



Research Paper

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ANTIBACTERIAL ACTIVITY OF EXTRACTS OF THREE *CROTON* SPECIES COLLECTED IN MPUMALANGA REGION IN SOUTH AFRICAS.C. Selowa¹, L.J. Shai^{2*}, P. Masoko³, M.P. Mokgotho³, S.R. Magano⁴¹Department of Biochemistry, University of Limpopo, P.O. Box 235, Medunsa, 0204; ²Department of Biomedical Sciences, Tshwane University of Technology, Private Bag X680, Pretoria, 0001;³Department of Biochemistry, Microbiology and Biotechnology, University of Limpopo, Private Bag X1106, Sovenga, 0727; ⁴Department of Life and Consumer Sciences, University of South Africa, Private Bag X6, Florida 1710*E-mail: ShaiLJ@tut.ac.za; Fax: +27 12 382 6262; Tel: +27 12 382 6342

Abstract

The antibacterial activities of three *Croton species* were compared using bioautography and the serial microdilution methods. The methanolic extracts of all the species had low activity against *Escherichia coli*. The highest activity was observed with *Croton megalobotrys* against *Enterococcus faecalis* with a minimal inhibitory concentration (MIC) value of 0.02 mg/ml. *Croton steenkapianus* extracts were the least active of the species investigated, only managing an MIC value of 0.625 mg/ml against *Pseudomonas aeruginosa*. *Croton megalobotrys* leaf powder was serially extracted using solvents of various polarities. The lowest MIC value (0.06 mg/ml) of the serially extracted fractions was observed with acetone against *Pseudomonas aeruginosa*. The liquid-liquid fractions of the methanol extract of *Croton megalobotrys* were also tested. The lowest MIC value of 0.02 mg/ml was observed with *n*-hexane fraction against *Enterococcus faecalis*. The carbon tetrachloride fraction was further fractionated using column chromatography with silica as the immobile phase. The resulting seven fractions were tested for activity following the bioassay-guided practice, and it emerged that the first three fractions had active compounds against *Staphylococcus aureus* when the bioautography method was used.

Key words: *Croton megalobotrys*; *C. steenkapianus*; *C. silvaticus*; antibacterial activity.

Introduction

Poor countries of Africa face serious primary healthcare challenges emanating from lack of resources, poor sanitation, corruption and recently, the scourge of human immuno-deficiency virus (HIV) and acquired immuno-deficiency syndrome (AIDS). For instance, cholera, an ancient disease, continues to affect populations in less developed countries in Africa. The cholera epidemic in Kwazulu-Natal province of South Africa in the year 2000-2001 resulted in over 100,000 cases. This disease is frequently associated with factors such as overcrowding and poor sanitation. The cholera causing pathogen, *Vibrio cholerae*, survives in environmental reservoirs (Mandelsohn and Dawson, 2008). People in most rural parts of Africa depend on traditional healing methods for the treatment of a variety of infectious diseases.

The past few decades have experienced an overwhelming increase in global interest on the practice of traditional medicine and its use of medicinal plants to treat illness (Akarele, 1994). Plant-derived preparations and isolated phytochemicals or their model derivatives may be potentially useful to treat infectious diseases, especially in the light of the emergence of drug-resistant microorganisms and the need to produce more efficacious and cost-effective antimicrobial agents (Ncube *et al.*, 2008). The use of antibiotics has revolutionized the treatment of various bacterial infections. However, their indiscriminate use has led to an alarming increase in antibiotic resistance among microorganisms (Hart and Karriuri, 1998). This necessitates the need for development of novel antimicrobials (Chopra *et al.*, 1997). One way of preventing antibiotic resistance of pathogenic species is development of new compounds that are not based on existing synthetic antimicrobial

agents (Rojas *et al.*, 2006). Plant-derived traditional medicines can be used to treat different diseases as they contain a variety of secondary metabolites to which the bacterial species may not be resistant. However, the safety, quality and efficacy of some of these preparations have not been validated scientifically.

Croton megalobotrys Mull. Arg. (*mohloko* in Pedi) is a medicinal plant that belongs to the Euphorbiaceae family. It is endemic to most parts of Africa including Tanzania, Malawi, Zambia, Zimbabwe, Botswana, Namibia and South Africa. It is mainly found on flood plains and along the river banks. The leaves are evergreen and the bark is poisonous, though various parts of the plants are used medicinally (Bolten, 1998). *Croton megalobotrys* is used to treat sexually transmitted diseases (Ndubani and Hojer, 1998) and malaria as a simple fever reducer (Bolten, 1998). The berries are crushed and applied as a poultice to skin infections and muscle aches or ingested as a purgative. The genus is known to contain powerful purgatives, one being the ingredient of the commercially-marketed croton oil. As this plant is widely used by traditional healers, the safety, quality and efficacy profiles of the medical formulations are extremely important and must be scientifically validated.

Another member of the genus, *C. sylvaticus* Hochst, which contains large quantities of tannins, is listed among the highly toxic plants that cause DNA damage and chromosomal aberrations (Taylor *et al.*, 2003). This species is used traditionally to relieve abdominal pains, uterine disorders and fever (Watt and Breyer-Brandwijk, 1962; Hutchings *et al.*, 1996). *Croton steenkampianus* is used to treat painful joints and back ache, as well as rheumatism (Watt and Breyer-Brandwijk, 1962; Hutchings *et al.*, 1996).

There is little information about the pharmacology and phytochemistry of *C. megalobotrys*, yet the plant continues to be used to prepare traditional remedies. The current study therefore investigated the efficacy of the extracts of three *Croton* species against bacteria.

Materials and Methods

Plant material

The leaves of *Croton megalobotrys*, *Croton steenkampianus* and *Croton salvaticus* were collected during May 2008 at the Lowveld National Botanical Garden in Nelspruit, South Africa. The plants are identified by name tags attached to each plant by the Lowveld National Botanical Garden staff. The leaves were left to dry at room temperature for a week. The dried leaves were then milled to fine powders using pestle and mortar. The powdered plant material was stored in glass bottles at room temperature in the dark.

Extraction

Plant material (2 g) from each species investigated was separately extracted three times with 20 ml of methanol (MeOH) on a shaking machine for 30 mins. The resulting extracts were filtered using Whatman No 1 filter paper before being transferred to pre-weighed glass beakers. The extracts were further concentrated to dryness under reduced pressure at 37°C using rotary evaporator (Büchi). The yields were weighed and dissolved in methanol to give a final concentration of 10 mg/ml.

Serial extraction of *C. megalobotrys*

Twenty grams of *Croton megalobotrys* leaf powder was serially extracted with *n*-hexane (Hex), dichloromethane (DCM), ethyl acetate (EAc), acetone (ACN) and MeOH. The resulting extracts were evaporated using a rotary evaporator at temperatures not exceeding 60°C. The extracts were weighed and tested for antibacterial activity using the serial microdilution assay.

Liquid-liquid fractionation of *C. megalobotrys* extract

Three hundred grams of plant material (*C. megalobotrys*) was extracted with MeOH and MeOH/water (4:1) mixture on a shaking machine. The extracts were filtered and concentrated as before. The resulting extract (20 g) was re-suspended in chloroform and an equal amount of water was added to a volume of 500 ml. This resulted in chloroform (C) and the water fraction (W). The W fraction was further extracted with *n*-butanol to give butanol (B) and water (W2) fractions. The C fraction was dried and dissolved in MeOH/water (9:1) and extracted with *n*-hexane to yield the *n*-hexane (Hex-2) and methanol/water fractions. The MeOH/water fraction was adjusted to 20% water (v/v) and extracted with carbon tetrachloride (CT), yielding the water/MeOH fraction and the CT fractions. The water/MeOH mixture was adjusted to 35% (v/v) water and extracted with chloroform to yield water/methanol and chloroform (C2) fraction. All the fractions were dried and weighed. Four fractions were used in bioassays, namely, C2, Hex-2, CT and B.

Column chromatography fractionation

Eight hundred grams of silica gel were mixed with Hex to form a homogenous suspension and stirred using a stirring rod to remove bubbles. The silica gel suspension was poured into a glass column. The sample was prepared by dissolving 4 g of CT fraction in 40 ml chloroform. To the solution, 8 g of silica was added and mixed by stirring with a glass rod. The mixture was dried using a rotary evaporator and the dried silica-extract mixture was carefully layered on the column gel. For elution chloroform was used as the mobile phase with the polarities varied by 10% increments of ethyl-acetate until 100% EAc was reached. Twenty milliliters of the fractions were collected in different test tubes. The test tubes were left to evaporate at room temperature to reduce the solvent. The column fractions collected were analyzed using thin layer chromatography (TLC) to determine the phytochemical profile of fractions. Fractions with the similar profiles were mixed and concentrated to yield seven major fractions. Fractions 1 through seven were tested using bioautography against the microorganisms.

Bacterial cultures

Bacterial cultures were kindly donated by Dr. Lyndy McGaw of the Phytomedicine Programme, University of Pretoria, South Africa. The cells were maintained in Luria agar (10 g/l Bacto tryptone, 5 g/l Bacto yeast extract, 10 g/l NaCl, pH 7.0) in Petri dishes or glass slant bottles. Bacterial cells which were used as test microorganism were the following, the gram negative: *Escherichia coli* and *Enterococcus faecalis*, the gram-positive: *Staphylococcus aureus* and *Pseudomonas aeruginosa*. These are the species which are often involved in nosocomial infection (Sosa *et al.*, 2002). The bacterial cultures were prepared by transferring 2 to 3 colonies into bacteria growth medium [Luria broth, prepared as per instructions of the manufacturer (Sigma)] and incubated at 37°C for 14 hrs before use.

Minimal inhibitory concentrations (MIC)

The MIC values of the three different *Croton* species were determined using the micro-dilution technique with 96 well micro-plates, as described by Eloff (1998).

Bioautography

For bioautography analysis, 200 µg of each extract was loaded on TLC plates. The plates were developed in mobile phases of varying polarities i.e., benzene: ethanol: ammonia (9:1:1) (BEA) (Kotze and Eloff, 2002) and DCM: EAc (4:1) (DE). The TLC plates were dried under a fume hood to evaporate the solvent. The developed plates were sprayed with concentrated bacterial cultures and incubated in a humidified container at 37°C overnight. The following morning the plates were sprayed with iodinitrotetrazolium chloride (INT) (Sigma) and incubated for 2 hrs. Clear zones indicate the antibacterial activity and the location of the active compound in different extract and the growth of the microorganisms indicated by the formation of the pink colour of the formazan.

Results and Discussion

The three *Croton* species have different effects on different microorganism and *C. megalobotrys* was more active against the test microorganisms as compared to the other two species. *Croton megalobotrys* was only able to inhibit *E. coli* at high concentrations. *Croton steenkapianus* was inactive even at high concentrations against *E. coli*, *S. aureus*, *E. faecalis* and was only able to inhibit *P. aeruginosa* with an MIC value of 0.625 mg/ml (Table 1). *Escherichia coli* was the most resistant test organism while *E. faecalis* was the most sensitive, as indicated by the MIC values. *Croton salvaticus* was weakly active against all the test organisms at the same concentration (1.25 mg/ml).

Powdered leaves of *C. megalobotrys* were extracted serially with Hex, DCM, EAc, ACN and MeOH, in the order mentioned. These extracts were tested for activity against four bacterial test organisms. All the extracts resulted in varying degrees of inhibition of bacterial growth. The ACN fraction inhibited the growth of *P. aeruginosa* with an MIC value of 0.06 mg/ml (Table 2). Since all the fractions resulted in similar inhibition profiles, we decided to use only MeOH as the extracting solvent in order to isolate the active compounds. The plates developed with BEA (9:1:1) showed activity against *S. aureus* by the presence of the clear zones on the plate. All the extract showed activity except in the Hex extract because of lack of clear zones on the TLC plate.

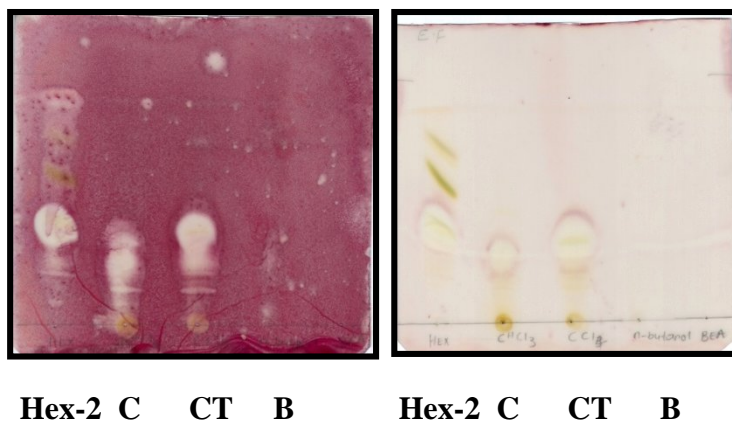


Figure 1: Bioautograms of various fractions of *C. megalobotrys* against *S. aureus* (Left) and *E. faecalis* (Right). The TLC plates were developed in BEA. Clear zones on bioautograms indicate antibacterial activity.

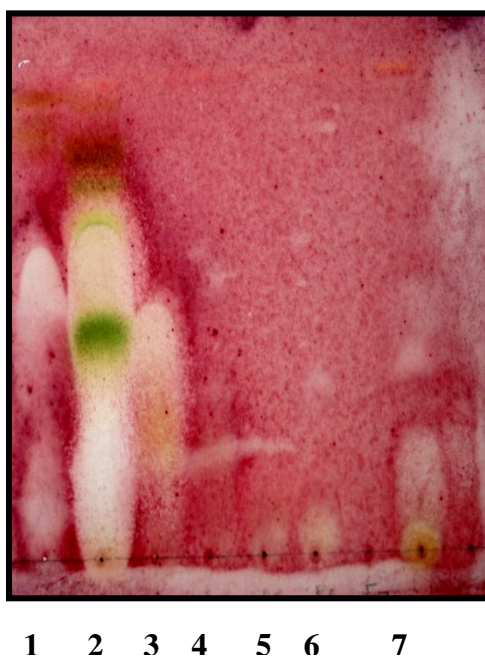


Figure 2: TLC bioautogram of different column fractions of CT extract of *C. megalobotrys* against *S. aureus*. TLC plates were developed in DE. Active compounds are currently being isolated from fractions 1, 2 and 3.

Table 1: MIC values (mg/ml) of the three *Croton* species. Extracts of MeOH of the three *Croton* sp. were compared using the microplate dilution method.

Species name	<i>E. coli</i>	<i>S. aureus</i>	<i>E. faecalis</i>	<i>P. aeruginosa</i>
<i>C. megalobotrys</i>	1.25	0.625	0.02	0.313
<i>C. steenkapianus</i>	-	-	-	0.625
<i>C. silvaticus</i>	1.25	1.25	1.25	1.25

- : not active

Table 2: The MIC values (mg/ml) of serially extracted fractions of *C. megalobotrys*.

Fractions	<i>E. coli</i>	<i>S. aureus</i>	<i>E. faecalis</i>	<i>P. aeruginosa</i>
ACN	0.332	0.312	0.196	0.060
MeOH	0.392	0.468	0.120	0.312
HEX	1.406	1.562	0.120	0.312
DCM	0.705	0.236	0.274	0.322
EAc	0.332	0.312	0.276	0.196

Table 3: MIC (mg/ml) of different fractions of *C. megalobotrys*. The fractions were obtained through liquid-liquid fractionation of the MeOH extract of the leaves of *C. megalobotrys*.

Fractions	<i>E. coli</i>	<i>S. aureus</i>	<i>E. faecalis</i>	<i>P. aeruginosa</i>
Hex	0.313	1.250	0.020	0.625
C2	0.625	0.937	0.313	0.313
CT	0.313	0.625	0.313	0.313
B	1.25	-	2.5	-

Croton megalobotrys was extracted with MeOH and MeOH/water mixture and then fractionated with various solvents to yield Hex-2, C, CT, B and W fractions. These fractions were further investigated using bioautography and serial dilution methods. The Hex-2, C and CT fractions resulted in visible zones of inhibition on TLC plates sprayed with *E. faecalis* or *S. aureus*. The B fraction did not lead to any observable zones of inhibition (Figure 1). The serial dilution assays further confirmed that the B fraction was the least active, failing to inhibit the growth of *S. aureus* and *P. aeruginosa* and only managing to inhibit growth of *E. coli* and *E. faecalis* only at high concentration (1.25 and 2.5 mg/ml, respectively) (Table 3). All the other fractions were active against all the bacterial species, with an MIC value of 0.02 mg/ml recorded against *E. faecalis* resulting from the hexane fraction.

The CT fraction was further separated using silica gel as the stationary phase. After combining fractions with similar components (as indicated by TLC), 7 major fractions resulted. Fractions 1-3 resulted in pronounced zones of inhibition (Figure 2). These fractions are presently being separated and the results will be reported in future reports.

Plants have been used for decades to treat different disease and in rural and poor communities people depend on the traditional healer for health care. *Croton megalobotrys* is a plant that has been used by traditional healers for many years to treat diseases such as malaria in Delta (Botswana) (Bolten, 1998; Mabogo, 1990) and sexually transmitted in Zambia (Ndubani and Hojer, 1999).

The evidence provided in this study supports the traditional use of this plant. It is likely that its use to treat sexually transmitted infections is attributable to its antibacterial activity. Antibacterial activity of *C. megalobotrys* extracts against some of the known sexually transmitted pathogens such as *Neisseria gonorhoea* should be determined and active compounds isolated. The toxicity studies will also be conducted.

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