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Full Length Research Paper

Isolation of an antibacterial stilbene from *Combretum woodii* (Combretaceae) leaves

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Acetone extracts of *C. woodii* leaf powder was separated by solvent-solvent partition into six fractions. The highest total activity was in the chloroform fraction. This fraction contained mainly one compound active against *S. aureus*. This compound was isolated by bioassay-guided fractionation using silica gel open column chromatography and identified by nuclear magnetic resonance (NMR) and mass spectroscopy (MS) as the stilbene 2',3',4-trihydroxyl-3,5,4'-trimethoxybibenzyl (combretastatin B5) previously isolated from the seeds of *C. kraussii.* It showed significant activity against *S. aureus* with an MIC of 16 ∝g/ml but with lower activity towards *P. aeruginosa* (125 ∝g/ml), *E. faecalis* (125 ∝g/ml) and slight activity against *E. coli*. This is the first report of the antimicrobial activity of combretastatin B5. Its concentration in the leaves was in the order of 5-10 mg/g which makes the use of non-polar leaf extracts a viable proposition in treating some infections, particularly in resource-poor settings.

Key words: Combretastatin B5, bibenzyl, stilbene, antibacterial, Combretaceae.

INTRODUCTION

With the increasing resistance of bacteria to antibiotics due to misuse and over prescription, there is a need to develop new antibiotics to delay or prevent the arrival of a post-antibiotic era (Leggadrio, 1995). Plants have been used for centuries to treat infectious diseases and present an obvious source of new antimicrobial compounds (Cowan, 1999). Thus far, there has been little success in developing new compounds from plants for use in the pharmaceutical industry, but more success in developing plant extracts to be used especially to treat skin infections is likely.

The Combretaceae is widely distributed in the tropical climes of Africa, South America and Asia (but not in Australia) and is an important resource in traditional medical practice. Species of the two main genera, *Combretum* and *Terminalia* have been used in the treatment of syphilis, abdominal pains, conjunctivitis, diarrhoea and toothache, among other ailments (Hutchings et al., 1996; Gelfand et al., 1985; Watt and Breyer-Brandwijk, 1962). Several species of *Combretum* have antibacterial activity (Eloff, 1999; Martini and Eloff, 1998; Martini et al., 2004a). Several compounds including flavonoids, phenanthrenes, stilbenes, cyclobutanes and triterpenoids have been isolated (Pettit et al., 1987a, 1987b, 1995; Rogers and Verotta, 1995; Katerere, 2001; Katerere et al., 2004; Martini et al., 2004b).

In a preliminary screening of 27 members of the Combretaceae, acetone leaf extracts of *C. woodii*, a deciduous tree found in northern South Africa and Swaziland had a reasonable antibacterial activity (Eloff, 1999). It contained antibacterial constituents that have different R_f values to the closely related species, *C. erythrophyllum* (unpublished results). More detailed investigation of *C. woodii* (Eloff et al., 2005) revealed that intermediate polarity extracts have substantial antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterococcus faecalis* with minimum inhibitory concentration (MIC) values in the order of 0.04 mg/ml. These activities were better than the two positive controls ampicillin and chloramphenicol. In many cases where antimicrobial compounds have been isolated, the compounds are present in low concentrations. In the acetone extracts of *C. woodii* one of the major

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compounds was also the principal antibacterial compound against *S. aureus*. This compound also had a distinctive colour reaction with vanillin-sulphuric acid (Eloff et al., 2005) and it seemed appropriate to isolate and characterise it.

We report here for the first time the isolation of a stilbene, the main antibacterial compound from the leaves of *C. woodii*. Although the compound has been previously isolated from the seeds of *C. kraussi*, this is the first report of its presence in high concentrations in leaves and of its antibacterial activity.

EXPERIMENTAL

Plant material

Leaf material was collected from two trees growing in the Lowveld National Botanical Garden, Nelspruit, South Africa. The origin of each tree is recorded in the database of the botanical garden and a voucher specimen was deposited in the garden's herbarium (Eloff, 1999).

Analysis of extracts by TLC

The following solvent systems were used to analyse fractions by thin layer chromatography (TLC) using Merck, Kieselgel 60 F_{254} plates : benzene : ethanol : ammonium hydroxide (BEA) (36:4:0.4), ethylacetate : methanol : water (EMW) (40:5.4:4) and chloroform : ethylacetate : formic acid (CEF) (20:16:4) . Separated compounds were visualized by spraying with freshly prepared vanillin spray reagent (0.1 g vanillin, 28 ml methanol, 1 ml sulphuric acid) (Stahl, 1969). The plates were carefully heated at 105 ^{0}C to optimal colour development.

Extraction and preliminary fractionation

Dried leaves (150 g) were milled into a fine powder with a Jankel and Kunkel model A10 mill and then extracted with acetone to yield 15.6 g. This was further fractionated using the solvent -solvent fractionation procedure recommended by the USA National Cancer Institute as described by Suffness and Douros (1979) and adapted by Eloff (1998a). This process leads to six fractions separated based on solubility characteristics of the constituents.

All extracts were then taken to dryness in a vacuum rotatory evaporator and weighed. Extracts were reconstituted in appropriate solvent to make up stock solutions of 20 mg/ml. A 5 ∝l aliquot of each was loaded on TLC plates (Merck) that were developed using the eluent systems described above.

Antibacterial activity

The number of antibacterial compounds present was determined by bioautography using the method described by Begue and Kline (1972). The minimum inhibitory concentration (MIC) of fractions was determined by a serial dilution microplate assay using tetrazolium violet to indicate growth of the bacteria (Eloff, 1998b). Four organisms recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 1992) as important causative agents of nosocomial infections were used. These were *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 25922), *Escherichia coli* (ATCC 27853) and *Enterococcus faecalis* (ATCC21212). The MIC of the isolated compound was compared with reference antibiotics (ampicillin and chloramphenicol) as positive controls.

Isolation by column chromatography

About 4 g of the chloroform fraction, which had the highest total antibacterial activity, was applied to a Silica gel 60 (63-200 ∝m) (Merck) column and eluted with varying mixtures of chloroform and ethyl acetate. Eluted fractions were collected and allowed to concentrate under an air stream at room temperature and analysed by TLC and bioautography. Compound **1** was purified by recrystallization from a mixture of methanol and chloroform. ^{1}H- and ^{13}C-NMR spectra were obtained at Medical University of Southern Africa (Medunsa) on a 300 MHz Varian NMR machine (Oxford instruments). Mass spectrometric analysis (HREIMS) was performed on a VG70-SEQ instrument at Cape Technikon.

RESULTS AND DISCUSSION

Acetone extracted about 10 % (15.60 g) of starting plant material. In the group separation technique, the quantity partitioning into the various solvents was determined. The highest percentage went into hexane (32%) and chloroform (26%). Water and 35% water in methanol had the lowest percentage (Figure 2). This was similar to the result obtained in a previous study on *C. erythrophyllum* (Martini and Eloff, 1998).

We assayed the various fractions for antibacterial activity using bioautography (Figure 3) and the micro- titre assays described above. Most of the activity was in the chloroform and hexane fractions. These fractions showed the highest total activity, calculated by dividing the mass by the MIC value (Table 1) as suggested by Eloff (2000, 2004), against the four test bacteria. The aim was to isolate the main antibacterial compound with an R_f of 0.74 in EMW. Inspection of the bioautogram (Figure 3) shows that this compound was present in five of the fractions. There were also at least six other antibacterial compounds. The chloroform fraction had a relatively simple composition containing predominantly the main antibacterial compound. This compound was yellow in visible light, fluoresced purple-blue in 365 nm light and was maroon turning to brown later after treating with vanillin-sulphuric acid spray reagent. Column chromatography of the chloroform fraction as described above led to the successful isolation of this compound (1).

The structure of compound **1** (Figure 1) was elucidated by NMR and MS as the bibenzylic compound, 2',3',4-trihydroxyl -3,5,4'-trimethoxyl bibenzyl (combretastatin B5) previously isolated from the seeds of *C. krausii* (Pellizzoni et al., 1992). HREI-MS gave a molecular ion at *m/z* 320 corresponding to $C_{17}H_{20}O_6$ and only two major fragments at *m/z* 153 ($C_8H_9O_3$) and 167 ($C_9H_{11}O_3$) . This is typical of tropylium derivatives associated with bibenzylic com-pounds (Letcher et al., 1972; Katerere, 2001). The fragmentation pattern was also suggestive of dihydroxy-

Table 1. MIC in mg/ml and total activity in ml of acetone extract of *C. woodii* leaves in hexane, chloroform, carbon tetrachloride, 35% water in methanol, butanol and water fractions obtained by solvent-solvent fractionation towards four bacterial pathogens

Bacteria	**H**	**$CHCl_3$**	**CCl_4**	**35% W/M**	**BuOH**	**Water**
Total quantity obtained in mg	320	256	64	65	117	72
MIC mg/ml						
S. aureus	0.6	0.3	0.1	0.2	0.2	> 5.0
E. coli	2.5	2.5	5.0	5.0	5.0	> 5.0
P. aeruginosa	0.3	0.3	0.3	2.5	2.5	5.0
E. faecalis	0.6	0.1	0.2	0.1	0.6	0.6
Total activity in ml						
S. aureus	533	853	640	325	585	<5
E. coli	128	102	13	13	23	<5
P. aeruginosa	1067	853	213	26	47	14
E. faecalis	533	2560	320	650	195	120
Total activity all organisms	2261	4360	1186	1014	850	134
Distribution of mass and total antibacterial activity						
% of total mass	35.8	28.6	7.2	7.3	13.1	8.1
% of total activity	23.1	44.5	12.1	10.3	8.7	1.4
ratio activity/mass	0.6	1.6	1.7	1.4	0.7	0.2

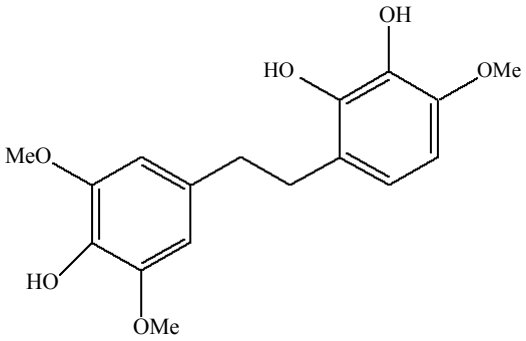

Figure 1. Structure of compound **1 (**2',3',4-trihydroxyl-3,5,4'-trimethoxyl bibenzyl, combretastatin B5).

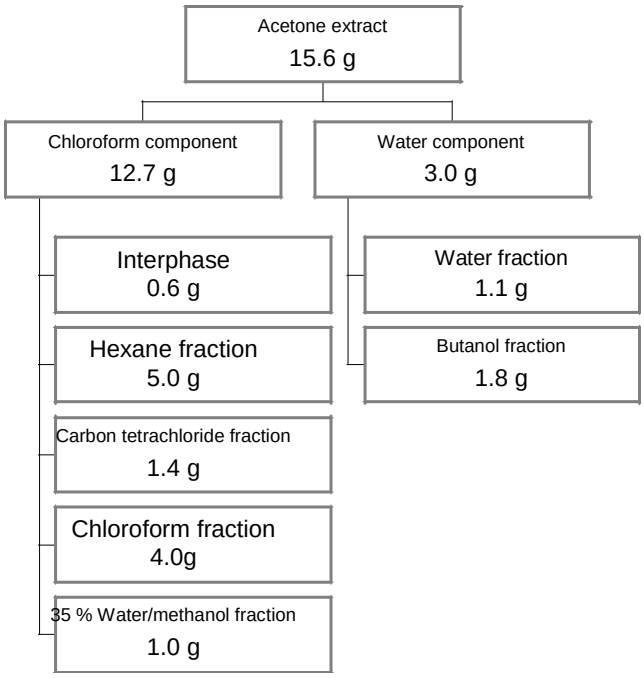

Figure 2. Solvent-solvent fractionation of 140 g of *C. woodii*. Quantity extracted in each fraction by group separation is indicated.

methoxy functions on one aromatic ring and dimethoxy-hydroxy moieties on the second. The ^{1}H -NMR spectra showed aromatic protons at 6.5 ppm (1H, d, J = 8.4 Hz) and 6.36 ppm (1H, d, J = 8.4 Hz) which are *ortho* coupled as well as the 2-proton singlet at 6.4 ppm. There is also a signal at 3.84 ppm integrating to three methoxyl groups and a complex multiplet at 2.82 ppm which is typical of the ethane bridge in bibenzylic compounds (Majumder et al., 1999; Katerere, 2001). By comparison with the literature and 2-D NMR, compound 1 was found to be 2',3',4-trihydroxyl-3,5,4'-trimethoxyl bibenzyl (combretastatin B5). Though previously isolated from the seed of *C. kraussi* (Pellizzoni et al., 1992), this is the first report of its presence in plant leaves.

Compound 1 had an *in vitro* antibacterial activity against all the four test organisms that compared favourably with ampicillin and chloramphenicol. *S. aureus* was the most sensitive of all the test organisms with an MIC value of 16 ∝g/ml followed by *E. faecalis* (125 ∝g/ml) and *P. aeruginosa* (125 ∝g/ml) and *E. coli* (250 ∝g/ml) (Table 2). This makes the compound a potent agent

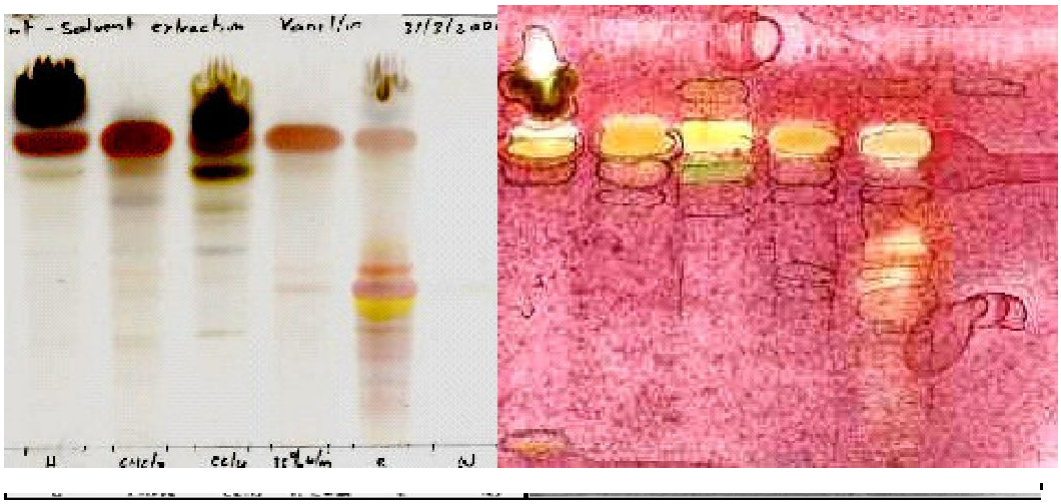

Figure 3. Chromatogram and bioautogram of acetone extract of *C. woodii* leaves separated into different fractions by solvent-solvent extraction. TLC plate developed in EMW sprayed with vanillin-sulphuric acid [left] and with *S. aureus* culture incubated overnight and then sprayed with INT [right]. Growth inhibition indicated by clearing zones on TLC plate. Lanes from left to right: hexane (H), chloroform ($CHCl_3$), carbon tetrachloride (CCl_4), 35% water in methanol (35% w/m), butanol (B) and water (W) fractions.

Table 2. MIC values in ∝ g/ml of Combretastatin B5, ampicillin and chloramphenicol towards four pathogens.

Bacteria	Combretastatin B5	Ampicillin	Chloramphenicol
S. aureus	16	80	160
E. coli	>250	160	40
P. aeruginosa	125	125	125
E. faecalis	125	160	160

against *S. aureus* with potential clinical application. Derivatives have been previously shown to possess potent anti-mitotic activity (Pellizzoni et al., 1992). There are no previous reports of its antibacterial activity.

Combretastatin B5 had a relatively low activity against *E. faecalis*, although the chloroform fraction was three times more active against *E. faecalis* than against *S. aureus* (Table 2). This can only be explained if other antibacterial compounds present in the chloroform extract had substantial activity against *E. faecalis* or if there were synergistic effects with other compounds that were removed on purification of combretastatin B5. The MIC of the pure compound towards *S. aureus* was 16 g/ml. The MIC of the crude acetone extract was 310 g/ml. This represents a c. 20-fold increase in potency with purification. The crude extract represented 100 mg/g leaf powder. From this it follows that, if there is only one antibacterial compound active towards *S. aureus* in the acetone extract, the concentration of combretastatin B5 should be 100/20 i.e. c. 5 mg/g leaf powder. With the inherent inaccuracy of the two-fold serial dilution assay there could be a doubling or halving of this value. Another way of roughly calculating the concentration in leaf material is to judge the concentration when 50 g of crude extract was separated by TLC. When we separated different masses of CB5 in the same volume (5 l), the size of the CB5 band in the crude extract was equivalent to c. 5 g of the pure compound. Because 50 g of the crude extract was chromatographed (5 l of 100 mg/ml), it means that the concentration was c. 100 x 5/50 i.e. c. 10 mg/g. This very high concentration makes the use of extracts for clinical purposes a viable possibility particularly in poor communities.

Summary of spectroscopic data

2',3',4-trihydroxyl-3,5,4'-trimethoxyl bibenzyl (combreta-tatin B5), $C_{17}H_{20}O_6$ (**1**). Yellow amorphous solid; ^{1}H- NMR (300 MHz, $CDCl_3$) 6.40 (2H, *s*), 6.54 (1H, *d*, J 8.4), 6.35, (1H, *d*, J 8.4), 3.84 (9H, *s*), 2.82 (2H, *m*) ^{13}C-NMR (75 MHz, $CDCl_3$): 133.4 (C-1), 105.1 (C-2),

146.8 (C-3), 132.2 (C-4), 146.8 (C-5), 105.1 (C-6), 36.5 (C- 1a), 32.1 (C-1'a), 121.5 (C -1'), 142.1 (C-2'), 132.7 (C-3'), 145.2 (C-4'), 102.3 (C-5'), 120.1 (C-6'), 56.2 (3,5-OMe), 56.1 (4'-OMe). HREIMS *m / z* (rel. int.): 320 $[M]^+$(41%), 153 $[M\text{-}C_9H_{11}O_3]^+$ (100%), 167 $[M\text{-}C_8H_9O_3]^+$

CONCLUSION

The leaves of *C. woodii* contain several antibacterial compounds of which the major compound active against *S. aureus* was isolated and characterized. This compound, though reported elsewhere previously, was isolated from the leaves of *Combretum* spp. for the first time and its potent antibacterial activity noted here for the first time. Further work should be directed towards testing the toxicity of CB5, expanding the assay to other micro-organisms and possible pre-clinical development.

The high concentration of this compound in leaves also make the use of leaf extracts a viable possibility. These results partly validate the ethnobotanical use of many *Combretum* species for conditions that may be of bacterial aetiology. *C. woodii* extracts also contain other antibacterial compounds with high antibacterial activity against *Escherichia coli, Pseudomonas aeruginosa* and *Enterococcus faecalis* (Eloff et al., 2005). This warrants further investigation of these extracts.

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