Antibacterial activity of selected medicinal plants used in ethnoveterinary medicine

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Five plant species (Balanites maughamii, Breonadia salicina, Dombeya rotundifolia, Hyperacanthus amoenus and Piliostigma thonningii) were extracted with acetone. Antibacterial activity of the leaf extracts of B. maughamii and D. rotundifolia, P. thonningii and bark extracts of B. salicina and H. amoenus were determined using serial dilution assay. The plant species were selected based on information provided by small scale farmers on the use of these plant species against various diseases in the domestic livestock. The crude extracts were tested for antibacterial activity against four selected bacterial strains, one gram positive (Bacillus cereus ATCC 14579) and three gram negative (Pseudomonas aeruginosa ATCC 27853, Escherichia coli ATCC 25922 and Enterococcus faecalis ATCC 29212). All plant extracts were active against the selected tested bacterial. Of the five plant species, leaf extracts of B. maughamii and P. thonningii had the best antibacterial activity against E. faecalis and P. aeruginosa (MIC value of 0.195 mg/ml). However, addition of polyethylene glycol (PEG) to the plant extracts resulted in reduction of antibacterial activity in all crude extracts, with PEG being more effective than polyvinyl polypryrolidone (PVPP). The highest percentage reduction (97.8%) was observed in extract of B. maughamii against E. faecalis. Bioautography assay was used to determine the number of antibacterial compounds in the extracts. No active compounds were observed in plant extract of P. thonningii with good antibacterial against microorganisms indicating possible synergism between separated metabolites. Based on our findings, selected plant species could be used by small scale farmers in ethnoveterinary medicine to combat bacterial infections in livestock.

Key words: Antibacterial activity, minimum inhibitory concentration, bioautography assay, ethnoveterinary medicine.

INTRODUCTION

Traditional medicine is undoubtedly the total knowledge, skills and practices based on theories, beliefs, and indigenous cultural experience (whether explicable or not) used in the maintenance of health, diagnosing, preventing, or eliminating physical, mental or social diseases (World Health Organization (WHO), 2006). Such knowledge may rely exclusively on past experience and observations handed from generation to generation.
verbally or in writing. The prime advantage of traditional medicine is that it is an immediate, existing source of health care for people where they live. According to WHO, more than 80% of the population in developing countries relies on plants as an integral part of their primary health care (Penso, 1980; Calixto, 2005). This also depends on whether the patients prefer to use biomedical or traditional health care (Gesler, 1984; Dauskardt, 1990).

In South Africa, the treatment of livestock diseases using traditional remedies is widely practised in rural communities (McGaw et al., 2007). In Limpopo province, Basani villages, several species of medicinal plants have been reported to be used by small scale farmers for the treatment of various ailments in domestic animals (personal communication). Ethnoveterinary medicine is developed by farmers in the fields and barns, rather than by scientists in laboratories and clinics. The need to validate ethnoveterinary medicine is important before they can be widely promoted (Mathias, 2001). Livestock owners have an excellent knowledge of ethnobotany, which has formed the basis for screening plant materials as potential sources of medical drugs. Traditional healers have less to offer in the treatment and control of epidemic and endemic infectious diseases and they can cope with a reasonable spectrum of common diseases such as diarrhoea, wounds, colds, worms, coccidiosis, and reproductive disorders (Matekaire and Bwakura, 2004). In recent years, increasing attention has been paid to ethnoveterinary knowledge and local veterinary practices (Martin et al., 2001). There is a need to encourage resource poor farmers to use available resources and methods of veterinary medicine to improve productivity, but these need to be assessed for effectiveness and safety.

Plants comprise the largest component of the diverse therapeutic elements of traditional livestock health care practices. In South Africa, plant remedies are prepared in various ways including infusions, decoctions, ground fresh plant material or sap expressed from fresh material, charring and drying. Application of a remedy is by different routes and methods, depending on the perceived cause of the disease and condition of the animal (Masika et al., 2000). Medicinal plants are widely used in some countries as a primary source of prevention and control of livestock diseases. The bark and fruits of Kigelia africana are boiled and the extract used as remedy for gastroenteritis in calves. Cereus jamacaru (cactus) is used to control worm infection in livestock (Vatta et al., 2011). Different plant parts leaves, roots, bark, flowers, fruits and seeds and other above ground plant parts are reported to be used for various treatments of livestock in Eastern Cape, South Africa (Mirutse, 2001). Dold and Cocks (2001) reported that the fresh leaves of Aloe ferox are put into poultry drinking water to prevent those contracting poultry disease and to prevent tick and lice infestation.

In this paper, we investigate the antibacterial activity of acetone extracts of selected plant species (Balanites maughmii, Breonadia salicina, Dombeya rotundifolia, Hyperacanthus amoenus and Pilostigma thonningii) against the Gram positive (Bacillus cereus) and gram negative bacteria (Pseudomonas aeruginosa, Escherichia coli and Enterococcus faecalis) and also the effect of polyethylene glycol (PEG) and polyvinyl polypyridilidone (PVPP) in plant extracts. To our knowledge, this is the first report to determine the antibacterial activity of the selected medicinal plants.

MATERIALS AND METHODS

Plant collection

Five plant species used for the treatment of various diseases in domestic animals by small scale farmers in Basani village, Giyani, Limpopo Province were identified and collected. Collected fresh plant material was examined and the old, insect and fungus-infected leaves were removed. Leaves were dried at room temperature (25°C) for about a week in a forced air draught in a purpose-built drying machine until the leaves were brittle enough to break easily. The dried plant material was ground to a fine powder (diameter = 0.1 mm) using a laboratory grinding mill (Telemecanique/MACSALAB model 200 LAB) and stored in airtight bottles in the dark until extraction.

Extraction procedure

Separate aliquots of finely ground plant material (2 g) were extracted with 40 ml acetone into a conical flask. All conical flasks were sonicated in an ultrasonic bath (Bransonic 220) at room temperature for 30 min followed by shaking of the extract on a Labcon platform horizontal shaker for 1 h. Extracts were then centrifuged at 1600 × g for 15 min. The supernatants were collected and placed in round bottom flasks and evaporated to dryness under reduced pressure on a rotavapor (Optolabor) at 40°C. Dry yields were dissolved in acetone, respectively, to a final concentration of 10 mg ml⁻¹.

Determining antibacterial activity

Bacterial strains and inoculums quantification

Four selected bacterial strains, one gram positive (B. cereus ATCC 14579) and three gram negative (P. aeruginosa ATCC 27853, E. coli ATCC 25922 and E. faecalis ATCC 29212) were obtained from the American type culture collection (ATCC). These were representatives of most common pathogens of infectious diseases, and were used for antimicrobial tests. Bacterial cultures and McFarland standards were obtained from Davies Diagnostic Company. All bacterial cultures were maintained on Mueller Hinton (MH) agar and subcultured before use in MH broth (Oxoid, Basingstoke, UK). Before the bacterial cultures were used, they were diluted with sterile nutrient broth to a turbidity that matches 0.5 McFarland standard (10⁵ Colony Forming Unit (CFU)/ml⁻¹) (Mulu et al., 2004).
Microdilution assay

The microplate method of Eloff (1998a) was used to determine the minimum inhibitory concentration (MIC) values for plant extracts. The plant extracts were tested in triplicate in each assay, and the assays were repeated in their entirety to confirm results. Residues of different extracts were dissolved in acetone to a concentration of 1 mg/ml. The extracts (100 μl) were serially diluted to 50% with water in 96 well microtitre plates (Eloff, 1998b), and 100 μl of bacterial culture was added to each well. Tetracycline and acetone were used as positive and negative control, respectively. It was previously shown (Eloff et al., 2007) that the final concentration of acetone in the microplate well that the bacteria are subjected to has no influence on the growth of fungi. As an indicator of growth, 40 μl of 0.2 mg/ml p-iodonitrotetrazolium violet (INT) dissolved in water was added to the microplate wells. The covered microplates were incubated for 24 h at 35°C at 100% relative humidity after sealing in a plastic bag to minimize bacterial contamination in the laboratory. The MIC was recorded as the lowest concentration of the extract that inhibited antibacterial growth. The colourless tetrazolium salt acts as an electron acceptor and is reduced to a red-coloured formazan product by biologically active organisms (Eloff, 1998b). Where bacterial growth is inhibited, the solution in the well remains clear or shows a marked reduction in intensity of colour after incubation with INT.

Bulk extraction

Finely ground plant materials (50 g), B. maughamii, B. salicina, D. rotundifolia, H. amoenus and P. thonninii were extracted with 1 liter methanol on a Labotec Model 20.2 shaking apparatus overnight. The extracts were filtered through Whatman No.1 filter paper using a Büchner funnel. The residue was washed three times with fresh solvent (1 liter) to extract the material. The resulting filtrate was dried under reduced pressure at 40°C in a rotavapor (Optolabor) and the reduced extracts were transferred into small (5 ml) beakers and allowed to dry. The masses of the extracts yields were determined.

Solvent-solvent fractionation

Each methanol extract (10 g) was dissolved in 500 ml hexane and transferred into a 1 L separatory funnel before being mixed with water (500 ml). When separation of the two layers occurred, the bottom layer was collected to yield hexane fraction, and the process was repeated three times by extracting the water fraction with hexane. Following this, 500 ml of 1 L ethyl acetate extracts was added to the water fraction and the top layer collected yielding the butanol fractions (Mahlo et al., 2013). The hexane, ethyl acetate and butanol fractions were evaporated to dryness at 45°C under reduced pressure using a rotavapor (Optolabor). The water fraction was evaporated using a Specht Scientific freeze dryer.

Phytochemical analysis

Chemical constituents of the extracts were analyzed using aluminium-backed thin layer chromatography (TLC) plates (ALIGRAM SIL g/UV 254-MACHEREY-NAGEL, Merck) that were developed in hexane: acetone (1:1, 2:1, 3:1 and 4:1), and benzene: ethanol: ammonium hydroxide: 90:10:1 (BEA) (nonpolar/basic) as eluent systems. Development of the chromatograms was under eluent saturated conditions. Samples (100 μg) were applied on the TLC plates in a 1 cm band and developed without delay to minimize the possibility of photo-oxidative change. The separated components were visualized under visible and ultraviolet light (254 and 360 nm, Camac Universal UV lamp TL-600). For the detection of chemical compounds not visible under UV light, vanillin-sulphuric acid spray reagent (Stahl, 1969) was used for detection.

Bioautography

TLC plates (10 x 10 cm) were loaded with 100 μg of each of the extracts with a micropipette in a line 1 cm wide. The prepared plates were developed using hexane: acetone (1:1, 2:1, 3:1 and 4:1) ratio. The chromatograms were dried at room temperature under a stream of air overnight to remove the remaining solvent. The plates were sprayed with concentrated suspension containing 106 colony forming unit (CFU) of actively growing bacteria. The plates were sprayed until wet, incubated overnight, sprayed with 2 mg/ml solution of p-iodonitrotetrazolium violet and further incubated overnight or longer at 35°C in a clean chamber at 100% relative humidity in the dark. Bacterial cultures were grown on Sabouraud (SD) agar for 24 h. Cultures were transferred into nutrient broth from agar with sterile swabs. The bacteria were centrifuged at 1600 x g for 10 min and the supernatant decanted. The pellet was diluted with sterile nutrient broth to a turbidity that matches 0.5 McFarland standard (105 CFU/ml) (Mulu et al., 2004). The developed TLC plates were sprayed with a concentrated suspension containing 106 CFU/ml of actively growing bacteria. The plates were sprayed until wet. Incubated here, reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of the test bacteria. The plates were sealed in plastic to prevent the spreading of the bacteria in the environment and to retain the humidity and then scanned to produce a record of the results.

RESULTS AND DISCUSSION

Acetone extracts of all four plant species had antibacterial activity with MIC value of 0.78 and 1.56 mg/ml against B. cereus, E. coli and P. aeruginosa. However, extracts had the lowest MIC value of 0.156 mg/ml against E. faecalis. Bark extracts of H. amoenus and B. salicina had the moderate antibacterial activity against B. cereus, E. coli and E. faecalis with MIC values of 0.39 and 0.78 mg/ml (Table 1). However, tetracycline inhibited the growth of B. cereus, E. faecalis and P. aeruginosa with MIC ranging between 0.01 and 0.03. Acetone extracts of B. salicina had good antifungal activity against P. janthinellum with MIC values of 0.08 mg/ml (Mahlo et al., 2010). B. salicina has been reported to have tannin content (Mahlo and Chauke, 2012). Gram-negative bacteria are relatively resistant to plant extracts owing to the presence of an outer membrane which is known to present a barrier to penetration of numerous antimicrobial molecules (Nikaido, 1996). In general, all
Table 1. Minimum inhibitory concentration (mg ml⁻¹) of crude acetone extracts of five plant species tested against four bacteria with the presence of PEG. The results show the average of three replicates with standard deviation 0 in all cases.

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Plant part</th>
<th>PEG (%)</th>
<th>B. cereus</th>
<th>E. coli</th>
<th>E. faecalis</th>
<th>P. aeruginosa</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>MIC</td>
<td>% increase in MIC</td>
<td>MIC</td>
<td>% increase in MIC</td>
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<tr>
<td>B. m</td>
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<td></td>
<td></td>
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<td>87.5</td>
<td>12.5</td>
<td>93.8</td>
</tr>
<tr>
<td>B. s</td>
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<td>0.78</td>
<td>0.00</td>
<td>1.56</td>
<td>0.00</td>
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<td></td>
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<td>1.0</td>
<td>6.25</td>
<td>87.5</td>
<td>3.13</td>
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<tr>
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<td></td>
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<tr>
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<td>1.0</td>
<td>1.56</td>
<td>75.0</td>
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<td>50.0</td>
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</table>

B. m = Balanites maughamii; B. s = Breonadia salicina; D. r = Dombeya rotundifolia; H. a = Hyperacanthus amoenus; P. t = Piliostigma thonningii. LF= leaf, BK= bark.

plant extracts had the moderate antibacterial activity against the tested microorganisms. This indicates that compounds with intermediate polarity have the highest activity. Plant extracts with low MIC values could be a good source of bioactive components with antimicrobial potency. Acetone was the best extractant to isolate antimicrobial compounds (Eloff, 1998a). Other reasons for its suitability include its volatility, miscibility with polar and nonpolar solvents and its relatively low toxicity against test microorganisms (Eloff, 1998a). Based on our findings, acetone was not toxic to the tested microorganisms. These findings were also confirmed by Eloff et al. (2007).

Addition of 1% PEG to the acetone extracts of plant material increased the MIC values which imply a reduction of antibacterial activity. The highest percentage reduction (97.8%) of antibacterial activity was achieved for B. maughamii against E. faecalis. On the other hand, the lowest percentage reduction (50%) of antibacterial activity for extracts of B. maughamii, B. salicina and P. thonningii was against E. coli and P. aeruginosa. PEG and PVPP are chemical reagents that bind tannins. In the current study, addition of PEG and PVPP was initiated to determine the effect of the two binding reagents on plant extracts against the tested microorganism. More importantly, the two chemical reagents resulted in the reduction of antibacterial activity in all plant extracts (Table 1). This implies that the plant extract with the presence of PEG and PVPP were not active against the tested microorganism. However, in the crude acetone extracts all plant extracts had shown a moderate antibacterial activity against B. cereus, P. aeruginosa, E. coli and E. faecalis. Previous researcher indicated that tannins bind to bacterial adhesions, and so interfere with the availability of receptors on the cell surface (Cowan, 1999). Tannins at low concentrations may also reduce bacteriophages (bacterial viruses) which can cause a reduction
in microbial efficiency through non-specific lysis of bacteria or have anti/protozoal activity (Makkar et al., 1995).

Bioautography assay was used to determine the number of active compounds of different plant extracts. A representative bioautogram is shown in Figure 1. The methanol crude extract, hexane, ethyl acetate and butanol fractions of B. maughamii, B. salicina, D. rotundifolia and H. amoenus were active against P. aeruginosa. Noticeably, no antibacterial compounds were observed in the fractions against B. cereus, E. coli and E. faecalis. Antibacterial compounds with Rf value of 0.37 were visible in the ethyl acetate fraction of D. rotundifolia. However, a distinct compound was visible at the origin of the spot. In general, most of antibacterial compounds were visible in the ethyl acetate fractions of the five plant species. Crude methanol and ethyl acetate of D. rotundifolia and P. thonningii had similar active compounds against P. aeruginosa at Rf value of 0.41. Active compounds with the same Rf value were observed in the crude methanol, and ethyl acetate of B. maughamii (Figure 1) against P. aeruginosa were the most promising since the crude methanol and fraction (ethyl acetate) displayed several antibacterial compounds inhibiting bacterial growth and could be used for further isolation.

Antibacterial compounds were also observed in the hexane, ethyl acetate and butanol fractions of B. salicina. This confirmed the results with fungal and animal fungal pathogens (Mahlo et al., 2013). The separated compounds in the crude methanol, hexane and ethyl acetate fractions had shown antibacterial compounds on bioautograms screening against the test microorganisms. However, no antibacterial compounds were observed in plant extracts of P. thonningii with good antibacterial activity in microdilution assay. Possible reasons maybe that some of the active compounds were volatile and evaporated or inactive by photo-oxidation during the drying period of the TLC plates (Mahlo et al., 2010).

Conclusion

All plant extracts tested had antibacterial activity against the gram-positive and gram-negative bacteria. Acetone
extract of \textit{B. maughamii} had the highest antibacterial activity against \textit{E. faecalis} and \textit{P. aeruginosa} with lowest MIC values. Based on MIC and bioautography results, \textit{B. maughamii} appear to be the best plant species for isolation of antibacterial compounds. The study demonstrated that the selected plant species could be used by small scale farmers in ethnoveterinary medicine to combat bacterial infections in livestock. Based on antibacterial activity, it is therefore important that the use of medicinal plants should be validated and their efficacy proven or investigated so that effective health products can be made from some plants. It is also important to identify effective plant species among those that are used. Such information can contribute to local empowerment and development of indigenous knowledge.

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