

**Potentising and application of a *Combretum woodii*
leaf extract with high antibacterial and antioxidant
activity**

By

Vincent Kudakwashe Zishiri
(S23332515)

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Faculty of Veterinary Science
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Promoter: Prof JN Eloff
Co-Promoter: Prof CJ Botha

Preface

This represents a record of the work carried out in the Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, under the supervision of Prof J.N Eloff and Prof C.J Botha.

The results in these studies have not been submitted in any form to any other University and represent work done by Vincent Kudakwashe Zishiri, except where the work of others is acknowledged.

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Abbreviations used

ABTS ⁺	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)
AFA	Antibiotic Feed Additive
ATCC	American type culture collection
BEA	Benzene/Ethanol/Ammonium hydroxide (90/10/1 v/v/v)
C ₁₈ column	18-Carbon reverse phase column
CB5	Combretastatin B5
CEF	Chloroform/Ethylacetate/Formic acid (5/4/1 v/v/v)
COX-1	Cyclooxygenase enzyme 1
COX-2	Cyclooxygenase enzyme 2
dH ₂ O	Distilled water
DMSO	Dimethylsulphoxide
DNA	Deoxyribose nucleic acid
DPPH	2, 2,diphenyl-1-picrylhydrazyl
ELISA	Enzyme linked immunosorbent assay
EMW	Ethylacetate/Methanol/Water (40/5.4/4 v/v/v)
FCR	Feed Conversion Ratio
HKI	Hans Knoll Institute
INT	Iodonitro-tetrazolium salts
LC ₅₀	Lethal concentration for 50% of the cells
LDL	Low density lipids
LNBG	Lowveld National Botanical Garden
MDA	Malondialdehyde
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye
NaCl	Sodium chloride
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCCLS	National Committee for Clinical Laboratory Standards
NMR (¹³ C and ¹ H)	Nuclear magnetic resonance (carbon 13 and proton)
PBS	Phosphate buffer saline

R _f	Retardation factor
ROS	Reactive oxygen species
rpm	revolutions per minute
SEE	Serial exhaustive extraction
TEAC	Trolox equivalent antioxidant capacity
TF2	Target fraction 2
TLC	Thin layer chromatography
UP	University of Pretoria
UV	Ultra violet radiation
v/v	volume per volume
VLC	Vacuum liquid chromatography
WHO	World Health Organisation

Summary

Given the drawbacks associated with the use of antibiotics as feed additives and the imminent banning of its use in the European Union, the aim of this project was to develop an extract that could be used as an alternative feed additive in poultry production. The desired extract preferably had to be rich in antibacterial activity to control proliferation of undesired microorganisms, and antioxidant activity to boost the immune system of the poultry.

A number of trial extraction procedures were employed on dried leaf material samples to identify the best extraction method. In preliminary extraction studies, direct extraction was performed on leaf samples from the Lowveld National Botanical Gardens (LNBG) and from University of Pretoria Botanical Garden (UP). The principle aim of preliminary studies was to identify the solvents that extracted high antibacterial and antioxidant activity while also extracting large quantities of material. The secondary objective was to test for differences in activities between samples collected from LNBG and UP. Five extractants of varying polarities; acetone, ethanol, ethylacetate, dichloromethane and hexane were used.

Antibacterial activity of all extracts was quantified by a serial dilution microplate technique while bioautography was used in qualitative analysis of the antibacterial active compounds. ATCC strains of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterococcus faecalis* were used as test organisms. Qualitative antioxidant activity was determined by using a DPPH assay on TLC plates.

Results from preliminary extraction studies showed larger quantities of material were present in extracts from the LNBG sample than in the UP sample. Two major antioxidant compounds (R_f values of 0.85 and 0.35 in EMW solvent system) were seen on DPPH sprayed TLC plates, while bioautography showed the presence of a number antibacterial active compounds in the acetone, ethanol and ethylacetate extracts with R_f values ranging between 0.85 and 0.56 on TLC plates developed in the EMW solvent system. MIC values of the extracts tallied with the results from bioautography. The acetone, ethanol and ethylacetate extracts had the highest antibacterial activity while the hexane extracts had the lowest activity with average MIC value of 0.55 mg/ml for both the LNBG and UP

samples. MIC values as low as 0.04 mg/ml were measured in the acetone and ethylacetate extracts of the LN BG sample against *S. aureus* and *E. faecalis*. Based on results from preliminary extraction studies, hexane was identified as a possible pretreatment solvent for application in enrichment procedures, acetone and ethanol were chosen as the main extractants and only the LN BG sample was used for future work.

Enrichment procedures were employed along two pathways; the first pathway involved the use of hexane “wash” as a pretreatment procedure prior to extraction with acetone or ethanol. The second pathway involved the use of various mixtures of acetone in water and ethanol in water as extractants. The rationale of using these various ratios was an attempt to identify solvent mixtures that would selectively extract the bioactive components or otherwise selectively remove inactive material.

A serial dilution microplate method was used to determine Minimum Inhibitory Concentrations (MICs) and the Trolox Equivalent Antioxidant Capacity (TEAC) assay was used to quantify antioxidant activity of all extracts. The optimal extract was the one developed by pretreatment with a single direct extraction with hexane prior to extraction with acetone. It had a TEAC value of 2.3, an increase in TEAC value of 283% compared to that of the crude acetone extract. The average MIC of the crude acetone extract against ATCC stains of *S. aureus*, *Ps. aeruginosa*, *E. coli* and *E. faecalis* had dropped from 0.15 mg/ml to 0.08 mg/ml in the optimal extract (an improvement in antibacterial activity of 87.5%).

Since the optimal extract is intended for commercial application in poultry production, its antibacterial activity was tested against *Campylobacter jejuni*, *Clostridium perfringens*, *Salmonella enteritidis*, *E. coli* and multi drug resistant *E. coli* isolated from chickens. Its *in vitro* toxicity was ascertained using the brine shrimp assay and the MTT cytotoxicity assay on monkey kidney cells. The optimal extract was effective against *Campylobacter jejuni* and *Clostridium perfringens* with MIC values ranging from 40 µg/ml to 80 µg/ml. It was also active against multi-resistant strains of *E. coli* and *Salmonella enteritidis* (MIC values of 125 µg/ml for both strains).

LC₅₀ results from the brine shrimp assay and the MTT cytotoxicity assay on monkey kidney cells gave values of 863 µg/ml and 226 µg/ml respectively indicating low toxicity.

These results meant that though in some cases the MICs of the optimal extract were higher than befitting of typical antibiotics, due to its relatively low toxicity, large quantities of the extract may possibly be feed to achieve the desired activity without causing any toxicity in the poultry.

The major antioxidant compound was isolated by silica gel column chromatography. The isolated compound 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. kraussii* and also from *C. woodii* leaves. Famakin (2002) showed this compound to be the major antibacterial compound in *C. woodii* leaves. Combretastatin B5 (CB5) demonstrated *in vitro* cytotoxicity in the MTT assay on monkey kidney cells with an LC₅₀ value of 10 µg/ml. *In vitro* cytotoxicity of CB5 could be due to its antimetabolic activity. The TEAC value of 7.9 found in this study means that combretastatin B5 has about 8 times the antioxidant capacity of vitamin E. This is the first report of the antioxidant activity of any of the combretastatins

Tolerance of broiler chickens to the optimal extract was assessed at clinically inferred doses of 2 mg/kg, 5mg/kg and 10 mg/kg. After 21 days of infeed-dosing with the optimal extract, none of the chickens died or showed any behavioral signs of toxicity. There were no statistically significant differences in weight gain between broilers fed the optimal extract and the positive and negative control. There was also no positive correlation between weight gain and amount of the optimal extract incorporated in feed.

Although the optimal extract did not result in significant growth promotion relative to the positive and negative control, 2 mg/kg dose regimens showed the best Feed Conversion Ratio (FCR), with a 6.2% improvement compared to the negative control. The positive control was the only other feed regimen to provide a positive FCR with an improvement of 1.73% compared to the negative control. Because purchase of feed could represent up to 80% of costs of broiler production, this is an important finding. If these results can be confirmed, the product may therefore have commercial value. Repetition of the experiment with lower doses of the optimal extract on poultry challenged with bacterial infections is required to confirm the commercial applicability of this product.