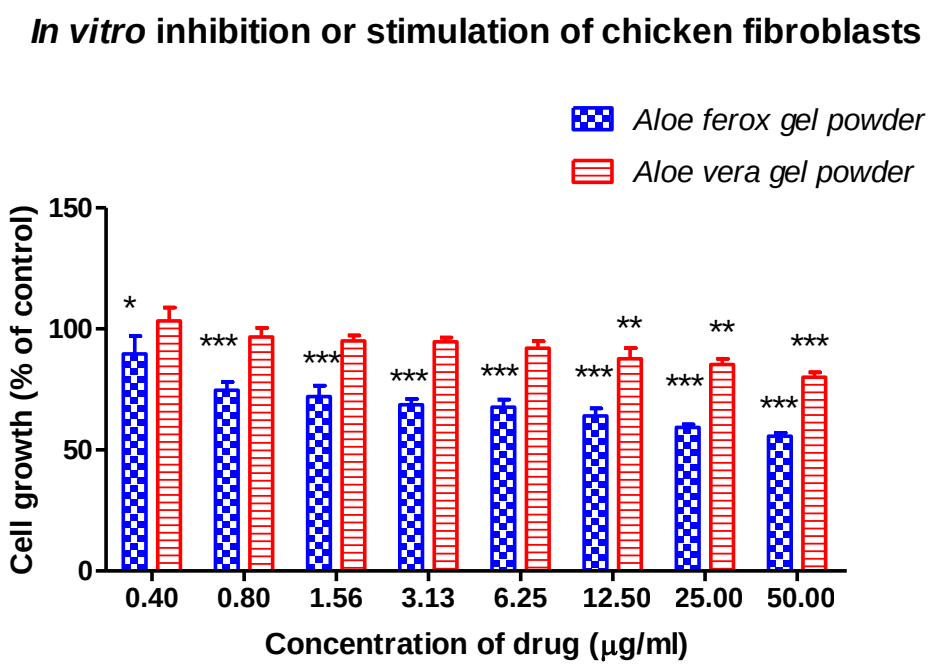


Figure 3.4.3: Effect of solvent extracted gel powder of *Aloe ferox* and *Aloe vera* on the proliferation of in vitro chicken fibroblast cultures compared to untreated control wells.

$P < 0.001$= ***
$P < 0.01$ = **
$P < 0.05$ = *

Solvent extracted gel powder of *Aloe ferox* showed very highly significant inhibitory effects at almost all concentrations tested, whereas solvent extracted gel powder of *Aloe vera* showed significant inhibitory effects only at the high concentrations tested.

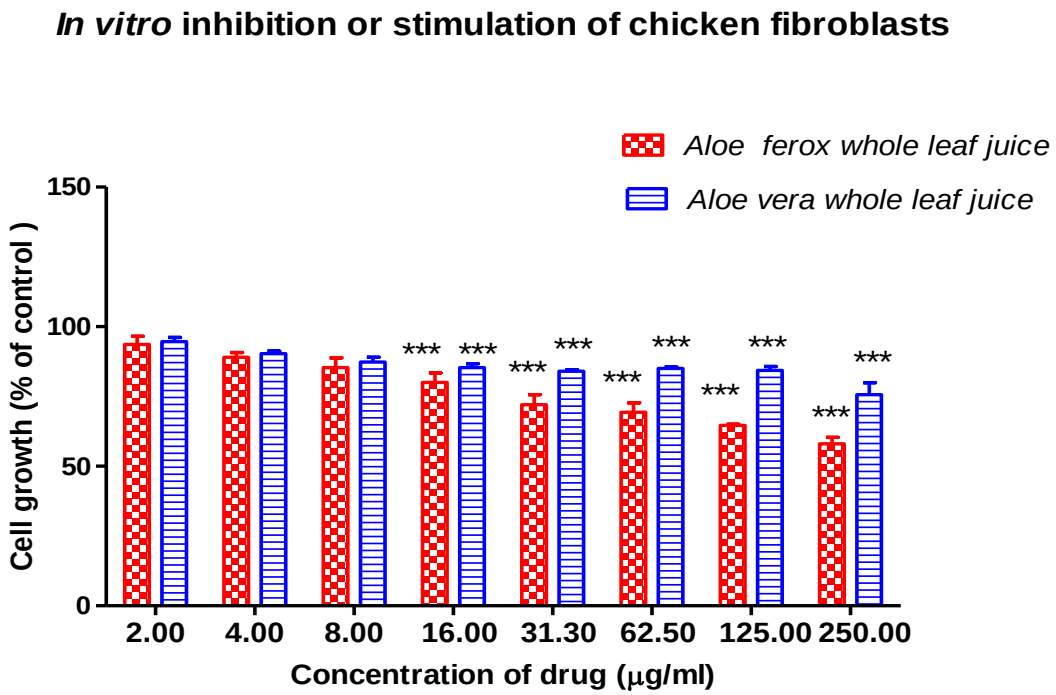


Figure 3.4.4: Whole leaf juice of *Aloe ferox* and *Aloe vera* on the proliferation of in vitro chicken fibroblast cultures compared to untreated control wells.

P < 0.001= ***
P < 0.01 = **
P < 0.05 = *

Whole leaf juice of *Aloe ferox* and *Aloe vera* has demonstrated a very highly significant inhibitory effect at almost all concentrations tested.

From all the 4 figures above it is evident that as the concentration increased for both whole leaf juice and solvent extracted gel powder from both Aloe species becomes increasingly cytotoxic. It was determined that the inhibition is statistically highly significant hence it is concluded that all four tested extract samples do have an inhibitory effect on chicken fibroblasts.

It is also evident that whole leaf juice and solvent extracted gel powder of *Aloe ferox* is more inhibitory than whole leaf juice and solvent extracted gel powder of *Aloe vera*, however it was determined that the difference between these two extracts is not statistically significant.

3.5. Discussion

According to the literature *Aloe vera* and *Aloe ferox* have excellent wound healing properties which implies a suitable environment for fibroblast proliferation [1, 10]. However according to the results obtained in this study, whole leaf gel and solvent extracted gel powder of *Aloe vera* and *Aloe ferox* water extract do not demonstrate any stimulating effect on cultured chicken fibroblasts, instead inhibitory effects have been demonstrated thus based on this, wound healing properties could not be confirmed.

However, Abdullah et al [54] reported *Aloe vera* (with bitter juice) stimulates proliferation of diabetic fibroblasts *in vitro.* This might mean that the different preparation methods of the *Aloe vera* extracts and products had an impact on the results reported in this study (other components could have been removed during the removal of bitter juice process). It has also been proven that the *Aloe vera* gel (with bitter juice) can be used to improve wound healing [56], thus removal of bitter components appears to have an influence on the wound healing results obtained. Furthermore water extract of *Aloe vera* has been proven to stimulate growth of rabbit kidney fibroblasts grown under tissue culture conditions[1].

Isolated components of *Aloe vera* have been shown to have growth promoting factors [54] e.g acemannan [57] and mannose-6-phosphate [58] that have demonstrated wound healing properties when applied topically on rats. Perhaps there is more efficacy in isolated component than in a crude extract, with many different unknown components, hence no proliferation of chicken fibroblast was observed using the test samples prepared to specifically remove the bitter components.

Fresh Aloe contains anthroquinones and polysaccharides, with the anthroquinones having cathartic effects and found in the bitter juice. The polysaccharides are reported to improve wound healing rates in a wound healing model [59]. As the anthroquinones were lost during the removal of bitter juice during the preparation of the samples used in this study, the effects of these compounds would not have been observed. Perhaps some active polysaccharide components were also lost during the bitter component removal process e.g. acemannan, aloeride, mannose and cellulosic glucose [36]. Furthermore it has been proven that the polysaccharides found in aloe gel are not stable, especially under stress conditions such as heat, the presence of acid and enzymatic activities [6]. Thus during the preparation phase,

some of the remaining components may have been destroyed and thus resulted in toxic compounds which could explain the inhibitory effects of all the samples tested. Taken together this might explain why no chicken fibroblast proliferation was observed for any of the test samples at any of the tested concentrations.

It has been recommended that Aloe gel should be used directly from the plant to yield best results [60]. This could imply that whole leaf juice and solvent extracted gel powder as prepared for this study were not stable and had lost activity or destroyed during the extraction process hence did not have a stimulatory effect on the chicken fibroblasts.

There are two preparations of the samples of *Aloe ferox* and *Aloe vera*. For the first preparation samples were filter sterilised whereas for second preparation samples were γ irradiated (for sterilization purposes). There is no significant difference between the two preparations with regards to results obtained from whole leaf juice of both the Aloe species. There is also no significant difference between the two preparations with regards to results obtained from solvent extracted gel powder of *Aloe ferox*. However there is a significant difference between the two preparations with regards to results obtained from solvent extracted gel powder of *Aloe vera*. With the first preparation solvent extracted gel powder has a significant inhibitory effect on all concentrations tested, whereas with the second preparation it has a significant inhibitory only at high concentrations. This result could imply that the γ Irradiation process may have caused some changes in the compounds such as crosslinking or potentially the complete destruction of labile compounds resulting in new toxic components.

3.6. Conclusion

Water extract of whole leaf juice and the solvent extracted gel powder of *Aloe ferox* and *Aloe vera* prepared to have no bitter components demonstrate an inhibitory effect on chicken fibroblast proliferation.

Chapter 4

4. Antimicrobial Activity

4.1. Introduction

The human immune system protects the body against the damaging effects of microbial agents through the innate (non-specific) and acquired (specific) immunity.

The innate immune system comprises amongst others of neutrophils, macrophages, natural killer cells, and antigen presenting cells as well as the protein based complement system,. Acquired immune response also comprises of humoral and cell mediated components [61] and is antigen specific.

Apart from the natural immunity, there are antimicrobial agents or drugs that have a selective toxicity towards invading microorganisms without harming the cells of the hosts. The selection of antimicrobial depends on the following: organism's identity, its susceptibility to a particular agent, the site of the infection, patient factors, the safety of the agent, the site of the infection, and the cost of therapy [62].

Currently, the following are used for treatment of micro-organisms:

1. Antibiotics derived from moulds and fungi: They are used for treatment of minor infections like conjunctivitis through to life threatening diseases like pneumonia.
   Common drugs: penicillins, cephalosporins, aminoglycosides, tetracyclines, macrolides and lincosamides [63].
2. Antibacterial derived from synthetic chemicals: They are usually used for treatment of urinary tract infections and also used for some middle ear infection.
   Common drugs: Sulphonamides (Co-trimoxazole), urinary antiseptics (nalidixic acid), Dapsone, Metronidazole and trimethoprim [63].
3. Antifungal drugs: these drugs are used in the treatment of subcutaneous and systemic mycoses.
   Common drugs: Drugs for subcutaneous and systemic mycoses: Amphotericin B, Caspofungi, fluconazole, Itraconazole,
   Drugs for cutaneous mycoses: Butconazole, clotrimazole, Econazole, Nystatin and griseofulvin [62]

In most of the antibiotics, antibacterial and antifungal drugs currently in use, drug resistance has been experienced [62], due to this there is an urgent need of more antimicrobial drugs of different origins. These will allow more choices of drugs, thus specific drugs for specific microorganism could be discovered.

Aloe ferox and *Aloe vera* in different preparations, conditions and concentrations have been claimed to have antimicrobial properties [3;61;64].

Freeze dried whole leaf juice of *Aloe vera* at a concentration of 20 mg/ml in distilled water heated for 15 minutes at 80°C has been proven to be effective against *Staphylococcus aureus, Streptococcus pyogenes* and *Corynebacterium xeros in vitro.* The agar diffusion technique was used [65] for that assay.

Ethanol extract of *Aloe vera* leaf has been proven to be effective against *Staphyloccus aureus, Pseudomonas aeruginosa* and *Candida albicans in vitro* at a concentration of 25 mg/ml. Antimicrobial effect was measured by the appearance of zone of inhibition [3].

The following compounds isolated from *Aloe ferox* have been proven to have anti-microbial properties on different species using microplate dilution method:
Aloe emodin: *Bacillus subtilis, Staphylococcus epidermidis*, and *Shigella sonnei* [66]
Aloin A: *Bacillus subtilis, Staphylococcus epidermidis, Shigella sonnei, Escherichia coli, Bacillus cereus* and *Staphyloccus aureus* [66].
Aloin: *Candida albicans* [64].

In this experiment water, chloroform, ethyl acetate, methanol and ethanol extracts of whole leaf juice and derived solvent extracted gel powder from both *Aloe ferox* and *Aloe vera* will be used to determine antimicrobial properties. Water extracts were only used for the other cells (lymphocytes, neutrophils and chicken fibroblasts) because the other extracts are toxic to the living cells (lymphocytes, neutrophils and chicken fibroblasts).

4.2. Aim:

To assess the antimicrobial properties of water, chloroform, ethyl acetate, methanol and ethanol extracts of whole leaf juice and derived solvent extracted gel powder from both *Aloe ferox* and *Aloe vera* using the disc diffusion method.

4.3. Materials and methods

Different methods of preparation were used for each of the Aloe species

Water extracts of the whole leaf juice extract two aloe species

A. First Preparation

Exactly 250 mg of each extract was added to 50 ml double deionised water .The 50 ml tube containing the mixture was then sonicated for 1 hour, incubated for 1 hour at 60°C, sonicated for 1 hour again, centrifuged at 800 g for 30 min and supernatant was collected into a tube.

Empty pre-marked and dried petri dishes were weighed. Exactly 1 ml of each extract was placed into weighed pre-marked and dried petri dishes then left to dry overnight in an oven at 90°C. Petri dishes were cooled in a desiccator with dry silica gel and weighed again to determine the difference (which gives the dry mass of extract derived from 1.0 ml of each extract.

B. Second preparation

Similar to the first preparation except that the 50 ml tube containing the mixture was then ultracentrifuged for 60 mins at 64 000 g. Only the supernatant was used for the further testing of the extracts

C. Third preparation

Similar to the first preparation except that the 50 ml tube containing the mixture was not incubated at 60°C but sonicated for 2 hours and centrifuged at 800 g for 30 min

Concentrations used for all anti-microorganism tests:

Whole leaf juice - 2.5 mg/ml

Solvent extracted gel powder juice - 0.5 mg/ml

Preparation of chloroform extracts, ethyl acetate extracts, methanol extracts and ethanol extracts

For each extract 250 mg dry material was added to 50 ml selected solvent. The 50 ml tube containing the mixture was then sonicated for 2 hours, centrifuged at 800 *g* for 30 min and supernatant was collected into a tube. Empty pre-marked and dried petri dishes were weighed. For each extract 1.0 ml was placed into weighed pre-marked and dried petri dishes and left to dry overnight in an oven at 90°C.
Petri dishes were cooled in a desiccator with dry silica gel and weighed again to determine the difference (which gives the dry mass of each extract) between the empty petri dishes and petri dishes with 1.0 ml of each extract.

Chloroform extracts
Whole leaf juice - 1.5 mg/ml
Solvent extracted gel powder - 1.5 mg/ml
Ethyl acetate extracts
Whole leaf juice - 3 mg/ml
Solvent extracted gel powder - 1.5 mg/ml
The concentration of the methanol and ethanol extracts was too low for testing studies and thus could not be used.

All extracts were tested for antimicrobial properties against *Candida albicans, Staphylococcus aureus* and *Pseudomona aeruginosa*.

Preparation of agar for bacterial and fungal growth

Clinical isolates of *Candida albicans, Staphyloccocus aureus* and *Pseudomonas aeruginosa* were obtained from the Department of Microbiology, University of Pretoria.

Bacterial and fungal cultures were prepared using the following method

A. Candida albicans

Mueller-Hinton media was made by adding 0.8 g media powder to 23 ml of distilled water. The bottle containing the agar was then incubated for 1 hour at 121°C, left to cool for 5 to 10 min, emptied into the petri dishes and left for 30-55 min.

Standardised *Candida* inoculum was plated onto the agar using a plating loop. Plate was covered and incubated at 37°C for 24 hours.

B. Staphyloccocus aureus

Mannitol salt agar at a mass of 1.74 g was added into 23 ml of distilled water.

The bottle containing the agar was then Incubated for 1 hour at 121°C, left to cool for 5 to 10 min, emptied in the petri dishes and left for 30-55 min.

Standardised concentrations of the relevant bacteria were plated onto the agar. Plate was covered and incubated at 37°C for 24 hours.

C. Pseudomonas aeruginosa

Pre-prepared blood agar petri dishes of 10 cm were purchased from the Department of Microbiology University of Pretoria. Standardised concentrations of the relevant bacteria were plated onto the agar. Plate was covered and incubated at 37°C for 24 hours

Preparation of standardised inocula

Microbiological inocula were prepared by transferring colonies from freshly prepared subcultures to sterile saline (0.85%) until a turbidity of 0.5 McFarland standard is reached.

0.5 McFarland standard is as follows:

Candida albicans: 1.0×10^8 CFU/ml $0.8A_{570}$

Staphylococcus aureus: 1.0×10^8 CFU/ml $0.3A_{570}$

Pseudomonas aeruginosa: 1.0×10^8 CFU/ml $0.3A_{570}$

Disc diffusion assay

A sterile filter-paper disc, was impregnated with 400 µl of respective plant extract (started with 50 µl with waiting periods of 30 min between successive applications until a volume was 400 µl had been applied per disk. The disc was then completely air dried so that no solvent remained on the disc). Twenty three milliliters of Mueller-Hinton agar was poured in each of the 10 cm petri dishes and allowed to set. Two hundred microliters of the specific culture inoculum was inoculated onto each agar plate, this created a "lawn" of microbial growth. The dried extract impregnated filter paper discs was placed in the center of the inoculated agar plates. Positive control antibiotic disc for the respective microorganism was placed in the center of a different agar plate. Plates were incubated at 37°C for 24 hours.
Antimicrobial activity was expressed in terms of the mean diameter of the zone of growth inhibition around the disc. The assay was performed in triplicates.

Table 4.3.1. Positive controls for microorganisms used

Control	**Microorganisms**
Itraconazole 10 µg	*Candida albicans* (ATCC 10231)
Ampicillin 2 µg	Gram-positive organisms (*Staphylococcus aureus*) (ATCC 12600)
Gentamicin 10 µg	Gram-negative organisms (*Pseudomonas aeruginosa*) (ATCC 27853)

Three preparations of water extracts, for solvent extracted gel powder and whole leaf juice of each of the two Aloe species were also tested by adding directly (instead of placing the drug on the disc) onto a lawn culture of *Candida albicans, Staphylococcus aureus* and *Pseudomonas aeruginosa* toassess antimicrobial properties as it was unknown whether the extracts would have been soluble enough to diffuse into the agar gel.

This was to verify whether the respective extract was antimicrobial in direct contact with the microorganisms and would eliminate any requirement for solubility or diffusion through a sugar based medium.
The control drugs for the direct application of extracts were the same impregnated disks used for the disc diffusion assays.

4.4. Results

A. Neither of the whole leaf juice or the solvent extracted gel powder or extracts of either of the two Aloe species showed any antimicrobial properties:
   Water extracts using the three different preparation methods, Chloroform extracts and Ethyl acetate extracts.

Due to none of the above extracts demonstrating any antimicrobial activity against actively growing *Candida albicans, Staphylococcus aureus* and *Pseudomonas aeruginosa* when using the diffusion disc assay an alternative application method was attempted where the water extracts of solvent extracted gel powder and whole leaf juice of the two aloe species were each tested by directly applying these extracts onto a lawn of actively growing microorganisms on an agar plate to determine the antimicrobial properties.
Even under these conditions, none of the water extracts prepared using the 3 preparation methods showed any antimicrobial properties.

The positive controls exhibited a significant inhibition of the respective microorganism in both the assay methods using disc diffusion or directly applied to the microorganims.

4.5. Discussion

According to the results none of the extracts tested showed any antimicrobial properties, this is in contrast with results from other studies as *Aloe ferox* and *Aloe vera* has been proven to have antimicrobial properties [3;61;64] although these positive antimicrobial results used different sample preparation methods that could have included different active compounds.

Since results obtained by applying the test extracts onto the actively growing microorganisms directly matched the results observed for the disc diffusion assay it could be concluded that the respective extracts did not show lack of activity due to diffusion limitations but were in fact not active against these microorganisms. Thus the reason for the lack of antimicrobial activity was not associated with solubility or diffusion characteristics.

The removal of the bitter juice from these plant extracts more probably had an effect on the antimicrobial results; anthroquinones are restricted to the bitter juice [67;68] and encompass

the following components: Aloe-emodin, aloetic-acid, anthranol, aloin A and B (or collectively known as barbaloin), isobarbaloin, emodin, ester of cinnamic acid [6]. These components with proven antimicrobial properties when isolated, had been removed from the extracts used as test compounds in this study.

Aloe emodin and Aloin A from *Aloe ferox* extract has shown activity as follows: Aloe emodin: *Bacillus subtilis*, *Staphylococcus epidermidis*, and *Shigella sonnei* [66]
Aloin A: *Bacillus subtilis*, *Staphylococcus epidermidis*, *Shigella sonnei*, *Escherichia coli*, *Bacillus cereus* and *Staphyloccus aureus* [66] . Aloin (at a concentration of 5 mg/ml): *Candida albicans* [64]. These experiments were all done using microplate dilution method.

This might perhaps be the reason for lack of antimicrobial activities on all tested extracts.

Solvent extracted gel powder of *Aloe ferox* and *Aloe vera* has been precipitated from methanol and isopropanol respectively. During the precipitation, methanol (for *Aloe ferox*) and isopropanol (for *Aloe vera*) has been used 3 successive times to ensure that all the bitter juice was removed.
Thus other active components which may be responsible for antimicrobial properties could also have been discarded.
Furthermore, the *Aloe vera* gel methanol extract using the agar well diffusion technique has demonstrated inhibitory activity on the following microorganisms
Gram positive: *Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, Streptococcus pyogenes.*
Gram negative*: Escherichia coli, Klebsiella pneumonia, Salmonella typhi* and *Pseudomonas aeruginosa* [69].
In addition, Lin et al [70] compared the extractability of different solvents and reported methanol to be a better solvent for more consistent extraction of antimicrobial substances from the Aloe plant.

In this project methanol extraction of *Aloe ferox* whole leaf gel (precipitated from methanol) and *Aloe vera* whole leaf solvent extracted gel powder (precipitate from isopropanol) was not successful as the methanol extract yielded insignificant extractable mass from the solvent extracted gel powder s from both the Aloe species.

Currently there are no studies reporting on the antibacterial properties of methanol extracts of *Aloe ferox*.

Ethanolic extracts of *Aloe vera* has been proven to have inhibitory activity against *Staphyloccus aureus* and *Pseudomonas aeruginosa* using the agar well diffusion assay where minimum inhibitory concentrations were determined against these pathogenic microorganisms [71]. In this study ethanol extraction was not successful as ethanol extract yielded extremely low concentrations from whole leaf juice and solvent extracted gel powder derived from the two Aloe species. This is probably due the solvent precipitation process used for the preparation of whole leaf juice and solvent extracted gel powder.

Aloe ferox water extracts have been shown that they are not active against *Candida albicans* at concentrations of as much as 20 mg/ml while methanol extract has been shown to have low activity against *Candida albicans* at 20 mg/ml in vitro [64].

It has been reported that *Candida albicans* is resistant to many plant extracts thus a concentration as high as 100 mg/ml was suggested [64] to be tested to assess anti-Candida activity.
This report supports the finding that the tested Aloe extracts do not exhibit antifungal activities (as the concentrations tested were very much lower than those suggested in the report). Also most of the results obtained for extracts from *Aloe ferox* and *Aloe vera* have only exhibited antimicrobial properties at high concentrations and most of the results were from assays done *in vitro*.

4.6. Conclusion

Water, chloroform, ethyl acetate, methanol and ethanol extracts of dried whole leaf juice or the derived solvent extracted gel powder prepared through a process of solvent precipitation and washing to remove the bitter components from either *Aloe ferox* or *Aloe vera* did not demonstrate measurable antimicrobial properties.

Chapter 5

Anti-oxidant Activity

5.1. Introduction

A by-product of oxygen uptake by the body is free radicals which if uncontrolled can damage cells by the process of oxidation [72]. It is thought that this damage is a major contributor to aging and degenerative diseases of aging such as cancer, cardiovascular disease, immune-system decline, brain dysfunction and cataracts [73] . Antioxidants hinder the process of oxidation by reacting with free radicals and other reactive oxygen species within the body. Thus their main function is to maintain a critical balance between the radical generating and radical scavenging systems [72].
Antioxidant defences against cell damage include ascorbate, urate, tocopherol, and carotenoids.

Free radicals can induce inflammation [2;74]. Thus antioxidants help in reducing inappropriate inflammation. Antioxidants can be classified into four categories depending on their function; preventive antioxidants, free radical scavenging antioxidants, repair and de novo antioxidants and adaptation [75]. In this study whole leaf juice and solvent extracted gel powder from *Aloe ferox* and *Aloe vera* were studied to determine if they have radical scavenging properties.
Various preparations of *Aloe vera* have been reported to have anti-oxidant properties [2].

Aloe ferox components include antioxidant polyphenols, indoles and alkaloids [30].

There are few studies that reveal the antioxidant activities of *Aloe ferox*, and it is unknown which radicals can be easily scavenged by extracts from this plant.

Four different assays were used to determine the antioxidant properties of whole leaf juice and solvent extracted gel powders from the two aloe species

1. Oxygen radical absorbance capacity (ORAC assay)
2. 2,2-diphenyl-1-picrylhydrazyl (DPPH assay)
3. Trolox equivalent antioxidant capacity (TEAC) assay
4. Chemiluminescence assay

ORAC, DPPH and TEAC use trolox as a standard. It is a water soluble vitamin E analogue which has a high antioxidant activity [76].

Oxygen radical absorbance capacity (ORAC assay)

5.1.1. Introduction

The ORAC assay is commonly used to measure the antioxidant capacities of different foods, supplements and nutritional compounds [77].
AAPH is a free radical generator [78]. Decomposition of AAPH produces molecular nitrogen and 2 carbon radicals. The carbon radicals may combine to produce stable products or react with molecular oxygen to give peroxyl radicals [79]. In this study AAPH generated radicals which reacted with fluorescein to quench the fluorescence. The aloe extracts were tested as antioxidants by their ability to react with the radicals leaving fewer radicals to react with the fluorescein thus giving an indication of the antioxidant activity [77].

Aloe ferox gel has demonstrated antioxidant properties using the ORAC method. It has also been proven to have the following possible antioxidant compounds: Various phenolic acids/polyphenols, phytosterols, indoles, alkaloids. [30]

Very few studies have determined the antioxidants effects of both *Aloe ferox* and *Aloe vera* using ORAC.

5.1.2. Aim

To determine the peroxyl radical scavenging effects of the water extract of whole leaf juice and solvent extracted gel powders solution of *Aloe ferox* and *Aloe vera*.

5.1.3. Materials and methods

For each extract 250 mg was added to 50 ml double distilled water. The tube with the mixture was then sonicated for 1 hour, incubated for 1 hour at 60 °C, sonicated for 1 hour again, centrifuged at 64000 *g* for 60 min and supernatant was collected into a tube.
Empty pre-marked and dried petri dishes were weighed. For each extract 1,0 ml was placed into weighed pre-marked and dried petri dishes and left to dry overnight in an oven.
Petri dishes were cooled in a desiccator with dry silica gel and weighed again to determine the difference (which gives the concentration of each extract) between the empty petri dishes and petri dishes with 1,0 ml of each extract.

Concentrations used:
Whole leaf juice: 71.4 µg/ml
Solvent extracted gel powder : 47.6 µg/ml

Preparations of reagents

a. Trolox

Anti-oxidant activity of the samples was reported as Trolox equivalents.
Trolox was dissolved in water and the following concentrations were used
0, 2.4 µg/ml, 4.8 µg/ml, 7.2 µg/ml, 9.6 µg/ml and 12 µg/ml

b. AAPH

2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) was used as a radical generator.
AAPH at a mass of 0.08 *g* was dissolved in 4 ml of deionised water.

AAPH generated radicals which reacted with fluorescein during heating and quenched the fluorescence of these molecules

c. Fluorescein

Fluorescein at a mass of 3.8 mg was dissolved in 10,0 ml of deionised water (solution A), 35 µl of solution A was added to 5 ml of a phosphate buffer solution (PBS) and the tube was filled with 45 ml of distilled water.

Test sample dilution

Exactly 100 µl of each sample was added to 1.4 ml of water

Experimental procedures

Experiments were performed using white 96 well culture plates for fluorescence assays

For the control

For the blank, 60 µl of water was added to 160 µl of fluorescein solution. For the standard, 20 µl of each Trolox concentration was added to 160 µl of fluorescein and 40 µl of AAPH

For the experiment

Ten microlitres of stock solutions of either *Aloe ferox* whole leaf gel or solvent extracted gel powder was added to 160 µl of fluorescein solution and finally 40 µl of AAPH solution added. The final concentrations of the aloe extracts were 71.4 µg/ml for the whole leaf juice extract and 47.6 µg/ml for the solvent extracted gel powder.

Twenty microliters either *Aloe vera* whole leaf gel or solvent extracted gel powder was added to160 µl flourescein solution and finally 40 µl of AAPH solution added. The final concentrations of the aloe extracts were 71.4 µg/ml for the whole leaf juice extract and 47.6 µg/ml for the solvent extracted gel powder.

5.1.4. Results

Results obtained from of anti-oxidant effects of *Aloe ferox* and *Aloe vera* (solvent extracted gel powder solution and whole leaf juice) using ORAC assy.

Table 5.1.4.1. Anti-oxidant activity of the samples reported as Trolox equivalents (TE).

Plant	**Exp1 umol TE/mg**	**Exp2 umol TE/mg**	**Exp3 umol TE/mg**	**MEAN**	**SD**	**SEM**
Aloe vera solvent extracted gel powder	16.9	14.8	11.9	14.5	2.51	1.42
Aloe vera whole leaf juice	27.4	33.1	26.7	29.1	3.50	1.98
Aloe ferox solvent extracted gel powder	358.6	386.0	371.5	372.0	13.72	7.75
Aloe ferox whole leaf juice	64.5	69.2	56.5	63.4	6.42	3.63

As seen from Table 5.1.4.1 and Figure 5.1.4.1, *Aloe ferox* solvent extracted gel powder has the highest anti-oxidant activity compared to *Aloe vera* solvent extracted gel powder.
Statistical analysis: Kruskal Wallis test for non-parametric data was used. There is a significant differences between the means of the two samples ($P<0.0001$).

Aloe ferox whole leaf juice has a higher anti-oxidant activity compared *Aloe vera* whole leaf juice. In this case the P- value obtained was 0.0012.

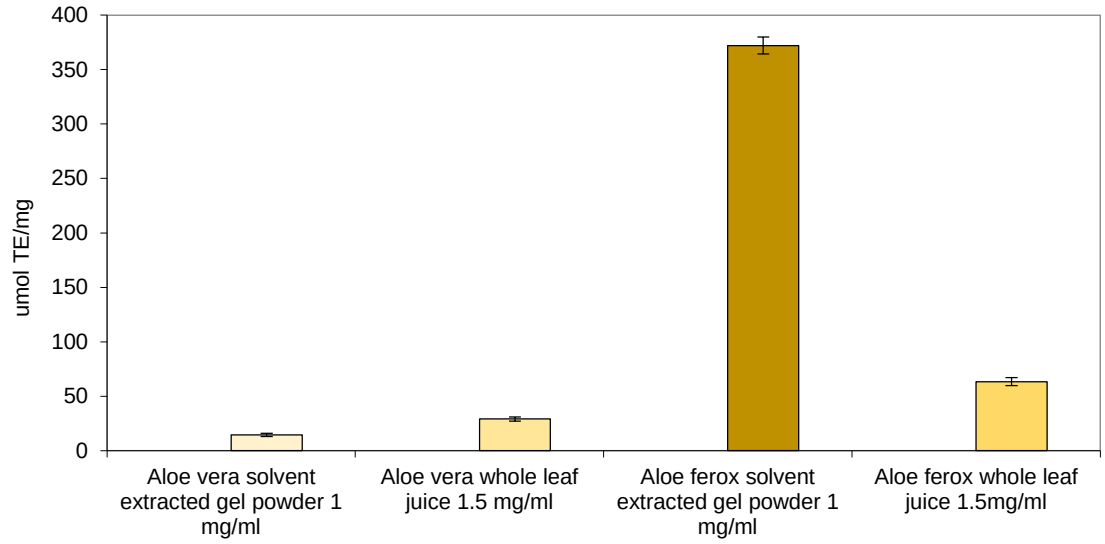


Figure 5.1.4.1. The antioxidant activities of the samples

Aloe ferox solvent extracted gel powder solution and whole leaf gel have a higher level of antioxidant activity compared to solvent extracted gel powder and whole leaf juice of *Aloe vera*.

5.1.5. Discussion

Aloe ferox and *Aloe vera* have been reported to contain relatively high concentrations of flavonoids [30] which have been proven to reduce peroxyl radicals [80]. *Aloe ferox* solvent extracted gel powder was more water soluble than the whole leaf juice or the equivalent *Aloe vera* extract which could imply that there were more flavonoids, that are water soluble. This could be the reason for the high ORAC value observed for the *Aloe ferox* gel powder.

Aloe vera solvent extracted gel powder was observed to have the lowest ORAC value. This is similar to the results obtained in a study where 38 different plants extracts were studied, *Aloe vera* gel was also found to have the lowest ORAC value (1.88 µmol TE/mg) in that study [81].

According to the literature, *Aloe ferox* (with bitter juice) has been shown to have antioxidant properties [30]. In this study solvent extracted gel powder of *Aloe ferox* still had very potent antioxidant properties even though bitter juice is removed. The antioxidant activity was almost 20 times larger than the equivalent *Aloe vera* sample.

5.1.6. Conclusion

Water extract of whole leaf juice and solvent extracted gel powders of *Aloe ferox* and *Aloe vera* have peroxyl radical scavenging effect at the concentrations used.
However *Aloe ferox* has shown a higher antioxidant level than *Aloe vera* for both preparations.
According to literature [29,30], *Aloe vera* loses its viscosity much more rapidly after extraction than *Aloe ferox*, this might be the reason *Aloe ferox* demonstrated higher antioxidant properties than *Aloe vera*. Thus *Aloe ferox* could have retained relevant components after precipitation with methanol because of its viscosity.
Aloe ferox has also been proven to contain more amino acid content than *Aloe vera*, [29,30] perhaps not all amino acids were discard during the preparation of *Aloe ferox* samples, thus those amino acids that could have remained could have reduced peroxyl radicals.

5.2. 2,2-Diphenyl-1-picrylhydrazyl (DPPH assay)

5.2.1. Introduction

DPPH is a radical with a violet colour in solution and it changes to colourless or pale yellow when reacting to an antioxidant compound [82]. DPPH has an absorption band at 515 nm in methanol and disappears upon reaction with an antioxidant [83].

The following aloesin derivatives in *Aloe vera* showed potent DPPH radical scavenging activities: Isorabaichromone, feruloylaloesin and p-coumaroylaloesin [84].

Ethanol extracts of *Aloe vera* at three different stages: two years, three years and four years showed DPPH radical scavenging activites. Extracts of a three years *Aloe vera* showed a higher activity than the other two [85].

The boiled leaf skin powder displayed a stronger level of DPPH Trolox radical scavenging activity than freeze-dried whole leaf powder and freeze-dried leaf skin powder [86].

It was proven that ethanolic extracts of *Aloe vera* of different fractions exhibit radical scavenging activity of DPPH at different rate. The following fractions were tested on DPPH for radical scavenging properties: Chloroform-ethanol fraction, ethyl acetate, butanol and hexane extracts. Chloroform-ethanol fraction exhibited the highest scavenging properties [87].

Ethanol extracts of *Aloe vera* have been proven to be effective against DPPH [88]

Aloe ferox extracted with 50% methanol (100 ml) followed by sequential extraction of 100 ml of each of petroleum ether, dichloromethane and ethanol has been proven to have inhibitory activity of DPPH [89].

5.2.2. Aim

To determine the DPPH scavenging effects of the whole leave gel and solvent extracted gel powders of *Aloe ferox* and *Aloe vera*

5.2.3. Materials and methods

Preparation of water extracts

For each extract 250 mg was added to 50 ml double distilled water. The 50 ml tube containing the mixture was then sonicated for 2 hours, centrifuged at 800 *g* for 30 min and supernatant was collected into a tube.

Empty pre-marked and dried petri dishes were weighed. For each extract 1.0 ml was placed into weighed pre-marked and dried petri dishes. They were left to dry off overnight in an oven 90˚C. Petri dishes were cooled in a desiccator with dry silica gel and weighed again to determine the difference (which gives the concentration of each extract) between the empty petri dishes and petri dishes with 1.0 ml of each extract.

Concentrations tested:

Whole leaf juice: 150 µg/ml

Solvent extracted powder: 25 µg/ml

Preparation of reagents

DPPH

DPPH at a mass of 12 mg was dissolved in 50 ml methanol and sonicated for 20 minutes. From this solution 10 ml was added to 40 ml methanol to give a working solution and kept for 20 minutes in the dark before using.

Trolox

Trolox stock solution made by adding a mass of 1,3 mg was dissolved in 5 ml of water (1000 µM /260 µg/ml). The following dilutions of Trolox were used for the calibration curve

0 µM, 50 µM, 100 µM, 200 µM, 400 µM, 600 µM, 800 µM, 1000 µM

Into a 96 well plate 10 µl of each Trolox dilution and 190 µl of the working solution were added. After addition of the standards the plate was incubated for 15 minutes in the dark. Absorbance was read at 570 nm

Aloe extracts

Into a 96 well plate 10 µl of each aloe extract test solution and 190 µl of the working solution were added. After addition of the standards the plate was incubated for 15 minutes in the dark. Absorbance was read at 570 nm.

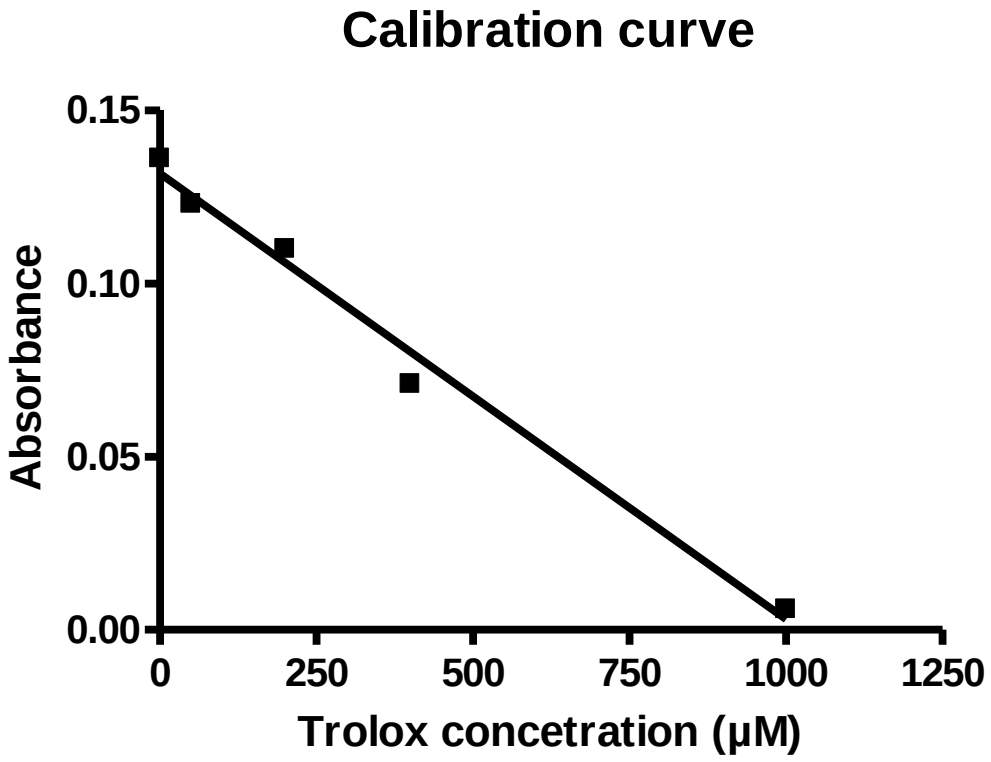
5.2.4. Results

One experiment was repeated 3 times, hence there are results for experiment A, B and C.

Experiment A

Table 5.2.4.1. Trolox results

Sample ID	**Concentration (µM)**	**Absorbance**
Trolox A	1000	0.006
Trolox B	400	0.071
Trolox C	200	0.110
Trolox D	100	0.130
Trolox E	50	0.123
Trolox F	0	0.136



5.2.4.1. Trolox calibration curve

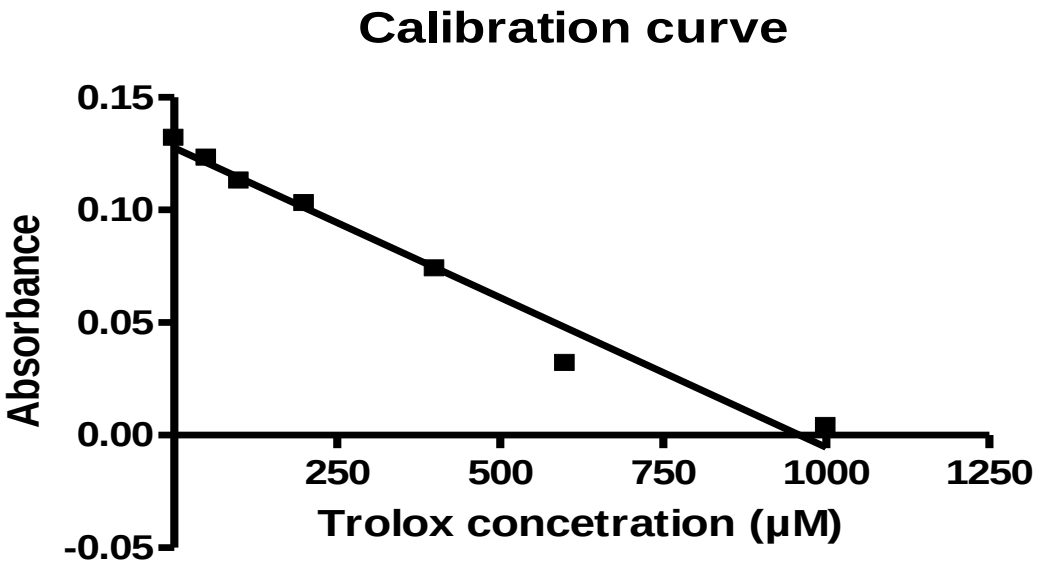
Table 5.2.4.2. Results in terms of Trolox equivalents for the different extracts tested at 150 µg/ml for whole leaf juice and 25 µg/ml for solvent extracted powder

Sample ID	**Concentration (µM)**	**Absorbance**
Aloe ferox whole leaf juice	-8.01172	0.135
Aloe ferox whole leaf juice	-21.4879	0.137
Aloe ferox whole leaf juice	-14.7499	0.136
Aloe vera whole leaf juice	-8.01172	0.135
Aloe vera whole leaf juice	-8.01172	0.135
Aloe vera whole leaf juice	-14.7499	0.136
Aloe ferox solvent extracted gel powder	-75.3931	0.145
Aloe ferox solvent extracted gel powder	-82.1312	0.146
Aloe ferox solvent extracted gel powder	-61.9169	0.143
Aloe vera gel solvent extracted powder	-61.9169	0.143
Aloe vera solvent extracted gel powder	-55.1787	0.142
Aloe vera solvent extracted gel powder	-41.7024	0.140

Experiment B

Table 5.2.4.3. Trolox calibration curve

Sample ID	Concentration (µM)	Absorbance
Trolox A	1000	0.004
Trolox B	800	0.005
Trolox C	600	0.032
Trolox D	400	0.074
Trolox E	200	0.103
Trolox F	100	0.113
Trolox G	50	0.123
Trolox H	0	0.132



5.2.4.2. Trolox calibration curve

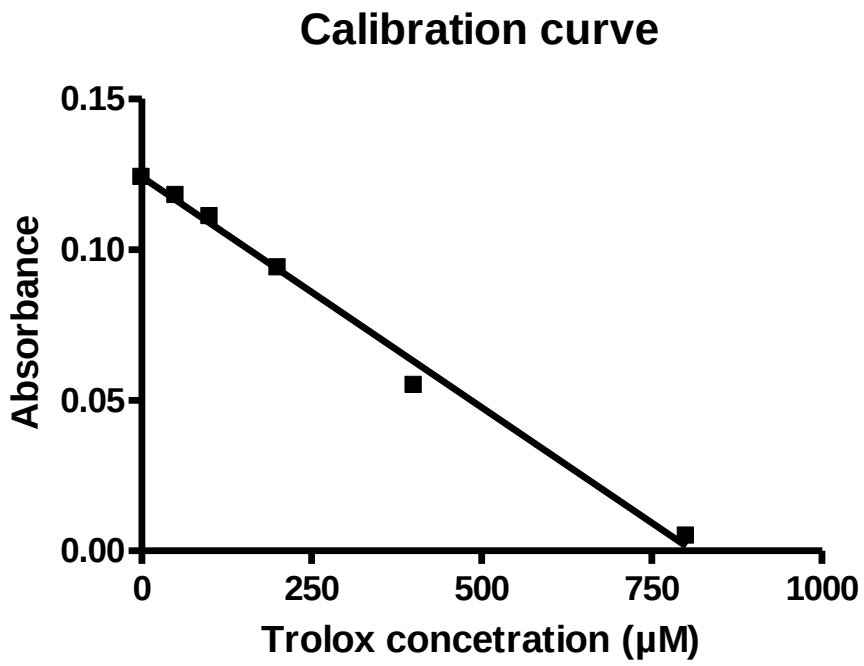
5.2.4.4. Samples results

Sample ID	**Concentration (µM)**	**Absorbance**
Aloe ferox whole leaf juice	-28.6589	0.131
Aloe ferox whole leaf juice	-55.7735	0.135
Aloe ferox whole leaf juice	-35.4376	0.132
Aloe vera whole leaf juice	-69.3307	0.137
Aloe vera whole leaf juice	-89.6667	0.140
Aloe vera whole leaf juice	-116.781	0.144
Aloe ferox solvent extracted gel powder	-69.3307	0.137
Aloe ferox solvent extracted gel powder	-55.7735	0.135
Aloe ferox solvent extracted gel powder	-55.7735	0.135
Aloe vera solvent extracted gel powder	-42.2162	0.133
Aloe vera solvent extracted gel powder	-48.9949	0.134
Aloe vera solvent extracted gel powder	-28.6589	0.131

Experiment C

Table 5.2.4.5. Trolox results

Sample ID	Concentration (µM)	Absorbance
Trolox A	1000	0.004
Trolox B	800	0.005
Trolox C	600	0.007
Trolox D	400	0.055
Trolox E	200	0.094
Trolox F	100	0.111
Trolox G	50	0.118
Trolox H	0	0.124



5.2.4.3. Trolox calibration curve

Table 5.2.4.6.Sample results

Sample ID	**Concentration (µM)**	**Absorbance**
Aloe ferox whole leaf juice	-21.7225	0.122
Aloe ferox whole leaf juice	-36.2368	0.124
Aloe ferox whole leaf juice	-7.20816	0.120
Aloe vera whole leaf juice	-7.20816	0.120
Aloe vera whole leaf juice	-36.2368	0.124
Aloe vera whole leaf juice	-36.2368	0.124
Aloe ferox solvent extracted gel powder	-65.2655	0.128
Aloe ferox solvent extracted gel powder	-21.7225	0.122
Aloe ferox solvent extracted gel powder	-43.494	0.125
Aloe vera solvent extracted gel powder	-36.2368	0.124
Aloe vera solvent extracted gel powder	-65.2655	0.128
Aloe vera solvent extracted gel powder	-65.2655	0.128

Compared to Trolox at the above concentrations, whole leaf juice and solvent extracted gel powder of both *Aloe ferox* and *Aloe vera* has no antioxidants properties but appeared to have pro-oxidant effects.

5.2.6. Discussion

Results obtained in this study are in contrast with what other researchers have found.

According to Jun Hu et al [85] the age of *Aloe vera* plant has an impact on the antioxidant properties, as extracts of *Aloe vera* that was three years old demonstrated higher antioxidant activity than that of two and that of four years. In this study, a combination of either *Aloe vera* or *Aloe ferox* at different ages have been mixed for preparation of whole leaf juice and solvent extracted gel powder , perhaps this could have had an impact. However it is more plausible that the growing conditions with respect to availability of nutrients, water, sunlight and other influences such as temperature attack by insects or other parasites would have a larger influence on the concentration of various compounds in the plant.

The aloesin derivatives: isorabaichromone, feruloylaloesin and p-coumaroylaloesin [84] and Aloin [83] which are the components of the bitter juice have been proven to have inhibitory properties on DPPH. This means bitter components forms an important part of the antioxidant properties of *Aloe ferox* and *Aloe vera*, thus removal of bitter components probably had a major impact on the total antioxidative effect of the extracts tested during this study.

Perhaps the preparation method had a negative impact on the overall activity of the extracts as it has been noted by Qiuhui Hu *et al* [86] that boiled leaf skin powder displayed a increased level of DPPH Trolox radical scavenging activity compared to freeze-dried whole leaf powder and freeze-dried leaf skin powder.

It can be concluded that *Aloe ferox* and *Aloe vera* have less antioxidant properties compared to Trolox and do in fact demonstrate pro-oxidative effects when using the radical scavenging effects against DPPH as the source of the oxidative species.

5.3. Trolox equivalent antioxidant capacity (TEAC) assay

5.3.1. Introduction

TEAC is an assay used to measure antioxidant activities of different substances, as compared to the synthetic water soluble vitamin E analogue, Trolox [77]. The TEAC assay is based on the scavenging of the 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical (ABTS(*)) converting it from the intense blue radical into a colourless reduced product. The degree of decolourization induced by the substance is compared to that induced by Trolox giving the TEAC value [90].
The ABTS radical is quenched by most antioxidants [91].

Aqueous extract of *Aloe vera* has been found to contain the following antioxidant compounds: phenols, flavonoids, ascorbic acid, β-carotene and α-tocopherol. The extract exhibited reductive capacity against ABTS. Using the same method the "inner gel" of Aloe vera did not show any antioxidant activity [76].

Currently there are no reports of experiments done on *Aloe ferox* and its components using ABTS to assess the antioxidant activity.

5.3.2. Aim

To determine the ABTS radical scavenging effects of whole leaf juice and solvent extracted gel powder from Aloe vera and Aloe ferox water extract.

5.3.3. Materials and methods

Sample preparation
For each extract 250 mg was added to 50 ml double distilled water. The tube with the mixture was then sonicated for 2 hour, centrifuged at 800 *g* for 30 min and supernatant was collected into a tube. Empty pre-marked and dried petri dishes were weighed. For each extract 1.0 ml was placed into weighed pre-marked and dried petri dishes and left to dry off overnight in an

oven at 90ºC. Petri dishes were cooled in a desiccator with dry silica gel and weighed again to determine the difference (which gives the concentration of each extract) between the empty petri dishes and petri dishes with 1.0 ml of each extract.

Concentrations used
Whole leaf juice -1 mg/ml
Solvent extracted gel powder– 1 mg/ml

Preparation of reagents

a. Trolox

Ten milligrams of Trolox was dissolved into 10 ml of distilled water

b. ABTS (2,2'- azinobis (3- ethylbenzothiazoline-6-sulfonate))

ABTS was used as the radical, it is commonly used to determine the antioxidant activity of different compounds [92].
Nineteen millilitre of ABTS and 3 mg of $K_2S_2O_8$ were added to 5 ml of water. ABTS was prepared 24 hours before use and allowed to stabilise at 4ºC.

Experimental procedures

ABTS was diluted with water until the absorbance was 0.709 at the wavelength of 734 nm.
Serial dilutions and two controls were made for the calibration curve. The following concentrations of trolox were used for the calibration curve:
0.5 mmolar, 0.75 mmolar 1 mmolar, 2 mmolar, 3 mmolar and 4 mmolar
From the above concentrations 20 µl each concentration was added to 2 ml of ABTS and for the control 20 µl of the first concentration of trolox was added to 2 ml of water.

To determine the antioxidant properties of the samples
For the control, 20 µl of each sample was added to 2 ml of water and for the experiment,
20 µl of each sample was added to 2 ml of ABTS. Concentration for all samples was
1 mg/ml.

5.3.4. Results

One experiment was repeated 3 times, hence there are results for experiment A, B and C.

Experiment A

Table 5.3.4.1 Trolox results

Wavelength(s)	Sample ID	Concentration (mg/ml)	Ordinate value
734	Trolox A	2.000	0.0005
734	Trolox B	1.000	0.0286
734	Trolox C	0.750	0.1526
734	Trolox D	0.500	0.3370
734	Trolox E	0.250	0.4900
734	Trolox F	0.125	0.6343
734	Trolox G	0.093	0.6824
734	Trolox H	0.063	0.6487
734	Trolox I	0.031	0.7316

Equation: y = 7.173362e- 01 + - 7.229537e – 01 x X

Residual error: 0.034776

Correlation coefficient 0.992503

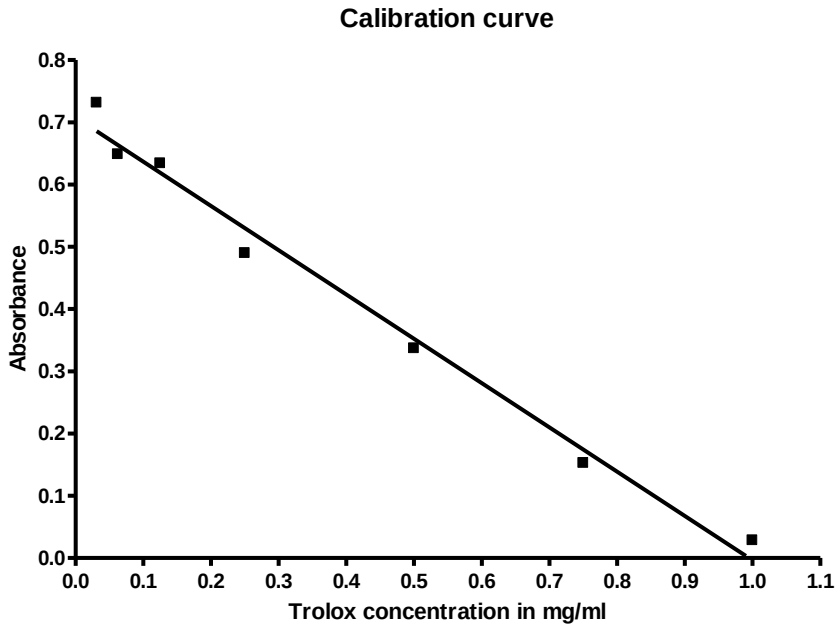


Figure 5.3.4.1. Trolox calibration curve

Table 5.3.4.2. Samples results

Wavelength(s)	Sample ID	Concentration (mg/ml)	Ordinate value
734	*Aloe ferox* whole leaf juice	- 0.056	0.7579
734	*Aloe ferox* whole leaf juice	- 0.016	0.7295
734	*Aloe ferox* whole leaf juice	- 0.047	0.6191
734	*Aloe vera* whole leaf juice	- 0.057	0.7590
734	*Aloe vera* whole leaf juice	- 0.059	0.7604
734	*Aloe vera* whole leaf juice	- 0.054	0.7565
734	*Aloe ferox* solvent extracted gel powder	- 0.045	0.7499
734	*Aloe ferox* solvent extracted gel powder	- 0.051	0.7542
734	*Aloe ferox* solvent extracted gel powder	- 0.051	0.7547
734	*Aloe vera* solvent extracted gel powder	- 0.062	0.7623
734	*Aloe vera* solvent extracted gel powder	- 0.056	0.7583
734	*Aloe vera* solvent extracted gel powder	- 0.055	0.7576

Experiment B

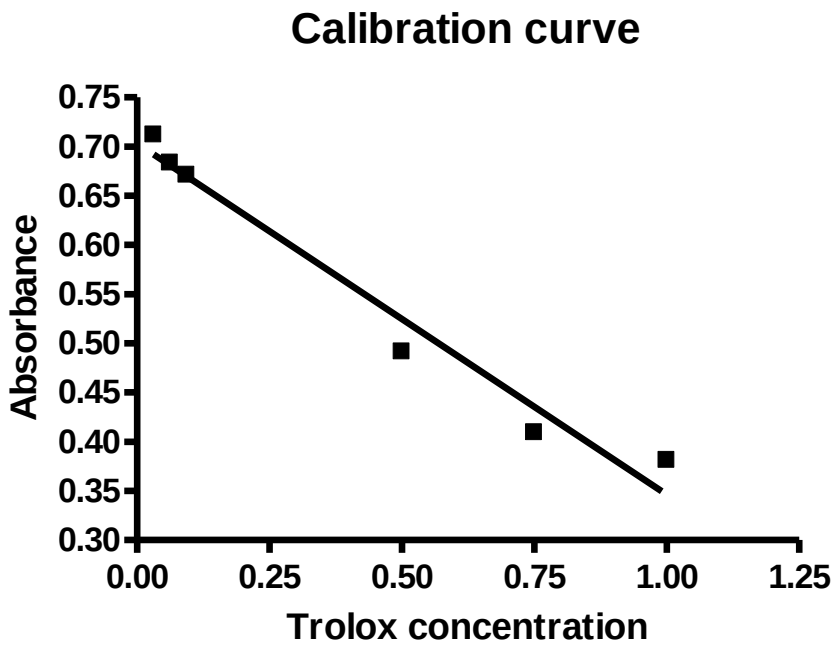
Table 5.3.4.3. Trolox results

Wavelength(s)	Sample ID	Concentration (mg/ml)	Ordinate value
734	Trolox A	**1.000**	**0.3812**
734	Trolox B	**0.750**	**0.4095**
734	Trolox C	**0.500**	**0.4916**
734	Trolox D	**0.250**	**0.5296**
734	Trolox E	**0.125**	**0.6291**
734	Trolox F	**0.094**	**0.6712**
734	Trolox G	**0.062**	**0.6836**
734	Trolox H	**0.031**	**0.7119**

Equation: y = 6.80991e – 01 + - 3.438434e – 01 x X

Residual error: 0.043242

Correlation coefficient: 0.956819



5.3.4.2. Trolox calibration curve

5.3.4.4. Samples results

Wavelength(s)	Sample ID	Concentration (mg/ml)	Ordinate value
734	*Aloe ferox* whole leaf juice	- 0.168	0.7441
734	*Aloe ferox* whole leaf juice	- 0.160	0.7413
734	*Aloe ferox* whole leaf juice	- 0.162	0.7418
734	*Aloe vera* whole leaf juice	- 0.172	0.7454
734	*Aloe vera* whole leaf juice	- 0.168	0.7441
734	*Aloe vera* whole leaf juice	- 0.171	0.7450
734	*Aloe ferox* solvent extracted gel powder	- 0.146	0.7364
734	*Aloe ferox* solvent extracted gel powder	- 0.149	0.7374
734	*Aloe ferox* solvent extracted gel powder	- 0.153	0.7390
734	*Aloe vera* solvent extracted gel powder	- 0.177	0.7471
734	*Aloe vera* solvent extracted gel powder	- 0.177	0.7473
734	*Aloe vera* solvent extracted gel powder	- 0.182	0.7489

Experiment C

Table 5.3.4.5. Trolox results

Wavelength(s)	**Sample ID**	**Concentration (mg/ml)**	**Ordinate value**
734	Trolox A	1.000	0.0298
734	Trolox B	0.750	0.3104
734	Trolox C	0.500	0.3832
734	Trolox D	0.250	0.4885
734	Trolox E	0.125	0.5937
734	Trolox F	0.094	0.6354
734	Trolox G	0.062	0.6503
734	Trolox H	0.031	0.6783

Equation : y = 6.836989e – 01 + - 6.043842e – 01 x X

Residual error: 0.043349

Correlation coefficient: 0.983557

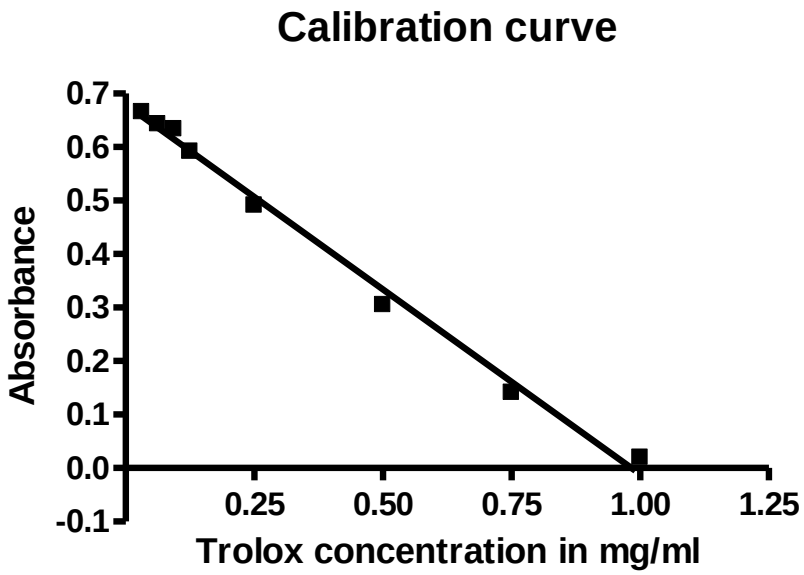


Figure 5.3.4.3. Trolox calibration curve

Table 5.3.4.6 Sample results

Wavelength(s)	**Sample ID**	**Concentration (mg/ml)**	**Ordinate value**
734	*Aloe ferox* whole leaf juice	- 0.011	0.6874
734	*Aloe ferox* whole leaf juice	- 0.011	0.6867
734	*Aloe ferox* whole leaf juice	- 0.006	0.6834
734	*Aloe vera* whole leaf juice	- 0.013	0.6885
734	*Aloe vera* whole leaf juice	- 0.014	0.6893
734	*Aloe vera* whole leaf juice	- 0.015	0.6895
734	*Aloe ferox* solvent extracted gel powder	- 0.013	0.6885
734	*Aloe ferox* solvent extracted gel powder	- 0.015	0.6900
734	*Aloe ferox* solvent extracted gel powder	- 0.013	0.6883
734	*Aloe vera* solvent extracted gel powder	- 0.014	0.6899
734	*Aloe vera* solvent extracted gel powder	- 0.016	0.6912
734	*Aloe vera* solvent extracted gel powder	0.017	0.6944

According to the results, compared to Trolox in all the above concentrations used whole leaf juice and solvent extracted gel powder of *Aloe ferox* and *Aloe vera* have no antioxidant properties according to the TEAC assay

5.3.5. Discussion

This is the first study done to determine the ABTS scavenging effects of whole leaf juice and solvent extracted gel powder of *Aloe ferox* as well as for the *Aloe vera* products where the bitter components had been removed.

Aloe vera has been proven to contain the antioxidant vitamins A, C and F [5], perhaps these are lost during the preparation of these extracts.
Thus lack of antioxidant activity against ABTS could be due to the method used to prepare the plants extracts.

5.3.6. Conclusion

Whole leaf juice and solvent extracted gel powders of *Aloe ferox* and *Aloe vera* have no effect on ABTS radical.

5.4. Chemiluminescence assay

5.4.1. Introduction

Neutrophils are white blood cells and they have a short half-life. They are phagocytic and they are the first immune cells to arrive at the site of infection [93]. When they are stimulated they release reactive oxygen species (ROS) through the enzyme system called neutrophil NADPH oxidase. NADPH oxidase reduces molecular oxygen to superoxide $O_2^{\cdot -}$ and hydrogen peroxide (H_2O_2) [94]. At high concentrations the released ROS can cause harmful effects to cell structures [95].
Chemiluminescence is a reaction that emits light [96]. It was found that chemiluminescence can selectively measure superoxide O_2^- a common reactive oxygen species available as a result of neutrophil activation [95] if the chemiluminescence enhancer lucigenin.

Lucigenin was used as a chemiluminescent enhancer molecule that amplified the detection of light generated by neutrophils. Lucigenin is a large molecule which reacts selectively with available $O_2^{\cdot -}$ [97].

Phorbol myristate acetate (PMA) is the active principal of croton oil that has been proven to stimulate human neutrophils in aspects of all cellular oxidative metabolism related to phagocytosis. It has been proven to increase hydrogen peroxide and superoxide anion through stimulation of NAPDH oxidase, thus it invokes a high chemiluminescence signal [98].

Hydrogen peroxide can be converted into hydroxyl radical by Harber–
Weis reaction or the Fenton reaction [99]. Harber reaction occurs in cells and hydroxyl radicals are generated from superoxide and hydrogen peroxide [100].

Aqueous extract of *Aloe vera* gel has been proven to contain two classes of chemically and functionally distinct immune-modulators. The aqueous extract was passed through ultra-filtration under nitrogen pressure in an Amicon chamber, low molecular and high molecular weight fractions were obtained using a Diaflo YM-10 membrane. Only the low molecular weight components inhibited reactive oxygen species produced by human polymorphnuclear leucocytes [101].

Low molecular constituents from *Aloe vera* gel have been proven to reduce hydrogen peroxide and superoxide from neurophils stimulated by PMA. Low molecular constituents were prepared as follows: The leaves of *Aloe vera* plants were harvested, the mucilaginous parenchymous tissue was excised from freshly cut leaves, lyophilized and kept in a sealed plastic bags until use. An aqueous extract of this dried powder was used for further experiments [102] .
In contrast to this, Jain and Basal [103] demonstrated that *Aloe vera* which was extracted in water and evaporated to dryness *in vacuo* had no scavenging properties on reactive oxygen species produced by polymorphonuclear leukocyte.

Currently no studies have reported on studies to determine reactive oxygen species inhibition properties of *Aloe ferox*.

5.4.2. Aim:

To determine the effects of the whole leaf juice and solvent extracted gel powder from *Aloe ferox* and *Aloe vera* on the NADPH oxidase system of phorbol myristate acetate (PMA) stimulated neutrophils.

5.4.3. Materials and methods

Extract preparation
For each extract 250 mg was added to 50 ml double distilled water. The tube with the mixture was then sonicated for 2 hours, centrifuged at 800 *g* for 30 min and supernatant was collected into a tube. Empty pre-marked and dried petri dishes were weighed. For each extract 1.0 ml was placed inside weighed pre-marked and dried petri dishes and left to dry off overnight in an oven at 90ºC. Petri dishes were cooled in a desiccator with dry silica gel and weighed again to determine the difference (which gives the concentration of each extract) between the empty petri dishes and petri dishes with 1.0 ml of each extract.

Concentration used:
Whole leaf juice: 125 µg/ml and 250 µg/ml
Solvent extracted gel powder : 25 µg/ml and 50 µg/ml

Preparation of neutrophils

Ten millilitre of blood was drawn from healthy human volunteers and was heparinised.
Five millilitre of blood was placed into a 50 ml tube and filled with cold 0.83% ammonium chloride solution, the tube was left in an ice for 10 min and then centrifuged at 250 *g* for 8 min.
The supernatant was carefully removed without disturbing the pellet, the tube was then filled with colour free Hanks Balance Salt Solution and then centrifuged at 250 *g* for 8 min.
The pellet was resuspended into 5 ml of Hanks. The tube was placed on ice and 2.5 ml of lucigenin was added to the resuspended pellet then left on the ice for 30 mins to 1 hour to allow loading of the cells.

Preparation of Reagents

Lucigenin: (Sigma Diagnostic catalogue number M-8010): (1 mg/ml) Fifty milligrams was added into 50 ml tube and dissolved in a 50 ml tube containing PBS (phosphate buffered saline). This dissolving was facilitated by warming up at 37°C. This was kept on ice for the duration of the experiment.

PMA :(Phorbol myristate acetate Sigma Diagnostics, cataloque number P 8139)
Vial of 25 mg was mixed with 25 ml of ethanol (1 mg/ml), then stored in a -80ºC freezer in 1 ml aliquots. Prior to use, PMA was diluted by adding 20 µl into 1 ml of HBSS (20 µg/ml), 100 µl of that was added into 1.9 ml of HBSS (1000 ng/ml).

Experimental procedure

A 96 well plate was used, to all wells 80 µl of the pre-incubated cells with lucigenin was added
The instrument dosing pump was preloaded with the following solutions:
To the positive control 20 µ1 of Phorbol myristate acetate (PMA) was added
To the experimental blank columns 20 µl of a drug was added
To the experimental columns 20 µl of a drug and 20 µl of PMA was added.
Negative control was the pre-incubated cells with 20 µl Hanks added.

The plate was read on chemiluminometer for 1 minute prior to addition of the stimulating PMA solution or an equivalent volume of Hanks for the negative control samples.
The results were collected as time verses chemiluminescent intensity curves and the area under the curve calculated till 800 seconds post dosing time.

5.4.4. Results

Whole leaf juice and solvent extracted gel powder from *Aloe ferox* and *Aloe vera* did not have any stimulating effect on resting neutrophils as the extracts alone did not induce any increase in chemiluminescence.

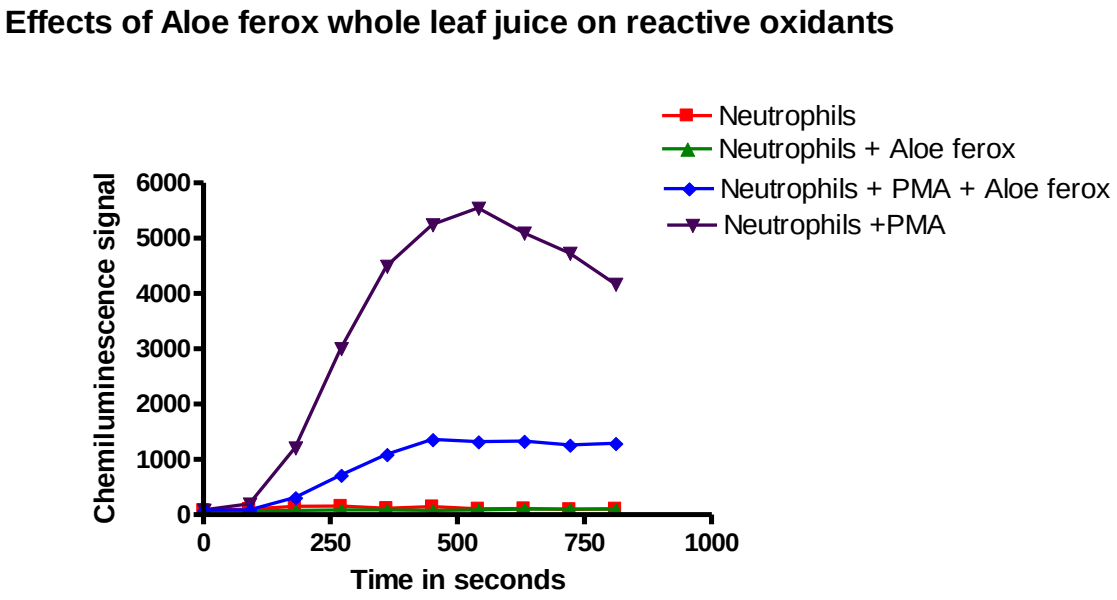


Figure 5.4.4.1.*Aloe ferox* whole leaf juice at a concentration of 250 µg/ml

Table 5.4.4.1.*Aloe ferox* whole leaf juice at a concentration of 250 µg/ml

	P value
Neutrophils vs Neutrophils + *Aloe ferox*	P > 0.05
Neutrophils vs Neutrophils +PMA	P < 0.001
Neutrophils vs Neutrophils + PMA + *Aloe ferox*	P > 0.05
Neutrophils + *Aloe ferox* vs Neutrophils +PMA	P < 0.001
Neutrophils + *Aloe ferox* vs Neutrophils + PMA + *Aloe ferox*	P > 0.05
Neutrophils +PMA vs Neutrophils + PMA + *Aloe ferox*	P < 0.001

Elaboration of the Figure 5.4.4.1 and Table 5.4.4.1

Whole leaf juice of *Aloe ferox* had no effect on neutrophils.

PMA had stimulated neutrophils to release reactive oxygen species.

Whole leaf juice of *Aloe ferox* had significantly reduced reactive species produced by PMA.

Whole leaf juice of *Aloe ferox* had no effect on neutrophils whereas PMA had a high effect on neutrophils, there is a significant difference between these two groups.

There is no significant difference between effects of whole leaf juice of *Aloe ferox* on neutrophils and effects of whole leaf juice of *Aloe ferox* and PMA on neutrophils.

There is a significant difference between effects of PMA on neutrophils and effects of whole leaf juice of *Aloe ferox* and PMA on neutrophils.

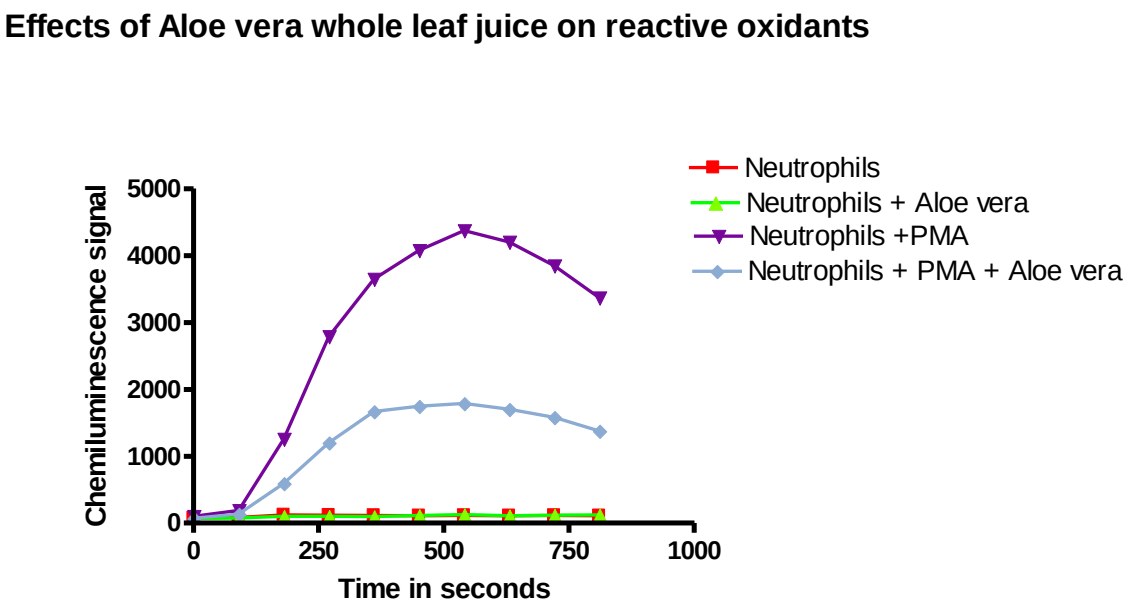


Figure 5.4.4.2. *Aloe vera* whole leaf juice at a concentration of 250 µg/ml

Table 5.4.4.2. *Aloe vera* whole leaf juice at a concentration of 250 µg/ml

	P value
Neutrophils vs Neutrophils + *Aloe vera*	P > 0.05
Neutrophils vs Neutrophils +PMA	P < 0.001
Neutrophils vs Neutrophils + PMA + *Aloe vera*	P > 0.05
Neutrophils + *Aloe vera* vs Neutrophils +PMA	P < 0.001
Neutrophils + *Aloe vera* vs Neutrophils + PMA + *Aloe vera*	P > 0.05
Neutrophils +PMA vs Neutrophils + PMA + *Aloe vera*	P < 0.01

Elaboration of the Figure 5.4.4.2 and Table 5.4.4.2

Whole leaf juice of *Aloe vera* had no effect on neutrophils.

PMA had stimulated neutrophils to release reactive oxygen species.

Whole leaf juice of *Aloe vera* reduced reactive species produced by PMA.

Whole leaf juice of *Aloe vera* had no effect on neutrophils whereas PMA had a high effect on neutrophils, there is a significant difference between these two groups.

There is no significant difference between effects of whole leaf juice of *Aloe vera* on neutrophils and effects of whole leaf juice of *Aloe vera* and PMA on neutrophils.

There is a significant difference between effects of PMA on neutrophils and effects of whole leaf juice of *Aloe vera* and PMA on neutrophils

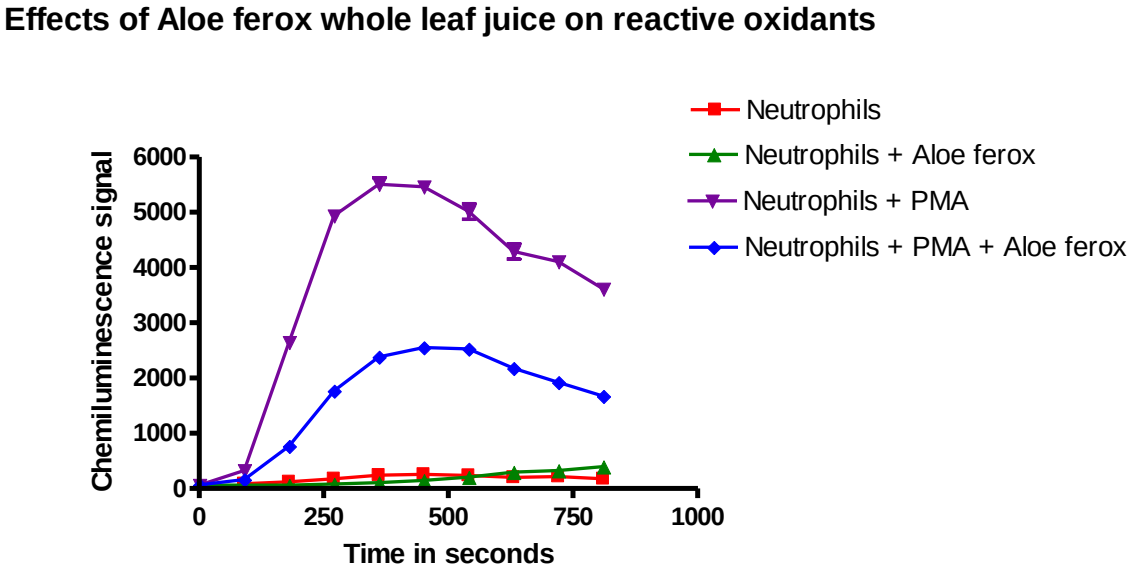


Figure 5.4.4.3. *Aloe ferox* whole leaf juice at a concentration of 125 µg/ml

Table 5.4.4.3.*Aloe ferox* whole leaf juice at a concentration of 125 µg/ml

	P value
Neutrophils vs Neutrophils + *Aloe ferox*	P > 0.05
Neutrophils vs Neutrophils +PMA	P < 0.001
Neutrophils vs Neutrophils + PMA + *Aloe ferox*	P > 0.05
Neutrophils + *Aloe ferox* vs Neutrophils +PMA	P < 0.001
Neutrophils + *Aloe ferox* vs Neutrophils + PMA + *Aloe ferox*	P < 0.05
Neutrophils +PMA vs Neutrophils + PMA + *Aloe ferox*	P < 0.01

Elaboration of the Figure 5.4.4.3 and Table 5.4.4.3

Whole leaf juice of *Aloe ferox* had no effect on neutrophils.

PMA had significantly stimulated neutrophils to release reactive oxygen species.

Whole leaf juice of *Aloe ferox* reduced reactive species produced by PMA. However this is not statistically significant.

Whole leaf juice of *Aloe ferox* had no effect on neutrophils whereas PMA had a high effect on neutrophils, there is a significant difference between these two groups.

There is significant difference between effects of whole leaf juice of *Aloe ferox* on neutrophils and effects of whole leaf juice of *Aloe ferox* and PMA on neutrophils.

There is a significant difference between effects of PMA on neutrophils and effects of whole leaf juice of *Aloe ferox* and PMA on neutrophils.

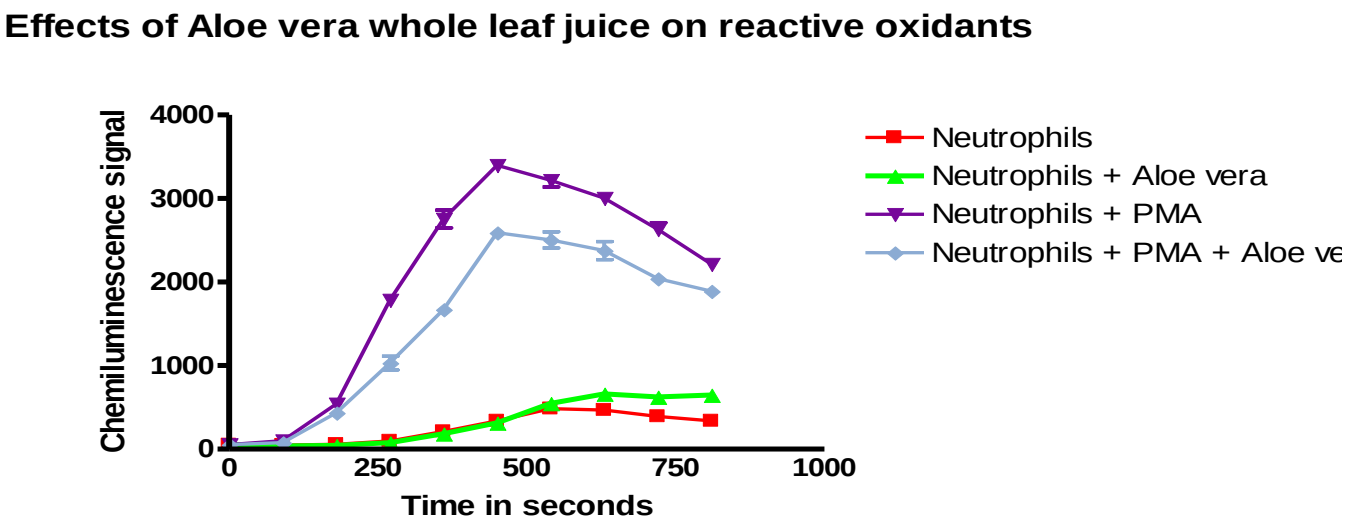


Figure 5.4.4.4. *Aloe vera* whole leaf juice at a concentration of 125 µg/ml

Table 5.4.4.4. *Aloe vera* whole leaf juice at a concentration of 125 µg/ml

	P value
Neutrophils vs Neutrophils + *Aloe vera*	P > 0.05
Neutrophils vs Neutrophils + PMA	P < 0.001
Neutrophils vs Neutrophils + PMA + *Aloe vera*	P < 0.05
Neutrophils + *Aloe vera* vs Neutrophils + PMA	P < 0.001
Neutrophils + *Aloe vera* vs Neutrophils + PMA + *Aloe vera*	P < 0.05
Neutrophils +PMA vs Neutrophils + PMA + *Aloe vera*	P > 0.05

Elaboration of the Figure 5.4.4.4 and Table 5.4.4.4

Whole leaf juice of *Aloe vera* had an insignificant effect on neutrophils.

PMA had significantly stimulated neutrophils to release reactive oxygen species.

Whole leaf juice of *Aloe vera* had significantly reduced reactive species produced by PMA.

Whole leaf juice of *Aloe vera* had no effect on neutrophils whereas PMA had a high effect on neutrophils, there is a significant difference between these two groups.

There is significant difference between effects of whole leaf juice of *Aloe vera* on neutrophils and effects of whole leaf juice of *Aloe vera* and PMA on neutrophils.

There is no significant difference between effects of PMA on neutrophils and effects of whole leaf juice of *Aloe vera* and PMA on neutrophils.

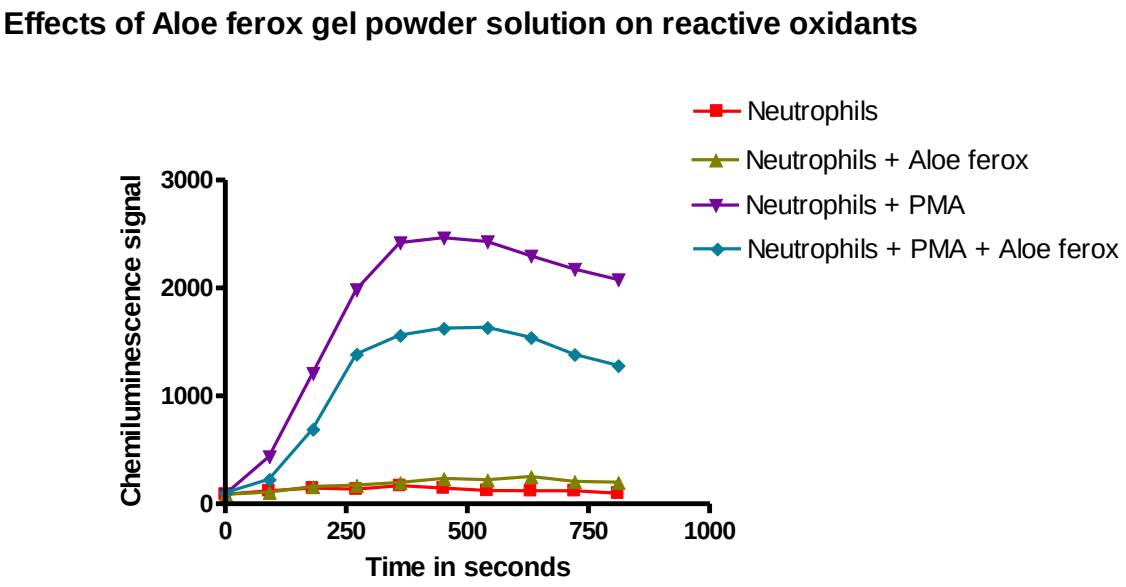


Figure 5.4.4.5. *Aloe ferox* solvent extracted gel powder at a concentration of 50 µg/ml

Table 5.4.4.5.*Aloe ferox* solvent extracted gel powder at a concentration of 50 µg/ml

	P value
Neutrophils vs Neutrophils + *Aloe ferox*	P > 0.05
Neutrophils vs Neutrophils +PMA	P < 0.001
Neutrophils vs Neutrophils + PMA + *Aloe ferox*	P < 0.001
Neutrophils + *Aloe ferox* vs Neutrophils +PMA	P < 0.001
Neutrophils + *Aloe ferox* vs Neutrophils + PMA + *Aloe ferox*	P < 0.01
Neutrophils +PMA vs Neutrophils + PMA + *Aloe ferox*	P > 0.05

Elaboration of the Figure 5.4.4.5 and Table 5.4.4.5

Solvent extracted gel powder of *Aloe ferox* had no effect on neutrophils.

PMA had significantly stimulated neutrophils to release reactive oxygen species.

Solvent extracted gel powder of *Aloe ferox* had significantly reduced reactive species produced by PMA.

Solvent extracedel powder s of *Aloe ferox* had no effect on neutrophils whereas PMA had a high effect on neutrophils, there is a significant difference between these two groups.

There is significant difference between effects of solvent extracted gel of *Aloe ferox* on neutrophils and effects of solvent extracted gel powder of *Aloe ferox* and PMA on neutrophils.

There is no significant difference between effects of PMA on neutrophils and effects of solvent extracted gel powder solution of *Aloe ferox* and PMA on neutrophils.

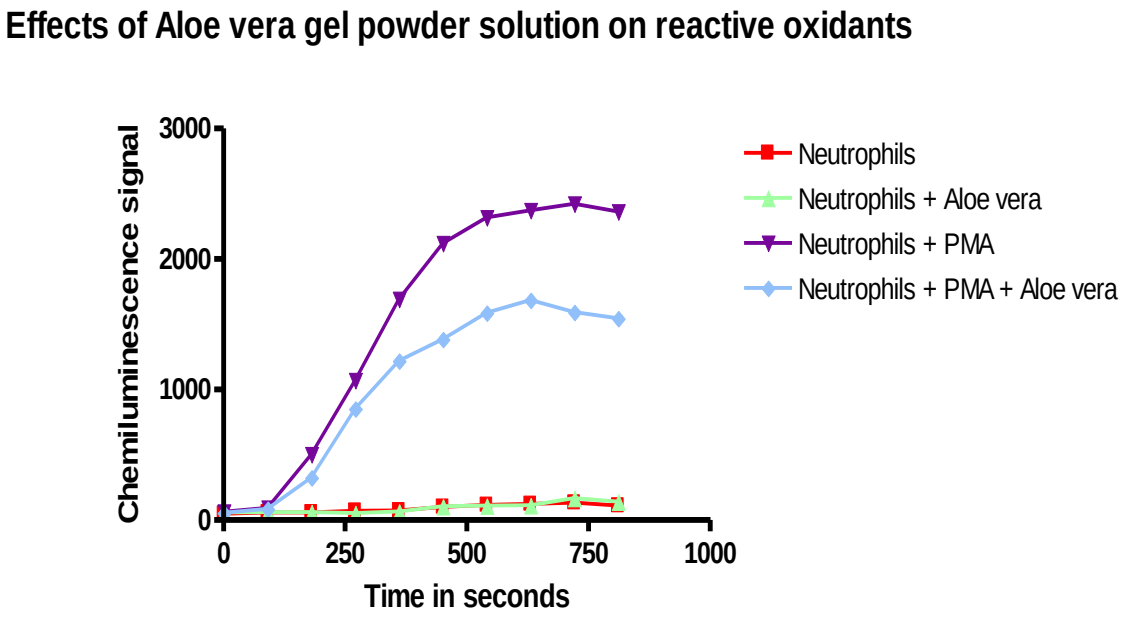


Figure 5.4.4.6. *Aloe vera* solvent extracted gel powder at a concentration of 50 µg/ml

Table 5.4.4.6.*Aloe vera* solvent extracted gel powder at a concentration of 50 µg/ml

	P value
Neutrophils vs Neutrophils + *Aloe vera*	P > 0.05
Neutrophils vs Neutrophils +PMA	P < 0.001
Neutrophils vs Neutrophils + PMA + *Aloe vera*	P < 0.01
Neutrophils + *Aloe vera* vs Neutrophils +PMA	P < 0.001
Neutrophils + Aloe vera vs Neutrophils + PMA + *Aloe vera*	P < 0.01
Neutrophils +PMA vs Neutrophils + PMA + *Aloe vera*	P > 0.05

Elaboration of the Figure 5.4.4.6 and table 5.4.4.6

Solvent extracted gel powder of *Aloe vera* had no effect on neutrophils.

PMA had significantly stimulated neutrophils to release reactive oxygen species.

Solvent extracted gel powder of *Aloe vera* had significantly reduced reactive species produced by PMA.

Solvent extracted gel powder of *Aloe vera* had no effect on neutrophils whereas PMA had a high effect on neutrophils, there is a significant difference between these two groups.

There is significant difference between effects of gel powder solution of *Aloe vera* on neutrophils and effects of solvent extracted gel powder of *Aloe vera* and PMA on neutrophils. There is no significant difference between effects of PMA on neutrophils and effects of solvent extracted gel powder of *Aloe vera* and PMA on neutrophils.

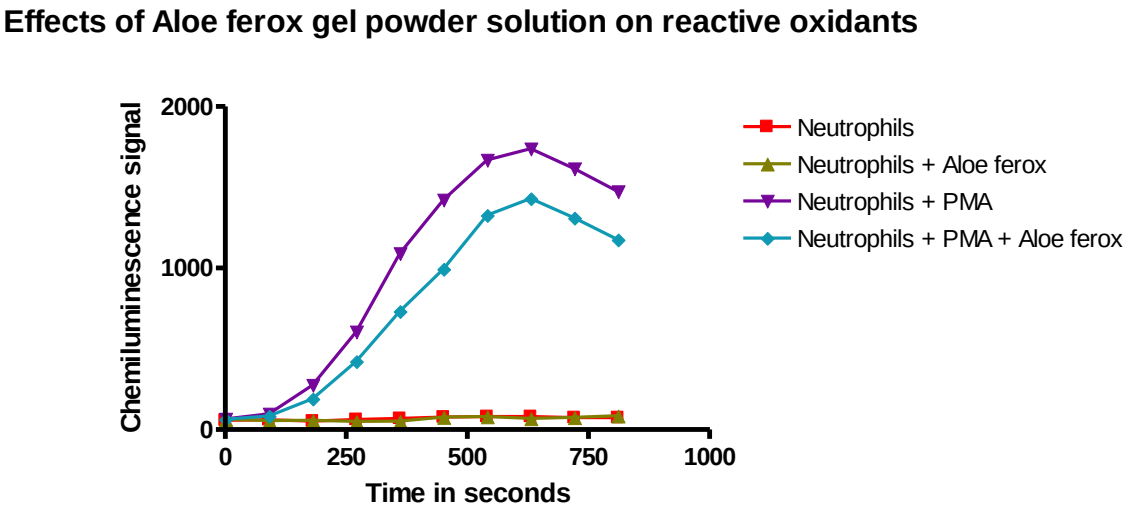


Figure 5.4.4.7.*Aloe ferox* Solvent extracted gel powder at a concentration of 25 µg/ml

Table 5.4.4.7.*Aloe ferox* solvent extracted gel powder at a concentration of 25 µg/ml

	P value
Neutrophils vs Neutrophils + *Aloe ferox*	P > 0.05
Neutrophils vs Neutrophils +PMA	P < 0.001
Neutrophils vs Neutrophils + PMA + *Aloe ferox*	P < 0.01
Neutrophils + *Aloe ferox* vs Neutrophils +PMA	P < 0.001
Neutrophils + *Aloe ferox* vs Neutrophils + PMA + *Aloe ferox*	P < 0.01
Neutrophils +PMA vs Neutrophils + PMA + *Aloe ferox*	P >0.05

Elaboration of the Figure 5.4.4.7 and table 5.4.4.7

Solvent extracted gel powder of *Aloe ferox* had no effect on neutrophils.

PMA had significantly stimulated neutrophils to release reactive oxygen species.

Solvent extracted gel powder of *Aloe ferox* had significantly reduced reactive species produced by PMA.

Solvent extracted gel powder of *Aloe ferox* had no effect on neutrophils whereas PMA had a high effect on neutrophils, there is a significant difference between these two groups.

There is significant difference between effects of gel powder solution of *Aloe ferox* on neutrophils and effects of solvent extracted gel powder of *Aloe ferox* and PMA on neutrophils.

There is no significant difference between effects of PMA on neutrophils and effects of solvent extracted gel powder of *Aloe ferox* and PMA on neutrophils.

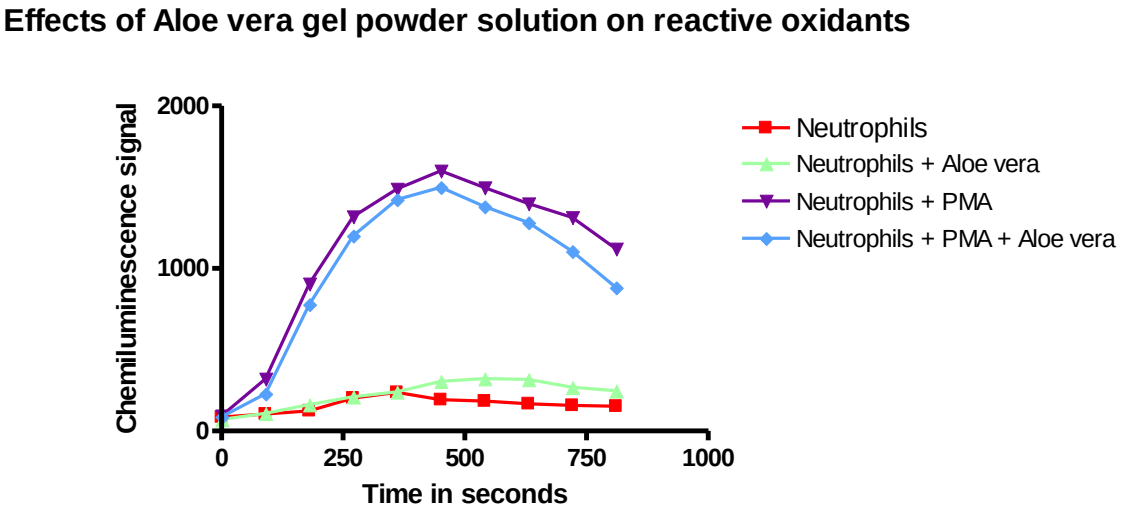


Figure 5.4.4.8. *Aloe vera* solvent extracted gel powder at a concentration of 25 µg/ml

Table 5.4.4.8.*Aloe vera* solvent extracted gel powder at a concentration of 25 µg/ml

	P value
Neutrophils vs Neutrophils + *Aloe vera*	P > 0.05
Neutrophils vs Neutrophils +PMA	P < 0.001
Neutrophils vs Neutrophils + PMA + *Aloe vera*	P < 0.001
Neutrophils + *Aloe vera* vs Neutrophils +PMA	P < 0.001
Neutrophils + *Aloe vera* vs Neutrophils + PMA + *Aloe vera*	P < 0.001
Neutrophils +PMA vs Neutrophils + PMA + *Aloe vera*	P > 0.05

Elaboration of the Figure 5.4.4.8 and table 5.4.4.8

Solvent extracted gel powder solution of *Aloe vera* had no effect on neutrophils.

PMA had significantly stimulated neutrophils to release reactive oxygen species.

Solvent extracted gel of *Aloe vera* had significantly reduced reactive species produced by PMA.

Solvent extracted gel of *Aloe vera* had no effect on neutrophils whereas PMA had a high effect on neutrophils, there is a significant difference between these two groups.

There is significant difference between effects of solvent extracted gel of *Aloe vera* on neutrophils and effects of solvent extracted gel of *Aloe vera* and PMA on neutrophils.

There is no significant difference between effects of PMA on neutrophils and effects of solvent extracted gel of *Aloe vera* and PMA on neutrophils.

5.4.5. Discussion

In all the experiments neither whole leaf juice nor solvent extracted gel of *Aloe ferox* or *Aloe vera* had an effect on neutrophils, thus whole leaf juice and solvent extracted gel of *Aloe ferox* and *Aloe vera* do not have stimulation effects, pro-oxidant effects on neutrophils and they are not cytotoxic towards these cells.

In all the experiments PMA has stimulated neutrophils to release reactive oxygen species, thus it has strong stimulating effects resulting in release of strong pro-oxidants by neutrophils as already shown by DeChaletelet LR *et al* [97].

Whole leaf juice of *Aloe ferox* at a concentration of 250 µg/ml and 125 µg/ml had significantly reduced the effects of PMA on neutrophils, thus is might have antioxidant properties or may interfere with the stimulation mechanism of the PMA used as the activator of the oxidative burst. Which process of reduction of reactive oxygen species cannot be determined from the data as there were no selective inhibitors of the process assessed.

Also if whole leaf juice of *Aloe ferox* have reacted with the reactive oxygen species directly, it is not known particularly which reactive oxygen species are reduced however, PMA has been proven to stimulate neutrophils to release superoxide anion, hydrogen peroxide [98] and

hydroxyl radical which can be obtained from a combination of these two radicals [99;100], thus it could be any of these radicals.

Solvent extracted gel of *Aloe ferox* at a concentration of 50 µg/ml and 25 µg/ml had reduced the effects of PMA on neutrophils however, this is not a statistically significant decrease.

Whole leaf juice of *Aloe vera* at a concentration of 250 µg/ml had significantly reduced the effects of PMA on neutrophils while there was no significant effect at a concentration of 125 µg/ml. The process of radical scavenging is also not clear.

Solvent extracted gel of *Aloe vera* at a concentration of 50 µg/ml and 25 µg/ml had reduced the effects of PMA on neutrophils slightly however, this is not statistically significant.

Based on the results obtained solvent extracted gel of *Aloe vera* and *Aloe ferox* at the above concentration have no radical scavenging properties.

Two dihydrocoumarin derivatives isolated from *Aloe vera* leaf juice showed antioxidant activity against superoxide generated by the hypoxanthine/ xanthine oxidase (XOD) system and hydroxyl radicals generated from Fenton's mixture that was composed of ferrous ion and hydroxyl peroxide [104].

APS-1 which is a polysaccharide isolated from *Aloe vera* leaf juice and composes of mannose and glucose showed antioxidant activity against superoxide generated in a Phenazin methosulfate (PMS)/ h-nicotinamide adenine dinucleotide system (NADH) [105].

A glycoprotein fraction isolated from *Aloe vera* also showed radical scavenging activity against superoxide anion generated by the xanthine-xanthine oxidase system [19].

Aloe vera gel showed radical scavenging activity against superoxide anion generated by the xanthine-xanthine oxidase system [2].

According to the literature different components of *Aloe vera* have superoxide and hydroxyl radical scavenging properties. Perhaps whole leaf juice of *Aloe vera* at a concentration of 250 µg/ml has reacted directly with reactive oxygen species and superoxide and hydroxyl

radical were scavenged. Solvent extracted gel of *Aloe vera* did not have any significant effect on reactive oxygen species, perhaps the required ingredients have been lost during the preparation of gel powder.

5.4.6. Conclusion

Whole leaf juice of *Aloe ferox* and *Aloe vera* have demonstrated radical scavenging properties at high concentrations, whereas solvent extracted gel powder solution of the two aloe species had no significant effect on reactive oxygen species released by neutrophils.

Chapter 6

General conclusion

Water extracts of whole leaf juice and solvent extracted gel powders from *Aloe ferox* and *Aloe vera* without bitter juice have significant inhibitory effects on human lymphocytes, these results are contrary to what has been reported in the literature.
As this is a unique study where the samples to be tested were specially prepared to have no bitter components, there is possibilities that major components that show some of the advantageous properties of Aloe extracts were removed, destroyed or converted into toxic compounds during the preparation phase. Highly concentrated test samples demonstrated a high cytotoxic effect, thus for future studies lower concentrations of the different preparations should be used.

Water extracts of whole leaf juice and solvent extracted gel powders from *Aloe ferox* and *Aloe vera* without bitter component have significant inhibitory effects on chicken fibroblasts at the concentrations tested.

According to the literature [54,56] the bitter component seems to contain more compounds which promote proliferation of chicken fibroblasts thus contributing positively to the wound healing process. In this experiment the aim was to determine if the samples can be used orally without laxative effects. Based on results obtained, samples may not be effective when used orally without the bitter components, unless a specific technique may be discovered that could retain all major components and yet the laxative effects are avoided.

Thus it will be more effective to prepare the samples without removing the bitter components and the samples can be used topically.

According to the results obtained all samples are inhibitory to chicken fibroblast at almost all concentrations tested, Thus the method used for the preparation of the tested samples should not be used for future experiments where wound healing is investigated as there is a high potential to inhibit or kill off fibroblasts thus aggravating the wounds.

According to the literature, different isolated components of the two Aloe species have been proven to have stimulatory effects on different cells in the body [43, 54, 57, 58 and 59]. Thus it is more effective to use isolated components than to use the crude extracts although this is not a generally applicable concept. Inhibitory effects seen during this study with respect to both human lymphocytes and chicken fibroblasts could be due to the combination of unknown components which could be working against each other.
Water, Chloroform and Ethyl acetate extracts of whole leaf juice and solvent extracted gel powders from *Aloe ferox* and *Aloe vera* without bitter components did not demonstrate any antimicrobial properties.

According to the literature [67 and 68] bitter components seem to contain more compounds which possess antimicrobial properties. Thus for bacterial or fungal treatment, it will be more effective to prepare the samples without removing the bitter components so the samples can be used topically to fight infections particularly during wound healing.

Different isolated compounds from the two Aloe species have been proven to have significant antimicrobial properties against different microorganisms [64, 66]. Thus it could be more effective to use isolated components. The lack of antimicrobial properties noted for whole leaf juice and solvent extracted gel powder from both the Aloe species could be from a lack of solubility but could also be due to the combination of several unknown components which could have been deactivated during the preparation phase.

Most of the results obtained for extracts from *Aloe ferox* and *Aloe vera* have only exhibited antimicrobial properties at high concentrations. In this experiment the concentrations obtained during the preparation were less than many of those reported in the literature, and perhaps this could be a reason for not finding antimicrobial properties.
Water extracts of whole leaf juice and solvent extracted gel powders from *Aloe ferox* and *Aloe vera* without bitter juice have partial antioxidant properties as demonstrated by the ORAC and chemiluminescence assay, however when using TEAC and DPPH assay, no antioxidant properties were observed and in fact there were indications of pro-oxidative effects.

Concentrations tested that have demonstrated significant antioxidant properties on ORAC assay have exhibited significant inhibitory effects on both human lymphocyte and chicken

fibroblast proliferation. Thus even though a high antioxidant level has been obtained, preparation methods and concentrations used in this study could not be achieved in vivo and should therefore not be used for future studies as they are toxic to body cells. Thus a specific method that will exhibit both antioxidant and cell proliferation effects is still required.

According to the literature [83, 84] most of the components that have antioxidant properties are found within bitter components. This could be the reason why TEAC and DPPH assays did not exhibit any antioxidant properties from all the samples tested.

Whole leaf juice and solvent extracted gel powder of the two Aloe species did not have any effect on measured oxidants when tested on neutrophils for any of the concentrations tested, this might mean that the samples in addition to not stimulating the neutrophils do not have any pro-oxidant properties as this would have increased the detected oxidants through the chemiluminescent method.

At the highest concentrations tested, whole leaf juice of the two Aloe species exhibited antioxidant properties when tested on reactive oxygen species produced from stimulated neutrophils, Thus even though bitter juice was removed there was still potent antioxidant properties.

At the highest concentrations tested, solvent extracted gel powder of the two Aloe species exhibited antioxidant properties when tested on reactive oxygen species produced from the respiratory burst stimulated neutrophils, however this was not statistical significant, thus at high concentration the results might be statistically significant.

Generally the preparation method for all the samples used exhibited less positive results which are in contrast to the current literature. The aim of the study was to determine the effects of the extracts on the following properties: human lymphocyte proliferation, chicken fibroblast proliferation (for a potential wound healing model), antimicrobial and antioxidant properties so that samples can be used orally without laxative properties. According to this study, the four samples could not be used orally to achieve the desired effects of a “drug” without laxative effects. This would mean that for future studies a different method for sample preparation will be required.

From this study it becomes evident that it would be difficult to obtain positive results from *Aloe ferox* and *Aloe vera* without bitter components being present in the extracts. According to the literature *Aloe ferox* and *Aloe vera* (with bitter components included), have growth stimulatory effects on different cells, however in this study all samples at most concentrations tested exhibited inhibitory effects. Perhaps bitter components contain certain compounds which protect cells rather than inhibiting them.

In this study, ORAC and chemiluminence assay exhibited potent antioxidant properties whereas DPPH and TEAC exhibited slight pro-oxidant properties. However the exhibited antioxidant properties when using the chemiluminescence only become significant at concentrations that would not be physiologically attainable and at those concentrations the extracts exhibited cytotoxic effects towards human lymphocytes and chicken fibroblasts.

Generally *Aloe ferox* is more toxic to human lymphocytes and chicken fibroblasts than *Aloe vera*. However *Aloe ferox* exhibited much greater antioxidant properties than *Aloe vera* as noted from ORAC and Chemiluminescence assays.

In this study, the objectives were to investigate the following properties: proliferative effects of human lymphocytes, potential wound healing effects by stimulating chicken fibroblasts, antimicrobial and antioxidants properties. All the objectives were met out as per the original protocol. However most of the results obtained were contrary to the bulk of the literature available about these beneficial plant's extracts. The removal of the bitter components, that have been reported to stimulate human lymphocytes and fibroblasts and to have antimicrobial and antioxidant properties has been suggested as the main reason why the effects of the tested extracts did not correspond to much of the reported literature. From the results obtained from various aspects of this study it could be concluded that the removal of bitter components contributed to the apparently contradictory results.

From this study it might be concluded that the four samples tested could not be used therapeutically for wound healing, antimicrobial or antioxidant properties. However they could still be effective for cosmetics purposes as noted from the literature.

6. References

1. Grindlay D, Reynolds T. The Aloe vera phenomenon: A review of the properties and modern uses of the leaf parenchyma gel. Journal of Ethnopharmacology 1986; 16: 117-51.

2. Langmead L, Makins RJ, Rampton DS. Anti-inflammatory effects of aloe vera gel in human colorectal mucosa in vitro. Alimentary Pharmacology & Therapeutics 2004; 19: 521-7.

3. Agarry OO. Comparative antimicrobial activities of Aloe vera gel and leaf. S.frican Journal of Biotechnology 2005; 4: 1413-4.

4. Steenkamp V, Stewart MJ. Medicinal Applications and Toxicological Activities of Aloe. Products. Pharmaceutical Biology 2007; 45: 411-20.

5. Joseph B and Justin S. Pharmacognostic and phytochemical properteis of Aloe vera Linn-An overview. International Journal of Pharmaceutical 2010; 4: 106-10.

6. Josias HH. Composition and Applications of Aloe vera Leaf Gel. Molecules 2008; 13: 1599-616.

7. Hutter JA, Salman M, Stavinoha WB *et al.* Antiinflammatory C-Glucosyl Chromone from Aloe barbadensis. Journal of Natural Products 1996; 59: 541-3.

8. Lee JK, Lee MK, Yun YP *et al.* Acemannan purified from Aloe vera induces phenotypic and functional maturation of immature dendritic cells. International Immunopharmacology 2001; 1: 1275-84.

9. Duansak D, Somboonwong J, , Patumraj S. Effects of Aloe vera on leukocyte adhesion and TNF-a and IL-6 levels in burn wounded rats. Clinical Hemorheology and Microcirculation 2003; 29: 239-46.

10. Choi SW, Son BW, Son YS, Park YI, Lee SK, Chung MH. The wound-healing effect of a glycoprotein fraction isolated from aloe vera. British Journal of Dermatology 2001; 145: 535-45.

11. Habeeb F, Stables G, Bradbury F *et al.* The inner gel component of Aloe vera suppresses bacterial-induced pro-inflammatory cytokines from human immune cells. Methods 2007; 42: 388-93.

12. Howland RD. Anti-inflammatory drugs and Autacoids. In: Pharmacology. Philadelphia: Lippincott Willliams and Wilkins, Series editors: Richard A Harvey and Pamela C Champe 2006; 495-9.

13. Bezakova L. Antilipoxygenase activity and the trace elements content of *Aloe vera* in relation to the therapeutical effect. Ceska a Slovenska Farmacie 2005; 54: 43-6.

14. Yusuf S, Agunu A, Diana M. The effect of Aloe vera A. Berger (Liliaceae) on gastric acid secretion and acute gastric mucosal injury in rats. Journal of Ethnopharmacology 2004; 93: 33-7.

15. Vízquez B, Avila G, Segura D, Escalante B. Antiinflammatory activity of extracts from Aloe vera gel. Journal of Ethnopharmacology 1996; 55: 69-75.

16. Ghannam N, Kingston M, Meshaal IA, Tariq M, Parman NS, Woodhouse N. The Antidiabetic Activity of Aloes: Preliminary Clinical and Experimental Observations. Hormone Research in Paediatrics 1986; 24: 288-94.

17. Okyar A, Can A, Akev N, Baktir G, Sutlupinar N. Effect of Aloe vera leaves on blood glucose level in type I and type II diabetic rat models. Phytotherapy Research 2001; 15: 157-61.

18. Rajasekaran S. Antioxidant effect of Aloe vera gel extract in streptozotocin-induced diabetes in rats. Pharmacol Rep 2005; 57: 90-6.

19. Yagi A. Radical scavenging glycoprotein inhibiting cyclooxygenase-2 and thromboxane A2 synthase from Aloe vera gel. Planta Medica. 2003; 69: 269-71.

20. Chen X, Zha D, Xiu J *et al.* A new hydrodynamic approach by infusion of drag-reducing polymers to improve left ventricular function in rats with myocardial infarction. International Journal of Cardiology 2011; 147: 112-7.

21. Herlihy JT, Bertrand HA, Kim JD, Ikeno Y, Yu BP. Effects of Aloe vera ingestion in the rat. I. Growth, food and fluid intake and serum chemistry. Phytotherapy Research 1998; 12: 183-8.

22. Ferreira M, Teixeira M, Silva E, Selores M. Allergic contact dermatitis to Aloe vera. Contact Dermatitis 2007; 57: 278-9.

23. Bottenberg MM. Oral Aloe vera-induced hepatitis. Ann.Pharmacother 2007; 41: 1740-3.

24. Lee A. Possible interaction between sevoflurane and Aloe vera. Ann.Pharmacother 2004; 38: 1651-4.

25. Van Wyk, BE and Smith G. *Guide to the Aloes of South Africa*. Briza Publications, Pretoria, 1996.

26. Shackleton CM, Gambiza J. Growth of Aloe ferox Mill. at selected sites in the Makana region of the Eastern Cape. South African Journal of Botany 2007; 73: 266-9.

27. van Wyk BE, van Rheede van Oudtshoorn M, Smith GF. Geographical variation in the major compounds of Aloe ferox leaf exudate. Planta Medica 1995; 61: 250-3.

28. Viljoen A. A chemotaxonomic study of phenolic leaf compounds in the genus Aloe. 1-1-1999. PhD Thesis, Rand Afrikaans University.

29. Mabusela WT, Stephen AM, Botha MC. Carbohydrate polymers from Aloe ferox leaves. Phytochemistry 1990; 29: 3555-8.

30. Loots DT, van der Westhuizen FH, Botes L. Aloe ferox Leaf Gel Phytochemical Content, Antioxidant Capacity, and Possible Health Benefits. Journal of Agricultural and Food Chemistry 2007; 55: 6891-6.

31. Kametani S, Oikawa T, Kojima-Yuasa A *et al.* Mechanism of Growth Inhibitory Effect of Cape Aloe Extract in Ehrlich Ascites Tumor Cells. Journal of Nutritional Science and Vitaminology 2007; 53: 540-6.

32. Chithra, P., Sajithlal, G. B., and Chandrakasan, G. Influence of Aloe vera on collagen characteristics in healing dermal wounds in rats. Molecular and Cellular Biochemistry 181[1/2], 71-76. 1998.

33. Chithra P, Sajithlal GB, Chandrakasan G. Influence of aloe vera on the healing of dermal wounds in diabetic rats. Journal of Ethnopharmacology 1998; 59: 195-201.

34. Speranza G, Morelli CF, Tubaro A, Altinier G, Manitto P. Aloeresin I, an Anti-Inflammatory 5-Methylchromone from Cape Aloe. Planta Medica 2005; 71: 79-81.

35. Wintola OA. Toxicological evaluation of aqueous extract of Aloe ferox Mill. in loperamide-induced constipated rats. Hum Exp Toxicol 2011; 30: 425-43.

36. Femenia A, Sínchez ES, Simal S, Rossell C. Compositional features of polysaccharides from Aloe vera (Aloe barbadensis Miller) plant tissues. Carbohydrate Polymers 1999; 39: 109-17.

37. Roderick N. Basic concepts and components of the immune system. In: Immunology for medical students. Matthew Helbert. 2002; 4.

38. Robbins. *Pathologic Basis of Disease*. Kumar AF 2004.

39. Ross R. The role of T lymphocytes in inflammation. Proceedings of the National Academy of Sciences of the United States of America 1994; 91: 2879.

40. *Aloe ferox. in Medicinal plants of South Africa*. Herbert, D Published by Briza Publishers Pretoria for the , Department of Agriculture, Forestry and Fisheries, 2006.

41. Yagi A, Egusa T, Arase M, Tanabe M, and Tsuji H. Isolation and characterization of the glycoprotein fraction with a proliferation -promotion activity on human and hamster cells *in vitro* from *Aloe vera* gel. 1997 Planta Medica 63 (91) 18-21..

42. Tim M. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods 1983; 65: 55-63.

43. Abdullah KM, Abdullah A, Johnson ML *et al.* Effects of Aloe vera on Gap Junctional Intercellular Communication and Proliferation of Human Diabetic and Nondiabetic Skin Fibroblasts. The Journal of Alternative and Complementary Medicine 2003; 9: 711-8.

44. Winters W, Benavides R, Clouse W. Effects of aloe extracts on human normal and tumor cells in vitro. Economic Botany 1981; 35: 89-95.

45. Nguyen D. The Pathophysiologic Basis for Wound Healing and Cutaneous Regeneration. In: *Biomaterials For Treating Skin Loss*. Woodhead Publishing (UK/Europe) & CRC Press (US), Cambridge/Boca Raton, 2009; 25-57.

46. Pattanayak SP, Sunita P. Wound healing, anti-microbial and antioxidant potential of Dendrophthoe falcata (L.f) Ettingsh. Journal of Ethnopharmacology 2008; 120: 241-7.

47. Stadelmann WK, Digenis AG, Tobin GR. Physiology and healing dynamics of chronic cutaneous wounds. The American Journal of Surgery 1998; 176: 26S-38S.

48. Ni Y, Turner D, Yates K, Tizard I. Stabilization of Growth Factors Relevant to Wound Healing by a Plant Cell Wall Biomaterial. Planta medica 2007; 73: 1260,1266.

49. Montesano R, Orci L. Transforming growth factor beta stimulates collagen-matrix contraction by fibroblasts: implications for wound healing. Proceedings of the National Academy of Sciences 1988; 85: 4894-7.

50. Heggers JP, Pelley RP, Robson MC. Beneficial effects of Aloe in wound healing. Phytotherapy Research 1993; 7: S48-S52.

51. Ratree M. The efficacy of *Aloe vera* used for burn wound healing: A systemic review. Burns 2007; 33: 713-8.

52. Choi SW, Son BW, Son YS, Park YI, Lee SK, Chung MH. The wound-healing effect of a glycoprotein fraction isolated from aloe vera. British Journal of Dermatology 2001; 145: 535-45.

53. Thomas DR, Goode PS, LaMaster K, Tennyson T. Acemannan hydrogel dressing versus saline dressing for pressure ulcers. A randomized, controlled trial. Advances in wound care. Journal for prevention and healing 1998; 11: 273-6.

54. Abdullah KM, Abdullah A, Johnson ML *et al.* Effects of Aloe vera on Gap Junctional Intercellular Communication and Proliferation of Human Diabetic and Nondiabetic Skin Fibroblasts. The Journal of Alternative and Complementary Medicine 2003; 9: 711-8.

55. Jia Y, Zhao G, Jia J. Preliminary evaluation: The effects of *Aloe ferox* Miller and Aloe arborescens Miller on wound healing. Journal of Ethnopharmacology 2008; 120: 181-9.

56. Bozzi A, Perrin C, Austin S, Arce Vera F. Quality and authenticity of commercial aloe vera gel powders. Food Chemistry 2007; 103: 22-30.

57. Roberts, D. B. and Travis, E. L. Acemannan-containing wound dressing gel reduces radiation-induced skin reactions in C3H mice. International Journal of Radiation Oncology 1995, 32[4], 1047-1052..

58. Davis RH, Donato JJ, Hartman GM, Haas RC. Anti-inflammatory and wound healing activity of a growth substance in Aloe vera. Journal of the American Podiatric Medical Association 1994; 84: 77-81.

59. Yao H, Chen Y, Li S, Huang L, Chen W, Lin X. Promotion proliferation effect of a polysaccharide from Aloe barbadensis Miller on human fibroblasts in vitro. International Journal of Biological Macromolecules 2009; 45: 152-6.

60. Monte T. The *Complete Guide to Natural Healing*, Berkley Publishing Group, New York.1997.

61. Tan B. K and Vanitha, J. Immunomodulatory and Antimicrobial Effects of Some Traditional Chinese Medicinal Herbs: A Review. Current Medicinal Chemistry 11[11], 1423-1430. 2004.

62. Richard DH. Principals of antimicrobial therapy. In: Pharmacology. Lippincott's Illustrated Review, Series Editors: Richard A Harvey and Pamela C Champe 2006; 341.

63. Medical Association of South Africa. Guide to medicines and drugs. Infections and Infestations 1992; 122-4.

64. Kambizi L. Extracts from *Aloe ferox* and *Withania somnifera* inhibit *Candida albicans* and *Neisseria gonorrhoea*. African Journal of Biotechnology 2008; 7: 12-15.

65. Lorenzetti LJ, Salisbury R, Beal JL, Baldwin JN. Bacteriostatic property of Aloe vera. Journal of Pharmaceutical Sciences 1964; 53: 1287.

66. Kambizi L, Sultana N, Afolayan AJ. Bioactive Compounds Isolated from Aloe ferox.: A Plant Traditionally Used for the Treatment of Sexually Transmitted Infections in the Eastern Cape, South Africa. Pharmaceutical Biology 2005; 42: 636-9.

67. Thiruppathi S. Antimicrobial activity of *Aloe vera* (L) Burm. F. against pathogenic microorganisms. J.Biosc.Res 2010; 4: 251-8.

68. Franz G. In vitro biosynthesis of the C-glycosidic bond in aloin. Planta 1981; 152: 562-4.

69. Lawrence R, Tripathi P, Jeyakumar E. Isolation, Purification and Evaluation of Antibacterial Agents from Aloe vera. Brazilian Journal of Microbiology 2009; 40: 906-15.

70. Lin J, Opoku AR, Geheeb-Keller M *et al.* Preliminary screening of some traditional zulu medicinal plants for anti-inflammatory and anti-microbial activities. Journal of Ethnopharmacology 1999; 68: 267-74.

71. Shubhi M A. Comparative antimicrobial activities of Neem, Amla, Aloe, Assam tea and clove extracts against *Vibrio Cholerae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Journal of Medicines Plants Research 2010; 4: 2393-8.

72. Rajasekaran S, Sivagnanam K, Subramanian S. Modulatory effects of Aloe vera leaf gel extract on oxidative stress in rats treated with streptozotocin. Journal of Pharmacy and Pharmacology 2005; 57: 241-6.

73. Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. Proceedings of the National Academy of Sciences 1993; 90: 7915-22.

74. Priya T, Sabu M, Jolly C. Free radical scavenging and anti-inflammatory properties of <i>Lagerstroemia speciosa</i> (L). Inflammopharmacology 2008; 16: 182-7.

75. Kountouras J, Chatzopoulos D, Zavos C. Reactive oxygen metabolites and upper gastrointestinal diseases. Hepato-gastroenterology 2001; 48: 743-51.

76. Ozsoy N, Candoken E, Akev N. Implications for degenerative disorders: antioxidative activity, total phenols, flavonoids, ascorbic acid, beta-carotene and beta-tocopherol in Aloe vera. Oxidative medicine and cellular longevity 2009; 2: 99-106.

77. Huang D, Ou B, Prior RL. The Chemistry behind Antioxidant Capacity Assays. Journal of Agricultural and Food Chemistry 2005; 53: 1841-56.

78. Noguchi N, Takahashi M, Tsuchiya J, Yamashita H, Komuro E, Niki E. Action of 21-Aminosteroid U74006F as an Antioxidant Against Lipid Peroxidation. Biochemical Pharmacology 1998; 55: 785-91.

79. Culbertson SM, Porter NA. Unsymmetrical Azo Initiators Increase Efficiency of Radical Generation in Aqueous Dispersions, Liposomal Membranes, and Lipoproteins. Journal of the American Chemical Society 2000; 122: 4032-8.

80. Kazuo K. Differential effects of flavonoid quercetin on oxidative damages induced by hydrophilic and lipophilic radical generators in hepatic lysosomal fractions of mice. Journal of health science 2000; 46: 509-12.

81. Zheng W, Wang SY. Antioxidant Activity and Phenolic Compounds in Selected Herbs. Journal of Agricultural and Food Chemistry 2001; 49: 5165-70.

82. Mark S.M.. *Polymer science dictionary*. Springer, 1997; 152-161.

83. Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. LWT - Food Science and Technology 1995; 28: 25-30.

84. Yagi A, Kabash A, Okamura N, Haraguchi H, Moustafa SM, Khalifa TI. Antioxidant, Free Radical Scavenging and Anti-Inflammatory Effects of Aloesin Derivatives in Aloe vera. Planta medica 2002; 68: 957,960.

85. Hu Y, Xu J, Hu Q. Evaluation of antioxidant potential of aloe vera (Aloe barbadensis miller) extracts. Journal of Agricultural and Food Chemistry 2003; 51: 7788-91.

86. Hu Q, Hu Y, Xu J. Free radical-scavenging activity of Aloe vera (Aloe barbadensis Miller) extracts by supercritical carbon dioxide extraction. Food Chemistry 2005; 91: 85-90.

87. Sonia Miladi MD. In vitro antioxidant activities of Aloe vera leaf skin extracts. Journal de la Société Chimique de Tunisie 2008; 10: 101-9.

88. Tin A.Khaing. Evaluation of the Antifungal and Antioxidant Activities of the Leaf Extract of *Aloe vera* (*Aloe barbadensis* Miller). World Academy of Science, Engineering and Technology 2011; 75:611

89. Fawole OA, Amoo SO, Ndhlala AR, Light ME, Finnie JF, van Staden J.
Anti-inflammatory, anticholinesterase, antioxidant and phytochemical properties of medicinal plants used for pain-related ailments in South Africa. Journal of Ethnopharmacology 2010; 127: 235-41.

90. Arts MJ, Haenen GR, Voss HP, Bast A. Antioxidant capacity of reaction products limits the applicability of the Trolox Equivalent Antioxidant Capacity (TEAC) assay. Food and Chemical Toxicology 2004; 42: 45-9.

91. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine 1999; 26: 1231-7.

92. Lee BW, Lee JH, Gal SW, Moon YH, Park KH. Selective ABTS radical-scavenging activity of prenylated flavonoids from Cudrania tricuspidata. Bioscience, biotechnology, and biochemistry 2006; 70: 427-32.

93. Segal AW. How neutrophils kill microbes. Annual review of immunology 2005; 23: 197-223.

94. Lundqvist H, Dahlgren C. Isoluminol-enhanced chemiluminescence: A sensitive method to study the release of superoxide anion from human neutrophils. Free Radical Biology and Medicine 1996; 20: 785-92.

95. Patel RP. The biochemistry of nitric oxide and peroxynitrite: implications for mitochondrial function. In: *Understanding the process of aging: the roles of mitochondria, free radicals, and antioxidants*. New York, N.Y: Marcel Dekker, 1999; 39-56.

96. Ian Weeks. Introduction. In: Chemiluminescence immunoassay. Libecap1992; 1.

97. Parij N, Nagy AM, Fondu P. Effects of non-steroidal anti-inflammatory drugs on the luminol and lucigenin amplified chemiluminescence of human neutrophils. European Journal of Pharmacology 1998; 352: 299-305.

98. DeChatelet LR, Shirley PS, Johnston RJ. Effect of phorbol myristate acetate on the oxidative metabolism of human polymorphonuclear leukocytes. Blood 1976; 47: 545-54.

99. Imada I, Sato EF, Miyamoto M. Analysis of Reactive Oxygen Species Generated by Neutrophils Using a Chemiluminescence Probe L-012. Analytical Biochemistry 1999; 271: 53-8.

100. Koppenol WH. The Haber-Weiss cycle--70 years later. Redox report : communications in free radical research 2001; 6: 229-34.

101. Hart LA, van Enckevort PH, van Dijk H, Zaat R, De Silva KTD, Labadie RP. Two functionally and chemically distinct immunomodulatory compounds in the gel of aloe vera. Journal of Ethnopharmacology 1988; 23: 61-71.

102. Hart LA, Nibbering PH, van den Barselaar MT, van Dijk H, van den Berg AJJ, Labadie RP. Effects of low molecular constituents from Aloe vera gel on oxidative metabolism and cytotoxic and bactericidal activities of human neutrophils. International Journal of Immunopharmacology 1990; 12: 427-34.

103. Jain A, Basal E. Inhibition of Propionibacterium acnes-induced mediators of inflammation by Indian herbs. Phytomedicine 2003; 10: 34-8.

104. Zhang Xf, Wang Hm, Song Yl *et al.* Isolation, structure elucidation, antioxidative and immunomodulatory properties of two novel dihydrocoumarins from Aloe vera. Bioorganic & Medicinal Chemistry Letters 2006; 16: 949-53.

105. Wu JH, Xu C, Shan CY, Tan RX. Antioxidant properties and PC12 cell protective effects of APS-1, a polysaccharide from Aloe vera var. chinensis. Life Sciences 2006; 78: 622-30.

Appendix

Ethics approval letters

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1908 - 2008

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee
Fakulteit Gesondheidswetenskappe Navorsingsetiekkomitee

DATE: 27/08/2009

Prof V Steenkamp
Department of Pharmacology
University of Pretoria

Best Prof Vanessa Steenkamp

RE.: Application for Blood Collection utilizing lymphocytes, macrophage, neutrophils and plasma.

Herewith acknowledgement that the above Application for blood collection has been received and tabled on 26/08/2009, and found to be acceptable by the Faculty of Health Sciences Research Ethics Committee.

With regards

DR R SOMMERS; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

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Gisela

Animal Use and Care Committee

Dr Roland Auer
Acting AUCC Co-ordinator: Faculty of Health Sciences

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University of Pretoria

Prof C Medlen
Department of Pharmacology
Faculty of Health Sciences
BMW Building, Prinshof Campus
PO Box 2034
Pretoria
0001

June 27, 2006

Dear Prof Medlen,

Protocol H22/06 - The use of chicken embryo fibroblast cultures in toxicity assays

The Animal Use and Care Committee approved the above-mentioned request at a meeting held on Monday, June 26, 2006.

The AUCC however requested that you report the number of embryonated eggs used at the end of the year.

Please contact this office should you have any questions.

Yours sincerely,

Dr Daan Verwoerd
Chair: AUCC

Cc Prof Medlen