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No evidence of antiseptic properties and low toxicity of selected *Aloe* species

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Abstract

Background and Aim: Closely related plant species often share similar secondary metabolites and bioactivities and are therefore good targets for bioactivity testing when one or more species within a genus are known to possess therapeutic properties. The genus *Aloe* has a long history of medicinal usage in many areas of the world. Many species are known to have therapeutic properties, several species of which have well-established antibacterial bioactivities. The current studies examine the toxicity of several *Aloe* species and their ability to inhibit bacterial growth and compare them to the most extensively studied species, *Aloe barbadensis*, which has well-established antibacterial bioactivities. **Results:** *A. barbadensis* methanolic extract displayed broad spectrum antibacterial activity, inhibiting the growth of 8 of the 14 bacteria tested (57%). It was effective against both Gram-positive and Gram-negative bacteria, inhibiting the growth of 4 of 4 Gram-positive bacteria (100%) and 4 of 10 Gram-negative (40%) bacteria tested, respectively. In contrast, *Aloe* elgonica, *Aloe* pruinosa, *Aloe* chabaudii, *Aloe* daiyana, *Aloe* marlothi and *Aloe* vryheidensis all showed no antibacterial activity toward any of the bacteria tested. All of the *Aloe* species displayed low toxicity similar to that of the *A. barbadensis* control. *A. daiyana* was the most toxic of the *Aloe* species tested with 24, 48 and 72 hours LC50 values of 1018.2, 517.0 and 405.7 μg/ml, respectively. **Conclusions:** Despite their close taxonomic relationship, *A. elgonica*, *A. pruinosa*, *A. chabaudii*, *A. daiyana*, *A. marlothi* and *A. vryheidensis* do not have the same antibacterial medicinal potential as *A. barbadensis*, but may still have other similar toxicity-related bioactivities. Testing against protozoa, fungi, virus and tumor cells is required to determine if this is the case.

Key words: *Aloe barbadensis* Miller, *Aloe vera*, antibacterial, medicinal plants, methanolic extracts, phytotoxicity

INTRODUCTION

The use of natural plant therapeutics is as old as human civilization and in many regions of the world is still the primary modality of health care. Ayurvedic medicine in India, for example, is still commonly practiced, with approximately 85% of Indians using crude plant preparations for the treatment of various diseases and ailments.[1] Even in Western civilizations, plants play an important role in medicine. At least 25% of pharmaceuticals prescribed worldwide are directly obtained from plants with many more drugs being semi-synthetic derivatives of natural plant precursors.[2-4] Examples of medicinally important plant-derived compounds include the anti-malarial drug quinine and its derivatives (from *Cinchona* spp.), the antitumor drugs vincristine and vinblastine (from *Catharanthus roseus*) along with the semi-synthetic analogue vindesine, the analgesics morphine and codeine (from *Papaver somniferum*), the anticholinergic drug atropine derived from plants of the family *Solanaceae* (*Atropa belladonna*, *Datura stramonium* and *Mandragora officinarum*), the anticancer drug taxol (derived from *Taxus brevifolia*) and the cardiac glycoside digoxin (from *Digitalis purpurea*).[5]

Despite the potential of plants to provide us with useful pharmaceutical agents, the field is still poorly studied. Only an estimated 5–10% of the approximately 300,000–500,000 plant species worldwide have been screened for one or

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more bioactivities.[5] With so many plant species yet to be tested, it is essential that plant selection processes narrow the field. The main selection criterion currently used is to select plants on the basis of their ethnobotanical usage as traditional medicines. Another important selection method is to examine plants closely related to plants for which medicinal potential is well established. Many plant secondary metabolites are regarded as family-, genus- or species-specific and investigation of species closely related to those used as traditional medicines may lead to natural therapeutic discovery.[2]

Plants from the genus *Aloe* (family *Asphodelaceae*) have a long history of medicinal use in the treatment of a wide variety of medical disorders and conditions. Four species, specifically *Aloe barbadensis* Miller (commonly known as *Aloe vera*),[6] *Aloe ferox* (Cape *Aloe*),[7] *Aloe arborescens* (Candelabra *Aloe*)[8] and *Aloe perryi baker* (Perry's *Aloe*),[9] have had their ethnopharmacological usage particularly well documented. Of these, *A. barbadensis* has been the most extensively studied. Along with anti-inflammatory,[10] immunomodulatory[11] and redox-state maintenance bioactivities,[12,13] it has also been reported to have good antimicrobial bioactivity.[14,15] *A. ferox* and *A. arborescens* have also been shown to inhibit the growth of various bacteria associated with wound infections and thus enhance wound healing.[16] Similarly, *A. perryi baker* has been shown to inhibit the growth of *Staphylococcus aureus* and *Enterococcus faecalis in vitro*.[17]

Although much of the scientific investigation into the antibacterial properties of *Aloe* species is dominated by *A. barbadensis*, *A. ferox*, *A. arborescens* and *A. perryi baker*, there have been a number of other fruitful investigations into the antibacterial properties of other *Aloe* species. *Aloe chinensis* and *Aloe succotrina* have been reported to be effective against *Mycobacterium tuberculosis*.[18] *Aloe secundiflora* has been shown to inhibit *Salmonella gallinarum* growth[19] and *Aloe excelsa* inhibits the growth of various bacteria *in vitro*.[20] This study was undertaken to determine whether other *Aloe* species display similar antibacterial activities and toxicity as *A. barbadensis* Miller.

MATERIALS AND METHODS

Plant material

Collection of plant samples

Aloe elgonica, *Aloe pruinosa*, *Aloe chabaudii*, *Aloe daiyana*, *Aloe marlothi* and *Aloe vryheidensis* leaves were obtained from Philip Cameron, Senior Botanic Officer, Brisbane Botanical Gardens, Mt Cootha. *A. barbadensis* leaves were obtained from John Gorringe of Aloe Wellness Australia. Leaves of each species were obtained from single plants, washed in deionized water and processed within 4 hours of collection.

Preparation of crude extracts

A. elgonica, *A. pruinosa*, *A. chabaudii*, *A. daiyana, A. marlothi, A. vryheidensis* and *A. barbadensis* leaves were dried in a sunbeam food dehydrator and the dried material was ground to a coarse powder. One gram of each of the dried plant materials was extracted extensively in 50 ml methanol (Ajax, AR grade) for 24 hours at 4°C with gentle shaking. 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 pellet was dissolved in 5 ml deionized water. The extract was passed through 0.22 µm filter (Sarstedt, Mawson Lakes, Australia) and stored at 4°C.

Antibacterial screening

Test microorganisms

All the microbial strains were obtained from Michelle Mendell and Tarita Morais, Griffith University, Australia. Stock cultures of *Aeromonas hydrophilia*, *Alcaligenes faecalis*, *Bacillus cereus*, *Citrobacter freundii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas fluorescens*, *Salmonella newport*, *Serratia marcescens*, *Shigella sonnei*, *Sta. aureus*, *Staphylococcus epidermidis* and *Streptococcus pyogenes* were subcultured and maintained in nutrient broth at 4°C.

Evaluation of antimicrobial activity

Antimicrobial activity of all plant extracts was determined using a modified Kirby-Bauer[21] disk diffusion method. Briefly, 100 µl of the test bacteria were grown in 10 ml of fresh media until they reached a count of approximately $10^{[8]}$ cells/ml. Then, 100 µl of microbial suspension was spread onto nutrient agar plates.

The *Aloe* leaf methanolic extracts were tested for antibacterial activity using 5 mm sterilized filter paper disks. Disks 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 faecalis*, *Aeromonas hydrophilia*, *Bacillus cereus*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Proteus mirabilis, Pseudomonas fluorescens*, *Serratia marcescens*, were incubated at 30°C for 24 hours, and then the diameters of the inhibition zones were measured in millimetres. Plates inoculated with *Escherichia coli*, *Salmonella newport*, *Shigella sonnei*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pyogenes* were incubated at 37°C for 24 hours, and then the diameters of the inhibition zones were measured. All measurements were to the closest whole millimeter. Each antimicrobial assay was performed in at least triplicate. Mean values are

reported in this study. Standard disks of ampicillin (2 μg) and chloramphenicol (10 μg) were obtained from Oxoid Ltd. and served as positive controls for antimicrobial activity. Filter disks impregnated with 10 μl of distilled water were used as a negative control.

Toxicity screening

Reference toxins for toxicity screening

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared as a 1.6 mg/ml solution in distilled water and was serially diluted in artificial seawater for use in the *Artemia franciscana* nauplii bioassay. Mevinphos (2-methoxycarbonyl-1-methylvinyl dimethyl phosphate) was obtained from Sigma-Aldrich as a mixture of *cis* (76.6%) and *trans* (23.0%) isomers and prepared as a 4 mg/ml stock in distilled water. The stock was serially diluted in artificial seawater for use in the bioassay.

Ar. franciscana nauplii toxicity screening

Toxicity was tested using the *Ar. franciscana* nauplii lethality assay developed by Meyer *et al.*[22] for the screening of active plant constituents with the following modifications. *Ar. franciscana* Kellogg cysts were obtained from North American Brine Shrimp, LLC, USA (harvested from the Great Salt Lake, Utah). Synthetic seawater was prepared using Reef Salt, AZOO Co, Whittier., USA. Seawater solutions in 34 g/l distilled water were prepared prior to use. Two grams of *Ar. franciscana* cysts were incubated in 1 l synthetic seawater under artificial light at 25°C, 2000 Lux with continuous aeration. Hatching commenced within 16–18 hours of incubation. Newly hatched *Ar. franciscana* (nauplii) were used within 10 hours of hatching. Nauplii were separated from the shells and remaining cysts and were concentrated to a suitable density by placing an artificial light at one end of their incubation vessel and the nauplii rich water closest to the light was removed for biological assays. Exactly 400 μl of seawater containing approximately 45 (mean 43.8, n = 145, SD 18.6) nauplii were added to wells of a 48-well plate and immediately used for bioassay. The plant extracts were diluted to 3000 μg/ml in seawater for toxicity testing, resulting in a 1500 μg/ml concentration in the bioassay. Four hundred microliters of diluted plant extracts and the reference toxins were transferred to the wells and incubated at 25 ± 1°C under artificial light (1000 Lux). A negative control (400 μl seawater) was run in at least triplicate for each plate. All treatments were performed in at least triplicate. The wells were checked at regular intervals and the number of dead counted. The nauplii were considered dead if no movement of the appendages was observed within 10 seconds. After 72 hours, all the nauplii were sacrificed and counted to determine the total number per well. The LC_{50} with 95% confidence limits for each treatment was calculated using probit analysis.[23]

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extraction of 1 g of the dried *Aloe* leaf material with methanol yielded dried plant extracts ranging from 27.8 (*A. pruinosa*) mg to 78.2 mg (*A. barbadensis*) [Table 1]. The dried extracts were resuspended in 5 ml of deionized water resulting in the extract concentrations shown in Table 1.

Antibacterial activity

Ten microliters of each extract was tested in the disk diffusion assay against a panel of 14 bacteria [Table 2]. Only *A. barbadensis* displayed antibacterial activity, inhibiting the growth of 8 of the 14 bacteria tested (57.1%). It was particularly potent against *Al. faecalis* and *Pr. mirabilis* as determined from the zone of inhibition (15.6 ± 1.5 mm and 14.3 ± 1.5 mm, respectively). The *A. barbadensis* methanolic extract displayed antibacterial activity toward both Gram-positive and Gram-negative bacteria, although Gram-positive bacteria appeared more susceptible. It inhibited the growth of all 4 of the Gram-positive bacteria tested (100%). Of the 10 Gram-negative bacteria tested, 4 (40%) were inhibited by the *A. barbadensis* methanolic extract. In contrast, none of the other *Aloe* species inhibited the growth of any of the bacteria tested.

Numbers indicate the mean diameters (mm) of inhibition of at least triplicate experiments ± standard deviation. — Indicates no growth inhibition. Ampicillin (2 μg) and chloramphenicol (10 μg) were used as the positive controls

Quantification of toxicity

The *Aloe* leaf extracts [Figures 1a-g] were diluted to 3000 μg/ml in artificial seawater for toxicity testing, resulting in a 1500 μg/ml concentration in the *Artemia* nauplii lethality bioassay. For comparison, the reference toxins potassium dichromate (800 μg/ml) [Figure 1i] and Mevinphos (2000

Table 1: The mass of *Aloe* leaf methanol extracts and the concentration after resuspension in deionized water

Plant leaf extracted	Mass of dried extract (mg)	Resuspended extract concentration (mg/ml)
A. elgonica	53.8	10.8
A. pruinosa	27.8	9.6
A. chabaudii	32	6.4
A. daiyana	56.4	11.2
A. marlothi	43.2	8.6
A. vryheidensis	69	13.8
A. barbadensis	78.2	15.6

Table 2: Antibacterial activity of *Aloe* methanolic extracts measured as zones of inhibition (mm)

	A. elgonica	*A. pruinosa*	*A. chabaudii*	*A. daiyana*	*A. marlothi*	*A. vryheidensis*	*A. barbadensis*	Ampicillin	Chloramphenicol
Gram negative rods									
Al. faecalis	—	—	—	—	—	—	15.6 ± 1.5	15.2 ± 1.2	6.3 ± 0.6
Ae. hydrophilia	—	—	—	—	—	—	10.5 ± 1.2	12.0 ± 1.0	28.7 ± 1.6
C. freundi	—	—	—	—	—	—	—	8.3 ± 0.6	15.7 ± 1.2
E. coli	—	—	—	—	—	—	—	14.7 ± 0.6	17.3 ± 0.6
K. pneumoniae	—	—	—	—	—	—	6.6 ± 0.3	10.3 ± 0.6	21.3 ± 1.5
Pr. mirabilis	—	—	—	—	—	—	14.3 ± 1.5	17.3 ± 0.6	8.7 ± 0.6
Ps. fluorescens	—	—	—	—	—	—	—	18.2 ± 0.5	21.2 ± 1.2
Sa. newport	—	—	—	—	—	—	—	18.7 ± 0.6	20.3 ± 0.6
Se. marcescens	—	—	—	—	—	—	—	0 ± 0	14.7 ± 0.6
Sh. Sonnei	—	—	—	—	—	—	—	14.0 ± 0	14.3 ± 0.6
Gram positive rods									
B. cereus	—	—	—	—	—	—	8.0 ± 0.9	26.7 ± 0.6	13.3 ± 1.2
Gram positive cocci									
Sta. aureus	—	—	—	—	—	—	7.2 ± 0.6	11.7 ± 2.1	16.0 ± 1.0
Sta. epidermidis	—	—	—	—	—	—	6.3 ± 0.6	26.3 ± 1.5	12.3 ± 0.6
Str. pyogenes	—	—	—	—	—	—	9.0 ± 1.2	17.0 ± 1.0	24.0 ± 1.0

Numbers indicate the mean diameters (mm) of inhibition of at least triplicate experiments ± standard deviation. — Indicates no growth inhibition. Ampicillin (2 µg) and chloramphenicol (10 µg) were used as the positive controls

µg/ml) [Figure 1j] were also tested in the *Ar. franciscana* lethality bioassay. Both reference toxins were rapid in their induction of the onset of mortality, inducing mortality within the first 3 hours of exposure. Also, 100% mortality was evident following 4–5 hours of exposure. Similarly, *A. barbadensis* [Figure 1g] exposure induced mortality rapidly, with 100% mortality induction achieved by 4 hours. *A. elgonica* [Figure 1a] also induced significant mortality by 24 hours (80.1 ± 8.1%). The induction of mortality reached 100% by 72 hours. *A. pruinosa* [Figure 1b] and *A. daiyana* [Figure 1d] also induced mortality significantly above that of the seawater control [Figure 1h] although neither induced ≥50% mortality by 24 hours. Exposure to all other *Aloe* species [Figures 1c, 1e] resulted in no increase in mortality above that of the seawater control [Figure 1h].

To determine the effect of toxin concentration on the induction of mortality, the extract was serially diluted in artificial seawater to test across the concentration range 2000–100 µg/ml in the *Artemia* nauplii bioassay at 24, 48 and 72 hours. Table 3 shows the LC_{50} values of the *Aloe* leaf extracts toward *Ar. franciscana*. No LC_{50} values are reported for the *A. pruinosa*, *A. chabaudii*, *A. marlothi* and *A. vryheidensis* leaf extracts at some times as less than 50% mortality was seen for all concentrations tested at these test periods. Indeed, all *Aloe* extracts tested displayed low toxicity (>1000 µg/ml) at 24 hours with *A. pruinosa* showing low toxicity also at 48 hours. *A. daiyana* was the most toxic of the *Aloe* species at all times tested. Potassium dichromate (24 hour LC_{50} 78.0; 48 hour LC_{50} 14.0; 72 hour LC_{50} 3.9) was substantially more toxic than all the *Aloe* leaf extracts or Mevinphos (24 hour LC_{50} 1320; 48 hour LC_{50} 495; 72 hour LC_{50} 115) at all times tested.

Table 3: LC_{50} (95% confidence interval) for brine shrimp nauplii exposed to *Aloe* extracts or the reference toxins potassium dichromate and Mevinphos

Sample	LC50 (µg/ml) at time		
	24 hours	48 hours	72 hours
A. elgonica	1094.3	923.9	906.8
A. pruinose	—	1786.7	654.5
A. chabaudii	—	—	—
A. daiyana	1018.2	517	405.7
A. marlothi	—	—	—
A. vryheidensis	—	—	—
A. barbadensis	1043	904	895
Mevinphos	1320	495	115
Potassium dichromate	78	14	3.9

— Denotes values that were not obtained as ≥50% mortality was not obtained at this time point

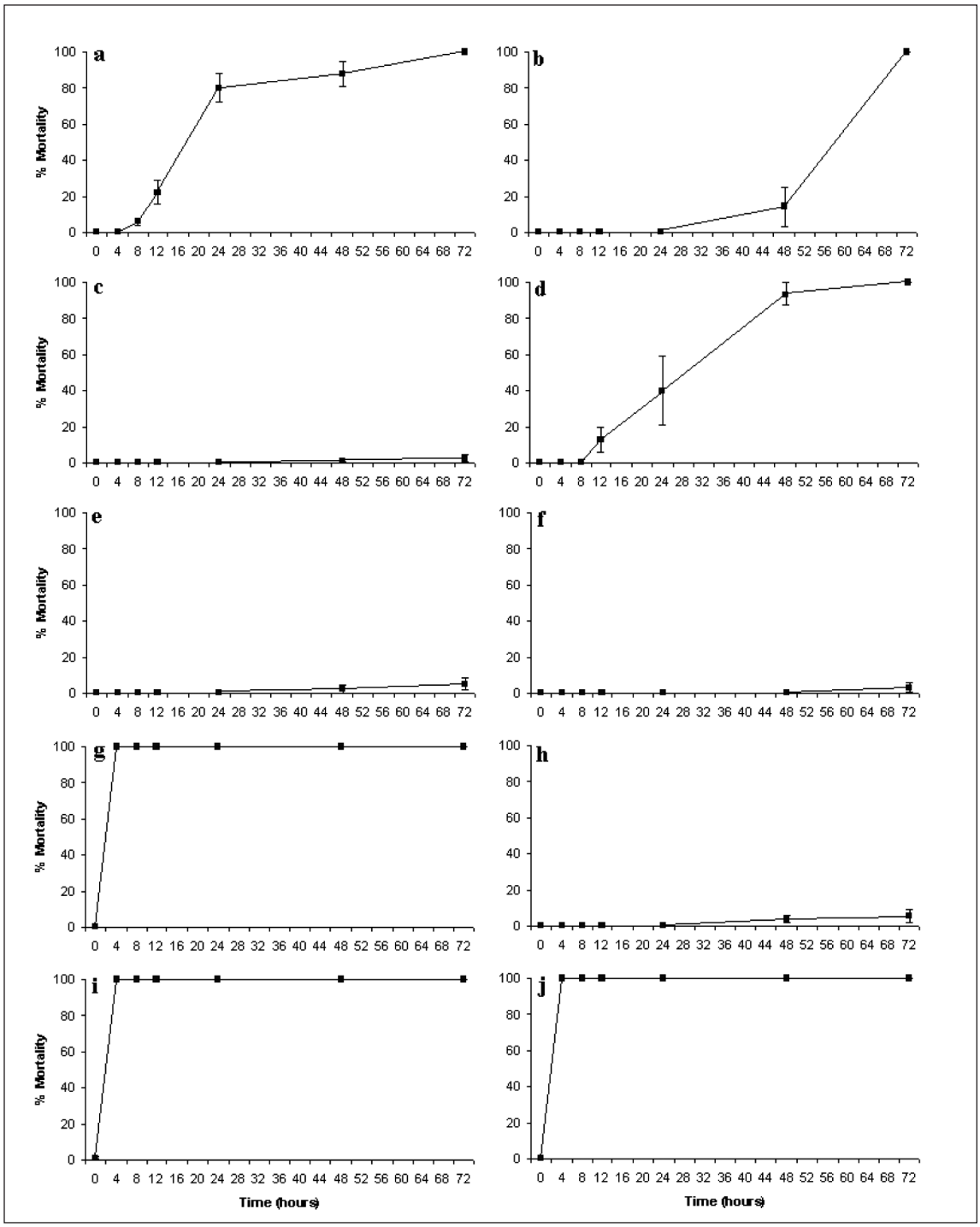

Figure 1: Brine shrimp lethality of (a) *A. elgonica* methanol extract (1500 µg/ml), (b) *A. pruinosa* methanol extract (1500 µg/ml), (c) *A. chabaudii* methanol extract (1500 µg/ml), (d) *A. daiyana* methanol extract (1500 µg/ml), (e) *A. marlothi* methanol extract (1500 µg/ml), (f) *A. vryheidensis* methanol extract (1500 µg/ml), (g) *A. barbadensis* methanol extract (1500 µg/ml), (h) artificial seawater negative control, (i) potassium dichromate (800 µg/ml), (j) Mevinphos (2000 µg/ml). All bioassays were performed in at least triplicate and are expressed as mean ± standard deviation

DISCUSSION

In agreement with previously published reports,[14,15] *A. barbadensis* methanolic extract displayed antibacterial inhibitory activity toward a broad spectrum of bacteria tested in this study. This extract inhibited the growth of both Gram-positive and Gram-negative bacteria although it was more effective at inhibiting Gram-positive bacteria. The greater susceptibility of Gram-positive bacteria seen in this study is in agreement with previously reported results for South American,[24] African[25,26] and Australian[27] plant extracts. The Gram-negative bacterial cell wall outer

membrane is thought to act as a barrier to many substances including antibiotics.[28] The uptake of the *A. barbadensis* extract antibiotic agents by Gram-negative bacteria is presumably affected by the cell wall outer membrane of some bacteria. Despite the close taxonomic relationship between *A. barbadensis* and the other *Aloe* species tested in this study, none of the other *Aloe* extracts displayed any ability to inhibit the growth of any of the bacteria tested.

Whilst individual antibacterial components were not identified in this study, *A. barbadensis* leaf gel and rind are known to contain the anthraquinones aloe emodin and aloin. Scientific studies have demonstrated the antibacterial activity of these anthraquinones[29] although the mechanism of their antibacterial bioactivity has not yet been fully established. Previous studies have reported on the antioxidant and pro-oxidant potential of *A. barbadensis* juice and extracts[12,13,30,31] and the high free radical scavenging activity of aloe emodin and aloin and their ability to inhibit lipid peroxidation.[32]

The seemingly conflicting antioxidant/pro-oxidant effects of these anthraquinones may be due to different concentrations present in these studies. Tian and Hua[33] have reported on the concentration-dependent effects of aloin and aloe emodin. Aloin has a pro-oxidant effect at low concentrations and has an antioxidant effect at higher concentrations. In contrast, aloe emodin was shown to function as a pro-oxidant only at high concentrations. Thus, the antibacterial bioactivity associated with the *A. barbadensis* extract in the current study may be due to a relatively high level of aloe emodin and/or a low concentration of aloin present in the extract. Interactions between the various components within the extract may also play a role in converting otherwise antioxidant molecules into pro-oxidants in the extract or vice versa. It is therefore still possible that aloin and/or aloe emodin are also present in the other *Aloe* extracts which did not display antibacterial activity in the current study, albeit at concentrations that would confer antioxidant rather than pro-oxidant bioactivities. Their presence/absence in these extracts was not examined in the current report. Further studies are needed to determine whether these compounds are present, and if they are present at concentrations that would favour antioxidant rather than pro-oxidant activity.

Studies within this laboratory have also established the presence of antibacterial coumaroyl chromones in *A. barbadensis* methanolic extracts and have demonstrated their antibacterial potential.[14] Other studies have demonstrated the antimicrobial activity of other chromones from diverse plant species such as *Origanum syriacum* Lauraceae,[34] *Chamaecyparis pisifera*[35] and *Ferula communis*.[36] Budzisz *et al*.[37] examined the antibacterial activities of various synthetic chromones, finding activity against various bacterial species. The Budzisz study found the synthetic chromones to be particularly active at inhibiting the growth of Gram-positive bacteria. Whether similar chromones are also present in the other *Aloe* species extracts was not established in the current study and further studies need to focus on the phytochemistry of these extracts. Due to the lack of antibacterial activity of the other *Aloe* extracts, it is likely that if present, these compounds are in much lower concentrations or are present as inactivated forms. Alternatively, compounds that inhibit their potential antibacterial mechanisms may be present in the other *Aloe* extracts, but are lacking or in low concentrations in the *A. barbadensis* extract.

CONCLUSIONS

Methanolic extracts of *A. elgonica, A. pruinosa, A. chabaudii, A. daiyana, A. marlothi* and *A. vryheidensis* displayed no antibacterial activity in the disk diffusion assay despite their close taxonomic relationship with *A. barbadensis*, a plant with well-known antibacterial properties. All *Aloe* species displayed low toxicity toward *Artemia* nauplii similar to that of *A. barbadensis*, indicating that the compounds responsible for this toxicity may not be responsible for the antibacterial activity of *A. barbadensis*.

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