ANTIMICROBIAL ACTIVITY OF CRUDE EXTRACTS OF *ISOLONA CAULIFLORA* (ANNONACEAE) AGAINST SOME BACTERIA OF VETERINARY IMPORTANCE

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SUMMARY

In this work, the growth inhibiting activity of crude leaf extracts of *I. cauliflora* against animal bacterial pathogens *E. coli*, *S. gallinarum*, and *S. aureus* was compared to commercially available antibiotics cephalexin, trimethoprin, and gentamycin. In standard disk assays, *I. cauliflora* extracts caused zones of growth inhibition against all three strains tested. The extract was most effective against *S. aureus*, displaying inhibition zones of greater magnitude than the antibiotics tested. The minimal inhibitory concentration (MIC) fell below 100 µg/ml for *S. aureus*, well within the effective clinical range for a therapeutic antimicrobial agent. Similarly, the minimal bactericidal concentration (MBC) for *S. aureus* was determined to be 31.25 µg/ml, well below the LC_{50} of 72.44 µg/ml as determined by the Brine Shrimp test. Crude *I. cauliflora* leaf extracts also displayed moderate bacteriostatic activity against *E. coli* and *S. gallinarum*.

INTRODUCTION

It has been estimated that 75 to 90% of the world's rural people rely on herbal traditional medicine for the primary health care of themselves and their animals (Hamann, 1991). There are several plants whose extracts are medicinally useful in a crude form (Farnsworth and Soejarto, 1991). They include *Atropa belladonna* (SOLANACEAE) whose tincture is an antispasmodic, *Rauvolfia serpentina* (APOCYNACEAE) whose roots are used for hypertension and as a tranquilliser and *Papaver somniferum* (Papaveraceae) whose extract or tincture is used as an analgesic. Within the family Annonaceae, several species are known, albeit at crude extracts level only, to be medicinal (Watt and Breyer-Brandwijk 1962; Kokwaro, 1976). However, so far, the medicinal properties of another member of the family Annonaceae, *Isolona cauliflora*, which is endemic to Tanzania (Verdcourt, 1971), have not been documented.

The development of antibiotic resistant pathogenic strains of

bacteria has intensified efforts to discover new antimicrobial agents that may function via novel cellular or biochemical mechanisms. This work was aimed at studying the antimicrobial effects of crude leaf extracts of *Isolona cauliflora* on some bacteria of veterinary importance namely, *Escherichia coli*, *Salmonella gallinarum* and *Staphylococcus aureus* (Kazwala, 1992, Minga *et al.* 1993, Maeda-Machangu *et al.* 1998).

MATERIALS AND METHODS

Plant material and extraction

Mature leaves of *Isolona cauliflora* were hand picked from plants growing at Longuza in Muheza district, Tanzania. The tissue was packed in polyethylene bags and transported to the laboratory on the same day. In the laboratory, the leaves were sliced into small pieces, spread on benches, and left to dry at room temperature (25 – 28° C) until a constant dry weight was reached in a week's time. Subsequently, the dried plant materials were ground in a grinding machine.

Crude extracts for analysis were prepared by extracting 100 g pulverised tissue with 300 ml analytical grade ethanol for 48 hours at room temperature. The crude extract was then filtered by vacuum filtration and the filtrate concentrated to dryness under reduced pressure at room temperature. Extracts were tested immediately or stored at -18° C until use.

Test microorganisms and sub-culturing.

Bacteria used in this study were *Escherichia coli*, *Salmonella gallinarum* and *Staphylococcus aureus*, all of them being of great veterinary importance (Kazwala, 1992, Minga *et al.* 1993, Maeda-Machangu *et al.* 1998). Bacterial species were obtained from the Department of Veterinary Microbiology and Parasitology, Sokoine University of Agriculture (SUA) in Morogoro, Tanzania. Bacteria were cultured on nutrient agar plates after inoculation from slant stock cultures. Plates were incubated at 37° C for 24 hours, then observed for contamination before use in antimicrobial assays.

Assay for antimicrobial activity

Three methods were used to test for the antimicrobial activity of the *I. cauliflora* crude extracts against *E. coli*, *S. gallinarum* and *S. aureus*. The methods were (i) the disc method, (ii) the Minimum Inhibitory Concentration (MIC) and, (iii) the Minimum Bactericidal Concentration (MBC) method.

Disc method

The antimicrobial activity of *I. cauliflora* crude extracts was compared to standard antibiotic activities by the disc method (Cremer 1976, Egorov 1985, Platt 1986, Brock and Madigan 1991, Carter and Chengappa 1991). Liquid bacterial cultures were prepared by inoculating 100 ml

nutrient broth from slant cultures, and incubating at 37° C for 24 hours with vigorous agitation. A 1:1000 dilution series was subsequently prepared from the overnight liquid culture and the dilution that yielded approximately 10^5 CFU/ml was identified by the viable cell count method (Claus 1989, Grigorova and Norris 1990). To prepare seeded test plates, 0.2 ml of the 10^5 CFU/ml inoculum was aseptically mixed into 15 ml molten Muller Hinton agar at 45° C in 90 mm diameter Petri dishes. For each strain, 12 plates were prepared and allowed to solidify at room temperature.

For antimicrobial activity tests, crude *I. cauliflora* extract was dissolved in 25% aqueous ethanol to a final concentration of 4 mg/ml. Paper disks with a 7 mm diameter were prepared from Whatman No. 1 filter paper, soaked for 5 minutes in the extract solution, then aseptically dried for 5 minutes. Disks were transferred to the center of the seeded Petri plates. Control plates contained ethanol-soaked disks treated as described above, or MASTERING-S M42 Multodisks (Mast Laboratories Ltd, Merseyside, U.K.) containing cephalexin (30 µg), trimethoprin (2.5 µg) or gentamycin (10 µg). All tests were performed in quadruplicate.

Plates were refrigerated for 6 hours prior to incubation at 37° C for 24 hours. Inhibition zone diameters were then measured with a transparent ruler, the paper disk diameter subtracted and final values for the area of inhibition were calculated in mm^2. Means, standard error, and confidence limits were determined by standard methods.

Determining Minimum Inhibitory Concentrations (MIC)

Minimum inhibitory concentrations (MIC) were determined according to previously described methods (Sherris 1984, Egorov 1985, Carter and Chengapa 1991). A concentrated stock of crude *I. cauliflora* extract was prepared in absolute ethanol at 4 mg/ml. One ml of the concentrated stock was diluted into 7 ml Muller Hinton broth to yield a 500 µg/ml working solution. This working solution was serially diluted 1:1 in sterile culture tubes to yield 2 ml samples spanning a concentration range from 500 µg/ml to 0.0038 µg/ml. Tubes were inoculated with 0.03 ml 10^5 CFU/ml culture of each strain, then incubated at 37° C for 24 hours. A control tube containing 2 ml Muller Hinton broth only was prepared in the same manner. Growth was evaluated visually by comparing culture turbidity to a control tube containing non-inoculated sterile broth. The MIC for each strain corresponded to the extract dilution at which no growth was observed. The assays for MIC were repeated four times.

Determination of Minimum Bactericidal Concentration (MBC)

From each culture tube in the dilution series corresponding to the MIC (i.e. no growth observed), 10 µl was subcultured on Muller Hinton agar plates at 37° C for 24 hours. Resulting colonies were

enumerated and counts compared to freshly inoculated control plates. Similar colony counts between the control- and MIC-inoculated plates was taken as an indication of bacteriostatic activity. The absence of colonies on the MIC-inoculated plates indicated bactericidal activity (complete killing). Assays for MBC were done four times.

Toxicity tests

Toxicity of the crude *I. cauliflora* extract was assayed using the Brine shrimp test of Meyer *et al* (1982). A 4 mg/ml stock solution of the extract was prepared in dimethyl sulphoxide (DMSO), then diluted into 5 ml of artificial sea water to yield test solutions at 240, 80, 24, and 8 µg/ml. Test solutions were prepared in quadruplicate. Control vials contained 5 ml artificial sea water with DMSO alone or with no additions. To each vial, 10 shrimp larvae were introduced, then incubated at room temperature for 24 hours. The number of surviving larva was recorded and average percent mortality determined. The LC_{50} was calculated as the extract concentration corresponding to 50% mortality as plotted on a log. graph.

RESULTS

From 1.0 Kg of dried ground leaves, 49.40 g (4.94 % recovery rate) of concentrated crude extract was obtained. The crude *I. cauliflora* extracts demonstrated antibacterial activity against all three strains, and inhibitory activity fell within the range of several control antibiotics tested. Average inhibition zones observed measured 21.7+/- 0.67 mm for *S. aureus*, 22 +/- 1.07 mm for *E. coli*, and 22.5 +/- 0.7 mm for *S. gallinarum*, with the largest inhibition zone observed against *S. aureus* seeded plates (Fig. 1). Crude *I. cauliflora* extracts yielded results against all three species that exceeded the inhibition observed with the antibiotics cephalexin and trimethoprin at standard concentrations. The crude extract demonstrated significantly stronger activity against *S. aureus* when compared to the antibiotic controls ($p=0.05$). Only gentamycin disks displayed larger inhibition zones than the crude extract (Fig. 1). Cephalexin and trimethoprin caused inhibition zones of 15 mm and 20 mm respectively, on all plates for all the test bacterial species. On the other hand, gentamicin caused inhibition zones of 35 mm on all plates with *E. coli*, 25 mm diameter inhibition zones on all plates with *S. gallinarum* and 20 mm diameter inhibition zones on all plates with *S. aureus* (Fig. 1)

The minimum inhibition concentrations (MIC) and the minimum bactericidal concentrations (MBC) observed with crude *I. cauliflora* extracts are given in Table 1. The extracts were significantly more effective against *S. aureus* than the other strains tested, in terms of both inhibitory and bactericidal activity (15.62 and

31.25 µg/ml respectively). Crude *I. cauliflora* extract exerted a moderate inhibitory effect on both *S. gallinarum* and *E. coli,* (62.5 µg/ml on both species) but only limited bactericidal activity against these strains (250.0 and 125.0 µg/ml respectively).

Results on the toxicity tests against Brine shrimp are graphed in Figure 2. The median lethal concentration (LC_{50}) of the crude leaf extracts of *I. cauliflora* extract was calculated as 72.44 µg/ml, a value higher than the MICs observed with all three bacterial species. The calculated LC_{50} also exceeded the MBC for *S. aureus*, but was significantly lower than MBCs observed with *S. gallinarum* or *E. coli.*

DISCUSSION

Isolona cauliflora is a plant that is found to grow in Tanzania only (Verdcourt, 1971). In this work, the antimicrobial activity of crude leaf extracts of *I. cauliflora* against some bacteria of veterinary importance has been demonstrated. The activity of extracts from *I. cauliflora* has not been previously reported, even in extensive reviews of antimicrobial and medicinal properties of plants (Watt and Breyer-Brandwijk, 1962; Kokwaro 1976; Taniguchi *et al* 1978 and Taniguchi and Kubo 1993).

The growth inhibition observed with crude leaf extracts of *I. cauliflora* was comparable to the activity of several standard antibiotics. In particular, the crude extract was particularly effective against the species *S. aureus*, producing inhibition zones of greater magnitude than standard effective concentrations of cephalexin, trimethoprin, or gentamycin. Since high activity was observed even in the relatively crude extract, it is likely that once purified, the active agent may be quite potent against several relevant bacterial species.

While the growth inhibition zones observed against *S. aureus*, *E. coli*, and *S. gallinarum* did not differ significantly in magnitude, differences were observed in the effective MIC and MBC values observed with the three strains. This may be due to differences in diffusion capability of active agents in the crude extract, a determining factor in the plate assay but not in the MIC or MBC tests. Thus, while *I. cauliflora* leaf extracts yielded similar inhibition patterns on plate growth assays, the MIC value observed against *S. aureus* was four-fold less than with the other two test strains. Similarly, MBC values were five- to eight-fold less for *S. aureus*, suggesting that the extract contain an agent that is highly effective against *S. aureus.*

For most clinically effective antimicrobial agents, the MICs for fully susceptible organisms range from 100 to 0.01 µg/ml or less (Sherris, 1984). Successful therapy usually requires working levels above the MIC at the site of infection, but safely below the LC_{50} for the host. The MICs of the crude extract reported here were all at

levels below 100 ug/ml (Table 1). Furthermore, the observed MBC values for all three strains were well above the MICs, suggesting the presence of a bacteriostatic agent in crude *I. cauliflora* leaf extracts. Against the pathogen *S. aureus*, crude extracts displayed an MBC of 31.25 µg/ml, well below the LC_{50} computed from Fig. 2. This suggests the potential use of the crude extracts as a bactericidal agent with particular effectiveness against *S. aureus*. Although the MIC values for the other two strains tested were close to the LC_{50}, it is possible that further characterization of the active agent in crude extracts may identify a component or components with high bacteriostatic activity that are effective at much lower working concentrations. Toxicity of extracts to host animal cells is observed if the extracts are applied at concentrations higher that the observed LC_{50} value (Eaton *et al.* 1995).

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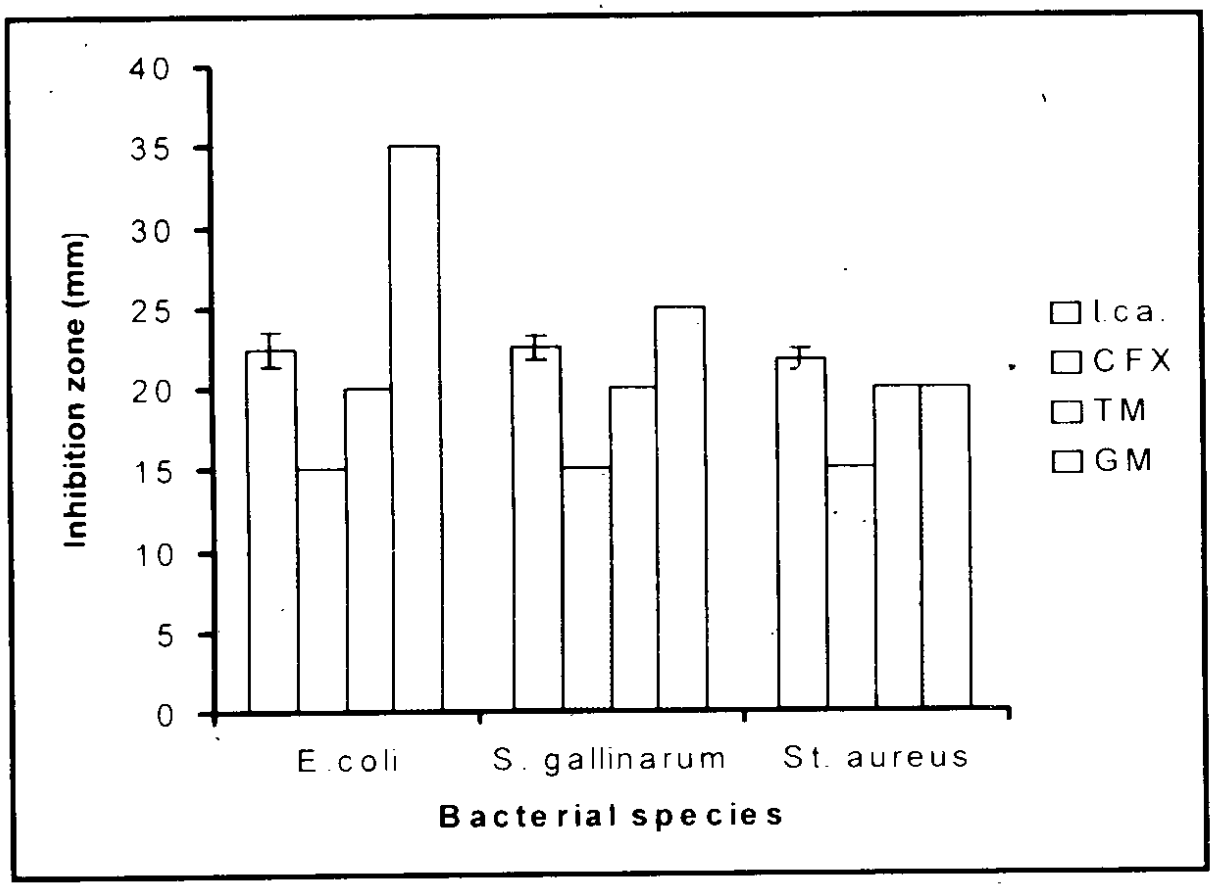

Fig. 1 Inhibition zones by crude leaf extracts of *Isolona cauliflora* (I. ca.), and the standard antibiotics cephalexin (CFX), trimethoprin (TM) and gentamicin (GM) on some bacteria of veterinary importance

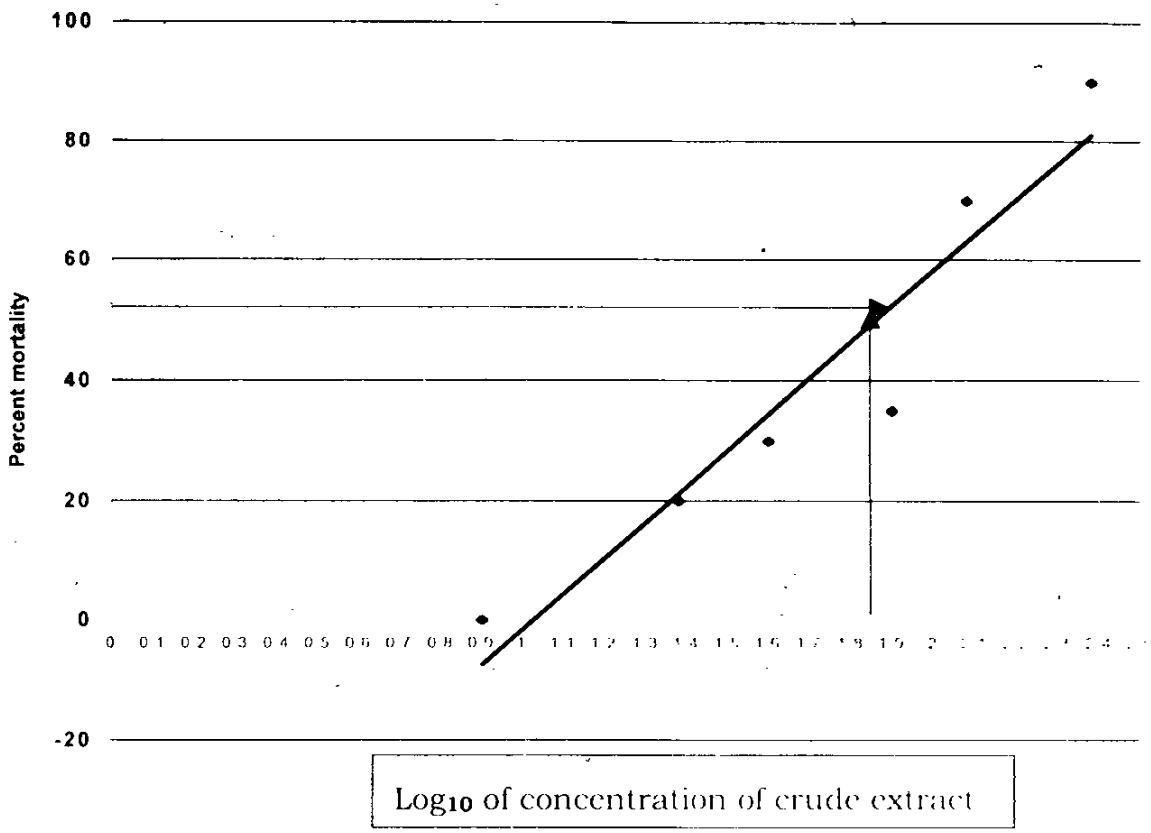

Fig 2. Toxicity of *Isolona cauliflora* crude extracts to *Artemia salina* after 24 hrs incubation

Table 1: Minimum inhibitory concentrations (MIC) and Minimum bactericidal concentrations (MBC) of *Isolona cauliflora* on *S. aureus*, *S. gallinarum* and *E. coli*.

Microorganism	MIC (µg/ml)	MBC ((µg/ml)
Staphylococcus aureus	15.62	31.25
Salmonella gallinarum	62.5	250.0
Escherichia coli	62.5	125.0