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Influence of annual rainfall on antibacterial activity of acetone leaf extracts of selected medicinal trees

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ABSTRACT

With the increasing demand for medicinal plants there is a danger that some populations may be destroyed by over collection. Cultivation of medicinal plants may address this problem, but many traditional healers believe that cultivated plants are not as active as plants growing in nature. There is uncertainty around the extent to which environmental stress affects the biological activity of plant extracts. We determined the effects of different annual rainfalls on antibacterial activity of tree leaf extracts of *Terminalia sericea*, *Combretum collinum* and *Sclerocarya birrea* growing under annual rainfalls of ≥870 mm, 650 mm and ≤480 mm in the Lowveld, South Africa. The minimum inhibitory concentration of acetone leaf extracts was determined by a serial microplate dilution technique using tetrazolium violet as growth indicator on four nosocomial bacteria *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. MICs of leaf extracts of different trees varied between 60 and 1460 μg/ml. Variations were for *T. sericea* (60–820 μg/ml), for *C. collinum* (70–820 μg/ml) and for *S. birrea* (70–1460 μg/ml). Total activity values of the same extracts ranged between 48 ml/g and 539 ml/g. Antibacterial activity and total activity of plant extracts against test bacteria generally significantly ($P \leq 0.05$) increased under lower rainfall. In some cases there were no clear correlations. There were varying sensitivities of different bacteria of leaf extracts of plants grown under different annual rainfalls. This variation suggests that possible water stress caused by different rainfalls did not exclusively affect antibacterial activity of plant extracts. It is possible that other factors such as genetic, edaphic, microclimate, herbivory and pathogens could also have had an effect. More experiments using clonal plant material under well controlled environmental conditions are required to provide a more definitive answer.

1. Introduction

Herbal medicines continue to be the mainstay of the local healthcare systems (Hoareau and DaSilva, 1999) especially those of developing countries. About 27 million South Africans use indigenous biomedicines that have an economic value of approximately R4 billion (Keirungi and Fabricius, 2005). The increased dependence on herbal medicines is largely attributed to escalating occurrence of microbial resistance to conventional antibiotics and prevalence of bacterial, viral, fungal and inflammatory diseases that affect human, livestock and other animals.

Many traditional healers prefer plants that grow under natural environment because they perceive them to possess superior medicinal properties (Cunningham, 1994). Some scientists believe that wild plants form biologically active compounds when under stress conditions and competition (Schippmann et al., 2002). This is certainly true in some cases especially where there are infections with pathogens or herbivory. There is uncertainty around the extent to which environmental stresses may affect the antimicrobial activity of plant extracts. It was against this backdrop that the study evaluated the effects of different rates of annual rainfall (and possibly other factors) on antibacterial activity of plant extracts. For the purpose of the study, three tree species were selected based on the following criteria:

- Easy to identify by a non-taxonomist
- Accessible and obtainable
- Abundance and widespread distribution
- Scientifically proven antimicrobial activity

The selected species were *Terminalia sericea* Burch. ex Dc. (Combretaceae), *Combretum collinum* Fresen. (Combretaceae) and *Sclerocarya birrea* (A. Rich) Hochst. subsp. caffra (Sond.) (Anacardiaceae or mango family). Several tree species that are members of these families are used for many purposes including herbal medicines (Shackleton et al., 2002) and also do possess antioxidant and antimicrobial activities (Fyhrquist et al., 2002; Eloff, 1999, 2001; Eloff et al., 2001, 2008).

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2. Materials and methods

2.1. Localities where selected tree species were collected

Leaf samples of *T. sericea*, *C. collinum* and *S. birrea* were collected from some localities in the Lowveld region of Limpopo Province of South Africa. Those localities were in Hazyview, Wits Rural Facility in Acornhoek and Manyeleti Game Reserve subjected to annual rainfalls of ≥c. 870 mm, c. 650 mm and ≤c. 480 mm, respectively (Shackleton, 1999). All localities had an altitude of about 550 m.

2.2. Collection and preparation of leaf samples

Voucher specimens of *T. sericea* (117134), *C. collinum* (117133) and *S. birrea* (117135) were collected. The curator, Mrs Elsa van Wyk, verified and stored specimens in the H.G.W.J. Schweickerdt Herbarium of the University of Pretoria.

Leaves from twelve trees (4 per species) at each annual rainfall level were collected. Only trees situated within a radius of about 50 m per site were considered in order to minimise genetic variability. Fully developed fresh leaves were collected from the lowest branches of 36 individual trees during spring (between October and November). Leaves were collected between 9 a.m. and 4 p.m. as study sites were situated far apart. After collection, leaves were separated from the stems and dried in a ventilated storeroom at room temperature and ground into a fine powder in a Jankel and Kunkel Model A10 mill. The powder was stored in the airtight containers and kept in the dark cupboards at room temperature until required. Keeping leaf samples in the dark ensures stable biological activity. Acetone was used to extract the leaf samples because it has proven to be a good extractant for several plant metabolites (Eloff and McGaw, 2006) and the least toxic to pathogenic microorganisms in bioassays (Masoko and Eloff, 2007). In studies, comparing many extractants with varying polarities, acetone proved to be the best for diversity of metabolites extracted from leaves (Kotze and Eloff, 2002; Eloff et al., 2005).

2.3. Extraction procedure

The finely ground air-dried leaves (1.0 g) of each tree was extracted with 10 ml acetone in 50 ml centrifuge tubes. The tubes were vigorously shaken in a Labotec model 20.2 machine for 3–5 min at high speed to ensure efficient extraction (Eloff, 1998a). The extracts were centrifuged at 3000 ×*g* for 10 min and the supernatant was filtered through Whatman No. 1 filter paper into a pre-weighed glass vials. The same process was repeated thrice in order to exhaustively extract the plant material and the extracts were combined. An aliquot of the extract (5 ml) was removed and placed into a pre-weighed vial under a stream of air at room temperature in a fume cupboard to remove the acetone and to determine the concentration of the combined extract. The required quantity of acetone in the combined extract was removed to yield a concentration of 10 ml/mg. This process limits problems experienced in redissolving dried extracts (Eloff, 2004).

2.4. Test bacterial strains

Gram-positive [*Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212)] and Gram-negative [*Pseudomonas aeruginosa* (ATCC 25922) and *Escherichia coli* (ATCC 27853)] bacterial strains were used to evaluate the antibacterial activity of both plant and tree species. These are the four most important nosocomial bacteria. The strains were obtained from the Central Microbiology Laboratory, Faculty of Veterinary Science at the University of Pretoria. The strains were grown at 37 °C in Mueller–Hinton broth (Merck Chemicals) (Eloff, 1998b).

2.5. Phytochemical analysis

Chemical constituents of the extracts were analysed by thin layer chromatography (TLC) using aluminium-backed plates (Merck, silica gel $60F_{24}$). The TLC plates were developed in the three mobile systems of differing polarity that gave excellent separation of many different compounds in a *Combretum* acetone leaf extract (Kotze and Eloff, 2002). The mobile systems used were; chloroform/ethyl acetate/formic acid (CEF: intermediate polarity) (5:4:1), benzene:ethyl acetate:ammonia (BEA: non-polar) (9:1:0.1) and ethyl acetate:methanol:water (EMW: polar) (40:5.4:5). The TLC plates were visualised under UV light (250 and 360 nm, Camac Universal lamp TL-600) to detect UV active absorbing sports or plant constituents. The plates were then sprayed with vanillin spray reagent (0.1% vanillin dissolved in 28 ml methanol and 1 ml sulphuric acid) and heated at 100 °C to optimal colour development. The position of the visible compounds on the TLC plate was established by calculating the retardation factor (R_f), which is the distance compound travelled divided by the distance the solvent had travelled from the origin.

2.6. Bioautography assay

The developed TLC chromatograms (not sprayed with vanillin spray reagent) were air-dried overnight in a stream of cold air and sprayed with a concentrated suspension of actively growing cells of test bacteria. This method relies on the direct growth inhibition or killing of pathogens on contact with the active band (Masoko and Eloff, 2006). The chromatograms sprayed with a suspension of test bacteria were incubated overnight at 38 °C in a chamber at 100% relative humidity to allow the pathogens to grow on the chromatograms. After the incubation bioautograms were sprayed with an aqueous solution of 2 mg/ml *p*-iodonitrotetrazolium violet (INT) (Sigma) and incubated overnight to observe clear zones on the bioautograms indicating growth inhibition of pathogens by bioactive compounds in the extracts (Begue and Kline, 1972). A set of TLC plates sprayed with vanillin was used as reference chromatograms for bioautography plates displaying areas of inhibition The R_f values of active zones were correlated with those bands on the reference chromatograms.

2.7. Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) values (mg/ml) were determined by two-fold serial dilution of extracts beyond where no inhibition of growth of test bacteria was observed (Eloff, 2001). This method was used to determine the antibacterial activity of extracts (Eloff, 1998a) with a concentration of 10 mg/ml. Plant extracts (100 μl) in triplicate for each experiment were serially diluted two-fold in a 96-well microlitre plates. A similar volume 100 μl of the actively growing test organism culture was added to each well and the cultures were incubated overnight at 37 °C under 100% relative humidity. As an indicator of bacterial growth, 40 μl of 0.2 mg/ml of *ρ*-iodonitrotetrazolium violet (INT) dissolved in water was added to each microplate well before being incubated for an hour or two (Eloff, 1998b). The MIC value was recorded as the lowest concentration that inhibited growth of bacteria. The colourless tetrazolium salt acts as an electron acceptor and is reduced to a red-coloured formazan product by biologically active pathogens (Eloff, 1998b). Clear zones on the chromatogram indicated inhibition of the growth of bacteria after incubation with INT. The experiment was repeated thrice to confirm the results, and three replicates were included in each experiment.

2.8. Total activity

The quantity extracted from different plants has to be taken into account and not only the MIC when comparing different plants (Eloff, 2000). The total activity of a plant is calculated by dividing the quantity