INVESTIGATION OF ANTIBACTERIAL COMPOUNDS PRESENT IN *COMBRETUM WOODII* DUEMMER

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PREFACE

The experimental work described in this dissertation was carried out by me in the Department of Pharmacology, Faculty of health science, University of Pretoria, Pretoria, under supervision of Prof. J. N. Eloff and Dr. D. R. P. Katerere.

These studies represent the work done by the author and have not otherwise been submitted in any form of degree or diploma to any other University. Where use has been made of the work of others it is duly acknowledged in the text.

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James Olusanya Famakin

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GLOSSARY OF ABBREVIATIONS

1	ACN	Acetone (extractant)
2	ATCC	American type Culture Collection
3	B	Butanol (extractant or fraction)
4	^{13}C	Carbon 13
4	BEA	Benzene:Ethanol:Ammonium hydroxide [36:5.4:4]
5	CC	Column chromatography
6	CCl_4	Carbon tetrachloride (extractant or fraction)
7	CEF	Chloroform:Ethyl acetate:Formic acid [5:4:1]
8	CF	Column chromatography fraction
9	$CHCl_3$	Chloroform (extractant or fraction)
10	EA	Ethyl acetate extractant.
11	*E. coli*	*Escherichia coli*
12	EE	Diethyl ether (extractant)
13	*E. faecalis*	*Enterococcus faecalis*
14	EMW	Ethanol : methanol : water [40:5.4:4]
15	ET	Ethanol (extractant)
16	H	Hexane (extractant or fraction)
17	^{1}H	Proton
18	I	Isopropyl ether
19	IB	Insoluble butanol fraction
20	INT	*p*-iodonitrotetrazolium violet
21	IW	Insoluble water fraction
22	IWM	Insoluble 35% water in methanol (fraction)
23	M	Methanol extractant
24	MDC	Methylene dichloride
25	MS	Mass spectroscopy
26	35% W/M	35% water in methanol (fraction)
27	NMR	Nuclear magnetic resonance
28	*Ps. aeruginosa*	*Pseudomonas aeruginosa*
29	R_f	fractional movement of a solute band, relative to the distance moved by solvent front
30	*S. aureus*	*Staphylococcus aureus*
31	THF	Tetrahydrofuran extractant
32	TLC	Thin layer chromatography
33	UV	Ultra-violet light
34	W	Water (extractant)

ABSTRACT

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 isolating and characterizing any compound(s) with antibacterial activity present. The antibacterial activity of all the extracts was tested against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterococcus faecalis*. All the extracts, with exception of the water extract, inhibited the growth of *S. aureus* and *E. faecalis* using bioautography of thin layer chromatography plates. Two major inhibitory compounds with R_f values of 0.74 and 0.88 were visible on the bioautograms of extracts sprayed with *S. aureus* and *E. faecalis* respectively in ethanol:methanol:water (40:5.4:4) solvent systems. There were at least three more polar inhibitory compounds against *E. faecalis* separated in benzene:ethanol:ammonium hydroxide (36:4:0.4) solvent system.

According to thin layer chromatography using *p*-anisaldehyde-sulphuric acid as spray reagent, most solvents extracted at least seven compounds but water extracted only one visible compound.

Tetrahydrofuran, methylene dichloride, and acetone extracted the largest quantity. The methylene dichloride and acetone extracts had the highest antibacterial activity against all the four test organisms. However, acetone was selected for extraction of *C. woodii* dried ground leaves because of its relatively low toxicity to test organisms and the ease of removal after extraction.

Acetone extracted 11% of 140 g of dried ground leaves. Group separation by solvent-solvent extraction was applied to the acetone extract. The complex extract was simplified by separating into six fractions and an interphase. The highest number of non-polar compounds was in the hexane fraction, followed by carbon tetrachloride and chloroform fractions. The highest quantity of extract, 32%, was also in the hexane fraction followed by chloroform (25.6%), butanol (11.7%), water (7.2%), 35% water in methanol (6.5%), and carbon tetrachloride (6.4%) fractions. The carbon tetrachloride fraction had the most complex mixture of compounds. The six fractions obtained inhibited the four test organisms to different degrees. Most of the bioactive compounds were in the chloroform and hexane fractions. The chloroform fraction had the highest relative antibacterial activity (almost 33 times higher than the water fraction). Generally, *S. aureus* was the most sensitive, followed by *E. faecalis*, *Ps. aeruginosa* and *E. coli*. There were at least six growth inhibitors of pathogenic

bacteria. A major active compound with R_f value of 0.67 in chloroform:ethylacetate:formic acid (20:16:4) and 0.74 in ethanol:methanol:water (40:5.4:4) solvent systems was present in all the fractions (except water fraction). Attempts were made to isolate and characterize this major active compound.

The chloroform fraction was subjected to silica gel 60 (63–200 μm) column chromatography using a chloroform and ethyl acetate mixture and 10% methanol in acetone to elute the column fractions. Further TLC analyses and column chromatographic procedures on the collected fractions led to the isolation of this compound. This was identified by nuclear magnetic resonance and mass spectroscopy as combretastatin B5 (2', 3', 4-trihydroxyl, 3, 5, 4'-trimethoxybibenzyl) previously isolated from the seeds of *C. krausii*. This compound has been found to have antimitotic activity. The closely related combretastatin A4, the first of a new class of anticancer agents, is currently undergoing clinical trials.

The antibacterial activity of combretastatin B5 showed significant activity against *S. aureus*, *Ps. aeruginosa*, *E. faecalis* and slight activity against *E. coli*. The MIC values of the isolated active compound for *S. aureus* was 16 μg/ml, which compares favourably to the MIC values of 80 μg/ml and 160 μg/ml for ampicillin and chloramphenicol in this test respectively.

The results obtained validate the use of *Combretum* species for the bacterial infections in traditional medicine. Further work, needs to be done to investigate the possible clinical value of combretastatin B5 and isolate and characterize other antibacterial compounds in *C. woodii*.

SAMEVATTING

Om te bepaal wat die beste ekstraheermiddel is vir gedroogde *C. woodii* blare is, is tien vloeistowwe (heksaan, di-isopropieleter, dietieleter, metileendichloried, etielasetaat, tetrahidrofuraan, asetoon, etanol, metanol en water) gebruik om antibakteriese verbindings te isoleer en karakteriseer. Die toetsorganismes was *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterococcus faecalis*. Volgens bio-outografieresultate, het al die ekstrakte behalwe die waterekstrak die groei van *S. aureus* and *E. faecalis* onderdruk. Daar was twee belangrike inhibeerders met R_f waardes van 0.74 en 0.88 in bio-outogramme ontwikkel in etanol:metanol:water (40:5.4:4). Daar was ten minste nog drie meer polêre inhibeerders van *E. faecalis* volgens bio-outogramme ontwikkel in benseen:etanol:ammoniumhidroksied (36:4:0.4). Met die uitsondering van die waterekstrak kon ten minste sewe verbindings na dunlaagchromatografie (DLC) en spuit met *p*-anysaldehied-swawelsuur aangetoon word.

Tetrahidrofuraan, metileen dichloried en asetoon het die grootste hoeveelheid ge-ekstraheer en laasgenoemde twee het die hoogste antibakteriese aktiwiteit teen die vier toetsorganismes gehad. Asetoon is gekies as ekstraheermiddel omdat dit 'n relatiewe lae toksisiteit vir die toetsorganismes gehad het en maklik verwyder kon word na ekstraksie.

Asetoon het 11% van die 140 g gedroogde fyngemaalde blare ge-ekstraheer. Die ekstrak is deur vloeistof-vloeistof groepskeiding vereenvoudig na ses fraksies en 'n interfase. Die grootste getal nie-polêre verbindings was in die heksaanfraksie gevolg deur die koolstoftetrachloried- en chloroformfraksies. Die grootste hoeveelheid, 32% van die totaal, was ook in die heksaanfraksie gevolg deur die chloroform- (25.6%), butanol- (11.7%), water- (7.2%), 35% water in metanol- (6.5%), and koolstoftetrachloriedfraksies (6.4%). Die koolstoftetrachloriedfraksie het die mees komplekse samestelling gehad en al ses fraksie het die groei van die toetsorganismes tot 'n mindere of meerdere mate geïnhibeer. Oor die algemeen was *S. aureus* die sensitiefste gevolg deur *E. faecalis, Ps. aeruginosa* en *E. coli.* Daar was ten minste ses groei-inhibeerders van die bakterieë teenwoordig. Die sterkste inhibeerder het 'n R_f-waarde van 0.67 in chloroform:etielasetaat: mieresuur (20:16:4) and 0.74 in etanol:metanol:water (40:5.4:4) gehad. Hierdie verbinding was teenwoordig in al die fraksies behalwe die waterfraksie. Pogings is aangewend om hierdie verbinding te isoleer.

Die komponente van die chloroformfraksie is deur silikagel chromatografie (silika gel 60 (63-200 µm) met 'n gradiënt van chloroform-etielasetaat en later 10% metanol in asetoon geskei. Die suiwer verbinding is deur verdere DLC analise en kolomchromatografie geïsoleer. Die verbinding is deur kernmagnetiese resonansspektroskopie en massaspektroskopie geïdentifiseer as combretastatin B5 (2',3',4-trihidroksiel, 3,5,4'-trimethoksiebibensiel) wat voorheen geïsoleer is uit die saad van *C. krausii.* Hierdie verbinding het antimitotiese aktiwiteit gehad. Die naverwante combretestatin A4, die eerste van 'n nuwe klas antikanker agense ondergaan tans kliniese proewe.

Combrestatin B5 het sterk antibakteriese aktiwiteit teen *S. aureus*, *Ps. aeruginosa*, *E. faecalis* en laer aktiwiteit teen *E. coli* gehad.. Die MIC-waardes vir *S. aureus* was 16 µg/ml, teenoor MIC waardes van 80 µg/ml en 160 µg/ml vir ampisillien en chlooramfenikol in hierdie eksperimente.

Die resultate ondersteun die etnobotaniese gebruik van *Combretum* spesies vir bakteriese infeksies. Verdere werk behoort uitgevoer te word op die moontlike kliniese waarde van combretastatin B5 en die isolering van ander antibakteriese verbindings in *C. woodii.*