AN INVESTIGATION OF ANTIBACTERIAL COMPOUNDS IN *Combretum microphyllum* [KLOTZSCH]

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UNIVERSITY OF PRETORIA

2000

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B. Pharm (PU vir CHO)

Dissertation submitted to the faculty of Medicine (Department of Pharmacology), University of Pretoria, Pretoria, in partial fulfilment of the requirements of the degree of

MAGISTER SCIENTIAE

Promoter: Prof JN Eloff

Date of submission: November 2000

PREFACE

I hereby confirm that this is my own work and have not been submitted to any other institution

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Magdeleen Kotze

ACKNOWLEDGEMENTS

I would like to extend a warm word of thanks to the following:

- Prof JN Eloff, my promoter, without whose support and wisdom I would not have been able to complete this investigation.
- The Department of Pharmacology, University of Pretoria, for making their facilities available at all hours throughout the three years of this research.
- My fellow students and Dr I von Teichman who assisted me in the laboratory and Julia Becker for all her help.
- My parents, brothers and sister for their love and encouragement.
- Francois for his patience and understanding.
- My Creator for His abundant blessings.

SUMMARY

Acetone, which efficiently removes compounds from finely ground *Combretum* spp leaves, was compared with 1 % aqueous sodium bicarbonate as extractants using intact dried leaves of *Combretum microphyllum*.

Acetone gave more promising results, than sodium bicarbonate. The acetone extract yielded the highest concentration, as well as the greatest diversity of compounds visible after TLC. The sodium bicarbonate extract inhibited Gram-positive bacteria to a larger extent than Gram-negative bacteria. *E. coli* was inhibited much more than *E. faecalis* by the acetone extract. This indicated that there may be some selectivity.

The total quantity extracted by acetone from intact leaves was substantially lower (3.8 %) than values found earlier (6.2 %) for finely ground material. The MIC values for the extracts of intact leaves were lower, indicating that fewer inactive compounds were co-extracted. I decided to continue working on finely ground leaf material. I experienced difficulties in the acid precipitation of the bicarbonate extracts and decided not to continue with sodium bicarbonate as extractant.

Eleven different extractants (hexane, carbon tetrachloride, isopropyl ether, diethyl ether, methylene dichloride, tetrahydrofurane, ethyl acetate, acetone, ethanol, methanol and water) were used on finely ground, dried leaf material to extract antibacterial compounds from *C. microhyllum*.

There were large differences in the chemical composition of compounds extracted. Methylene dichloride and ethanol extracted the most compounds. The extracts of the intermediate polarity extractants isopropyl ether, ethyl ether and methylene dichloride gave the best separation with BEA as eluent, whereas the carbon tetrachloride and tetrahydrofurane extracts gave pronounced streaking. Carbon tetrachloride surprisingly extracted a large quantity of polar compounds (low Rf-value) and a small quantity of non-polar compounds in comparison to the more polar isopropyl ether and diethyl ether.

On the other hand, a relatively polar extractant such as ethanol, extracted several non-polar compounds. With CEF as eluent the carbon tetrachloride and tetrahydrofurane extracts gave serious streaking. Acetone extracted the highest concentration and most diverse number of compounds that reacted with the vanillin spray reagent. Although it did not extract the most antibacterial activity, I decided to continue with acetone as extractant due to its relatively low toxicity to test organisms and the good TLC separation of compounds with this extractant.

Solvent/solvent fractionation was used to simplify extracts because of the initial complexity of the extracts. The most non-polar fractions (hexane, carbon tetrachloride and chloroform) gave the best separation of compounds as well as having the greatest diversity of components when separated by TLC. The highest quantity, c. 42 %, was present in the hexane fraction and nearly two-thirds of the total antibacterial activity was also present in the hexane fraction.

Frequently Gram-negative bacteria are more resistant to plant extracts than Gram-positive bacteria, but this was not true for the compounds present in *C. microphyllum*.

Bioautography worked well with *S. aureus*, but was not as reproducible with the other test organisms. Unfortunately *S. aureus* was the least sensitive of the four test organisms, especially for the hexane and carbon tetrachloride fractions.

Because the hexane fraction had the highest antimicrobial activity by far for all four test organisms used, I chose this fraction for the next steps in the isolation of bioactive compounds with column chromatography.

Attempts to use Extrelut, a diatomaceous earth product, instead of the solvent/solvent fractionation, were not successful.

Column chromatography on silica gel 60 [15-40 μm] gave good results. Four volatile eluents, which worked well on TLC, were used on the column in the following order: BEA, CEF, acetone and methanol.

Fractions were combined based on the analysis by TLC. Fraction ABC gave the most definite bands and best separation on TLC. By far the largest antibacterial activity (ABC: 66 % and DEF: 15 %) was in the first highly non-polar pooled fractions. *S. aureus* was not as sensitive as the other test organisms.

I developed a TLC system, using volatile eluents that would separate the components of fractions ABC and DEF well in order to employ this system in a next silica gel chromatographic separation. Good results were obtained with a 2:1 hexane-acetone mixture. I decided to continue with only the ABC fraction using this eluent. To increase the resolution, a finer grade silica gel [15-25μm] with a narrower size range was used as packing material.

The fractions were analyzed by TLC and were combined based on the TLC

analysis and the quantity present in each fraction. Some of the pooled minor fractions seemed to be relatively pure based on the TLC analysis of these fractions and had significant antibacterial activity based on bioautography with *S. aureus* as test organism.

When these active pooled fractions were dried under vacuum in a vacuum dessicator prior to NMR spectroscopy, c. 80 % was lost. According to the NMR analysis, the remaining sample consisted mainly of phthalate, a common component of plasticizers used in making plastics and also a common contaminant in certain solvents such as ethyl acetate.

Phthalic acid did not react with the vanillin spray reagent, but it gave clear quenching in UV light after TLC. According to the Rf-value, phthalic acid was not responsible for the antibacterial activity. It was also not volatile under high vacuum.

High concentrations of phthalic acid had a slight antibacterial activity, possibly due to a pH effect.

The procedures developed therefore led to the isolation of an antibacterial compound from *C. microphyllum*. Unfortunately personal circumstances made it impossible to repeat the last part of the work or expand on any other part of the results.

A major avenue for further work exists in the non-polar fractions of the final separation containing most of the dry mass and biological activity. There is also the possibility to capitalize on the suspected volatility of the bioactive compound to purify it by vacuum distillation.

SAMEVATTING

Asetoon, wat verbindings baie goed verwyder vanuit fyngemaalde *Combretum* spp blare, is vergelyk met 1 % waterige natriumbikarbonaat, as ekstraheermiddels deur heel, gedroogde blare van *Combretum microphyllum* te gebruik.

Asetoon het meer belowende resultate gelewer as natriumbikarbonaat. Die asetoonekstrak het die hoogste konsentrasie asook die meeste verskillende verbindings sigbaar na dunlaagchromatografie gelewer. Die natriumbikarbonaat ekstrak het Gram-positiewe bakterieë meer geinhibeer as Gram-negatiewe bakterieë, maar *E. coli* was meer onderdruk as *E. faecalis* deur die asetoonekstrak. Dit het daarop gedui dat daar wel selektiwiteit mag bestaan.

Die totale hoeveelheid geëkstraheer deur asetoon vanaf heel blare, was aansienlik laer (3.8 %) as waardes wat vroeër gevind is (6.2 %) vir fyngemaalde blare. Die minimum inhiberende konsentrasie waardes vir heel blare was laer, wat daarop gedui het dat minder onaktiewe verbindings geëkstraheer is. Ek het besluit om eerder op fyngemaalde blare te werk. Ek het ook probleme ondervind met die suur presipitering van die bikarbonaat ekstrakte en het besluit om nie verdere studies op natriumbikarbonaat as ekstraheermiddel te doen nie.

Elf verskillende ekstraheermiddels [heksaan, koolstoftetrachloried, isopropieleter, diëtieleter, metileendichloried, tetrahidrofuraan, etielasetaat, asetoon, etanol, metanol en water) is gebruik op gedroogde, fyngemaalde blare om antibakteriële verbindings vanuit *C. microphyllum* te ekstraheer.

Daar was groot verskille in die chemiese samestelling van verbindings wat geëkstraheer is. Metileendichloried en etanol het die meeste verskillende verbindings geëkstraheer. Die ekstrakte van die intermediêre polariteit ekstraheermiddels, isopropieleter, etieleter en metileendichloried het die beste skeiding met BEA as elueermiddel gegee, terwyl die koolstoftetrachloried en tetrahidrofuraan ekstrakte onbevredigend gestreep het. Koolstoftetrachloried het tot my verbasing 'n groot hoeveelheid polêre verbindings (lae Rf-waarde) geëkstaheer en 'n klein hoeveelheid nie-polêre verbindings in vergelyking met die meer polêre isopropieleter en diëtieleter.

Aan die ander kant het 'n relatiewe polêre ekstraheermiddel soos etanol verskeie nie-polêre verbindings geëkstraheer. Met CEF as elueermiddel het die koolstoftetrachloried en tetrahidrofuraan ekstrakte duidelik gestreep. Asetoon het die hoogste konsentrasie en die grootste hoeveelheid verbindings geëkstraheer wat met die vanillien spuitstof gereageer het. Alhoewel asetoon nie die meeste antibakteriële aktiwiteit geëkstraheer het nie, het ek besluit om steeds voort te gaan met asetoon as ekstraheermiddel as gevolg van asetoon se relatiewe lae toksisiteit vir die toetsorganismes asook die duidelike skeiding van verbindings.

Die oplosmiddel-fraksionerings proses is gebruik om ekstrakte te vereenvoudig na aanleiding van die aanvanklike kompleksiteit van die ekstrakte. Die mees nie-polêre drie fraksies (heksaan, koolstoftetrachloried en chloroform) het die beste skeiding van verbindings asook die meeste verskillende verbindings gegee deur dunlaagchromatografie. Die hoogste hoeveelheid, ongeveer 42 %, was teenwoordig in die heksaan fraksie en amper twee derdes van die totale antibakteriële aktiwiteit was ook teenwoordig in die heksaan fraksie.

Gewoonlik is Gram-negatiewe bakterieë meer weerstandbiedend vir plant ekstrakte as Gram-positiewe bakterieë, maar dit was nie die geval met verbindings wat teenwoordig is in *C. microphyllum* nie.

Bioautografie het goed gewerk met *S. aureus*, maar nie so goed met die ander toetsorganismes nie. Ongelukkig was *S. aureus* die minste sensitief van die vier die toetsorganismes, veral vir die heksaan en koolstoftetrachloried fraksies.

Omdat die heksaan fraksie verreweg die hoogste antimikrobiese aktiwiteit vir al vier die toetsorganismes gehad het, het ek besluit om verder te gaan met eksperimente vir die isolering van bio-aktiewe verbindings deur kolom chromatografie deur die heksaan fraksie te gebruik.

Pogings om Extrelut, 'n diatoomaarde produk te gebruik in plaas van die oplosmiddel-fraksionerings proses, was nie suksesvol nie.

Kolom chromatografie op silika gel 60 [15-40 μm] het goeie resultate gelewer. Vier vlugtige elueermiddels, wat goed gewerk het met dunlaagchromatografie, is gebruik in die volgorde BEA, CEF, asetoon en metanol.

Fraksies is gekombineer, gebaseer op die analise deur dunlaagchromatografie. Fraksies ABC het die duidelikste bande en beste skeiding gegee op dunlaagchromatografie Die meeste antibakteriese aktiwiteit (ABC: 66 % en DEF: 15 %) was in die eerste hoogs nie-polêre gekombineerde fraksies. *S. aureus* was nie so sensitief soos die ander organismes nie.

Ek het 'n dunlaagchromatografie stelsel ontwikkel met vlugtige elueermiddels, wat die verbindings in fraksies ABC en DEF goed sou skei met die doel om hierdie stelsel dan te gebruik op 'n volgende silikagel kolomskeiding. Goeie resultate is verkry met 'n 2:1 heksaan-asetoon mengsel. Ek het besluit om slegs met die ABC fraksie voort te gaan deur hierdie elueermiddel kombinasie te gebruik. Om die resolusie te verhoog is 'n fyner graad silika gel [15-25 μm] met 'n smaller grootte verspreiding te gebruik as pakkingsmateriaal.

Die fraksies is met dunlaagchromatografie geanaliseer en gekombineer, gebaseer op die dunlaagchromatografie analise en die hoeveelheid teenwoordig in elke fraksie. Sommige van die gekombineerde kleiner fraksies het redelik suiwer voorgekom volgens die dunlaagchromatografie resultate van hierdie fraksies en het 'n beduidende antibakteriese aktiwiteit gehad volgens bioautografie met *S. aureus* as toetsorganisme.

Ongeveer 80 % van hierdie gekombineerde fraksies het verlore gegaan toe hierdie fraksies gedroog is onder vakuum in 'n vakuumdessikator net voor KMR spektroskopie uitgevoer is.

Volgens die KMR resultate het die oorblywende monster hoofsaaklik bestaan uit ftalsuur, 'n algemene komponent van plastiseerders wat gebruik word in die maak van plastiek. Dit is ook 'n algemene kontaminant in sekere oplosmiddels byvoorbeeld etielasetaat.

Ftalsuur het nie met die vanillien spuitstof gereageer nie, maar die het baie duidelike blussing van UV lig gegee na dunlaagchromatografie met F_{254} plate. Volgens die Rf-waarde kon ftalsuur nie verantwoordelik gewees

het vir die antibakteriese aktiwiteit nie. Dit was ook nie vlugtig onder hoë vakuum nie.

Die prosedures wat dus ontwikkel is, het gelei tot die isolasie van 'n antibakteriese verbinding uit *C. microphyllum*. Ongelukkig het persoonlike omstandighede dit vir my onmoontlik gemaak om die werk te herhaal of om uit te brei op enige ander deel van die resultate.

Groot ruimte vir verdere studies bestaan in die nie-polêre fraksies van die finale skeiding wat die meeste van die droë gewig en biologiese aktiwiteit besit het. Daar is ook 'n moontlikheid om te kapitaliseer op die vermoedelike vlugtigheid van die bio-aktiewe verbinding deur dit te suiwer deur middel van vakuumdistillasie.

CONFERENCE ATTENDED

M. Kotze and J.N. Eloff: Extraction and investigation of antibacterial compounds from *Combretum microphyllum*. Indigenous Plant Use Forum. Nelspruit, July 2000.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

1.	A	Acetone extractant
2.	ATCC	American Type Culture Collection
3.	B	Butanol fraction
4.	BEA	Benzene/ethanol/ammonium hydroxide [90/9/1, v/v/v]
5.	CCl_4	Carbon tetrachloride extractant
6	CEF	Chloroform/ethyl acetate/formic acid [5/4/1, v/v/v]
7.	CF	Chloroform fraction
8.	CT	Carbon tetrachloride fraction
9.	E	Ethanol extractant
10.	EA	Ethyl acetate extractant
11.	*E. coli*	*Escherichia coli*
12.	EE	Diethyl ether extractant
13.	*E. faecalis*	*Enterococcus faecalis*
14.	H	Hexane fraction
15.	HE	Hexane extractant
16	HCl	Hydrochloric acid
17.	H_2O_2	Hydrogen peroxide
18.	INT	*p*-iodonitrotetrazolium violet
19.	IPE	Isopropyl ether extractant

20.	M	Methanol extractant
21.	MA	Methylene dichloride/acetone [3/2, v/v]
22.	MDC	Methylene dichloride extractant
23.	MIC	Minimum inhibitory concentration
24.	MW	35 % water in methanol fraction
25.	$NaHCO_3$	Sodium bicarbonate
26.	O_2	Oxygen
27.	*P. aeruginosa*	*Pseudomonas aeruginosa*
28.	*S. aureus*	*Staphylococcus aureus*
29.	THF	Tetrahydrofurane extractant
30.	TLC	Thin layer chromatography
31.	UV	Ultraviolet
32.	W	Water fraction
33.	WA	Water extractant

LIST OF FIGURES

LIST OF TABLES

Combretum microphyllum