CHAPTER 12

General discussion and Conclusion

Six *Terminalia* species and twenty-four *Combretum* species were evaluated for antifungal activity and antioxidant screening based on the use of the two genera in traditional medicinal treatments for both domestic animals and humans in southern Africa, as well as their availability. The total percentages of the *Terminalia* species extracted using different solvents (acetone, hexane, DCM and methanol) were determined. Methanol extracted a greater quantity of plant material. There was a major difference in the methanol extractability of *T. gazensis* leaves compared with all the other species. This difference is not related to the sectional division of the species (Carr, 1988).

The leaves of Combretaceae family are known for their pharmacological activity and in this study, I have shown that many extracts also contain several anti-oxidant compounds. Methanol and acetone extracted the largest number of different antioxidant compounds based on DPPH TLC. *In vitro* studies coupled with the phytochemical analysis confirm that the extracts possessed potential antioxidant activity.

The solvent tolerance of the microorganisms was tested using the following solvents; DMSO, acetone, methanol and ethanol. In order to determine the maximum concentration at which different solvents would allow the test microorganisms to reach normal growth, different concentrations from 10 to 100% were used. Based on MIC DMSO was the least toxic of the solvents used with an average MIC of 616 mg/ml (56%) followed by acetone 512 mg/ml (64%), methanol 320 mg/ml (40%) and ethanol 304 mg/ml (38%). The danger of using ethanol or methanol is evident from the inhibition by 20% ethanol or methanol of *M. canis* and *S. schenckii*. In general the two moulds (*M. canis* and *S. schenckii*) appeared to be most resistant. Acetone was the only extractant that could be used with a safety margin at a 50% concentration.

The serial dilution microplate method used for detecting antibacterial activity worked well with fungi after slight modifications. The antifungal activity of some of the extracts were at concentrations that could be therapeutically useful already, leading to the distinct possibility that some of the extracts may be applied clinically for dermatophyte infections e.g. *M. canis.* If one extrapolates from *in vitro* to *in vivo* activity especially in topical applications, it means that an acetone leaf extract from 1 g of *T. sericea* leaves diluted to 2.7 L would still inhibit the growth of *M. canis*. Extracts from other species had values as high as 6.05 L/g. The results of this study indicate that the *Combretum* species assayed possess substantial antifungal properties. If there are no synergistic effects and the antifungal compounds comprise 0.1 % of the total mass, the antifungal compound should have an MIC of 0.02 to 0.2 µg/ml. The results of this study support several of the traditional medicinal uses of *Combretum* species all over Africa. I found that adding of INT from the beginning of the MIC experiment allowed the early detection of growth of the fungi tested, which overcomes the difficulties of previous methodologies. The acetone extracts with the highest activity were from *C. imberbe*, *C. nelsonii*, *C. albopunctactum* and *T. sericea*, and were considered to be good candidates for the *in vivo* tests.

T. sericea holds promise for isolating antifungal compounds, because hexane and DCM extracts of *T. sericea* have compounds inhibiting growth of all pathogens, especially the compound at R_f value of 0.46. It is followed by *T. brachystemma*, which also had compounds (R_f = 0.46), which inhibited growth of all tested pathogens.

Extracts of *Combretum* species in the Section *Hypocrateropsis* had high number of total active antifungal compounds, ranging from 56 to 62. These values were high because, in some instances, the same compound was active against different fungi and it was observed in more than one of the three TLC solvent systems used and was present in more than one extract. For comparative purposes we counted all visible compounds. That explains high numbers reported. *C. celastroides* ssp. *celastroides* and *C. clelastroides* ssp. *orientale* possess 62 active compounds each. Section *Angustimarginata* follows section *Hypocrateropsis* with active compounds ranging from 37 to 43, more active compound being in *C. nelsonii* (43). *Metallicum* section, which is made up of *C. collinum* ssp. *suluense* and *C. collinum* ssp. *taborense* did not contain active compounds against all tested microorganisms. *C. zeyheri* in *Spathulipetala* section contained 26 active compounds. In *Ciliatipetala* section, *C. albopunctactum* contained more active compounds than other species in the same section, with 43 compounds. It is followed by *C. apiculatum* ssp. *apiculatum* and *C. petrophilum* with 20 and 14 compounds respectively. In *Breviramea* section we had only one species, *C. hereroense,* with 16 active compounds. *Connivetaia* section species, C. *microphyllum* and *C. paniculatum* had a similar number of active compounds, 7 and 8 respectively. *C. mossambicense* and C. *acutifolium* in *Poivrea* section, had the most active compounds, which were 25 for each species, and *C. bracteosum* had 14. That was the biggest difference in the section as compared to others where species in the section had almost the same number of compounds.

C. neoformans was the most sensitive organism against all *Combretum* species, with 367 compounds active against it. This was followed by *C. albicans* and S*. schenckii* with 339 and

314 compounds active against them respectively. *M. canis* had 298 compounds active against it, followed by *A. fumigatus* with only 192 compounds being active against it.

The results indicate that bioautography is probably the most important detection method for new or unidentified antifungal compounds, because it is based on the biological effects of the substances under study. In the study of biologically active compounds from natural sources, it is evident that rapid and sufficient detection of such compounds is a critically important aspect of the discovery process. Bioautography is a method that makes it possible to localise antifungal activity on the chromatogram. Bioautography results confirmed the low MIC values obtained in the previous chapters (Masoko *et al*., 2005).

Bioassay-guided fractionation on silica gel 60 (63-200 µm) in column chromatography resulted in the successful isolation of the highly active compound I (later shown to be a 3:4 mixture of asiatic acid and arjunolic acid) from the leaves of *C. nelsonii*. This very active "Compound **1**" was used in the *in vivo* assay.

The *in vitro* cytotoxicity of three *Combretum* and one *Terminalia* species extract were investigated. Responses varied for the different extracts and between the two assays, but brine shrimps were less sensitive than the Vero monkey kidney cells. Only acetone extracts were used, because it was found not to be toxic on fungi. In this study it was used as control and it was found not to have effect on *A. salina* nauplii and Vero cells at the concentrations used. These values were all higher than the berberine chloride control of 4.35 µg/ml. The value was higher than that of berberine in the literature of 0.141-0.148 µg/ml (Vennestrom and Klayman, 1988). *C. imberbe* extracts was the least toxic with an LC_{50} of 168.6 µg/ml and *C. nelsonii* extracts were the most toxic with an LC_{50} of 75.7 µg/ml, LC_{50} values for *C. albopunctactum* and, *T. sericea* were 102.9 and 121.7 µg/ml respectively (Masoko, 2006)

The relative safety margins (RSM) in the MTT assay were high compared to those in the brine shrimp assay. RSM of *C. imberbe*, *T. sericea* and *C. albopunctactum* in *C. albicans* were 0.09, 0.16 and 0.19 respectively. *C. nelsonii* and *T. sericea* had high RSM values against *S. schenckii* and *M. canis*, therefore high amount of the material must be used in treatment.

In the *in vivo* experiments the mixture of asiatic acid and arjunolic acid (Compound **1)** and *C. nelsonii* extracts were very active on *M. canis* and *S. schenckii.* Wounds infected with *C. neoformans* took longer time to reduce in size. Amphotericin B had almost same activity against all tested pathogens. *C. imberbe* and *T. sericea* extracts had similar impact on pathogens. *C. albopunctactum* extracts were very active against *M. canis*. Plants used for the treatment of wounds can have different properties like anti-inflammatory, antimicrobial, healing, analgesic, haemostatic and immuno-modulating activities. The immune system is an important factor in the process of healing of wound.

Acetone extracts of leaves of *C. nelsonii*, *C. albopunctactum*, *C. imberbe* and *T. sericea* possess remarkable growth inhibitory activities against fungal pathogens. Acetone extracts of leaves and isolated compound demonstrated wound healing properties comparable with that of the antibiotic amphotericin B. Even the untreated wound healed but not at the same rate as the treated wounds. As earlier suggested, healing in this untreated group may be due to a strong immune system. It is important to note that throughout the period of wound treatment, the extracts did not cause irritation or pain to the animals as the rats neither showed any signs of restlessness nor scratching/biting of wound site when the extracts were applied.

After structure elucidation, the isolated compound were terminolic acid and "Compound **1**". Terminolic acid was not studied further as "Compound **1**" was present in a high concentration and was very active. The structure of Compound **1** was elucidated by using NMR and MS. It was found that Compound **1** is a mixture (3:4) of two isomers by intensities and number of its NMR signals. The MS gave a molecular ion peak at *m*/*z* 487 (M–H) relating to $C_{30}H_{48}O_5$. The isomers were labeled Compound **1a** and **1b**. ^{1}H NMR data of **1a** and **1b** were similar, with the main differences noted in the DEPT experiment, where **1a** and **1b** possessed ten and nine CH_2, six and eight CH, respectively, with seven methyl groups for each compound. The ^{13}C NMR spectra showed 30 carbon atoms for each. The chemical shift of the carbon atoms C_{12} and C_{13} at δ 122.4; 144.4 and δ 125.7; 138.8 for **1a** and **1b**, respectively, suggested the presence of two classes of triterpenes, the oleanane and ursane. The ^{1}H and ^{13}C NMR data of Compound **1** were similar to those recorded for arjulonic acid **1a** and asiatic acid **1b** (Facundo et al., 2005). Asiatic acid and arjunolic acid are well known but it is the first that the two compounds are isolated from *C. nelsonii*.

A variety of triterpenoids have been isolated from *Combretum spp.* (Rogers and Verotta, 1996). Terpenes or terpenoids are active against bacteria and fungi (Taylor *et al.*, 1996). From our group, Martini *et al*., (2004a) isolated and characterized seven antibacterial compounds. Four were flavanols: kaemferol, rhamnocitrin, rhamnazin, quercitin 5,3 -dimethylether] and three flavones apigenin, genkwanin and 5-hydroxy-7,4'-dimethoxyflavone. All test compounds had good activity against *Vibrio cholerae* and *E. faecalis*, with MIC values in the range of 25-50 μg/ml. Angeh (2005) isolated 8 compounds with antibacterial activity from *Combretum* section, Hypocrateropsis. All eight compounds had moderate (MIC of 60 µg/ml) to strong (10 µg/ml) antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis* and *Mycobacterium vaccae*.

The mixture of asiatic acid and arjunolic acid had excellent antifungal activity against all tested pathogens i.e. Mixture of asiatic acid and arjunolic acid had the lowest MIC value against *M. canis* and *S. schenckii* which was 0.2 µg/ml, followed by *C. neoformans* which was 0.4 µg/ml. *A. fumigatus* was the most resistant with the highest MIC value of 1.6 µg/ml. LC_{50} of compound A was 10.58 µg/ml, a value that is similar to the reported LC_{50} value of the berberine standard (10 µg/ml), this is an indication that it is as toxic as berberine. Because it is effective at such a low dose the therapeutic index of 50 may be acceptable.

This study indicates that the C*ombretum* and *Terminalia* species assayed possess substantial antifungal properties. This explains the use of these plants in folk medicine for the treatment of various diseases related to fungal infections. The aims of the project have largely been attained and it appears that there may be scope for continuing work on plant extracts of *C. nelsonii* or *C. imberbe* due to its higher therapeutic index.