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Full Length Research Paper

Combretum woodii (Combretaceae) leaf extracts have high activity against Gram-negative and Gram-positive bacteria

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Dried ground leaves of *Combretum woodii* were extracted with 10 different solvents (hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethyl acetate, tetrahydrofuran, acetone, ethanol, methanol and water) to determine the best extractant for subsequent isolation and characterization of antibacterial compounds. With the exception of the water extract, which had no antibacterial activity, the other extracts were bioactive with at least one of them exhibiting minimum inhibitory concentration values of 0.04 mg/ml against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* or *Enterococcus faecalis*. Intermediate polarity solvents extracted c. 10% of the dry mass compared to c. 3% with the more polar or non-polar solvents. These solvents also had higher antibacterial activity than more polar or non-polar extractants. Ethyl acetate was the best extractant with an average minimum inhibitory concentration (MIC) value of 0.08 mg/ml for the four pathogens followed by acetone and methylene dichloride with values of 0.14 mg/ml. The average MIC values for the positive controls were 0.13 (ampicillin) and 0.12 mg/ml (chloramphenicol). By taking the quantity extracted from the leaf powder into consideration, the total activity, a measure of potency, was highest for methylene dichloride (1309 ml/g) followed by acetone (1279 ml/g) extracts. The antibacterial activity was high enough to consider the use of extracts for clinical application and to isolate and characterise antibacterial compounds from the extracts. Based on the R_f values of the antibacterial compounds determined by bioautography, the antibacterial compound was not a polyphenol or a tannin.

Key words: Extraction, antibacterial, MIC, acetone, total activity.

INTRODUCTION

Resistance to antimicrobial agents is a major global public health problem. Infective diseases account for approximately one-half of all deaths in tropics. Despite the progress made in the understanding of microorganisms and their control in industrialized nations, incidents due to drug resistant microorganisms and the emergence of hitherto unknown disease-causing microbes, pose enormous public health concerns (Iwu et al., 1999).

Streptococcus pneumonia, Streptococcus pyogenes and Staphylococci, the organisms that cause respiratory and cutaneous infections, as well as Pseudomonads and members of the Enterobacteriaceae, causing diarrhoea, urinary tract infections, and sepsis, are now resistant to virtually all of the older antibiotics (Harold, 1992). The search for new antibacterial compounds with improved activity to replace those that have become inactive is therefore necessary. Traditional healers use many plants to treat diseases that appear to be of bacterial aetiology and plants may afford valuable antimicrobial compounds (Cowan, 1999).

Many Combretaceae species are widely distributed in Africa and are used in traditional medicine for treating a myriad of conditions including pneumonia, syphilis, abdominal pains, conjunctivitis, diarrhoea, leprosy, scorpion bites and mumps (Hutchings et al., 1996; Gelfand et al., 1985; Watt and Breyer-Brandwijk, 1962). Infective agents may cause many of these conditions and the efficacy of the plant may be due to the presence

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of antimicrobial compounds.

Acetone leaf extracts of 27 southern African members of the Combretaceae, had antibacterial activity with MIC values of 1.6 mg/ml or lower against at least one of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* or *Enterococcus faecalis* (Eloff, 1999). There is a large variation in the antibacterial activity and chemistry of antibacterial compounds among different genera and species in the Combretaceae (Eloff, 1999; and unpublished data). *Combretum erythrophyllum* has at least 14 antibacterial compounds and some of these compounds that were isolated but not characterized, have activities higher than chloramphenicol and ampicillin (Martini and Eloff, 1998). Seven of these compounds have been isolated, their structure determined (Martini *et al*., 2004a) and several biological activities of five of these flavonoids were determined (Martini et al., 2004 b).

This report focuses on *C. woodii*, which belongs to the same section Angustimarginata of the subgenus *Combretum* as *C. caffrum, C. erythrophyllum and C. zeyheri* (Carr, 1988). Antineoplastic combretastatins with promising therapeutic properties currently evaluated in clinical trials were isolated from *C. caffrum* (Pettit et al., 1987). Seven antibacterial flavonoids were isolated and characterized from *C. erythrophyllum* and investigated for biological activity (Martini et al., 2004a, b) In contrast, little has been reported on *C. woodii*. The MIC of acetone leaf extracts to *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* or *Enterococcus faecalis* varied from 0.4 to 1.6 mg/ml (Eloff 1999). The acetone extract also had antibacterial compounds with different R_f values than those found in *C. erythrophyllum* (unpublished data). We could not find any record of its use in traditional medicine but these taxa are difficult to distinguish and rural people possibly use *C. woodii* interchangeably with *C. erythrophyllum*. In a taxonomic survey Rodman (1990) concluded that *C. caffrum* and *C. woodii* are synonyms for *C. erythrophyllum*. Although her view is not generally accepted among plant taxonomists, it shows the close relationship based on morphology.

As a first step in investigating *C. woodii*, we examined the efficacy of different extractants towards the quantitative antibacterial activity (MIC values) and qualitative antibacterial activity (number and R_f value of antibacterial compounds). These results should indicate if extractants could be used to selectively extract antibacterial compounds or remove inactive compounds. Some members of the Combretaceae have high concentrations of tannins or polyphenolic compounds. These compounds are known to have *in vitro* antimicrobial activity, but have limited *in vivo* application due to low bioavailability and strong binding to proteins. If high antimicrobial activity resides in the polar extracts, it may indicate that tannins or polyphenolic compounds are responsible for the activity and limit interest in continuing to study this species. The results may also indicate if extracts with sufficient activity for clinical use could be prepared.

MATERIALS AND METHODS

Collection of plant material

C. woodii Dümmer is a deciduous tree or shrub growing to a height of 8 - 12 m found in North- eastern South Africa and Swaziland at low to medium altitudes. It is commonly known as the Bastard Forest Bush willow, Basterbosvaderlandswilg (Afrikaans), iWaphu (Zulu) or Mbondvo sehlatsi (Siswati). Leaves were collected in early summer from two trees growing in the Lowveld National Botanical Garden, Nelspruit. The origin of the tree is recorded in the database of the botanical garden and a voucher specimen is deposited in the garden's herbarium (Eloff, 1999) . The leaves were dried in the shade at room temperature (20ºC) and milled into a fine powder with a Jankel and Kunkel model A10 mill. The powder was stored at room temperature in the dark in tightly closed glass containers.

Extraction of leaf powder

To determine the efficiency of different extractants, 0.5 g samples of finely ground leaves were extracted in 5 ml in each of 10 technical grade solvents (Merck) of varying polarity: hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethyl acetate, tetrahydrofuran, acetone, methanol and water with vigorous shaking (Kotze and Eloff, 2002). The extracts were decanted after centrifuging at 5300 x g for 5 min. The process was repeated twice. The solvents were removed under a cold air stream at room temperature. The extracts were weighed and re-dissolved in acetone to give 10 mg/ml stock solutions.

TLC analysis of extracts

The following solvent systems were used to analyse 50 ∝g of extract by thin layer chromatography (Merck, Kieselgel 60 F_{254}): Benzene : ethanol : ammonium hydroxide (BEA) (36:4:0.4), Ethylacetate : methanol : water (EMW) (40:5.4:4), Chloroform : ethylacetate : formic acid (CEF) (20:16:4). Visible bands were marked under daylight and ultraviolet light (254 nm and 360 nm, Camac Universal UV lamp TL-600) before spraying with freshly prepared *p*-anisaldehyde (1 ml *p*-anisaldehyde, 18 ml ethanol, 1 ml sulphuric acid) or vanillin (0.1 g vanillin, 28 ml methanol, 1 ml sulphuric acid) spray reagents (Stahl, 1969). The plates were carefully heated at 105ºC for optimal colour development.

Qualitative antibacterial activity assay by bioautography

The bioautography procedure described by Begue and Kline (1972) was used. TLC plates were prepared and developed in the different solvent systems, dried overnight under a stream of air to remove residual solvent, which might inhibit bacterial growth. One of the plates was sprayed with the vanillin spray reagent and the others with one of four bacterial cultures - *Staphylococcus aureus* (ATCC 29213) , *Pseudomonas aeruginosa* (ATCC 25922), *Escherichia coli* (ATCC 27853) or *Enterococcus faecalis* (ATCC21212). These species are the major cause of nosocomial infections in hospitals (Sacho and Schoub, 1993) and these are the strains recommended for use by the National Committee for Clinical Laboratory

Table 1. Quantity in mg extracted from 500 mg of *C. woodii* leaf powder with different solvents arranged in order of polarity. Hexane (H), diisopropyl ether (I), diethyl ether (EE), methylene dichloride (MDC), ethyl acetate (EA), tetrahydrofuran (THF), acetone (ACN), ethanol (ET), methanol (M) and water (W).

Extraction	H	I	EE	MDC	THF	EA	ACN	ET	M	W
1st	8	14	18	44	47	29	43	36	23	2
2nd	1	2	2	7	6	2	4	7	4	1
3rd	1	1	0	2	2	1	2	2	2	1
Total	10	17	20	53	55	32	48	45	29	4
% extracted	2	3.4	4	10.6	11	6.4	9.6	9	5.8	0.8

Standards (NCCLS, 1992). 10 ml of highly dense fresh bacteria culture was centrifuged at 5300 x g for 20 min to concentrate the bacteria. The supernatant was discarded and the combined pellet resuspended in 2 – 4 ml of fresh Müller-Hilton broth. The plates were sprayed with the concentrated suspension until they were just wet, dried in air to remove excess liquid, and incubated overnight at 37ºC in 100% relative humidity. After incubation, plates were sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet (Sigma Chemicals). Clear zones on chromatograms indicated inhibition of growth after incubating for about 1 h (Begue and Kline, 1972).

Quantitative antibacterial activity assay by minimum inhibitory concentration (MIC) and total activity

MIC values were determined by serial dilution microplate bioassay and indicating growth with tetrazolium violet (Eloff, 1998a) using the same test organisms as for bioautography. Ampicillin and chloramphenicol were used as positive controls. The total activity in ml/g was calculated by dividing the MIC value with the quantity extracted from 1 g of plant material. The value indicates the volume to which the extract can be diluted and still inhibit the growth of the bacterial isolate (Eloff, 2004).

RESULTS AND DISCUSSION

Extraction efficacy

There were substantial differences in the quantity of material extracted. Tetrahydrofuran (THF) extracted 11%, nearly 14 times as much as water (W) (0.8%) (Table 1). As was the case with *C. microphyllum* (Kotze and Eloff, 2002), extractants with intermediate polarity extracted a much higher quantity than polar or non-polar extractants. With the six intermediate polarity extractants, c. 87% of the total quantity was extracted with the first extraction and c. 3% with the third extraction. For the two most polar and two most non-polar extracts, the rate of extraction was much lower with c. 12% of the total mass still extracted during the third extraction.

TLC analysis

More compounds were visible with vanillin-sulphuric acid than with the *p*-anisaldehyde spray reagent. *C. woodii* leaves appear to have a preponderance of non-polar compounds that are coloured by the vanillin spray reagent. Approximately 17 bands were separated with the non-polar BEA compared to 11 bands with the intermediate polarity CEF and nine bands with the more polar EMW solvent system (Figure 1). There were similarities between the chromatograms of the intermediate polarity extracts but the hexane, di-isopropylether and water extracts were very different. Especially with BEA, the non-polar solvent system, substantial differences between the other extracts were visible, but the differences were less apparent with CEF and EMW as solvent systems.

Qualitative analysis of antibacterial activity

The bioautography method worked well with *S. aureus* and *E. faecalis* but not as well with the Gram-negative *E. coli* and *P. aeruginosa*. EMW and BEA yielded better results in bioautography probably due to the difficulty in removing residual formic acid in CEF chromatograms. There was one major antibacterial compound (R_f value of 0.74 in EMW and 0.28 in BEA) against *S. aureus* with the intermediate polarity extractants (Figure 2). This main antibacterial compound had a distinctive colour with the vanillin (brown when BEA was used as TLC eluent and red with CEF and EMW) and purple with the anisaldehyde spray reagent (Figure 1). This compound had a lower activity against the other pathogens, but there were compounds with other R_f values active against the other bacteria (results not shown) including two to three minor antibacterial compounds against *S. aureus* present in the non-polar extractants (Figure 2).

Quantitative analysis of antibacterial activity

In 18 cases, MIC values of the extracts were better or the same as the values for the two positive controls Table 2, ampicillin and chloramphenicol. If we ignore the results for the three most polar extracts, the average MIC for *E. coli* was 0.05; for *E. faecalis*, 0.06; for *S. aureus*, 0.48;

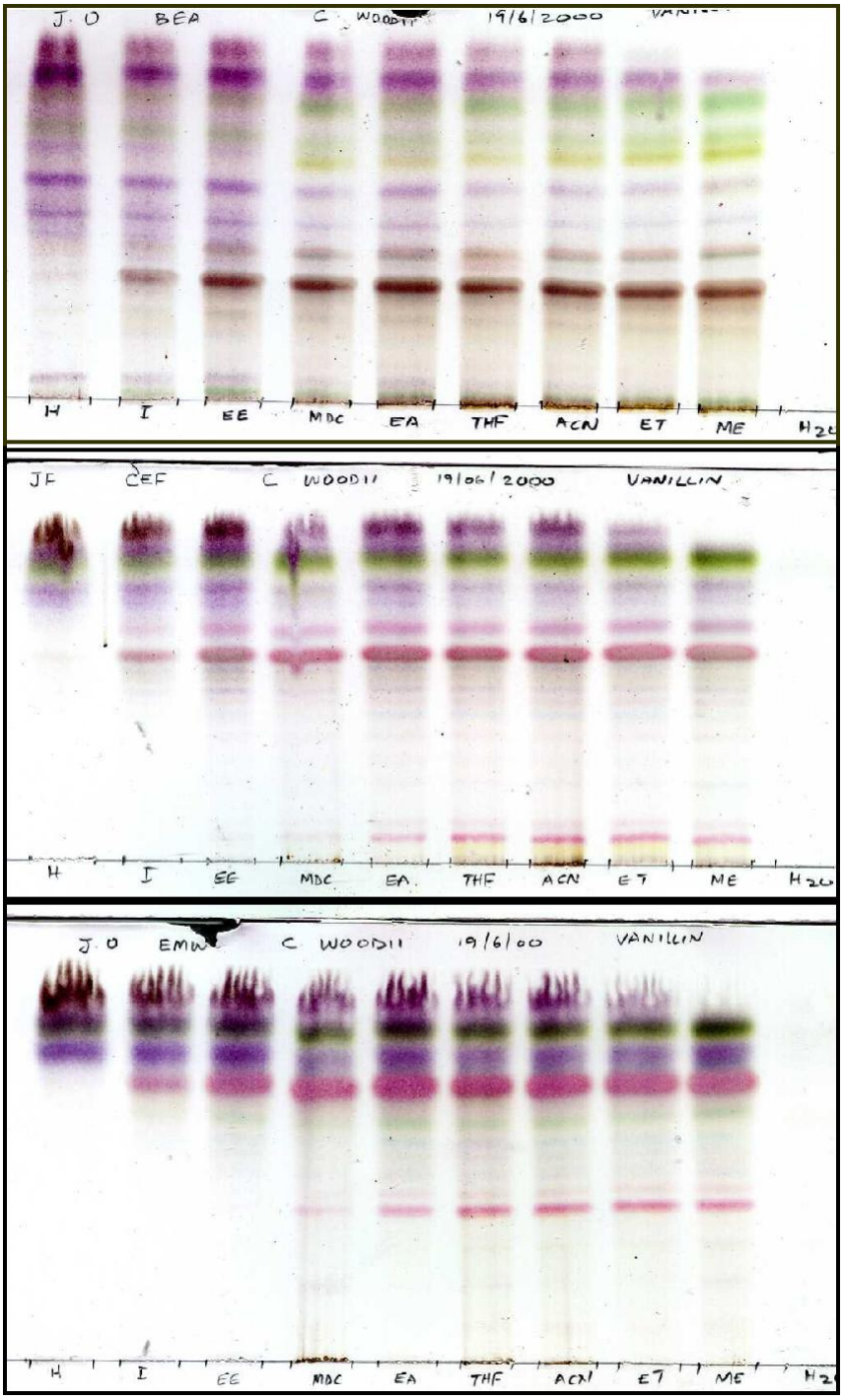

Figure 1. Separation of components present in 50 ∝g of 10 different extracts with BEA, CEF and EMW as eluents and vanillin-sulphuric acid spray reagent Lanes from left to right: hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethyl acetate, tetrahydrofuran, acetone, ethanol, methanol and water.

and for *P. aeruginosa*, 0.19 mg/ml. These values compared well with the respective average values for the two positive controls; 0.16, 0.12, 0.13 and 0.10 mg/ml, respectively. Ethyl acetate was the most active with an average MIC value for the four pathogens of 0.08 mg/ml followed by acetone, and methylene dichloride with values 0.14 mg/ml. The average values for ampicillin was 0.13 and for chloramphenicol, 0.12 mg/ml. When the

Table 2. Quantity extracted MIC values in mg/ml and total activity in ml (Eloff, 2000) of *C. woodii* per gram leaves extracted with hexane (H), diisopropyl ether (I), diethyl ether (EE), methylene dichloride (MDC), ethyl acetate (EA), tetrahydrofuran (THF), acetone (ACN), ethanol (ET), methanol (M) and water (W). Positive controls; ampicillin (AMP) and chloramphenicol (CAM).

	H	I	EE	MDC	EA	THF	ACN	ET	M	W	AMP	CAM
Total quantity in mg extracted from 1g	16	28	36	88	58	94	86	72	46	4		
MIC in mg/ml												
E. faecalis	0.16	0.04	0.04	0.04	0.04	0.08	0.04	0.31	0.63	> 2.5	0.16	0.16
S. aureus	1.25	0.63	0.63	0.16	0.08	0.31	0.31	0.31	0.31	> 2.5	0.08	0.16
P. aeruginosa	0.04	0.16	0.31	0.31	0.16	0.16	0.16	> 2.5	> 2.5	> 2.5	0.13	0.13
E. coli	0.08	0.08	0.04	0.04	0.04	0.04	0.04	0.04	0.31	>2.5	0.16	0.04
Average	0.38	0.23	0.26	0.14	0.08	0.15	0.14	>0.79	>0.94	> 2.5	0.53	0.49
Total activity in ml/g												
S. aureus	13	44	57	550	725	303	277	232	148	<1.6		
P. aeruginosa	400	175	116	284	363	588	538	<1.6	<1.6	<1.6		
E. coli	200	350	900	2200	1450	2350	2150	1800	148	<1.6		
Average	178	317	493	1309	997	1104	1279	567	93	<1.6		

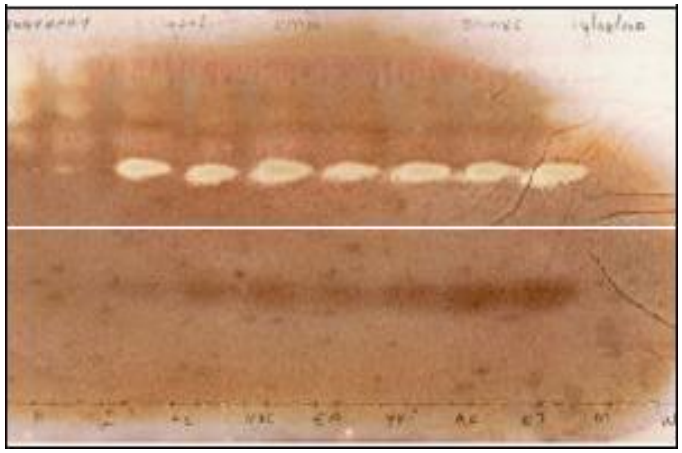

Figure 2. Bioautogram of *C. woodii* leaves extracted by 10 different extractants TLC developed in EMW and sprayed with *S. aureus* culture incubated overnight then sprayed with INT. Growth inhibition indicated by lighter zones on TLC plates. Lanes from left to right: hexane (H), diisopropyl ether (I), diethyl ether (EE), methylene dichloride (MDC), ethyl acetate (EA), tetrahydrofuran (THF), acetone (ACN), ethanol (ET), methanol (M) and water (W).

quantity extracted from the leaf powder was calculated the total activity was highest for methylene dichloride (1309 ml) followed by acetone (1279 ml) . The inactivity of the water extract is shown by the total activity value of <1.6 ml. If the water extractable compounds present in 1 g of leaf powder were dissolved into 1 ml, it would, therefore, not inhibit the growth of any of the bacteria tested.

CONCLUSION

The chemical composition of the extracts based on the TLC chromatograms differed very much from that of *C. erythrophyllum*, which supports the conclusion of most taxonomists that Rodman (1990) is wrong in suggesting that *C. woodii* should be sunk in *C. erythrophyllum*.

The very limited activity of the aqueous and methanol extracts indicates that tannins are not responsible for antibacterial activity in the leaves of *C. woodii*. Although a similar pattern (i.e. most activity in the intermediate polarity extracts) was present as in *C. microphyllum*, *C. woodii* extracts were substantially more active than extracts of *C. microphyllum* (Kotze and Eloff, 2002).

Although there was not much difference in the vanillin positive components in the chromatograms of the intermediate polarity extracts, there were substantial differences in their antibacterial activity. In most cases plant extracts are active against Gram -positive pathogens (Vlietinck et al., 1995), but *C. woodii* extracts had substantial activity against Gram-negative bacteria as well.

Because the activity of most of the plant extracts were higher than that of the positive controls, it is reasonable to consider the possible use of extracts at least for topical application. Unfortunately, aqueous decoctions or infusions would have no antibacterial activity. Users in poor areas e.g. rural dwellers would have to use a leaf

paste, express sap, or ingest the powder due to the unavailability of non-polar extractants to them. The use of animal fat, vegetable oil or formulation into petroleum jelly may increase the bioavailability of the bioactive constituents shown in this study.

Since all the extracts consisted of many different compounds, it is clear that unless there are substantial synergistic effects, these plant extracts must contain compounds with much higher activity than the value obtained for the extract as a whole. In addition to investigating the use of extracts it seems to be worthwhile to isolate and characterise the antibacterial compounds present in these extracts. The big difference in antibacterial activity and vanillin-visualised chromatograms may indicate that the antibacterial compounds are present in low concentrations or that they do not react with the vanillin spray reagent. The one major active compound against *S. aureus* occurs in a high concentration in several extracts. The different colour of this compound on treatment with the vanillin spray reagent (red or brown depending on the pH of the eluent used) would be useful in the bioassay-guided isolation of this compound. In the case of the other bacteria, there was not one major antibacterial compound but several antibacterial compounds present.

Any of the intermediate polarity extractants would be useful to prepare active extracts or to use in isolating the bioactive compounds based on the quantity extracted and the antibacterial activity. The average total activity of four extracts were much higher than the other six i.e. methylene dichloride (1309 ml/g), acetone (1279 ml/g), tetrahydrofuran (1104 ml/g) and ethyl acetate (997 ml/g). Due to the water miscibility of acetone and the relatively low toxicity, this is the obvious choice as an extractant and confirms results found with other plants (Eloff, 1998b). We are in the process of isolating antibacterial compounds from *C. woodii* extracts.

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