A RATIONAL IN VITRO EVALUATION OF 53 MEDICINAL PLANTS USED IN THE TREATMENT OF DIARRHOEA AND THE POTENTIAL USE OF *DEINBOLLIA OBLONGIFOLIA* (SAPINDACEAE) EXTRACTS

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Thesis submitted in fulfilment of the requirements for the degree

Philosophiae Doctor

In the Phytomedicine Programme
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University of Pretoria

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Co-promoter: Dr L.J. McGaw

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DECLARATION

This represents an experimental record for the work carried out in the Department of Pharmacology, University of Pretoria, under the supervision of Prof. J.N. Eloff and Dr L.J. McGaw.

I, the undersigned Gabriele Würger, present this document as my authentic material and acknowledge that it has not been submitted in any other form to any other institution. I also acknowledge that I have consulted many publications in compiling this work and the references are all listed.

Gabriele Würger
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<td>Albumin/Globulin ratio</td>
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<td>ALP</td>
<td>Alkaline Phosphatase</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
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<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>BEA</td>
<td>Benzene/Ethanol/Ammonia</td>
</tr>
<tr>
<td>C</td>
<td>crude extract</td>
</tr>
<tr>
<td>Ca</td>
<td>Calcium</td>
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<td>CEF</td>
<td>Chloroform / Ethyl acetate/ Formic acid</td>
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<td>CF</td>
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<td>CNMR</td>
<td>Carbon nuclear magnetic resonance</td>
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<td>COSY</td>
<td>Correlated spectroscopy</td>
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<tr>
<td>Crea</td>
<td>Creatinine</td>
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<tr>
<td>CSIR</td>
<td>Council for Scientific and Industrial Research</td>
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<td>DB</td>
<td><em>Deinbollia oblongifolia</em> n-butanol fraction</td>
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<td>EA</td>
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<td>E. coli</td>
<td><em>Escherichia coli</em></td>
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<td>EMW</td>
<td>Ethyl acetate/Methanol/Water</td>
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<td>F</td>
<td>Fraction</td>
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<td>Gamma glutamyl transferase</td>
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<td>GLOB</td>
<td>Globulin</td>
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<td>H</td>
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<tr>
<td>HMBC</td>
<td>Heteronuclear bond correlation</td>
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<td>HMQC</td>
<td>Heteronuclear multiple quantum correlation</td>
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<tr>
<td>HNMR</td>
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<td>INT</td>
<td>p-Iodonitrotetrazolium violet</td>
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<td>K</td>
<td>Potassium</td>
</tr>
<tr>
<td>LC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Lethal cell toxicity</td>
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<td>MEM</td>
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<tr>
<td>MIC</td>
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<td>MTT</td>
<td>3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide</td>
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<tr>
<td>Na</td>
<td>Sodium</td>
</tr>
<tr>
<td>NIR</td>
<td>Near-Infrared Reflectance</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>OECD</td>
<td>Organisation for Economic Cooperation and Development</td>
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<td>P.a.</td>
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<td>R&lt;sub&gt;f&lt;/sub&gt;</td>
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<td>TA</td>
<td>Total activity</td>
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<td>T.e.</td>
<td>Trichilia emetica</td>
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<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
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<tr>
<td>TSP</td>
<td>Total serum protein</td>
</tr>
<tr>
<td>Urea</td>
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UV  Ultraviolet
WHO  World Health Organization
Z.m.  Ziziphus mucronata
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ABSTRACT

Antibiotic Feed Additives (AFA) have been used to prevent many bacterial infections during weaning of livestock. The use of these AFA’s resulted in the development of multiresistant bacterial strains and was therefore banned by the European Union. The United States also restricted the use of these feed additives considerably. Many scientists have started to search for alternatives in the prophylactic and therapeutic treatment of bacterial infections. Plants have been used traditionally by resource poor people all over the world to treat many infections. Diarrhoea not only causes many problems in the high intensity production of livestock but also leads to many human deaths. A large number of plants have been used to treat diarrhoea in humans and animals. Several authors have selected one or two species based on traditional use to evaluate in depth. In this project several different relevant parameters of 53 plant species used traditionally to treat diarrhoea were investigated in order to develop a model that would identify the species with the highest chance of delivering a useful antibacterial product.

Antibacterial activities against two pathogens important in diarrhoea (Staphylococcus aureus and Escherichia coli) were positive parameters in selecting species. Because tannins frequently have antibacterial activity, but are not useful as prophylactic agents due to their effect on production, high tannin content was considered to be a negative indication. Cellular toxicity was also used as a negative parameter at a later stage.

In addition to the in vitro assays there are also other parameters to be investigated to evaluate the potential use of plants. The influence of season of collection was determined on the antibacterial activity and tannin content of acetone leaf extracts of five plant species traditionally used to treat diarrhoea. They were Acacia karroo, Acacia sieberiana var. woodii, Peltophorum africanum, Trichilia emetica and Ziziphus mucronata.

The antibacterial activity varied depending on the season of collection with the best activity generally in the months of late summer to autumn (January to April). The activity of Acacia karroo against E. coli was best in the month of April (MIC = 0.11 mg/ml (TA = 332 ml/g)) and against S. aureus in the month of March (MIC = 0.06 mg/ml (TA = 334 ml/g)). Acacia sieberiana subsp. woodii extracts had the best activity against E. coli (MIC = 0.10 mg/ml (TA = 303 ml/g)) in March against S. aureus in April (MIC = 0.08 mg/ml (TA = 303 ml/g)). Peltophorum africanum extracts were most active against E. coli in February (MIC = 0.05 mg/ml (TA = 1188 ml/g)) and against S. aureus in February and March (MIC = 0.04 mg/ml (TA = 1188 ml/g and 1075 ml/g)). Trichilia emetica extracts were generally not very active against the bacterial strains (best activity: MIC = 0.22 mg/ml (TA = 74 ml/g) against E. coli in May and MIC = 0.28 mg/ml (TA = 26 ml/g) against S. aureus in December). Ziziphus mucronata was most active
against both bacterial strains and in May (E. coli: MIC = 0.10 mg/ml (TA = 589 ml/g); S. aureus: MIC = 0.04 mg/ml (TA = 1099 ml/g)). The tannin content varied in the extracts as well. The antibacterial activity however did not seem to be directly correlated to the tannin content.

Another important parameter in the use of plant species is to determine the interspecies variation of plants based on genetic or environmental influences. Leaves from 42 plants of Combretum molle were collected at different locations during the same season. The average MIC against E. coli was 0.227 mg/ml. The low standard deviation of 0.07 indicates that there was very little variation in activity. The average value against S. aureus was 0.399 mg/ml with a slightly higher standard deviation of 0.16. However due to the fact that the samples from different areas extracted different amounts, the total activity varied. The tannin assays revealed that there was with one exception no correlation between the antibacterial activity and the tannin content. So it can be safe to assume that genetic variation does not influence the activity too much at least in C. molle leaves.

Fifty three plant species traditionally used to treat diarrhoea in published literature were then ranked using a novel system in order to determine which species had the most potential value. Ranking was based on the lowest MIC value against E. coli, lower activity against S. aureus (to limit selecting for general metabolic toxins), low tannin concentration and high extract yield.

From this ranking, five plants were chosen to investigate their potential value further: Acacia sieberiana var. woodii (E. coli: MIC = 0.13 mg/ml, TA = 108 ml/g; S. aureus: MIC = 0.13 mg/ml, TA = 108 ml/g; Yield = 14 mg), Albizia adianthifolia (E. coli: MIC = 0.14 mg/ml, TA = 239 ml/g; S. aureus: MIC = 0.04 mg/ml, TA = 765 ml/g; Yield = 34 mg), Deinbollia oblongifolia (E. coli: MIC = 0.17 mg/ml, TA = 158 ml/g; S. aureus: MIC = 0.08 mg/ml, TA = 338 ml/g; Yield = 27 mg), Spirostachys africana (E. coli: MIC = 0.13 mg/ml, TA = 300 ml/g; S. aureus: MIC = 0.09 mg/ml, TA = 438 ml/g; Yield = 38 mg) and Tetradenia riparia (E. coli: MIC = 0.09 mg/ml, TA = 214 ml/g; S. aureus: MIC = 0.13 mg/ml, TA = 149 ml/g; Yield = 20 mg). None of the plants contained any tannin.

The next step towards the recommendation of a plant for the development of a commercial product was to evaluate the cytotoxicity of the selected five species. The following values were obtained: Acacia sieberiana var. woodii LC50 = 0.026 mg/ml, Albizia adianthifolia LC50 = 0.068 mg/ml, Deinbollia oblongifolia LC50 = 0.078 mg/ml, Spirostachys africana LC50 = 0.025 mg/ml and Tetradenia riparia LC50 = 0.028 mg/ml.

Deinbollia oblongifolia (for its low LC50 value) and Spirostachys africana (for its good antibacterial activity and total activity) were potentized by removing inactive compounds through solvent-solvent fractionation. The antibacterial activity against E. coli was increased this way (MIC = 0.08 mg/ml for...
*Deinbollia oblongifolia* (chloroform fraction) and MIC = 0.08 mg/ml for *Spirostachys africana* (chloroform fraction)). The LC$_{50}$ values for both chloroform fractions were determined (LC$_{50}$ = 0.188 mg/ml for *Deinbollia oblongifolia* and LC$_{50}$ = 0.062 mg/ml for *Spirostachys africana*). The selectivity index (SI) was also determined and proved that the potentization was indeed successful (*Deinbollia oblongifolia* SI = 2.35 compared to a value of 0.45 for the crude extract; *Spirostachys africana* SI = 0.78 compared to a value of 0.19 for the crude extract). Based on these values, the chloroform fraction of *Deinbollia oblongifolia* was chosen as the less toxic one with similar activity and a higher selectivity index to be worked on further. One of the active compounds was isolated and evaluated for its activity against *E. coli* (MIC = 0.74 mg/ml) The LC$_{50}$ value of 0.042 mg/ml indicated that the activity of the extract was a result of synergism rather than being due to a single active compound (the selectivity index (SI) was 0.06 compared to the values of 0.45 for the crude and 2.35 for the potentized extract).

The plant extracts should of course be as effective against pathogenic strains as they were against the ATCC strains and so the extracts and pure compound of *Deinbollia oblongifolia* were tested for their activity against four different pathological *E. coli* strains. The results showed that the crude extract and the fraction were as active as in the preliminary screening results against only one of the four pathological strains. The pure compound on the other hand was more active against all four pathological strains than against the ATCC strain.

The next step was to test the safety of the extracts of *Deinbollia oblongifolia* in mammals. Unfortunately neither the crude extract nor the chloroform fraction of *Deinbollia oblongifolia* could be used safely in a living organism or in an isolated organ study. A part of the problem may have been caused by the vehicle used in the study despite reports in the literature that an acetone water mixture is safe to use.

In general all the species investigated had good antibacterial activity against *E. coli* this supports the traditional use of these species although we used acetone as extractant rather than the water used traditionally. There were major differences in antibacterial activity over a season indicating that mature leaves were more active than young leaves before senescence started. At least in the case of *Combretum molle* there was little difference in the antibacterial activity of many plants collected at different locations during the same season.

The results obtained in this study could be useful in further studies to develop extracts that can be used to control diarrhoea in animals. Possibly more emphasis should be put on the difference in activity towards *E. coli* and *S. aureus* to eliminate the presence of general metabolic toxins. Such an approach would lead to a different priority order for species to examine. A major first step would probably be to test the *in vitro* and *in vivo* toxicity of selected species.