The presence of antibacterial compounds in *Anthocleista grandiflora* (Loganiaceae)

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In response to an unpublished report that *Anthocleista grandiflora* extracts had antimicrobial activity, leaves were dried, extracted and fractionated by a mild liquid/liquid extraction process into six fractions. Activity of components separated by thin layer chromatography was tested by bioautography using *Staphylococcus aureus, Pseudomonas aeruginosa* and *Bacillus subtilis* as test organisms. Growth of all three organisms were inhibited by compounds in the chloroform and carbon tetrachloride soluble fractions. One or two compounds had a high degree of inhibition. Up to eight other compounds with a lower level of inhibition were also separated. There was little or no activity in the highly polar (water) or non-polar (hexane) fractions.

Keywords: *Anthocleista grandiflora*, antibacterial, antimicrobial, solvent/solvent fractionation, bioautography.

Introduction

Due to the increasing resistance of bacterial isolates for antibiotics, efforts to find alternative antimicrobial components have been intensified (Berkowitz 1995). Leggiadro (1995) stated that effective regimens may not be available to treat some enterococcal isolates and that it is critically important to develop new antimicrobial compounds for these and other organisms before we enter the post-antibiotic era. Many scientists have consequently investigated plants for the presence of antimicrobial components. Much effort went into screening plants used medicinally in different regions e.g. Rwanda (Vlietinck *et al.* 1995), Nepal (Taylor *et al.* 1995) and southern Mexico (Meckes *et al.* 1995).

The pharmaceutical company Noristan has also been doing research on South African medicinal plants for a number of years (Fourie *et al.* 1992). Hundreds of plants were screened for several parameters including antimicrobial activity. After the Noristan programme was terminated due to a change in management policy, we started a research programme on antimicrobial components in plants. The Noristan scientists provided us with a list of promising plants as far as antimicrobial activity was concerned (Dr T.G. Fourie, personal communication). *Anthocleista grandiflora* leaf extracts had a significant antimicrobial activity against *Staphylococcus aureus* and a slight activity against *Streptococcus pyogenes* strains in a preliminary screening and we decided to investigate this plant in more detail.

Anthocleista grandiflora Gilg (Loganiaceae), the forest fever tree, is a tall slender tree 6 to 30 m in height with large leaves and grows in rainfall forests at a medium to low altitude in North-eastern South Africa, Swaziland and Zimbabwe. A decoction of the leaves is used as a remedy for malaria and the bark is chewed to relieve diarrhoea (Palgrave 1983).

No published reports of antimicrobial activity of this plant were found, but seed and bark of *A. djalonensis, A. nobilis* and *A. procera* have been used for antipyretic, stomachic and purgative action and a decoction of *A. nobilis* leaves is used as a remedy for epilepsy (Watt 1967) and as a diuretic (Oliver-Bever 1986). The seed and bark of *A. vogelii* has antipyretic, tonicum and laxative activity and contains oxidised xhanthones (Ghosal *et al.* 1973).

Various authors have isolated swertia macroside or swertiamin (bitter monoterpenic heteroside), anthocleistin (a triterpene pentacyclic acid) and gentianin from *A. procera* (Oliver-Bever, 1986). *A. nobilis* is used against liver diseases, malaria and gastro-intestinal worms in Nigeria and anthocleistol (a secoiridoid) was isolated from it (Madubunyi *et al.* 1994). Djelonelol, a new monoterpene diol was isolated from *A. djalonensis* (Onocho *et al.* 1995).

With the exception of gentianin, the antimicrobial activity of these isolated compounds was apparently not investigated. Gentianin, however, had no effect on *S. aureus, Bacillus subtilis* or several other micro-organisms although it had analgesic, antihistamine and anti-inflammatory activity (Ghosal *et al.* 1973).

This study was undertaken to follow up on the report of antimicrobial activity of *A. grandiflora* leaf extracts and to attempt to isolate the active component(s) using three test organisms also used by many other authors.

Materials and Methods

Leaves were collected from a tree on the campus of the University of Pretoria. Prof A.E. van Wyk, Curator of the Albert Seweicker Herbarium, University of Pretoria confirmed the identity of the tree. A

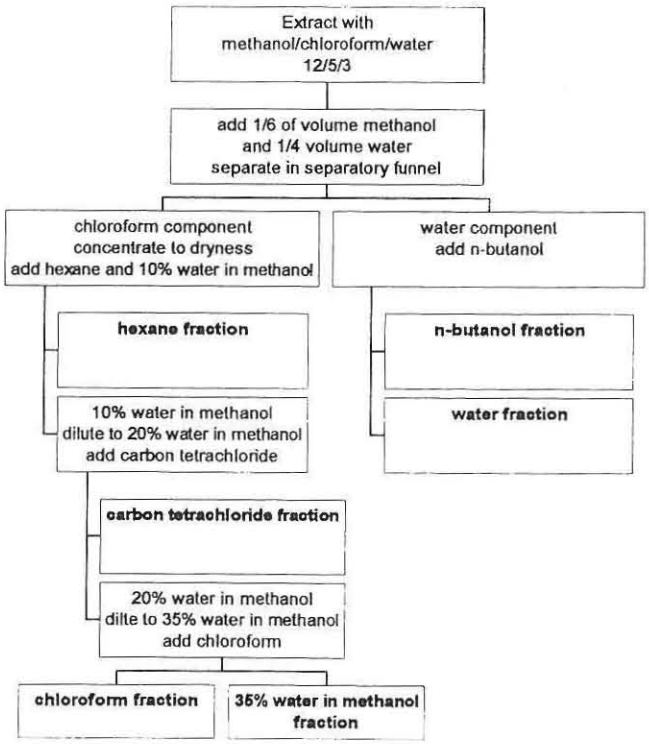

Figure 1 The procedure used for the solvent/solvent fractionation of the components in the *A. grandiflora* extract.

Table 1 The inhibition of *B. subtilis* growth by different fractions of *A. grandiflora* separated by TLC with choloroform:acetone 2:1 as eluant. R_f values of active components and relative degree of inhibition are shown (X = slight inhibition, XXXX = very high inhibition)

R_f	Inhibit.	R_f	Inhibit.	R_f	Inhibit.	R_f	Inhibit.	R_f	Inhibit.
				0.82	XX	0.80	XX	0.82	XX
				0.74	XXXX	0.73	XX		
				0.69	X				
				0.63	X				
				0.58	X				
				0.45	X				
				0.33	X				
				0.21	XX				
				0.15	X				
0.02	XXX	0.02	XX	0.02	XX				
35% W in MeOH		n-BuOH		Chloroform		Carbon tetrachloride		Hexane	

voucher specimen, Eloff 501 (PRU), is deposited in this herbarium.

Leaves were dried in the shade at room temperature or freeze-dried overnight. Stems and thick veins were removed before grinding to a fine powder in a hammer mill. Five different portions of the the powder were extracted for 2 hours on a shaking machine respectively with acetone, ethanol, methanol:methylenedichloride 1:1 (MMDC), methanol and a one-phase mixture of methanol:chloroform:water 12:5:3 (MCW) (Merck technical grade). The extractant to dry weight ratio was 10:1. The process was repeated two more times and the extracts were combined.

Further work was done only on the MCW-extract. The extract was fractionated by a variation of the solvent/solvent group separation procedure used by the USA National Cancer Institute as described by Suffness and Douros (1979). The protocol is illustrated in Figure 1. The combined MCW extract was separated into two fractions by adding $^1/_6$ of the total volume of methanol and $^1/_4$ of the total volume of water. The water fraction was extracted with an equal volume of n-butanol in a separatory funnel to yield the water and butanol fractions. The chloroform fraction was taken to dryness in a rotary evaporator under reduced pressure and dissolved in a 1:1 mixture of hexane and 10% water in methanol. The hexane fraction was recovered with a separatory funnel. The 10% water in methanol extract was diluted to 20% water in methanol and extracted with carbon tetrachloride to yield the carbon tetrachloride fraction. The 20% water in methanol extract was diluted to 35% methanol in water and extracted with chloroform to yield the chloroform fraction and the 35% water in methanol fractions. In all cases equal volumes of the solvents were used and the extraction was repeated with a small volume three more times or until all the colour was extracted. All extracts were taken to dryness in a rotary evaporator under reduced pressure. Extracts were dissolved in acetone or an acetone water mixture before chromatography.

Thin layer chromatography (10 µl of extract adjusted to c. 100 mg extract/ml solution) was on Merck TLC F254 plates with methylenedichloride:acetone 2:3 or chloroform:acetone 2:1 as eluant. Separated components were visualised under visible and ultra-violet

Table 2 The inhibition of *P. aeruginosa* and *S. aureus* growth by different fractions of *A. grandiflora* separated by TLC with chloroform:acetone 2:1 as eluant. R_f values of active components and relative degree of inhibition shown (X = slight inhibition, XXXX = very high inhibition)

P.aeruginosa						*S.aureus*			
R_f	Inhibit.	R_f	Inhibit.	R_f	Inhibit.	R_f	Inhibit.	R_f	Inhibit.
0.80	XXXX	0.79	XXXX	0.84	X	0.83	X	0.80	XXX
0.72	XXXX	0.69	XXXX			0.75	XXXX	0.74	XXX
0.66	XX					0.70	X		
0.61	X					0.62	X		
0.54	XX	0.56	XXX			0.50	X		
0.46	XX	0.45	XX						
0.39	X								
0.31	X								
0.24	XX	0.26	X						
0.16	X	0.18	XX			0.12	X		
Chloroform		Carbon tetrachloride		Hexane		Chloroform		Carbon tetrachloride	

Figure 2 Bioautogram of different extracts of *A. grandiflora* leaf powder. TLC plate developed in acetone:methylenedichloride 2:3, dried, sprayed with *S. aureus* cell suspension, incubated and sprayed with INT. Colourless areas denote inhibition of bacterial growth. Lanes from left to right extracted with acetone, methanol, ethanol, MCW upper and lower phase of concentrated extract, methylenedichloride. Lines indicate fluorescing compounds.

light (254 and 360 nm, Camac Universal UV lamp TL-600) and with iodine vapour.

The bioautography procedure described by Begue and Kline (1972) was used. Chromatography plates were dried overnight and sprayed with a concentrated suspension of actively growing cells of *Staphylococcus aureas* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 25922) or *Bacillus subtilis*, before being incubated overnight at 38°C in a chamber at 100% relative humidity. Plates were sprayed with a 2 mg/ml solution of p-iodonitrotetrazolium violet (Sigma Chemicals). Inhibition of growth was indicated by clear zones on the chromatogram after incubating for one hour.

Results and Discussion

No differences could be detected in the TLC chromatograms of extracts from freeze-dried or air dried leaf material. Acetone, methanol, MDC and MCW extracted similar quantities of an inhibitor for *S. aureus* (Figure 2). Ethanol extracted less of the inhibitor with the same Rf value and a water control extracted no inhibiting components from the dried leaf powder (results not shown). Concentrating the MCW extract led to a phase separation and all of the activity was in the organic phase. For the experiments described in this paper only MCW was used because it facilitated the subsequent handling of the extracts. In experiments carried out subsequently in which many different parameters were evaluated, however, acetone was found to be the best solvent (Eloff 1998).

No problems were experienced with the solvent/solvent fractionation process. The acetone:chloroform TLC system gave better separation of components over a wide range of Rf values and was used for analysing the different fractions. The bioautography technique worked better with the *S. aureus* than with the *B. subtilis* or the *P. aeruginosa* cultures because zones of inhibition were clearer. Nevertheless the presence of growth inhibitors could be ascertained with all three cultures (Tables 1 and 2).

Because high concentrations of the extracts could be chromatographed, additional inhibitors were found in the bioautography of the different fractions. Most of the biological activity was in the chloroform and carbon tetrachloride fractions with all of the test organisms. There was little or no activity in the more polar or non-polar fractions. Only in the case of *B. subtilis*, was there any activity visible in the more polar fractions. The bioautography technique has a drawback in that coloured compounds may mask the growth inhibition of the bacteria. Some of the more polar components have a yellow or brown colour and it is possible that some of these compounds may also inhibit the growth of one or more of the test organisms because the colour may mask the absence of a reaction of the bacteria with the INT in the bioautography. In subsequent experiments with *Combretum erythrophyllum* extracts, this did in fact happen (Martini & Eloff 1998).

The solvent/solvent fractionation did not separate the bioactive components well between the carbon tetrachloride and the chloroform fractions. These two components represented 0.5 and 0.9% of the original dry weight indicating that many non-active components were removed by this mild procedure. When scaling up this procedure to isolate the active components, it makes sense to combine the CT and CF extracts by diluting the 10% water in methanol directly to 35% water in methanol. Most of the activity should be in the lower organic phase.

The Gram-negative organism *P. aeruginosa* was inhibited to a higher degree than the two Gram-positive organisms *S. aureus* and *B. subtilis*. A compound with a similar Rf value (0.72–0.75) was the most active to all three bacterial isolates. In addition to this there were up to 8 other components that separated in the TLC system and inhibited the growth of one or more of the test organisms.

The procedure that was developed used mild techniques and provides useful preliminary information on the diversity of the bioactive components present and also of the polarity of these compounds. The results confirm the observation by the Noristan scientists that *A. grandiflora* leaves contain antimicrobial inhibitors. Attempts are under way to isolate and to chemically and microbiologically characterise some of these compounds. Initial results indicate that the compound with the highest activity may be unstable under the conditions subsequently used for isolation and/or storage.

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