

Full Length Research Paper

Antimicrobial potency of the ethanolic crude bark extract of *Ziziphus mucronata* Willd. subsp. *mucronata* Willd.

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***Ziziphus mucronata* Willd. subsp. *mucronata* Willd. is a medicinal plant widely used in South Africa for the treatment of various diseases. Like many medicinal plants to which traditional claims including the treatment of ailments of infectious origin but without scientific reports have been associated, *Z. mucronata* is not an exemption. The ethanolic stems bark extract of *Z. mucronata* against medically important pathogens was investigated for the antimicrobial activity by the agar dilution method while the degree of its antimicrobial activity was determined by the macrobroth dilution method. The extract showed good antibacterial and antifungal activities. Both Gram-negative and Gram-positive bacteria were highly inhibited by the extract at very low concentrations. All the bacteria had inhibition zones greater than 20 mm at a concentration of 5 mg/ml with the exception of *Pseudomonas aeruginosa* (ATCC 19582) and *Bacillus pumilus* (ATCC 14884). For the bacteria, the minimum inhibitory concentration (MIC) values ranged between 0.078 and 0.625 mg/ml. For the fungi, the MIC values were between 2.5 to 10 mg/ml. The bacteria were more susceptible to the extract than the fungi. The antimicrobial activity of the extract indicated a broad spectrum, great therapeutic potential and justified the use of this plant by the rural communities in the treatment of microbial infections.**

Key words: Agar dilution, antimicrobial, broad spectrum, ethanol extract, fungicidal concentration.

INTRODUCTION

Medicinal plants constitute an effective source of effective therapy for both traditional and modern medicine. They are important for pharmacological research and drug development. Their constituents are used directly as therapeutic agents as well as starting materials for the syntheses or as models for pharmacologically active compounds. Due to a rapid increase in the rate of infections, antibiotic resistances in microorganisms and side effects of synthetic antibiotics, uses of medicinal plants in the treatment of microbial infections are gaining fast recognitions over antibiotics (Babu and Subhasree, 2009). Hence, plants have turned out to be an important

source of new potent antibiotics, new drug leads and new chemical entities (Saklani and Kutty, 2008). They are considered cheapest and safer alternative sources of antimicrobials (Sharif and Banik, 2006; Doughari et al., 2007) to which pathogens are not resistant (Fabricant and Farnsworth, 2001).

Consequently, phytomedical investigations have revealed several plants with anti-infective properties (Stafford et al., 2005; Kambizi et al., 2007; Anwar et al., 2011). The effects of many different plant extracts on pathogenic microorganism have been indicated by numerous studies (Vundac et al., 2007; Háznagy-Radnai et al., 2008). Pharmacologically bioactive compounds such as alkaloids, flavanoids, tannins and phenolic compounds (Nawrot et al., 2007; Beltrame et al., 2011; Ullah et al., 2011; Samali et al., 2012) producing definite physiological actions on the humans have been

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identified. Antimicrobials of plant origin effective in the treatment of infectious diseases and simultaneously mitigating many of the side effects often associated with synthetic antimicrobial agents have been discovered (Iwu et al., 1999; Rajeshwar et al., 2005).

Ziziphus mucronata Willd. subsp. *mucronata* Willd, a small to medium-sized tree with a spreading canopy, is distributed throughout summer rainfall areas of sub-Saharan Africa, extending from South Africa northwards to Ethiopia and Arabia. In ethnomedicine, pastes of the roots and leaves are applied to treat boils, swollen glands, wounds and sores while steam baths from the bark are used to purify and improve complexion (Palmer and Pitman, 1972). In East Africa, the roots are used for treating snake bites, gonorrhoea, diarrhoea and dysentery (Hutchings et al., 1996). In South Africa, ethnobotanical survey indicated that it is used for gastrointestinal disorder, dysentery and diarrhoea. Unlike some members of the *Ziziphus* genus, there is a lack of scientific reports to indicate the pharmacological importance and antimicrobial activities of this plant. Hence, this study was aimed at investigating the antimicrobial activities of ethanolic bark extract of *Z. mucronata* and justifies its ethnomedicinal importance in complementary and alternative medicine.

MATERIALS AND METHODS

Collection of plant material

The bark materials of *Ziziphus mucronata* subsp. *mucronata* were collected in August, 2010, from the plant growing within the University of Fort Hare campus in Alice, South Africa. The plant was authenticated in the Department of Botany and a voucher specimen was prepared and deposited in the Griffen Herbarium of the University.

Extract preparation

The bark sample was air-dried at room temperature and pulverized using a milling machine. About 100 g of the pulverized sample was extracted with 500 ml of ethanol for 72 h. The extract was filtered through Whatman No. 1 filter paper and evaporated to dryness under reduced pressure at 40°C using a rotary evaporator. The extract was redissolved in ethanol to the required concentrations for the bioassay analysis.

The reconstituted ethanolic extract solution was sterilized by filtering through 0.45 µm membrane filter and tested for sterility after membrane filtration by introducing 2 ml of the extract into 10 ml of sterile nutrient broth and incubated at 37°C for 24 h. A sterile extract was indicated by the absence of turbidity in the broth after the incubation period (Ronald, 1995).

Test organisms

The microorganisms used in this study included *Escherichia coli* (ATCC 25922), *Bacillus cereus* (ATCC 10702), *Bacillus pumilus* (ATCC 14884), *Proteus vulgaris* (KZN), *Serratia marcescens* (ATCC 9986), *Enterococcus faecalis* (KZN), *Staphylococcus aureus* (OK3), *Pseudomonas aeruginosa* (ATCC 19582), *Klebsiella*

pneumoniae (KZN), *Staphylococcus aureus* (OK1), *Salmonella typhi* (ATCC 13311), *Acinetobacter calcoaceticus* (UP), *Candida krusei*, *Candida albicans*, *Cryptococcus neoformans*, *Cryptococcus rugosa*, *Penicillium notatum*, *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus terreus*. These organisms were obtained from the Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa. The organisms were maintained on nutrient broth, nutrient agar (Biolab), potato dextrose agar and sabouraud dextrose broth. The antibacterial assays were carried out using Mueller Hinton II Agar (Biolab) and broth. The antifungal assays were carried out using sabouraud dextrose broth and potato dextrose agar.

Antimicrobial assay by agar diffusion method (Inhibition zones)

The susceptibility screening of the test bacteria to the ethanolic bark extract of *Z. mucronata* and ciprofloxacin, used as control, was done in accordance with the methods described previously (Irobi et al., 1994; Akinpelu et al., 2008). For the antibacterial assay, the inoculum of each test bacterial strains was standardized at 5×10^6 cfu/ml using McFarland Nephelometer standard (NCCLS, 1993). Sterile Mueller Hinton agar plates were seeded with each adjusted test bacterial strains and allowed to stand at 37°C for 1 h. For the antifungal assay, 1 cm² of seven day old fungal cultures was dropped in sterile distilled water and vortexed for 2 min to release the fungal spores. Potato dextrose agar plates were seeded with 200 µl of the fungal spore solutions, allowed to stand for 1 h on the laboratory bench. Wells were then bored into the agar media using a sterile 6 mm cork borer. The wells were filled with 100 µl of different concentrations of the extract and ciprofloxacin taking care not to allow spillage of the solutions onto the surface of the agar. The plates were allowed to stand on the laboratory bench for 30 min to allow proper diffusion of the extract and antibiotics before being incubated at 37°C for 24 h for bacterial cultures and 27°C for 72 h for fungal cultures. Wells in blank Mueller Hinton agar and potato dextrose agar plates containing 10% ethanol representing the final ethanol concentration in the test plates without the extract served as positive controls. After 24 h incubation period, antimicrobial activities were determined by measuring the inhibition zones against the test organisms using a calibrated transparent meter rule. Both antibacterial and antifungal assays were carried out in triplicates.

Macrobroth dilution for minimum inhibitory concentration (MIC)

The activity of the extract and minimum inhibitory concentration were determined by the macrobroth dilution methods (NCCLS, 1993). For antibacterial assay, different concentrations of the extract ranging from 0.02 to 10 mg/ml were prepared by serial dilutions in Mueller Hinton broth medium. Different concentrations of ciprofloxacin ranging from 0.01 and 5 µg/ml used as positive control were also prepared by serial dilution in Mueller Hinton agar. The tubes were inoculated with 100 µl of each of the bacterial strain. Blank Mueller Hinton broth was used as negative control.

For the antifungal assay, different concentrations of the extract ranging from 0.2 and 20 mg/ml were prepared in sabouraud dextrose broth by serial dilutions. Each broth concentration was inoculated with 100 µl of the prepared fungal spores' solution. Two control tubes were included: One with spores and broth but no plant extract, and one with broth and plant extract but no spores. The bacterial containing tubes were incubated aerobically at 37°C for 24 h. The fungal containing tubes were incubated at 27°C for 3 to 5 days. The first tube in the series with no visible growth after incubation period was taken as the MIC.

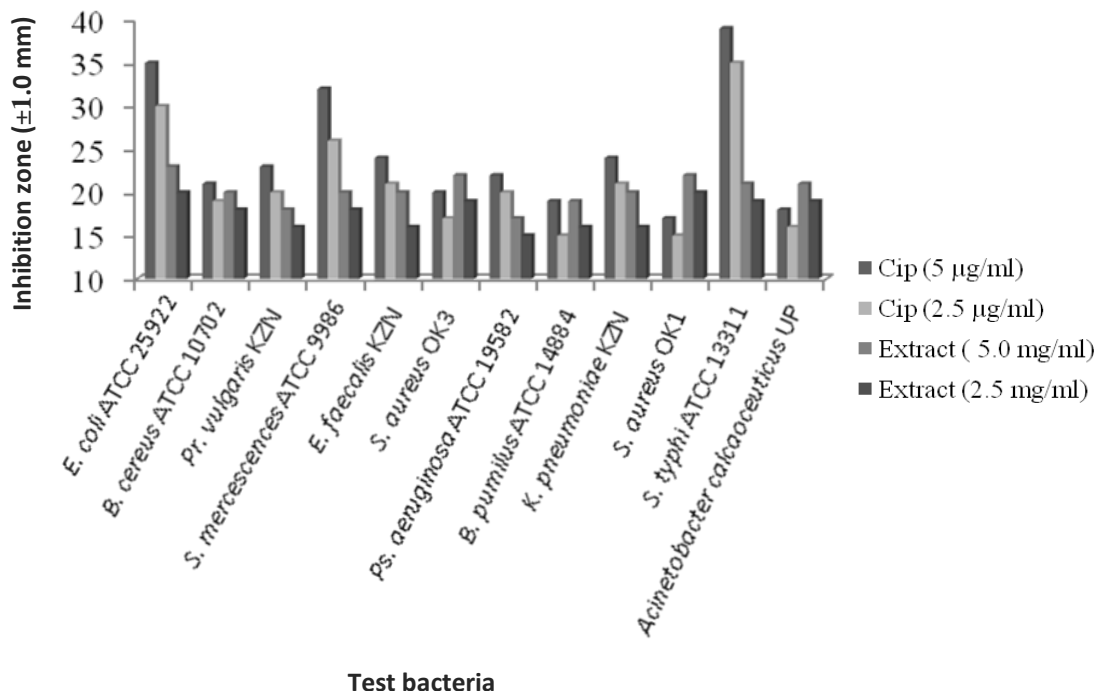


Figure 1. Antibacterial activity of ethanolic extract of *Z. mucronata* and ciprofloxacin against tested bacteria.

Determination of minimum bactericidal and fungicidal concentrations (MBC/MFC)

For the determination of the MBC and MFC, fresh nutrient agar and potato dextrose agar plates were inoculated with one loopful of culture taken from each of the first three broth cultures that showed no growth in the MIC tubes. While MBC assay plates were incubated for 24 h, MFC assay plates were incubated for 5 days. After the incubation periods, the lowest concentration of the extract that did not produce any bacterial or fungal growth on the solid medium was regarded as MBC and MFC values for this extract (Irkin and Korukluoglu, 2007). This observation was matched with the MIC test tube that did not show evidence of growth after 48 h of incubating the bacteria or spore germination for the fungi after five days of incubation.

Statistical analysis

All the data were subjected to one way analysis of variance (ANOVA) and the mean values were separated at ($p < 0.05$) using Duncan's multiple range test. The one way ANOVA test was used to determine if there was any statistically significant difference in the diameter of the zones of inhibition obtained from the different concentrations of the extract tested against the microorganisms. All statistical analyses were done using SAS software (1999) model.

RESULTS

The antibacterial activities of the stem bark extracts of *Z. mucronata* against medically important pathogens were assessed by the presence or absence of inhibition zones. To determine the degree of antibacterial activity, the extract was subjected to minimum inhibitory concentration (MIC) assay by serial two fold dilution method

(Florey et al., 1989). The result revealed that the ethanolic extract of *Z. mucronata* possess good antibacterial activity against both Gram-positive and Gram-negative bacteria (Figure 1) and antifungal activity against all the tested fungal isolates (Figure 2). The antibacterial activity assessed in terms of inhibition zone indicated that *E. coli* ATCC 25922 had the highest inhibition zone and *P. aeruginosa* ATCC 19582 had the least inhibition zone from 5 mg/ml concentration of the extract (Figure 1). The minimum inhibitory concentrations assay indicated that the two groups of bacteria were susceptible to the extract at very high concentrations with *S. aureus* OK₁ having the highest minimum inhibitory concentration of 0.0781 µg/ml (Figure 3). With the exception of *P. aeruginosa* ATCC 19582 and *B. pumilus* ATCC 14884, all the bacteria had inhibition zones greater than 20 mm at a concentration of 5 mg/ml. The extract was less potent than the standard antibiotic, ciprofloxacin, to which the bacteria were highly susceptible at 5 µg/ml.

The antifungal assay indicated that all the fungal isolates were highly susceptible to different concentrations of the extract and their susceptibilities were concentration dependent. With an increase in the concentration of the extract, there are increases in the inhibition zones of each of the microorganisms. Minimum fungicidal concentrations of the plant extracts were expectedly higher than the minimum inhibitory concentration while the MIC values were found ranging from 2.5 to 10 mg/ml for fungi. The MIC values of *P. notatum* and *A. flavus* was 2.5 mg/ml, *A. niger*, *A. terreus*

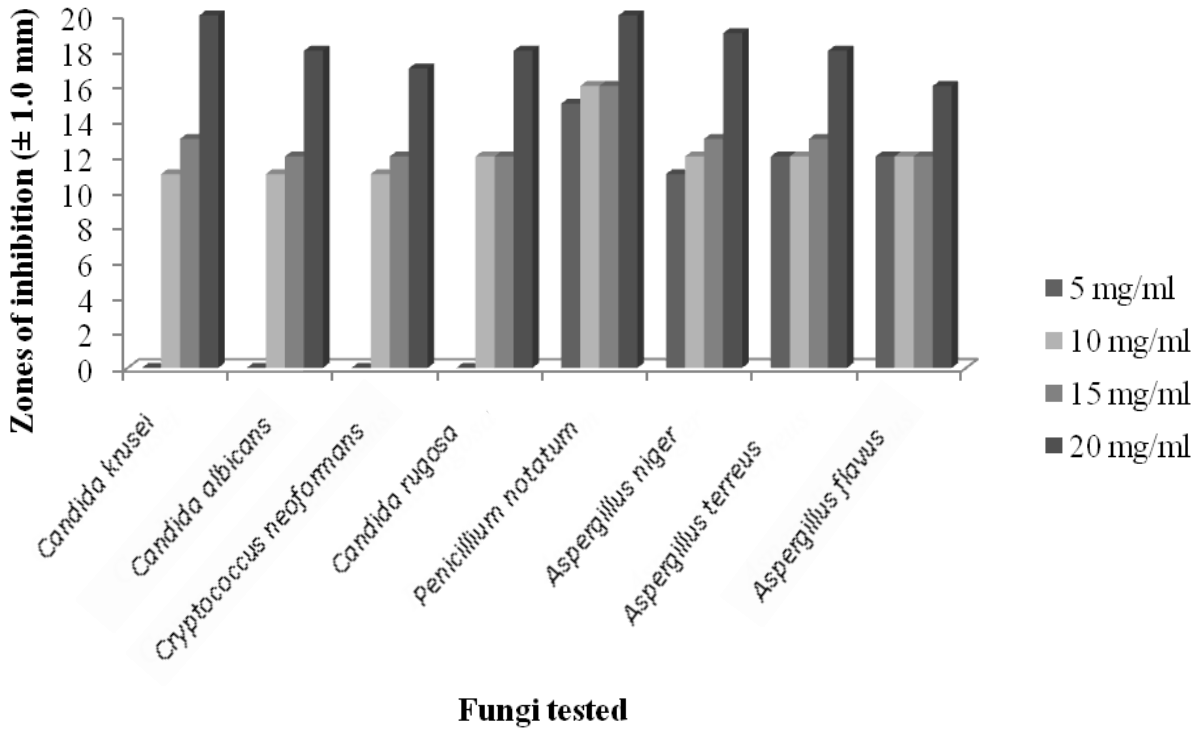


Figure 2. Antifungal activities of different concentration of ethanolic extract of *Z. mucronata* subsp. *mucronata*.

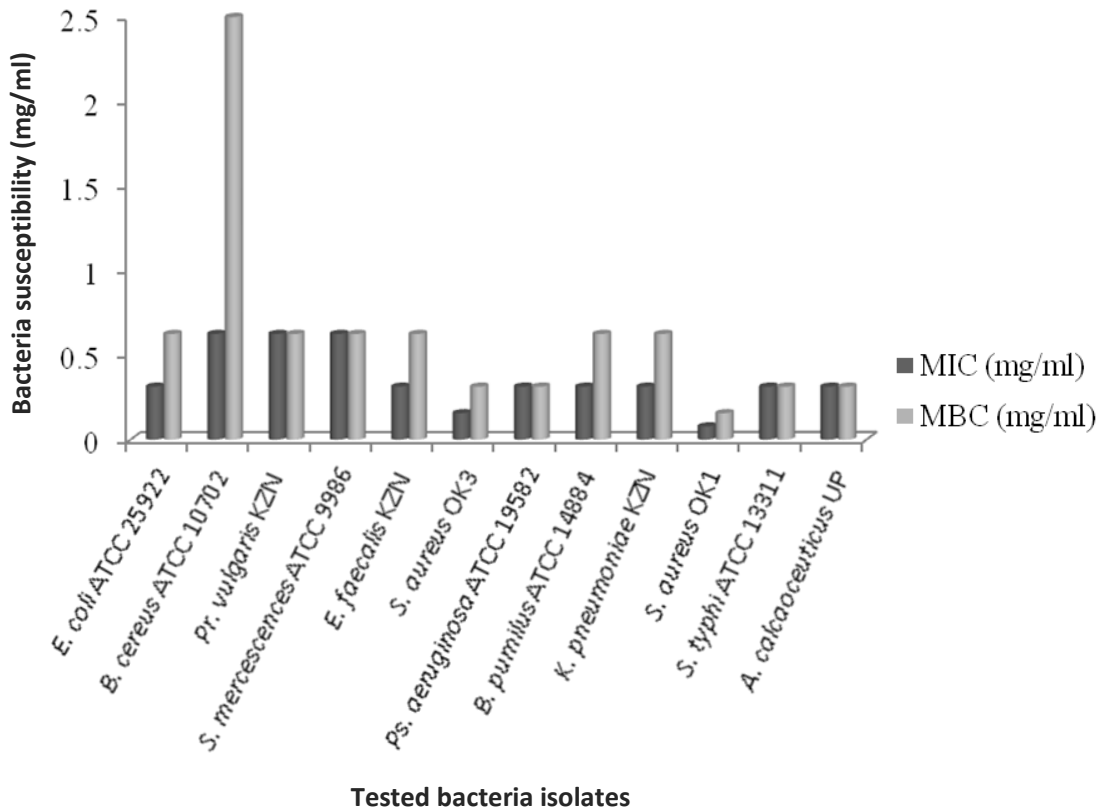


Figure 3. MIC and MBC of ethanolic extract of *Z. mucronata* subsp. *mucronata* against tested bacteria.

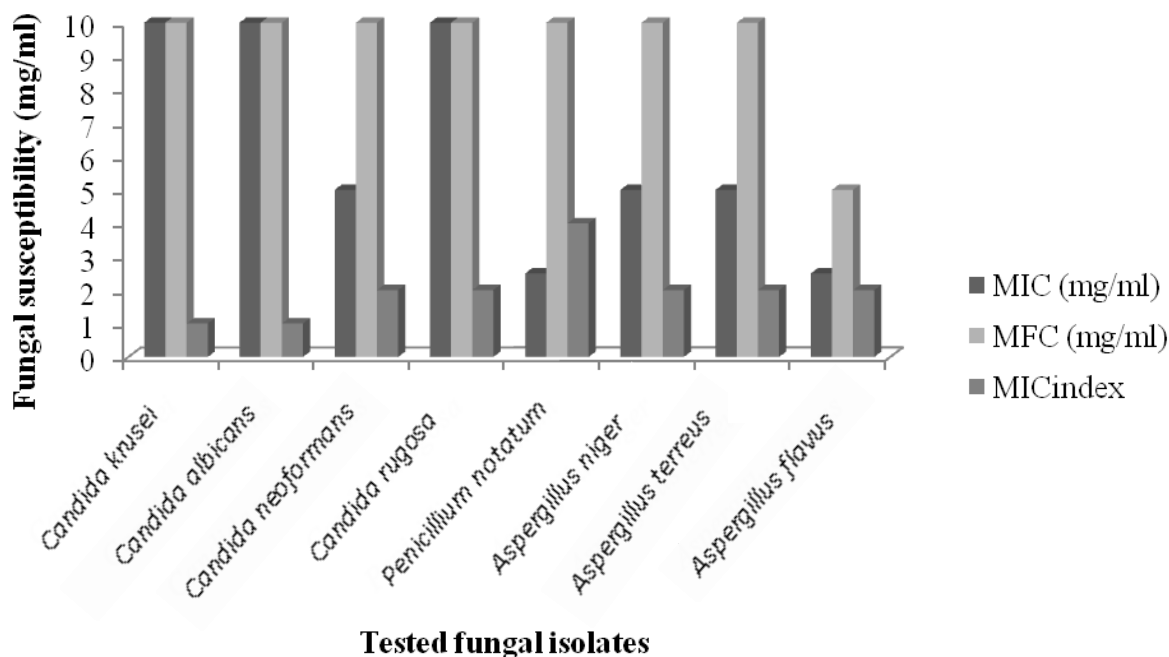


Figure 4. MIC and MFC of ethanolic extract of *Z. mucronata* subsp. *mucronata* against tested fungi.

and *Cryptococcus neoformans* was 5 mg/ml, *C. krusei*, *C. albicans* and *C. rugosa* was 10 mg/ml (Figure 4). Although the MIC values of the extract against bacteria were considerably low, they were higher than those obtained for the fungi indicating that the bacteria were more susceptible to the extract than the fungi. The MIC/MBC and MIC/MFC ratios indicated that the extract is bacteriostatic and fungistatic at lower concentrations while at higher concentrations, it is bactericidal and fungicidal. The activity of the extract on the fungal species is significant as these fungi are usually implicated in atrophic candidiasis, vaginal thrush and aflatoxin production. Where MIC equals MBC/MFC, the similarity revealed that the plant bark possesses potent antibacterial and fungicidal components against the test isolates. The low MIC and MBC values showed that the extract has strong antimicrobial activity. The antimicrobial activity of the extract, however, indicated a broad spectrum and great therapeutic potential of the plant.

DISCUSSION

The antibacterial and antifungal activities of the ethanol extract of *Z. mucronata* were investigated in this study. The results showed that the extract had significant antimicrobial potency against both bacteria and fungi tested. While the active components in the crude extract may be acting synergistically to produce good antimicrobial effects (Eloff, 1998), the disparity between the activities of the extract and the standard antimicrobial

drug may be due to the mixtures of bioactive compounds present in the extract compared to the pure compound contained in the standard antibiotics (Gatsing et al., 2010).

Screening of plants for their pharmacological activity has indeed been the vast source of innumerable therapeutic effects representing molecular diversity engineered by nature. Plant extracts and their purified compounds have shown antibacterial and fungicidal effects both *in vitro* and *in vivo* (Saglam and Arar, 2003). Extracts with known antimicrobial properties can be of great importance in therapeutic treatments (Erdogru, 2002; Rojas et al., 2006) and their active principles have been considered alternative cheap and effective herbal drugs against common microbial infections (Kareru et al., 2008). In agreement with several reports on antimicrobial activities of many medicinal plants (Afolayan et al., 2002; Mathabe et al., 2006; Lategan et al., 2009; Gavanji et al., 2011; Govindappa et al., 2011), the significant antimicrobial activities of *Z. mucronata* indicated its medicinal potential that could be used against infectious pathogens. Its ethanolic extract, at different concentrations, possesses inhibitory effects indicating a broad spectrum of antimicrobial activity.

Although the mechanism of action of this extract is not understood, its action against the bacteria and fungi may be due to the inhibition of cell wall through pore formation in the cell and leakage of cytoplasmic constituents by the bioactive components of the extract (Bais et al., 2002; Hassan et al., 2007). While phytochemical compounds such as tannin coagulate the wall proteins, saponins

facilitated the entry of toxic material or leakage of vital constituents from the cell (Onwuliri and Wonang, 2005). Flavonoids inhibit the activity of enzymes (Dathak and Iwu, 1991) by forming complexes with bacterial cell walls, extracellular and soluble proteins, more lipophilic flavonoids disrupt cell wall integrity (Kurtz et al., 1994) or microbial membranes (Tsuchiya et al., 1996) at low concentrations.

Since the MIC values indicated the definite nature of the antimicrobial activities of this plant, the inhibition zones values, only, indicated extent of effectiveness of the extract with increasing concentration. The difference in the antibacterial activities between the macrobroth and agar diffusion assays may be attributed to the degree of exposure of each of the tested organisms and the rate of diffusion of the extract into the surrounding agar from the wells. In broth assay, the organisms have direct contacts with the extract. The diffusion in agar assay, in effect, created an extract gradient around the wells with microbial inhibition being achieved only when the extract concentration in the agar approximately reached that of the tube MIC. Progressively, higher extract concentrations yielded progressively greater degrees of inhibition (larger inhibition zones). The tube assay provided a more accurate determination of the MIC and antibacterial activity of the extract than the well diffusion assay. Under liquid conditions, the microbial cells are in direct contact with the extract which was more homogeneously present throughout the culture liquid and no differential gradient was established. The obtained result is similar to the findings of Banso et al. (1999) and Rasooli et al. (2006) that showed that higher concentrations of antimicrobial substance yielded greater degrees of growth inhibition.

In conclusion, this study demonstrated that crude extract of *Z. mucronata* exhibited good antimicrobial activity against different infectious agents but not as effective as the standard antibiotic used for comparison. The activity of the extract against both bacteria and fungi has confirmed its broad spectrum antimicrobial activity. The present study, therefore, justifies the rationale for the traditional use of the ethanolic stem bark extract of *Z. mucronata* in the treatment of microbial infections by rural communities in South Africa.

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