

Antimicrobial Activity of *Aloe barbadensis* Miller Leaf Gel Components

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Table of Contents

Abstract

Introduction

Material and Methods

Results and Discussion

References

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Abstract

Methanolic extracts of *Aloe barbadensis* Miller inner leaf gel were fractionated by RP-HPLC and the resultant fractions were tested for inhibitory activity against a panel of bacteria and fungi. Five fractions were identified as having antimicrobial activity. Fraction 1 had the broadest antibacterial activity, being capable of inhibiting growth of both Gram-positive and Gram-negative bacteria as well as inhibiting

growth of a nystatin resistant strain of the fungus *Aspergillus niger*. Fraction 1 had similar UV spectral properties as aloe emodin and was chromatographically identical to the pure compound. The other fractions tested were much more selective in their antimicrobial activities, being only capable of inhibiting the growth of specific Gram-negative rod bacteria. Two of these antimicrobial fractions were identified by ESI mass spectroscopy as being isomers of 8-C- β -D-[2-O-(E)-coumaroyl] glucopyranosyl - 2-[2-hydroxy]-propyl-7-methoxy-5-methylchromone.

Key words: *Aloe barbadensis* Miller, Aloe vera, antibacterial, antifungal, aloe emodin, anthraquinone

Introduction

Bacterial resistance to antibiotics is increasingly becoming a concern to public health. Currently used antibiotic agents are failing to bring an end to many bacterial infections due to super resistant strains. For this reason the search is ongoing for new antimicrobial agents, either by the design and synthesis of new agents or through the search of natural sources for as yet undiscovered antimicrobial agents. Herbal medications in particular have seen a revival of interest due to a perception that there is a lower incidence of adverse reactions to plant preparations compared to synthetic pharmaceuticals. Coupled with the reduced costs of plant preparations, this makes the search for natural therapeutics an attractive option.

Aloe barbadensis Miller (Aloe vera) has a long history of use as a therapeutic agent with many reported medicinal properties. Amongst its therapeutic properties, it has been shown to have anti-inflammatory activity (Azfal et al., 1991; Malterud et al., 1993) immunostimulatory activity (Ramamoorthy and Tizard, 1998), and cell growth stimulatory activity (Tizard et al., 1994; Rodriguez-Bigas, 1988). Furthermore, activity against a variety of infectious agents has been attributed to Aloe vera; for instance, antibacterial (Ferro et al., 2003), antiviral (Kahlon et al., 1991) and anti fungal (Kawai et al., 1998).

Despite the therapeutic possibilities of this plant, there have been limited reports on the antimicrobial effects of isolated Aloe vera components. Ferro et al. (2003) have shown that Aloe vera leaf gel can inhibit the growth of the two Gram-positive bacteria *Shigella flexneri* and *Streptococcus progenes*. Specific plant compounds such as anthraquinones (Garcia-Sosa et al., 2006; Dabai et al., 2007) and dihydroxyanthraquinones (Wu et al., 2006), as well as saponins (Reynolds and Dweck, 1999), have been proposed to have direct antimicrobial activity. Acemannan, a polysaccharide component from whole plant material, has been proposed to have indirect antimicrobial activity through its ability to

stimulate phagocytic leukocytes (Pugh et al., 2001). Wang et al. (1998) have reported on the effect of the anthraquinone aloe emodin on arylamine N-acetyl transferase activity in *Helicobacter pylori*, and hence its antimicrobial activity. The purpose of this study is to test HPLC separated Aloe vera gel components against a comprehensive panel of microbes to characterise their antimicrobial activities. Furthermore, this report details the extraction, fractionation and partial identification of the antimicrobial leaf gel fractions.

Materials and Methods

Chemical Reagents

Aloe emodin (Sigma, purity >95%) was prepared freshly before use by dissolving in distilled water to give a concentration of 500 $\mu\text{g ml}^{-1}$. Aloe vera juice was obtained from Aloe Wellness Pty Ltd, Australia and was stored at 4 °C until use. Aloe vera juice was used undiluted in the antimicrobial assay.

Plant Material

Fresh clean whole *A. barbadensis* leaves were supplied by Aloe Wellness Pty Ltd, Australia. The leaves were cut and the outer green rind was discarded. The mucilaginous inner pulp was minced and thoroughly homogenised with a hand held blender. Each leaf produced approximately 120 ml of gel. The homogenised gel was lyophilised in vacuo at 22 °C and the resultant lyophilised material was stored frozen until further extraction.

Preparation of Crude Extracts

1 g of lyophilised *A. barbadensis* gel was extracted extensively in 50 ml methanol (Ajax, AR grade) for 2 hours at 22 °C. The extract was filtered through filter paper (Whatman No. 54) under vacuum followed by drying by rotary evaporation in an Eppendorf concentrator 5301. The resultant waxy red pellet was dissolved in 1 ml 20 % methanol giving a dark red extract. The extract was passed through 0.22 μm filter (Sarstedt) and stored at 4 °C.

HPLC Separation of Extract Components

The extract was analysed and further fractionated by RP-HPLC using a Shimadzu HPLC system. The system consisted of twin LC-10AT pumps, a DGU-12A degasser, a SIL-10AD automatic injector using a 20 μ l injector loop and a SPD-M10A diode array detector, all under the control of a SCL-10A system controller. All solvents were of HPLC grade and were obtained from Lab-Scan.

HPLC separations were performed using a Waters Spherisorb® C₁₈ column (5 cm \times 4.6 mm). 20 μ l samples of Aloe gel extract were injected and chromatographed using a gradient from 20 % methanol to 60 % methanol as follows: 2 minutes isocratically at 20 % methanol followed by an 8 minute gradient to 40 % methanol. This was followed by isocratic elution at 40 % methanol for a further 5 minutes. The methanol was increased to 60 % over a further 10 minutes. The column was washed with 100 % methanol before reequilibrating to 20 % methanol for further chromatograms. Samples from multiple chromatograms (30 repeats) were collected and pooled. These samples were dried by rotary evaporation in an Eppendorf concentrator 5301 and were resuspended in 1 ml distilled water and stored at 4 °C for further analysis. For comparison, 20 μ l of aloe emodin (500 μ g ml⁻¹) was chromatographed using the same HPLC gradient.

Electrospray Ionisation (ESI) Mass Spectroscopy

Electrospray ionisation (ESI) mass spectroscopy was performed using a single quadrupole VG platform 2 mass spectrometer equipped with an electrospray (ES) ionisation source at an electrospray energy of 3.5 kV. Mass spectra were recorded in both the positive mode (cone voltage = 30 V) and negative mode (cone voltage = 50 V). Samples (100 μ g ml⁻¹) were introduced into the spectrometer at a flow rate of 1 μ l min⁻¹ in 80 % acetonitrile.

Test Microorganisms

All microbial strains were obtained from Tarita Morais, Griffith University. Stock cultures of *Aeromonas hydrophilia*, *Alcaligenes faecalis*, *Bacillus cereus*, *Bacillus subtilis*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Salmonella salford*, *Serratia marcescens*, *Staphylococcus aureus* and *Yersinia enterocolitica* were subcultured and maintained in nutrient broth at 4 °C. *Aspergillus niger*, *Candida albicans*, and *Saccharomyces cerevisiae* were maintained in Sabouraud media at 4 °C.

Evaluation of Antimicrobial Activity

Antimicrobial activity of Aloe vera juice, extract and of the HPLC purified compounds was determined using a modified Kirby-Bauer (Bauer et al. 1966) disc diffusion method. Briefly, 100 µl of the test bacteria/fungi were grown in 10 ml of fresh media until they reached a count of approximately 10^8 cells ml^{-1} for bacteria, or 10^5 cells ml^{-1} for fungi. 100 µl of microbial suspension was spread onto agar plates corresponding to the broth in which they were maintained.

The extract and fractionated components were tested using 6 mm sterilised filter paper discs. Discs were impregnated with 10 µl of the test sample, allowed to dry and placed onto inoculated plates. The plates were allowed to stand at 4 °C for 2 hours before incubation with the test microbial agents. Plates inoculated with *Alcaligenes feacalis*, *Aeromonas hydrophilia*, *Bacillus cereus*, *Bacillus subtilis*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Serratia marcescens*, *Yersinia enterocolitica*, *Candida albicans* and *Saccharomyces cerevisiae* were incubated at 30 °C for 24 hours, then the diameters of the inhibition zones were measured in millimetres. Plates inoculated with *Enterobacter aerogenes*, *Escherichia coli*, *Salmonella Salford* and *Staphylococcus aureus* were incubated at 37 °C for 24 hours, then the diameters of the inhibition zones were measured. *Aspergillus niger* inoculated plates were incubated at 25 °C for 48 hours then the zones of inhibition were measured. All measurements were to the closest whole millimetre. Each antimicrobial assay was performed in at least triplicate. Mean values are reported in this report. Standard discs of ampicillin (2 µg), penicillin (2 units), chloramphenicol (10 µg) or ciprofloxacin (2.5 µg) were obtained from Oxoid Ltd. and served as positive controls for antimicrobial activity. For fungi, nystatin discs (100 µg, Oxoid Ltd.) were also used as a positive control. Filter discs impregnated with 10 µl of distilled water were used as a negative control.

Results and Discussion

The antimicrobial activity of Aloe vera juice was investigated by agar disc diffusion against a panel of bacteria, fungi and yeast (Table 1). Aloe vera juice showed antibacterial activity against only the Gram-negative bacteria *A. hydrophilia* and *E. coli*. It did not show any inhibitory activity against any of the fungi or yeast tested.

Table 1: Zones of microbial growth inhibition of crude Aloe vera juice, gel extract and RP-HPLC separated fractions against a panel of microbial agents. Inhibition of growth was determined by the agar disc diffusion method. Antimicrobial discs used as positive controls were Amp (ampicillin, 2µg), Chl (chloramphenicol, 10µg), Nys (nystatin, 100µg) and Cip (ciprofloxacin, 2.5µg).

Microbial Species	Mean Zone of Inhibition ± SD (mm)								
	Antibiotic	Juice	Gel extract	Aloe emodin	Fraction 1	Fraction 2	Fraction 5	Fraction 8	Fraction 9
Gram negative rods									
<i>Aeromonas hydrophilia</i>	18.0±0.8 (Chl)	10 ± 0	10.8 ± 0.5	11 ± 0.8	9 ± 0	-	-	10.3 ± 0.5	-
<i>Alcaligenes faecalis</i>	13.3±0.5 (Amp)	-	15.8 ± 1.0	-	-	-	-	12.8 ± 0.5	-
<i>Citrobacter freundii</i>	25.3±1.5 (Chl)	-	-	-	8.8 ± 0.5	8.3 ± 0.5	8.3 ± 0.5	-	-
<i>Enterobacter aerogenes</i>	17.5±0.6 (Chl)	-	-	-	7.3 ± 0.5	-	-	-	-
<i>Escherichia coli</i>	16.8±0.5 (Amp)	8.8 ± 0.5	-	10.3 ± 1.0	-	-	-	8.3 ± 0.5	10 ± 1.4
<i>Klebsiella pneumoniae</i>	18.3±1.0 (Amp)	-	6.8 ± 0.3	-	7.3 ± 0.5	10.5 ± 0.6	-	-	-
<i>Pseudomonas aeruginosa</i>	32.5±0.6 (Cip)	-	-	-	-	-	-	-	-
<i>Pseudomonas fluorescens</i>	21.0±0.8 (Chl)	-	-	-	7.8 ± 0.5	-	-	-	-
<i>Salmonella salford</i>	25.5±0.6 (Amp)	-	-	-	-	-	-	-	-
<i>Serratia marescens</i>	25.8±0.5 (Chl)	-	-	-	7.5 ± 0.6	6.8 ± 0.3	-	-	-
<i>Yersinia enterocolitica</i>	16.5±0.6 (Amp)	-	-	-	-	-	-	-	-
Gram positive rods									
<i>Bacillus cereus</i>	25.8±1.3 (Chl)	-	-	-	-	-	-	-	-
<i>Bacillus subtilis</i>	23.5±2.6 (Amp)	-	-	9 ± 0	10 ± 1.2	-	-	-	-
Gram positive cocci									
<i>Staphylococcus aureus</i>	16.3±0.5 (Amp)	-	-	-	-	-	-	-	-
Fungi									
<i>Aspergillus niger</i>	18.3±0.5 (Cip)	-	11.8 ± 0.5	-	8.3 ± 0.5	-	-	-	-
<i>Candida albicans</i>	26.0±0.8 (Nys)	-	-	-	-	-	-	-	-
Yeast									
<i>Saccharomyces cerevisiae</i>	21.5±0.6 (Nys)	-	-	-	-	-	-	-	-

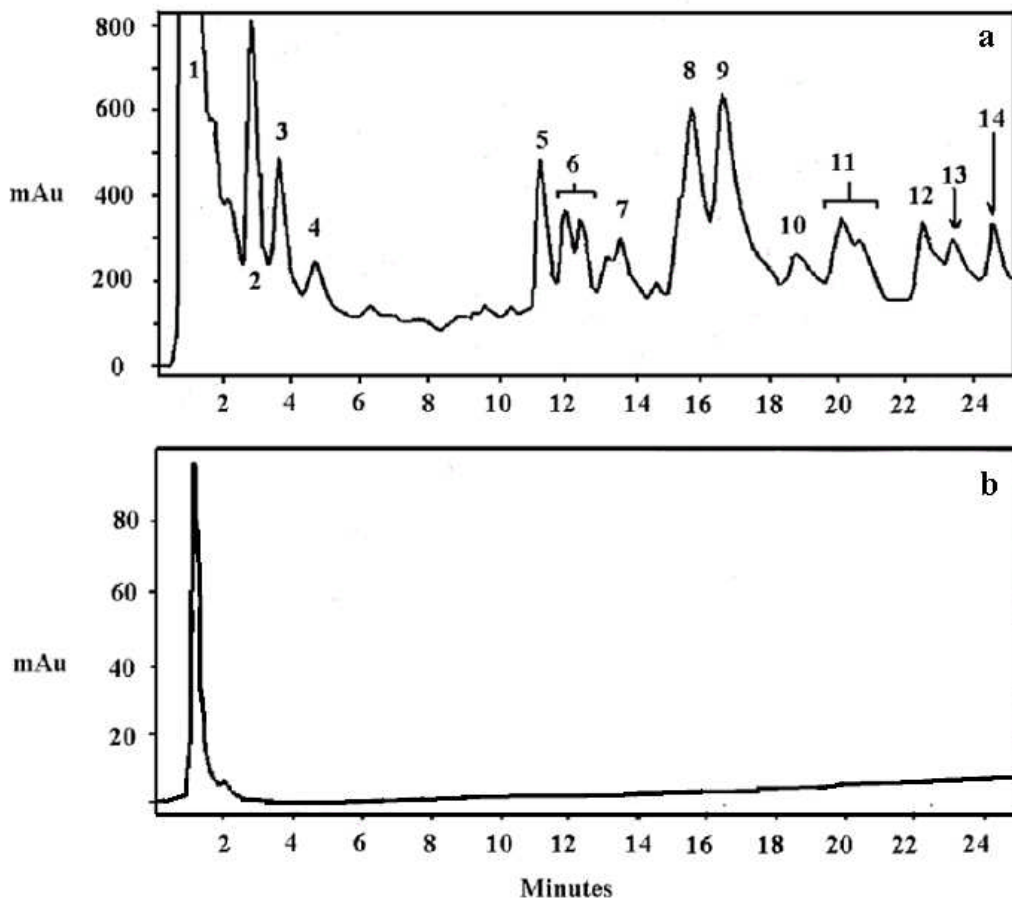
The components of lyophilised *A. barbadensis* inner leaf gel were also screened for antimicrobial activity. 1 g of lyophilised Aloe vera gel was extracted extensively with methanol. The resultant extract was dried by rotary evaporation resulting in 426 mg of dried extract material. The dried extract was resuspended in 1 ml of distilled water resulting in approximately 1.2 ml of concentrated extract. The

extract was tested for its antimicrobial activity (Table 1). The antimicrobial profile of the gel extract differed from that of Aloe vera juice. Whilst both the juice and the gel extract had antibacterial activity against *A. hydrophilia*, only the juice was able to inhibit *E. coli* growth. It is possible that component(s) essential for *E. coli* antibacterial activity is/are lost in the methanol extraction procedure. Conversely, only the gel extract was able to inhibit *K. pneumoniae* and *A. faecalis*, possibly indicating that components blocking/counteracting the inhibitory activity against these bacteria are also lost through the methanol extraction procedure. The gel extract also showed antifungal activity against *A. niger* whilst no such antifungal activity was seen for the juice. This was of particular interest as the *A. niger* strain used was resistant to the majority of other antimicrobial agents tested (nystatin, ampicillin, penicillin and chloramphenicol). Of the antimicrobial agents tested, only ciprofloxacin and the Aloe vera gel extract fractions were capable of inhibiting the growth of the otherwise resistant strain of *A. niger*. The current studies, therefore, identify Aloe vera leaf gel extract as possible antifungal agent to combat otherwise resistant strains of *A. niger*.

Aloe vera juice and gel are known to contain the anthraquinone aloe emodin which has previously been shown to have antimicrobial activity (Wu et al., 2006). For this reason, the antimicrobial activity of pure aloe emodin was also examined in the current study. Pure aloe emodin possessed similar antimicrobial activities as the juice, being able to inhibit the growth of both *A. hydrophilia* and *E. coli* (Table 1). However, pure aloe emodin also showed antibacterial activity towards the Gram-positive bacteria *B. subtilis*. It is possible that further components exist in Aloe vera juice and in the gel extract that can block/mask the antibacterial activity of aloe emodin towards *B. subtilis*.

To further study the antimicrobial growth inhibitory activity of the individual components of the Aloe vera leaf gel, the extract was further fractionated by reverse phase HPLC. 20 µl samples of the leaf gel extract were injected onto the column and fractions were collected and pooled. In total, 30 chromatograms were run and their fractions were pooled for further analysis. Figure 1(a) shows a typical HPLC profile. All 14 of the HPLC separated fractions were tested for antimicrobial activity. Only fractions 1, 2, 5, 8 and 9 showed any antimicrobial activity (Table 1).

Figure 1: (a) A typical RP-HPLC chromatogram of an Aloe vera gel extract fraction with detection at 210 nm. Chromatography conditions were as described in Materials and Methods. Fractions collected for further analysis are indicated with numbers on the chromatogram. (b) A typical chromatogram of pure aloe emodin. 20 µl of 0.5 mgml⁻¹ aloe emodin was dissolved in distilled water and injected onto the RP-HPLC column.



Fraction 1 had the broadest antibacterial activity of any of the fractions, inhibiting the growth of the majority of the Gram-negative rod bacteria tested (*C. freundii*, *S. marescens*, *K. pneumoniae*, *A. hydrophilia*, *E. aerogenes* and *P. fluorescens*). Fraction 1 was also capable of inhibiting the growth of the Gram-positive rod bacteria *B. subtilis*. No activity was observed against the single species/strain (*S. aureus*) of gram positive cocci tested, however, further testing against this class of bacteria may reveal sensitivity in other species/strains. Fraction 1 also possessed antifungal activity, being capable of inhibiting the growth of a nystatin resistant strain of *A. niger*.

Electrospray ionisation (ESI) mass spectroscopy of fraction 1 showed complicated patterns that indicated the presence of multiple molecular species, thus a definitive structural characterisation was not possible. However, when dry, this fraction was a crystalline solid with an orange red colour and a slightly tacky consistency. A methanolic solution absorbed UV light between 250 and 290 nm with a maxima at 265 nm in the UV region. For comparison, anthraquinones are characterised by their orange red colour and their absorbances in the UVB range. Hirata and Suga (1977) have listed the UV

absorbance peaks for aloe emodin as 221, 253, 266 and 289 nm. Furthermore, pure aloe emodin had a similar elution volume to fraction 1 when chromatographed under identical conditions (Figure 1b). Thus it is likely that whilst by no means pure, fraction 1 contains aloe emodin. This fraction has a broader antimicrobial specificity than pure aloe emodin, indicating other antimicrobial compounds may also be present in fraction 1. More work is necessary to fully characterise the molecular composition of this fraction.

The other fractions tested were much more selective in their antimicrobial activities, being capable of only inhibiting the growth of specific Gram-negative rod bacteria. Fraction 2 inhibited the growth of *C. freundii*, *K. pneumoniae* and *S. marescens*. Structural identification of this fraction was also not possible due to complicated mass spectra indicating the presence of multiple molecular species. However, due to the overlapping peaks between this fraction and fraction 1 (Figure 1(a)), it is likely that this fraction may also contain aloe emodin and that the antimicrobial activity of fraction 2 may also be due to this compound. It is likely that this fraction also contains aloin A. Previous studies in our laboratory (data not shown) have shown that pure aloin A elutes at the same elution volume as fraction 2 when chromatographed under identical conditions. Furthermore, a methanolic solution of fraction 2 gave a similar UV spectra to that of pure aloin A, absorbing UV light between 250-290 nm with a peak at 260 nm. However, the antimicrobial activity of this fraction can not be attributable to aloin A. Studies in our laboratory have found pure aloin A to be incapable of inhibiting the growth of any of the tested microbial agents (data not shown).

Fraction 5 only inhibited *C. freundii*. Structural identification of this fraction was also not possible due to complicated ESI mass spectra patterns. This fraction was notable for its pink colour. A methanolic solution of this fraction absorbed UV light between 240 and 300 nm with maxima at 252 nm, 272 nm and 296 nm in the UV region. Whilst the UV spectra of this fraction suggests it may be an anthraquinone or a dihydroanthraquinone, its identity remains unknown. Further studies are needed to elucidate the structure of this fraction.

Fraction 8 had antibacterial activity against *A. faecalis*, *A. hydrophilia* and *E. coli*. Analysis by mass spectrometry revealed an intense $[M+Na]^+$ peak at 579 nm, consistent with a molecular weight of 556 Da. ESI analysis revealed the following fragments at m/z 538 $[M-H_2O]^+$ ($[M-18]^+$), m/z 512 $[M-CH_3CHO]^+$ ($[M-44]^+$), m/z 410 $[M-coumaroyl]^+$ ($[M-146]^+$), m/z 392 $[M-(146+18)]^+$, m/z 366 $[M-(146+44)]^+$, m/z 259 $[M-(133+146+18)]^+$, m/z 193 $[M-(133+146+84)]^+$, m/z 147 $[coumaroyl]^+$ and m/z 119 $[HOC_6H_4CHCH]^+$. This spectra is consistent with the spectra reported by Lee et al. (2000) for

aloeresin D (8-C- β -D-[2-O-(E)-coumaroyl] glucopyranosyl -2-[2-hydroxy]-propyl-7-methoxy-5-methylchromone).

Previous studies have demonstrated the antimicrobial activity of other chromones from diverse plant species such as *Origanum syriacum* Lauraceae (Alma et al., 2003), *Chamaecyparis pisifera* (Xiao et al., 2001) and *Ferula communis* (Al-Yahya et al., 1998). Budzisz et al. (2001) examined the antibacterial activities of various synthetic chromones, finding activity against various bacterial species. This study found the synthetic chromones to be particularly active at inhibiting the growth of Gram-positive bacteria. The current study indicated greater activity towards Gram-negative bacteria. This may be in part due to differences in the bacterial species examined in these two studies, but may also be due to the fact that all the chromones used in the Budzisz et al. study were phosphonic derivatives of chromone and would be likely to have very different chemical properties to the Aloe vera chromones examined in the current study.

Fraction 9 only inhibited *E. coli* growth. The ESI mass spectroscopy analysis of this fraction was almost identical to that obtained for fraction 8, indicating that these two fractions are possibly isomers of each other. Lee et al. (2000) have also reported the structure of an isomeric form of aloeresin D, differing only in the configuration of the single hydroxyl group on the 2-hydroxypropyl side chain of the chromone ring. It is likely therefore that fractions 8 and 9 represent the isomers aloeresin D and isoaloeresin D. The present study did not distinguish which of these fractions contain each of these isomers. Accordingly, further studies are needed for absolute structural determination of these fractions.

The fungi tested in the present study were shown to have limited susceptibility to Aloe vera gel and extracted fractions. *A. niger* growth was inhibited by fraction 1 and the extract. This is an important result as this strain of *A. niger* was resistant to all other antimicrobial agents tested except ciprofloxacin. The only yeast tested in these studies (*S. cerevisiae*), was not inhibited by any of the tested fractions.

The findings of this study have established the susceptibilities of a broad range of bacteria to fractions isolated from Aloe vera inner leaf gel. Gram-negative rod bacteria were found to be particularly susceptible to Aloe vera gel components. Of the bacterial classes tested, only the Gram-positive cocci bacteria were resistant to the Aloe vera components.

It is evident that fractions derived from Aloe vera inner gel have potential as antimicrobial agents, especially against Gram-negative bacteria and against resistant strains of *A. niger*. Many previous

studies such as that of Ferro et al. (2003) have focussed on the antimicrobial activity of Aloe vera whole gel. Other reports have attempted to determine the antimicrobial activity of purified fractions such as anthraquinones (Garcia-Sosa et al., 2006; Wu et al., 2006; Dabai et al., 2007) and saponins (Reynolds and Dweck, 1999). Studies in this field are necessary in the face of increasing microbial antibiotic resistance. The identification of natural antimicrobial compounds and the future development of these compounds through structure/activity studies provides a promising avenue of research for novel antimicrobials.

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