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Antibacterial evaluation of South African *Aloe ferox* (Mill)

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Background: There has been an alarming increase in infections because of multidrug resistance. Several multidrug-resistant microbes are becoming more resistant to medications with several side effects. There is an urgent requirement for natural, safer and inexpensive antimicrobial agents.

Aim: The study sought to examine the effects of the antimicrobial activity of *Aloe ferox (A. ferox)* in different regions across South Africa.

Method: The antimicrobial activity was determined using the agar disk diffusion method. The minimum inhibitory concentration was determined using the microplate dilution method. 100 mg of dry extract was dissolved in 1 mL of ethanol, ethyl acetate and aqueous to obtain the concentration of 100 mg/mL dilutions (1:10, 1:100 and 1:500), respectively.

Results: The aqueous extracts showed antibacterial activity against all Gram-positive bacteria. Ethanol extracts showed greater inhibition of Gram-positive bacteria than ethyl acetate extracts. Ethanol showed inhibition on Gram-negative bacteria, that is, *Protea vulgaris*, *Escherichia coli* and *S. sonnei*. Aqueous and ethanol extracts displayed strong inhibition at the concentration of 1:10 against all fungal species across regions.

Conclusion: *A. ferox* showed inhibition to varying degrees across all the regions. The concentrations of *A. ferox* active ingredient used in traditional medicine differ by region. The study validates the potential difference in the antimicrobial activity of *A. ferox* across different geographical regions.

Contribution: This study adds to existing knowledge about the remarkable antimicrobial activity of *A. ferox* as a traditional medicinal plant because of differences in regions.

Keywords: *Aloe ferox*; minimum inhibitory concentration; Gram-positive bacteria; Gram negative bacteria; plant extract.

Introduction

The constant rise in drug resistance, the emergence of incurable diseases and rising medical costs have necessitated the bioprospecting of plants as an alternative source of therapeutic compounds (Maliehe et al. 2022). Terreni, Taccani and Pregnolato (2021) allude that there has been an alarming increase in infections because of multidrug resistance, a phenomenon in which microorganisms develop resistance to more than one class of antimicrobials. Many antibiotics and antimicrobial agents are available on the market that control microbes or inhibits their growth, assisting in the control of pathogenic microorganisms (Danish et al. 2020). Even though many microbes are multidrug-resistant, these microbes are becoming more resistant to medications (Catalano et al. 2022). Santos et al. (2023) pointed out that medication can also have several side effects; hence, natural, safer and less expensive antifungal and antibacterial agents are required. The challenge of microbial resistance is a growing concern, and the future use of antimicrobial drugs remains uncertain (Andersson et al. 2020). Årdal et al. (2020) draw attention to actions that must be taken to address this challenge by limiting the use of antibiotics, conducting more research to better understand the mechanisms of resistance and developing more studies for the discovery of new drugs. Multidrug resistance poses a grave threat to human health and the economy as it is responsible for over 7 million deaths worldwide and a total economic loss of more than 20 billion dollars per year (Maliehe et al. 2022).

Roy et al. (2022) alluded to medicinal plants known to produce diverse secondary metabolites with a wide range of pharmaceutical applications. Tannin, terpenoids, ferulic acid, cinnamic, phenols and

flavonoids are some compounds that are characterised by profound antimicrobial action and have been isolated from different endophytic fungi (Maliehe et al. 2022). *Aloe ferox* (*A. ferox*) has been reported to have pharmacological activities including anti-inflammatory, immunomodulatory, antibacterial, antifungal, antiviral, antiproliferative, antidiabetic, laxative, wound healing, moisturising, anti-aging and skin protection (Nalimu et al. 2021; Singh et al. 2021).

Even though little is known about the extent of geographical differences in the antimicrobial properties of *A. ferox*, generalisations about product quality persist. However, the current investigation has shown that the antimicrobial properties of *A. ferox* vary across different geographical locations. The biologically active compounds are known as phytochemicals derived from every part of the plant including roots, stems, leaves, flowers, fruits and seeds (Balamurugan, Fatima & Velurajan 2019). They have been reported to defend plants against harmful agents like insects and microbes, as well as stressful events like ultraviolet (UV) radiation and extreme temperatures (Saivinayak & Santhosh 2022). According to the literature, biological properties may be attributed to several compounds in the phytochemical profile of *A. ferox* extracts rather than a single class of compounds (Andrea et al. 2020). Medicinal plants do not consistently produce the same chemicals in the same quantities; therefore, the effectiveness of medicinal plants may be affected by the biochemical factors within the individual species such as plant parts extracted and external factors such as climate, geographical location, season and growth conditions (Buwa & Staden 2006).

Aloe species are increasingly being incorporated into different cosmetic products, health drinks, foods and beverages because of the beneficial biological activities of the phytochemicals found mainly in the leaves (Singh et al. 2021) Variation in the concentration of the chemical constituents is based on the plant part used, the extraction process, the solvent, the stage of growth and the plant source (López-Malo et al. 2020). Many studies have been conducted to determine the potential toxicity and risks associated with various plants and vegetables, particularly hepatotoxicity, nephrotoxicity and cancer (Aboufaras, Selmaoui & Ouzennou 2023; Chang et al. 2023; Liu et al. 2021). Because of the risks involved, the toxicological evaluation of medicinal plants has become one of the main concerns to assure their safe use (Nalimu et al. 2021).

Aloe ferox has a diverse distribution throughout South Africa and it is abundant in the Free State, KwaZulu-Natal, Eastern Cape and Western Cape provinces of South Africa (Viljoen et al. 2023). The 'plant' is the most neglected part of plant-based medicine (Cock, Mavuso & Van Vuuren 2021). Since the dawn of time, people have been searching for, collecting, and effectively using plants to treat ailments of the body, spirit and mind. Khan and Ahmad (2019) allude that the vast majority of people around the world consume plant-based medicines for a range of medical disorders. In South Africa, most people especially the rural communities still depend, to a large extent on medicinal plants to treat different ailments (Cock & Van Vuuren 2020). This is not surprising since South Africa is home to over 24000 higher plant species with approximately 3000 plant species recorded by various cultural groups as part of their materia medica (De Canha et al. 2021).

Studies indicate that the first step in antimicrobial discovery is usually to screen library candidate drugs against the minimum inhibitory concentration (MIC) of the target bacteria (Miethke et al. 2021). The study sought to investigate the effects of antimicrobial (antibacterial and antifungal) activity of *A. ferox* (Mill) across various regions of the Republic of South Africa.

Methods

Study area and plant collection

The study areas were selected in the Eastern Cape (EC), Free State (FS) and KwaZulu-Natal (KZN) provinces, Republic of South Africa. Six regions in the **EC** were selected: Sarah Baartman (SBR-EC), Amathole (AR-EC), Chris Hani (CHR-EC), Joe Gqabi (JGR-EC), OR Tambo (ORT-EC) and Alfred Nzo (AN-EC). In **FS,** the regions selected were: Xhariep (XR-FS), Mangaung (MR-FS), Thabo Mofutsanyana (TMR-FS), Fezile Dabi (FDR-FS) and Lejweleputswa (LJR-FS). In **KZN:** Ethekwini (ER-KZN), Ugu (UR-KZN), uMgungundlovu (UMR-KZN), iLembe (IR-KZN), Harry Gwala (HG-KZN) and uThukela (TR-KZN) regions were selected. The study sites represent different geographic locations where the natural population of *A. ferox* exists.

The collected plants were deposited at the University of Fort Hare, Agriculture and Environmental Science Herbarium for identification, voucher specimens: GOG EC 1, GOG EC 2, GOG EC 3, GOG EC 4, GOG EC 5, GOG EC6, GOG KZN1, GOG KZN 2, GOG KZN 3, GOG KZN 4, GOG KZN 5, GOG KZN 6, GOG FS 1, GOG FS 2, GOG FS 3, GOG FS 4, GOG FS 5.

Plant preparation

Fresh *A. ferox* leaves were rinsed and subsequently chopped at 4 cm piece, weighed and air-dried for 30 days. The dried plant leaves were ground to a fine powder and subsequently stored in a sealed clear plastic container in the dark at room temperature (25°C) until further processing.

Antibacterial assay

The antibacterial activity was carried out according to Coopoosamy et al. (2010). The fresh *A. ferox* leaf material (approximately 1 kg of each species) was collected from the wild population. The leaf material was dried in an oven at 60°C. The dried material of *A. ferox* was divided into three portions of equal mass and placed in separate conical flasks containing one of three mediums, that is, boiled water, ethyl acetate and ethanol, for extraction. All media except the boiled water were left for 72 h in an orbital shaker at 20 shakes per minute. After 72 h, the extracts were filtered. The boiled water extracts were placed on a hot plate and left for

extraction for over 4 h at 60°C. The extracts were stored at room temperature (25°C) for further analysis.

The crude extracts were then tested for antibacterial properties against five Gram-positive (*Bacillus subtilis* ATCC11744, *Micrococcus kristinae* ATCC 700405, *Bacillus cereus* ATCC19524, *Staphylococcus epidermis* ATCC 29728 and *Staphylococcus aureus* ATCC 29737) and four Gram-negative (*Escherichia coli* ATCC13706, *Protea vulgaris* ATCC 49132, *Shigella sonnei* ATCC28172 and *Enterobacter aerogenes* ATCC 35029). Each organism was prepared by diluting in 24-h-old broth cultures with sterile nutrient broth. The cultures were then diluted 100-folds to give approximately 106 bacteria mL^{-1}.

Agar-disc diffusion method

Nine bacterial cultures were selected from the stock collection in the Department of Microbiology, University of KwaZulu-Natal. Gram-positive cultures were: *S. epidermis, S. aureus B. cereus, B. subtilis* and *M. kristinae.* Gram-negative cultures were: *E. coli, Proteus vulgaris, S. sonnei, E. aerogenes*, and cultures were plated out, verified by using Gram stains, and stock cultures were stored in micro bank vials (Davies Diagnostics, South Africa) using 50% glycerol. A suspension (1 mL of 10^8 cfu/mL) of the test bacteria was spread on Mueller Hinton Agar plates (Biolab, Mereck, South Africa). A volume of 50 µL of the dissolved extracts of concentrations, 1000 µg/mL, 500 µg/mL, 250 µg/µL, 100 µg/mL, 10 µg/mL and 1 µg/mL was transferred onto sterile 9 mm discs made from Whatman No. 1 filter paper. Each concentration was tested in triplicate. Controls were chloramphenicol[a] and streptomycin sulfate[b]. The impregnated discs were allowed to evaporate in an open sterile Petri dish in a biological safety cabinet with a vertical laminar flow (Labtec Bioflow II, South Africa). These were placed onto inoculated agar plates and incubated at 37°C for 24 h. The inhibition was determined from the diameter of clearing around the disks in mm. The MIC was taken as the lowest concentration that inhibited growth after incubation. All tests were conducted in triplicate and the average has been presented.

Minimum inhibition concentration

The MIC was determined using the microplate dilution method. Sterile 96-well microplates were used for the assay (0.5 mL volume, Fisher Scientific) according to Eloff (1998). Test samples were dissolved in a minimal amount of dimethyl sulfoxide (DMSO). Stock solutions before dilution were no more than 5% DMSO so that final concentrations in the microwells were typically less than 1% DMSO and solvent controls were run at these concentrations. Samples were diluted to twice the desired initial test concentration with total serum bilirubin (TSB); samples that were difficult to dissolve were sonicated. All wells, except the first, were filled with TSB (50 µL). Test sample (100 µL) was added to the first well and serial two-fold dilutions were made down to the desired minimum concentration. Day-old cultures of bacteria grown on blood agar plates were suspended in TSB until turbidity was equal to a 0.5 McFarland Standard. The plates were inoculated with the bacterial suspension (50 µL per well) and incubated at 37°C overnight. A solution of p-INT (40 µL) was then added to each well, and plates were incubated for an additional 1 to 2 h. The MIC^{INT} was determined as the lowest sample concentration at which no red colour (signifying live growth) appeared.

Antifungal assay

Microdilution method

Aloe ferox leaves from different regions (approximately 1 kg each) were cut into small pieces and crushed in a homogenizer. The plant material was soaked in ethanol (95% v/v) in a 2-litre conical flask for 2 weeks. A separate conical flask containing distilled water was placed on a hot plate at 60°C for over 4 h. The extracts (aqueous and ethanol) obtained were evaporated at reduced pressure (45°C) to a residue. Plant extracts for testing ethanol, and aqueous extract were prepared in three different concentrations. The stock solutions were prepared by dissolving 100 mg of dry extract in 1 mL of ethanol, and water separately in order to obtain a concentration of 100 mg/mL dilutions (1:10, 1:100 and 1:500). These stock solutions were then used in phosphate buffer at pH 6.0 to evaluate the antifungal activity (Coopoosamy & Magwa 2007). The solutions were then tested for antifungal activity using the following fungal cultures: *Aspergillus flavus, Aspergillus glaucus, Candida albicans, Candida tropicalis, Trichophyton mentagrophytes* and *Trichophyton rubrum.* Plates containing potato dextrose agar were used as controls.

Ethical considerations

This article followed all ethical standards for research without direct contact with human or animal subjects.

Review findings

Antibacterial activity

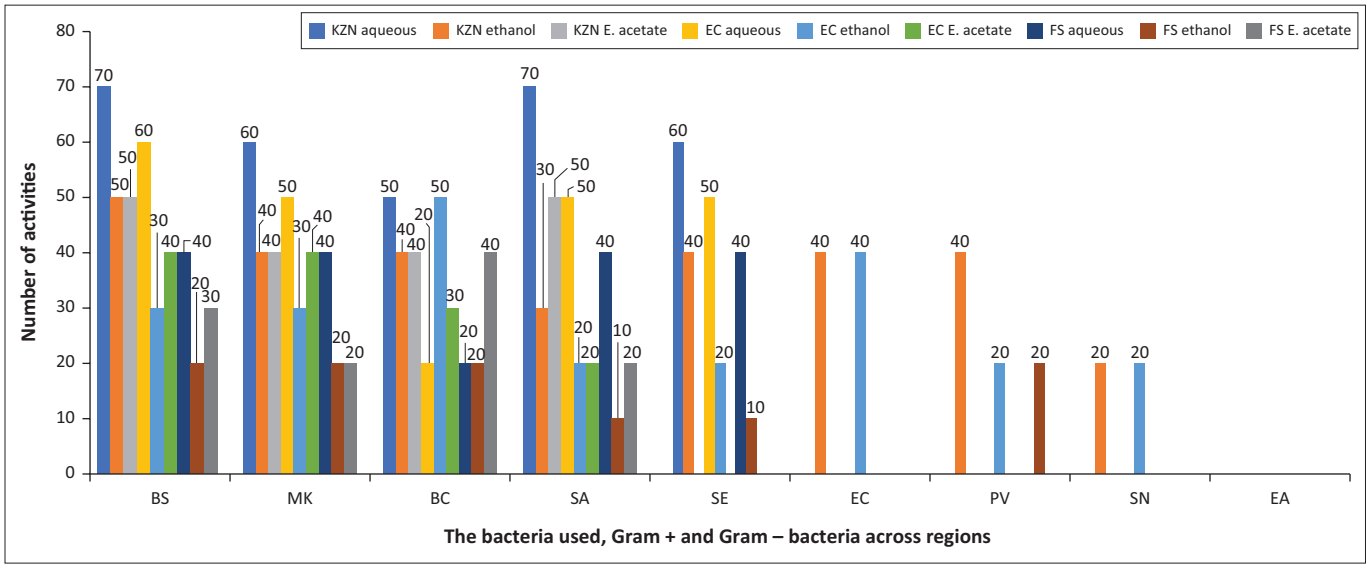
The aqueous extracts showed antibacterial activity against all Gram-positive bacteria except ethyl acetate extracts across regions (Table 1). The aqueous extract of all regions showed greater inhibition of Gram-positive bacteria, that is, *B. subtilis* and *S. aureus* 7.0 mm for KZN, 6.0 mm for EC, and 4.0 mm for FS regions, respectively. Furthermore, Gram-positive bacteria across regions exhibited greater antibacterial activity than the Gram-negative bacterial strains. Ethanol extracts showed greater inhibition of Gram-positive bacteria than ethyl acetate extracts, whereas ethyl acetate exhibited activity across regions except Gram-positive bacteria, that is, *S. epidermis,* and all Gram-negative bacterial strains. Ethanol also showed low inhibition of *S. epidermis* in the FS regions. Aqueous and ethyl acetate extracts of regions had effects on *E. coli* (Figure 1). Ethanol was the only extracting medium capable of showing inhibitory effects on three Gram-negative bacteria, that is, *P. vulgaris, E. coli and S. sonnei* across the regions.

Danish et al. (2020) reported the inhibitory effect of the root and leaf extract of *Aloe vera* (*A. vera)* to have shown varying degrees of inhibition of growth against bacterial and fungal strains. The ethanol gel extracts of *A. vera* root and

TABLE 1: Minimal inhibitory concentration (MIC) of *A. ferox* across different regions.

Bacteria	Gram -/+	Plant species and treatment *(A. ferox)*										
		KwaZulu-Natal regions			Eastern Cape regions			Free State regions			Controls	
		Aqueous (mm)	Ethyl Acetate (mm)	Ethanol (mm)	Aqueous (mm)	Ethyl Acetate (mm)	Ethanol (mm)	Aqueous (mm)	Ethyl Acetate (mm)	Ethanol (mm)	Chlor *a* µg/mL	Strept *b* µg/mL
Bacillus subtilis	+	7.0	5.0	5.0	6.0	4.0	3.0	4.0	3.0	2.0	< 2.0	< 2.0
Micrococcus kristinae	+	6.0	4.0	4.0	5.0	4.0	3.0	4.0	2.0	2.0	< 2.0	< 2.0
Bacillus cereus	+	5.0	4.0	4.0	2.0	3.0	5.0	2.0	4.0	2.0	< 2.0	< 2.0
Staphylococcus aureus	+	7.0	5.0	3.0	5.0	2.0	2.0	4.0	1.0	2.0	< 2.0	< 2.0
Staphylococcus epidermis	+	6.0	N/A	4.0	5.0	N/A	2.0	4.0	N/A	1.0	< 2.0	< 2.0
Escherichia coli	-	N/A	N/A	4.0	N/A	N/A	4.0	N/A	N/A	N/A	< 2.0	< 2.0
Proteus vulgaris	-	N/A	N/A	4.0	N/A	N/A	2.0	N/A	N/A	20	< 2.0	< 2.0
Shigella sonnei	-	N/A	N/A	2.0	N/A	N/A	2.0	N/A	N/A	2.0	< 2.0	< 2.0
Enterobacter aerogenes	-	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	< 2.0	< 2.0

Na, no activity; Chlor *a*, Chloramphenicol; Strept *b*, Streptomycin sulphate.
$N = 3$.



Gram-Positive Bacteria – BS, *Bacillus subtilis*; MK, *Micrococcus kristinae*; BC, *Bacillus cereus*; SA, *Staphylococcus aureus*; SE, *Staphylococcus epidermis.*
Gram-Negative Bacteria – EC, *Escherichia coli*; PV, *Proteus vulgaris*; SN, *Shigella sonnei*; EA, *Enterobacter aerogenes.*
FIGURE 1: Antibacterial activity of *A. ferox* across different regions against Gram -ve and Gram+ ve bacteria.

leaves showed the highest degree of activity around 19 mm and ranged from 11 mm to 19 mm at the highest concentration against the selected pathogens. The methodology and standard applied have shown similar antimicrobial results which correlate with the findings of this study.

Aloe ferox has been reported to be 'the most widely distributed *Aloe* species occurring from the Swellendam area in the Western Cape through the dry parts of the Western and Eastern Cape Provinces'; to be very common in the EC; occurring in the dry valleys of Southern-Natal in KZN (Smith & Figueiredo 2020). The species is restricted to the Zastron district along the Lesotho border in FS region (UNEP-WCMC, I.U.C.N. 2012).

Antifungal activity

Alcohol extracts provide a more complete extraction with fewer polar compounds, and many of these extracts have antifungal properties (Blondeau et al. 2020). Whereas alcohol extracts have previously demonstrated antifungal activity, the presence of activity in an aqueous extract of the same plant suggests that the active components may be more polar compounds, according to the literature (Zreen et al. 2022). According to Al-Ameedy and Omran (2019), if the aqueous extract does not have the same activity as the alcohol extract, this indicates that the less polar compounds are the active components.

The antifungal activity (Table 2) of the ethanol extracts as well as the aqueous extracts displayed strong inhibition at the concentration of 1:10 against all fungal species under investigation from all regions. Aqueous extract of the KZN regions was shown to be particularly effective in inhibiting the fungi tested, especially at 1:10 dilution. However, against the test organisms, the ethanol extract demonstrated greater antifungal activity than the aqueous extract. This could be because of the difference in polarity of the extraction media.

TABLE 2: The effect of *A. ferox* aqueous and ethanol extracts on various fungal species at different concentrations across different regions (N = 3).

Fungal strains	Plant species and treatment *(A. ferox)*																	
	KwaZulu-Natal regions						Eastern Cape regions						Free State regions					
	Aqueous extract			Ethanol extract			Aqueous extract			Ethanol extract			Aqueous extract			Ethanol extract		
	1:10	1:100	1.500	1:10	1:100	1:500	1:10	1:100	1:500	1:10	1:100	1:500	1:10	1:100	1:500	1:10	1:100	1:500
A. flavus	++++	+++	++	++++	+++	++	++++	+++	++	++++	+++	++	++++	+++	++	++++	+++	++
A. glaucus	+++	+++	+++	++++	+++	+++	+++	+++	+++	++++	+++	+++	+++	+++	+++	++++	+++	+++
C. albicans	++	+	-	++	+	-	++	+	-	++	+	-	++	+	-	++	+	-
C. tropicalis	+++	++	+	+++	++	++	+++	++	+	++++	++	++	+++	++	+	+++	++	++
T. mentagrophytes	++	++	+	+++	++	++	++	++	+	++++	++	++	++	++	+	+++	++	++
T. rubrum	++	+	+	++	++	+	++	+	+	++	++	-	++	+	+	++	++	+

- = Negative antifungal activity.

+ = Positive antifungal activity (low inhibition), ++ = Positive antifungal activity (medium inhibition), +++ = Positive antifungal activity (high inhibition), ++++ = Positive antifungal activity (very high inhibition).

Note: Plates containing Potato dextrose agar served as controls. Controls did not show any inhibition of any of the test fungal species.

Growth inhibition (zone of inhibition) was recorded as very high (++++), high (+++), medium (++) and low (+), which indicated zones of inhibition between 41 mm – 50 mm, 31 mm – 40 mm, 21 mm – 30 mm and 11 mm – 20 mm, respectively (Coopoosamy et al. 2010). The high zones of inhibition noted in the ethanol extracts (using a 1:500 concentration) suggest further investigation on the possibility of using *Aloe* species in alternative uses against diseases caused by the above fungal organisms.

A. flavus and *A. glaucus* showed positive antifungal activity with very high inhibition on ethanolic and aqueous extracts in concentrations ranging from 1:10 to 1:500, respectively. *C. tropicalis* and *T. mentagrophytes* demonstrated high antifungal activity on ethanolic extract with varying inhibition at different concentrations, whereas *T. rubrum* demonstrated positive antifungal activity with low inhibition on ethanolic extract at 1:500. *C. albicans* showed positive antifungal activity with low inhibition at 1:10 up to 1:100 concentrations, but low antifungal activity at 1:500 concentrations on both ethanolic and aqueous extracts observed across all the regions.

At various concentrations, *A. flavus*, *A. glaucus*, *C. tropicalis*, *T. mentagrophytes* and *T. rubrum* demonstrated strong antifungal potential in ethanolic and aqueous extracts. The presence of the extract's active components in both the aqueous and ethanolic extracts suggests that they are among the more polar compounds. The study found antimicrobial trends to be in line with the previous studies conducted (Anju et al. 2021; Chaudhary et al. 2019; Danish et al. 2020). The pathogen strains used in the studies explain the differences in the findings. The study discovered that a single extract has varying degrees of activity against different strains of a common species.

The differences between the same studies could be attributed to the extraction methods used. The aqueous extracts may contain some of the less polar compounds; alcohol is a general solvent that provides a more thorough extraction of compounds with varying polarities. It is also possible that climate variations and seasonal differences and the time of harvesting account for some of the observed differences. The quantity and quality of active compounds vary with the growing conditions, and compounds tend to concentrate in different parts of the plant during different growth cycles and seasons. Kowalska-Krochmal and Dudek-Wicher (2021) reported that the MIC value is currently the best available parameter for determining an antibiotic's effectiveness against bacterial strains. Despite the standardisation of approved methods, it should be noted that the actual MIC value obtained in the investigation may differ by +/- double dilution from the one obtained in the investigation. However, this difference usually does not affect the clinical interpretation (Kowalska-Krochmal & Dudek-Wicher 2021).

Antibiotic resistance is a problem that continues to challenge the healthcare sector in a large part of the world in both developing and developed countries (Manandhar, Luitel & Dahal 2019). Reynolds et al. (2022) allude that the emergence and spread of multidrug-resistant pathogens have substantially threatened the current antibacterial therapy. This has necessitated a search for a new source of antimicrobial substances such as plants as they produce a variety of bioactive compounds with known therapeutic properties (Mustafa et al. 2017). This study sought to investigate the effects of antimicrobial (antibacterial and antifungal) activity of *A. ferox* (Mill) across various regions of the Republic of South Africa.

Although some extracts of *A. forex* exhibited good antibacterial activity towards different tested bacterial strains, some extracts have exhibited a very slight or limited antibacterial activity against the test bacterial strains as arbitrated by their MIC values.

Conclusion

The study revealed variations in antimicrobial activity across geographical regions in the Republic of South Africa, namely KZN, EC and FS provinces. The concentrations of *A. ferox* active ingredient used in traditional medicine differ by regions and within a region. This necessitates further investigation into the possible isolation, and quantification of active constituents across different regions and seasons, determining the effects of each of these constituents. Furthermore, it is worth noting that for many years, rural communities, traditional healers or *sangomas* have relied on

boiling the plant material in water to extract the relative ingredients for wide use in traditional medicine and practices because of a lack of adequate resources for extraction.

There are many factors that can influence the results obtained and the great variation often observed in testing crude plant extracts in antimicrobial assays. The extraction technique, the culture medium, the strain of bacteria used for testing, the botanical source of the plant, the age of the plant, the state of the plant material used and the amount of extract being tested are factors to consider.

The challenge of microbial resistance is a growing concern, and the future use of antimicrobial drugs remains uncertain. Actions must be taken to address this issue, including limiting antibiotic use, conducting more research to better understand the genetic mechanisms of resistance, and continuing studies to develop new drugs, both synthetic and natural. Finally, the use of *Aloe* species, particularly *A. ferox*, by indigenous peoples of the rural areas cannot be disputed. *A. ferox* showed inhibition to varying degrees across all regions. Therefore, the study validates the potential differences in the biological activity of *A. ferox* across different geographical regions.

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Competing interests

The authors declare that they have no financial or personal relationships that may have inappropriately influenced them in writing this article.

Authors' contributions

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Data availability

The authors confirm that the data created or analysed in this study are included in this manuscript.

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