Screening of 16 poisonous plants for antibacterial, anthelmintic and cytotoxic activity in vitro

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Acetone extracts of the leaves of 16 plants known to have toxic effects when ingested by livestock were screened for biological activity, on the basis that toxic plants have proven pharmacological activity. The plants were tested for antibacterial effects against two Gram-negative (Escherichia coli and Pseudomonas aeruginosa) and two Gram-positive (Enterococcus faecalis and Staphylococcus aureus) bacteria. Anthelmintic activity against the free-living test nematode Caenorhabditis elegans was assessed. The potential cytotoxicity of the extracts was investigated using the brine shrimp lethality assay. Gossypium herbaceum, Lantana camara, L. rugosa, Thevetia peruviana and Sorghum bicolor inhibited the growth of all test bacteria with MIC values ranging from 0.39mg ml–1 to 6.3mg ml–1. A few plants, namely Gossypium herbaceum, Hertia pallens, Jatropha multifida and Lantana rugosa, showed notable effects against the nematodes at a concentration of 1mg ml–1. Only Hertia pallens (LC50 = 0.54mg ml–1) and Lantana rugosa (LC50 = 0.69mg ml–1) exhibited cytotoxic activity as indicated by the brine shrimp assay. These results establish the limited applicability of the brine shrimp assay to determine the toxicity of plant extracts.

Introduction

In many parts of the world, poisonous plants cause significant economic losses to the livestock industry (Radeleff 1970). The economic impact of these toxic plants is difficult to quantify, as both direct and indirect losses must be incorporated. Animal poisoning by plants often results from management errors and forage conditions, and depends upon the type of animal rather than just the presence of toxic plants (James 1994). Forage availability can be influenced by overgrazing and drought, which decrease the occurrence of palatable, nutritious forage plants and allow the spread of less palatable, possibly poisonous, plant species that are often well adapted to adverse conditions (James 1994). Toxic plants may grow together with forage plants, but animals allowed free choice generally tend to avoid poisonous plants, as they are often unpalatable and are usually only eaten in the absence of alternative food sources. Some poisonous plants act as acute toxins, while others must be consumed over a considerable period of time to produce harmful effects (Radeleff 1970).

A collection of plants known to be toxic to grazing and browsing animals is growing in the toxic plant demonstration garden of the Division of Pharmacology and Toxicology (Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria). Because toxic plants have at least one pharmacological activity, they have interested scientists investigating other pharmacological effects. In this investigation, acetone extracts of 16 plants toxic to livestock were tested for various biological activities. The reasoning for this approach is the widely accepted premise that pharmacology is merely toxicology at a lower dose, and that toxic substances may elicit interesting pharmacological effects at a lower non-toxic dose (McLaughlin 1991, Vlietinck and Apers 2001). Toxicity expressed in grazing animals may not necessarily preclude the use of extracts or isolated compounds especially for topical application.

Acetone was selected as the extracting solvent as it has a number of advantages over other commonly used solvents when the intention is to screen a number of plants. These advantages include the relatively low toxicity of acetone to the test organisms, as well as the solvent’s ability to extract a broad range of both polar and non-polar inhibitory compounds in the plants under study (Elloff 1998a). The extracts were subjected to in vitro biological assays designed to detect antibacterial, anthelmintic and brine shrimp lethality effects.

Materials and Methods

Preparation of plant extracts

Leaves of the following 16 plants growing in the Toxic Plants Garden at the Department of Paraclinical Sciences, University of Pretoria, were collected in May 2002: Albizia versicolor, Amaranthus hybridus, Asclepias fruticosa,
Cestrum laevigatum, Gossypium herbaceum, Hertia pallens, Jatropha multifida, Lantana camara, Lantana rugosa, Leucaena leucocephala, Medicago sativa, Melianthus comosus, Melianthus major, Nicotiana glauca, Sorghum bicolor and Thevetia peruviana.

The leaves were dried in an oven at 40–50°C under a stream of air and ground to a powder using a Janke and Kunkel mill. Herbarium specimens were prepared and deposited in the Department of Paraclinical Sciences’ herbarium. Extracts of the dried ground material were prepared using 2g plant material and 20ml extracting solvent. The extracts were filtered through Whatman No. 1 filter paper and dried to a residue in front of a stream of cold air before being resuspended in acetone to a concentration of 50mg ml⁻¹.

Antihelmintic screening

The test organism used in the anthelmintic screening was the free-living nematode Caenorhabditis elegans var. Bristol (N2), which was cultured on nematode growth (NG) agar seeded with E. coli, according to the method of Brenner (1974). The plant extracts were tested at concentrations of 0.5mg ml⁻¹, 1mg ml⁻¹ and 2mg ml⁻¹ following the protocol of Rasoanaivo and Ratsimamanga-Urverg (1993), as modified by McGaw et al. (2000). In this assay, approximately 500 nematodes (7–10 day-old cultures) in M9 buffer (Brenner 1974) were incubated with the different concentrations of plant extract for 2h at 25°C in the dark. The anthelmintic drug levamisole (Sigma, 10µg ml⁻¹) was used as a positive control, and a Pasteur pipette. The plant extracts were tested at concentrations of 0.1mg ml⁻¹, 1mg ml⁻¹, 2mg ml⁻¹ and 5mg ml⁻¹ as was an acetone solvent control. In addition to the MIC determination of the minimal inhibitory concentration (MIC) value for the plant extract against the particular bacterial strain. The antibiotic neomycin (Sigma) was included as a standard in each assay, as was an acetone solvent control. In addition to the MIC value, the extent of the antibacterial activity depends on the total quantity of the specific extract obtained from the plant. The total activity can be calculated from the quantity in mg extracted from 1g divided by the MIC in mg per ml (Eloff 2000). The resultant value indicates the volume in ml to which the antibacterial compound extracted from 1g can be diluted and still inhibit the growth of the test organism.

Toxicity testing

To gain an indication of their potential cytotoxicity, the plant extracts were tested against the larvae of Artemia salina (brine shrimp), using the method of Solis et al. (1993). Brine shrimp eggs were obtained from a local pet shop and hatched in artificial sea water (3.8g NaCl per 100ml distilled H₂O). After 48h, the phototropic nauplii were collected using a Pasteur pipette. The plant extracts were tested at concentrations of 0.1mg ml⁻¹, 1mg ml⁻¹, 2mg ml⁻¹ and 5mg ml⁻¹. For each concentration, the plant extract solution was placed in two replicate wells of a 96-well microtitre plate. Nauplii suspension (100µl, containing approximately 10–15 nauplii) was added to each well. The microplate was covered and incubated for 24h at room temperature. The number of dead and live nauplii in each well was counted using a stereomicroscope. If control deaths occurred, the percent death values were corrected using Abbott’s formula:

\[
\text{Corrected mortality percentage} = \frac{m - M}{S} \times 100
\]

where \( m \) = mean percentage of dead larvae in treated tubes; \( M \) = mean percentage of dead larvae in controls; and \( S \) = mean percentage of living organisms in controls. Podophyllotoxin (Sigma) was used as a positive control at a concentration of 5µg ml⁻¹ and solvent blanks were included in each assay.

Antimicrobial screening

The plant extracts were tested for antibacterial activity using the two-fold serial dilution microplate method of Eloff (1988b), which allows the determination of the minimal inhibitory concentration (MIC) values of each plant extract against the bacterial species used in the assay. The test bacteria included two Gram-positive bacteria, Enterococcus faecalis (ATCC 29212) and Staphylococcus aureus (ATCC 29213), and two Gram-negative species, Pseudomonas aeruginosa (ATCC 27853) and Escherichia coli (ATCC 35219). The bacterial cultures were incubated in Muller-Hinton (MH) broth overnight at 37°C and diluted 1:100 with fresh MH broth prior to use in the microdilution assay. A two-fold serial dilution of plant extract (100µl) was prepared in 96-well microtiter plates, and 100µl bacterial culture was added to each well before overnight incubation at 37°C. The presence of bacterial growth was detected by adding to each well 40µl p-iodonitrotetrazolium violet (INT) (Sigma), which is reduced to a red-coloured formazan by biologically-active organisms, in this case the dividing bacteria. When the solution in the well remained clear after an hour at 37°C, bacterial growth was completely inhibited by that concentration of plant extract. There was no evidence to suggest that the MIC was a minimal inhibitory concentration (MIC) value for the plant extract against the particular bacterial strain. The antibiotic neomycin (Sigma) was included as a standard in each assay, as was an acetone solvent control. In addition to the MIC value, the extent of the antibacterial activity depends on the total quantity of the specific extract obtained from the plant. The total activity can be calculated from the quantity in mg extracted from 1g divided by the MIC in mg per ml (Eloff 2000). The resultant value indicates the volume in ml to which the antibacterial compound extracted from 1g can be diluted and still inhibit the growth of the test organism.

Results and Discussion

The anthelmintic assay using the free-living nematode Caenorhabditis elegans is a simple and rapid in vitro assay that provides an idea of broad-spectrum anthelmintic activity of test extracts or compounds. Most commercially available anthelmintics have detectable effects on C. elegans, which provided impetus for its development as a model for parasitic nematodes for drug screening (Simpkin and Coles 1981). However, the limitations of using a free-living nematode to replicate parasitic nematode systems are inescapable; for example, there is no accounting for the complexity of the infectious process (Geary and Thompson 2001). Additionally, the screen has not produced valuable new leads since its development (Geary et al. 1999).

Of the 16 acetone leaf extracts screened in this investigation, only four were active against the C. elegans nematodes, and only three extracts showed a lethal effect against the brine shrimp larvae (Table 1). Gossypium herbaceum and Jatropha multifida showed anthelmintic but no cytotoxic activity. Hertia pallens and Lantana rugosa had both anthelmintic and cytotoxic activity. Interestingly, Lantana camara displayed no activity against C. elegans or Artemia salina. At a high concentration (5mg ml⁻¹),
Melianthus major killed 35% of brine shrimps but the related species M. comosus was not active at any of the concentrations tested.

The brine shrimp assay is a simple, inexpensive screening test for cytotoxic or pharmacologically active constituents, but it is unable to detect compounds requiring metabolic activation in animals or man. This is possibly the reason why few plant extracts displayed activity against the brine shrimps in this study. For example, Amaranthus species are known to contain nitrate, which is relatively non-toxic, but its conversion to nitrite by micro-organisms in the rumen results in toxicity to the animal (Allison 1978). However, in the present study, the lowest LC50 value was 0.54 mg ml–1, shown by Hertia pallens extracts.

The bacterial strains employed in this study are responsible for most nosocomial diseases in hospitals (Sacho and Schoub 1993), and are those recommended for antibacterial activity testing by the National Committee for Clinical Laboratory Standards (NCCLS 1990). Eleven of the 16 acetone extracts tested possessed antibacterial activity (Table 2), particularly against the Gram-positive Staphylococcus aureus and Enterococcus faecalis. Seven extracts displayed activity against both Gram-negative and Gram-positive bacteria. Gram-positive bacteria are generally more susceptible to antimicrobial substances than are Gram-negative species (Vlietinck et al. 1995), owing to differences in the bacterial cell wall structure. The two Melianthus species showed a similar pattern of activity, being antibacterial against the Gram-positive but not the Gram-negative bacteria. The Lantana species tested also revealed comparable activity, with notable effects against all the bacteria in the study. The highest total activity value of 82.1 ml g–1 for the Lantana rugosa extract against S. aureus.

### Table 1: Anthelmintic activity and brine shrimp toxicity in acetone leaf extracts

| Plant examined   | Anthelmintic activitya | Brine shrimp lethality assay |
|------------------|------------------------|-----------------------------|
|                  | (mg ml–1 plant extract) | LC50 (mg ml–1) |
| Albizia versicolor | 0 0 0 N                  |                            |
| Amaranthus hybridus    | 0 0 0 N               |                            |
| Asclepias fruticosa     | 0 0 0 N               |                            |
| Cestrum laevigatum       | 0 0 0 N               |                            |
| Gossypium herbaceum      | 0 1 1 N               |                            |
| Hertia pallens          | 0 1 1 0.54             |                            |
| Jatropha multifida       | 0 1 2 N               |                            |
| Lantana camara          | 0 0 0 N               |                            |
| Lantana rugosa          | 0 1 1 0.69             |                            |
| Leucaena leucocephala    | 0 0 0 N               |                            |
| Medicago sativa         | 0 0 0 N               |                            |
| Melianthus comosus       | 0 0 0 N               |                            |
| Melianthus major         | 0 0 0 >5               |                            |
| Nicotiana glauca        | 0 0 0 N               |                            |
| Sorghum bicolor         | 0 0 0 N               |                            |
| Thevetia peruviana      | 0 0 0 N               |                            |

a Anthelmintic testing scoring system: 0 = nematode number and movement same as blank (distilled water only); 1 = 80% of nematodes alive; 2 = 70% of nematodes alive
b N: indicates no lethal effect exhibited against the brine shrimp larvae by the plant extract

### Table 2: Antibacterial activity of acetone leaf extracts

| Plant examined   | E. coli MIC (mg ml–1) | S. aureus MIC (mg ml–1) | P. aeruginosa MIC (mg ml–1) | E. faecalis MIC (mg ml–1) | Total activity (ml g–1) |
|------------------|------------------------|--------------------------|-----------------------------|--------------------------|-------------------------|
| Albizia versicolor | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 |
| Amaranthus hybridus    | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 |
| Asclepias fruticosa     | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 |
| Cestrum laevigatum       | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 |
| Gossypium herbaceum      | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 |
| Hertia pallens          | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 |
| Jatropha multifida       | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 |
| Lantana camara          | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 |
| Lantana rugosa          | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 |
| Leucaena leucocephala    | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 |
| Medicago sativa         | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 |
| Melianthus comosus       | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 |
| Melianthus major         | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 |
| Nicotiana glauca        | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 |
| Sorghum bicolor         | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 |
| Thevetia peruviana      | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 | >6.3 6.3 >6.3 6.3 |

a MIC is taken as lowest concentration of plant extract which completely inhibited bacterial growth
b nc: not calculated

* Neomycin 1.56 x 10–3 6.3 x 10–3 25 x 10–3 0.78 x 10–3 0.78 x 10–3

*Table 1: Anthelmintic activity and brine shrimp toxicity in acetone leaf extracts

*Table 2: Antibacterial activity of acetone leaf extracts*
compares poorly with values of 182 ml g⁻¹ found for Combretum erythrophyllum extracts on S. aureus and E. coli (Eloff 1999) and of values higher than 258 ml g⁻¹ for Cassine peragua var. peragua (Eloff 2000).

Gossypium herbaceum was active against all species of bacteria tested, and these results are supported by Essenberg et al. (1990), who noted the accumulation of antibacterial sesquiterpenoids in Gossypium leaves and cotyledons inoculated with bacteria. Saleh et al. (1999) isolated the triterpenoids lantic acid, camaric acid, camaraminic acid and lantanic acid from Lantana camara, reporting on the broad spectrum antibacterial activity of lantic acid. It is possible that L. rugosa possesses similar compounds on a chemotaxonomic basis, but no reports on the chemical constitution of this species are available.

In the case of some plants, components other than leaves are responsible for toxicity; for example, many livestock animals such as sheep and goats are killed by ingesting the seed pods of Albizia versicolor (Soldan et al. 1996). Other plants affect particular organs in the animal; for instance, Cestrum species in southern Africa are hepatotoxic to livestock (Kellerman et al. 1988). Hertia pallens affects mainly the liver and lungs of sheep but the toxic principles do not appear to have been investigated (Kellerman et al. 1988). Leguminous plants, including lucerne (Medicago sativa), are known to be capable of producing bloat, particularly in cattle (Cheeke and Shull 1985). The bloat-inducing constituents of plants have been reported to be saponins (Clarke et al. 1981) and soluble proteins (Howarth et al. 1977). Although in vitro experiments are essential for some investigations, there are clearly cases where the results of these tests are not relevant to the disease in vivo (Pass 1992). For example, the major toxins of Lantana camara are the pentacyclic triterpene acids lantadene A and lantadene B. These compounds are absorbed from the rumen and small intestine, and are metabolised in the liver, causing damage to this organ (Pass 1992).

As noted earlier, livestock generally tend to avoid poisonous plants as they are often unpalatable and are usually only eaten in the absence of alternative food sources. For example, Asclepias fruticosa is known to contain toxic cardenolides (Warashina and Noro 1994), but is not acknowledged to be of veterinary importance as it is extremely unpalatable and is only eaten when other food is not available (Kellerman et al. 1988). Many other plants with toxic effects to livestock are known to contain poisonous substances. Anabasine, an alkaloid chemically related to nicotine, is the toxic principle of Nicotiana glauca and death from this plant is thought to result from respiratory paralysis (Kellerman et al. 1988). Toxic bufadienolides have been isolated from the root bark of Melianthus comosus (Anderson and Koekemoer 1969). Sorghum species contain the cyanogenetic glycoside dhurrin, and the plants are sometimes associated with nitrate poisoning (Kellerman et al. 1988). Thevetia peruviana contains the cardenolide thevetin (Watt and Breyer-Brandwijk 1962) and Leucaena leucocephala contains the toxic non-protein amino acid mimosine (D'Mello 1992). Jatropha multifida contains high levels of cyanogentic glycosides and cases of suspected prussic acid poisoning have been diagnosed in ruminants browsing on the leaves. The seeds contain curcin, a toxalbumin with an action similar to ricin (Steyn 1949, cited by Kellerman et al. 1988). The yellow polyphenolic pigment gossypol is a constituent of Gossypium species. The toxicity of gossypol varies with the age, species and breed of animal. Acute poisoning of livestock may occur with a very high intake of gossypol, but small quantities of the compound over prolonged periods have a cumulative effect in animals. Death is thought to result from cardiac and circulatory failure (Kellerman et al. 1988).

Poisoning of livestock by various plant species arises from several causes. There exist complexities in screening for toxicity in vitro as this process does not reveal toxic effects following metabolism of plant components, the long-term or cumulative effects of toxins, or detrimental effects on certain organs of the animal. Other consequences such as plant-induced bloat may be responsible for the harmful effects of some plants. Only plants containing toxic principles with noticeable activity in in vitro screens such as the brine shrimp lethality assay will be detected. The limitations of the free-living anthelmintic and brine shrimp assays, including the lack of data correlating activity in the assays with activity in vivo, restrict the value of these techniques. Other methods such as cell line cytotoxicity assays may provide a more useful indication of direct toxicity in vivo and this approach is being investigated for future studies. Mechanism-based in vitro screens such as isolated organ or cell assays are useful in deducing how a toxin causes its effects, but activity in vivo would have to confirm these effects.

As toxicity is associated with pharmacological activity in lower doses, there is a possibility that toxic plants may contain active constituents with useful biological activities. This proposal was demonstrated in this situation where a number of plant extracts exhibited marked antibacterial activity. However, this was not confirmed by the brine shrimp assay. As suggested by Meyer et al. (1982), the brine shrimp lethality assay is possibly useful as a convenient probe for pharmacological activities in plant extracts which may be manifested as toxicity towards the crustacean larvae. However, it is evident from this investigation that some plants with known or proposed alternative mechanisms of toxicity do not show activity in the brine shrimp assay. In this study only leaves were tested, since grazing or browsing animals generally ingest leaf material rather than other less easily available plant parts such as bulbs or tubers, which may contain an array of toxic principles. Depending on the mechanism of toxicity, extracts with substantial antibacterial activity such as those of Lantana species, which occur widely as alien invasive plants, may be useful for treating external infections in animals.

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